Chakravarthi Mohan *Editor*

Sugarcane Biotechnology: Challenges and Prospects

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ISBN 978-3-319-58945-9 ISBN 978-3-319-58946-6 (eBook)
DOI 10.1007/978-3-319-58946-6

Library of Congress Control Number: 2017947513

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The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

This book is dedicated to my mentor,
Dr. N. Subramonian, Emeritus Scientist,
ICAR–Sugarcane Breeding Institute, who has
always been encouraging and supportive and
is an inspiration for young researchers.

Preface

Sugarcane is an important cash crop grown throughout the tropical and subtropical regions of the world. It serves as the major source for sugar and also used for ethanol and biomass production. The demand for sugarcane and its by-products is set to increase in recent years due to increasing population, higher demand for sugar and climate change. Moreover, sucrose yield has been unchanged for the past decades. Owing to these factors, there exists the need for sugarcane improvement through biotechnology which would inevitably improve the yield as well as the sustainability of sugar industries. With the advent of next generation sequencing technologies and genome editing tools, the realization of sugarcane improvement through biotechnology is not very far. Several transcriptomic studies have been carried out in sugarcane and whole genome sequencing is in progress. Transgenic sugarcane for several traits has been reported, the highlight being the commercialization of drought-tolerant transgenic sugarcane in Indonesia and others in pipeline. Sugarcane is being used as a platform to produce several recombinant proteins and products. Very recently, transcription activator-like effector nucleases (TALENs) have been used in sugarcane initiating genome editing approach in this complex polyploid genome.

In this volume, a collection of 11 chapters is presented by experienced researchers working on sugarcane biotechnology. This book provides exhaustive information on several recent technologies that are employed for sugarcane improvement through biotechnology. An array of topics such as genomics and transcriptomics, transgenic sugarcane for trait improvement, potential candidate promoters, new strategies for transformation, molecular farming, sugarcane as biofuel, chloroplast transformation and genome editing which are currently employed in sugarcane for trait improvement has been discussed comprehensively in this book which will serve as an encyclopaedia for graduates, postgraduates and researchers who work on sugarcane. This book will also be of great interest to plant scientists, biotechnologists, molecular biologists and breeders who work on sugarcane crop. As editor of this book, I am grateful to the contributors of various chapters for writing their chapters meticulously and enabling to produce this book on time and in a great manner. I also thank the editorial staff of Springer, New York, who were very generous

and helpful to initiate this book project. I am also grateful to the São Paulo Research Foundation (FAPESP, Proc. 2015/10855-9) for the postdoctoral research grant. Finally, special thanks to Springer, Switzerland, for publishing this book. I firmly believe that the information covered in this volume will make a sound contribution to sugarcane research.

São Carlos, SP, Brazil Chakravarthi Mohan

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About the Editor

Dr. Chakravarthi Mohan is presently a postdoctoral fellow at the Department of Genetics and Evolution, Federal University of São Carlos, Brazil, where he aims to develop transgenic sugarcane for weevil resistance through RNAi approach. He received his Ph.D. in biotechnology in 2015 for his work on 'Isolation and characterization of constitutive and wound inducible promoters and validation of designed synthetic stem/root specific promoters for sugarcane transformation' from Bharathiar University, Coimbatore, and the study was carried out at the ICAR–Sugarcane Breeding Institute, Coimbatore, India. He has considerable expertise on sugarcane genetic engineering, transcriptome analysis and sugarcane molecular farming which is evident from his publications. He has published 13 research papers in international peer-reviewed journals and 5 book chapters. He is a life member of Indian Science Congress Association and also serves as reviewer for several international peer-reviewed journals. He has also presented his research in international and national conferences.

Chapter 1
Potential Health Benefits of Sugarcane

Chinnaraja Chinnadurai

Abstract Sugarcane is a perennial grass belonging to Poaceae family and it has been cultivated worldwide more than 90 countries because of its economical and medicinal value of high-yielding products. Refined sugar is obtained as a primary product from sugarcane juice, an eminent raw material of sugarcane. Other commercial value-added by-products such as brown sugar, molasses, and jaggery are also obtained during the process in an unrefined form. The expensive carnauba wax is produced from sugarcane wax and utilized in cosmetics and pharmaceutical applications. Sugarcane juice is widely used in traditional medicine system of several countries mainly in India, to treat several health issues such as jaundice, hemorrhage, dysuria, anuria, and other urinary diseases. In this chapter, various types of phytoconstituents and health benefits of sugarcane and its valuable products are summarized. The phytochemistry of sugarcane juice, sugarcane wax, leaves, and its products also established the occurrence of various fatty acids, alcohol, phytosterols, higher terpenoids, flavonoids, -O- and -C-glycosides, and phenolic acids. Necessity on advanced research for the production of various medicinal products from sugarcane and its phytopharmacological study has been summarized.

Keywords Medicine • Molasses • Pharmacological properties • Phytochemical profile • Sugarcane juice

1.1 Introduction

Sugarcane is a tall perennial true grass belonging to the genus *Saccharum* and tribe Andropogoneae. It originated in Southeast Asia and is now cultivated in tropical and subtropical countries throughout the world for sugar and by-products. The genus *Saccharum* contains five important species, viz., *Saccharum officinarum, Saccharum sinense, Saccharum barberi, Saccharum robustum*, and *Saccharum spontaneum*.

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© Springer International Publishing AG 2017

C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_1

The cultivation of *S. officinarum* and its hybrids is mostly used for the production of sugar and ethanol and other industrial applications in more than 90 countries around the world. The stems and the by-products of the sugar industry are also used for feeding livestock. *S. officinarum* was originally grown in Southeast Asia and Western India. Around 327 B.C. it was an important crop in the Indian subcontinent. It was introduced to Egypt around 647 A.D. and about one century later, to Spain (755 A.D.). Since then, the cultivation of sugarcane extended to nearly all tropical and subtropical regions around the world. Portuguese and Spaniards introduced sugarcane to the New World early in the sixteenth century. *S. officinarum* L. more recently is utilized as a replacement of fossil fuel for motor vehicles.

Worldwide, sugarcane inhabits 20.42 million ha area with a total production of 1900 million metric tons (FAO 2014). Sugarcane area and productivity differ widely from country to country. Brazil occupies the highest sugarcane-growing area (5.343 million ha) followed by India, China, Thailand, Pakistan, and Mexico. Sugarcane is a best example for renewable natural agricultural resource since it provides sugar, besides biofuel, fiber, fertilizer, and a myriad of by-products/coproducts with ecological sustainability. White sugar, brown sugar (Khandsari), jaggery (Gur), and ethanol are obtained from sugarcane juice and bagasse and molasses are the main by-products of the sugar industry. Molasses are the chief by-products used as main raw material for the production of alcohol. Excess bagasse is now being used as raw material in the paper industry. In addition, cogeneration of power using bagasse as fuel is considered feasible in most sugar mills.

Sugarcane holds potential health benefits and generally most of them are not aware of it. Sugarcane can be edible in the form of either pieces of stem or juice. Sugarcane juice extracted from the cane is nutritious and refreshing. It contains about 15% natural sugar that helps to rehydrate the human body and gives instant energy. Sugarcane juice is rich in minerals such as phosphorus, potassium, calcium, iron, and magnesium and vitamins such as vitamin A, B1, B2, B3, B5, B6, C, and E. About 100 mL of sugarcane juice contains 39 calories of energy and 9 g of carbohydrates.

1.2 Health Benefits of Sugarcane

Sugarcane juice is used to cure several types of human diseases in different parts of the world. It has been used in Ayurveda and Unani systems of medicine in India since time immemorial either as single drug or in combination with other plant products. Sugarcane extracts were established with a wide range of biological effects such as immunostimulation (El-Abasy et al. 2002), anti-thrombosis activity, anti-inflammatory activity, vaccine adjuvant, modulation of acetylcholine release (Barocci et al. 1999), and anti-stress effects. Sugarcane juice has broad biological effects on raising innate immunity to infections (Lo et al. 2005).

Jaundice patients and people having liver-related disorders have been encouraged to consume sugarcane extract in traditional system of medicine in curing

diseases. Sugarcane juice is also used as aphrodisiac, laxative, demulcent, antiseptic, and tonic (Xu et al. 2005). According to the Unani system of medicine in India, sugarcane juice is considered beneficial for the liver by regulating the bilirubin levels and it is recommended that consumption of large amount of sugarcane juice helps for an immediate relief from jaundice. These assumptions have also been supported by modern pharmacological studies, which revealed that sugarcane contains various bioactivities like anti-inflammatory, analgesic, antihyperglycemic, diuretic, and hepatoprotective effects. Although apigenin, tricin, and luteoline glycosides like orientin, vitexin, schaftoside, and swertisin were reported as the main constituents in sugarcane juice, various policosanols and steroids were also reported in different parts of *S. officinarum.* Based on these bioactivities and chemical constituents of sugarcane, great attention has been given for the investigation of some lead molecules of this cheapest crop for various diseases.

Sugarcane juice regulates natural immunity of host cells against different microbial infections such as viral, bacterial, and protozoan having effects on the levels of macrophages, neutrophils, and natural killer cells (El-Abasy et al. 2002, 2003; Lo et al. 2005). A wide range of biological activities are observed with by-products of sugarcane juice including antioxidant activities (Tanaki et al. 2003), prophylactic activities, and other physiological functions (Takara et al. 2002).

Sugarcane juice is a rich source of antioxidants. Free radicals have been concerned in the etiology of several human ailments and many antioxidants are being considered as potential therapeutic agents (Sies 1996; Spiteller 2001). The mechanism involved in many human diseases such as hepatotoxicities, hepatocarcinogenesis, diabetes, malaria, acute myocardial infarction, and skin cancer includes lipid peroxidation as a main source of membrane damage (Yoshikava et al. 2000). Antioxidants are molecules capable of terminating the chain reaction of free radicals before vital molecules are damaged. Supplementation of these antioxidants became an attractive therapeutic strategy for reducing the risk of diseases caused by free radicals (Brash and Harve 2002). Recent studies on the role of phenolic compounds from foods and beverages against free radical-mediated diseases became more significant due to the finding of association between lipid peroxidation of LDL and arthrosclerosis. Antioxidant properties of phenolic compounds can be attributed to a wide range of pharmacological activities. These compounds in general act by quenching free radicals, inhibiting the activation of pro-carcinogens, or binding carcinogens to macromolecules. The phenolic and flavonoid contents of sugarcane juice were found with equal proportion of antioxidant effects (Krishnaswamy 1996).

The polyphenols in sugarcane juice also induce metabolism and help keep weight gain during pregnancy and its low glycemic index helps to maintain energy levels. A glass of sugarcane juice with a dash of ginger helps to reduce morning sickness of pregnant women. Small doses of sugarcane juice more than twice a day are recommended for morning sickness, a common complaint among pregnant women. Since sugarcane juice is a rich source of calcium, magnesium, and iron, regular consumption can help boost immunity and keep mineral deficiency at bay during pregnancy. Constipation is also an issue with pregnancy. The juice can also be used

to boost digestion and treat constipation due to the presence of potassium. It helps in proper functioning of digestive system and prevents stomach infections.

Sugarcane juice has been recommended for its diuretic property (Karthikeyan and Simipillai 2010; Cáceres et al. 1987). Regular use of sugarcane juice leads to clear urinary flow since it aids kidneys to perform their function properly. With addition of lime juice and coconut water, sugarcane juice helps in reducing burning sensation which is commonly associated with urinary tract infections, sexually transmitted diseases, kidney stones, and prostatitis.

Intake of sugarcane juice is recommended for diabetic patients. It comprises natural sugar which has low glycemic index that prevents steep rise in blood glucose levels in diabetics. Noni fruit juice was mixed with sugarcane juice and kukui nuts (*Aleurites moluccana* (L.) Wild, Euphorbiaceae) to be used as purgative, or diluted with spring water to treat diabetes and high blood pressure or prevent intoxication from kava (McClatchey 2002; Chun 1994). However, type 2 diabetes patients are recommended to consume it in moderate levels after doctor consultation.

Cancer cannot survive in an alkaline environment. Sugarcane juice comprises high concentration of calcium, magnesium, potassium, iron, and manganese since it is proven that regular consumption of sugarcane juice is effectively fighting against cancer, especially prostate and breast cancer.

Studies established that sugarcane juice protects against tooth decay and bad breath due to its high mineral content. Deficiency of nutrients in the body can easily be recovered by including sugarcane juice in our diet. Febrile disorder is quite common in infants and children resulting in fevers, which can lead to seizures and loss of proteins in the body. Sugarcane juice helps in compensating the lost protein and helps in recovery.

Alpha hydroxy acids help fight acne, reduce blemishes, prevent ageing, and keep the skin hydrated. One of the most effective alpha hydroxy acids is glycolic acid and is present in sugarcane and considered as one of its few natural sources. Even though sugarcane juice has many advantages, it is also important to consume the juice as soon as it is extracted because it tends to get oxidized within 15 min. As it is rich with medicinal values, sugarcane juice is considered as a miracle drink.

1.3 Phytochemical Profile of Sugarcane and Its By-products

1.3.1 Sugarcane Leaves

Sugarcane leaves are naturally coated with waxes which are considered as an important source of various policosanols and D-003. In addition, various flavones -*O*- and -*C*- glycosides were isolated from methanolic extracts of sugarcane leaves through HPLC microfractionation techniques.

1.3.2 Sugarcane Wax

Sugarcane wax deposits on the surface of stalks and leaves seem whitish to dark yellow in color and are extracted from the sugarcane filter residue, the so-called bagasse, during sugar production and utilized for industrial, cosmetic, and pharmaceutical applications (Hoepfner and Botha 2004). It is one of the important commercial sources of long-chain fatty alcohols, acids, esters, aldehydes, and ketones. Apart from that policosanols and D-003, some steroids and terpenoids have also been isolated as by-products from sugarcane wax. Policosanols range from 2.5 to 80% and are a blend of long-chain primary aliphatic alcohols. Octacosanol constitutes 50–80% of the total policosanols (Awika and Rooney 2004). Other active components of sugarcane wax are long-chain aliphatic fatty acids that occur at lower concentrations. The blend of these acids is known as D-003 (Mas 2004). Several phytosterols, steroids, and higher terpenoids were also reported (Georges et al. 2006; Bryce et al. 1967) apart from the major constituents of fatty acid and fatty alcohol in sugarcane wax (Goswami et al. 1984). The quantity of wax derived from sugarcane is between the range of 0.1 and 0.3% and it differs from variety to variety (Laguna Granja et al. 1999). The sugarcane wax is considered as a possible substitute for the expensive carnauba wax.

1.3.3 Sugarcane Juice

Sugarcane juice is extracted by grinding the sugarcane stems for the production of white/brown sugar, jaggery, and molasses. Sugarcane juice holds water (70–75%), sucrose (13–15%), and fiber (10–15%). Several color components with chlorogenic acid, cinnamic acid, and flavones were identified from sugarcane juice during 1971 (Farber et al. 1971). Further, all the colored components were categorized into four major classes: plant pigments, polyphenolic compounds, caramels, and degradation products of sugars condensed with amino derivatives.

The presence of phenolic acids such as hydroxycinnamic acid, sinapic acid, and caffeic acid, along with flavones such as apigenin, luteolin, and tricin, was also identified in high-performance liquid chromatography with diode array detection (HPLC-DAD) analysis of phenolic compounds from sugarcane juice. In that, tricin derivatives were obtained with highest concentration (Maurício Duarte-Almeida et al. 2006). Further, detailed chromatographic and spectroscopic studies established the presence of various -*O*- and -*C*- glycosides of the above-mentioned flavones (Vila et al. 2008). Apart from that few minor flavones swertisin, tricin-7-*O*-neohesperoside-4′-*O*-rhamnoside, tricin-7-*O*-methylglucuronate-4′-*O*-rhamnoside, and tricin-7-*O*-methylglucuronide (Colombo et al. 2009) and some novel acylated flavone glycosides, such as tricin-7-*O*-β-(6′-methoxycinnamic)-glucoside, luteolin-8-*C*-rhamnosyl glucoside, and

tricin-4′-*O*-(erthroguaicylglyceryl)-ether, were isolated, along with orientin, from sugarcane juice (Duarte-Almeida et al. 2007).

1.3.4 Sugarcane Products

Brown sugar, molasses, syrups, and non-centrifugal sugar are the several important by-products of sugarcane (Balasundaram et al. 2006). Apart from some identified compounds of sugarcane juice, three new flavonoid glycosides, tricin7-(2′-rhamnosyl)-α-galacturonide, orientin-7,3′-dimethyl ether, and iso-orientin-7,3′-*O*-dimethyl ether, were isolated from mill syrups (Mabry et al. 1984). Along with the already stated isoorientin-7 and 3′-*O*-dimethyl ether, a novel O-glycoside and dehydroconiferylalcohol-9′-*O*-β-D-glucopyranoside were also isolated from sugarcane molasses and have been validated as antibacterial compounds (Takara et al. 2007). Through liquid chromatography-mass spectrometry (LC-MS) analysis of aqueous and dichloromethane extracts of brown sugars, the presence of various phenolic acids and eight major volatile constituents has been described.

1.4 Pharmacological Properties of Sugarcane and Its By-products

Various phytochemicals including phenolic compounds, plant sterols, and policosanols are present in sugarcane and help in defense against pest and diseases. Several studies have proven the biological activities of sugarcane products including antioxidant activity, cholesterol-lowering properties, and other potential health benefits.

1.4.1 Antithrombotic Activity

Antithrombotic activity was examined with policosanols and D-003 for their platelet aggregation and in rats. Plasma level of 6 keto-PGF1-α (a stable metabolite of prostacyclin PGI) was significantly increased with oral administration of D-003 at a single dose of 200 mg/kg and policosanols at a concentration of 25 mg/kg in rats, compared to control. In addition, D-003 significantly reduced the thromboxane plasma levels and weight of venous thrombus in collagen-stimulated whole blood of rats (Molina et al. 2002). Also, the pharmacokinetic study established that the effect of D-003 was detected after 30 min of dosing and the maximal effect exhibited after 1–2 h of treatment (Molina et al. 2000).

1.4.2 Diuretic Activity

Intragastric application of ethanol extracts (50%) of fresh leaves to rats at a dose of 40 mL/kg was found with diuretic activity, while its decoction was not found with any diuretic activity (Ribeiro Rde et al. 1986; Cáceres et al. 1987).

1.4.3 Analgesic and Antihepatotoxic Activity

Ethanol extracts (95%) from sugarcane leaves and shoots were recorded with analgesic activity in mice with intragastric application at a dose of 1 g/kg. The ethanol extract of sugarcane shoots was found active only against the tail-flick method while leaf extracts were active against benzoyl peroxide-induced writhing and tail-flick response (Costa et al. 1989).

Intraperitoneal application of aqueous extract of dried stems to mice, at a dose of 25 mg/kg, was found active against chloroform-induced hepatotoxicity (Jin et al. 1981).

1.4.4 Antihypercholesterolemic Effect

Oral administration of sugarcane policosanols (5–200 mg/kg) on normocholesterolemic New Zealand rabbits revealed a significant decrease in the level of total cholesterol and low-density lipoprotein cholesterol (LDL-C) in a dose-dependent manner. It also reduced the level of serum triglyceride, but it was not found as dose dependent. However, the high-density lipoprotein levels remained unchanged (Arruzazabala et al. 1994).

Policosanols also prevented atherosclerosis in male New Zealand rabbits fed on a cholesterol-rich diet for 60 days at doses of 25 or 200 mg/kg. Interestingly, hypercholesterolemia was not found in policosanol-treated rabbits and the intima thickness was also found significantly less compared to control animals (Arruzazabala et al. 2000).

1.4.5 Antihyperglycemic Activity

Intragastrical application of ethanol extract of leaves at a dose of 1 g/kg and 60 mg/animal, respectively, produced weak activity against alloxan-induced hyperglycemia (Arruzazabala et al. 1994). Further, intraperitoneal application of juice of dried stems exhibited hypoglycemic activity at a dose of 200 mg/kg (Takahashi et al. 1985).

1.4.6 Anti-inflammatory Effect

Oral administration of the mixture of fatty acids isolated from sugarcane wax showed anti-inflammatory activity in the cotton pellet granuloma assay and in the carrageenan-induced pleurisy test, both in rats and in the peritoneal capillary permeability test in mice (Ledón et al. 2003).

1.4.7 Acetylcholine Release

The study on the effect of policosanols on the release of acetylcholine (ACh) at the neuromuscular junction in mice revealed that policosanols enhanced a slight extent of either the spontaneous or the evoked ACh release. Additionally, it was found that increment in the level of conformational changes induced at the nicotinic receptor channel complex, which established the release of Ach (Re et al. 1999).

1.5 Toxicity Profile of Sugarcane Juice

Incomplete combustion of the organic matter develops polycyclic aromatic hydrocarbons (PAHs) in sugarcane juice at harvesting season and their presence originates mainly from processing and cooking of food. The presence of four PAHs, benz(*a*)anthracene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, and benzo(*a*) pyrene, was confirmed in HPLC analysis of sugarcane juice collected during different harvesting period (Silvia Tfouni et al. 2009).

1.6 Conclusion and Future Perspectives

This chapter provides a detailed analysis on health benefits of sugarcane, its phytochemical profile, and pharmacological applications. Sugarcane extract is utilized as a regular nutritional drink in several Asian countries since it comprises significant amount of minerals, vitamins, and hydrophilic compounds with essential biological activities. The presence of pharmacological activities is proven in sugarcane juice and its unrefined products such as brown sugar, molasses, and jaggery are considered as richest sources of phenolic compounds, such as phenolic acids, flavonoids, and different glycosides. The lipophilic compounds including various policosanols, D-003, and phytosterols are the important components of sugarcane wax present in sugarcane leaves and shoots are observed with several pharmacological effects such as sympathomimetic, antihypercholesterolemic, and antithrombotic activities.

Further there is a wide scope for investigation to identify the presence of new compounds with more activities in *S. officinarum* and its products. Even though the presence of carcinogenic compounds such as polycyclic aromatic hydrocarbons was reported in *S. officinarum*, yet advanced research associated with recent technologies has to be made. Further, a detailed examination has to be made in future in sugarcane and its products since there is a lack of comprehensive investigation on the large number of identified compounds and their pharmacological activities. Although the chemical composition is known for several compounds of sugarcane, future research has to be made to understand the metabolic pathways of these compounds. Additional verification is needed to understand the phytochemistry of sugarcane products such as jaggery and thermostable chemical components of sugarcane juice.

There is a need for further improvement on sugarcane production since there is a product diversification and sugarcane has the potential to supply high-value niche markets with a variety of products (Hildebrand 2002). Hence**,** recent researches have been made to achieve cane improvement and industry diversification through the application of biotechnology to make more profitable sugarcane production. Such new approaches to plant improvement might enable the cane plant to store higher levels of sucrose or to produce and store new products with wider markets than sugar.

Transgenic plants have been developed with new genes incorporated by genetic engineering for the improvement of yield and enhance resistance to pests, diseases, and herbicides and production of value-added traits (James 2011; Potrykus 2001). In case of sugarcane, the first successful transformation of sugarcane with reporter genes using particle inflow gun appeared in 1992 (Bower and Birch 1992). Later, there has been several reports of genetically engineered sugarcane plants using particle gun and agrobacterium-mediated gene transformation methods including with improved disease, pest and herbicide resistance to sugarcane mosaic virus (Joyce et al. 1998), leaf scald (Zhang et al. 1999), stalk borers (Arencibia et al. 1999), and herbicide resistance (Enriquez-Obregon et al. 1998; Manickavasagam et al. 2004) were produced.

