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PLANT-BASED GENETIC TOOLS FOR BIOFUELS PRODUCTION

Editors:
Daniela Defavari do Nascimento

William A. Pickering

Bentham e Books

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Edited by

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PREFACE

The growing dependency on energy derived from depleting supplies of fossil fuels no longer has a future. The combustion of oil as an energy source is one of the biggest causes of air pollution, due to the releasing of CO_2 in the environment. Solar energy, on the other hand, when captured by plants through photosynthesis, promotes the assimilation of CO_2 and results in the opposite of the "greenhouse effect". Increased demand for fuels of vegetable origin, in addition to their environmental appeal, has made these fuels an appealing alternative and increased their production possibilities. This is where the need has arisen for the application of different biotechnological techniques, such as viable biomass production, development of cell wall degrading tools, and efficient fermentation technology.

The choice of biomass species is a key step in the production of biofuels with high oil and carbohydrate content. There is a great potential for the use of different kinds of crop residues for the production of bioenergy. After sugarcane juice is extracted, the remaining biomass (bagasse), rich in cellulose, can be used for second generation biofuel. A similar process can also be performed using corn stover, municipal solid wastes, and forestry residues. Evidence suggests that the current production of biofuels is at less than capacity. This is considered to be due to a confluence of factors, including high feedstock and processing costs, regulatory frameworks, risk avoidance, and limits to the amount of biofuel that can be blended with conventional fuels in major markets.

The use of cell suspension, somatic embryogenesis, gametic embryogenesis, and protoplast fusion, presented in chapters 1 and 2, give some insight into the ways that these techniques can be used to produce renewable fuels. Biomass feedstock production may benefit from the identification and characterization of key proteins involved, for example, in the biosynthesis of cell wall components and oil bodies. In this context, as seen in chapter 6, the exploration of subcellular proteomes also shows great promise in the characterization of new protein families and regulation mechanisms for improved biofuel crops. As discussed in chapter 4, new plant breeding techniques comprise a group of methods that offer the possibility of performing precise editing, replacement, or insertion of genes, targeting specific genomic regions without the use of any selectable marker. These features minimize the probability of undesirable random gene disruption, thereby providing interesting alternative tools for genetic transformation. The new tools are more predictable and less prone to position effects than are conventional methods.

All living organisms are constantly exposed to a variety of DNA-damaging agents. Chapter 3 shows that mutations in DNA are frequently observed in several diseases, a factor which is reflected in metabolic changes of great importance in biotechnology and biofuels production. It is seen in Chapter 5 that viruses constitute very powerful targets or tools for plant biotechnology applications. Plant viruses can be efficient vehicles for heterologous gene expression in plants used as biofactories or biofuels sources. Biotechnology is playing a major role in new advances in the fermentation of different substrates and in the production, not only of ethanol, but of biodiesel, butanol, and many other biofuels, as described in chapter 9. Genomic resources and bioinformatic tools are available for plant species with bioenergy and biofuels potential, and these are presented in chapter 7.

Plant biomass is the main feedstock for biofuels production. Efforts to maximize yield per unit of production area are of crucial importance in meeting the rising demand for renewable energy sources. In chapter 8, a broad overview is given of the factors influencing biomass yield, of advances in cultivation technologies, and of the relation of these conditions to the

physiology of energy crops. The chapter also presents innovative technologies that can support management decisions, focusing on sugarcane as a model for bioenergy crops. Microalgae biomass has also been described by several authors as an alternative with great potential for accomplishing the goal of the replacement of diesel by biodiesel, while at the same time not competing with fertile land useable for food production. However, the technology must still overcome a number of obstacles in order to be widely deployed. The advances and challenges of the technologies used, including procedures for obtaining biomass, are presented in chapter 10.

As is clear from the above discussion, this eBook is aimed at addressing sustainable biotechnological techniques that have been applied in the search for significant increases in biofuel productivity, without affecting food production. It is expected that the lessons from the sustainability criteria applied to first generation biofuels will be incorporated into advanced biofuels production, not necessarily focusing on the type of supply, but instead on existing local demands and domestic development strategies.

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CHAPTER 1

Biotechnology and its Impact on Vegetative Propagation of Plant Species

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Abstract: The application of biotechnology has had great impact on the agricultural sciences. Micropropagation, in particular, is one of the biotechnological methods whose major achievements have contributed to the development of agriculture in Northeast Argentina, and it is used in the mass production of aromatic, medicinal, fruit, ornamental, and forest plant species. It is normally applied to certified cultivars with good productive performance, providing significant development to the sector. Micropropagation also provides significant production and economic benefits, and an unprecedented environmental contribution.

Keywords: Biotechnology, Tissue culture, Vegetal micropropagation.

BIOTECHNOLOGY

After its first application in the cattle sector, the term biotechnology evolved in association with industrial fermentation. In 1961, a Swedish microbiologist defined it as the industrial production of goods and services through the use of organism systems or biological processes. The use of microorganisms thus became reflected in the concept. Yeasts were used to allow fermentation processes in the production of wine and beer, and antibiotics were obtained from fungi. Insulin and vaccines against hepatitis B are also produced by microorganisms, encompassing what is called industrial biotechnology [1, 2].

The development of recombinant DNA technology in the 1970s allowed plants and animals to behave as new gene product bioreactors. This opened a new horizon of great impact on agricultural and animal sciences that would complement the advance and release of genome projects for several species, where countless coding sequences of interest were discriminated and categorized

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by functionality. This generated possibilities for the construction of different transformation vectors [2].

Recently, biotechnology has been defined as the application of science and technology to living organisms, as well as to the parts, products, and models thereof, so as to alter living or non-living materials for the production of knowledge, goods, and services. This general framework allows us to include or add various techniques such as cell/tissue culture, biological pest control, biological supply production (pesticides, fertilizers, and fungicides of biological origin), genomics, and gene expression profiling, as well as techniques that allow direct and targeted modification of DNA, genetic engineering, and the introduction of new features in natural genomic sequences. A new field is thus opened with an unprecedented production potential, one that is especially relevant for the agricultural and forestry sector due to the characteristics and qualities of plants used in this type of study [2 - 4].

PLANT BIOTECHNOLOGY

Within what is called sustainable agriculture, the social, ecological, and economic aspects are crucial and prerogative. According to various researchers and economists, it is estimated that the world population will increase by about a third between 2009 and 2050 [5, 6]. This translates into an increase of 2300 million people, with growth occurring mainly in developing countries. Therefore, particularly in these countries, there will be a greater demand for food. To meet this demand there is a priority for road construction, increase of arable land, and improvement in performance and/or crop adaptation to marginal conditions. The first factor is insufficient for, and even detrimental to, the protection of natural environments [7]. However, the last factor is more desirable and points to South America as a great producer, as well as to its developmental potential for biotechnology and agricultural sciences.

The contributions of recombinant DNA technology, coupled with the progress of advanced genomics, formed what was previously called modern biotechnology [4], often supplemented by contributions from mass clonal propagation [2, 8]. In this context, companies such as Genentech [9], Monsanto [10], Syngenta [11], and Amgen [12], have been developing varieties of corn, tomatoes, and soybeans, among other species, and have improved various features such as herbicide tolerance, insect and virus resistance, and tolerance to abiotic stresses [2, 9, 12].

With the introduction of biotechnological techniques, new products, and new markets, a new economy has been generated, leading to greater production per unit area. This innovative concept of bioeconomy enables sustainable production

with reduced costs, while improving product quality and the development of less aggressive environmental practices [13].

PLANT BIOTECHNOLOGY IN ARGENTINA

Argentina can be divided into five major distinct regions, whose soil and climatic characteristics determine their production profile: NEA (Northeast Argentina), NOA (Northwest Argentina), Cuyo, the Pampean region, and Patagonia [2]. As the country is an efficient and diverse producer of high-quality food due to its deep and rich soils, mild climate, adequate rainfall, and good access to maritime transport, it has great potential for the application of modern technologies in the value chain and in processes [6, 14].

Since 1996, many producers in Argentina have been steadily growing genetically modified plants (GMOs) [15]. In 2003, the Argentinian position in the world market was second among the eighteen countries that extensively cultivate GMOs, due to its fourteen million cultivated hectares. In 2007, some GMO varieties tolerant to insects and herbicides, such as soybean and maize, were released on the Argentinian market, and in 2009 cotton varieties were introduced. In 2012, twenty-four million hectares were used for GMO cultivation, ranking Argentina as third in the world in GMO use [14, 16].

