Review: Towards much more efficient biofuel crops – can sugarcane pave the way?

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Abstract

Triple challenge is confronting world plant production in a few forthcoming decades: population increase, worsening of growth conditions, and changeover from fossil-based to renewable energy and raw materials. The challenge cannot be met without utilizing best modern biological techniques, genetic modification included. In the current era of rapid environmental changes, plant breeding should take even greater responsibility for food, feed, fiber and fuels than in the past. Though; there are good prospects for remarkable improvements in yield level and energy efficiency of plant production, as is exemplified with the cases of modern crop and especially sugarcane improvement in consideration. For example sugar content, biomass yield, pest and disease resistance, environmental safety and resource use efficiency of biofuel crop production can be essentially improved on the basis of new genetic know-how and taking advantage of the richness of genetic resources available in the Plant Kingdom. Especially the natural reservoir of 10,000 wild grass species should be exploited in the most pure way possible by means of modern and precise GM methods. Consequently, our vital needs in biofuel crop production can be fulfilled without increasing crop production areas untenably at the expense of the remaining wilderness, or compromising food security in the world.

Running title: Towards much more efficient biofuel crops

Introduction

The merits of the huge increases in agricultural production efficiency during the 10,000 latest years are attributed about fifty-fifty between the developments in crop husbandry, crop protection etc. *versus* plant breeding. Now that we live in rapidly changing and possibly hard times, the responsibilities of plant breeding may surge. But then also the potentials of breeding are greater than ever before, thanks to the revolution in genetic knowledge and know-how in this millennium.[1]

Consequently, for example China has allocated 3.5 billion US dollars in its GM Crops Initiative, and the release of the long-awaited domestic GM crops on the market is written into the government's short-term focal goals. In addition, the development of new GM crops is one of the 16 major projects listed in China's plan for scientific and technological development until 2020. The government's plans include the development of pest- and disease-resistant GM rice, rapeseed, maize and soy, with research focusing on yield, quality, nutritional value and drought tolerance.[2,3]

Current bioenergy crops are often criticized in the media for competing untenably with food, feed and fiber production in the fields. Such a new source of competition may tend to enhance future price speculations, and it may thus fuel the spiking of food prices in international markets. Indeed, due to the very low efficiency characteristic of the maize-based production of bioethanol in USA, a large proportion of maize production area has to be redirected from food and feed purposes to fuel uses even if the very first quantitative goals set down by legislators for biofuel production during forthcoming decades in USA are to be fulfilled. Whereas, high-yielding crops (50 tn⁄ha) that have high conversion efficiency (75 %) would require a global land footprint of around 100 million ha to replace current (2008) oil consumption in the world. Such increase in cultivated area might somewhat soundly be achieved, provided that lands abandoned due to overuse or salinization in traditional agriculture and less favorable areas not much used in agriculture hitherto in certain continents, e.g. South America, could be taken in bioenergy production.[4]

International plant science organizations point out that great improvements are required in current bioenergy crops for achieving sustainable systems of biofuel production.[5] On the other hand great prospects for such improvements exist, because relatively little breeding for such special traits has been done previously. Accordingly, genetic variation in certain "energy" traits may still be found in the breeding populations of the crop species. Further genetic diversity is available in the Nature. The 10,000 wild grass species in the world harbor riches of highly efficient solutions available for improving the productivity and ecological tolerance to environmental stresses of crop plants, as soon as the genetic basis of the desirable traits is being unraveled by modern genome research.

The efficiency of the bioenergy crops measured in savings in fossil inputs such as fertilizers and tractor fuels as well as in biofuel yields produced per hectare depends much on the methods used in their production. Therefore, essential improvements in ecological and carbon efficiency can be reached, if bioenergy crops can be bred to manage with lower fossil inputs without compromising their high yield levels. When more efficient plant varieties become available, sustainable production of bioenergy and renewable products can be obtained without jeopardizing food security and wildlife.

Sugarcane is very efficient in assimilating solar energy into carbohydrates, and according to various evaluations tropical sugarcane production is sustainable both in terms of carbon efficiency and in ethanol yield per hectare. International Energy Agency states that ethanol from sugar cane produced in the tropical/sub-tropical regions such as Brazil, Southern Africa and India, for example, has excellent characteristics in terms of economics, CO_2 reductions and low land use requirements.[6] Other studies also confirm that tropical sugarcane ethanol yields the highest savings achieved hitherto (85–98 %) in fossil energy use and greenhouse gas emissions.[7]

Regarding biodiesel production, oil palm (*Elaeis guineensis*) is far superior to any oil crops produced in Europe. It produces nine times more oil per ha than soybean and six times more than oilseed rape,[8] which means much less wastage of natural resources in agriculture. Oil palm requires tropical climate. However, contrary to certain "activist" campaigns, palm oil needs not be produced in rainforests but certified oil palm plantations can be founded on set-aside and waste lands. Political doubts still occur at least in European public as regards the reliability of such certification systems in the circumstances when the overall demand for palm oil is increasing dramatically.[9]

Though all kinds of animal or plant fats can be used in the manufacturing process for NExBTL biodiesel of the Finnish company Neste Oil,[10,11] such food waste materials are only available in minor quantities that could provide for no more than a few percentages of the biodiesel volumes to be re-

quired. Accordingly, the decision by the company of using palm oil in its biodiesel process for the time being is justified on environmental grounds.

Biofuel production is quite new field of application in plant cultivation, and consequently the potentials of many unconventional plant species are in consideration as well,[4] e.g. jatropha (*Jatropha curcas* L.). Such species are often not domesticated hitherto, jatropha included. Consequently, much work and time would still be needed before the practicability of such neo-domesticated species may be proven. Reliable cultivation methods must be developed and the adaptability of their diverse genetic origins for large-scale cultivation and efficient biofuel production in different regions and various environments shall be tested in practice. If proven successful, however, a novel species may offer one substantial advantage: its genetic diversity is not yet exhausted by the centuries of selection breeding as is often the case with old crop species. Anyhow, substantial experience in plant breeding has shown that the amendment of a wild plant species to an important crop plant tends to take centuries rather than decades.[12,13]

Jatropha regarded, such evidence is still to be awaited, though large cultivation tests have already been planted in the tropics lately. Even if the species has high oil content in its seed (27–40 %) and can tolerate poor conditions such as drought, its true productivity in such environments remains to be seen.[14] Adequate ecological caution may also be needed before such ecologically "overly" tolerant species are distributed for large-scale cultivation everywhere. Namely, some of the most infamous tropical weeds have originated from the introductions of alien trees which have thereafter become highly invasive in certain local ecosystems. Additionally, jatropha belongs to a highly toxic plant family (Euphorbiaceae) and might fight its controlling pests too well off in some new environments. Toxicity could also limit the potential uses of its side products, which may erode its net economic sustainability.

Consequently, sugarcane and ethanol were selected for the case of a detailed analysis in the present paper, because a) there are many scientifically interesting developments going on in sugarcane, and b) our most important crop plants are cereals, i.e. grass species, and not palm plants.

Though, regarding the important new traits in question, information from other plant species is also considered when necessary. Namely, in spite of the great differences in chromosome number and size between grass species, all 10,000 species of the grass family (Poaceae) are closely related genetically, and the gene content of different grass species does not vary greatly.[15,16,17] In spite of the great amount of DNA in sugarcane cells, due to its high polyploidy, the basic *Saccharum* genome is only twice the size of rice and significantly smaller than maize genome.[18] As a consequence, achievements in genetic research and trait development in other grasses can quite likely be also utilized in sugarcane, and *vice versa*.

The morphological structure, water use, fertilizer intake, sucrose content, and the very nature of sugar production in sugarcane are likely to undergo major changes with the modern tools of genetic modification. Scientists predict that the ethanol yield of sugarcane per ha can be doubled in practical cultivations within the next 15 years.[19] Additionally, prospects for remarkable enhancements in resource use efficiency also exist in sugarcane, at least regarding water and nitrogen.

Locally well-adapted and highly productive biomass grasses are under development in temperate and cool climates, e.g. switchgrass (*Panicum virgatum*) in North America, Miscanthus (*Miscanthus x giganteus*) both in Europe and N. America,[20,21] and reed canarygrass (*Phalaris arundinacea*) e.g. in Finland and UK (though mainly aimed at combustion use hitherto).[22,23] What lessons could possibly be learned for their breeding from the experiences in sugarcane?

Current sugarcane production

Sugarcane (*Saccharum officinarum*) is cultivated in 22 million ha, and its average cane yield is 70.9 tn/ha. World production is 1,560 million tn of cane, which yields 68 million tn of sugar annually. World sugarcane production has increased by a quarter from the turn of the century onwards. The greatest cane producers are Brazil and India, with 33 % and 22.3 % share of world sugarcane production, respectively. Other great producers are China, Thailand, Pakistan, Mexico, Colombia and Australia, which in combination share 22.6 % of world sugarcane production.[24]

The bulk of sugarcane is produced in a zone surrounding equator: between 35 °S and 35 °N. Depending on varieties and growth conditions, yield is harvested in 9–24 month intervals by cutting the cane stalks. Sugarcane is a perennial crop, and it is economically viable to take 3–8 crops from the same cane roots in recurrent years. Commercial sugarcane is propagated by vegetative means, and new cultivations are established by burying segments of stalks in furrows in the field. Furrow interval is 1.1–1.4 m, and one hectare of sugarcane cultivation contains 21,000–35,000 cane stalks.

Sugarcane is an efficient assimilator and may according to a comparative study produce more than 200 tn of biomass (in fresh weight) per ha in the best experimental conditions, though the respective commercial maximum and commercial average yields remain to three quarters and one half of that, respectively. Though, even higher yields have been reported, but their comparability is hard to ascertain. A huge figure of 381 tn/ha has been estimated as a theoretical maximum annual yield of sugarcane in the most favorable conditions.[18] Typical values in informal sources for average cane yield may range between 50–150 tn/ha – in wet tropics good rainfed cane yield is 70–100 tn/ha, whereas in dry tropics and subtropics good cane yield using irrigation may often be 110–150 tn/ha.