Transgenic sugarcane plants with altered metabolic pathways were developed with a view to improve sucrose accumulation (Botha et al. 2001), sugar characteristics (Vickers et al. 2005), as well as novel sugars (Basnayake et al. 2012) but none are commercially available. Nevertheless, transgenic canes could have a key role in industrial applications and in crop improvement.

References

Arencibia AD, Carmona ER, Cornide MT, Castiglione S, O'Relly J, Chinea A, Oramas P, Sala F (1999) Somaclonal variation in insect-resistant sugarcane (*Saccharum* hybrid) plants produced by cell electroporation. Transgenic Res 8(349):360

Arruzazabala ML, Carbajal D, Mas R, Molina V, Valdes S, Laguna A (1994) Cholesterol-lowering effects of policosanol in rabbits. Biol Res 27(3–4):205–208

Arruzazabala ML, Noa M, Menéndez R, Más R, Carbajal D, Valdés S et al (2000) Protective effect of policosanol on atherosclerotic lesions in rabbits with exogenous hypercholesterolemia. Braz J Med Biol Res 33(7):835–840

Awika JM, Rooney LW (2004) Sorghum phytochemicals and their potential impact on human health. Phytochemistry 65(9):1199–1221

Balasundaram N, Sundram K, Samman S (2006) Phenolic compounds in plants and agri-industrial by-products: antioxidant activity, occurrence, and potential uses. Food Chem 99(1):191–203

Barocci S, Re L, Capotani C, Vivani C, Ricci M, Rinaldi L et al (1999) Effects of some extracts on the acetyl-choline release at the mouse neuromuscular joint. Pharm Res 39:239–245

Basnayake SWV, Morgan TC, Wu L, Birch RG (2012) Field performance of transgenic sugarcane expressing isomaltulose synthase. Plant Biotechnol J 10(2):217–225

Botha FC, Sawyer BJB, Birch RG (2001) Sucrose metabolism in the culm of transgenic sugarcane with reduced soluble acid invertase activity. Proc Int Soc Sugar Cane Technol 24:588–591

Bower R, Birch RG (1992) Transgenic sugarcane plants via microprojectile bombardment. Plant J 2(3):409–416

Brash DE, Harve PA (2002) New careers for antioxidants. Proc Natl Acad Sci USA 99(22):13969–13971

Bryce TA, Martin-Smith M, Osske G, Schreiber K, Subramanian G (1967) Sterols and triterpenoids-XI. Isolation of arundoin and sawammilletin from Cuban sugarcane wax. Tetrahedron 23(3):1283–1296

Cáceres A, Girón LM, Alvarado SR, Torres MF (1987) Screening of antimicrobial activity of plants popularly used in Guatemala for the treatment of dermatomucosal diseases. J Ethnopharmacol 20(3):223–237

Chun M (1994) Native Hawaii medicines. First People's Productions, Honolulu

Colombo R, Yariwake JH, Queroz EF, Ndjoko K, Hostettmann K (2009) On-line identification of minor flavones from sugarcane juice by LC/UV/MS and post-column derivatization. J Braz Chem Soc 20(9):1574–1579

Costa M, Di Stasi LC, Kirizawa M, Mendaçolli SL, Gomes C, Trolin G (1989) Screening in mice of some medicinal plants used for analgesic purposes in the state of São Paulo. J Ethnopharmacol 27(1–2):25–33

Duarte-Almeida JM, Negri G, Salatino A, de Carvalho JE, Lajolo FM (2007) Antiproliferative and antioxidant activities of a tricin acylated glycoside from sugarcane (*Saccharum officinarum*) juice. Phytochemistry 68(8):1165–1171

El-Abasy M, Motobu M, Na KJ, Sameshina T, Koge K, Onodera T et al (2002) Immunostimulating and growth promoting effects of sugarcane extracts (SCE) in chickens. J Vet Med Sci 64(11):1061–1063

El-Abasy M, Motobu M, Na KJ, Shimura K, Nakamura K, Koge K et al (2003) Protective effect of sugarcane extracts (SCE) on *Eimeria tenella* infections in chickens. J Vet Med Sci 65(8):865–871

Enriquez-Obregon GA, Vazquez-Padron RI, Prieto-Samsonov DL, Riva GA, Selman-Housein G (1998) Herbicide-resistant sugarcane (*Saccharum officinarum* L.) plants by *Agrobacterium*-mediated transformation. Planta 206:20–27

Farber L, Carpenter FG, McDonald EJ (1971) Separation of colorants from cane sugar. Int Sugar J 69:323–328

Georges P, Sylvestre M, Ruegger H, Bourgeois P (2006) Ketosteroids and hydroxyketosteroids, minor metabolites of sugarcane wax. Steroids 71(8):647–652

Goswami PC, Singh HD, Bareuh JN (1984) Isolation of phytosterols from sugarcane press mud and microbial conversion of the phytosterols to 17-ketosteroids. Curr Sci 53:917–919

Hildebrand C (2002) Independent assessment of the sugar industry, Report to the Hon. Warren Truss MP Minister for Agriculture, Fisheries and Forestry, Canberra

Hoepfner SW, Botha FC (2004) Purification and characterisation of fructokinase from the culm of sugarcane. Plant Sci 167(3):645–654

James C (2011) Global status of commercialised biotech/GM crops. ISAAA Brief No. 43. ISAAA: Ithaca

Jin YF, Liang HZ, Cao CY, Wang ZW, Shu RS, Li XY (1981) Immunological activity of bagasse polysaccharides (author's transl). Zhongguo Yao Li Xue Bao 2(4):269–275

Joyce PA, McQualter RB, Bernard MJ, Smith GR (1998) Engineering for resistance to SCMV in sugarcane. Int Symp Biotechnol Trop Subtrop Spec 461:385–391

Karthikeyan J, Simipillai SS (2010) Sugarcane in therapeutics. J Herb Med Toxicol 4:9–14

Krishnaswamy K (1996) Indian functional food: role in prevention of cancer. Nutr Rev 54(11 Pt 2):S127–S131

Laguna Granja A, Hernandez JM, Quintana DC, Valmana LA, Ferreiro RM, Mesa MG (1999) Mixture of higher primary aliphatic alcohols, its obtention from sugar cane wax and its pharmaceutical uses. US Patent No. 5856316

Ledón N, Casacó A, Rodríguez V, Cruz J, González R, Tolón Z et al (2003) Anti-inflammatory and analgesic effects of a mixture of fatty acids isolated and purified from sugarcane wax oil. Planta Med 69(4):367–369

Lo DY, Chen TH, Chien MS, Koge K, Hosono A, Kaminogawa S et al (2005) Effects of sugarcane extract on modulation of immunity in pigs. J Vet Med Sci 67(6):591–597

Mabry TM, Liu YL, Pearce J, Dellamonica G, Chopin J, Markham KR et al (1984) New flavonoids from sugarcane (Saccharum). J Nat Prod 47(1):127–130

Manickavasagam M, Ganapathi A, Anbazhagan VR et al (2004) *Agrobacterium*-mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharum* species hybrids) using axillary buds. Plant Cell Rep 23(3):134–143

Mas R (2004) D-003: a new substance with promising lipid modifying and pleiotropic effects for atherosclerosis management. Drugs Future 29(8):773–786

Maurício Duarte-Almeida J, Novoa AV, Linares AF, Lajolo FM, Inés Genovese M (2006) Antioxidant activity of phenolic compounds from sugarcane (*Saccharum officinarum* L.) juice. Plant Foods Hum Nutr 61(4):187–192

McClatchey W (2002) From Polynesian healers to health food stores: changing perspectives of *Morinda citrifolia* (Rubiaceae). Integr Cancer Ther 1(2):110–120

Molina V, Arruzazabala ML, Carbajal D, Más R, Valdés S (2000) Antiplatelet and antithrombotic effect of D-003. Pharmacol Res 42(2):137–143

Molina V, Arruzazabala ML, Carbajal D, Más R (2002) D-003, a potential antithrombotic compound isolated from sugar cane wax with effects on arachidonic acid metabolites. Prostaglandins Leukot Essent Fatty Acids 67(1):19–24

Potrykus I (2001) Golden rice and beyond. Plant Physiol 125(3):1157–1161

Re L, Barocci S, Capitaini C, Vivani C, Ricci M, Rinaldi L et al (1999) Effects of some natural extracts on the acetylcholine release at the mouse neuromuscular junction. Pharmacol Res 39(3):239–245

Ribeiro Rde A, Fiuza de Melo MM, De Barros F, Gomes C, Trolin G (1986) Acute antihypertensive effect in conscious rats produced by some medicinal plants used in the state of São Paulo. J Ethnopharmacol 15(3):261–269

Sies H (ed) (1996) Antioxidants in disease, mechanism and therapy. Academic, New York

Silvia Tfouni AV, Souza NG, Neto MB, Loredo ISD, Leme FM, Furlani RPZ (2009) Polycyclic aromatic hydrocarbons (PAHs) in sugarcane juice. Food Chem 116(1):391–394

Spiteller G (2001) Peroxidation of linoleic acid and its relation to aging and age dependent diseases. Mech Ageing Dev 122(7):617–657

Takahashi M, Konno C, Hikino H (1985) Isolation and hypoglycemic activity of saccharin a, B, C, D, E and F glycans of *Saccharum officinarum* stalks. Planta Med 3:258–260

Takara K, Matsui D, Wada K, Ichiba T, Nakasone Y (2002) New antioxidative phenolic glycosides isolated from Kokuto non-centrifuged cane sugar. Biosci Biotechnol Biochem 66(1):29–35

Takara K, Ushijima K, Wada K, Iwasaki H, Yamashita M (2007) Phenolic compounds from sugarcane molasses possessing antibacterial activity against carcinogenic bacteria. J Oleo Sci 56(11):611–614
Tanaki H, Man SL, Ohta Y, Katsuyama N, Chinen I (2003) Inhibition of osteoporosis in rats fed with sugarcane wax. Biosci Biotechnol Biochem 67(2):423–425
Vickers JE, Grof CPL, Bonnett GD, Jackson PA, Knight DP, Roberts SE, Robinson SP (2005) Overexpression of polyphenol oxidase in transgenic sugarcane results in darker juice and raw sugar. Crop Sci 45(1):354–362
Vila FC, Colombo R, de Lira TO, Yariwake JH (2008) HPLC microfractionation of flavones and antioxidant (radical scavenging) activity of *Saccharum officinarum* L. J Braz Chem Soc 19(5):903–908
Xu F, Sun RC, Sun JX, Liu CF, He BH, Fan JS (2005) Determination of cell wall ferulic and p-coumaric acids in sugarcane bagasse. Anal Chim Acta 552(1–2):207–217
Yoshikava T, Toyokuni S, Yamamoto Y, Naito Y (eds) (2000) Free radicals in chemistry, biology and medicine. OICA International, London
Zhang LH, JL X, Birch RG (1999) Engineered detoxification confers resistance against a pathogenic bacterium. Nat Biotechnol 17(10):1021–1024

Chapter 2
Sugarcane Genomics and Transcriptomics

Lovejot Kaur, S. Dharshini, Bakshi Ram, and C. Appunu

Abstract Sugarcane is an important commercial crop belonging to Poaceae family and is a major source of sucrose and ethanol production worldwide. Sugarcane's large genome size, aneuploidy of commercial cultivars, and polyploidy of interspecific hybrids have always imposed a challenge for generation of genomic and transcriptomics resources for crop improvement. Despite of these hurdles, linkage maps based on different segregating populations has been constructed. Efforts to map QTLs controlling various traits are being carried out and map-based cloning has also been tried. Available EST data can now be used for SNP mining, expression profiling, discovering new genes, etc. The comparative analysis of sugarcane and sorghum genome revealed high similarity between the two genomes. This information will further expedite sugarcane improvement initiatives. The advent of high-throughput sequencing technologies such as Roche/454 and Illumina/Solexa is being used to gain knowledge on transcriptome of the cell under different stress conditions. RNA-seq can provide the sequences of all RNA molecules, including mRNA, rRNA, tRNA, and noncoding RNAs, produced in one or a population of cells. The data generated can be used to measure transcript levels, to find novel genes, fusion transcript, and splice junctions. Knowledge of the sugarcane transcriptome can provide information about synthesis of various biomolecules and their interactions with other metabolic pathways in the complex sugarcane genome. Both genomic and transcriptome resources of sugarcane are immensely important for improving yield as well as quality of sugarcane; this will help sugarcane farming community to a great extent.

Keywords Genome • Microarray • Polyploid • RNA-seq • *Saccharum* • Transcriptome

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C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_2

2.1 Introduction

Sugarcane is a complex crop species with each gene represented by a number of alleles in the genome, high polyploidy and aneuploidy. About 70–80% of genome composition of modern sugarcane cultivars has been derived from *S. officinarum* and 10–20% from *S. spontaneum.* It has been reported by Berkman et al. (2014) that proportion of repeated sequences in sugarcane hybrids ranged from 63.74 to 78.37% and that increased proportion may be because of ploidy level of sugarcane genome rather than repetitive content. Since it has been reported that it has synteny with grasses like sorghum and maize, the future of sugarcane improvement seems promising. The sorghum genome is the closest genome to sugarcane which has been fully sequenced and annotated and is widely accepted as reference genome for comparative analysis of sugarcane sequences.The origin of modern sugarcane cultivars raises issues not only related to the extent and nature of the divergence of the sugarcane and sorghum genomes, but also about the relationships (meiosis and expression dosage) among hom(e)ologous loci (De Setta et al. 2014). Association mapping experiments are being carried out in sugarcane to detect marker-trait associations as well as validating the position of different important genes. Sugarcane transcriptomic experiments have led to identification of large number of genes which are involved in controlling important biological functions. Further, various international efforts are concentrating on studying the effect of complex genome on transcript expression in sugarcane. The genes identified through transcriptomic studies can be used either as DNA markers or to develop transgenic sugarcane. Even if sugarcane is a crop of immense importance its genetics has lagged behind and plant scientists has shown little interest in studying it. One of the major reason being the complexity of the sugarcane genome, which exceeds that of any other important crop. This chapter focuses on recent advances in sugarcane genomics and transcriptomics that will further enhance our knowledge of the challenges faced in its improvement.

2.2 Molecular Cytogenetics

Sreenivasan et al. (1987) made initial attempts to elucidate sugarcane genome and its taxonomy by using classical cytogenetic methods. It indicated that basic chromosome set of *Saccharum* sp. could be $x = 5, 6, 8, 10$, or 12 and chromosome number of *S. officinarum* is considered to be $2n = 80$. Bremer (1923, 1961) reported the occurrence of $2n$ gamete transmission in hybrids of *S. officinarum* and *S. spontaneum.* This study further supported the hypothesis that modern sugarcane cultivars are derived from crosses between *S. officinarum* ($2n = 80$) and *S. spontaneum* ($2n =$ 36–128). *S. officinarum* clones that had a nuclear DNA content different from those having 80 chromosomes, i.e., outliers were relisted as hybrids (Aitken et al. 2006a). *S. spontaneum* has five major cytotypes: $2n = 64, 80, 96, 112$, or 128 (Panje and Babu 1960) and basic chromosome number of $x = 8$ (hypothesized) (D'Hont et al.

1996, 1998; Ha et al. 1999). The FISH (florescent in situ hybridization) experiments conducted physically mapped the 45S rRNA and the 5S genes on chromosomes of *S. officinarum* and *S. robustum.* Classical cytogeneticists thought that no exchange of chromosomes has occurred between *S. officinarum* and *S. spontaneum* (Price 1963, 1965; Berding and Roach 1987). But, further studies (D'Hont et al. 1996) using FISH of complete genomic DNA of *S. officinarum* and *S. spontaneum* in sugarcane cultivar R570 showed that 10% of the chromosomes appeared to be contributed by *S. spontaneum* and 80% from *S. officinarum* and remaining 10% were recombinants. These results were further confirmed by molecular mapping of cultivar R570 (Grivet et al. 1996; Hoarau et al. 2002). Molecular cytogenetic techniques have also been used to study other members of *Saccharum* complex, especially *Erianthus* and *Miscanthus*. FISH along with DNA markers has been used to identify true hybrids formed with *E. arundinaceus* and to track *E. arundinaceus* genes introgressed into *Saccharum* (D'Hont et al. 1995; Piperidis and D'Hont 2001; Jing et al. 2009). Specific repeated sequences from *Erianthus* and *Miscanthus* were cloned and FISH was used to analyze their chromosome distribution; this analysis revealed two subtelomeric, one centromeric, and one apparently dispersed family along the genome (Alix et al. 1998, 1999). FISH was also used to find out the chromosome composition of fertile *S. officinarum* × *E. arundinaceus* hybrids in F_1, BC_1, and BC_2. Recombinants were not observed in either BC1 or BC2 clones (Piperidis et al. 2010).

2.3 Genetic Diversity Analysis

Present day sugarcane cultivars have been derived from interspecific hybridizations of domesticated species *S. officinarum* known for high sugar and the wild species *S. spontaneum* characterized for resistant to biotic and abiotic stresses. The genome complexity in *Saccharum* spp. has made sugarcane and energy cane breeding cumbersome. Use of only few limited genotypes of *S. spontaneum* and *S. officinarum* clones in earlier breeding experiments has resulted in a narrow genetic base of present sugarcane cultivars. Characterization of genetic variation among different *Saccharum* genotypes has been carried out in both the organelle genome and nuclear genomes (D'Hont et al. 1993; Sobral et al. 1994). Diversity in chloroplast genome was initially studied by Takahashi et al. (2005); it was reported that analysis of genomic sequence of 26 regions in the chloroplast clearly distinguished *S. spontaneum* from the other five species of *Saccharum.* To study genetic variability among the chloroplast genomes of sugarcane (*Saccharum* spp.) and its wild progenitor species *Saccharum spontaneum* L. (Zhu et al. 2014), 19 primer pairs were designed targeting various chloroplast DNA (cpDNA) segments with a total length ranging from 4781 to 4791 bp. Ten out of 19 cp DNA segments were polymorphic, with 14 mutation sites. This demonstrated that the chloroplast genome of *S. spontaneum* was maternally inherited. Also, comparative sequence homology analyses clustered sugarcane cultivars into a distinctive group away from *S. spontaneum* and its progeny.

Large numbers of DNA markers have been used to assess the genetic diversity in the nuclear genome of *Saccharum* species. In recent years, genetic diversity has been investigated for sugarcane cultivars or ancestral species by using several molecular methods, such as restriction fragment length polymorphism (RFLP) (Lu et al. 1994; Besse et al. 1997), random amplified polymorphic DNA (RAPD) (Huckette and Botha 1995; Nair et al. 2002), amplified fragment length polymorphism (AFLP) (Aitken et al. 2005), inter simple sequence repeats (ISSR) (Virupakshi and Naik 2008), sequence-related amplified polymorphism (SRAP) (Li and Quiros 2001; Chang et al. 2012), target region amplification polymorphism (TRAP) (Alwala et al. 2006; Que 2009), genomic in situ hybridization (GISH) (D'Hont 2005; D'Hont et al. 2002), fluorescence in situ hybridization (FISH) (D'Hont 2005; D'Hont et al. 1996; Jenkin et al. 1995), simple sequence repeats (Aitken et al. 2005), and expressed sequence tag-SSR (EST SSR) markers (Cordeiro et al. 2001).

Nayak et al. (2014) evaluated all the 1002 accessions in World Collections of Sugarcane and Related Grasses (WCSRG) germplasm using SSR markers. The population structure analysis and principal coordinate analysis revealed three clusters with all *S. spontaneum* in one cluster, *S. officinarum* and *Saccharum* hybrids in the second cluster, and non-*Saccharum* spp. in the third cluster. A core collection of 300 accessions was selected that represented the majority of diversity in the WCSRG.

2.4 Molecular Genetic Maps

Aneuploidy, double genome structure, and homologous and homoeologous chromosomes of sugarcane have hindered the progress of constructing linkage maps with large coverage. For last 25 years different researchers from various labs have been involved in mapping sugarcane genome. Earlier maps made utilized the single dose (SD) markers generated mostly using RFLP, while the more recent maps used AFLP and SSRs. But the coverage provided is not satisfactory enough.

RFLP markers were used to map AP85-0068 and SES 208 (Silva et al. 1993). Same population was used (Al-Janabi et al. 1993) to map using arbitrary primed PCR. Integration of the data from these studies lead to construction of linkage map with 64 linkage groups (LGs) assembled in eight homologous groups. RAPD and AFLP markers were also used subsequently. Mudge et al. (1996) used LA-Purple and Molokai for linkage map construction using RAPD markers. A total of 160 RAPD markers and a morphological marker were assembled into 51 linkage groups. AFLP markers along with RFLP markers and arbitrary primers were used (Guimaraes et al. 1997, 1999) to generate maps. RFLP markers were further used to map R570; this map contained 96 linkage groups and ten putative HGs (Grivet et al. 1996). The extensive use of RFLP markers were made by Ming et al. (1998), the group generated four different maps using four different parents and the number of linkage groups varied from 69 to 72. Earliest, most extensive maps were constructed by Aitken et al. (2005) and Garcia et al. (2006). AFLP, randomly amplified DNA fingerprints (RAF), and SSR markers were mapped into 136 linkage groups (Aitken

et al. 2005). A linkage map was constructed using RFLP, SSR, and AFLP markers (Garcia et al. 2006); this map comprised 131 linkage groups with larger proportion of markers unlinked. Hybrid cultivar R570 and an old Australian clone MQ76-53 were used for linkage map construction. These maps contained AFLP, SSR, and RFLP markers, which were assembled into 86 linkage groups for R570 and 105 linkage groups for MQ76-53 (Raboin et al. 2006). Segregating F2 population of a hybrid cultivar LCP 85-384 was used for linkage map construction with AFLP, SSR and TRAP markers; in this map nine HGs contained 108 linkage groups (Andru et al. 2011).

In a major stride in sugarcane mapping Aitken et al. (2014) generated a comprehensive sugarcane genetic map of Q165; this map contained 2267 markers generated from Diversity Array Technology (DArT) markers, amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), single nucleotide polymorphism (SNP), restriction fragment length polymorphism (RFLP), and random amplified polymorphism (RAPD) markers. Usage of large numbers of different markers allowed most of the LGs to be placed into the eight homology groups; this number is consistent with the basic chromosome number of the ancestral species of *Saccharum* and so far the lowest basic chromosome number reported in the *Saccharum* genus. The use of DArT markers will allow the development of consensus genetic maps in sugarcane which would improve genome coverage and allow integration with other genomic resources (Aitken et al. 2014).

2.5 Mapping of QTLs

As discussed in previous section, molecular markers have been used to develop genetic maps; these are being used to trace the position of genes valuable for sugarcane improvement and also to identify quantitative trait loci (QTL) associated with different traits so as to speed up marker-assisted selection efforts. These efforts are slow since multiple QTLs control most of the traits of agronomic importance and individual contribution of these QTLs is very small. Sugarcane QTL mapping is mostly based on single marker analysis or interval mapping.