The incorporation of biotechnology tools into agricultural production in the 1990s led producers from the perception of potential profits to the reality of actual earned profits. Currently, there is empirical support for the economic benefits, such as higher yields, of various species treated with biotech tools. The following results have been obtained: increased income; reduced production costs (reduced tillage, cheaper herbicides, fewer pesticide applications); agronomic benefits, such as synergistic complementarities with direct seeding; health benefits (reduced application of herbicides and insecticides); and environmental benefits that allow the incorporation of technologies having less environmental impact and promoting carbon sequestration [16 - 18].

In the past ten years, Argentina has had the highest agricultural growth in its history. The new technologies have allowed for a threefold increase in productivity and acreage, and have led to a sevenfold increase in productivity. The highest impact factor for this leap in Argentinian productive agribusiness was change. It has been estimated that two thirds of the increase was due to the incorporation of new technologies [19, 20].

In productive agricultural regions outside the Pampas, there is a wide range of ecological conditions and a variety of crops. These conditions demand management policies that favor competitiveness, such as public policies for the

CHAPTER 2

Gametic Embryogenesis, Somatic Embryogenesis, Plant Cell Cultures, and Protoplast Fusion: Progress and Opportunities in Biofuel Production

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Abstract: Biofuel production represents an important alternative for replacing fossil fuels and reducing the emission of greenhouse gases into the atmosphere. With increasing demands for renewable fuel to replace fossil fuels, research on new energy sources is becoming more popular and new approaches in research techniques are occurring. In this context, the uses of somatic and gametic embryogenesis, cell suspension, and protoplast fusion in biofuels production will be presented in this chapter. Gametic embryogenesis is a convenient alternative in plant breeding because it makes possible the development of homozygous lines, increasing efficiency and speed in conventional breeding programs. Somatic embryogenesis is an important tool for plant cloning, looking toward the obtaining of improved plants by cell suspension culture or protoplast fusion. Suspension of plant cell cultures has several uses and applications for improving agronomical traits, and it is widely used in biotechnology for micropropagation, for the production of secondary metabolites or other substances, for obtaining somatic hybrids through protoplast fusion, and for modifying plants through genetic transformation. Protoplast fusion has been used by plant breeders to overcome the genetic barriers of outcrossing in incompatible plants, producing hybrid plants with different degrees of ploidy for improved agronomic and horticultural traits. In this chapter, current research with species that have potential to improve biofuel production is presented, with the aim of giving insights on the ways that these techniques can be used to produce renewable fuels.

Keywords: Bioenergy, Biotechnology, Cell suspension, Embryogenesis, Haploid technology, Somatic hybridization.

INTRODUCTION

Global climatic change and demographic pressure will continuously increase the demand for agronomic resources, and proving food and energy will therefore be

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one of the biggest challenges in plant production [1]. Along with this, the increasing consumption of fossil fuels and the reduction of resources are expected to increase oil prices. At the same time, the intensive and increasing use of fossil fuels has accelerated environmental degradation [2]. The most common plant species for producing biofuels in Europe and the United States is soybean; in Indonesia, rapeseed; in Malaysia and Thailand, palm oil; in Brazil, sugarcane is the most commonly used. All of them can compete with food production and bring about environmental problems [3].

In light of these problems [4], it is expected that microalgae will be the most promising alternative to replace agricultural crops producing sustainable biodiesel. However, according to Chen *et al*. [5], production costs are high and basic studies are necessary for elucidating microorganism characteristics and for developing microalgal biotechnology. In this context, C4 grasses from the Panicoideae clade must be included in the second generation production of bioethanol, for example: *Zea mays*, *Sorghum bicolor*, *Saccharum officinarum*, *Panicum virgatum*, and *Setaria viridis* [6, 7]. In addition, *Pennisetum purpureum*, *Arundo donax*, *Phalaris arundinacea*, *Mischantus* x *giganteus*, *M. sinensis*, *M. sacchariflorus*, *Eucaliptus globules*, *Jatropha curcas*, and *Pueraria Montana* species are listed as candidates for biofuel production, with research now in progress [8, 9]. Another sustainable pathway that has been exploited is the ability of *Mucor circillenoides* to convert single-cell oil into ethyl esters [10]. Another alternative is *Jatropha curcas* oil, which is less expensive for producing biodiesel [11, 12] and can be grown in poor environments [3]. *Populus deltoids* and *P. nigra* oils are also environmentally viable alternatives for reducing global warming, ozone depletion, and photochemical oxidation impact [13].

Biotechnology techniques present efficient alternatives for the large-scale production of clones for plant breeding and mutant selection. Using *in vitro* techniques, there are two alternatives for regenerating an entire plant from explants: organogenesis or somatic embryogenesis. Organogenesis can be obtained by inducing somatic explants to regenerate shoots and roots in appropriate culture conditions. On the other hand, somatic embryogenesis normally has a callus transition phase before embryo formation, and afterwards plant regeneration can be achieved on solid medium or by cell suspension in liquid medium. Cell suspension culture is an *in vitro* technique applied to isolated and multiple callus cells, where it is possible to produce large-scale plantlets or metabolic products of medicinal/industrial interest. For plant breeding, regarding the production of improved plants with different ploidy levels, it is possible to use the protoplast fusion technique. This procedure permits the production of hybrids for different purposes such as increased resistance, increased metabolism, seedless fruits, bigger fruits and flowers, *etc*. On other hand, if the breeding purpose is to

obtain pure lines, selected mutants, and/or to produce diploid hybrids, the haploid technology can be used through gametic embryogenesis (Fig. **1**).

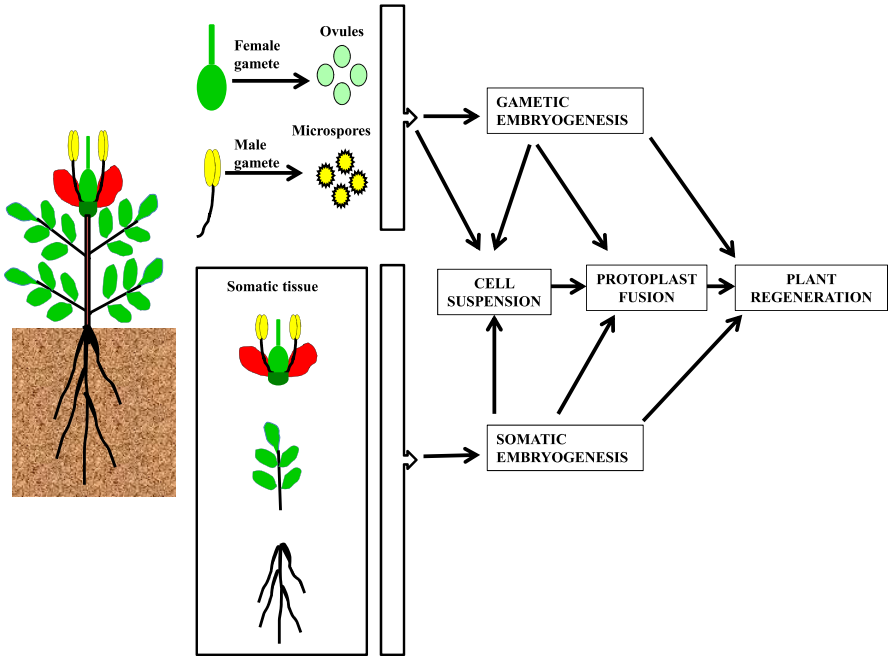

Fig. (1). Schematic representation showing a summary of the different ways of application of the techniques described in this chapter.

The purpose of this chapter is to expound the details of each technique and application as they relate to biofuel production. A hitherto unpublished procedure obtained with the oil producing plant *Olea europea* is presented in this article. Also presented is a protocol (see Appendix) for producing embryos from isolated gametes, with special attention given to the gametic embryogenesis (GE) technique.