Sugarcane processing products and byproducts

Sugarcane stalks are pressed to produce syrups (molasses), which are then processed further in a few purification steps to yield purified cane sugar. Remaining molasses fractions still contain some sugars and can be utilized for alcoholic fermentation. Brazil produces the bulk of its ethanol from sugarcane molasses. Additional uses for the molasses fractions are feed additives and fertilizers in sugarcane cultivation.

Bagasse is the highly fibrous residue remaining after cane is pressed to remove sucrose. Bagasse is high in lignocellulose, and it is being burnt for energy in sugar mills or used for paper production. Regarding feed uses the disadvantage of bagasse is its low digestibility (25 %) because of the presence of lignin which protects carbohydrates from being digested by the rumen microbes. Consequently, chemical, biological or thermo-mechanical treatments are required to improve the digestibility to approximately 65 %.[25]

Following harvest quite a lot of harvest residues (e.g. leaves) are left in cane fields. Their quantity roughly resembles that of bagasse remaining after cane pressing. According to certain estimations up to 80 % of the harvest residues could be utilized for raw materials without compromising sustainable sugarcane production.[26]

Bagasse and harvest residues would be suitable raw materials for the future production of cellulosic ethanol. In sugarcane-cultivating countries the quantity of biomasses available from sugar production may vastly exceed that any other potential biomass sources combined together, municipal

wastes included. For example in Australia, four times more biomass is available from sugar industry wastes than all other sources in combination.[26]

Alternatively, part of the wastes could be burnt in special furnaces into coal to be used for agriculture. Namely, such coal degrades extremely slowly in the soil, and it could therefore be applied for improving soil structure and organic matter content in cultivation.[27]

Growth requirements

Water

Water is often the limiting factor in sugarcane production. During their growth stage sugarcane varieties need much water (in total 1500–2500 mm, evenly distributed in the period) as well as warmth.[28,29,30] Cane yield is directly proportional to the amount of water used by sugarcane in the prevailing climatic conditions. About 37–330 kg of water is used for producing one kg of cane and 1,000–2,000 kg of water for producing one kg of sucrose, respectively.[29,30,31,32,33]

Sugarcane is being cultivated both rainfed and applying irrigation. Irrigation has been traditionally based on furrows, but recent trends favor sprinklers and drip irrigation (especially in Hawaii). Much water and work is saved using drip irrigation. Therefore, its use is economically sustainable, even if the drip hoses damaged by the burning treatments of the plantations must be replaced after harvest.[34]

Temperature

When harvesting period is approaching sugarcane needs dry, sunny and cool conditions in order to ripen to harvest state and boost its sugar content to 10–12 %. Rooting and sprouting of the planted stem pieces occurs at its best in 32–38 °C, and stalk growth reaches its optimum in 22–30 °C, but ripening of stems and their sugar enhancement proceeds most successfully in 10–20 °C.[25]

Soil

Sugarcane has no requirements for a special soil type. Optimum soil pH for sugarcane is 6.5 but the plant can be grown in soils with pH 5–8.5. Sugarcane grows best in more than one meter deep layer of soil, and parts of its root system may extend into the depth of five meters. However, the bulk of its roots (85 %) typically harbor the uppermost 60 cm layer of soil, especially if the plant is irrigated often and with small doses of water at a time.[34]

Deeper root systems could be generated by irrigating the plants less frequently and with greater doses at a time. Deeper-rooting varieties could presumably be developed with plant breeding and at least with genetic modification. Deeper root systems would diminish the susceptibility of the canes to damages caused by occasional drought periods in certain areas. Though, the metabolic costs of root growth and maintenance can be a significant drain on reproductive output.[29,35]

Nutrient requirements

In order to be productive sugarcane needs quite a lot of nitrogen (100–200 kg/ha, referring to yield level 100 tn/ha) as well as potassium (125–160 kg/ha), but rather little amount of phosphorus (20–90 kg/ha) is sufficient. Though, in the ripening period nitrogen content in the soil should be as low

as possible in order to reach high sucrose content in the stems (especially in hot and wet conditions).

For reducing the amount of harvest residues sugarcane stalks or plantations are often being burned before harvest or after having cut the stalks down in the field. However, at least the Australian sugar industry is trying to get rid of such a traditional procedure, because burning pollutes air with particles harmful to human health.[36]

Leaving harvest residues on the plantation as green mulch and for decomposition might beat burning also as regards soil nutrients. However, not much is known about the effects of such cultivation method on the nitrogen or carbon balance of the soil. It may apparently not have much effect on improving nitrogen availability of the next cane vegetation or rising permanent carbon content in the soil.

In studies in wet tropical Australia less than 6 % of the nitrogen in the harvest residue was utilized, i.e. found its way to the next harvested cane yield. The bulk of the carbon in the harvest residue was burnt to CO_2 due to microbial activities and lost in the air. Though, in wet tropical areas only about 6 % of fertilizer nitrogen is utilized by the cane plant as well, whereas in temperate regions 20–40 % of fertilizer nitrogen is being utilized by sugarcane for yield production.[37,38,39]

Classic cane breeding

Sugarcane originated in Asia. Sugarcane varieties in cultivation are species hybrids between the primitive cultivated sugarcane *Saccharum officinarum* (2n=80) and a wild cane species *S. spontaneum* (2n=40–128). Sugarcane varieties are highly polyploid plants i.e. they contain each of the cane basic chromosomes in 5 to 14 copies in their cells. Many varieties are even aneuploid, which means that different basic chromosomes may occur in different numbers. Therefore sugarcane varieties are often quite sterile.

Actually even *S. officinarum* itself is of complex species-hybrid origin and may have received whole chromosomes intact from as far as other plant genera (*Erianthus* and *Miscanthus*).

High sugar content came from *S. officinarum*. Unfortunately, the species also harbors many poor traits unsuitable for cultivation: it is very susceptible for diseases, devoid of ecological adaptability and lacks sprouting ability necessary for the perennial cropping system. Thus, *S. officinarum* cannot usually manage without human help, and its few ephemeral occurrences outside cane plantations cannot spread further in Nature.

Vigor, disease resistance, tolerance to poor cultivation conditions, and great biomass production has been introduced into sugarcane varieties from the wild cane, *S. spontaneum*. As a trade-off, the sugar content of the wild species is negligible. Much genetic variation occurs in its populations and the species is a troublesome weed in certain areas of the world. Though, its weedy characteristics have not been carried along to cultivated sugarcane varieties.[25]

In order to retain the sugar content high enough in sugarcane cultivation, primary species hybrids have been crossed back to *S. officinarum* for several generations. Consequently 80–90 % of the genes in currently cultivated sugarcane varieties originate from that high-sugar but primitive ancient cane species.

Sugarcane breeding takes decades

For genetic reasons considered above, the bulk of sugarcane varieties are more or less sterile. Furthermore, sterility is favored, because flower formation decreases sugar content in the stalks. When viable seed is rare, breeding via crosses becomes more difficult. In addition, seeds are tiny and their growth to adult canes may take years which retards the progress in selection.

High level of polyploidy remarkably complicates traditional sugarcane breeding. Because each allele may occur in 5–14 copies in the genome, replacing poor alleles with desirable ones can often prove much more unreliable and take a lot more of time than in a diploid plant species such as rice. Namely, simple Mendelian heritability rules do not apply in general but ought to be replaced with much more complicated segregation statistics typical of polyploid plants.

If a recessive allele is being introduced in sugarcane using crossing, the trait it encodes does not express itself in plant phenotype until every single original allele has been replaced with the introduced one in plant's genome. The probability of finding such a fortunate genetic recombination among cross progeny may be practically zero.

For example, though wheat is substantially less complicated genetically than sugarcane, it is impossible to breed aromatic wheat using conventional methods.[40] Wheat is a hexaploid species so that the harmful cereal gene for scentless grains occurs in altogether six copies. It is statistically impossible to switch all these copies off simultaneously (or even sequentially) with traditional, non-targeted means such as mutagenic treatments using radiation and chemicals. Whereas, all the six copies can easily be silenced simultaneously using new genetic modification methods such as RNA interference or targeted mutagenesis.[41,42,43]

Accordingly, very high numbers of progeny are often screened through, in the hope for finding a lucky hit in the stochastic lottery of traditional plant breeding. In clonally regenerated crops such as sugarcane, apple, pear, grape, potato, strawberry etc. it is enough to find one superior genotype which is thereafter being multiplied by vegetative means into millions of genetically identical shoots for cultivation as a new variety.