One of the first studies was done by Sills et al. (1995). Seven different traits namely stalk number, tasseled stalks, smutted stalks, stalk diameter, POL%, fiber content, and plot weight. Of the traits studied, epistatic interaction between two markers associated with stalk diameter was found to be significant. Daugrois et al. (1996) used self-fertilized population of R570, single marker analyses was performed and found a marker linked with brown rust resistant gene (at 10 cM). Extensive work has been done by Ming et al. (2001, 2002a, b, c). The group has used different mapping populations, with RFLP as a marker of choice, and studied different traits like sugar content, sugar yield, fiber content, POL, ash, stalk number, stalk weight, flowering time, and plant height. The analyses were performed using single marker analysis and interval mapping approach. Significant associations between markers and QTLs were identified for these traits. Marker-trait association for stalk number and sucker number identified seven and six RFLP markers, respectively

(Jordan et al. 2004). Aitken et al. (2006b) identified 37 marker–QTL associations for brix and POL. In this study phenotypic variation explained varied from 3 to 9%. In another study Aitken et al. (2008) used AFLP and SSR markers and found that 46% of the marker–QTL associations were consistent across different years of evaluation. This study identified 27 genomic regions significantly associated with traits like cane yield, stalk weight, stalk number, stalk length, and stalk diameter. Pinto et al. (2010) identified putative QTLs as well as their epistatic interactions for fiber content, cane yield, POL, and tonnes of sugar per hectare. A total of 120 associations were found and 50 digenic epistatic marker interactions were identified for the four traits evaluated. Singh et al. (2013) constructed linkage maps to identify QTLs for seedling, brix, sucrose percent, stalk number, stalk length, stalk diameter, internodes, and number of green leaves, at three crop cycles across seven environments in a segregating population with 207 individuals derived from a biparental cross of sugarcane elite cultivars. Thirty-one QTLs were identified, out of these 7 QTLs had stable effect across crop year and locations. Racedo et al. (2016) tried to establish an appropriate genome-wide association analysis (GWAS) tool in a sugarcane breeding population. In this study clones were genotyped with DArT (Diversity Array Technology) and TRAP (Target Region Amplified Polymorphism) markers, and evaluated for cane yield and sugar content. A total of 43 and 38 markers significantly associated with cane yield and sugar content, respectively.

2.6 Comparative Genomics

Sugarcane and sorghum share the same subtribe, i.e., *Saccharinae*, and reported to have diverged from a common ancestor approximately 8 million years ago. The sorghum genome, the closest related fully sequenced and annotated genome to sugarcane, is considered as reference genome for comparative analysis. Ten pairs of chromosomes has been sequenced; this effort has covered 90% genome of sorghum and 99% of protein coding region (Paterson et al. 2009). Sugarcane is highly complex and polyploid. Despite of this fact it shares high degree of synteny with diploid *Sorghum bicolor.* Also, sugarcane and sorghum genomes share extensive microcollinearity with each other, thus strengthening the fact that sorghum genome can be used as reference for assembly of sugarcane genome (Ming et al. 1998; Okura et al. 2012). Sugarcane genome analysis can use the sorghum genomic resources like sorghum gene indices/models (Hoang et al. 2015). BLASTp searches against the NCBI nonredundant database have confirmed that most of the sugarcane protein sequences are most similar to those of sorghum (Setta et al. 2014). Also, using the chromosomal locations of the 935 sorghum–sugarcane orthologous, the group was able to localize 265 sugarcane BACs onto sorghum chromosomal arms. Sequences of four DArT markers (analysis of sugarcane DArT marker sequences associated to important traits) showed high similitude and e-value with coding sequences of *Sorghum bicolor* (Racedo et al. 2016). This study further confirms that *Sorghum bicolor* share high gene microcollinearity between sorghum and sugarcane.

2.7 Sugarcane as an Important Source of Biomass

Tremendous phenotypic variation in *Saccharum* germplasm and advances in sugarcane genomic tools has assisted in characterization of traits important for use of sugarcane as biofuel. The fast growth and high yield of sugarcane makes it a suitable candidate for production of second generation biofuels. For use as biofuel, the genetic potential of sugarcane can be improved by screening the germplasm for biofuel characters, cell wall composition modifications, and utilizing the potential of next generation sequencing technologies (NGS). These approaches will help to pin point the important genes involved in biomass production in sugarcane and hence these genes can be manipulated to enhance sugarcane's potential as biofuel. Sugarcane along with other grasses such as *Miscanthus* species (*Miscanthus giganteus*), *Erianthus* species (*Erianthus arundinaceus* Retz.), and switch grass (*Panicum virgatum*) is an efficient converter of solar energy into chemical energy and biomass accumulation (Tew and Cobill 2008; Furtado et al. 2014). Soluble sugar as well as residues in sugarcane production (bagasse and trash) can be used for biofuel production (Seabra et al. 2010; Alonso Pippo et al. 2011; Macrelli et al. 2012). In sugarcane germplasm, along with other traits, variation can be seen in biomass yield and fiber content within species and within genera. More variations can be seen in wild sugarcane species as compared to the domesticated sugarcane. Moreover, the genetic diversity of *S. officinarum* has been used in sugarcane improvement programs but the diversity of *S. spontaneum* have not been used much (Aitken and McNeil 2010). The cell wall of sugarcane and other grasses are categorized as type II (Souza et al. 2013). This type of cell wall is usually characterized as having little pectin and lesser lignin and structural proteins (Carpita 1996; Henry 2010; Saathoff et al. 2011). Understanding the detailed composition and fine structure of sugarcane cell wall will help in optimizing the tissue pretreatment and cell wall hydrolysis protocol (Hoang et al. 2015). Altering the carbohydrates of the cell walls is the key of improving the biomass composition for biofuel production (Harris and DeBolt 2010). Use of biotechnology can help in producing sugarcane plants genetically modified to have favorable cellulose to non-cellulose content. Different studies have indicated that the efforts for improving sugarcane biomass is impeded by highly complex genome, low transformation efficiency, transgene inactivation, somaclonal variations, and problems during backcrossing (Ingelbrecht et al. 1999; Hotta et al. 2010; Arruda 2012; Dal-Bianco et al. 2012). About 25% of the total lignocellulosic biomass in sugarcane is composed of lignin; this high percentage affects the efficiency of saccharification during conversion to ethanol (Canilha et al. 2012, 2013). Altering sugarcane biomass composition for biofuel production can be done by downregulating some genes involved in lignin pathway. In sugarcane, a minimum of ten enzymes have been reported to be involved in lignin pathway (Higuchi 1981; Whetten and Ron 1995). Jung et al. (2012) reported that when RNA interference (RNAi) suppression was used to downregulate caffeic acid O-methyltransferase (COMT) lignin content and lignin S/G ratio was reduced. Species like *Miscanthus, Erianthus*, *S. officinarum*, and *S. spontaneum* have lot of allelic diversity and can be exploited for improving sugarcane biomass (Hoang et al. 2015).

2.8 Sugarcane Transcriptomics

In the past decade many large scale array-based studies of gene expression have been performed in sugarcane (Manners and Casu 2011). Differentially expressed genes study using microarray during sugarcane leaf and culm development provides information that it is not necessary to observe high abundance of sucrose metabolism transcript when high concentration of sucrose accumulates in tissue (Carson et al. 2002). Identification of novel genes associated with cold tolerance mechanism in sugarcane is extensively studied using microarray experiments (Nogueira et al. 2003). Sugarcane transcriptome profiling based on signal transduction-related genes using microarray were determined and it revealed that, in sugarcane, 3500 genes are reported to be involved in signal transduction including genes coding for 600 transcription factors, 477 receptors, 114 calcium and inositol metabolism proteins, 107 protein phosphatases, 510 protein kinases, 75 small GTPases, and 17 G proteins (Papini-Terzi et al. 2005). The study on expression pattern of sugarcane culm is done and information on genes associated with maturation shows differential gene expression in cellulose synthases and cellulose synthase-like genes (Casu et al. 2007). cDNA microarray analyses revealed by elevation of CO_2 on sugarcane leaves, 22 genes were upregulated and 14 genes were downregulated which mainly related to photosynthesis and development and finally showed an increase of about 29% in sucrose content (De Souza et al. 2008). Using microarray technique the identification of genes when ethanol is applied on sugarcane leaf is studied and reported that 70 transcripts show differential gene expression pattern which comes under categories like gene regulation and abiotic stress (Camargo et al. 2007). Gene expression profiling using arrays were used to identify genes specific to a tissue, e.g., stems (Damaj et al. 2010).

For both low and high sucrose producing cultivars, transcriptome microarray studies on signal transduction pathway involved during sucrose synthesis were done and the study revealed differential expression of 24 genes and of them 19 were reported in low sucrose producing plants. Three of these genes are involved in reducing sucrose phosphate synthase (de Maria Felix et al. 2009). Sugarcane plants were subjected to polyethylene glycol stress for 2–4 h and transcriptome analysis was done, results show upregulation of sucrose transporter 1, sodium proton antiporter, proline dehydrogenase, and catalase-2. When the salt stress were given to plants there was a downregulation in all these genes and indicating that sugarcane response differently to different kind of stress (Patade et al. 2012). Transcriptome studies of sugarcane reported that during stress conditions plant accumulate osmoprotectants and 56 clusters of candidate gene classified to osmoprotectant were upregulated (Dos Santos et al. 2011).

2.9 Next Generation Sequencing Technologies

Next generation sequencing (NGS), also known as high-throughput sequencing, is the catch-all term used to describe a number of different modern sequencing technologies including Illumina sequencing, Roche 454 sequencing, Ion torrent

sequencing, and SOLiD sequencing. Illumina NGS utilizes a fundamentally different approach from the classic Sanger chain-termination method. Illumina sequencing instruments and reagents support massively parallel sequencing using a proprietary method that detects single bases as they incorporated into growing DNA strands using sequencing by synthesis (SBS) and it has many applications such as whole genome sequencing, de novo sequencing, candidate region targeted resequencing, DNA sequencing, RNA sequencing for applications such as transcriptome and small RNA analysis, methylation analysis and protein-nucleic acid interaction analysis (ChIP-seq).

2.9.1 RNA-Seq Technology and Its Application in Sugarcane

RNA sequencing (RNA-Seq) technology allows you to discover and profile the transcriptome in any organism. Illumina RNA-Seq technology records the numerical frequency of sequences in a library population, and offers a number of advantages compared to other technologies such as microarrays.

Analysis of the total RNA complement of the cell is included in transcriptomic studies. This helps in making meaningful comparisons between tissues of the same organism at different developmental stages and tissues exposed to various stresses or treatments (Schnable et al. 2004; Brady et al. 2006; Galbraith 2006). In sugarcane, for large-scale expression profiling techniques involving hybridization of RNA samples with nucleotide probes or generation of sequence tags are used. Different research groups worldwide have developed multiple sugarcane EST databases; these databases collectively contain more than 300,000 ESTs.

With an aim to generate a panel of differentially expressed stress responsive genes Kido et al. (2012) generated four Super SAGE libraries, using bulked root tissues from four drought tolerant accessions as compared with four bulked sensitive genotypes. Most relevant BlastN matches comprised 567,420 tags, 75,404 uni tags with 164,860 different ESTs. Oloriz et al. (2012) used a sugarcane mutant, obtained by chemical mutagenesis of the susceptible variety B4362; it showed a post-haustorial hypersensitive response (HR)-mediated resistance to the pathogen and was used to identify genes differentially expressed in response to *P. melanocephala* via suppression subtractive hybridization (SSH). It was also found that genes involved in glycolysis and C4 carbon fixation were upregulated in both interactions while genes related with the nascent polypeptide associated complex, post-translational proteome modulation, and autophagy were transcribed at higher levels in the compatible interaction. Genes coding for a putative no apical meristem protein, S-adenosyl methionine decarboxylase, nonspecific lipid transfer protein, and GDP-L-galactose phosphorylase involved in ascorbic acid biosynthesis were upregulated in the incompatible interaction. Wu et al. (2013) used high-throughput tag-sequencing (tag-seq) analysis by Solexa technology on sugarcane infected with *Sporisorium scitaminea*, 2015 genes expressed differentially, of these 1125 were upregulated and 890 downregulated were obtained after mapping to sugarcane EST

databases in NCBI. To study small RNA transcriptome complexity and explore their roles in sugarcane development, Sternes and Moyle (2015) obtained almost 50 million small RNA reads from suspension cells, embryogenic calli, leaf, apex, and a developmental series of stem internodes. The complexity of the small RNA component of the transcriptome varied between tissues. The undifferentiated and young tissue type libraries had lower redundancy levels than libraries generated from maturing and mature tissues. Su et al. (2015) made a study to find out the role of sugarcane chitinase gene family. Ten differentially expressed chitinase genes were obtained from RNA-seq analysis of both incompatible and compatible sugarcane genotypes during *Sporisorium scitamineum* infection. Seven chitinases showed more positive with early response and maintained increased transcripts in the incompatible interaction than those in the compatible one. Their results suggest that sugarcane chitinase family exhibit differential responses to biotic and abiotic stress. Park et al. (2015) performed transcriptome analysis of sugarcane hybrid CP72-1210 (cold susceptible) and *Saccharum spontaneum* TUS05-05 (cold tolerant) using Sugarcane Assembled Sequences (SAS) from SUCEST-FUN Database and showed that a total of 35,340 and 34,698 SAS genes, respectively, were expressed before and after chilling stress. The analysis revealed that more than 600 genes are differentially expressed in each genotype after chilling stress. Blast2GO annotation revealed that the major differences in gene expression profile between CP72-1210 and TUS05-05 after chilling stress are present in the genes related to the transmembrane transporter activity.

Vicentini et al. (2015) performed a high-throughput transcriptome evaluation of two sugarcane genotypes contrasting for lignin content. This study generated a set of 85,151 transcripts of sugarcane using RNA-seq and de novo assembly. More than 2000 transcripts showed differential expression between the genotypes, including several genes involved in the lignin biosynthetic pathway. This provided important information on the lignin biosynthesis and its interactions with other metabolic pathways in the complex sugarcane genome. Casu et al. (2015) examined tissue-specific expression patterns to explore the spatial deployment of pathways responsible for sucrose accumulation and fiber synthesis within the stalk. They performed expression profiling of different tissues (storage parenchyma, vascular bundles and rind dissected from a maturing stalk, internode of sugarcane). They identified ten cellulose synthase subunit genes and examined significant differences in the expression of their corresponding transcripts and those of several sugar transporters. Overall, their study indicates that there is spatial separation for elevated expression of these important targets in both sucrose accumulation and cell wall synthesis. Zeng et al. (2015) used a customized microarray to analyze the changes in the level of transcripts of sugarcane genes 8, 24, and 72 h after exposure to low-K conditions. The group identified a total of 4153 genes that were differentially expressed in at least one of the three time points. The number of genes responding to low-K stress at 72 h was almost twofold more than the numbers at 8 and 24 h. Gene ontology (GO) analysis revealed that many genes involved in metabolic, developmental, and biological regulatory processes displayed changes in the level of transcripts in response to low-K stress. Also, differential expression of transcription factors,

transporters, kinases, oxidative stress-related genes and genes in Ca+ and ethylene signaling pathways was detected. Ferreira et al. (2016) performed a comparative expression profiling of sugarcane ancestral genotypes: *S. officinarum, S. spontaneum*, and *S. robustum* and a commercial hybrid: RB867515, linking gene expression to phenotypes to identify genes for sugarcane improvement. Oligoarray experiments of leaves, immature and intermediate internodes, detected 12,621 sense and 995 antisense transcripts. For all tissues sampled, expression analysis revealed 831, 674, and 648 differentially expressed genes in *S. officinarum, S. robustum*, and *S. spontaneum*, respectively, using RB867515 as reference. Co-expression network analysis identified 18 transcription factors possibly related to cell wall biosynthesis and in silico analysis detected cis-elements involved in cell wall biosynthesis in their promoters. Dharshini et al. (2016) performed transcriptome profiling of the low temperature (10 °C) tolerant *S. spontaneum* clone IND 00-1037 collected from high altitude regions of Arunachal Pradesh, North Eastern India. The Illumina Nextseq500 platform yielded a total of 47.63 and 48.18 million reads corresponding to 4.7 and 4.8 gigabase pairs (Gb) of processed reads for control and cold stressed (10 °C for 24 h) samples, respectively. These reads were de novo assembled into 214,611 unigenes with an average length of 801 bp. The study revealed that about 2583 genes were upregulated and 3302 genes were downregulated during the stress.

Huang et al. (2016) performed the transcriptome analysis of a high-sucrose sugarcane variety, GT35, using high-throughput Solexa technology. A KEGG pathway analysis of 30,756 unigenes revealed more than 30 pathways in the sugarcane transcriptome and 3420 simple sequence repeats were identified in 3185 unigenes. Santa Brigida et al. (2016) have produced a de novo transcriptome assembly (TR7) from sugarcane RNA-seq libraries submitted to drought and infection with *Acidovorax avenae* subsp. *avenae.* The libraries presented 247 million of raw reads and resulted in 168,767 reference transcripts. Mapping in de novo transcriptome assembly of reads obtained from infected libraries revealed 798 differentially expressed transcripts, of which 723 were annotated and corresponded to 467 genes. Differential analysis revealed that genes in the biosynthetic pathways of ET and JA PRRs, oxidative burst genes, NBS-LRR genes, cell wall fortification genes, SAR induced genes, and pathogenesis-related genes (PR) were upregulated (Table 2.1).

2.10 Different Databases and Sequence Sources

For bioinformatics analysis major available resources include expressed sequence tags (ESTs); more than 2.5 lakh sequences, genome survey sequences (GSS); approximately 9500 bacterial artificial chromosome sequences. The chloroplast genome of sugarcane has been sequenced and is of 141,182 bp (Asano et al. 2004; Junior Tercilio Calsa et al. 2004).

Since the complete genome sequencing of sugarcane is going to take few more years, comparative databases can prove to be excellent tools for sugarcane genome analysis. Bioinformatics portals like Gramene and portal at J. Craig Venter Institute

Table 2.1 List of transcriptomic studies in sugarcane

S. No.	Trait studied for transcriptome analysis	Author and year of publication
1	Infection caused by *Audovorax avenae* sub sp. *avenae*	Santa Brigida et al. (2016)
2	Low temperature tolerance gene profiling	Dharshini et al. (2016)
3	Genes associated with leaf abscission	Li et al. (2016)
4	Cell wall biosynthesis	Ferreira et al. (2016)
5	High-sucrose sugarcane varieties	Huang et al. (2016)
6	Maturing sugarcane stalk	Casu et al. (2015)
7	Genotypes contrasting for lignin content	Vicentini et al. (2015)
8	Biotropic interaction with *Sporisorium scitamineum*	Taniguti et al. (2015)
9	Response to low potassium stress	Zeng et al. (2015)
10	Cold responsive gene profiling	Park et al. (2015)
11	*Sporisorium scitamineum* challenge in sugarcane	Que et al. (2014)
12	Contrasting sugarcane varieties	Cardoso-Silva et al. (2014)
13	Sugarcane response to *Sporisorium scitamineum*	Wu et al. (2013)
14	Small RNA transcriptome analysis	Bottino et al. (2013)
15	Drought stress tolerance	Kido et al. (2012)

have genomic resources from different grasses that can be utilized for comparative genome analyses. As described earlier, sugarcane shares maximum similarity with *Sorghum bicolor*. Sorghum has a genome size of approximately 736 bp, similar to the monoploid genome of *Saccharum*. Paterson et al. (2009) sequenced *S. bicolor* genome and its chromosome-based assembly and annotation can be found in Phytozome (http://www.phytozome.org) and Joint Genome Institute (http://genome.jgi-psf.org/Sorbi1/Sorbi1.info.html).

The second most related crop to sugarcane is maize (*Zea mays*). Its sequencing has been completed and information about Maize Genome Sequencing Project can be found out on http://www.maizesequence.org/. The Maize Genome Database (Lawrence et al. 2004) available at http://www.maizegdb.org/ contains information like maps, QTLs, genetic stocks, cytogenetic and variations for alleles and polymorphisms, molecular markers, probes, gene products, images, metabolic pathways, and mutant phenotypes. The SUCEST initiative generated largest collection of ESTs from 26 different cDNA libraries (Vettore et al. 2001, 2003). Additionally, other groups (Casu et al. 2003, 2004; Bower et al. 2005; Ma et al. 2004) also generated >10,000 ESTs. Center for Genomic Research (TIGR) clustered these ESTs as Sugarcane Gene Index 2.1, while Sugarcane Gene index 2.1 was released by Computational Biology and Functional Genomics Laboratory at Dana-Farber Cancer Institute. The updated Sugarcane Gene Index contains theoretical contigs (formed by clustering ESTs and expressed transcripts), singleton ESTs, and singleton expressed transcripts. For transcript expression analysis, data from sugarcane high-throughput profiling experiments have been deposited in Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) (Edgar et al. 2002; Barrett et al. 2007, 2008).

An important Gene Ontology enrichment tool for sugarcane is EasyGO-http://bioinformatics.cau.edu.cn/easygo/ (Zhou and Su 2007). EasyGo can identify enriched GO terms for gene models, gene loci, protein coding genes, Ensembl, RefSeq and Uniport gene products, Gene Index entries and microarray oligonucleotides or probe sets for up to 17 organisms, also including the Sugarcane Gene Index and the Sugar Cane Affymetrix Genome Array probe sets. Efforts have been made to develop molecular marker and genetic Map Databases in sugarcane. TropGENE-DB (http://tropgenedb.cirad.fr/) is publicly available mapping resource (http://tropgenedb.cirad.fr/) (Ruiz et al. 2004). Nine different modules are included in this database. In addition to sugarcane, information about banana, cocoa, coconut, coffee, cotton, oil palm, rice, and rubber tree is contained in this database. Information of six genetic maps, with all maps sharing at least one parent is included in sugarcane module. Also, database can be searched for molecular markers, QTL, and clones. As far as metabolome of sugarcane is concerned KNApSAcK (http://kanaya.naist.jp/KNApSAcK/) tool contains information on different metabolites. This database provides information about the biological origins of the compounds and provides a tool for mass spectrum data.

2.11 Conclusion and Future Perspectives

To summarize, although several significant advancements in sugarcane genomics have been made, there exists a small incompleteness due to the unavailability of complete sugarcane genome information. However, the day is not so far as several research groups from Brazil and Australia are striving hard to sequence this complex polyploid for several years. Future researches would be greatly improved once the genome is sequenced. In addition, functional genomics will largely benefit leading to a great improvement in genetic engineering of sugarcane for value-added traits.

References

Canilha L, Chandel AK, Suzane dos Santos Milessi T, Antunes FA, Luiz da Costa Freitas W, das Graças Almeida Felipe M, da Silva SS (2012) Bioconversion of sugarcane biomass into ethanol: an overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. BioMed Research International. 2012.

Canilha L, Rodrigues RC, Antunes FA, Chandel AK, Milessi T, Felipe MD, Silva SS. (2013) Bioconversion of hemicellulose from sugarcane biomass into sustainable products. Sustainable degradation of lignocellulosic biomass-Techniques, applications and commercialization,15-45.

Casu RE, Rae AL, Nielsen JM, Perroux JM, Bonnett GD, Manners JM (2015) Tissue-specific transcriptome analysis within the maturing sugarcane stalk reveals spatial regulation in the expression of cellulose synthase and sucrose transporter gene families. Plant molecular biology. 89(6):607-28.

Chang D, Yang FY, Yan JJ, Wu YQ, Bai SQ, Liang XZ, Zhang YW, Gan YM (2012) SRAP analysis of genetic diversity of nine native populations of wild sugarcane, Saccharum spontaneum, from Sichuan, China. Genetics and Molecular Research. 11(2):1245-53.

D'Hont A, Lu YH, Feldmann P, Glaszmann JC. (1993) Cytoplasmic diversity in sugar cane revealed by heterologous probes. Sugar Cane (United Kingdom).

D'hont A, Paulet F, Glaszmann JC.(2002) Oligoclonal interspecific origin of 'North Indian'and 'Chinese'sugarcanes. Chromosome Research.10(3):253-62.

D'Hont A, Rao PS, Feldmann P, Grivet L, Islam-Faridi N, Taylor P, Glaszmann JC. (1995) Identification and characterisation of sugarcane intergeneric hybrids, Saccharum officinarum x Erianthus arundinaceus, with molecular markers and DNA in situ hybridisation. Theoretical and Applied Genetics. 91(2):320-6.

Silva JA, Sorrells ME, Burnquist WL, Tanksley SD(1993) RFLP linkage map and genome analysis of Saccharum spontaneum. Genome. 36(4):782-91.