GAMETIC EMBRYOGENESIS

Haploid technology, through gametic embryogenesis, is a promising and convenient alternative [14] that is being recognized as an important tool for plant breeding, making it possible to develop, in only one generation, completely homozygous plants from heterozygous parents [15]. The instantaneous production of homozygous plants through androgenesis (male gamete) or ginogenesis (female gamete) is highly appreciated as a practical perspective in research and plant breeding [16]. It makes it possible to shorten breeding cycles and fix agronomic traits [17] in pure lines, increasing the efficiency and speed of conventional

CHAPTER 3

DNA Repair: Its Molecular Basis for Use in Biotechnology

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Abstract: All living organisms are constantly exposed to DNA-damaging agents, leading to the accumulation of chemical and structural modifications which can affect key processes such as replication and transcription. Various DNA repair pathways have evolved for dealing with these modifications, thus preventing their toxic and mutational potential. Mutations in mtDNA are frequently observed in several diseases, which are reflected in metabolic changes or even in the attenuation of apoptotic response to anticancer therapies. To the integrity of the mitochondrial genome, repair mechanisms are recruited to the organelle. Among these, the BER pathway is the main pathway localized to mitochondria. The identification of mechanisms that prevent the accumulation of DNA damage in plants with agricultural interest can lead to improvements of these crops, including sugarcane, thus leading to improvements in sugarcane processing and consequently in the production of biofuels.

Keywords: Biochemistry, BER pathway, Bioenergy, Biofuel, Biotechnology, DNA, DNA damage, DNA repair, DRR pathway, LP-BER, Mitochondria, MtBER, Mutations, NER pathway, Oligonucleotides, Oxidative damage, Plants, Repair enzymes, SP-BER, Sugarcane.

INTRODUCTION

"We totally missed the possible role of ... [DNA] repair although ... I later came to realize that DNA is so precious that probably many distinct repair mechanisms would exist."

Francis Crick, writing in Nature, 26 April 1974 [1].

With this quote, we would like to introduce the reader to a very important aspect of genome stability in every single living organism: DNA repair mechanisms. The acknowledgment by the scientific community of the relevance of these "ancient

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molecular copyists" yielded the 2015 Nobel Prize in chemistry to Thomas Lindahl, Aziz Sancar, and Paul Modrich. The copyist analogy seems suitable in terms of the replication and maintenance of original genetic information, even when taking into consideration that stochastic genetic alteration is regarded as one of the core dogmas of biological evolution.

From the natural point of view, there is a dialectic conflict between replication error tolerance with consequent mutation-driven evolution, and replication nonsense error with consequent annihilation of information existence. DNA repair regulates the rates of random mutation, postponing widespread information decay, favoring control, and skewing the effects of randomness.

In 1949, Renato Dulbecco [2] and Albert Kelner [3] independently reported the first DNA repair mechanism in bacteriophage and in the gram-positive bacteria *Streptomyces*, a phenomenon now known as photoreactivation. However, neither researcher characterized the mechanism, nor was it their intention to do so.

Molecular mechanisms could be finally addressed after Beukers and Berends [4], Richard Setlow [5, 6], and Wulff and Fraenkel [7] described UV-induced thymine dimer formation in DNA. There was subsequently a flourishing in research on DNA damage and repair. Rupert led the research on the enzymatic properties of photoreactivation [8 - 11]. Hanawalt, Carrier, Howard-Flanders, and Boyce led the research on the "dark repair mechanism", which was later described as excision repair [12 - 16].

DNA repair as we know it today can be divided didactically into eight distinct molecular pathways: a) direct reversal repair (DDR); b) base excision repair (BER); c) nucleotide excision repair (NER); d) mismatch repair (MMR); e) homologous recombination repair (HRR); f) non-homologous end joining (NHEJ); g) translesion synthesis (TLS); and h) DNA damage response (DDR) [17]. These pathways deal with the vast chemically distinct DNA modifications. Considering only the nitrogenous bases of DNA, they can give rise to the following DNA modifications: 1) oxidation, alkylation, or deamination driven; 2) UV-photoproducts; 3) covalent adducts with nitroamines, polycyclic aromatic hydrocarbon epoxides, reactive aldehydes, ketoaldehydes, and lipid peroxides; and 4) DNA protein, inter- and intrastrand crosslink. Furthermore, DNA modification in 2′-deoxyribose (2′-dR) moiety brings forth: i) abasic sites; ii) single-strand breaks (SSB); and iii) double-strand breaks (DSB). Finally, and also important, DNA replication mechanisms themselves are not intrinsically flawless, as they can generate: a) base pair mismatches (non-canonical); b) DNA loops due to small insertions or deletions; and c) DSB due to premature replication fork collapse [18, 19].

The core logic of DNA repair pathways, with the exception of DRR, NHEJ, and TLS, consists of four steps: i) lesion recognition (and verification, depending on the pathway); ii) lesion excision; iii) repair synthesis; and iv) DNA ligation. DDR, NHEJ, and TLS may execute some of these steps, but not all of them. In the next section, the DRR, BER, and NER pathways will be briefly described. The DNA repair pathways were chosen according to the most relevant types of DNA damage that are inflicted in Plantae organisms, *i.e.,* UV-induced, alkylation, and oxidative DNA damage. We will discuss DNA repair pathways with regard to the eukaryote model, as well as introducing the historical importance of the prokaryote model *Escherichia coli* in gene discovery.

DIRECT REVERSAL REPAIR (DRR)

Direct reversal repair is the simplest DNA repair pathway. This is due to the unique feature of recognition and reversal of DNA damage by the same protein. There are three types of DDR proteins: i) DNA photolyases; ii) DNA alkyltransferases; and iii) oxidative demethylases of DNA.

DNA photolyase was the first enzyme attributed to a DNA repair phenomenon, and it was reported in the late 1940s [2, 3]. This molecular event was called photoreactivation at the time. Very briefly, photoreactivation is the reversal of the effects of UV on DNA by exposure of an organism to near-UV [20]; it was later discovered to be catalyzed by DNA photolyases.

DNA photolyases are closely related to cryptochromes, a family of flavoproteins largely distributed across eukaryotes responsible for blue-light-mediated plant development and mammalian circadian rhythm [21]. DNA photolyases possess a reduced flavin adenine dinucleotide ($FADH_2$), and a folate or deazaflavin [22]. In the dark (*i.e.,* light-independently), DNA photolyases bind with high affinity and specificity to pyrimidine dimers on dsDNA, a DNA lesion that arises from far-UV irradiation (200-300 nm). They absorb near-UV (300-500 nm) photons in order to split pyrimidine dimers back into two regular pyrimidines.

DNA photolyases are ubiquitous in almost every living organism [23]. The exception to this rule are the placental mammals, which, of course, include humans. The accepted proposition is that placental mammals lost DNA photolyase genes after switching from diurnal to nocturnal activity, a change which exercised low selective pressure on this class of genes.

The second class of DDR proteins are the DNA alkyltransferases. Some alkylation damage is repaired by direct removal [24]. These alkylation-induced DNA modifications – more specifically methylation – are directly reverted by the transferring of the extra methyl group in the DNA base to a cysteine residue in the

CHAPTER 4

New Breeding Techniques

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Abstract: A growing world population is demanding food and energy at the highest pace in the history of human kind. Plant biotechnology oriented to sustainable and competitive agriculture should lead a trail of innovation in order to address these emerging needs. The last decade has been characterized by the explosive accumulation of vast amounts of discoveries in the field of molecular biology. These achievements have paved the way for the advent of novel developments in plant biotechnology. Several new tools are being applied for genetic crop improvement every day, based on breakthrough versatile platforms that are increasing effectiveness and speed in the generation of new varieties of crops. The latest achievements are not only adaptations or improvements of modern techniques such as intra- and cis-genesis and accelerated breeding based on transgenics and null-segregants, meganucleases, and zinc finger nucleases; they are also the dawn of new disruptive technologies that are reshaping the paradigm of genetic improvement, as is the case with TALEN and CRISPR/Cas. Site-directed genome editing is becoming precise, cost-effective, versatile, and fast. These new breeding platforms are leading the way to next generation biotechnology. This chapter discusses the most recent updates and developments of new breeding techniques, paradigmatic achievements, future perspectives, and challenges in the context of plant biotechnology.

Keywords: Accelerated breeding, CRISPR/Cas, Genome editing, Intra-cis-genesis, Meganucleases, Oligonucleotide directed mutagenesis, Reverse breeding, Site-specific nucleases, Targeted mutagenesis, Transcription activator-like effectors nucleases, Zinc finger nucleases.