In traditional sugarcane breeding programs, progress is slower than with most staple crops, as rationalized above. Typically, a cross is made and its progeny are scrutinized for valuable genetic recombinants combining the best traits of both parents. Selection work usually starts with 100,000 progeny seedlings and proceeds in 4–6 stages (Table 1). Finally a single one new sugarcane variety may be released for cultivation, typically in 12–15 years' time after the cross was made.[44,45]

In the first two stages seedlings are picked for further selection stages according to their visual scoring in vigor and disease resistance. During the later selection stages individual seedlings are being multiplied into clones to be used for measuring their cane yield in consequent harvests during 2–3 years (primary cane crop and 1–2 re-growth or 'ratoon' crops in the subsequent years). Since then productivity is also taken into account in selecting the rather limited number of progeny genotypes to be kept for the final field test stages. The final production tests are performed in several regions of cultivation, because results only based on one district cannot usually be generalized to the whole area of sugarcane cultivation.[46]

Table 1. Summary of the decision process leading to the release of sugarcane cultivar CP 00-1101 in Florida.[44]

Year	Month	Stage and selection decision	Genotypes in stage	Locations
1998	Jan.	Cross made at USDA-ARS Sugarcane Field Station	–	Canal Point, FL
1999	May	Germinated true seed transplanted into field (seedlings)	100,000	Canal Point, FL
2000	Jan.	Advanced from plant-cane seedlings to Stage 1	15,000	Canal Point, FL
2000	Nov.	Advanced from plant-cane Stage 1 to Stage 2	1,238	Canal Point, FL
2001	Nov.–Dec.	Advanced from plant-cane Stage 2 to Stage 3	135	4 farms in Florida
2003	Nov.–Dec.	Advanced from first-ratoon Stage 3 to Stage 4	14	11 farms in Florida
2007	Sept.	Cultivar release	1	

The multi-phased and arduous process of selection is the most important and expensive stage in traditional breeding programs also in sugarcane.[47] Whereas 1,000 times fewer plant individuals are started with when an established sugarcane variety is being improved with one desirable new trait applying genetic modification. Consequently, the modern plant breeder may proceed directly to the penultimate or last stage of field tests, saving much costs and time.

Conventional sugarcane improvement is a Sisyfos task

When a clonal plant variety with a highly heterozygous genetic constitution is being crossed further, its fortunate gene combination inevitably disintegrates due to sexual reproduction. Once lost, the unique genetic combination cannot be reassembled in the progeny generations in practice.

Thus, traditional sugarcane breeding is a Sisyfos task: previous achievements are lost to a major degree each time new improvements are being pursued.

No wonder that e.g. sucrose content in sugarcane has not increased in several decades, even though studies show that genetic variation for the trait occurs in its breeding populations.[48] On the contrary, sucrose content even slightly decreased during 1970–90 in Australia, though 50 new sugarcane varieties were released for cultivation in the period.[25] Main focus was on biomass production and disease resistance.

When major progress is tried for, new genes or alleles must be retrieved from other cane species. E.g. genes for higher biomass production exist in S. *robustum* or S. *spontaneum*. However, for winning back the bulk of the desirable traits achieved hitherto in cultivated sugarcane, each species cross should be complemented with consequent backcrosses (usually with *S. officinarum*). Accordingly, the time required for breeding would be multiplied in proportion.

Even if such completing crosses and progeny selection would be made during 10–20 generations, which is possible in grain crops with shorter generation intervals, hundreds of undesired arrival genes might still remain in the progeny plants. E.g. five hundred alien genes still remained in maize

progeny after 14 generations of backcrosses and selection following the original cross of maize with gamagrass (*Tripsacum*).[49]

In traditional plant breeding such compromises are a commonplace, however, and a new (though impure) variety is being released so long as it looks better than old ones.

Better focusing is available with genetic modification

A major advantage of genetic modification is its high degree of focusing. Not thousands of unknown genes but one desired gene without any hitchhiking ones is introduced from a wild plant species. The transferred gene is added to the genome of the recipient plant variety in its vegetative phase of life cycle, and consequently its superior genotype is retained and not disrupted by meiosis.

That is why the Sisyfos task can be avoided and the achievements of prior breeders conserved and developed further. Furthermore, there is no need for subsequent crosses for purging the variety of unwanted alien alleles.

Consequently, using genetic modification 1,000 times fewer plant individuals have to be scrutinized than in traditional breeding. Therefore, much time and costs can be saved, especially in tall species with long generation interval such as sugarcane.

Though, making one improved plant individual is usually not enough in genetic modification, either, but some degree of selection is carried out. In practice the desired gene has usually been transferred to 50–200 individual plant lines. After comparisons in the laboratory, a few best-functioning plant lines are then being selected for the final field trials.

Namely, the site of fixation of the gene in plant's genome may also have influence on how well the gene functions in the plant cell. In classic techniques of genetic modification the site of transgene fixation could usually not be determined in advance (but in any case it was always specified afterwards). Thanks to recent scientific breakthroughs, however, that limitation has just expired, so that even fewer individual GM lines may now be enough.[42,43] On the other hand there are thousands of locations in the chromosomes where the transferred gene is able of functioning well. It is therefore sensible to screen through a modest number of individual transformation events in order to optimize the modification results.[50]

Doubling of sugar content in one step of genetic modification

There are several obstacles in raising the sucrose content in sugarcane. One basic reason is that a great number of genes are involved in sugar content, each with a fairly modest effect.

Alleles for high sucrose content originate from *S. officinarum*. In polyploid hybrids it is a demanding task to enrich such "sugary" alleles in one genotype, because there may occur up to 14 copies of the gene in the cell. Furthermore, if the bulk of efforts are concentrated on improving one trait, other traits may often deteriorate as a trade-off.

Other obstacles to rising sugar content in the plant with traditional breeding methods are its homeostasis and sugar sink systems. Though, there is very little knowledge of the regulation of the sugar accumulation process in sugarcane.[18] Sugar is stored in stalk cells in the amounts that may prove beneficial for the plant individual in its further development. If that level is exceeded, the homeostasis systems of the plant may start using the sugar more for other than storage purposes.

Therefore, major improvements in sucrose content may call for finding such homeostasis genes and optimizing their functioning according to human needs.

Transcription factors

When gene expression was compared among sugarcane genotypes with high and low sucrose content, more than 20 transcription factors were found associated with sucrose content. Furthermore, one third of the genes previously found to be responsive to drought also showed such correlation with sucrose content.[51]

Transcription factors are sequence-specific DNA-binding proteins involved in regulating gene expression.[52] Since transcription factors naturally act as master regulators of cellular processes, they are expected to be excellent candidates for modifying complex traits in crop plants.[53]

In fact, certain key traits during the history of plant domestication have arisen due to shifts in expression patterns and transcription factor activity. Examples of such traits are the dramatically altered inflorescence architecture from the open panicle in teosinte (the ancestor of maize) to the compact cob in maize, and the reduced grain scattering typical of all cultivated cereals today.[54]

Simpler command with an extra system

In order to bypass such troubles with complex control systems, it may prove easier to breed sugarcane cells for producing in addition to sucrose some kind of sugar that the plant is not able of utilizing itself. Such novel production might not be governed by the innate regulation mechanisms of the plant.

Accordingly, the sugar content of sugarcane was doubled in one step of genetic modification by introducing a gene for sucrose isomerase enzyme in the plant.[55,56] The modified cane produces normal amounts of sucrose in its cells but on top of that also similar amounts of isomaltulose, which is an isomeric form of sucrose. Because sugarcane is not able of utilizing that type of sugar itself, isomaltulose is readily accumulated in its storage tissues. It was channeled by the breeder to find its way to the vacuoles, which are membrane-bound organelles that perform certain storage and removal functions in plant cells. Accordingly, in the vacuoles such novel bioproducts can be stored in confinement, without disturbing cell functions.

Isomaltulose is a slowly-degrading sugar produced in growing amounts for functional food using microbial cultivations. The present production via microbial fermentation is quite costly, however. Isomaltulose can also be used as an acariogenic sweetener, because mouth bacteria cannot usually break it down. Regarding biofuels, isomaltulose can be exploited for a raw material in alcoholic fermentation just as sucrose.

Potential for ecological harms?

May the trait, in this case isomaltulose production, cause unfavorable effects in natural ecosystems? According to a long-standing consensus view in the Life Science community, it is not the methods of breeding but the traits bred in the plant variety that determine the resultant benefits or disadvantages in regard to man or Nature.[57,58,59,60]

However, GM legislation in EC is not based on such ecologically sound foundations, but its heavy requirements are launched merely on the grounds of the breeding method instead.[61] Similar principle has

thereafter been adopted in the genetic legislation of many other countries as well. One exception is Canada, whose breeding legislation is based on the trait itself, and specifically on its novelty, regarding the crop in question.[62] Though, that biologically valid principle may be bypassed in practice, as stated by the relevant authority: "To date in Canada, all plants that have been modified through modern biotechnology techniques are considered to be plants with novel traits, or PNTs, *because* they have new traits".[63]

On ecological grounds, special attention would only be reasoned in case that the trait in question is adaptive, i.e. the trait gives selection advantage to the plant or its relatives in local natural ecosystems. Namely, contrary to common beliefs, modified genes in plants are neither transported more efficiently nor retained more permanently in the Nature than all other genes but obey the established principles of population and ecological genetics.[64] Though, such adaptive potentials are always evaluated with biological studies in the laboratory and in controlled field trials before any new GM varieties are released for commercial cultivation.[65,66,67]

Whereas in practice, the traits 'improved' by us to serve our special needs in crop plants are often detrimental to the plants themselves, at least in natural ecosystems. In case of being carried in the Nature, either as escaped crop individuals or by hybridization with wild relatives, such adaptively disadvantageous traits are readily lost from natural ecosystems due to natural selection.[64,68]

So, might the capacity of isomaltulose synthesis transform sugarcane to an invasive species? That is highly unlikely, because the plant itself cannot utilize the produced new sugar at all. On the contrary, the in-physiological increase in sugar content might turn the plant to a more energy-rich and tempting resource for its herbivores and pathogens in general, causing its more rapid elimination from natural ecosystems.

Field trials

According to GM regulations in general, the release of GM crops in commercial production is a stepwise process, irrespective of the trait in question. Accordingly, after successful laboratory studies, the safety of the GM lines regarding their open use is to be first tested in restricted and strictly controlled field trials. Permission for such trials is licensed, if they would not cause undue risks based on a relevant environmental safety assessment.[66] Any application for their commercial release or 'deregulation' may only proceed if the safety records obtained from such field trials prove satisfactory.

Though the statutory purpose of such official field trials is safety assessment, they are naturally being utilized by the breeders also in checking their primary laboratory records of the trait in more realistic, outdoor conditions. Even if such performance figures are still but indicative, at best, they can be utilized for the selection of the chosen few GM lines to be possibly forwarded to commercial release.