Dal-Bianco M, Carneiro MS, Hotta CT, Chapola RG, Hoffmann HP, Garcia AA, Souza GM. (2012) Sugarcane improvement: how far can we go?. Current opinion in biotechnology 23(2):265-70.

Daugrois JH, Grivet L, Roques D, Hoarau JY, Lombard H, Glaszmann JC, D'Hont A (1996) A putative major gene for rust resistance linked with a RFLP marker in sugarcane cultivar 'R570'. Theoretical and Applied Genetics. 92(8):1059-64.

Furtado A, Lupoi JS, Hoang NV, Healey A, Singh S, Simmons BA, Henry RJ (2014) Modifying plants for biofuel and biomaterial production. Plant biotechnology journal. 12(9):1246-58.

Garcia AA, Kido EA, Meza AN, Souza HM, Pinto LR, Pastina MM, Leite CS, Da Silva JA, Ulian EC, Figueira A, Souza AP (2006) Development of an integrated genetic map of a sugarcane (Saccharum spp.) commercial cross, based on a maximum-likelihood approach for estimation of linkage and linkage phases. Theoretical and Applied Genetics. 112(2):298-314.

Grivet L, D'Hont A, Roques D, Feldmann P, Lanaud C, Glaszmann JC (1996) RFLP mapping in cultivated sugarcane (Saccharum spp.): genome organization in a highly polyploid and aneuploid interspecific hybrid. Genetics.142(3):987-1000.

Guimarães CT, Honeycutt RJ, Sills GR, Sobral BW (1999) Genetic maps of Saccharum officinarum L. and Saccharum robustum Brandes & Jew. ex grassl. Genetics and Molecular Biology. 22(1):125-32.

Hoarau JY, Offmann B, D'Hont A, Risterucci AM, Roques D, Glaszmann JC, Grivet L (2001) Genetic dissection of a modern sugarcane cultivar (Saccharum spp.). I. Genome mapping with AFLP markers. Theoretical and Applied Genetics. 103(1):84-97.

Hotta CT, Lembke CG, Domingues DS, Ochoa EA, Cruz GM, Melotto-Passarin DM, Marconi TG, Santos MO, Mollinari M, Margarido GR, Crivellari AC (2010) The biotechnology roadmap for sugarcane improvement. Tropical Plant Biology. 3(2):75-87.

Huang DL, Gao YJ, Gui YY, Chen ZL, Qin CX, Wang M, Liao Q, Yang LT, Li YR (2016) Transcriptome of High-Sucrose Sugarcane Variety GT35. Sugar Tech. 18(5):520-8.

Jing YF, Tao LA, Liu XL, An RD, Chen XK (2009) Use of simple sequence repeats for authentication of sugarcane hybrids generated from Yunnan Erianthus rockii. Sugar Tech. 11(3):296-9.

Jordan DR, Casu RE, Besse P, Carroll BC, Berding N, McIntyre CL (2004) Markers associated with stalk number and suckering in sugarcane colocate with tillering and rhizomatousness QTLs in sorghum. Genome. 47(5):988-93.

Kido EA, Ferreira Neto JR, Silva RL, Pandolfi V, Guimaraes AC, Veiga DT, Chabregas SM, Crovella S, Benko-Iseppon AM (2012) New insights in the sugarcane transcriptome responding to drought stress as revealed by supersage. The Scientific World Journal,2012.

Lu YH, D'Hont A, Walker DI, Rao PS, Feldmann P, Glaszmann JC(1994) Relationships among ancestral species of sugarcane revealed with RFLP using single copy maize nuclear probes. Euphytica.78(1):7-18.

Ming R, Wang Y, Draye X, Moore P, Irvine J, Paterson A (2002b)Molecular dissection of complex traits in autopolyploids: mapping QTLs affecting sugar yield and related traits in sugarcane. TAG Theoretical and Applied Genetics.105(2):332-45.

Ming R, Del Monte TA, Hernandez E, Moore PH, Irvine JE, Paterson AH (2002c) Comparative analysis of QTLs affecting plant height and flowering among closely-related diploid and polyploid genomes. Genome. 45(5):794-803.
Ming R, Liu SC, Bowers JE, Moore PH, Irvine JE, Paterson AH (2002a) Construction of a consensus genetic map from two interspecific crosses. Crop Science. 42(2):570-83.
Ming R, Liu SC, Moore PH, Irvine JE, Paterson AH (2001) QTL analysis in a complex autopolyploid: genetic control of sugar content in sugarcane. Genome Research. 11(12):2075-84.
Mudge J, Andersen WR, Kehrer RL, Fairbanks DJ(1996) A RAPD genetic map of Saccharum officinarum. Crop Science. 36(5):1362-6.
Nair NV, Selvi A, Sreenivasan TV, Pushpalatha KN (2002) Molecular diversity in Indian sugarcane cultivars as revealed by randomly amplified DNA polymorphisms. Euphytica. 127(2):219-25.
Park JW, Benatti TR, Marconi T, Yu Q, Solis-Gracia N, Mora V, da Silva JA (2015) Cold responsive gene expression profiling of sugarcane and Saccharum spontaneum with functional analysis of a cold inducible Saccharum homolog of NOD26-like intrinsic protein to salt and water stress. PloS one. 10(5):e0125810.
Pinto LR, Garcia AA, Pastina MM, Teixeira LH, Bressiani JA, Ulian EC, Bidoia MA, Souza AP. (2010) Analysis of genomic and functional RFLP derived markers associated with sucrose content, fiber and yield QTLs in a sugarcane (Saccharum spp.) commercial cross. Euphytica. 172(3):313-27.
Raboin LM, Oliveira KM, Lecunff L, Telismart H, Roques D, Butterfield M, Hoarau JY, D'Hont A (2006) Genetic mapping in sugarcane, a high polyploid, using bi-parental progeny: identification of a gene controlling stalk colour and a new rust resistance gene. Theoretical and Applied Genetics. 112(7):1382-91.
Racedo J, Gutiérrez L, Perera MF, Ostengo S, Pardo EM, Cuenya MI, Welin B, Castagnaro AP (2016) Genome-wide association mapping of quantitative traits in a breeding population of sugarcane. BMC Plant Biology.16(1):142.
Santa Brigida AB, Rojas CA, Grativol C, de Armas EM, Entenza JO, Thiebaut F, Lima MD, Farrinelli L, Hemerly AS, Lifschitz S, Ferreira PC (2016) Sugarcane transcriptome analysis in response to infection caused by Acidovorax avenae subsp. avenae. PloS one. 11(12):e0166473.
Sills GR, Bridges W, Al-Janabi SM, Sobral BW (1995) Genetic analysis of agronomic traits in a cross between sugarcane (Saccharum officinarum L.) and its presumed progenitor (S. robustum Brandes & Jesw. ex Grassl). Molecular Breeding.1(4):355-63.
Singh RK, Singh SP, Tiwari DK, Srivastava S, Singh SB, Sharma ML, Singh R, Mohapatra T, Singh NK (2013) Genetic mapping and QTL analysis for sugar yield-related traits in sugarcane. Euphytica. 191(3):333-53.
Sobral BW, Braga DP, LaHood ES, Keim P (1994) Phylogenetic analysis of chloroplast restriction enzyme site mutations in the Saccharinae Griseb. subtribe of the Andropogoneae Dumort. tribe. Theoretical and Applied Genetics.87(7):843-53.
Su Y, Xu L, Wang S, Wang Z, Yang Y, Chen Y, Que Y (2015) Identification, phylogeny, and transcript of chitinase family genes in sugarcane. Scientific reports,5.
Takahashi S, Furukawa T, Asano T, Terajima Y, Shimada H, Sugimoto A, Kadowaki K(2005) Very close relationship of the chloroplast genomes among Saccharum species. Theoretical and Applied Genetics.110(8):1523-9.
Vettore AL, da Silva FR, Kemper EL, Souza GM, da Silva AM, Ferro MI, Henrique-Silva F, Giglioti ÉA, Lemos MV, Coutinho LL, Nobrega MP (2003) Analysis and functional annotation of an expressed sequence tag collection for tropical crop sugarcane. Genome research. 13(12):2725-35.
Vicentini R, Bottcher A, dos Santos Brito M, dos Santos AB, Creste S, de Andrade Landell MG, Cesarino I, Mazzafera P (2015) Large-scale transcriptome analysis of two sugarcane genotypes contrasting for lignin content. PloS one. 10(8):e0134909.
Zeng Q, Ling Q, Fan L, Li Y, Hu F, Chen J, Huang Z, Deng H, Li Q, Qi Y (2015) Transcriptome profiling of sugarcane roots in response to low potassium stress. PloS one. 10(5):e0126306.

Zhu JR, Zhou H, Pan YB, Lu X (2014)Genetic variability among the chloroplast genomes of sugarcane (Saccharum spp.) and its wild progenitor species Saccharum spontaneum L. Genetics and Molecular Research. 13(2):3037-47.

Aitken KS, Jackson PA, McIntyre CL (2005) A combination of AFLP and SSR markers provides extensive map coverage and identification of homo(eo)logous linkage groups in a sugarcane cultivar. Theor Appl Genet 110(5):789–801

Aitken KS, Li JC, Jackson P, Piperidis G, McIntyre CL (2006a) AFLP analysis of genetic diversity within *Saccharum officinarum* and comparison with sugarcane cultivars. Aust J Agr Res 57(11):1167–1184

Aitken K and McNeil M (2010) "Diversity analysis," in Genetics, Genomics and Breeding of Sugarcane, eds Henry R., Kole C., editors. (Enfield, NH: Science Publishers;), 19–42.

Aitken KS, Hermann S, Karno K, Bonnett GD, McIntyre LC, Jackson PA Genetic control of yield related stalk traits in sugarcane (2008).Theoretical and Applied Genetics 117(7):1191-203.

Alwala S, Kimbeng CA, Gravois K A ,Bischoff KP (2006) TRAP, a new tool for sugarcane breeding: comparison with AFLP and coefficient of parentage. Journal American Society Sugar Cane Technologists 26, 62-86.

Andru S, Pan YB, Thongthawee S, Burner DM, Kimbeng CA Genetic analysis of the sugarcane (Saccharum spp.) cultivar 'LCP 85-384'. I. Linkage mapping using AFLP, SSR, and TRAP markers(2011). Theoretical and Applied Genetics 123(1):77-93.

Bottino MC, Rosario S, Grativol C, Thiebaut F, Rojas CA, Farrineli L, Hemerly AS, Ferreira PC. (2013) High-throughput sequencing of small RNA transcriptome reveals salt stress regulated microRNAs in sugarcane. PloS one 8(3):e59423.

Cardoso-Silva CB, Costa EA, Mancini MC, Balsalobre TW, Canesin LE, Pinto LR, Carneiro MS, Garcia AA, de Souza AP, Vicentini R (2014) De novo assembly and transcriptome analysis of contrasting sugarcane varieties. PloS one 9(2):e88462.

Casu RE, Dimmock CM, Chapman SC, Grof CP, McIntyre CL, Bonnett GD, Manners JM. (2004) Identification of differentially expressed transcripts from maturing stem of sugarcane by in silico analysis of stem expressed sequence tags and gene expression profiling. Plant molecular biology 54(4):503-17.

Cordeiro GM , Casu R, McIntyre CL, Manners JM, and Henry RJ(2001) Microsatellite markers from sugarcane (Saccharum spp.) ESTs cross transferable to Erianthus and sorghum. Plant Sci 160: 1115-1123.

De Setta N, Monteiro-Vitorello CB, Metcalfe CJ, Cruz GM, Del Bem LE, Vicentini R, Nogueira FT, Campos RA, Nunes SL, Turrini PC, Vieira AP (2014) Building the sugarcane genome for biotechnology and identifying evolutionary trends. BMC genomics 15(1):540.

Hoang NV, Furtado A, Botha FC, Simmons BA, Henry RJ (2015) Potential for genetic improvement of sugarcane as a source of biomass for biofuels. Frontiers in bioengineering andbiotechnology 3.

Huckett BI and Botha FC(1995) Stability and potential use of RAPD markers in a sugarcane genealogy. Euphytica 86.2 : 117-125.

Jenkin MJ, Reader SM, Purdie KA, Miller TE (1995) Detection of rDNA sites in sugarcane by FISH. Chromosome Research 3(7), 444-445.

Li M, Liang Z, Zeng Y, Jing Y, Wu K, Liang J, He S, Wang G, Mo Z, Tan F, Li S. (2016) De novo analysis of transcriptome reveals genes associated with leaf abscission in sugarcane (Saccharum officinarum L.). BMC genomics 17(1):195.

Ming R, Liu SC, Lin YR, Da Silva J, Wilson W, Braga D, Van Deynze A, Wenslaff TF, Wu KK, Moore PH, Burnquist W (1998) Detailed alignment of Saccharum and Sorghum chromosomes: comparative organization of closely related diploid and polyploid genomes. Genetics 150(4):1663-82.

Nayak SN, Song J, Villa A, Pathak B, Ayala-Silva T, Yang X, Todd J, Glynn NC, Kuhn DN, Glaz B, Gilbert RA (2014) Promoting utilization of Saccharum spp. genetic resources through genetic diversity analysis and core collection construction. PloS one 9(10):e110856.

Okura V, da Silva FR, da Silva MJ, Kudrna D, Ammiraju JS, Talag J, Wing R, Arruda P (2012) A BAC library of the SP80-3280 sugarcane variety (saccharum sp.) and its inferred microsynteny with the sorghum genome. BMC research notes 5(1):185.

Que Y, Chen T, Xu L, Chen R (2009) Genetic diversity among key sugarcane clones revealed by TRAP markers. Journal of Agricultural Biotechnology 17(3), 496-503.

Taniguti LM, Schaker PD, Benevenuto J, Peters LP, Carvalho G, Palhares A, Quecine MC, Nunes FR, Kmit MC, Wai A, Hausner G (2015) Complete genome sequence of *Sporisorium scitamineum* and biotrophic interaction transcriptome with sugarcane. PloS one. 10(6):e0129318.

Aitken KS, Jackson PA, McIntyre CL (2006b) Quantitative trait loci identified for sugar related traits in a sugarcane (*Saccharum* spp.) cultivar × *Saccharum officinarum* population. Theor Appl Genet 112(7):1306–1317

Aitken KS, McNeil MD, Hermann S, Bundock PC, Kilian A, Heller-Uszynska K, Henry RJ, Li J (2014) A comprehensive genetic map of sugarcane that provides enhanced map coverage and integrates high-throughput diversity Array technology (DArT) markers. BMC Genomics 15(1):152

Alix K, Baurens FC, Paulet F, Glaszmann JC, D'hont A (1998) Isolation and characterization of a satellite DNA family in the Saccharum Complex. Genome 41(6):854–864

Alix K, Paulet F, Glaszmann JC, D'Hont A (1999) Inter-Alu-like species-specific sequences in the Saccharum complex. Theor Appl Genet 99(6):962–968

Al-Janabi SM, Honeycutt RJ, McClelland M, Sobral BW (1993) A genetic linkage map of *Saccharum spontaneum* L. 'SES 208'. Genetics 134(4):1249–1260

Arruda P (2012) Genetically modified sugarcane for bioenergy generation. Curr Opin Biotechnol 23(3):315–322

Asano T, Tsudzuki T, Takahashi S, Shimada H, Kadowaki KI (2004) Complete nucleotide sequence of the sugarcane (*Saccharum officinarum*) chloroplast genome: a comparative analysis of four monocot chloroplast genomes. DNA Res 11(2):93–99

Barrett T, Troup DB, Wilhite SE, Ledoux P, Rudnev D, Evangelista C, Kim IF, Soboleva A, Tomashevsky M, Edgar R (2007) NCBI GEO: mining tens of millions of expression profiles—database and tools update. Nucleic Acids Res 35:D760–D765

Barrett T, Troup DB, Wilhite SE, Ledoux P, Rudnev D, Evangelista C, Kim IF, Soboleva A, Tomashevsky M, Marshall KA, Phillippy KH (2008) NCBI GEO: archive for high-throughput functional genomic data. Nucleic Acids Res 1(37):D885–D890

Berding N, Roach BT (1987) Germplasm collection, maintenance, and use. In: Heinz DJ (ed) Sugarcane improvement through breeding. Elsevier, Amsterdam, pp 143–210

Berkman PJ, Bundock PC, Casu RE, Henry RJ, Rae AL, Aitken KS (2014) A survey sequence comparison of Saccharum genotypes reveals allelic diversity differences. Tropical Plant Biol 7(2):71–83

Besse P, McIntyre CL, Berding N (1997) Characterisation of Erianthus sect. Ripidium and Saccharum germplasm (Andropogoneae-Saccharinae) using RFLP markers. Euphytica 93(3):283–292

Bower NI, Casu RE, Maclean DJ, Reverter A, Chapman SC, Manners JM (2005) Transcriptional response of sugarcane roots to methyl jasmonate. Plant Sci 168(3):761–772

Brady SM, Long TA, Benfey PN (2006) Unravelling the dynamic transcriptome. Plant Cell 18:2101–2111

Bremer G (1923) A cytological investigation of some species and species-hybrids of the genus Saccharum. Genetica 5:273–326

Bremer G (1961) Problems in breeding and cytology of sugarcane. Euphytics 10:59–78

Calsa JT et al (2004) Structural features and transcript-editing analysis of sugarcane (*Saccharum officinarum* L.) chloroplast genome. Curr Genet 46(6):366–373

Camargo SR, Cançado GM, Ulian EC, Menossi M (2007) Identification of genes responsive to the application of ethanol on sugarcane leaves. Plant Cell Rep 26(12):2119–2128

Carpita NC (1996) Structural and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol 47:445–476

Carson DL, Huckett BI, Botha FC, van Staden J (2002) Differential gene expression in sugarcane leaf and internodal tissues of varying maturity. S Afr J Bot 68(4):434–442
Casu RE, Grof CPL, Rae AL, McINtyre CL, Dimmock CM, Manners JM (2003) Identification of differentially expressed transcripts from maturing stem of sugarcane by in silico analysis of stem expressed sequence tag and microarray analysis. Plant Mol Biol 52:371–386
Casu RE, Jarmey JM, Bonnett GD, Manners JM (2007) Identification of transcripts associated with cell wall metabolism and development in the stem of sugarcane by Affymetrix GeneChip Sugarcane Genome Array expression profiling. Funct Integr Genomics 7(2):153–167
D'Hont A (2005) Unraveling the genome structure of polyploids using FISH and GISH; examples of sugarcane and banana. Cytogenet Genome Res 109:27–33
D'Hont A, Grivet L, Feldmann P, Ra S, Berding N, Glaszmann JC (1996) Characterization of the double genome structure of modern sugarcane cultivars (*Saccharum* spp.) by molecular cytogenetics. Mol Gen Genet 250:405–413
D'Hont A, Ison D, Alix K, Roux C, Glaszmann JC (1998) Determination of basic chromosome numbers in the genus Saccharum by physical mapping of ribosomal RNA genes. Genome 41:221–225
Damaj MB, Kumpatla SP, Emani C, Beremand PD, Reddy AS, Rathore KS, Buenrostro-Nava MT, Curtis IS, Thomas TL, Erik Mirkov T (2010) Sugarcane DIRIGENT and O-methyltransferase promoters confer stem-regulated gene expression in diverse monocots. Planta 231:1439–1458
De Souza AP, Gaspar M, Da Silva EA, Ulian EC, Waclawovsky AJ, Dos santos RV, Teixeira MM, Souza GM, Buckeridge MS (2008) Elevated CO_2 increases photosynthesis, biomass and productivity, and modifies gene expression in sugarcane. Plant Cell Environ 31(8):1116–1127
Dharshini S, Chakravarthi M, Manoj VM, Naveenarani M, Kumar R, Meena M, Ram B, Appunu C (2016) De novo sequencing and transcriptome analysis of a low temperature tolerant *Saccharum spontaneum* clone IND 00-1037. J Biotechnol 231:280–294
Dos Santos PB, Soares-Cavalcanti NM, Vieira-de-Melo GS, Benko-Iseppon AM (2011) Osmoprotectants in the sugarcane (*Saccharum* spp.) transcriptome revealed by in silico evaluation. Comput Intel Meth Bioinfo Biostat 6685:44–58
Edgar R, Domrachev M, Lash AE (2002) Gene expression omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30:207–210
Ferreira SS, Hotta CT, de Carli Poelking VG, Leite DC, Buckeridge MS, Loureiro ME, Barbosa MH, Carneiro MS, Souza GM (2016) Co-expression network analysis reveals transcription factors associated to cell wall biosynthesis in sugarcane. Plant Mol Biol 2:15–35
Galbraith DW (2006) DNA microarray analyses in higher plants. OMICS 10:455–473
Guimaraes CT, Sills GR, Sobral BWS (1997) Comparative mapping of Andropogoneae: *Saccharum* L. (sugarcane) and its relation to sorghum and maize. Proc Natl Acad Sci 94(26):14261–14266
Ha S, Moore PH, Heinz D, Kato S, Ohmido N, Fukui K (1999) Quantitative chromosome map of the polyploid *Saccharum spontaneum* by multicolor fluorescence in situ hybridization and imaging methods. Plant Mol Biol 39:1165–1173
Harris D, DeBolt S (2010) Synthesis, regulation and utilization of lignocellulosic biomass. Plant Biotechnol J 8:244–262. doi:10.1111/j.1467-7652.2009.00481.x
Henry RJ (2010) Plant resources for food, fuel and conservation. Eartscan, London
Higuchi T (1981) In: Tanner W, Loewus F (eds) Biosythesis of lignin in plant carbohydrates II. Springer, Berlin, pp 194–224
Ingelbrecht IL, Irvine JE, Mirkov TE (1999) Posttranscriptional gene silencing in transgenic sugarcane. Dissection of homology-dependent virus resistance in a monocot that has a complex polyploid genome. Plant Physiol 119:1187–1198. doi:10.1104/pp.119.4.1187
Jung JH, Fouad WM, Vermerris W, Gallo M, Altpeter F (2012) RNAi suppression of lignin biosynthesis in sugarcane reduces recalcitrance for biofuel production from lignocellulosic biomass. Plant Biotechnol J 10:1067–1076. doi:10.1111/j.1467-7652.2012.00734.x
Lawrence CJ, Dong Q, Polacco ML, Seigfried TE, Brendel V (2004) MaizeGDB, the community database for maize genetics and genomics. Nucleic Acids Res 32:D393–D397

Li G, Quiros CF (2001) Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in brassica. Theor Appl Genet 103:455–461

Ma HM, Schulze S, Lee S, Yang M, Mirkov E, Irvine J, Moore P, Paterson A (2004) An EST survey of the sugarcane transcriptome. Theor Appl Genet 108:851–863

Macrelli S, Mogensen J, Zacchi G (2012) Techno-economic evaluation of 2nd generation bioethanol production from sugarcane bagasse and leaves integrated with the sugar-based ethanol process. Biotechnol Biofuels 5:22. doi:10.1186/1754-6834-5-22

Manners JM, Casu RE (2011) Transcriptome analysis and functional genomics of sugarcane. Trop Plant Biol 4:9–21

de Maria Felix J, Papini-Terzi FS, Rocha FR, Vêncio RZ, Vicentini R, Nishiyama MY, Ulian EC, Souza GM, Menossi M (2009) Expression profile of signal transduction components in a sugarcane population segregating for sugar content. Trop Plant Biol 2:98–109

Pippo WA, Luengo CA, Alberteris LA, Garzone P, Cornacchia G (2011) Energy recovery from sugarcane-trash in the light of 2nd generation biofuels. Part 1: current situation and environmental aspects. Waste Biomass Valorization 2(1):1–6

Nogueira FT, De Rosa VE, Menossi M, Ulian EC, Arruda P (2003) RNA expression profiles and data mining of sugarcane response to low temperature. Plant Physiol 132(4):1811–1824