INTRODUCTION

The last decade has been marked by the constant emergence of breakthrough discoveries in molecular biology. We are experiencing a paradigm shift in experimental plant science, illustrated by the dynamic transformation of knowledge into innovative technological platforms and tools. New breeding

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techniques are emerging as incremental advances in traditional practices and as disruptive technologies that generate extraordinary progress at the fastest pace ever. This chapter will focus on the most innovative recent developments in a context of profound developmental shift. Several technologies devoted to crop improvement are presented, such as zinc finger nucleases (ZFN), transcription activator–like effectors nucleases (TALEN), and CRISPR/Cas 9, among others. The benefits and challenges of several technological processes and tools are discussed, highlighting some aspects related to the associated regulatory framework and focusing on the most advanced and promising technological developments.

Zinc Finger Nucleases

Shukla *et al*. [1] inaugurated the use of ZFNs (Fig. **1b**) for editing endogenous genes in plants. The process was mediated by the simultaneous expression of ZFNs and the delivery of heterologous donor molecules for targeting a herbicide tolerant gene in *Zea mays* plants. The procedure resulted in the insertional disruption of the IPK1 locus. The IPK1 interference was accompanied by both herbicide tolerance and alteration of the inositol phosphate profile in developing seeds.

Using ZFN, Schneider *et al*. [2] were able to insert a gene expression cassette into a target region flanked by two ZFN cutting sites. The 7 kb target site was replaced by a 4 kb unit with a selection and a visual marker. The homology directed repair event was implemented and evaluated in tobacco BY-2 cells. This proof of principle may result in the generation of transgenic crops, site-specifically modified at high frequency.

Arabidopsis plants expressing ZFNs were transformed *via* floral dip with a repair T-DNA with an incomplete protoporphyrinogen oxidase (PPO) gene. This repair T-DNA, in conjunction with the expressed ZFN, induced double-strand break of the PPO gene. The repair T-DNA harboring a PPO gene, missing the 5′ coding region and containing two mutations, resulted the generation of plants harboring an insensitive PPO gene and thus resistant to the herbicide butafenacil [3].

Tandemly arrayed genes (TAG) represent almost 17% of the Arabidopsis gene repertoire. Reverse genetics of these regions is usually complex and limited. Qi *et al*. [4] engineered ZFNs to target seven genes from three TAGs regions on two Arabidopsis chromosomes. One of these targets was the RPP4 gene cluster, which contains eight resistance genes. ZFN editing experiments resulted in gene cluster deletions ranging from 2 kb to 55 kb at high frequencies in somatic cells. Moreover, large chromosomal deletions of ~9 Mb and targeted chromosome rearrangements were also accomplished.

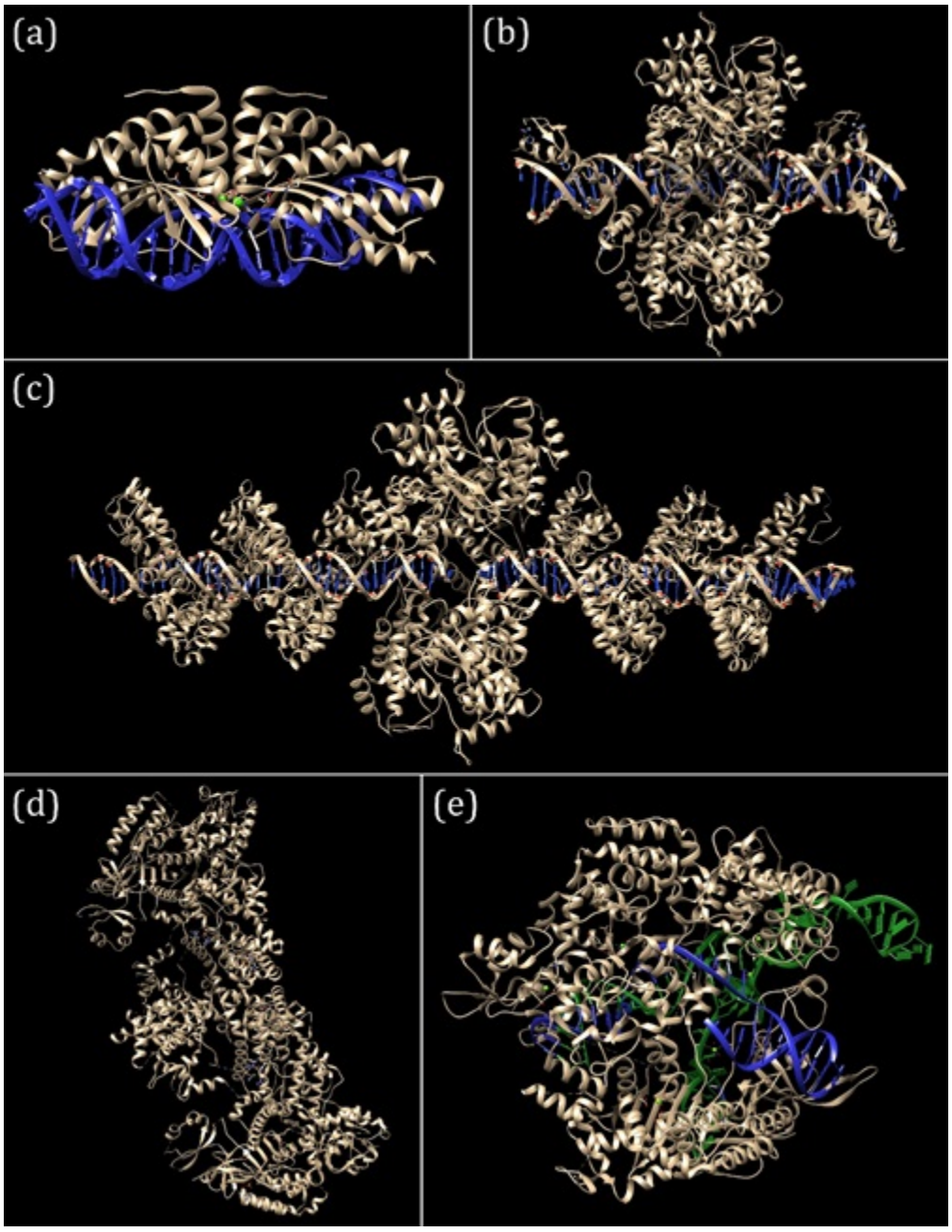

Fig. (1). 3D rendering of composite structures of diverse nucleases bound to their corresponding DNA targets: (a) meganuclease I-Cre; (b) zinc finger nuclease/FOK; (c) TALEN/FOK; (d) Cas9; (e) Cas9 + DNA/RNA. The figures were developed, compiled, and edited based on available crystal structures corresponding to PDB IDs of the protein data bank # 4AQU, 1AAY, 2FOK, and 4UN3.

CHAPTER 5

Use of Plant Virus and Post-Transcriptional Gene Silencing for Plant Biotechnological Applications

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Abstract: The current interest in green technologies has directed attention to the use of plant systems for several applications, including traditional crop plant systems used for biomass production, large-scale synthesis of a great number of recombinant proteins, and biofuels production. In this context, plant viruses are very useful instruments for plant biotechnology applications, constituting suitable tools for heterologous gene expression. Virions are particles with a complex composition, but their stability allows them to be used for the development of numerous biotechnological applications and as research tools for plant functional genomics studies. The development of infectious full-length viral clones is a strategy extensively employed as an alternative tool for introducing viruses into plants *via* inoculation with *Agrobacterium tumefaciens*. Another strategy, called RNA interference, a plant gene expression regulation mechanism based on post-transcriptional gene silencing, has extensively been employed to down-regulate the expression of endogenous transcripts and displays a number of biotechnological applications. Additionally, transgenic expression of viral proteins has been used to achieve pathogen-derived resistance, a mechanism that confers resistance to viral infections in agricultural crops. In this chapter we will discuss several strategies and methods for plant gene expression which employ plant viruses developed over the past decade.

Keywords: Agroinfiltration, Infectious clones, Plant virus, Post-transcriptional gene silencing, RNA interference, Viral expression vectors, Virus-induced gene silencing.