Altogether 120 different lines of isomaltulose sugarcane are being tested in field trials in Australia in 2005–10.[69] Diverse regulatory elements (promoters) obtained from sugarcane or maize are being tested for controlling the functioning of the sucrose isomerase enzyme in sugarcane. In the plant lines, the enzyme is being produced in different amounts and it has been channeled to different parts of the plant. Different combinations of regulatory elements are being compared with each other in their ability of accumulating isomaltulose in sugarcane without harming plant growth in customary growing conditions.

After field test stage clearance for commercial cultivation as sugarcane varieties may be applied for the most promising experimental lines. Varieties may be available for cultivation at the earliest in five years' time.[70] From the biological point of view the novel sugarcanes could be taken in use fairly rapidly after the field tests. Nonetheless, forecasts for the start of isomaltulose cane cultivation vary from three to seven years depending on how obstructive the permission bureaucracy may prove to be in practice.

In another field trial in 2009–15,[71] sucrose accumulation is expected to be modified with RNAi constructs containing "gene fragments from a common crop plant designed to alter sucrose transport, carbohydrate metabolism or osmotic stress tolerance" (details are declared confidential).

Cellulosic ethanol from self-degrading cane varieties

Sugarcane produces biomass up to 200 tn/ha (fresh weight), but on average less than 100 tn of cane is being harvested per ha annually. The bulk of the biomass is water, but about 10 % of it is cellulose which remains as bagasse after the pressing process. Similar amounts of cellulose also remain on the fields in harvesting residues, 80 % of which could be utilized as raw materials without compromising the sustainability of sugarcane cultivation.

Cellulose is a polysaccharide which could in principle become degraded into sugars to be fermented into alcohols. If the cellulose in sugarcane bagasse could also be utilized for ethanol, current ethanol yields per ha of sugarcane would be approximately doubled.

At present degrading cellulose into sugars is far too expensive to be economically viable.[72,73,74,75] Owing to the importance of the potential new resource, however, much research on cellulosic ethanol is going on. Apparently, the use of lignocellulosic ethanol as a viable alternative to petroleum-based transportation fuels largely depends on plant biotechnology breakthroughs.[75]

Though, providing for the case that the technology for converting cellulose into ethanol would become profitable in the near future, researchers in USDA-ARS have even released low-sugar (!) but high-fiber and cold-tolerant "energy sugarcanes" developed by crosses with Himalayan sugarcanes.[76]

To enable an economical process for bioenergy, deconstruction of the plant cell walls into fermentable sugars is considered to be the key step for biomass conversion to biofuels.[77] It is known that cell walls in the grass family (Poaceae) are in general very different to those found in other higher plants and suggest different processing requirements for conversion to biofuel.[4,74] However, the detailed structure of sugarcane cell wall is only inadequately known today, because studies on the sugar linkages and overall architecture of the wall have not been reported yet.[18] One further complication comes of the fact that a significant proportion of the hemicellulose fraction of lignocellulose is pentose sugars.[78] Sugarcane fibers are known to contain relatively high proportion of arabinoxylan, a pentose-sugar based polysaccharide, with cellulose.[79] Regarding fermentation of the released sugars into ethanol, pentose sugars have constituted an unused waste, because baker's yeast (*Saccharomyces cerevisiae*), unlike certain other yeasts,[80] has not been able of utilizing pentose sugars in the process. However, GM baker's yeast lines able of fermenting also pentose sugars into alcohol are available today.[78,81]

Plant cell walls need expensive pretreatments in hard process conditions in order to loosen the structure of the walls so that cellulose-degrading enzymes could have sufficient access to cellulose molecules in the walls later on.[82] One approach which may simplify or even allow omission of the

pretreatment step is to ‘digest from within’ through the production of cellulolytic and hemicellulolytic enzymes in GM plant feedstock.[83,84]

The successful degradation of lignocellulosic biomass requires more than ten enzymes.[83] Fairly large amounts of such enzymes are needed (15–25 kg cellulase per ton of biomass) and their purchasing from the markets would be very costly.[85,86,87]

Therefore, sugarcane is now being modified genetically to produce the necessary cell wall degrading enzymes itself, free of charge, in its cells. When produced from inside the cells the enzymes are also more efficient, having better access to the cell walls, and there is less need for expensive pretreatments as well. Thermal stability would be of importance also in plant-produced cellulases, regarding the harsh processing conditions. In fact, examples of a number of different cellulases with improved thermal stability and modified enzymatic activities for use in bioreactors are described in the literature.[88,89,90] If heat-activated cellulase enzymes are being used in the modification, they should have no detrimental effects to plants growing in typical ambient temperatures.[75]

Based on an inducible promoter cellulase production in GM sugarcane cells is being started with a special treatment not earlier than 2–3 days before harvest. That is why plant growth is not affected.[26] The utilization of such treatment-inducible promoters is not yet a commonplace in GM crops,[91] but a more in-depth understanding of individual signaling components and the mechanisms of their interactions will enable the general development of novel crops able of sensing and reacting to specific chemical or environmental signals.[92]

As a consequence of the complexity of plant cell walls, mixtures of several enzymes acting synergistically are generally required for their efficient breakdown in the Nature. Thus, it is likely that such combinations of various enzymes will also be needed, when cellulolytic enzymes for biofuel production are to be produced in plants.[77]

Recently, cocktails of several enzymes (e.g. endoglucanases, exoglucanase, and pectate lyases) useful in the degradation of plant cell wall materials into sugars were produced in GM tobacco chloroplasts. Typically, plastid GM results in high levels of expression, without measurable effects in plant growth rate or photosynthesis, and with minimal concerns of transgene silencing or position effect.[93] Chloroplast-derived crude-extract enzyme cocktails show so high enzyme activities that the extracts can be used directly without purification. Their production cost using plants is 1,000–3,000-fold lower than the costs of respective enzymes produced commercially using microbial fermentation. Such chloroplast-derived crude-extract enzyme cocktails yielded up to 36 times more glucose from cellulosic materials than commercial enzyme mixtures do.[94]

In the degradation of lignocellulose, even better results than with various enzyme mixtures can be obtained by combining several of the enzymes into a multifunctional chimaeric enzyme. So far, chimaeras with up to five functional components have been generated. If necessary for preventing detrimental consequences to the cell, the chimaeric combination of hydrolase enzymes can be targeted for being carried to an appropriate cellular compartment by linking a respective signal peptide to the protein. An alternative strategy, avoiding any phytotoxic effects and need for compartmentalizing the enzymes, relies on keeping the cellulose degradation gene silent until its expression is being induced by an artificial treatment near or even after harvesting.[26,83]

Such chimaeric genes encoding a complete set of lignocellulosic hydrolase activities may be successfully introduced in biofuel plants without apparent effects on the plant development, as shown by GM studies with a chimaeric hemicelluloses-degrading enzyme in tobacco.[83]

Self-degrading sugarcane for cellulosic ethanol production is being developed in a broad-based Australian–Brazilian research coalition. GM varieties already occur in field tests, and varieties may be released for cultivation in 2–5 to seven years' time depending on how slow the bureaucracy of its clearance for cultivation is evaluated to be.[87]

Genetic modification of lignin

Lignin is an integral and abundant part of the secondary cell walls of plants. It is a complex and heterogeneous mixture of polymers constituting up to one third of the dry mass of wood. Lignin is hydrophobic, resilient and hard to remove from fibers without harsh chemical treatments (chlorine- or oxygen-based bleaching). Lignin protects cell wall polysaccharides from microbial degradation, making them resistant to decay, and accordingly it constitutes an important limiting factor in the conversion of plant biomass to pulp or biofuels.[95] Even after its successful removal, the presence of residual lignin in cell walls can act as a steric hindrance to cellulolytic enzymes, thus preventing their effective binding to cellulose. Furthermore, cellulases are also bound non-productively by lignin, which limits the efficiency of bioethanol production from cellulose.[74,96,97]

Hence, the structure of lignin in sugarcane cell walls is being modified genetically in Brazil (Allelyx SA) to a better-degrading type consisting almost exclusively of syringyl instead of the more recalcitrant guaiacyl lignin.[98] It is interesting that shifts in guaiacyl and syringyl levels generally have only minor effects on plant development.[95] Cell-wall lignin resulting from such structural alterations developed with GM can be much more easily processed.[99,100]

Genetic modification of lignin for improving fermentable sugar yields from cell walls is also studied e.g. in alfalfa, where some GM lines with silenced lignin biosynthesis genes have yielded nearly twice as much fermentable sugar as wild-type plants. Though, part of the benefit was lost due to a reduction in overall biomass produced.[101]

Field trials

Field trials are going on in Australia in 2009–15 with sugarcanes genetically modified for improved cellulosic ethanol production from cane biomass.[71] Genes derived from two species of bacteria and a common plant are expected to modify the plant cell wall chemical structure or cause sub-cellular accumulation of cell wall degrading enzymes (details are declared confidential).

Halving N-fertilization with NUE cane?

Sugarcane needs quite a lot of nitrogen fertilizers, which impairs its production economy and carbon efficiency and pollutes environment. Grain crops can usually utilize less than half of the nitrogen administered to them in fertilizers (the reminder finds its way to air, groundwater and waterways).[102] In temperate regions, sugarcane may utilize 20–40 % of fertilizer nitrogen but in wet tropics only 6 %.[69]

Role of biologic nitrogen fixation

It is often told that sugarcane especially in Brazil may obtain a notable part of its nitrogen demand from nitrogen-fixing bacteria living in its root system. However, there is not much convincing evidence available, and most studies even lack systems of measurement reliable enough for the problem.[103]

Though, in reliable new studies small but positive (5–16 %) shares of biological nitrogen fixation have been recorded in sugarcane in Australia. However, securing favorable conditions in cane root system seems to be difficult in practice, and more research knowledge would be needed.[104]

Deficiencies may occur e.g. in the availability of efficient nitrogen-fixing bacteria for the plant species. Sugarcane roots cannot be inoculated with optimum nitrogen-fixing bacterial strains in advance, because plantations are founded from rootless pieces of sugarcane stalk.