Oloriz MI, Gil V, Rojas L, Portal O, Izquierdo Y, Jimenez E, Hofte M (2012) Sugarcane genes differentially expressed in response to *Puccinia melanocephala* infection: identification and transcript profiling. Plant Cell Rep 31(5):955–969

Panje R, Babu C (1960) Studies in *Saccharum spontaneum* distribution and geographical association of chromosome numbers. Cytologia 25:152–172

Papini-Terzi FS, Rocha FR, Vêncio RZ, Oliveira KC, de Maria Felix J, Vicentini R, de Souza Rocha C, Simoes AC, Ulian EC, di Mauro SM, Da Silva AM (2005) Transcription profiling of signal transduction-related genes in sugarcane tissues. DNA Res 12(1):27–38

Patade VY, Bhargava S, Suprasanna P (2012) Transcript expression profiling of stress responsive genes in response to short-term salt or PEG stress in sugarcane leaves. Mol Biol Rep 39:3311–3318

Piperidis G, D'Hont A (2001) Chromosome composition analysis of various *Saccharum* interspecific hybrids by genomic *in situ* hybridization (GISH). Proc Int Soc Sugar Cane Technol 11:565–566

Piperidis N, Chen JW, Deng HH, Wang LP, Jackson P, Piperidis G (2010) GISH characterization of Erianthus Arundinaceus chromosomes in three generations of sugarcane intergeneric hybrids. Genome 53(5):331–336

Price S (1963) Cytogenetics of modern sugar cane. Econ Bot 17:97–106

Price S (1965) Interspecific hybridization in sugarcane breeding. Proc Int Soc Sugar Cane Technol 12:1021–1026

Ruiz M, Rouard M, Raboin LM, Lartaud M, Lagoda P, Courtois B (2004) TropGENE-DB, a multi-tropical crop information system. Nucleic Acids Res 32:D364–D367

Saathoff A, Sarath G, Chow E, Dien B, Tobias C (2011) Downregulation of cinnamyl-alcohol dehydrogenase in switchgrass by RNA silencing results in enhanced glucose release after cellulose treatment. PLoS One 6:e16416. doi:10.1371/journal.pone.0016416

Schnable PS, Hochholdinger F, Nakazono M (2004) Global expression profiling applied to plant deveopmet. Curr Opin Plant Biol 7:50–56

Seabra JEA, Tao L, Chum HL, Macedo IC (2010) A techno-economic evaluation of the effects of centralized cellulosic ethanol and co-products refinery options with sugarcane mill clustering. Biomass Bioenergy 34:1065–1078. doi:10.1016/j.biombioe.2010.01.042

Souza A, Leite DC, Pattathil S, Hahn M, Buckeridge M (2013) Composition and structure of sugarcane cell wall polysaccharides: implications for second-generation bioethanol production. Bioenergy Res 6:564–579

Sreenivasan TV, Ahloowalia BS, Heinz DJ (1987) Cytogenetics. In: Heinz DJ (ed) Sugarcane improvement through breeding. Elsevier, Amsterdam, pp 211–253

Sternes PR, Moyle RL (2015) Deep sequencing reveals divergent expression patterns within the small RNA transcriptomes of cultured and vegetative tissues of sugarcane. Plant Mol Biol Report 33(4):931–951

Takahashi S, Furukawa T, Asano T et al (2005) Very close relationship of the chloroplast genomes among Saccharum species. Theor Appl Genet 110:1523–1529

Tew T, Cobill R (2008) In: Vermerris W (ed) Genetic improvement of sugarcane (*Saccharum* spp.) as an energy crop in Genetic Improvement of Bioenergy Crops. Springer, New York, pp 273–294

Vettore AL, da Silva FR, Kemper EL, Arruda P (2001) The libraries that made SUCEST. Genet Mol Biol 24:1–7

Virupakshi S, Naik GR (2008) ISSR analysis of chloroplast and mitochondrial genome can indicate the diversity in sugarcane genotypes for red rot resistance. Sugar Tech 10(1):65–70

Whetten R, Ron S (1995) Lignin biosynthesis. Plant Cell 7:1001–1013. doi:10.2307/3870053

Wu Q, Xu L, Guo J, Su Y, Que Y (2013) Transcriptome profile analysis of sugarcane responses to *Sporisorium scitaminea* infection using Solexa sequencing technology. Biomed Res Int 2013:1

Zhou X, Su Z (2007) Easy GO: Gene ontology-based annotation and functional enrichment analysis tool for agronomical species. BMC Genomics 8:246

Chapter 3
Unraveling the Sugarcane Genome: Progress Made So Far and Challenges Ahead

J. Ashwin Narayan, V.M. Manoj, Lovejot Kaur, and C. Appunu

Abstract Sugarcane (*Saccharum* spp.) is a major crop grown for sugar and biofuel in tropical and subtropical regions around the world. Sugarcane has a high level of polyploidy, large and complex genome. There is a constant increase in demand of sugarcane worldwide, to meet this demand there is a need in improving the sugarcane yield, sucrose content, increasing growth rate, abiotic and biotic stress tolerance, etc. Researchers have been using conventional breeding efficiently to improve the sugarcane for many years. Present situation demands the improvement in sugarcane varieties at faster rate than which the conventional breeding technique can provide. It is possible to achieve faster improvement only when researchers understand the genome of the plant. Genetics and genome studies have given a better path to develop better varieties. Understanding of sugarcane genome can help breeders to support the conventional breeding in selecting the parents and traits needed. In spite of the complexity, sugarcane genome is been successfully studied and in recent past good progress have been made by genome sequencing strategy, i.e., bacterial artificial chromosome (BAC) libraries. Study on genetic diversity among the species of sugarcane was carried out by RFLP, AFLP, RAPD, SRAP, TRAP and so on. In late 1990s fluorescent in situ hybridization (FISH) technique was used to physically map two *S. officinarum* and three *S. robustum* clones. Later using molecular cytogenetic technique of FISH, many other clones were studied. Quantitative trait loci (QTLs) have been used to screen variety with sugar content, sugar yield, disease resistance, etc. Researchers in Brazil have developed SUCEST database which consist of over 230,000 Expressed Sequence Tags (ESTs) which can be used for detection of molecular polymorphisms, gene expression profiles and gene discovery. In this chapter we discuss about the progress made so far and challenges faced during the study of sugarcane genome.

Keywords Genome • Molecular markers • *Saccharum* • Sequencing • SUCEST

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C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_3

3.1 Introduction

Sugarcane is an important food and energy crop worldwide. It accumulates sucrose in the stem which is used for the production of sugar and ethanol production. Sugarcane supplies more than 80% of the world's raw sugar and is increasingly used for biofuel production (De Setta et al. 2014). Use of fossil fuel has caused global warming and climate change; the best substitute for this is biofuel, which can also be obtained from sugarcane. The world sugar production is expected to reach 170 million tons (2016–2017); Brazil tops the list with 37 million tons followed by India with 23.9 million tons. Same time the world consumption will reach 173 million tons; India has the highest human consumption of about 27 million tons followed by European Union (19 million tons) and is expected to increase year after year. To meet the increasing sugar demand improvement in quality and yield of sugarcane must be doubled. Sugarcane belonging to genus *Saccharum* is a tall perennial grass which comes under Poaceae family along with *Zea mays, Sorghum* and other grasses. Different species likely originated in different locations, such as *Saccharum barberi* in India and *S. edule* and *S. officinarum* in New Guinea. It is theorized that sugarcane was first domesticated as a crop in New Guinea around 6000 BC (Hossain and Abdulla 2015). *S. sinense* in China and *S. barberi* in India were the two major clones used for production of sugar from prehistoric times. Introduction of selected *S. officinarum* clones, a more productive variety compared to previously grown Creole clone, sugar production became a large scale factory from cottage industries during sixteenth century (Roach 1989). Until the end of the nineteenth century most cultivated sugarcanes were clones of *Saccharum officinarum*, which contains $2n = 80$ chromosomes. A major breakthrough in breeding occurred with the development of the first hybrids between *S. officinarum* and the wild vigorous species *Saccharum spontaneum*. A series of backcrosses to *S. officinarum* resulted in cultivars with higher yields, improved ratooning ability and disease resistance. The modern cultivars are developed from these initial hybrids and chromosome number ranged between $2n = 100–130$. Only a few clones of *S. officinarum* and *S. spontaneum* are thought to have been involved in the development of these early hybrids. Most modern sugarcane breeding programs rely on extensive intercrossing of elite cultivars derived from these early hybrids (Lakshmanan et al. 2005). Other genera, such as *Erianthus*, *Miscanthus*, *Narenga* and *Sclerostachya* are closely related to the *Saccharum* genus and constitute with it an interbreeding group which is termed as "*Saccharum* complex."

To develop or to improve a plant the understanding of the plant genome is the first and necessary step. This stands good for sugarcane also. This chapter will discuss about progress made so far in understanding sugarcane genome and challenges faced by the researchers while trying to unravel the genome of sugarcane.

3.2 Sugarcane Diversity

Based on conventional taxonomy, the genus *Saccharum* includes six species including *S. spontaneum*, *S. robustum*, *S. officinarum*, *S. barberi*, *S. sinense* and *S. edule*. The most primitive of *Saccharum* species is *S. spontaneum* that has its origin and center of diversity in India and its chromosome number ($2n = 40$) is least of all other *Saccharum* species. *S. robustum* is said to have derived from introgression of *S. spontaneum* with other genera in Wallacea and New Guinea. *S. officinarum* ($2n$ = 80, noble canes) cultivated for thick juicy canes with high sucrose and low fiber is distributed in South East Asia and New Guinea. The center of diversity is New Guinea where nearly 1000 clones have been collected. However, center of origin is doubtful, there being two opinions: (a) that it originated from *S. robustum* due to natural and human selection in Wallacea/New Guinea (Grassl 1974, 1977) and (b) that it evolved from *S. spontaneum*, *Miscanthus* and *Erianthus arundinaceus* (Daniels and Roach 1987). *S. barberi* ($2n$ = 81–154) in India and *S. sinense* ($2n$ = 116–120) in China were used for production of sugar for very long time because these species included clones which well adapted to climate and had tolerance to extreme temperature, drought and water logging. It is believed that extraction of sugar was developed from these canes (Daniels and Daniels 1975). *S. edule*, a separate species, a group of clones in which if the inflorescencey is aborted, results in a cauliflower like delicacy (Sreenivasan et al. 1987)**.** Number of native species identified in different places are viz., 25 (Asia), 6 (North America), 4 (Central America), 2 (Africa) and 1 (Australia). The native Brazilian species were identified as *S. villosum, S. asperum* and *S. baldwinii* (Kumar and Kumar 2016).

3.3 Complexity of Sugarcane Genome

Sugarcane is an economically important crop used as production of sugar and ethanol (Hofsetz and Silva 2012) and electricity generation and paper production (Hassuani et al. 2005) and in recent years sugarcane is also used to produce other bio-products like paper (Chandel et al. 2012). There is an increase in demand for renewable sources of energy. To meet this increasing demand, the production of sugarcane should increase without compromising other land uses (Valdes 2011), thus requiring new and better varieties. Researchers around the world had been working for improving the cane yields, sugar content, rationing ability, maintained or improved biotic and abiotic resistance and maintaining acceptable fiber levels for milling (Jackson 2005). To achieve this many crosses between *S. officinarum* and *S. spontaneum* were carried out; as a result of this, modern sugarcane cultivars are polyploidy and aneuploid hybrids with unequal genome contribution of about 80–90% from *S. officinarum*, 10–20% from *S. spontaneum* parental genomes and a small percentage of recombinant chromosomes (Piperidis et al. 2010; D'Hont 2005).

Sugarcane hybrids have ploidy levels of 10 or more and have a large and complex genome size of about 10GB. The genome structure is highly polyploidy and aneuploid with complete set of homologous genes predicted to range from 10 to 12 copies (alleles) (Souza et al. 2011). Sugarcane cultivars have a relatively high level of genetic diversity probably due to their heterozygous nature and high polyploidy. The introgression of the highly polymorphic *S. spontaneum* genome is the major cause of genetic variability among modern sugarcane cultivars (D'Hont et al. 1996).

Even though sugarcane being an economically important plant very little genome-based research has been carried out by the scientist due to its large and complex genome. During the past two decades progress was made in the field of sugarcane genomics to overcome the difficulties in studying a complex genomics of non-model plant.

3.4 Achievements in Sugarcane Genomics

Conventional breeding technique to improve or develop sugarcane varieties takes long time (almost 14 years) and selection of required trait is very difficult. Genetics and genome studies have given a better path to develop a better variety. Sugarcane genetics has received comparatively little interest compared to other crops, because of its heterozygous nature, complex genome, poor fertility and the long breeding/selection cycle (Singh et al. 2014). In spite of these complexities, sugarcane genome is successfully studied and in recent past good progress has been made in genome sequencing strategy. A vast array of genomic tools has been developed; these have opened new ways to study the genetic architecture of sugarcane and to analyze its functional system (Ul Haq et al. 2016).

Molecular marker is the first molecular tool used and developed to understand the genetic constitution and gene information of an organism. Molecular markers, namely Restriction fragment length polymorphisms (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplification of Polymorphic DNA (RAPD), Sequence-Related Amplified Polymorphism (SRAP), Target Region Amplification Polymorphism (TRAP) and Simple Sequence Repeats (SSR), are valuable tool in the studies of complex genomes such as sugarcane (Daugrois et al. 1996). Molecular markers are the best assistants a breeder can get as it can screen the plant at any stage and for any desirable traits in a breeding program (Ardiel et al. 2002). Molecular genetic markers are valuable tool which reduces the time for developing new varieties by selecting important or required traits during early stages of a breeding program and by allowing the selection of best parents in a crossing program (Pinto et al. 2004). Reports show the importance of markers in assessment of sugarcane resistance to diseases, evaluation of genetic diversity and construction of genetic maps (Hotta et al. 2010). Other methods to study the genome of the sugarcane are FISH, GISH, bacterial BAC libraries, identification of quantitative trait loci (QTLs) and EST collections. Day by day different methods are employed and researchers around the world are successfully able to study the complex genome of sugarcane.

In 1998 a work based on molecular analysis was initiated to study genetic relationship among *Saccharum* species clones (Hemaprabha 1998). Restriction fragment length polymorphisms (RFLP) can be used for construction of genetic map, gene tagging, map-based cloning and assessment of genetic variability (Prince et al. 1992). D'Hont et al. (1995) studied sugarcane hybrids (*Saccharum officinarum* × *Erianthus arundinaceus*) using RFLP and genomic DNA in-situ hybridization (GISH). These techniques were used to characterize and differentiate the parental genomes in interspecific hybrids and then application to the analysis of the contribution of these two species to the sugarcane cultivar R570. *Saccharum* complex diversity was studied using isozyme variation and RFLPs in 1980s. After studying 39 wild and noble sugarcane clones it was seen that *Erianthus* clone was strongly differentiated from all *Saccharum* clones, it displayed a unique pattern for most enzymes. A strong molecular differentiation was detected between the *Saccharum* species and *E. arundinaceus*, allowing for the identification of *E. arundinaceus* specific bands. Then it was confirmed that, using these techniques, intergeneric hybrids can be efficiently identified by the presence of *Saccharum* and *Erianthus* specific isozyme or DNA markers (Glaszmann et al. 1989). It was also proved that the RFLPs are efficient for sugarcane genetic diversity and taxonomy study (Lu et al. 1994).

RAPD was used to analyze genetic diversity of *Saccharum* complex in which it was seen that *Saccharum*, *Narenga* and *Sclerostachya* were less diverse and at the same time *Erianthus* was significantly diverse in the *Saccharum* complex. This result proved that RAPD markers can be used in divergence study in closely related *Saccharum* complex as it went hand in hand with already existing data generated by RFLP markers in early 1990s (Nair et al. 1999). RAPD banding patterns usually represent the entire genome, whereas the STMS patterns are generated from the microsatellite regions only and both markers don't require any prior information about the target genome. A comparative study with both RAPD and STMS primers on 23 high biomass producing sugarcane hybrids showed that polymorphic information content (PIC) varied from 0.121 to 0.631, with an average of 0.447 and genetic similarity between cultivars varied from 0.542 to 0.844 for RAPD. At the same time PIC varied 0.195 to 0.663, with an average of 0.526 and genetic similarity between cultivars varied from 0.478 to 0.874 for STMS markers. This study showed STMS markers as a good and more efficient tool to discriminate genotypes of sugarcane hybrids with unique DNA fingerprints (Saravanakumar et al. 2014). Twenty-three sugarcane hybrids were analyzed using STMS and RAPD markers for identification of high biomass hybrids (Kumar et al. 2014). AFLP was used to understand the molecular diversity among 421 clones of cultivated sugarcane and wild sugarcane (*S. officinarum*) in which five AFLP primers generated 614 polymorphic markers out of 657 markers. This study also stands as a proof for the hypothesis that New Guinea is the center of origin of *S. officinarum* (Aitken et al. 2006a).

Using AFLP markers genetic fingerprinting of Indian commercial sugarcane cultivars were preformed to distinguish tropical and subtropical Indian sugarcane cultivars; as a result, it was shown that a single AFLP marker can be effective to fingerprinting of different varieties (Selvi et al. 2005). Another study involving 30 clones belonging to *Saccharum* complex showed that AFLP gave enhanced grouping

than that of RAPD and RFLP results since AFLP can resolve closely related members of *Saccharum* complex into distinct groups (Selvi et al. 2006).

Different molecular markers technologies such as RAPD, AFLP, RFLP and SSR have employed to develop genetic maps for *S. spontaneum*, *S. officinarum* and *S. robustum*, the ancestral species. Simple Sequence Repeats (SSRs) or Microsatellites are most preferred among the molecular markers due to its multi-allelic nature, high reproducibility, cross transferability, co-dominant inheritance, abundance and extensive genome coverage (Agarwal et al. 2008; Ul Haq et al. 2014; Parida et al. 2010). SSRs are short repeated nucleotide sequences from one to six bases in length which produces high degree of polymorphism based on the difference in the number of DNA repeat motif at loci. In both animal and plant kingdoms SSRs are used for genome mapping, fingerprinting, population and evolutionary studies. Since SSRs have the ability to reveal high allelic diversity it is widely used to distinguish between genotypes. SSRs proved to be more effective than RAPD marker in identifying intergeneric hybrids, especially in characterizing *Saccharum* × *Erianthus* hybrids (Nair et al. 2006a, b). Using SSR markers a set of *Erianthus* specific markers were developed which can be used to study and monitor the introgression of *Erianthus* genome in hybrids of sugarcane (Selvi et al. 2006). Using STMS markers 36 sugarcane varieties were analyzed; as a result, new STMS markers for different varieties were obtained and it was also seen that less genetic alterations were caused due to somaclonal variation compared to induced mutation (Hemaprabha et al. 2006).

TRAP is a simple PCR-based marker technique which uses EST or gene information to generate polymorphism (Hu and Vick 2003). A fixed primer of about 18 nucleotides is designed from EST sequences or genes of interest and another primer will be an arbitrary primer of about the same length designed with either an AT- or GC-rich motif to anneal with an intron or exon, respectively (Hu and Vick 2003). TRAP marker was used to evaluate their effectiveness for assessing genetic diversity among 30 genotypes from the *Saccharum* complex (Alwala et al. 2006). Six fixed primers along with three arbitrary primers were used in this study. Three arbitrary primers used were designed from sucrose and cold tolerance related EST sequences. Analysis proved that the taxonomical classification of *Erianthus* spp. and *Miscanthus* spp. as different genera is accurate and *S. spontaneum* falls in one group since it is low sucrose and cold tolerant species. As a result of this study it was clear that TRAP can be useful marker technique for genetic diversity studies in sugarcane. TRAP was also used to identify sucrose specific candidate genes by studying *Erianthus* spp., *Saccharum officinarum* and *Saccharum spontaneum* clones (Hemaprabha and Lavanya 2015).

Day by day there is new advancement in biotechnological technique to understand the genome of sugarcane. One such advancement in the field of molecular markers is Sequence-Related Amplified Polymorphism (SRAP) markers. In this method primers targeting to open reading frames for selective amplification of coding regions of DNA are used (Li and Quiros 2001). This method gained attention within short time because it is highly robust and efficient with less technical demand and suitable for direct application in crops where the genome sequence is not available. For that reason it has been widely applied and validated across genera (Aneja

et al. 2012; Robarts and Wolfe 2014). Combinatorial application of TRAP and SRAP markers has been demonstrated to study genetic variability and facilitated parental selection in breeding programs in a complex polyploidy guarana plant (da Silva et al. 2016). In sugarcane TRAP and SRAP were combined together for the first time to characterize the mutant germplasm developed through radiation induced mutagenesis in sugarcane. Total of 57 markers (27 TRAP and 30 SRAP markers) were used for molecular marker profiling that was carried out to validate the extent of genetic variability in the sugarcane mutants induced by gamma rays (Mirajkar et al. 2017).

In late 1990s fluorescent in situ hybridization (FISH) technique was used to physically map two *S. officinarum* and three *S. robustum* clones (D'Hont et al. 1996, 1998; Ha et al. 1999). To physically map 45SrRNA and 5 s genes were used. Later using molecular cytogenetic technique of FISH many other clones were studied. FISH is used to study the basic chromosome number of sugarcane (Thumjamras et al. 2013). FISH analyses on different cultivars revealed that they contain 10–23% of *S. spontaneum* chromosomes and 5–17% of recombinant chromosomes from parental genome. Using genomic in situ hybridization (GISH) analysis the introgression of the *E. arundinaceus* genome into sugarcane for more than one generation was studied. Chromosome composition in three generations of *Saccharum* × *Erianthus* intergeneric hybrids: F1 (*Saccharum officinarum* × *Erianthus arundinaceus*), BC1 (F1 × sugarcane cultivar) and BC2 (BC1 × sugarcane cultivar) (Piperidis et al. 2010) were studied. More recently Huang et al. (2015) investigated the introgression of the *E. arundinaceus* genome into sugarcane in the higher generations, intergeneric BC_2 and BC_3 progeny generated between *Saccharum* spp. and *E. arundinaceus* using GISH. This showed that BC2 and BC3 generations resulted from $n + n$ chromosome transmission. GISH has proved itself as a powerful and useful tool to differentiate the chromosomes of different genomes and to identify the true interspecific/intergeneric hybrids (Kumar and Kumar 2016).

Genetic linkage map construction and quantitative trait loci (QTL) mapping provide information about the genetic architecture of traits, linkage and pleiotropy (Zeng et al. 1999). Quantitative trait loci (QTLs) have been used to screen variety with high sugar content, sugar yield, disease resistance, etc. First QTL mapping study in sugarcane was carried by Sills et al. (1995). Since then continuous report of QTL mapping was studied around the world (Ming et al. 2001, 2002a, b; Hoarau et al. 2002; McIntyre et al. 2005; da Silva and Bressiani 2005; Reffay et al. 2005; Aitken et al. 2006b, 2008; Piperidis et al. 2008; Pastina et al. 2012; Singh et al. 2013). An important contribution to a better understanding of the genetic basis of economically useful traits in sugarcane was made possible when stable-effect QTL could be identified from different interaction effects such as QTL-by-location, QTL-by-harvest and QTL-by-harvest-by-location. Latest study in QTL mapping opened a new approach allowing the 1:2:1 segregation ratio (Costa et al. 2016). AFLP and SSR markers were used to generate 688 molecular markers. The genetic linkage map covered 4512.6 cM and had 118 linkage groups corresponding to 16 putative homology groups. Six QTL for stalk diameter, five QTL for stalk weight, four QTL for stalk height, five for fiber, two QTL for sucrose content, and three QTL for soluble solid content (BRIX), a total of 25 QTL were detected.