INTRODUCTION

Plant systems constitute advantageous systems in comparison with other conventional systems used to express recombinant proteins (mammals, bacterial or yeast cells). They offer lower production costs, and they also synthesize

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proteins that are structurally and functionally equivalent to those produced by mammalian cells. They thus present the advantage of being secure and with no risks of animal pathogen contamination, and also have the possibility of easily scaling up the purification technologies [1 - 5]. Furthermore, the use of specific promoters may allow the delivery of recombinant proteins (*e.g.,* vaccines) through different plant organs such as fruits, tubers, leaves, or seeds. In this way, the cold chain required for storage and transport of purified recombinant products can be avoided, as well as administration procedures by injection, in the case of orally administrated products like vaccines [1].

On the other hand, viruses constitute very powerful tools for plant biotechnology applications. Plant viruses can be efficient vehicles for heterologous gene expression in plants, which can be used as biofactories or biofuels sources. Virions are highly complex and stable nanostructures and constitute the basis for the development of multiple biotechnological applications. Plant viruses have also been used as research tools for plant functional genomics studies (by means of the virus-induced gene silencing strategy, which targets and down-regulates specific host transcripts). RNA interference is a plant protection approach based on post-transcriptional gene silencing. It has been developed to interfere with virus infections in plants and also to down-regulate the expression of endogenous transcripts. In addition, transgenic expression of viral proteins has been employed as a means to achieve pathogen-derived resistance to viral infection. In this sense, the development of infectious full-length viral clones is a strategy extensively employed as an alternative tool for introducing virus into plants *via* inoculation with *Agrobacterium tumefaciens*. In this chapter, we will discuss several plant-virus systems used for expression of proteins and other biotechnological applications involving transgenic expression of viral proteins and induction of post-transcriptional gene silencing.

Plant Viruses are Vehicles for Heterologous Gene Expression

Stable genetic transformation protocols are required for classical exogenous and foreign protein expression systems in plants. This strategy is based on the incorporation of a candidate gene into a plant genome and the subsequent expression of active proteins. Although many such proteins are produced by this strategy, this process is highly expensive and time consuming. Plant viral vectors constitute very useful and efficient alternatives for expressing foreign proteins [6]. A virus designed and engineered to express a candidate transcript is subsequently inserted in a host plant to initiate replication and consequently to produce significant amounts of the desired protein. The rapid viral multiplication rates in plant cells result in accumulation of exogenous transcripts into the complex viral expression system. Thus, the advantages achieved with the employment of viral

vectors are these: a relatively small and easy construct amenable for manipulation; the proteins can be produced in a very fast manner, achieving huge yields; the gene is never inserted into the plant genome [7]. However, the size and complexity of the inserted transcripts are factors implicated in the success of the stable expression. Additionally, there are some concerns related to the ability of modified plant viruses to spread in the environment. Overall, in spite of the aforementioned difficulties, there are numerous plant virus vectors developed and already employed in foreign protein expression plant systems. This technology is relatively more attractive to the public because of the absence of the negative connotations usually related to genetically modified plants [6].

Nowadays, two different strategies are possible for the application of plant-made biologics in the field: first generation vectors, that use complete or full versions of the viruses, and second generation vectors, composed of partial or deconstructed viral vectors. The elimination of some functions is employed to reduce or limit undesired features of the expression system. The subsequent rebuild of the virus is achieved by replacing the eliminated required functions in a genetically modified host that is able to supply the lost functions in the virus, or, alternatively, supply similar functions derived from other systems different from viruses [7]. Some advances have been made in both strategies, for example, full virus vectors are employed to produce long polypeptides directly fused to the viral capsid protein (polypeptides of 140 amino acids). Additionally, a novel viral vector generation has been developed to expose reactogenic amino acids on the viral surface with the aim of permitting easy chemical conjugation over the viral surface, with other separately produced proteins. This interesting tool is being used to synthesize new vaccines by exposing antigens attached to the surface of the virus [7].

It is worth mentioning that technical advances in the field of plant virus expression vectors have also been accompanied by important progress in the means of introducing viruses into host plants. Rather than merely infecting plants with the appropriate viral vectors, plant leaves can now be inoculated by agroinfiltration, that is, by incorporating the virus vector into *Agrobacterium tumefaciens* and infiltrating the leaf using a syringe or a vacuum treatment [8].

Different Plant Viruses used for Plant Biofactories Development

One of the major prerequisites for the construction of plant biofactories with good exogenous gene expression levels is the appropriate selection of viral vectors. There are several plant virus expression systems that have been developed to date, mainly based on positive sense RNA viruses.

For instance, *Tobacco Mosaic Virus* (TMV), an extensively studied and described plant virus, was the first one to be employed for vector development. While

CHAPTER 6

Proteomics for Bioenergy Production

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Abstract: This chapter reviews the importance and application of proteomics tools for studying bioenergy crops and microorganisms employed in sugar fermentation and/or degradation of cell wall compounds. The large volume of information from genome sequencing projects has allowed the development of many new platforms for profiling each step of the genetic information flow from DNA to protein to many molecular interactions involved in phenotype determination. In this context, proteomics is a comprehensive package of tools dedicated to identifying and characterizing protein expression in different biological systems. In this article, the application of proteomics in bioenergy production from feedstock is summarized, citing studies associated with the reduction of lignocellulose biomass recalcitrance and the discovery of more efficient enzymes for cell wall disruption and of potent microorganisms for sugar fermentation.

Keywords: Bioenergy, Biofuel, Biomass, Enzymes, Feedstock, Lignocellulose, Lignin, Maize, Mass spectrometry, Microorganisms, Oil, Proteomics, Proteome, Renewable, Saccharification, Subcellular, Sugarcane, Sorghum, Trees, 2D gel electrophoresis.

INTRODUCTION

Pressures arising from climate change and the future lack of fossil fuel are impacting the direction of the modern agriculture. Associated with an expanding population and decreased land areas for crop production, one of the major concerns in agriculture today is the need for sustainable energy sources. This panorama has challenged agriculture production and innovation, especially in the field of bioenergy supply, to find a way to a sustainable future.

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Bioenergy refers to the energy produced from biological sources, mainly plants and photosynthetic algae [1]. There are two types of bioenergy sources: the primary one employs crops; the other source is the lignocellulosic residue, discarded during food or wood production [2]. A large variety of biomass feedstocks can be used to generate bioenergy (including biofuels) and bioproducts (lignocellulose derivatives). The generation of biofuels can be achieved in different ways, such as the utilization of the sugar content in biomass *via* fermentation to produce ethanol (first generation ethanol/biodiesel). Biofuel generation can also directly originate from the whole lignocellulosic biomass (second generation ethanol), from lipids extracted from algae and oil crops, or from the use of syngas generated from the gasification of biomass [1]. Thus many crops are now seen as promising bioenergy feedstock candidates in addition to their nutritional property as food. Examples of conventional (sugar/starch) feedstock are sugarcane, sugar beet, sweet sorghum, and corn starch. On the other hand, crop residues left in the field, such as sugarcane straw, corn stover, and eucalyptus bark, can serve as biomass for second generation bioenergy production. In addition, species with high-yielding biomass and broad climatic/soil adaptation (switchgrass, miscanthus, energy cane) also serve as sources for cellulosic ethanol production.

In this context, bioenergy and biomass research have become the apple of the investor's and the researcher's eye. Currently, advancements in biotechnology, including the "omics" technologies (genomics, transcriptomics, proteomics, and metabolomics), can facilitate the identification of key genes and proteins associated with lignocellulose biosynthesis, as well as those involved in the processes of biomass degradation, biomass fermentation, and plant adaptation in adverse environments. The focus of this chapter is on pointing out the role of proteomics and its major contributions to the bioenergy sector.

THE VALUE OF PROTEOMICS FOR BIOENERGY PRODUCTION

Historically, plant genetics and breeding have led to significant improvements in agriculture production, through selection of desired phenotypes related to higher tolerance to abiotic and biotic stresses, better composition, and production efficiency. Advances at the molecular level have launched plant genetics into a new level of knowledge. Since the discovery of the DNA molecular structure and the elaboration of the central dogma of molecular biology, our understanding in genetics has evolved continuously, first with the development of marker-assisted selection allowing the association of molecular markers with agronomic traits of interest, and then with numerous DNA sequencing projects initiating the genomics era. Subsequent large-scale analyses of transcripts (transcriptomics), proteins (proteomics), and metabolites (metabolomics) associated with specific

environment effects have demonstrated the dynamic response of genes in determining different phenotypes. Advancements in "omics" technologies have thus contributed to the understanding of the complex dynamics and non-linearity of biological systems [3], in disagreement in some aspects with what was stated initially by the central dogma of molecular biology. The linear flux of genetic information from DNA > RNA > proteins, suffers from the influence of diverse factors and processes that alter the genetic information translated into a specific phenotype. To cite only a few of these interfering processes: RNA editing, alternative splicing, protein-protein interactions, post-translational modifications, and non-coding RNAs. It is clear that phenotype definition is not as simple as earlier thought.