In the long run breeders aim at developing grain crops capable of fixing their required nitrogen in their roots. That could be achieved most reliably in symbiosis with *Rhizobium* bacteria in plant root nodules. Several plant genes necessary for root nodule formation have been cloned, and early root nodule development can already be induced in legumes without the presence of rhizobia.[105] Though, many years may still be required for developing efficient nitrogen fixation in major crop grasses.

Reducing nitrogen fertilization with NUE crops

Crop plants with much higher Nitrogen Use Efficiency (NUE) are under development with genetic modification e.g. in maize, oilseed rape, wheat, rice, barley and sugarcane. In the applications advanced most far in the pipelines, either a gene from barley (*AlaAT*) or from maize (*ZmDof1*) has usually been utilized.[106,107] In wheat and barley, NUE lines based on a 'metabolic gene' from barley (details are confidential) are being field-tested in Australia in 2009–12.[108] Though, scores of other genes involved in nitrate uptake and metabolism have been found recently e.g. in corn. Their promoters often respond to nitrogen status, i.e. the functioning of the gene is either up- or down-regulated by nitrogen.[109]

The *AlaAT* gene from barley codes for the enzyme alanine aminotransferase. That enzyme is not directly involved in primary nitrogen uptake from the soil but it is functioning in a later stage in the nitrogen metabolic pathway. It deals with the metabolism of alanine, which can be a major storage amino acid in plants under certain stresses. Interestingly, even if *AlaAT* is such a "downstream" gene, enhancing its functioning in plant roots in a stress-induced and tissue-specific way finally results in more efficient uptake of nitrogen from the soil under low-nitrogen conditions.[106,107]

The particular NUE gene from barley is also being bred in sugarcane at least in India.[110] NUE sugarcane is under development also in Brazil, where a project has been started by Monsanto Company in collaboration with local breeding companies for improving the resource use efficiency of sugarcane.[111]

Nitrogen uptake and utilization into various nitrogen compounds in different plant parts is an example of a complicated biochemical pathway influenced by a multitude of different genes. The availability of the complete genome sequences of both thale cress (*Arabidopsis*) and rice has offered an unprecedented opportunity to identify regulatory genes and networks that control such important polygenic traits.[53]

The *ZmDof1* gene from maize codes for a transcription factor that up-regulates genes participating in the production of the carbon skeletons needed in amino acid syntheses. A study in *Arabidopsis* modified with a *Dof1* gene has shown a remarkable rise in amino acid concentrations, enhanced nitrogen assimilation, and increased growth under low-nitrogen conditions.[112,113]

According to a company (Arcadia Biosciences) developing the trait, field tests conducted hitherto in maize, oilseed rape and African rice have given indications that the first generation NUE crops are able of producing customary yield levels with significantly reduced nitrogen inputs in cultivation – the customary N-fertilization levels could even be decreased to one third without yield loss in canola. Though, no scientific reference for such remarkable figures is given.[114]

However, even much smaller reductions in the necessary amounts of nitrogen fertilizer inputs would improve both economical and carbon efficiency of field crops. Namely, industrial production of fixed nitrogen is a major use of energy, accounting for almost 2 % of all human energy uses, and consequently nitrogen fertilizers constitute one of the most expensive inputs in crop production.[85] Additionally, the more efficient intake of nitrogen from the soil would prevent environmental pollution caused by nitrogen wastage in the water systems or in the air.[110,115] Furthermore, with lower nitrogen levels in the soil, nitrogen-fixing bacteria thrive better. Thus, NUE sugarcanes would create more favorable conditions for taking advantage of microbial nitrogen fixation in cane production as well.

In the above crop plants, NUE varieties are just in Phase 1 of development, and first varieties are estimated to be released for cultivation in 8–10 years' time. Though, the necessary yield evaluations in a perennial crop such as sugarcane may require a few more years than in annual crop species.

Field trials

Field trials are going on with GM sugarcanes expressing enhanced nitrogen use in nitrogen-poor conditions in Australia in 2007–10[113] and 2009–15[71]. The trait is expected to result from expression of a maize transcription factor (*ZmDof1*). Its expression will be controlled with a variety of regulatory sequences, with the aim of optimizing expression patterns.

Improving drought tolerance

Provided climates warm up, water deficiencies are getting worse in large areas. Consequently, the necessity of irrigation also increases in cultivation. Though, in dry and hot regions traditional systems of irrigation result in soil salinization.[116] Such harms could be avoided by developing drought-tolerant plant varieties.

Drought-tolerant varieties would produce customary yields using less water. One important type of drought tolerance helps the plant to survive occasional periods of drought without permanent damages. Accordingly, its yield level does not collapse but the plant is rapidly recovering after the dry fortnight.

In a breeding program in Egypt, a single gene for drought tolerance was introduced in wheat. The gene was isolated from barley and transferred to wheat using genetic modification. Cultivation experiments showed that the number of irrigations necessary in wheat cultivation can be reduced from eight to one using these drought-tolerant wheat lines. Consequently, based on such drought-tolerant varieties wheat cultivation could be extended to areas of low rainfall lacking adequate systems of irrigation.[117]

Drought tolerance is a highly complex trait that often overlaps in part with salt tolerance (cf. below). For example, over expressing NAC transcription factors enhance both drought and salt tolerance in rice. *NAC* is a plant-specific gene family with probably 75 members in rice genome. One of them, *SNAC1*, is induced by drought predominantly in guard cells, which control water transpiration

through leaf stomata in plants. Its induction is followed by the up-regulation of a large number of stress-related genes. Under severe drought conditions in the field at the reproductive stage, GM lines over expressing *SNAC1* gene produced 22–34 % higher seed setting than the conventional controls while showing no phenotypic changes or yield penalty in normal conditions.[118]

In thale cress (*Arabidopsis*), transcription factor DREB2A is induced strongly by drought and high salinity, and study results indicate that it functions in stress responses to both insufficient water and high salinity.[119] Hence, DREB2-type genes are considered as one possible target of breeding, aiming at improving drought tolerance in crop plants. High constitutive expression of CBF/DREB proteins may produce undesirable phenotypes such as stunted growth, whereas more specific phenotypes for drought tolerance have been obtained by the use of drought-responsive promoters to induce CBF/DREB expression.[120]

Increased expression of a maize transcription factor, ZmNF-YB2, has been shown to confer drought tolerance and enhanced photosynthetic capacity under drought stress with improvements in grain yield observed across several growing seasons in maize. In relatively severe drought conditions, the best-performing GM maize line yielded 50 % more than the non-GM controls.[121]

In another study, conducted by Monsanto Company, rice and maize transformed to express bacterial cold shock proteins (CSP) showed tolerance for a number of abiotic stresses, including cold, heat and water deficits. CSP proteins are shown to have RNA binding properties, and they are supposed to rescue misfolded messenger-RNA molecules and help in coupling transcription with translation, allowing for a rapid post-transcriptional reaction to a stress situation. Interestingly, expression of CSP proteins in maize is not associated with undesired effects in other plant traits, indicating that stress tolerance does not come at a cost to crop productivity under well-watered conditions. In controlled water-deficit conditions, the selected GM maize line produced 12–21 % higher yields than elite non-GM hybrid maize controls.[122]

Breeding for enhanced drought tolerance has been started in many crop plants particularly using genetic modification. Field tests are going on e.g. in maize and rice as well as in wheat, cotton and oilseed rape in various countries. E.g. the GM wheat lines being field-tested for drought tolerance under rain-fed, drought-prone conditions in Australia in 2008–10 contain one of 15 different candidate genes derived from thale cress, maize, a moss, and Baker's yeast, "regulating gene expression or modulating biochemical and signal transduction pathways in the wheat plants (details are confidential)".[123] In 2007, 24 lines of GM wheat were tested, and seven of these provided higher yields under drought stress. Two best ones exceeded the yield of the control experimental variety by 20 %, with no apparent yield penalty under irrigated conditions.[124] Another field test deals with drought-tolerance in wheat and barley tried by transforming these crops with one of two drought-responsive transcription factors (*TaDREB2* and *TaDREB3*) derived from wheat.[125]

First drought-tolerant varieties are estimated to be released for cultivation in 4–5 years' time, though Monsanto has announced that it aims at releasing its first-generation drought-tolerant corn already in 2012.

Sugarcane

Water is the primary limiting factor in sugarcane production in many regions, India included.[28] Drought tolerance is under development in sugarcane in Brazil, Australia and Mauritius using genetic modification.[70,126] The bulk of the projected new sugarcane cultivations would be founded in

worn-out pasture areas. These are notably drier than traditional sugarcane cultivation regions. Consequently, improvements in drought tolerance would be welcomed.[127]

Trehalose is a sugar protecting cell structures from damages caused by dehydration in many organisms. A gene necessary for trehalose production was introduced in sugarcane from a mushroom species in China. The GM sugarcanes grew well and accumulated high concentrations of trehalose in their cells. Trials in laboratory and in the field showed that these trehalose sugarcanes tolerate periods of drought better, recover faster thereafter, grow better than conventional ones in dry conditions, and produce higher concentrations of sugar than customary sugarcanes.[128]

In the above application, the tolerance gene is functioning non-stop in all plant cells. Another Chinese research group has modified sugarcane with marker genes controlled by a promoter sequence which turns the gene on only in dry conditions. The promoter was found from thale cress.[129] Such inducible genes may protect the plant against periods of drought more economically in certain cases, because they do not retard plant development in favorable conditions.