Diversity Arrays Technology (DArT) has allowed to generate and map large number markers in sugarcane. As DArT works better in complex polyploid crops and does not require any kind of sequence information, it is very useful in plants such as sugarcane where whole genome is yet to be sequenced (Wenzl et al. 2008). Genetic mapping using DArT markers produces accurate and reproducible data at lower cost (Schouten et al. 2012). DArT technology was first established in rice; following its success it was developed in more complex genome plants such as wheat, oat, barely and sugarcane (Heller-Uszynska et al. 2011). In DArT, a frequent cutting restriction enzyme such as PstI is used to restrict the genomic DNA, after which the restricted DNA fragment is ligated with an adapter and amplification is carried out using primer complementary to the adapter. DArT genotyping efficiently can discover and score hundreds of polymorphisms in the complex genome of sugarcane. Both genetic relationships and construction of genetic maps in sugarcane can be analyzed using DArT fingerprint (Heller-Uszynska et al. 2011).

A new approach to analyze the genomes of higher organisms was developed by cloning of exogenous DNA into bacterial artificial chromosome (BAC) (Shizuya et al. 1992). Compared to other large insert libraries, the advantages of BAC libraries are that they are relatively simple to develop, easy to handle and have a low frequency of chimerical clones. BAC libraries containing large genomic DNA inserts are important tools for positional cloning, physical mapping and genome sequencing (Choi and Wing 2000). Since BAC vectors have been developed it has been widely used in construction of large insert libraries from plant genomic DNA such as apple (Vinatzer et al. 1998), *Arabidopsis thaliana* (Choi et al. 1995), barley (Yu et al. 2000), cotton (Tomkins et al. 2001), lettuce (Frijters et al. 1997), maize (Tomkins et al. 2001), *Medicago truncatula* (Nam et al. 1999), pearl millet (Allouis et al. 2001), rice (Yang et al. 1997), *Sorghum* (Woo et al. 1994), soybean (Danesh et al. 1998) and tomato (Folkertsma et al. 1999). BAC library for the sugarcane cultivar R570 was carried out; as a result, 103,296 clones were obtained and it was analyzed with ten different RFLP mapping probes (Tomkins et al. 1999). Most recently a team of researchers from Brazil (de Mendonça Vilela et al. 2017) sequenced and analyzed 27 BACs of sugarcane variety R570. This study analyzed genomic regions of Leafy (LFY), Phytochrome C (PHYC) and target of rapamycin kinase (TOR) as they play vital role on plant development and are members of complex regulatory networks. All three genes LFY, PHYC and TOR are present in sugarcane as single-copy genes similar to several grasses.

The use of next generation sequencing (NGS) such as 454 Life Sciences Genome Sequencer FLX is more cost effective than earlier sequencing for SNP identification and can significantly increase the identification of SNPs in sugarcane (Bundock et al. 2009). Whole genome of sugarcane being not yet well established, RNA-sequencing (transcriptome study) is the best way to understand the mechanisms involved in different stress responses. In sugarcane transcriptome study was first undertaken in South Africa (Carson and Botha 2000, 2002). Since then there were many group using transcriptomics as a tool to study the sugarcane genome. Recently transcriptome profiling of *S. spontaneum* clone IND 00-1037 under low

Table 3.1 The approximate number of clones that have been sequenced within SUGESI (Aitken et al. 2016)

Institution	Project lead	Country	Number of BAC clones sequenced
CSIRO	Karen Aitken	Australia	987
UQ-QAAFI	Robert Henry	Australia	500
University of São Paulo	Marie-Anne Van Sluys	Brazil	300
SASRI	Bernard Potier	South Africa	450
CIRAD	Angelique D'Hont	France	530
Total			**2767**

temperature was studied. In this study, there was 214,611 unigenes with an average length of 801 bp and differential gene expression analysis revealed that during the stress 2583 genes were upregulated and 3302 genes were downregulated (Dharshini et al. 2016).

The Sugarcane Genome Sequencing Initiative (SUGESI) was formed in 2009 by a group of researchers from different countries especially from Australia, Brazil, South Africa and France in a meet held at Port Douglas, Queensland, to develop a strategy for generation of a sugarcane genome sequence. It was determined that R570 variety genome will be sequenced by using BAC library method (Aitken et al. 2016). Table 3.1 shows the list of approximate number of clones that have been sequenced within SUGESI.

Whole genome shotgun (WGS) was a relatively new technique which is being used for sequencing. WGS methods pose a hindrance in sequencing sugarcane because of its complex genome and high proportion of repetitive sequences. These issues can be minimized by using gene enrichment strategies which is done by using methyl filtration with McrBC endonuclease digestion and the methyl-filtered along with unfiltered libraries can be compared, assembled and studied (Palmer et al. 2003). This strategy was used to sequence gene regions of maize and *Sorghum* (Palmer et al. 2003; Bedell et al. 2005). In sugarcane, methyl filtration allowed a better assembly by filtering out 35% of the sugarcane genome and by producing 1.53 more scaffolds and 1.73 more assembled length compared with unfiltered data-set (Grativol et al. 2014).

Researchers in Brazil have developed SUCEST database which contains large number of sugarcane expressed sequence tags (ESTs) around the world. Small collection of sugarcane ESTs generated from both meristematic region and stem from the cultivar NCO376 (Carson and Botha 2000, 2002) in South Africa gave a start to sugarcane EST collection. The largest collection of sugarcane ESTs was generated by SUCEST project. This project was started by Brazil ONSA consortium (Organization for Nucleotide Sequencing and Analysis) (Simpson and Perez 1998). As a result of this project, 237,954 sugarcane ESTs from 27 cDNA libraries were obtained which provided the researchers with preliminary view into the gene expression profile of sugarcane (Vettore et al. 2001, 2003). Apart from researchers in Brazil and South Africa, other researchers from different countries have also collected sug-

arcane ESTs from other sugarcane varieties such as Australia from cultivar Q117 (Casu et al. 2003), the United States of America from CP72-2086 (Ma et al. 2004) and from CoS 767 and Co 1148 by researchers in India (Gupta et al. 2010).

3.5 Bioinformatics

As on 25th January 2017 an organism search, using "*Saccharum*," has retrieved 13,231 nucleotide sequences, 285,216 expressed sequence tags (ESTs) and 83,138 genome survey sequences (GSS) whereas on 31st August 2009 there were only 1472 nucleotide sequences, 256,895 ESTs and 10,699 GSSs (Henry and Kole 2010). This shows the increasing data generated by the researchers around the world. Day-to-day advancement in technologies and new approaches to study the sugarcane and other plant genome made possible to generate a vast amount of molecular data. These large amounts of data have to be stored, organized, analyzed and made available for researchers around the world. Bioinformaticians around the world are working to develop and maintain databases in which the plant genome can be organized properly and easy to retrieve for future use.

There are different types of database which provides genome information of different plants along with sugarcane genome; following are some database in use:

National Center for Biotechnology Information (NCBI) was established on 1988 at National institutes of Health (NIH). NCBI contains gene information, nucleotide sequence, protein sequence, all other information about a gene. Researchers around the world submit their newly identified sequence in NCBI (Genbank) which can be accessed around the world. Phytozome is a plant comparative genomics portal maintained by Department of Energy's Joint Genome Institute. Other comparative genomics databases are GreenPhylDB, Plaza and PlantGDB. Plant genome databases for specific plant have been developed such as TAIR, Gramene, SGN, GDR and LIS specific for *Arabidopsis*, grasses, *Solanaceae*, *Rosaceae* and legumes (Swarbreck et al. 2008; Liang et al. 2008; Bombarely et al. 2011; Jung et al. 2008; Gonzales et al. 2005).

Same way SUCEST-FUN database is specific database for sugarcane. The SUCEST-FUN database is therefore being developed to give access to genomic and EST gene sequences, gene expression studies and make available tools that will allow a Systems Biology approach in sugarcane and the identification of regulatory networks. The SUCEST-FUN database has become a new tool for analyzing the sugarcane genome and functional genomics studies (Nishiyama et al. 2010). The SUCEST-FUN database will integrate the SUCEST sequences, promoters, CREs, expression data, agronomical, physiological, and biochemical characterization of sugarcane cultivars. It assembles different sugarcane databases such as the Sugarcane Expressed Sequence Tags (SUCEST) Genome Project (Vettore et al. 2003), the Sugarcane Gene Index (SGI), the SUCAST and the SUCAMET Catalogues, which include expression data, the GRASSIUS database (Yilmaz et al. 2009) and records of the agronomic, physiological, and biochemical characteristics of sugarcane culti-

vars. This database is part of the SUCEST-FUN Regulatory Network Project, which aims to study gene expression regulation through the use of tools that will allow a Systems Biology approach to the study of sugarcane.

3.6 Conclusion

Sugarcane is an important tropical and subtropical crop which is the major source of sugar for many years. Most of the development in the sugarcane crop is due to conventional breeding carried out by breeders. But in recent years sugarcane is been used in the production of biofuel too, which in turn increases its demand not only in food or domestic sector but as well as in industrial sector. Sugarcane yield needs to be doubled without increasing its cultivable area so that it doesn't affect the production of other crops. To meet these demands it is essential to understand sugarcane crop at its genome level. There is a tremendous growth in genome level study in sugarcane plants in last two decades due to advancement in biotechnological tools such as BAC library data and transcriptome. But still there is hindrance in studying this complex crop such as restricted access to already sequenced and available data in SUCEST database. Even though SUCEST database is the major database for sugarcane, it can be accessed only by certain researchers in certain countries. If it is made globally available, it will be very useful for scientists all around the world to exploit those data for the betterment of sugarcane research. Genetic fingerprints have limitations, large number of polymorphic markers are required to cover full genome, present day fingerprinting technique is labor intensive, result can vary and be expensive. To overcome these limitations DArT is the better method as it is sequence-independent genotyping method and it can generate genome-wide genetic fingerprints. DArT also works well in polyploidy crops like sugarcane. Whole genome shotgun sequencing is less costly method to sequence the whole genome of plants but this sequencing method is not effective in sugarcane due to its aneuploidy nature. With multiple homo/homeoalleles at each locus it is very difficult to assemble using shotgun sequencing, as reads arising from homeoalleles would collapse, making it difficult to recover large consensus sequences or contigs and only partial genome sequence can be aligned. These problems have to be solved in a better way or a new approach to sequence whole genome of sugarcane has to be developed and only then whole genome sequencing could be possible.

References

Agarwal M, Shrivastava N, Padh H (2008) Advances in molecular marker techniques and their applications in plant sciences. Plant Cell Rep 27(4):617–631

Aitken K, Paul B, Anne R (2016) The first sugarcane genome assembly: how can we use it? In: Proceedings of the Australian Society Sugar Cane Technology, p 38

Aitken KS, Hermann S, Karno K, Bonnett GD, McIntyre LC, Jackson PA (2008) Genetic control of yield related stalk traits in sugarcane. Theor Appl Gen 117(7):1191–1203
Aitken KS, Li JC, Jackson P, Piperidis G, McIntyre CL (2006a) AFLP analysis of genetic diversity within *Saccharum officinarum* and comparison with sugarcane cultivars. Crop Pasture Sci 57(11):1167–1184
Aitken KS, Jackson PA, McIntyre CL (2006b) Quantitative trait loci identified for sugar related traits in a sugarcane (*Saccharum* spp) cultivar × *Saccharum officinarum* population. Theor Appl Gen 112(7):1306–1317
Allouis S, Qi X, Lindup S, Gale MD, Devos KM (2001) Construction of a BAC library of pearl millet, *Pennisetum glaucum*. Theor Appl Gen 102(8):1200–1205
Alwala S, Suman A, Arro JA, Veremis JC, Kimbeng CA (2006) Target region amplification polymorphism (TRAP) for assessing genetic diversity in sugarcane germplasm collections. Crop Sci 46(1):448–455
Aneja B, Yadav NR, Chawla V, Yadav RC (2012) Sequence-related amplified polymorphism (SRAP) molecular marker system and its applications in crop improvement. Mol Breed 30(4):1635–1648
Ardiel GS, Grewal TS, Deberdt P, Rossnagel BG, Scoles GJ (2002) Inheritance of resistance to covered smut in barley and development of a tightly linked SCAR marker. Theor Appl Gen 104(2–3):457–464
Bedell JA, Budiman MA, Nunberg A, Citek RW, Robbins D, Jones J, Flick E, Rohlfing T, Fries J, Bradford K, McMenamy J (2005) Sorghum genome sequencing by methylation filtration. PLoS Biol 3(1):13
Bombarely A, Menda N, Tecle IY, Buels RM, Strickler S, Fischer-York T, Pujar A, Leto J, Gosselin J, Mueller LA (2011) The sol genomics network (solgenomics net): growing tomatoes using Perl. Nucleic Acids Res 39(Database):D1149–D1155
Bundock PC, Eliott FG, Ablett G, Benson AD, Casu RE, Aitken KS, Henry RJ (2009) Targeted single nucleotide polymorphism (SNP) discovery in a highly polyploid plant species using 454 sequencing. Plant Biotechnol J 7(4):347–354
Carson D, Botha F (2002) Genes expressed in sugarcane maturing internodal tissue. Plant Cell Rep 20(11):1075–1081
Carson DL, Botha FC (2000) Preliminary analysis of expressed sequence tags for sugarcane. Crop Sci 40(6):1769–1779
Casu RE, Grof CP, Rae AL, McIntyre CL, Dimmock CM, Manners JM (2003) Identification of a novel sugar transporter homologue strongly expressed in maturing stem vascular tissues of sugarcane by expressed sequence tag and microarray analysis. Plant Mol Biol 52(2):371–386
Chandel AK, da Silva SS, Carvalho W, Singh OV (2012) Sugarcane bagasse and leaves: foreseeable biomass of biofuel and bio-products. J Chem Technol Biotechnol 87(1):11–20
Choi S, Wing RA (2000) The construction of bacterial artificial chromosome (BAC) libraries in plant molecular biology manual. Springer, Dordrecht, pp 1–28
Choi S, Creelman RA, Mullet JE, Wing RA (1995) Construction and characterization of a bacterial artificial chromosome library of *Arabidopsis thaliana*. Plant Mol Biol Rep 13(2):124–128
Costa EA, Anoni CO, Mancini MC, Santos FRC, Marconi TG, Gazaffi R, Pastina MM, Perecin D, Mollinari M, Xavier MA, Pinto LR (2016) QTL mapping including codominant SNP markers with ploidy level information in a sugarcane progeny. Euphytica 211(1):1–16
D'Hont A (2005) Unraveling the genome structure of polyploids using FISH and GISH; examples of sugarcane and banana. Cytogenet Genome Res 109(1–3):27–33
da Silva EF, de Sousa SB, da Silva GF, Sousa NR, do Nascimento Filho FJ, Hanada RE (2016) TRAP and SRAP markers to find genetic variability in complex polyploid *Paullinia cupana* var *sorbilis*. Plant Gene 6:43–47
Danesh D, Penuela S, Mudge J, Denny RL, Nordstrom H, Martinez JP, Young ND (1998) A bacterial artificial chromosome library for soybean and identification of clones near a major cyst nematode resistance gene. Theor Appl Gen 96(2):196–202
Daniels J, Daniels C (1975) Geographical, historical and cultural aspect of the origin of the Indian and Chinese sugarcane *S barberi* and *S sinense*. Sugarcane Breed Newsl 36:4–23

Daniels J, Roach BT (1987) Taxonomy and evolution sugarcane improvement through breeding, vol 7. Elsevier, Amsterdam, pp 7–84

Daugrois JH, Grivet L, Roques D, Hoarau JY, Lombard H, Glaszmann JC, D'Hont A (1996) A putative major gene for rust resistance linked with a RFLP marker in sugarcane cultivar 'R570'. Theor Appl Gen 92(8):1059–1064

de Mendonça Vilela M, Del-Bem LE, Van Sluys MA, de Setta N, Kitajima JP, Cruz GMQ, Sforça DA, de Souza AP, Ferreira PCG, Grativol C, Cardoso-Silva CB (2017) Analysis of three sugarcane homo/homeologous regions suggests independent polyploidization events of *Saccharum officinarum* and *Saccharum spontaneum*. Genome Biol Evol 9(2):266–278

De Setta N, Monteiro-Vitorello CB, Metcalfe CJ, Cruz GMQ, Del Bem LE, Vicentini R, Nogueira FTS, Campos RA, Nunes SL, Turrini PCG, Vieira AP (2014) Building the sugarcane genome for biotechnology and identifying evolutionary trends. BMC Genomics 15(1):540

Dharshini S, Chakravarthi M, Ashwin Narayan J, Manoj VM, Naveenarani M, Kumar R, Meena M, Ram B, Appunu C (2016) De novo sequencing and transcriptome analysis of a low temperature tolerant *Saccharum spontaneum* clone IND 00-1037. J Biotechnol 231:280–294

D'Hont A, Grivet L, Feldmann P, Glaszmann JC, Rao S, Berding N (1996) Characterisation of the double genome structure of modern sugarcane cultivars (*Saccharum* spp.) by molecular cytogenetics. Mol Gen Genet 250(4):405–413

D'Hont A, Ison D, Alix K, Roux C, Glaszmann JC (1998) Determination of basic chromosome numbers in the genus *Saccharum* by physical mapping of ribosomal RNA genes. Genome 41(2):221–225

D'Hont A, Rao PS, Feldmann P, Grivet L, Islam-Faridi N, Taylor P, Glaszmann JC (1995) Identification and characterisation of sugarcane intergeneric hybrids, *Saccharum officinarum* × *Erianthus arundinaceus* with molecular markers and DNA in situ hybridization. Theor Appl Gen 91(2):320–326

Folkertsma RT, Spassova MI, Prins M, Stevens MR, Hille J, Goldbach RW (1999) Construction of a bacterial artificial chromosome (BAC) library of *Lycopersicon esculentum* cv Stevens and its application to physically map the Sw-5 locus. Mol Breed 5(2):197–207

Frijters ACJ, Zhang Z, van Damme M, Wang GL, Ronald PC, Michelmore RW (1997) Construction of a bacterial artificial chromosome library containing large Eco RI and Hin dIII genomic fragments of lettuce. Theor Appl Gen 94(3-4):390–399

Glaszmann JC, Noyer JL, Fautret A, Feldmann R, Lanaud C (1989) Biochemical genetic markers in sugarcane. Theor Appl Gen 78(4):537–543

Gonzales MD, Archuleta E, Farmer A, Gajendran K, Grant D, Shoemaker R, Beavis WD, Waugh ME (2005) The legume information system (LIS): an integrated information resource for comparative legume biology. Nucleic Acids Res 33(1):D660–D665

Grassl CO (1974) The origin of sugarcane. Sugarcane Breed Newsl 34:1018

Grassl CO (1977) The origin of the sugarcane producing cultivar of *Saccharum*. Sugarcane Breed Newsl 39:8–33

Grativol C, Regulski M, Bertalan M, McCombie WR, Silva FR, Zerlotini Neto A, Vicentini R, Farinelli L, Hemerly AS, Martienssen RA, Ferreira PCG (2014) Sugarcane genome sequencing by methylation filtration provides tools for genomic research in the genus *Saccharum*. Plant J 79(1):162–172

Gupta V, Raghuvanshi S, Gupta A, Saini N, Gaur A, Khan MS, Gupta RS, Singh J, Duttamajumder SK, Srivastava S, Suman A (2010) The water-deficit stress-and red-rot-related genes in sugarcane. Funct Integr Genomics 10(2):207–214

Ha S, Moore P, Heinz D, Kato S, Ohmido N, Fukui K (1999) Quantitative chromosome map of the polyploidy *Saccharum spontaneum* by multifluorescence in situ hybridization and imagine methods. Plant Mol Biol 39(6):1165–1173

Hassuani SJ, Da Silva JEAR, Neves JLM (2005) Sugarcane trash recovery alternatives for power generation. Proc Int Soc Sug Cane Technol 25:394–402

Heller-Uszynska K, Uszynski G, Huttner E, Evers M, Carlig J, Caig V, Aitken K, Jackson P, Piperidis G, Cox M, Gilmour R (2011) Diversity arrays technology effectively reveals DNA polymorphism in a large and complex genome of sugarcane. Mol Breed 28(1):37–55

Hemaprabha G (1998) Genetic relationship among *Saccharum* species clones through RAPD analysis. In: First national plant breeding congress, Coimbatore, India, p 78
Hemaprabha G, Lavanya DL (2015) Sucrose specific TRAP markers as genus and species specific markers in *Saccharum* and *Erianthus* spp. Indian J Genet Plant Breed 75(1):99–104
Hemaprabha G, Govindaraj P, Singh NK (2006) STMS markers for fingerprinting of varieties and genotypes sugarcane (*Saccharum* spp). Indian J Genet Plant Breed 66(2):95–99
Henry RJ, Kole C (2010) Genetics, genomics and breeding of sugarcane. CRC Press, Boca Raton
Hoarau JY, Grivet L, Offmann B, Raboin LM, Diorflar JP, Payet J, Hellmann M, D'Hont A, Glaszmann JC (2002) Genetic dissection of a modern sugarcane cultivar (*Saccharum* spp.) II detection of QTLs for yield components. Theor Appl Gen 105(6–7):1027–1037
Hofsetz K, Silva MA (2012) Brazilian sugarcane bagasse: energy and non-energy consumption. Biomass Bioenergy 46:564–573
Hossain MM, Abdulla F (2015) Forecasting the sugarcane production in Bangladesh by ARIMA model. J Stat Appl Probab 4(2):297–303
Hotta CT, Lembke CG, Domingues DS, Ochoa EA, Cruz GM, Melotto-Passarin DM, Marconi TG, Santos MO, Mollinari M, Margarido GR, Crivellari AC (2010) The biotechnology roadmap for sugarcane improvement. Trop Plant Biol 3(2):75–87
Hu J, Vick BA (2003) Target region amplification polymorphism: a novel marker technique for plant genotyping. Plant Mol Biol Report 21(3):289–294
Huang Y, Wu J, Wang P, Lin Y, Fu C, Deng Z, Wang Q, Li Q, Chen R, Zhang M (2015) Characterization of chromosome inheritance of the intergeneric BC 2 and BC 3 progeny between *Saccharum* spp. and *Erianthus arundinaceus*. PLoS One 10(7):0133722
Jackson PA (2005) Breeding for improved sugar content in sugarcane. Field Crop Res 92(2–3):277–290
Jung S, Staton M, Lee T, Blenda A, Svancara R, Abbott A, Main D (2008) GDR (genome database for Rosaceae): integrated web-database for Rosaceae genomics and genetics data. Nucleic Acids Res 36(1):D1034–D1040
Kumar SS, Govindaraj P, Appunu C (2014) Morphological and molecular characterization of high biomass IGH, ISH and *Saccharum* hybrids. Sugar Tech 17(3):243–251
Kumar U, Kumar S (2016) Genetic improvement of sugarcane through conventional and molecular approaches. In: Molecular breeding for sustainable crop improvement. Springer, Cham, pp 325–342
Lakshmanan P, Geijskes RJ, Aitken KS, Grof CL, Bonnett GD, Smith GR (2005) Sugarcane biotechnology: the challenges and opportunities. In Vitro Cell Dev Biol Plant 41(4):345–363
Li G, Quiros CF (2001) Sequence related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor Appl Gen 103(2–3):455–461
Liang C, Jaiswal P, Hebbard C, Avraham S, Buckler ES, Casstevens T, Hurwitz B, McCouch S, Ni J, Pujar A, Ravenscroft D (2008) Gramene: a growing plant comparative genomics resource. Nucleic Acids Res 36(1):D947–D953
Lu YH, D'Hont A, Walker DIT, Rao PS, Feldmann P, Glaszmann JC (1994) Relationships among ancestral species of sugarcane revealed with RFLP using single-copy maize nuclear probes. Euphytica 78(1):7–18
Ma HM, Schulze S, Lee S, Yang M, Mirkov E, Irvine J, Moore P, Paterson A (2004) An EST survey of the sugarcane transcriptome. Theor Appl Gen 108(5):851–863
McIntyre CL, Whan VA, Croft B, Magarey R, Smith GR (2005) Identification and validation of molecular markers associated with Pachymetra root rot and brown rust resistance in sugarcane using map-and association-based approaches. Mol Breed 16(2):151–161
Ming R, Del Monte TA, Hernandez E, Moore PH, Irvine JE, Paterson AH (2002a) Comparative analysis of QTLs affecting plant height and flowering among closely-related diploid and polyploid genomes. Genome 45(5):794–803
Ming R, Wang Y, Draye X, Moore P, Irvine J, Paterson A (2002b) Molecular dissection of complex traits in autopolyploids: mapping QTLs affecting sugar yield and related traits in sugarcane. Theor Appl Gen 105(2–3):332–345