Considering the modern thinking of molecular biology, the proteome, that is, the entire set of proteins expressed by a genome under certain conditions and at a specific time [4], represents the key player in biochemical processes, being closer to the phenotype than DNA markers [5]. For this reason, proteome investigation may provide great insights into the modulation of biochemical processes.

Protein abundance, protein-protein interactions, PTMs, subcellular localization, and protein turnover, are important protein properties that should be explored for understanding the dynamics of biological processes [6]. Since the year 2000, plant proteomics has made great progress, as evidenced by the increasing number of publications. Progress has been made in multiple bases of proteomics: i) new technology, with the development of highly sensitive and accurate mass spectrometers; ii) new algorithms for confident protein assignments; iii) targeted protein approaches; iv) quantitative proteomics approaches for relative and absolute quantification; v) subcellular proteomics; and vi) the development of enrichment techniques for isolation and mapping of PTMs. All this progress has been applied to the study of different issues associated with biodiversity, nutrition, crop improvement, safety, and energy sustainability [7, 8].

In the current bioenergetics world scenario, the study of energy crops and microorganisms has also gained the attention of the proteomics field. Among crops, sugarcane, sorghum, and maize have been featured as high-yield energy crops. The main research focus is on the conversion of the lignocellulose biomass and/or saccharide plant content into simple sugars or ethanol. The conversions are highly dependent on microorganisms or enzymatic reactions. Thus intense research is being conducted on the production of high biomass and sugar yield throughout crop breeding, and on the isolation of enzymes capable of efficient conversions. Plant proteomics has contributed a lot to the field, but it really is only beginning to do so with regard to bioenergy crops. Below we discuss the

CHAPTER 7

Genomics as a Tool for Bioenergy and Biofuel Crops

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Abstract: The quest for a renewable and inexpensive source of energy is one of the greatest challenges of the 21st century. Plants have already been used as a renewable source of energy, and continue to be one of the greatest hopes in this area. In order for plants to continue providing a cost-effective and renewable source of energy, it is imperative that their biomass growth and quality be constantly improved. Recent advances in the genomics field, led by the development of high-throughput sequencing and genotyping platforms, have opened up new strategies for accelerating plant breeding and aiding biotechnology development. Analyses of the vast amount of data generated by these modern genomics platforms are only possible with the constant development of computers and bioinformatics tools. In this chapter, we will present the genomic resources available for the most important plant species with bioenergy potential. Bioinformatics tools for gene expression analyses with RNA-Seq and for SNP genotyping are also presented.

Keywords: Bioinformatics, Gene expression, Plants, Phytozome, RNA-seq, Sugarcane, SNP genotyping.

INTRODUCTION

One of the greatest challenges of the 21st century revolves around finding a renewable and inexpensive source of energy [1]. Energy is essential for world economic development and for sustaining our modern and ever growing conveniences (transportation, air conditioning, appliances, *etc.*). A renewable energy source is urgently needed, given the disastrous effects of climate change we and other species have been facing. There is a substantial body of scientific evidence linking climate change to the relatively recent and extensive emissions of CO_2 from the use of non-renewable fossil fuels [2].

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It is estimated that by the year 2035, carbon dioxide emissions will be increased from 31 gigatons (Gt) to 37 Gt [3]. Distributed, cost-effective, and renewable sources of energy are thus resources that are much needed in order to face the challenges posed by climate change. Currently, renewable energies account for only 13% of total energy consumption worldwide, with bioenergy representing 10% of this value. Bioenergy derives from biomass (biological raw material), in the form of solid, liquid, or gas products [4].

Plants have already been used as a renewable source of energy, and they continue to be one of the greatest hopes in relation to this matter. These organisms have the capacity to absorb CO_2 from the atmosphere and to store sunlight energy through photosynthesis. The energy accumulated in their biomass can be released by burning or can be converted into much needed liquid biofuel [5–7].

In order for plants to continue providing a cost-effective, renewable source of energy, it is imperative that their biomass growth and quality be constantly improved. Traditional plant breeding has proven to be a successful method of doing so [8]. However, it is considered to be a slow process, especially with perennial species such as forest trees, for example, the fast-growing species of the *Eucalyptus* and *Pinus* genera.

With recent advances in the genomics field, led by the development of high-throughput sequencing and genotyping platforms, scientists have been focused on a strategy for accelerating the breeding process. Genomic selection is a method that uses thousands of molecular markers, well distributed throughout the genome, to predict the breeding value of genotypes from a breeding population [9]. By having a well-adjusted genomic selection model, breeders can predict the value of a genotype very early, as soon as the plant produces its first leaves for DNA extraction (in the nursery). With this technology, breeders can thus perform early selection and faster advance the breeding cycle. The power of genomic selection has its greatest potential, arguably, in perennial plant species [10].

Sequencing and functional analyses of plant genomes are also very powerful for discovering genes and/or regulatory elements for biotechnology. This is especially important for the challenge of converting biomass into biofuels, where plant cell wall degradation is still a major challenge that can potentially be met with biotechnology [2].

In this chapter, we will present: a) the genomic resources available for the most important species with bioenergy potential; b) Phytozome as a genomics database of bioenergy crops; and c) some bioinformatics tools for exploiting the genomic resources available for bioenergy crops. More specifically, we will show bio-

informatics pipelines for gene expression analyses with RNA-Seq and for SNP genotyping with the Genome Analyses Toolkit [11].

PLANT SPECIES WITH BIOENERGY AND BIOFUEL POTENTIAL

The main crops used globally for bioethanol production include corn (produced primarily in the United States), sugarcane (Brazil and South Africa), beets (European Union), and wheat (European Union and China). For biodiesel production, the main species are canola and sunflower (European Union), soy (United States and Brazil), and palm oil (Southeast Asia) [12, 13].

A comparative study on the productivity of corn and sugarcane as raw materials for bioenergy at different latitudes has shown that sugarcane produces on average three times more energy per hectare than corn [6]. The planted area in Brazil has increased from 1.4 to 7 million hectares between 1960 and 2007 [14]. Even with this high-efficiency and large sugarcane planted area, Brazil still lags behind the US, the largest producer of biofuel. Production in the US is largely based on corn. After Brazil, the European Union is the third largest producer, mostly based on biodiesel production from rapeseed and sunflower [4].

In addition to starch and sugar for the production of first generation biofuels, sugarcane bagasse, wood, corncobs, and other plant residues are also potential sources (lignocellulosic biomass) for energy production. Energy derived from this type of biomass, that is, from the cell wall instead of starch or sugar, is classified as a second generation technology [6]. This biomass originates from non-food lignocellulosic materials such as woody energy crops (eucalyptus, poplar, alfalfa, reed canary grass, elephant grass, switchgrass, among others), agricultural wastes (wheat husk, stems, cobs), forest residues (cuttings, wood fuel), and wood processing residues, as well as urban and industrial solid wastes (such as paper and cardboard) [15 - 18]. New policies stipulate that bioenergy must be generated from different biomass combinations, preferentially including non-food crops, to avoid the potential of increasing food prices. Next generation fuel production will enable middle and long-term solutions for sustaining economic development in a scenario of global climate changes [18].

GENOMIC RESOURCES AVAILABLE FOR PLANT SPECIES WITH BIOENERGY AND BIOFUEL POTENTIAL

There is a significant effort on the part of agriculture to develop more efficient crops to meet global demand. The search for more efficient crops to produce bioenergy and biofuels requires advances in agronomics and plant breeding. The daunting challenge is to select plants, with potential as energy crops, that possess increased productive efficiency. Among the characteristics needed to enhance

CHAPTER 8

Challenges in Biomass Production for Biofuels

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Abstract: Plant biomass is the feedstock for biofuel production. Efforts to maximize yield per unit of production area are of crucial importance in meeting the rising demand for renewable energy sources. Plantation, irrigation, use of fertilizers and pesticides, together with harvest, represent the major costs involved in biomass production. In this chapter we give a broad overview of (i) the factors influencing biomass yield (such as water and nutrients) and advances in cultivation technologies, discussing sustainability issues and the link of these practices with industry needs, and (ii) the relation of these conditions to the physiology of energy crops, presenting innovative technologies that can support management decisions. We will focus on sugarcane as a model for bioenergy crops.