Novel transcription factor (*SodERF3*) was recently found from sugarcane In Cuba. It is induced in cane leaves e.g. by ethylene and salt stress, and thereby participates in the regulation of many stress-responsive genes. GM tobacco plants expressing *SodERF3* displayed increased tolerance to drought and osmotic stress, without any visible phenotypic change in growth and development. Thus, the factor might be utilized in engineering drought and salt tolerance in crop plants.[130]

Water availability for the sugarcane plant could probably be enhanced by improving the structure of plant root system as well. E.g. in rice, following the identification of four major quantitative gene loci influencing root traits, marker assisted backcrossing was successfully used to transfer the alleles for greater root length and thickness from a rice variety from Philippines into an Indian upland rice variety.[131] The bulk of sugarcane roots populate the uppermost 60 cm layer of soil, whereas a few roots may grow even to the depth of 5 meters. Deep rooting could be pursued by breeding so that water reservoirs deeper in the soil would become available for the plant in dry conditions. If not truly necessary, however, the construction of such great root mass in deep root systems may involve higher construction, maintenance and transport costs and constitute a physiological burden for the plant, channeling uselessly resources away from stem growth and sugar yield.[132]

Regarding water use – and its wastage – stomatal pores in plant leaves are key actors in plants. Knowledge of their formation and control is accumulating, and a breakthrough was made recently, paving the way for the breeding of better drought-tolerant crops. Namely, guard cells close stomatal pores in the event of excess ozone or drought, and the activity of a gene (*SLAC1*) encoding a membrane protein was shown to be required for such stomatal closing in response to various stresses.[133]

The bulk of the higher plants apply the C_3 system of CO_2 assimilation which works well in temperate and moist environmental conditions. However, C_3 plants are devoid of a CO_2 storage system, and consequently they are inevitably losing much water by keeping their stomata open in sunlight for the acquisition of CO_2 for assimilation in real time. Therefore, plants with the C_4 system of assimilation, such as maize and sugarcane, are better adapted to sun-baked conditions. Namely, they can load their CO_2 reserves for assimilation in advance at night, when water transpiration rates are lower, and accordingly avoid water stress. Though, despite of the better water use efficiency of C_4 plants, C_4 photosynthesis is equally or even more sensitive to water stress, if it falls on, than its C_3 counterpart.[134]

A great international research consortium is developing rice to a C_4 plant within a decade. The estimated benefits of such amendment in photosynthesis are: 50 per cent higher yield level plus doubly better efficiency in water use.[135]

Field trials

Field trials with three different drought-tolerant GM sugarcanes are going on in Australia in 2007–10.[113] Their water use efficiency has been improved either by producing various extra sugars in sugarcane cells or utilizing a regulator gene controlling other genes' activities in the plant. Genes have been retrieved from thale cress (*AtMYB2*), *E. coli* (*EcTPSP*) or apple (*MdS6PDH*). In another field trial in 2009–15,[71] enhanced drought tolerance is expected as a result of the expression of genes "from a common plant and a common bacterium" (*WUE1*, *WUE2*) involved in plant hormone biosynthesis, or by expression of a transcription factor from rice (*OsDREB1A*).

Breeding for salt-tolerance

Provided climate conditions change as forecasted, shortage of fresh water will limit crop production severely in hot regions in the world. About one half of the readily accessible fresh-water reserves are already in use.[136] That fact has to be taken into account by developing more salt-tolerant crops, especially in areas where remarkable increases in crop production are being planned, whether for food, feed, fiber of biofuel.[137]

Fresh water constitutes only one per cent of all water in the Earth, and the same holds true for brackish water. Accordingly, 98 per cent of our water reserves are marine salt-water. One quarter of the global land area is salinized, and due to salinization the area of irrigated lands is reduced by 1–2 % annually.[138]

In coastal regions saline water could be utilized for irrigation – provided that our crops could be adapted to salinity. Though, the bulk of our staple crops cannot tolerate salinity (Table 2).[139] No more than one per cent of current land plants are able of growing and reproducing in saline soils, and only a few ones can tolerate the salt concentrations occurring in seawater.

Table 2. Soil salinity classes in terms of electrical conductivity (EC_e).[140]

Salinity class	**EC_e (dS/m)**	**Salinity effects on crops**
Non-saline	< 2	Salinity effects are negligible
Slightly saline	2–4	Yields of very sensitive crops may be restricted
Moderately saline	4–8	Yields of many crops restricted
Very saline	8–16	Only tolerant crops yield satisfactorily
Extremely saline	> 16	Only a few very tolerant crops yield satisfactorily

Quite the opposite was true in the far-off past. The first plant species grew in the sea, and consequently all of these were halophytes, i.e. adapted to high salt concentrations. Notably, in addition to salt, seawater contains richly of all the indispensable micro and macro nutrients that are often lacking in the fields.

Sensitive plants (such as papaya, mango and banana) are affected at about $EC_e = 2$, whereas tolerant ones (e.g. coconut, tamarind) are only affected at 8–10 or more.[141]

A chromosomal region connected with salt-tolerance during seedling stage has been localized in wild rice. The region has been transferred to several cultivated rice varieties using traditional spe-

cies crosses followed by backcrosses with cultivated rice – an old method burdened with genetic contaminations.[142] Though being fairly slight, such tolerance can help rice cultivation in the soils (such as in Pakistan) that are only temporarily salinized for short times during seedling stage, e.g. following sea flooding, but are thereafter rapidly desalinized thanks to monsoon rains.

In permanently salinized soils, additional genes for salt-tolerance would be needed. African rice varieties are being developed with GM for tolerating irrigation with saline water, and first varieties are expected to be available by 2016.[115]

Salt-tolerance is under development in cultivated plants by bringing in tolerance genes from naturally salt-tolerant plant species using genetic modification. Tolerance genes have been found e.g. from common seashore plants, such as Annual Sea-blite or Seepweed (*Sueada salsa*, Fig. 1), or mangrove trees growing in brackish water.[143,144]

In a mangrove plant (*Bruguiera gymnorhiza*), altogether 44 salt-tolerance gene candidates were originally identified using functional screening in *Agrobacterium*. When tested further, at least two of these gene candidates also provided GM *Arabidopsis* with enhanced salt tolerance, one of them up to 150 mM NaCl.[145]

Figure 1. Annual Sea-blite or Seepweed (*Sueada salsa*) is a halophyte even able of growing on the floor of salt-collection basins. Golden Sands, Bulgaria. ©J.Tammisola 2006

Seepweed plants not only survive but in fact thrive better in saline soils (100–300 mM NaCl). They have strong ability of accumulating Na^+ ions and sequestrating them mainly in their stems and leaves. Though, enzymes in plants have generally been found to be sensitive to Na^+ ions. Accordingly, in Seepweed cells, Na^+ is not accumulated in cell cytoplasm but compartmentalized into vacuoles, where it can be stored in confinement, without troubling cell functions.[146]

The transport of Na^+ ions into vacuoles through their bordering membrane in *S.salsa* is governed by a vacuolar Na^+/H^+ antiporter gene (*SsNHX1*). Over expression of Seepweed *SsNHX1* improved both salt and cold tolerance in GM thale cress plants, and the increased salt tolerance was correlated with Na^+ accumulation in their vacuoles under salt stress. [146]

Improved salt tolerance can also be achieved by limiting Na^+ accumulation in plant cells. That is shown by over expressing a plasma membrane gene (*SOS1*), coding for a Na^+/H^+ antiporter protein, which controls the transport of Na^+ ions in and out of the cell through plasma membrane in thale cress. Such GM plants accumulated less Na^+ in the xylem transpirational stream and in the shoot, and about half of the seedlings of certain GM lines still had green cotyledons when grown in 150 mM NaCl, whereas all control seedlings were severely bleached.[147]

In rice, over expression of a stress-responsive gene (*SNAC1*) encoding a NAC transcription factor provided the GM plants both with significantly improved drought tolerance (see above) and strong tolerance to salt stress. After treatment with 200 mM NaCl for 12 days, 80 % of transgenic seedlings survived, whereas almost all of the control seedlings died. None of the genes up-regulated in the GM rice showed homology to any reported ion transporter or antiporter genes, indicating that the salt tolerance due to *SNAC1* gene is caused by another type of mechanism.[118]

A sodium pump of a type not existing in higher plants was found in a moss (*Physcomitrella patens*). Its gene (*PpENA1*), coding for a Na^+-pumping ATPase protein, has been isolated and introduced in rice and barley. Based on a constitutive promoter, the gene was expressed in all GM plant tissues. In consequence, the concentrations of many metabolites were changed, as shown in a detailed analysis, though the GM plants did not show any abnormal growth phenotypes.[148] However, aiming at improving salt tolerance without unnecessary changes in other plant parameters, the gene pumping salt back out of the cell should preferably be expressed specifically in roots, where the leakage of sodium into most crop plants is primarily occurring. When expressed particularly in the roots, the gene under study has pretty big effects on plant salt tolerance (oral communication by Dr. Tester).

Field trials on salt tolerance among other abiotic stress tolerances have been made e.g. in GM wheat,[149] and new ones are going on for example in wheat and barley in Australia during 2010–15.[150] Altogether 1161 lines of GM wheat and 1179 lines of GM barley modified to contain one of 35 genes obtained from wheat, barley, maize, thale cress, moss or yeast are being tested. Some of the genes are expected to enhance tolerance to a range of abiotic stresses including drought, cold, salt and low phosphorus. The lines are grown under drought, rain fed or saline field conditions.