Ming R, Liu SC, Moore PH, Irvine JE, Paterson AH (2001) QTL analysis in a complex autopolyploid: genetic control of sugar content in sugarcane. Genome Res 11(12):2075–2084

Mirajkar SJ, Rai AN, Vaidya ER, Moharil MP, Dudhare MS, Suprasanna P (2017) TRAP and SRAP molecular marker based profiling of radiation induced mutants of sugarcane (*Saccharum officinarum* L.) Plant Gene 9:64–70

Nair NV, Selvi A, Sreenivasan TV, Pushpalatha KN, Mary S (2006a) Characterization of intergeneric hybrids of Saccharum using molecular markers. Genet Resour Crop Evol 53(1):163–169

Nair NV, Selvi A, Ramraj SS, Pandian KS (2006b) Use of SSR markers for the identification of interspecific and intergeneric hybrids of Saccharum in plant breeding in post genomics era. In: Proceedings of second national plant breeding congress, Coimbatore, India, Indian Society of Plant Breeders 1–3 March, pp 310–318

Nair NV, Nair S, Sreenivasan TV, Mohan M (1999) Analysis of genetic diversity and phylogeny in *Saccharum* and related genera using RAPD markers. Genet Resour Crop Evol 46(1):73–79

Nam YW, Penmetsa RV, Endre G, Uribe P, Kim D, Cook DR (1999) Construction of a bacterial artificial chromosome library of *Medicago truncatula* and identification of clones containing ethylene-response genes. Theor Appl Gen 98(3–4):638–646

Nishiyama MY Jr, Vicente FFR, Lembke CG, Sato PM, Dal-Bianco ML, Fandino RA, Hotta CT, Souza GM (2010) The SUCEST-FUN regulatory network database: designing an energy grass. Proc Int Soc Sugar Cane Technol 27:1–10

Palmer LE, Rabinowicz PD, O'Shaughnessy AL, Balija VS, Nascimento LU, Dike S, de la Bastide M, Martienssen RA, McCombie WR (2003) Maize genome sequencing by methylation filtration. Science 302(5653):2115–2117

Parida SK, Pandit A, Gaikwad K, Sharma TR, Srivastava PS, Singh NK, Mohapatra T (2010) Functionally relevant microsatellites in sugarcane unigenes. BMC Plant Biol 10(1):251

Pastina MM, Malosetti M, Gazaffi R, Mollinari M, Margarido GRA, Oliveira KM, Pinto LR, Souza AP, Van Eeuwijk FA, Garcia AAF (2012) A mixed model QTL analysis for sugarcane multiple-harvest-location trial data. Theor Appl Gen 124(5):835–849

Pinto LR, Oliveira KM, Ulian EC, Garcia AAF, De Souza AP (2004) Survey in the sugarcane expressed sequence tag database (SUCEST) for simple sequence repeats. Genome 47(5):795–804

Piperidis G, Piperidis N, D'Hont A (2010) Molecular cytogenetic investigation of chromosome composition and transmission in sugarcane. Mol Gen Genomics 284(1):65–73

Piperidis N, Jackson PA, D'Hont A, Besse P, Hoarau JY, Courtois B, Aitken KS, McIntyre CL (2008) Comparative genetics in sugarcane enables structured map enhancement and validation of marker-trait associations. Mol Breed 21(2):233–247

Prince JP, Loaiza-Figueroa F, Tanksley SD (1992) Restriction fragment length polymorphism and genetic distance among Mexican accessions of capsicum. Genome 35(5):726–732

Reffay N, Jackson PA, Aitken KS, Hoarau JY, D'Hont A, Besse P, McIntyre CL (2005) Characterisation of genome regions incorporated from an important wild relative into Australian sugarcane. Mol Breed 15(4):367–381

Roach BT (1989) Origin and improvement of the genetic base of sugarcane. Proc Australian Soc Sugar Cane Technol 10:34–47

Robarts DW, Wolfe AD (2014) Sequence-related amplified polymorphism (SRAP) markers: a potential resource for studies in plant molecular biology. Appl Plant Sci 2(7):1400017

Saravanakumar K, Govindaraj P, Appunu C, Senthilkumar S, Kumar R (2014) Analysis of genetic diversity in high biomass producing sugarcane hybrids (*Saccharum* spp complex) using RAPD and STMS markers. Indian J Biotechnol 13:214–220

Schouten HJ, Van de Weg WE, Carling J, Khan SA, McKay SJ, van Kaauwen MP, Wittenberg AH, Koehorst-van Putten HJ, Noordijk Y, Gao Z, Rees DJG (2012) Diversity arrays technology (DArT) markers in apple for genetic linkage maps. Mol Breed 29(3):645–660

Selvi A, Nair NV, Noyer JL, Singh NK, Balasundaram N, Bansal KC, Koundal KR, Mohapatra T (2005) Genomic constitution and genetic relationship among the tropical and subtropical Indian sugarcane cultivars revealed by AFLP. Crop Sci 45(5):1750–1757

Selvi A, Nair NV, Noyer JL, Singh NK, Balasundaram N, Bansal KC, Koundal KR, Mohapatra T (2006) AFLP analysis of the phenetic organization and genetic diversity in the sugarcane complex, *Saccharum* and *Erianthus*. Genet Resour Crop Evol 53(4):831–842

Shizuya H, Birren B, Kim U-J, Mancino V, Slepak T, Tachiri Y, Simon M (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using F-factor-based vector. Proc Natl Acad Sci U S A 89(18):8794–8797

Sills GR, Bridges W, Al-Janabi SM, Sobral BW (1995) Genetic analysis of agronomic traits in a cross between sugarcane (*Saccharum officinarum* L.) and its presumed progenitor (*S robustum* Brandes & Jesw ex Grassl). Mol Breed 1(4):355–363

da Silva JA, Bressiani JA (2005) Sucrose synthase molecular marker associated with sugar content in elite sugarcane progeny. Genet Mol Biol 28(2):294–298

Simpson AJ, Perez JF (1998) ONSA, the São Paulo virtual genomics institute. Nat Biotechnol 16(9):795–796

Singh R, Srivastava S, Verma AK, Singh B, Singh R (2014) Importance and progresses of microsatellite markers in sugarcane (*Saccharum* spp hybrids). Indian J Sugarcane Technol 29(01):1–12

Singh S, Singh RP, Bhavani S, Huerta-Espino J, Eugenio LVE (2013) QTL mapping of slow-rusting, adult plant resistance to race Ug99 of stem rust fungus in PBW343/Muu RIL population. Theor Appl Gen 126(5):1367–1375

Souza GM, Berges H, Bocs S, Casu R, D'Hont A, Ferreira JE, Henry R, Ming R, Potier B, Van Sluys MA, Vincentz M (2011) The sugarcane genome challenge: strategies for sequencing a highly complex genome. Trop Plant Biol 4(3–4):145–156

Sreenivasan TV, Ahloowalia BS, Heinz DJ (1987) Cytogenetics. In: Heinz DJ (ed) Sugarcane improvement through breeding. Elsevier, Amsterdam, pp 211–253

Swarbreck D, Wilks C, Lamesch P, Berardini TZ, Garcia-Hernandez M, Foerster H, Li D, Meyer T, Muller R, Ploetz L, Radenbaugh A (2008) The *Arabidopsis* information resource (TAIR): gene structure and function annotation. Nucleic Acids Res 36(1):D1009–D1014

Thumjamras S, de Jong H, Iamtham S, Prammanee S (2013) Sugar cane genome numbers assumption by ribosomal DNA FISH techniques. J Med Bioeng 2(4):248–251

Tomkins JP, Peterson DG, Yang TJ, Main D, Wilkins TA, Paterson AH, Wing RA (2001) Development of genomic resources for cotton (*Gossypium hirsutum* L) BAC library construction, preliminary STC analysis, and identification of clones associated with fiber development. Mol Breed 8(3):255–261

Tomkins JP, Yu Y, Miller-Smith H, Frisch DA, Woo SS, Wing RA (1999) A bacterial artificial chromosome library for sugarcane. Theor Appl Genet 99(3–4):419–424

Ul Haq S, Jain R, Sharma M, Kachhwaha S, Kothari SL (2014) Identification and characterization of microsatellites in expressed sequence tags and their cross transferability in different plants. Int J Genomics 2014(3):863948

Ul Haq S, Kumar P, Singh RK, Verma KS, Bhatt R, Sharma M, Kachhwaha S, Kothari SL (2016) Assessment of functional EST-SSR markers (sugarcane) in cross-species transferability, genetic diversity among poaceae plants and bulk segregation analysis. Genet Res Int 2016:7052323

Valdes C (2011) Brazil's ethanol industry: looking forward. BIO-02 outlook. United States Department of Agriculture, Economic Research. Service http://usda.mannlib.cornell.edu/usda/ers/BioEnergy/2010s/2011/BioEnergy-06-27-2011_Special_Report.pdf

Vettore AL, da Silva FR, Kemper EL, Souza GM, da Silva AM, Ferro MIT, Henrique-Silva F, Giglioti ÉA, Lemos MV, Coutinho LL, Nobrega MP (2003) Analysis and functional annotation of an expressed sequence tag collection for tropical crop sugarcane. Genome Res 13(12):2725–2735

Vettore AL, Silva FRD, Kemper EL, Arruda P (2001) The libraries that made SUCEST. Genet Mol Biol 24(1–4):1–7

Vinatzer BA, Zhang HB, Sansavini S (1998) Construction and characterization of a bacterial artificial chromosome library of apple. Theor Appl Gen 97(7):1183–1190

Wenzl P, Huttner E, Carling J, Xia L, Blois H, Caig V, Heller-Uszynska K, Jaccoud D, Hopper C, Aschenbrenner-Kilian G, Evers M (2008) Diversity Arrays Technology (DArT): a generic

high-density genotyping platform in safflower: unexploited potential and world adaptability 7th international safflower conference, Australia, Agri-MC marketing and communication, pp 1–7

Woo SS, Jiang J, Gill BS, Paterson AH, Wing RA (1994) Construction and characterization of bacterial artificial chromosome library of *Sorghum bicolor*. Nucleic Acids Res 22(23):4922–4931

Yang D, Parco A, Nandi S, Subudhi P, Zhu Y, Wang G, Huang N (1997) Construction of a bacterial artificial chromosome (BAC) library and identification of overlapping BAC clones with chromosome 4-specific RFLP markers in rice. Theor Appl Gen 95(7):1147–1154

Yilmaz A, Nishiyama MY, Fuentes BG, Souza GM, Janies D, Gray J, Grotewold E (2009) GRASSIUS: a platform for comparative regulatory genomics across the grasses. Plant Physiol 149(1):171–180

Yu Y, Tomkins JP, Waugh R, Frisch DA, Kudrna D, Kleinhofs A, Brueggeman RS, Muehlbauer GJ, Wise RP, Wing RA (2000) A bacterial artificial chromosome library for barley (*Hordeum vulgare* L.) and the identification of clones containing putative resistance genes. Theor Appl Gen 101(7):1093–1099

Zeng ZB, Kao CH, Basten CJ (1999) Estimating the genetic architecture of quantitative traits. Genet Res 74(3):279–289

Chapter 4
Methods of Sugarcane Transformation

S. Radhesh Krishnan and Chakravarthi Mohan

Abstract A considerable change has been evolved over the past two decades in the field of sugarcane (*Saccharum* spp.) research to improve its chief traits like sweetness, fiber content, and resistance to biotic and abiotic stresses through tissue culture and genetic engineering. With the increase in the global demand for sugar and bioethanol there exists a need for sustainable production necessitating research on crop improvement to resist abiotic and biotic stresses. These genetic improvisations for superior traits are of ultimate success only when suitable vector cassettes, gene transfer methods, and regeneration of whole plants are in place. This review discusses the recent advancements in sugarcane transformation methods with particular emphasis on biolistics and *Agrobacterium*-mediated methods in the recalcitrant monocot system, *Saccharum* spp. Moreover, recent advances like gene targeting by site-specific double-stranded nucleotide breaks for zinc-finger nucleases (ZFN), TAL effector nucleases (TALENs), and CRISPR (clustered regularly interspaced short palindrome repeats)/Cas 9 (CRISPR-associated) systems require a well-established transformation method which signifies the need for an efficient genetic transformation system in sugarcane.

Keywords *Agrobacterium* • Biolistic • Gene transfer • Selectable markers • Sugarcane • Transformation

4.1 Introduction

Sugarcane (*Saccharum officinarum* L.) is a monocot stick or bamboo composed of rich source of sugar. The complex aneuploidy nature with an approximate 80–120 chromosomes (D'Hont et al. 1998) makes it complex for genetic manipulation.

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C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_4

Wild species (*S. barberi*, *S. officinarum* L., and *S. sinense* Roxb.) of sugarcane provide a good source of novel genes with characteristic features like disease and abiotic stress resistance. Remarkable progress has been observed in the improvement of cane sugars by conventional methods of breeding (Berding et al. 1997; Hogarth et al. 1997). The cane sugar and its traits could be improved by involving elite cultivars (Cox and Hansen 1995). Integrated strategies with molecular and conventional breeding are prerequisite to develop sugarcane with desired traits so as to face the global challenge.

4.2 Sugarcane Transformation

Reliable and reproducible plant tissue culture protocol is necessary to develop transgenic crops with appropriate gene transfer system. Unlike monocots, tissue culture and transformation methods are well established in most of the dicot plants due to their in vitro responsiveness. Monocots generally require a stringent method and optimization of various factors for efficient delivery of desired gene and selection of positive transformants without any false positives.

The transformation protocol as such is the method to transfer the gene of interest to a desired host system without disturbing its genetic or metabolic functions. Wide applications are offered by biotechnology for selecting an efficient method or strategy to develop a reproducible and valid protocol for sugarcane crop improvement. Numerous factors are prerequisite for a successful transformation. Several factors like genotype of sugarcane, *Agrobacterium* strain, type of vector, method for transformation, and culture condition are influential which would be described in detail in Chap. 5. Use of embryogenic callus or auxiliary buds (Brumbley et al. 2008), age and type of sugarcane explant used (Arencibia et al. 1998), preconditioned meristematic sections (Enriquez-Obregon et al. 1998), and an efficient reporter system like *Gus* (Elliot et al. 1998) play a key role in effective transformation. Joyce et al. (2013) reported that cocultivation medium and selection system are critical in *Agrobacterium*-mediated transformation. The success of a protocol is decided based on its efficacy in transgene integration, number of integrated copies, and stability in subsequent generations.

4.3 Methods of Sugarcane Transformation

To obtain a high efficiency with a low-cost transformation numerous researches have been reported in the recent past (Rakoczy-Trojanowska 2002). Several genes have been transferred to sugarcane using a number of methods including particle bombardment (biolistic), *Agrobacterium*-mediated transformation, electroporation,

and PEG treatment although the first two methods are popular and widely used. Recently, Mayavan et al. (2015) demonstrated an *in planta* technique for sugarcane using *Agrobacterium*-mediated transformation. This section describes the various methods of sugarcane transformation.

4.3.1 Biolistic™ or Microprojectile-Mediated Transformation

One of the most widely used techniques for cereal transformation is biolistic or microprojectile-mediated particle bombardment by direct physical gene transfer which was first developed by Sanford (1988). DNA-coated microscopic metals like gold or tungsten (1–4 μm dia) are used as a carrier by hitting the explants explosively with a high velocity ranging in 300–600 m/s. Biolistic bombardment can be used for both transient and stable transformation. Modern machines use precise technology by using helium-driven, particle bombardment apparatus (PDS-1000/He) which is most commonly being used (Slater et al. 2003). This technique is simple and applicable to a diverse range of tissues targeted for sugarcane transformation (Birch and Franks 1991; Franks and Birch 1991; Lakshmanan et al. 2005). Being one of the most commonly used methods it has many advantages over others like rapid gene transfer to specific/nonspecific tissues with higher efficiency and no host limitations. It does not require any intrinsic vector requirements; hence transgene of any size can be used to bombard. However, it also has few demerits which include environmental safety concerns (unwanted vector sequences and antibiotic-resistance genes are introduced), multiple transgene insertions, and incidence of escapes. Nevertheless, this method has been the most popular method for sugarcane transformation so far.

4.3.2 Agrobacterium-*Mediated Sugarcane Transformation*

Agrobacterium-mediated transformation has been used in more than 90 plant species. The use of acetosyringone in the medium enabled the use of this method in monocots as well. The ease in use, low copy insertion, and low expenditure make this method popular for genetic transformation. Arencibia et al. (1998) produced the first sugarcane transgenics followed by several researchers for an array of input and output traits (Beyene et al. 2013). A simple well-established protocol (adapted from Arvinth et al. 2010) including the time required since transformation until transgenic sugarcane is described in Fig. 4.1. Based on this protocol, transgenic sugarcane lines could be obtained after 6 months from the day 1 of transformation. However, several critical factors determine the efficiency of this method including genotype, cocultivation method, vector, selectable marker, regeneration efficiency, use of additional techniques such as vacuum infiltration,

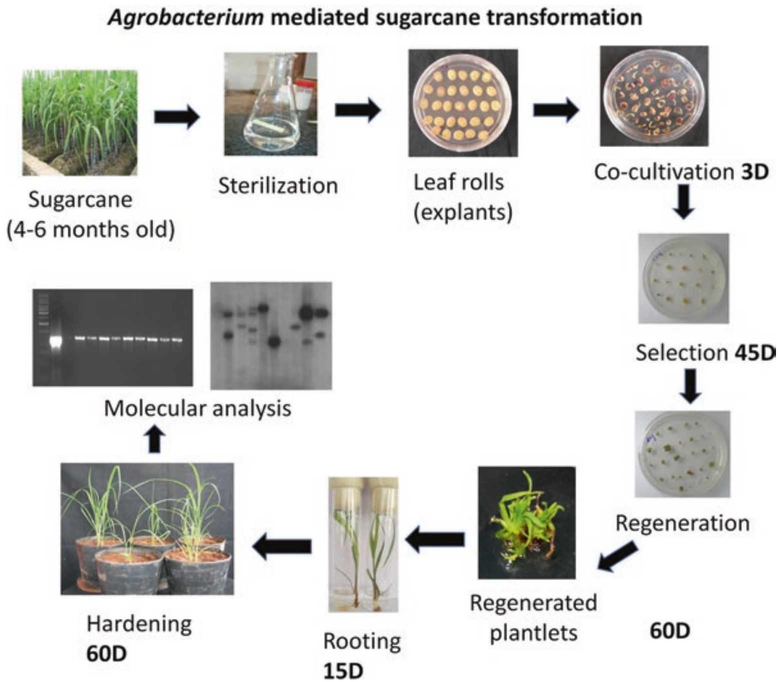

Fig. 4.1 Schematic representation of *Agrobacterium* mediated transformation in sugarcane

and promoters which have been well studied recently (Joyce et al. 2010, 2014). Several novel strategies and improved techniques have been reported in the recent years (described in Chap. 6) which would provide an enhanced transformation efficiency using this protocol.

4.3.3 Electroporation

In electroporation, the cells are subjected to an electric impulse with a high voltage for milliseconds so as to bring in a reverse permeability on the cell/plasma membrane eventually leading to uptake of a naked DNA or plasmid. Stable transformation in crops like sugarcane (Chen et al. 1987) using cell protoplast has been reported but failed in its regenerative ability to form a whole plant. They performed transformation using both electroporation and PEG treatment and observed that electroporation was more suitable. Stable transformation was successfully reported using electroporation (Chowdhury and Vasil 1992; Rathus and Birch 1992; Arencibia et al. 1992, 1995). A major advantage of this protocol is that the amount of DNA delivered to individual cells is low which reduces the copy numbers. Due to generation of electric impulse there exists an increase in the DNA uptake as the cells become hyperactive eventually

leading to an increased efficiency of the positive transformants. Thus the efficiency depends purely on the tissue treatment condition and electroporation. However, lack of regeneration from protoplasts remains a major disadvantage of this method.

4.3.4 *Microinjection*

Gene transfer to nucleus of the explant by immobilizing the protoplasts to substrates like agarose and thereby capillary-injection pipette-mediated delivery of foreign DNA was reported by Morikawa and Yamada (1985). Similarly efficient protoplast transformation was demonstrated by Reich et al. (1986) in *Medicago sativa* L. with 15–26% transformation frequency. Recently, Baskaran and Dasgupta (2012) demonstrated the delivery of transgene using *A. tumefaciens* using microinjection, directly to shoot apical meristems of rice. But there is a practical difficulty in the time and laborious method of choosing individual explant to inject the gene of interest unlike the biolistic/particle bombardment method. Bower and Birch (1992) reported the inefficacy to inject DNA injection or DNA-pollen mixtures to young floral sugarcane tillers. Apart from this, the presence of thick lignins and cellulose makes it harder to produce an efficient transformation. The release of vacuolar toxins like hydrolases hinders the cell to uptake and metabolize, eventually leading to cell death.

4.3.5 *Polyethylene Glycol-Mediated Transformation*

Polyethylene glycol (PEG)-mediated gene delivery system is one of the most widely used direct gene transfer methods. Plant protoplast, as it is devoid of cell walls, tends to uptake the naked DNA when treated with PEG. PEG is used with divalent cations that destabilize the cell membrane and eventually the naked DNA is taken up by the cell. Many groups reported development of transgenics using protoplast as the chief host system (Zhang and Wu 1988; Toriyama et al. 1988). This method requires careful optimization, as it has a low efficiency in producing transgenics with regenerable cell suspensions (Slater et al. 2003). In sugarcane, Chen et al. (1987) employed this method but it yielded very low transformation efficiency and poor reproducibility and so didn't receive much attention.

4.4 Promoter and Gene Expression

The success of plant transformation is decided by active markers, but the expression of a marker gene is controlled by the promoter under which the genes are linked. The use of an appropriate promoter is a prerequisite for an efficient gene expression and determines the strength and specificity of the gene expression (Chakravarthi

et al. 2015). CaMV35S promoter, though routinely used for heterologous transformation (Fang et al. 1989), confers lower expression in monocots (Jang et al. 2002). Rice *Act1* (Mcelroy et al. 1990; McElroy et al. 1991) and maize *Ubi1* (Christensen et al. 1992) are the predominantly used monocot promoters. These promoters could be changed, modified, and truncated or can be cloned at different sites depending upon the gene to be regulated. As far as sugarcane is concerned, maize *Ubi1* has been the workhorse promoter for over two decades due to its constitutive and consistent expression. Wei et al. (2003) reported ubiquitin promoters of sugarcane (*ubi4* and *ubi9*); however *Gus* expression was silenced in regenerated tissues using the promoters indicating their inability to regulate expression in stably transformed tissues. Several other promoters have also been isolated from sugarcane and its wild relatives and validated for expression. A detailed list of sugarcane-based promoters has been described in Chap. 6. Truncated promoters or promoters with intron sequences in between are of huge importance. Introns between the promoter sequence and coding sequence of ubiquitin genes have enhanced gene expression to a greater extent, in sugarcane (Philip et al. 2013; Chakravarthi et al. 2015). Recently, several reports on the use of high-expressing promoters and combinatorial gene expression by using different introns or terminators have gained importance. Some of the novel strategies to enhance gene expression have been described in Chap. 6.