Keywords: Agricultural productivity, Bioenergy crops, Biomass accumulation, Energy cane, First and second generation ethanol, Harvest, Innovative technologies, Management, Metabolomics, Photosynthetic rates, Plant breeding, Plant physiology, Soil mechanization, Straw maintenance, Sucrose, Sugarcane bagasse, Sustainability, Tillage, Water and nutrient uptake, Yield.

INTRODUCTION

Agricultural productivity has a strong correlation with local edaphoclimatic conditions and management. Soil quality, *i.e.,* nutrient and water availability, are key indicators for inferring environmental sustainability. They somehow influence

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accumulation. Agricultural management tools can be used to improve soil fertility, and are also capable of increasing the yield harvested. Complementarily, the understanding of how nutrient and water uptake occur until their incorporation into the plant biomass can assist both in management decisions in the field and in biotechnologies development. The discoveries produced should then go beyond the laboratory scale and be applied not only at the industry level, but also in production fields.

Brazilian ethanol production is favored by several conditions, such as vast areas for planting, geographic position, sunlight incidence, and plenteous rainfall. In addition, the economic and environmental concerns that arose in the last century promoted an increase in the use of Brazilian ethanol in the energy matrix worldwide, resulting in environmental, economic, and social gains. The Brazilian National Alcohol Program (Proálcool) contributed to the increase of the sugarcane harvest area. The area increased from 1.7 Mha in the 1970s and to 9.8 Mha in 2014 [1], and the number of ethanol mills has also increased during more than forty years of technology development.

One of the most productive plants worldwide, sugarcane is also considered the most efficient material for first generation ethanol production [2]. In addition, two-thirds of sugarcane's energy potential lies in bagasse and straw [3], materials that can be used to produce so-called second generation ethanol (E2G). However, as is widely known, the costs of sugarcane E2G production are not yet competitive with first generation ethanol. Along with more established strategies such as increasing the efficiency of enzymes, studies from the biomass perspective related to plant fiber content can offer an extra advantage to E2G feasibility. For all of the reasons mentioned above, therefore, this discussion will be focused on sugarcane production in Brazil.

Due to more recent concern about energy sources, and the resulting increased demand for ethanol, sugarcane production is expanding worldwide [2]. However, inherent to the monoculture model, the occupation of large areas associated with certain agricultural practices raises concerns of possible environmental impacts. Different issues should be integrated in order to find a reasonable solution that maximizes field production with minimal negative impacts. Through the tools used in molecular biology, plant genetics, and physiology, it is possible to reach into new technologies and approaches in order to support biomass field production. One of these innovative technologies is metabolomics, an approach that may be able to unravel the link between plant metabolism and biomass accumulation.

In this chapter, the following topics will be addressed: management and other environmental aspects related to plant yield, such as water and nutrients; management decisions used to eliminate possible bottlenecks; how these measures adopted in the field can affect plant physiology and biomass accumulation, and the other way round; how knowledge and innovative technologies in plant physiology can influence field production.

FACTORS INFLUENCING BIOMASS YIELD AND ADVANCES IN CULTIVATION TECHNOLOGIES

Brazilian sugarcane yields increased dramatically from 46 ton.ha^{-1} in 1970 to 83 ton.ha^{-1} in 2010 [4]. The increment can be attributed to large-scale genetic breeding programs supported by the government and the private sector, particularly in the 1970s and 1980s, but also by better agricultural techniques which in some cases are intrinsically related to mechanization.

In São Paulo State, the largest producer with around 370 million tons annually, a semi-mechanized system is used. Mechanical harvesting is used in 83% of the production area, but mechanized planting is used in only around 45% of the area [5]. Despite the advantages of mechanical harvesting, it is claimed that due to harvest losses, soil compaction, and ratoon damage, mechanical harvesting reduces plant productive life and sugarcane yield as compared with the manual harvesting of burned cane [6].

Heavy soil mechanization due to tillage (plowing, disking, and subsoiling) and excessive traffic associated with other agricultural practices such as mulching, irrigation, and fertilization, particularly those in which byproducts or residues are used, have great potential to affect local water resources.

For centuries, sugarcane was harvested manually and the fields were burned to remove sugarcane straw (the tops and dry leaves), driving away snakes and other potentially poisonous animals and facilitating harvesting. In the last decade, the harvest of sugarcane by human cane cutters has been gradually reduced, and was changed to harvesting with the use of machines that maintained the straw on the ground [7]. The green management of sugarcane requires the maintenance of vegetal residues on the ground, resulting in 10-20 tons of dry matter/hectare. At present, research experiments are addressing the advantages of maintaining plant residues on the soil [8]. An example of these studies is illustrated in Fig. (**1**).

The maintenance of sugarcane residues on the ground can enhance nutrient cycling [9], provide higher water-holding capacity [10], higher aggregate stability [11], increased soil organic carbon stocks [12], and can reduce greenhouse gas emissions [13] (Fig. **2**).

CHAPTER 9

Industrial Use of Yeast (*Saccharomyces cerevisiae*): Biotechnology in Ethanol Biofuel Production Control in Brazil

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Abstract: The production of biofuels by fermentation is as old as mankind itself. The use of yeast (*Saccharomyces cerevisiae*) in the production of wine and beer, as well as bread, has been known from the time of the Rosetta Stone and the Bible. Nowadays, biotechnology is playing a major role in new advances in the fermentation of different substrates and in the production of ethanol, as well as in the production of biodiesel and many other biofuels. Advances in biotechnology have shed new light on ancient and well established technologies, bringing biofuels and mankind to a new, clean, sustainable, and bright future. A single and simple biotechnology method (electrophoretic karyotyping of intact chromosomes) has brought the entire ethanol industry in Brazil to new horizons.

Keywords: Bacteria contamination, Ethanol, Foam, Fingerprinting, Fructose, Glucose, Glycerol, Sucrose, Sugarcane, Yeast, Yield, Yeast monitoring.

INTRODUCTION

The production of biofuels by fermentation is as old as mankind itself. The use of yeast (*Saccharomyces cerevisiae*) in the production of wine and beer, as well as bread, has been known since the time of the Rosetta stone and the Bible. At present, biotechnology plays a major role in new advances in the fermentation of different substrates and in the production of ethanol, as well as in the production of biodiesel and many other biofuels. Advances in biotechnology have shed new light on ancient and well-established technologies, bringing biofuels and mankind to a new, clean, sustainable, and bright future.

"What cannot be measured cannot be managed." This maxim is particularly important when talking about biofuel production on a large scale, as in the case of the ethanol production sector in Brazil. Small increases in fermentation yields

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represent large amounts of additional ethanol produced.

In the last thirty years, many techniques and protocols have been developed for understanding how to manage and improve yeast fermentation yields. Most of these techniques focus on the fermentation environment, but few are directly related to the yeast population itself.

The use of antibiotics to control bacterial infection is well known, but the infection of the fermentation by other "wild" yeasts, the ecology of the fermenting tank, and the relationship between different strains of yeast and between different strains of yeast and bacteria, are factors that are only now being brought to light by science.

THE ROLE OF YEAST (*SACCHAROMYCES CEREVISIAE*) IN ETHANOL FERMENTATION

The main physiological characteristic of yeast is its ability to degrade six-carbon carbohydrate (C6) molecules, such as glucose and fructose, to ethanol two-carbon (C2) components, without completely oxidizing them to CO_2, even in the presence of oxygen.

Yeast started to become a major ethanol producer about 80 million years ago, at the end of the Cretaceous period. A large amount of fruits were available, offering many fermentable substrates. Yeast developed the ability to accumulate and tolerate ethanol, inhibiting competing organisms from growing.

As a unicellular organism that clones itself, yeast has been "alive" at the surface of the earth ever since then, which indicates that it is very well adapted to the role of ethanol fermentation [1].