Sugarcane

Salinized and acidic soils are widespread in sugarcane growing areas of the world.[29] Irrigation waters with high salt concentrations are a commonplace in semidry areas of Brazil.[151] Those areas could be utilized fairly productively for sugarcane cultivation provided salt-tolerant varieties were available (Fig. 2).[152]

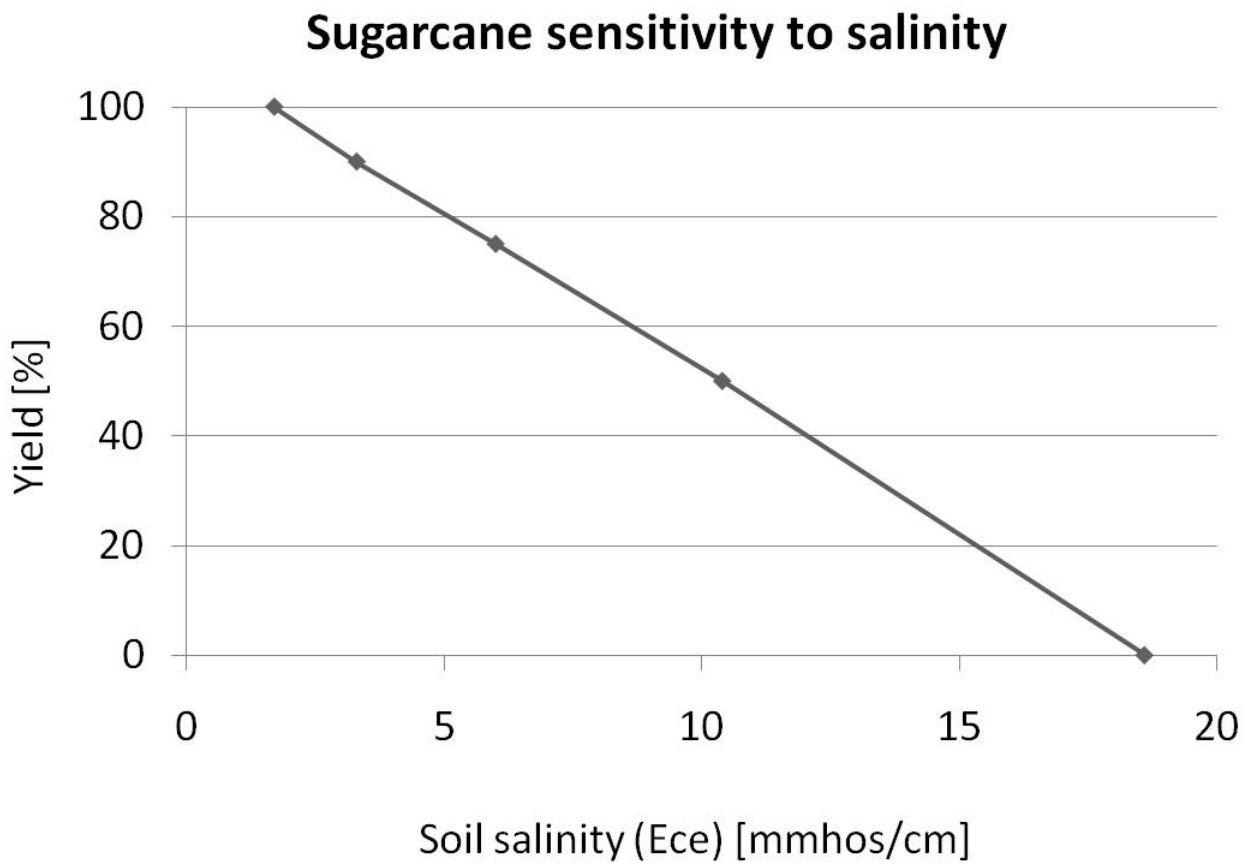

Figure 2. Sugarcane is moderately sensitive to soil salinity, and its yield is rapidly reduced with increasing salt concentrations in the soil.[34]

Certain variation in the sensitivity to salt, and some ability of avoiding the intake of Cl^- ions or transferring them to older leaves has been recorded in a few sugarcane varieties.[153,154,155,156,157,158,159] Even though, breeding sugarcanes for substantial salt tolerance would most probably call for genetic modification methods. A score of the currently best sugarcane varieties should be chosen for starting materials. In genetic modification these popular varieties could largely retain their assured characteristics and be only supplemented with the novel salt-tolerance trait, because their superior genotypes are not broken apart as is the rule in meiosis.

In addition to the ones mentioned above, more than a dozen of other genes influencing salt-tolerance have been found in studies in experimental plants.[144] Some of these candidate genes may prove feasible in developing salt-tolerance in sugarcane. Salt-tolerant sugarcane is reported to be under development in Mauritius, in cooperation with Queensland University in Australia.[160]

Breeding for cold tolerance

Poor cold tolerance seriously limits the possibilities of extending the production area e.g. of rice, wheat, oil palm, sugarcane, and other important crop species to cooler regions. It is estimated that 5–15 % of the world's agricultural production is lost to frost each year, and the number is estimated to be even higher in USA.[161]

Accordingly, breeding for cold tolerance is being intensified today, and in our changing climates the trait deserves extra attention. For example, cold-tolerant GM eucalypti are being field-tested in USA.[162]

Antarctic Hairgrass (*Deschampsia antarctica*) is the sole grass species colonizing the Antarctic Peninsula. It can tolerate frosts down to -30 °C in wintertime and periods of -15 °C during the growing season, thanks to its gene family coding for ice recrystallization inhibition proteins (IRIPs). Such proteins inhibit the growth of small ice crystals into potentially damaging large ones. The transcription levels of *D. antarctica IRIP* genes are greatly enhanced in leaf tissues following cold acclimation.[163]

The gene family was isolated from *D. antarctica* and characterized. When expressed in thale cress (*Arabidopsis thaliana*), the gene *DaIRIP4* rendered the recipient plants tolerant to freezing, even though thale cress is a dicotyledonous plant. Hence, the gene family constitutes a potential resource for improving freezing tolerance in sensitive crops in general, including cultivated grasses such as rice, wheat and sugarcane.

In sugarcane, 20 new cold-responsive genes were found by comparing its gene expression profiles in normal and low temperatures.[164] Further studies, using GM, are needed for finding out the functioning of such cold-responsive genes, and testing whether their adjustment could improve cold tolerance in sugarcane.

So far cold-tolerance is being retrieved to cultivated sugarcanes by crosses with cold-adapted wild relatives from Himalayan mountain regions. Though, inherently in the old method, harmful traits such as low sugar content always lift with to cultivated sugarcanes as well.[76]

Classic GM traits

Since the introduction of GM crops in 1996, considerable experience has accumulated on the use of a few "classic" GM traits such as herbicide tolerance (HT) or insect resistance (IR) in soybean, corn and cotton. Extensive records now prove beyond dispute that such traits have produced substantial net environmental and economic benefits to farmers compared with non-GM crops in conventional agriculture in USA.[165] Similar positive experiences also accumulate from small-scale GM farming, developing countries included, as regards environmental effects and household incomes.[166,167,168]

Classic IR and HT traits are now under development in many other crops as well, also in developing countries and even Africa. Both conventional breeding and classic or new GM methods may be applied in those programs today.[169,170,171] Clearly; such traits are worth developing also in biofuel crops, sugarcane included.

The breakdown of insect resistance in Bt crops due to evolution in insect populations, rendering them unaffected by such crop trait, has so far been low and of little economic or agronomic consequence.[165] Though, in order to greatly prolong the economical life span of such important resistance traits, double protection should preferably be bred in sugarcane from the start. I.e. at least two functionally independent IR genes should be stacked in its genotypes – and preferably in adjacent loci to facilitate keeping them ideally together during further cycles of breeding.[172,173] The latest version of Bt corn (Dow AgroSciences), to be released in 2010, has altogether six Bt genes.[174]

Through the acquisition of two Brazilian sugarcane breeding and technology companies, CanaVialis SA and Allelyx SA, Monsanto Company is applying classic Bt technology to develop resistance in sugarcane against certain economically important pests in Brazil, sugarcane borer included.[111,175]

Resistance to plant viruses (e.g. SrMV) through viral gene silencing is one of the early traits also tried in sugarcane. In this classic approach, DNA sequences for viral coat protein are introduced in plant genome.[176] Much new knowledge of the various systems of natural RNA silencing has been accumulated thereafter, and the 2006 Nobel prize in physiology or medicine was awarded for research performed on RNA interference.[41] In plants, RNA silencing is now regarded as a powerful though so far underutilized means of breeding virus resistant crops in general.[177]

Actually, in the course of further studies, RNA silencing may also prove universally applicable in the control of bacterial and fungal pathogens, parasitic nematodes and insect pests in plant production.[177] Namely, selected genes of root-knot and cyst nematodes can be silenced through feeding. Accordingly, a crop plant can be bred nematode resistant by modifying it genetically to produce short specific RNA sequences targeted at silencing certain vital genes of the pest.[178] Similar results have been obtained in herbivorous insects.[179,180]

Reliable and safe pest control would help in rendering biofuel production better economically and ecologically efficient. In addition to herbivorous insects, e.g. parasitic nematodes may also grow into a severe problem in bioenergy crops as well, as indicated by recent studies.[181]

RNA interference would likely offer more selective and safer control measures than most of the methods being used today. Though, any workable method for nematode control did not even exist hitherto, because all available nematicides are being banned due to their toxicity or ozone depleting properties.[178]

Herbicide-tolerant varieties could bring along significant savings in labor and fossil fuel use also in biofuel crops. The presence of weeds in the fields is one of the main causes of productivity loss in sugarcane production. Though, only tolerance to glyphosate has been utilized in great majority of HT crops hitherto. Due to such bias, the application of rotations or mixtures of different types of herbicides in the crop is unduly complicated, even if such diversity of measures would be advisable for minimizing and delaying the emergence of herbicide tolerance in weeds.[165,182] Not quite unexpected, glyphosate tolerance is in product pipeline in sugarcane as well,[175] though also tolerance to glufosinate has been studied.[183]

In sugarcane, the same plant clones are being re-grown and harvested during several years in tandem. Therefore, in order to enable pertinent diversity in herbicide use in sugarcane, tolerance to at least two different herbicides should preferably be bred in each of its HT varieties.

Field trials are going on with up to 6,000 GM sugarcane lines in 2009–15 in Australia. The genes conferring herbicide tolerance have been obtained from "a common bacterium and a plant species which have been consumed safely by humans and animals for centuries".[184]

How to combine GM with conventional breeding?

Molecular techniques such as marker-assisted selection and GM are becoming in ubiquitous use in 21st century crop improvement for enhancing its precision and efficiency.[185] Though, such techniques cannot replace conventional plant breeding in general but, for best results, various methods are to be used in tandem.

Regarding the biological virtues of various breeding methods, they could be used in combination based on two essential strategies:

1. Improving established top varieties, i.e. the GM trait is added to each pre-existing traditional top variety separately, one by one, which very likely yields an array of superior varieties in a short time, or

2. Basic GM breeding or improving the breeding population of the crop species, which may result in slower advance but greater number of derived GM varieties in the near future. That is, the GM trait is introduced somewhere in the breeding population, and an array of new varieties with GM trait(s) is thereafter produced e.g. using conventional crosses and selection.

The former strategy would be biologically ideal for clonally multiplied, highly heterozygous varieties in slow-bred crops such as sugarcane or trees. It does not play havoc with well-established popular varieties but keeps their virtues as untouched as possible, only adding a couple of necessary traits in a highly precise way.

However, due to the outdated GM legislation of today, strategy No. 1 is economically very hard to follow in a large scale, because of unnecessarily costly bureaucracy. Namely, any such enhanced top variety now formally represents a separate genetic modification event (i.e. independent insertion of a transgene into a crop genome), so that a separate permission is being required for each one hitherto in the legislation of EC, USA and other countries. The expense of gaining regulatory approval for commercial release of a novel GM event is estimated to be 7–15 million US dollars, counting only direct compliance costs.[186] Though, exactly identical modifications can be produced in each top variety today by using up-to-date gene targeting methods.[42,43] Accordingly, on the grounds of science, such genetically equivalent GM events ought to be approved collectively instead, so that the groundless multiplication of expenses could be avoided.

Experience in EC shows, however, that no essential improvements in its GM legislation can be expected within quite a few years. Thus, strategy No. 2 must often be chosen in practice until now, aiming at minimizing the number of transformation events and consequent deregulation applications. Namely, even if the GM permission obtained according to EC legislation only pertains to one specific transformation event, it at the same time also covers any number of varieties derived thereof using conventional breeding methods.

Hence, once introduced in a few top varieties based on strategy No. 1 the GM trait can be crossed further in the breeding population, which actually means transition to the latter strategy of breeding. Anyway, in a medium time scale, basic breeding is necessary for taking any important new trait in a general use in future varieties of a crop species, sugarcane included.

How to obviate narrowing of genetic diversity?

One further undesirable consequence of such outdated GM legislation is that genetic diversity will be unnecessarily narrowed in cultivation. When only a couple of top GM varieties can be released in the beginning, due to high bureaucracy costs, a genetic bottleneck is caused in the fields for years, when most farmers try to cash on the best few varieties available by that time.

Troubles with ecological tolerance and especially disease resistance are often worsened due to overly extensive cultivation of genetically narrow-based plant materials in the fields. Mixtures of different varieties might help, but they are as a rule too difficult to manage technically, and their mixed yield cannot usually meet the high standards of uniform quality claimed by the end users.

Such problems in ecological tolerance may likely be met also in sugarcane cultivation, when only a few superior varieties are available for biofuel production in the beginning. High-precision GM could now provide an interesting new solution to the problem – provided unnecessary legislative obstacles were reduced. Namely, desirable genetic diversity in important resistance traits could be generated *within* a top variety using GM, without compromising its uniformity in other traits such as product quality. Hence, though quite homogenous morphologically, the variety could consist of a mixture of plant lines differing from each other only as regards their important resistance characteristics.* Consequently, the field would be turned into a patchwork in immunologic sense, which could slow down the rate of evolution of new pathogen races as well as epidemic pest spread in cultivation.[187,188] That could confer more durable disease resistance, which is especially important in slow-bred species such as sugarcane.

Contrary to common beliefs, intellectual property rights (IPR) do not in principle prevent the use of valuable new traits in further breeding programs, at least in Europe. Namely, EC patent legislation[189] provides for compulsory licensing of important breeding traits. Consequently, the IPR owner cannot refuse licensing her patented gene to any other breeder interested in utilizing it for the development of derived plant varieties in her own breeding programs.

Could sugarcane research be applied to the development of other bioenergy crops?

Can the achievements in sugarcane be adapted to the breeding of other crops as well? The answer is: likely yes. Though, tolerance to drought or salinity may not prove useful in production regions lacking such problems even in the future, e.g. Northern Europe. Unlike in traditional breeding, the progress achieved using GM methods can often be transferred to many other crop species as such or suitably adapted to their specific conditions where necessary. Certain new traits enhancing the carbon- and eco-efficiency as well as fuel productivity of the future sugarcanes, self-degrading cellulose included, could probably be successfully introduced also in other bioenergy plants, especially grasses such as e.g. switchgrass, Miscanthus or reed canary grass.[20,21,22,23]

Prospects in the near future

Developments in rapidly advancing fields, such as modern biology, are hard to forecast. Unforeseen new discoveries may redirect the main course of the field of research anytime, as shown by the history of science.

Even so, it is possible to make a couple of general inferences regarding the near future. The above mentioned breeding efforts may probably result in an array of more efficient biofuel crop varieties to be released for cultivation within a decade. Even if significant enhancements may be achieved, these novel varieties still usually represent single trait improvements.

During the subsequent decade, however, the established new traits are being combined together, both using traditional crosses and *de novo* GM events. For example, varieties combining isomaltulose/trehalose or high-sucrose traits with drought and salt tolerance, successful lignin constitution or cellulose-degrading capacity may be commonly cultivated in various niches of sugarcane production area in the world.

* The idea was proposed by the author in his plant breeding lectures already in the 80's

Combinations of improved traits may in some instances show multiplicative effects and result in quantum leaps in biofuel crop efficiency. Such sustainable production would allow for retaining our food security even if the production conditions may widely deteriorate.

Breakthrough in precision and efficiency of GM in plants

The age-old hopes in plant breeding came true in April 2009, when the development of an efficient and precise method for targeted genetic modification of plant genes *in situ*, i.e. in their native location in plant chromosomes, was announced by two independent research groups.[42,43] Double-strand DNA breaks are generated in breeder-specified loci in plant genome, and the plant is stimulated and guided to make the desired genetic modification itself with the help of its own DNA repair enzymes. The need of using specific selection markers for finding out the few successfully transformed cells from among the masses of untransformed ones may be going out in the future, because modification rates are rising so high (up to 4 %) that the successfully modified plant individuals may be recognized amongst the progeny plants simply by screening their DNA for the presence of the desired gene form in their genotype.

Regarding highly polyploid crops, such as sugarcane, one further great advantage is gained with these brand new gene targeting methods. Namely, many or all undesirable alleles of the targeted gene in a crop variety can now be replaced with the desired allele simultaneously or by means of just a couple of successive modification cycles.

In the near future, more efficient gene forms for a trait, e.g. freezing tolerance, need not any further be added to plant chromosomes, but the plant's endogenous (inferior) gene form can be *replaced* precisely and efficiently with the desired one. In addition, the fine structure of any endogenous gene can be optimized *in situ*, or a harmful gene can readily be blocked from being expressed.

Meanwhile, the European Community is but lost with its outdated GM legislation which was built largely on lay beliefs in the 80's, omitting the viewpoints of European Nobelists and scientific community.[50,59] Not even the experts[†] are able of explaining what those statutes may try to denote with 'genetic modification' – and why.[190] The mere definition of the concept is a patchwork based on various lists of included, excluded or omitted items (all without relevant safety justification), covering a full page when pulled together in printing.[191] As confirmed by two decades of biological research, that mess seems to have no true relationship with biological risk evaluation.

EC Directorate General Environment has recently founded an expert working group for puzzling out whether the recent breakthroughs in precision, efficiency and command in plant improvement should still be punished with overly burdensome and costly GM regulation,[61] or is there any possible way of re-interpreting the letter of the Regulation in order to exclude the newest precision methods from its scope. Meanwhile, "dirty" old methods of breeding are fully exempted from regulatory and financial burden, whereas the regulatory oppression on modern life sciences continues despite declarations announced recurrently by the scientific community during decades.[57,58,59]

Clearly, the cutting edge of plant breeding in general and biofuel crop development in particular still stays in other continents for quite some time. One of the above research groups is making its gene targeting method available publicly and will be offering training sessions in the technique. Consequently, brand new GM plant varieties invaluable for our changing world in regard to bio-

[†] The author has got to know the development and functioning of the European GM legislation in his duties as a biotechnology advisor in the Ministry of Agriculture and Forestry of a EC Member State in 1997–2009

energy, food security and more balanced nutrition,[192] may start pouring from small plant laboratories in the Third World in 15–20 years' time.

Are these "GM" or "non-GM" plants? That may in many cases be mutating to a rhetoric question or sophism. Namely, regarding a plant line with its native genes adjusted in a targeted way by short DNA-base substitutions, insertions or deletions, there will be no scientific means whatsoever of proving that such improved plant line was not, or could not be, a creation of the Nature itself ("found from behind the barn"). Nor will there be any scientific reason for wrestling with such biologically empty distinctions.[193] Regarding the Nature, it is the outcome that matters, i.e. the genetic constitution of the resulting crop variety, and not the means utilized in (the early steps of) its creation.

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