SUGARCANE ETHANOL PRODUCTION IN BRAZIL

In Brazil, the ethanol production industry is based on sugarcane and its large amounts of sugars (sucrose, glucose, and fructose) directly fermentable by yeast. The production of ethanol is conducted in sugar mills that produce only ethanol, or both ethanol and sugar. Each mill has a very different type of wort, depending on the availability of feedstocks such as sugarcane juice, sugarcane syrup, raw sugar, and molasses. The different amounts of each feedstock in wort production and the contamination of each component have direct implications for the population of bacteria and the different strains of yeast. The industry in Brazil does not sterilize the wort, and for this reason many different strains ("wild" yeast) are brought into the fermentation process. Any strain of yeast that is not

selected or not known to the ethanol industry is commonly referred to as "wild" yeast.

"Wild" yeast is, 90% of the time, a bad strain for industrial ethanol fermentation due to foam production, low ethanol yields, residual sugar, etc. However, 10% of the time "wild" yeast can be a good new strain and thus receives attention from the industry and from academic experts.

THE FERMENTATION PROCESS IN THE SUGARCANE ETHANOL INDUSTRY IN BRAZIL

The main fermentation process used in Brazil is batch feed fermentation, with recirculation of the yeast. It consists of a very simple and efficient process.

The wort is produced using a mix of water, sugarcane juice, sugarcane syrup, raw sugar, and molasses (any of them in various proportions), reaching approximately 20% fermentable sugars.

The wort is then added to a fermenting vessel already containing treated yeast as 20% of its volume. In Brazil, there exist vessels of approximately one million liters.

The addition of the wort is done slowly in order to prevent the formation of foam and to allow the large yeast population to rapidly consume the sugar available in the wort, thus producing large amounts of ethanol. When the filling of the vessel is complete, two or three more hours are requited for the fermentation process to finish. The entire fermentation process totals about eight to twelve hours.

The so-called "wine" (fermented wort) is sent to centrifuges that will split the yeast cream from the wine. The yeast cream is sent to the treatment vessel, and the wine is sent to the distilling sector of the industry.

In the treatment vessel, the yeast receives water and sulfuric acid until reaching pH 2.5. The yeast stays for about two hours in this acid treatment, and is then sent back for a new fermentation.

This recirculation of yeast in the fermentation process is very important, because yeast consumes about 2 kg of sugars for each 1 kg of biomass produced. The loss of yeast biomass in the process reduces the ethanol yield.

"WILD" YEAST CONTAMINATION

The entire sugarcane ethanol process is conducted in a non-sterile environment and is directly affected by bacteria and "wild" yeast contamination. Bacteria are

CHAPTER 10

Biodiesel from Microalgae: Third Generation Biofuel

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Abstract: At present, fuels derived from biomass play an important role in scenarios for the expansion of renewable energy worldwide. Considering the liquid biofuels, biodiesel is an interesting alternative that minimizes mineral diesel consumption. Among the raw materials available for biodiesel production, microalgae biomass has been described as an alternative with great potential for accomplishing the goal of replacing diesel by biodiesel without competing with fertile land for food production. This paper presents advances and challenges in the technologies presently used for the production of biodiesel from microalgae, including the procedures used to obtain biomass and the evolving technologies for reducing production steps (and consequently time and process costs). It was found that microalgae is currently still not a viable option for large-scale biodiesel production, as it has a negative energy balance. On the other hand, microalgae indicates substantial earning potential in biomass and lipid fractions, being a good alternative because of its high fat content. Therefore, research directed toward the production of biofuels from microalgae should receive greater attention and investment. Microalgae can become a competitive alternative and a commercial reality in the biofuels sector with the development of genetic improvement and technological production systems and with hoped-for reductions in costs, and also because of its sustainable qualities in contrast to other raw materials.

Keywords: Biofuel, Biodiesel, Biomass, Microalgae.

INTRODUCTION

The growing demand for energy on the world stage, predictions of high trends in oil prices, concerns about the environmental impact of the use of fossil fuels, energy security, and governmental and social incentives, all justify the prospects for expansion of renewable energy worldwide. Fuels derived from biomass play an important role in this scenario, and, among the available biofuels, biodiesel stands out among the most promising. It consists of alkyl esters of long chain carboxylic acids produced from the transesterification and/or esterification of raw

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greases and fats of vegetable and animal origin. This biofuel is an attractive alternative to petroleum-derived liquid fuel and can be used without major changes in current engine design, generating sustainability in the sector by reducing greenhouse gas emissions. It can also be produced from different sources, such as vegetable oils (soybean, palm, sunflower, cotton, peanut, *etc*.), animal fat, and reused oil (fry oil).

Recent studies have also shown the potential of unusual oil sources, especially the production of oil from microalgae. Microalgae are a diverse group of prokaryotic and eukaryotic microorganisms with chlorophylls and/or other photosynthetic pigments, which have the ability to perform photosynthesis. Due to their simple structures and their efficient photosynthetic systems for conversion of solar energy into organic compounds, they have the ability to grow rapidly and store large amounts of lipids. In general, microalgae present themselves as an alternative for biodiesel production due to high productivity of lipid biomass in a very short production cycle. Microalgae also do not require fertile and/or arable land, and thus do not compete with food production and do not cause deforestation. Furthermore, they can even be used as mitigating sources of greenhouse gases from stationary sources. However, microalgae biomass production and the subsequent production of biodiesel consist of several stages toward attaining the final product, with various technical and economic barriers that need to be analyzed and controlled before the raw material becomes a viable alternative to large-scale production of biomass for energy purposes. The steps of the process are presented in this paper, showing the different methods and technological systems employed, as well as their challenges. The present paper analyzes a variety of studies on this topic, evaluating the use of microalgae for biodiesel production and studying its potential as a renewable energy source and its environmental and economic viability.

BIOFUELS

Biofuels are alternative biodegradable fuels produced from biomass (organic raw materials) derived from renewable energy sources. They have become a sustainable alternative to “traditional” fuels, as they are able to bring significant energy security and environmental benefits [1]. Their production has become a solution for reducing dependency on fossil fuels currently used in transportation vehicles and various other industrial processes [2]. Primary biofuels such as firewood, for example, are used without processing, mainly for heating, cooking, or electricity production. Secondary biofuels, such as bioethanol and biodiesel, are produced from the processing of biomass [3]. Secondary biofuels can be classified into four generations based on different parameters such as type of raw material, processing technology, and level of development. Third generation biofuels are

produced from microalgae cultivation, where cells accumulate lipids which, after extraction, pass through the transesterification process for obtaining biodiesel [4 - 7].

BIODIESEL HISTORY

In 1895, about twenty-five years after the beginning of the oil industry, Rudolf Diesel created the compression ignition engine, which was later named in his honor [8]. In 1900, during the Paris Exposition, the French company Otto, at the request of the French government, presented a small diesel engine running on peanut oil. Peanuts were a crop that was widespread in French colonies in Africa and could be easily cultivated locally, thus meeting the energy demands of colonies and industries. In the 1940s, several European countries that had African colonies such as Belgium, France, Italy, the UK, and even Germany, showed interest in the production and development of fuel through vegetable oils. During World War II, vegetable oils were used as emergency fuel for various applications in countries like Brazil, Argentina, China, India, Japan, and the United States. At this time, concerns about excessive use of oil products, and about their possible scarcity, stimulated the development of alternatives such as mixtures (biofuels) using cottonseed oil, corn, and mixtures with conventional diesel [9]. However, at the end of the war, the return of a plenteous and low priced supply of imported oil, mainly from the Middle East, ended up discouraging the use, and thus the development, of alternative fuels.

Biodiesel in the World

Despite the decline suffered by the sector after the war, advances in the development of biofuels have been gradually expanding around the world. According to the United States Energy Information Administration (EIA) [10], world production of biodiesel increased steadily from 213 million gallons in 2000 to 6289 million gallons in 2013. The biodiesel market worldwide arose from the need for reducing the use of fossil diesel. In addition, the anthropogenic causes of global warming described by the Kyoto Protocol decreed the urgent need for new energy technology. Another variable is the unquestionable political and social instability in major oil producing countries [11, 12].

Developing countries have a great opportunity to rise in the biofuels market, and these emerging countries can stimulate this segment of their economies, the development of industrial parks, the use of technology, the exploration of new markets, and the forming of partnerships. According to BP Global, biodiesel has been widely used in the EU (mainly Germany and France) from 2014 on [13], using the rapeseed oil surplus and rapeseed. The alcohol used in Europe is methanol, which can be purchased at extremely competitive prices due to the

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