4.5 Selectable Markers and Reporter Genes

The success of a genetically engineered/modified organism relies on the selection system to a great extent. At times there exists a situation wherein the transgene is (1) either overexpressed, (2) silent, or (3) not inserted/integrated into the specific/desired site of the host. It is easier to test by recent advanced molecular biological experiments, if a gene is present, absent, upregulated, or downregulated. But it remains tedious and laborious to screen each and every transgenic event for the presence of transgene. The ultimate aim is to reduce the labor and screen the positive transgenics through physical screening. Hence arose the requirement/importance of a specific tag-like gene called "marker." A marker is a gene that is an integral part of an expression vector, which codes for an enzyme or a product allowing the transformed cells or tissues to survive or resist and proliferate. In other words, to select a positive clone with our desired gene, selection markers like antibiotic resistance (ampicillin or kanamycin) are used. The gene is engineered with these resistant markers so as to select the positive transformed colonies. These markers must inhibit the growth of the non-transformants without affecting the transformed cells. Most of the plant selection markers are linked to the gene of interest that is expressed in a transgenic plant. The two most widely used plant selectable markers are Tn5 encoding *neomycin phosphotransferase* (*nptII*/*aph* 3′ *II*) and *hygromycin phosphotransferase* (*hpt*/*hph*/*aphIV*) that confer resistance to neomycin and hygromycin, respectively, in the transgenic plants. In sugarcane, *bar* and *pat* genes (herbicide resistance), *nptII* and *hptII*, are commonly used

selectable markers. In addition, a positive selection system (PMI) has also been reported (Jain et al. 2007).

Reporter genes are used as scorable markers for selecting transformed cells. In sugarcane, beta-glucuronidase (GUS), GFP, yellow fluorescent protein (EYFP), luciferase (LUC), and maize anthocyanin genes have been used for the optimization of transformation to allow visual selection of transient and stable integrations (Arencibia et al. 1995; Bower and Birch 1992; Bower et al. 1996; Elliot et al. 1998), functional analysis (Braithwaite et al. 2004; Damaj et al. 2010; Gallo-Meagher and Irvine 1993; Liu et al. 2003; Wei et al. 2003), and subcellular targeting (Gnanasambandam and Birch 2004; Gnanasambandam et al. 2007; Palanisamy et al. 2016), to detect transgene silencing (Birch et al. 2010) and determination of terminator efficiencies of constructs (Beyene et al. 2011).

4.6 Conclusion and Future Perspectives

Current advancements in biolistic gene transfer permit an efficient gene delivery with loci specificity and single copy of the transgene with similar effect as *Agrobacterium*-mediated gene transfer (Jackson et al. 2013; Wu et al. 2015). Despite a few stress-inducible promoters being used to produce desirable traits, there exists the need for novel promoters that precisely regulate expression of the transgene. With the whole-genome sequence available it will be easier for the researchers to edit genes and annotate their function, thereby validating the efficacy of their promoters. Through functional genomics it is possible to employ copious tools including global transcript profiling that are coupled with studying the mutants and transgenics to portray/distinguish novel genes in short periods. Though stringent rules on genetically modified crops are prevailing, the scarcity for food and the increasing demand and population explosion will all lead to acceptance of transgenics in the near future. As far as sugarcane is concerned, several nations have their transgenics under pipeline. Well-established protocols for transformation will enable crop improvement to a greater extent using biotechnological tools thereby facilitating food security in the near future.

References

Arencibia A, Molina P, Gutiérrez C, Fuentes A, Greenidge V, Menéndez E, De la Riva G, Selman-Houssein G (1992) Regeneration of transgenic sugarcane (*Saccharum officinarum* L.) plants from intact meristematic tissues transformed by electroporation. Biotechnol Aplicada 9:156–165

Arencibia A, Molina PR, de la Riva G, Selman-Housein G (1995) Production of transgenic sugarcane (*Saccharum officinarum* L.) plants by intact cell electroporation. Plant Cell Rep 14:305–309

Arencibia A, Carmona E, Tellez P et al (1998) An efficient protocol for sugarcane (Saccharum spp.) transformation mediated by *Agrobacterium tumefaciens*. Trans Res 7:213–222

Arvinth S, Arun S, Selvakesavan RK et al (2010) Genetic transformation and pyramiding of aprotinin-expressing sugarcane with cry1Ab for shoot borer (*Chilo infuscatellus*) resistance. Plant Cell Rep 29(4):383–395. doi:10.1007/s00299-010-0829-5

Baskaran P, Dasgupta I (2012) Gene delivery using microinjection of *Agrobacterium* to embryonic shoot apical meristem of elite indica rice cultivars. J Plant Biochem Biotechnol 21:268–274. doi:10.1007/s13562-011-0078-x

Berding N, Moore PH, Smith GR (1997) Advances in breeding technology for sugarcane. In: Intensive sugarcane production: meeting the challenge beyond 2000. CAB International, Wallingford, pp 189–220

Beyene G, Buenrostro-Nava MT, Damaj MB, Gao S, Molina J, Mirkov TE (2011) Unprecedented enhancement of reporter gene expression from minimal cassettes using a double terminator. Plant Cell Rep 30:13–25

Beyene G, Curtis IS, Damaj MB, Buenrostro-Nava MT, Mirkov TE (2013) "Genetic engineering of saccharum." Genomics of the saccharinae. Springer, New York, pp 223–254

Birch RG, Franks T (1991) Development and optimization of microprojectile systems for plant genetic transformation. Aust J Plant Physiol 18:453–469

Birch GR, Shen B, Sawyer BJB, Huttner E, Tucker WQJ, Betzner AS (2010) Evaluation and application of a luciferase fusion system for rapid *in vivo* analysis of RNAi targets and constructs in plants. Plant Biotechnol J 8:465–475

Bower R, Birch RG (1992) Transgenic sugarcane plants via microprojectile bombardment. Plant J 2(3):409–416

Bower R, Elliott AR, Potier BAM, Birch RG (1996) High-efficiency, microprojectile-mediated cotransformation of sugarcane, using visible or selectable markers. Mol Breed 2:239–249

Braithwaite KS, Geijskes RJ, Smith GR (2004) A variable region of the sugarcane bacilliform virus (SCBV) genome can be used to generate promoters for transgene expression in sugarcane. Plant Cell Rep 23:319–326

Brumbley SM, Snyman SJ, Gnanasambandam A et al (2008) Sugarcane. In: A compendium of transgenic crop plants: sugar, tuber and fiber crops. Blackwell, Oxford, pp 1–58

Chakravarthi M, Philip A, Subramonian N (2015) Truncated ubiquitin 5′ regulatory region from *Erianthus arundinaceus* drives enhanced transgene expression in heterologous systems. Mol Biotechnol 57:820–835. doi:10.1007/s12033-015-9875-0

Chen WH, Gartland KMA, Davey MR, Sotak R, Gartland JS, Mulligan BJ, Power JB, Cocking EC (1987) Transformation of sugarcane protoplasts by direct uptake of a selectable chimaeric gene. Plant Cell Rep 6(4):297–301

Chowdhury MKU, Vasil IK (1992) Stably transformed herbicide resistance callus of sugarcane via microprojectile bombardment of cell suspension cultures and electroporation of protoplasts. Plant Cell Rep 11:494–498

Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18:675–689. doi:10.1007/BF00020010

Cox MC, Hansen PB (1995) Productivity traits in southern and central regions and the impact of new varieties. Proc Australian Soc Sugarcane Technol 17:1–7

D'Hont A, Ison D, Alix K, Roux C, Glaszmann JC (1998) Determination of basic chromosome numbers in the genus Saccharum by physical mapping of ribosomal RNA genes. Genome 41:221–225

Damaj MB, Kumpatla SP, Emani C, Beremand PD, Reddy AS, Rathore KS, Buenrostro-Nava MT, Curtis IS, Thomas TL, Mirkov TE (2010) Sugarcane dirigent and O-methyltransferase promoters confer stem-regulated gene expression in diverse monocots. Planta 231:1439–1458

Elliot A, Campbell J, Brettell I, Grof P (1998) *Agrobacterium* mediated transformation of sugarcane using GFP as a screenable marker. Aust J Plant Physiol 25:739–743

Enriquez-Obregon G, Vazquez P, Prieto S et al (1998) Herbicide resistant sugarcane (*Saccharum officinarum* L.) plants by Agrobacterium-mediated transformation. Planta 206:20–27

Fang RX, Nagy F, Sivasubramaniam S, Chua NH (1989) Multiple cis regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell 1(1):141–150

Franks T, Birch RG (1991) Gene transfer into intact sugarcane cells using microprojectile bombardment. Aust J Plant Physiol 18:471–480

Gallo-Meagher M, Irvine J (1993) Effects of tissue type and promoter strength on transient GUS expression in sugarcane following particle bombardment. Plant Cell Rep 12:666–670

Gnanasambandam A, Birch RG (2004) Efficient developmental mistargeting by the sporamin NTPP vacuolar signal to plastids in young leaves of sugarcane and *Arabidopsis*. Plant Cell Rep 23:435–447

Gnanasambandam A, Polkinghorne IG, Birch RG (2007) Heterologous signals allow efficient targeting of a nuclear-encoded fusion protein to plastids and endoplasmic reticulum in diverse plant species. Plant Biotechnol J 5:290–296

Hogarth DM, Cox MC, Bull JK (1997) Sugarcane improvement: past achievements and future prospects. In: Kang MS (ed) Crop improvement for the 21st century, 1st edn. Research Signpost, Trivandrum, pp 29–56

Jackson MA, Anderson DJ, Birch RG (2013) Comparison of *Agrobacterium* and particle bombardment using whole plasmid or minimal cassette for production of high-expressing, low-copy transgenic plants. Trans Res 22:143–151

Jain M, Chengalrayan K, Abouzid A, Gallo M (2007) Prospecting the utility of a PMI/mannose selection system for the recovery of transgenic sugarcane (Saccharum spp. hybrid) plants. Plant Cell Rep 28:581–590

Jang I-C, Choi W-B, Lee K-H et al (2002) High-level and ubiquitous expression of the rice cytochrome c gene OsCc1 and its promoter activity in transgenic plants provides a useful promoter for transgenesis of monocots. Plant Physiol 129:1473–1481. doi:10.1104/pp.002261.1

Joyce P, Kuwahata M, Turner N (2010) Selection system and co-cultivation medium are important determinants of Agrobacterium-mediated transformation of sugarcane. Plant Cell Rep 29(2):173–183. doi:10.1007/s00299-009-0810-3

Joyce PA, Dinh SQ, Burns EM, O'Shea MG (2013) Sugar from genetically modified sugarcane: tracking transgenes, transgene products and compositional analysis. Int Sugar J 115:864–867

Joyce P, Hermann S, Connell AO et al (2014) Field performance of transgenic sugarcane produced using Agrobacterium and biolistics methods. Plant Biotechnol J 12(4):411–424. doi:10.1111/pbi.12148

Lakshmanan P, Geijskes RJ, Aitken KS, Grof CLP, Bonnett GD, Smith GR (2005) Sugarcane biotechnology: the challenges and opportunities. In Vitro Cell Dev Biol Plant 41:345–363

Liu DW, Oard SV, Oard JH (2003) High transgene expression levels in sugarcane (*Saccharum officinarum* L.) driven by the rice ubiquitin promoter RUBQ2. Plant Sci 165:743–750

Mayavan S, Subramanyam K, Jaganath B (2015) *Agrobacterium* -mediated in planta genetic transformation of sugarcane setts. Plant Cell Rep 34:1835–1848. doi:10.1007/s00299-015-1831-8

McElroy D, Zhang W, Cao J, Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2:163–171. doi:10.1105/tpc.2.2.163

McElroy D, Blowers AD, Jenes B, Wu R (1991) Construction of expression vectors based on the rice actin 1 (Act1) 5′ region for use in monocot transformation. Mol Gen Genet 231:150–160. doi:10.1007/BF00293832

Morikawa H, Yamada Y (1985) Capillary microinjection into protoplasts and intranuclear localization of injected materials. Plant Cell Physiol 26:229–236

Palaniswamy H, Syamaladevi DP, Mohan C, Philip A, Petchiyappan A, Narayanan S (2016) Vacuolar targeting of r-proteins in sugarcane leads to higher levels of purifiable commercially equivalent recombinant proteins in cane juice. Plant Biotechnol J 14(2):791–807

Philip A, Syamaladevi DP, Chakravarthi M, Gopinath K, Subramonian N (2013) 5′ regulatory region of ubiquitin 2 gene from *Porteresia coarctata* makes efficient promoters for transgene expression in monocots and dicots. Plant Cell Rep 32(8):1199–1210

Rakoczy-Trojanowska M (2002) Alternative methods of plant transformation—a short review. Cell Mol Biol Lett 7:849–858

Rathus C, Birch R (1992) Stable transformation of callus from electroporated sugarcane protoplasts. Plant Sci 82:81–89

Reich TJ, Iyer VN, Miki BL (1986) Efficient transformation of alfalfa protoplasts by the intranuclear microinjection of Ti plasmids. Nat Biotechnol 4(11):1001–1004

Sanford J (1988) The biolistic process. Trends Biotechnol 6:299–302

Slater A, Scott NW, Fowler MR (2003) Plant biotechnology: the genetic manipulation of plants, second. Oxford University Press, New York

Toriyama K, Arimoto Y, Uchimiya H, Hinata K (1988) Transgenic rice plants after direct gene transfer into protoplasts. Bio/Technology 6:1072–1074. doi:10.1038/nbt0988-1072

Wei H, Wang M, Moore PH, Albert HH (2003) Comparative expression analysis of two sugarcane polyubiquitin promoters and flanking sequences in transgenic plants. J Plant Physiol 160:1241–1251. doi:10.1078/0176-1617-01086

Wu H, Awan FS, Vilarinho A et al (2015) Transgene integration complexity and expression stability following biolistic or Agrobacterium-mediated transformation of sugarcane. In Vitro Cell Dev Biol Plant 51:603–611

Zhang W, Wu R (1988) Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. Theor Appl Genet 76:835–840. doi:10.1007/BF00273668

Chapter 5
Factors Affecting Genetic Transformation Efficiency in Sugarcane

Pushpanathan Anunanthini, Sarma Rajeev Kumar, and Ramalingam Sathishkumar

Abstract Sugarcane (*Saccharum officinarum*) is an important cash crop cultivated across the world. Conventional breeding methods are used to cross different *Saccharum* spp. to develop sugarcane hybrids with high sucrose content and for other novel traits including increased tolerance to various biotic and abiotic stresses. Two major factors that limit conventional breeding method are that it is highly time consuming and difficulty in getting the desirable trait in the hybrid. These limitations can be overcome by genetic transformation method in which specific gene(s) are used to generate stable transgenic lines expressing specific trait. Compared to conventional breeding methods, generation of stable lines takes less time. In addition, complications associated with backcross and testcross during breeding program can be avoided. There are several reports since 1990s mentioning generation of transgenic sugarcane by different methods of transformation such as electroporation, particle bombardment method, and *Agrobacterium*-mediated transformation. Transient expression systems have also been developed in sugarcane. Nevertheless, all transformation methodologies have their own limitation which hinders the stable expression of the transformed gene. Here, we discuss about the complications and factors affecting efficiency of genetic transformation in sugarcane.

Keywords *Agrobacterium* • Cocultivation • Explants • Genotype • Sugarcane • Transformation efficiency

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C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_5

5.1 Introduction

Sugarcane (*Saccharum* sp) belongs to grass family Poaceae, which is the most widely cultivated cash crop all over the world (Singh et al. 2013). It matures in 12–18 months and has the ability to acclimatize in both tropical and subtropical climatic conditions. The most profitable part of sugarcane is its stem where it accumulates high amount of sucrose, which is used for the production of sugar and its by-products. Due to their high economic value, improvement of sugarcane varieties is most important to fulfill the desired needs. *Saccharum spontaneum* and *Saccharum officinarum* are the two subspecies mainly used for producing hybrids or for genetic engineering approaches for improving different traits. *Saccharum officinarum* contains high sucrose content and *Saccharum spontaneum* is a resistant variety that exhibits tolerance to various biotic and abiotic stresses (Cheavegatti-Gianotto et al. 2011).

Conventional breeding method for producing improved varieties is laborious and time consuming (Mohan 2016). In addition, crossing two species with complex genome to produce specific varieties is much complicated as backcross and test-cross need longer time period. Genetic engineering technology helps to overcome these problems (Lakshmanan et al. 2005), wherein the desired gene of interest can be easily transferred either from interspecific or intraspecific species. Interspecific hybridization or producing transgenic plants has become a modern practice for generating improved varieties (Vacher et al. 2011). In early 1990s, researchers started working on producing genetically engineered/modified transgenic monocotyledonous plants/crops for improving different traits.

Different transformation methods in sugarcane have been standardized by researchers across the world (Enriquez-Obregon et al. 1997, 1998; Arencibia et al. 1998). Chapter 4 of this book describes various methods used for sugarcane transformation. Bower and Birch (1992) and Gallo-Meagher and Irvine (1996) attempted particle bombardment method using herbicide-resistant *bar* gene to generate transgenic sugarcane. Jain et al. (2007) reported marker-free antibiotic selection system for sugarcane transgenic plants. Electroporation technique was attempted using meristematic tissues from sugarcane (Arencibia et al. 1992, 1995). An efficient protocol for *Agrobacterium*-mediated genetic transformation from meristematic sugarcane tissue was reported (Enriquez-Obregon et al. 1997, 1998). The above mentioned are only few examples among other reports on sugarcane genetic transformation.

Despite these, generating transgenic sugarcane using *Agrobacterium*-mediated transformation is more difficult as *vir* gene induction and transfer of T-DNA are not simple in monocots as compared to dicots. Although the use of acetosyringone removed this barrier to some extent, success rate was still less when compared with direct gene transfer method. Further, after successful transformation and selection, transgenic plants may fail to express the trait due to phenomena associated with gene silencing (insertion of multiple copies) or epigenetic (posttranscriptional gene silencing/DNA methylation) mechanisms (Rajeevkumar et al. 2015). Expression of foreign genes in transgenic plants is often influenced by many factors including explants type, infection media, T-DNA stability, temperature of cocultivation, photoperiod, construct stability and size (Manfroi et al. 2015).

5.2 Tissue Culture

Tissue culture techniques refer to the multiplication of plants using different explants under in vitro conditions. It is a well-established technique to conserve plant species especially, to produce identical copies of mother plants. The choice of explants, sterilization techniques, surfactants, culture medium and hormones determines the response of explants in the media. Generally, a surfactant is used for the sterilization process to make the explants free from microorganisms. Sodium hypochlorite (NaClO), mercuric chloride ($HgCl_2$) and ethanol (EtOH) are the commonly used surface-sterilizing agents. Long exposure to sterilizing chemical leads to the death of explants and also affects the biological character of the plant genome.

Tissue culture for sugarcane crop is successfully applied nowadays for producing genetically modified biotic and abiotic resistant plant and high-sugar-content varieties. In case of breeding with the genetically modified crop, the offspring may lose its transgene for resistance/gene of interest (GOI).

In sugarcane, explants such as apical meristem, young leaf sheath and seeds are commonly used for genetic transformation. In vivo-grown plants are surface sterilized using 70% ethanol for 45s to 1 min followed by NaClO for 2–3 min or 0.1% $HgCl_2$ for 3–5 min followed by water washes for removal of NaClO/$HgCl_2$ traces. It has been reported that if tissues are exposed to surfactants for longer duration than normal, it affects the growth of the plants leading to death of the explants by necrosis (Clough and Bent 1998). Genetic transformation methodologies differ for apical meristem in which the explants are cut into whorls and are infected with *Agrobacterium* culture with different light and dark incubation for efficient transformation. In case of young leaf sheath, they are cut into small leaf bits and infected with *Agrobacterium* culture, whereas seeds are directly infected and grown (Kalunke et al. 2009; Mayavan et al. 2013). Explants can be used directly for infection or else further they can be cultured to develop callus tissue and developed calli can be used for transformation. Increased duration of explants, apical meristem, young leaf sheath, seeds or callus to *Agrobacterium* can suppress the growth of new dividing cells and also leads to false-positive results of selection in producing transgenic plants (Taylor et al. 1992; Geijskes et al. 2003; Song et al. 2013).

Antibiotics and selectable markers are generally used in the tissue culture medium for selection of transformed cells and to avoid false-positive transgenic plants. Antibiotics such as streptomycin, tetracycline, cefotaxime, chloramphenicol and penicillin are used for suppressing the overgrowth of bacterial culture on the explants and help for the growth and regeneration of explants. Selectable marker genes such as neomycin phosphotransferase (kanamycin resistance), hygromycin phosphotransferase (hygromycin resistance), gentamicin acetyltransferase (gentamicin resistance), bleomycin-resistance (ble) gene (bleomycin-resistance), phleomycin resistance (sh) gene (phleomycin resistance), methotrexate-insensitive dihydrofolate reductase (methotrexate resistant), aminoglycoside-3-adenyltransferase (streptomycin resistance) and phosphinothricin acetyl transferase (glufosinate-resistance)

(Hille et al. 1986; Hauptmann et al. 1988) have been used for selecting transgenic plants. Concentration of antibiotics ranges from 250 to 300 mg/L and selectable maker about 50 to 100 mg/L. Growth factors and regulators used in the media have the ability to trigger whole-plant regeneration from the explants (harboring the transgene). 2,4-Dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP) and kinetin are the growth regulators commonly used in sugarcane regeneration media for efficient growth. Supplements including coconut water have been previously reported to enhance regeneration in sugarcane (Asad et al. 2007). The concentration of growth regulators exceeding either higher or lower leads to necrosis producing transient expression of transgenic plants.

5.3 *Agrobacterium*-Mediated Genetic Transformation

Agrobacterium spp. is a rod-shaped gram-negative soil bacteria that have the natural ability to transfer the bacterial genes into plant genome (Gelvin 2003; Tzfira and Citovsky 2006). Two types of subspecies mostly used in genetic engineering of plant system are *Agrobacterium tumefaciens* (causing crown gall and frequently used for producing transgenic plants) and *Agrobacterium rhizogenes* (causing hairy roots, subsequently used for producing secondary metabolites and rarely used for producing transgenic plants). In dicots acetosyringone, a phenolic wounding signaling molecule, is naturally produced at the wound site, so transfer of T-DNA is easy and producing transgenic plant has been successful (De La Riva et al. 1998; Gelvin 2003; Shrawat and Lorz 2006; Dong et al. 2014a, b; Joyce et al. 2014; Mayavan et al. 2015). Enriquez-Obregon et al. (1998) reported *Agrobacterium*-mediated transformation in sugarcane using *A. tumefaciens* strains for different explants to produce herbicide-resistant, drought/salinity-tolerant varieties. Sugarcane is a polyploidy species and has been previously reported that loss of T-DNA during selection and regeneration is a common phenomenon. In 1990s well-established protocol for sugarcane regeneration was reported (Franks and Birch 1991; Bower and Birch 1992; Taylor et al. 1992; Arencibia et al. 1998; De La Riva et al. 1998; Arencibia and Carmona 2006).

Although there are many reports mentioning generation of transgenic sugarcane lines expressing different traits (Weng et al. 2011; Gao et al. 2016), there are reports regarding failure of transgene expressing in sugarcane transgenic plants (Snyman et al. 1996 and Ingelbrecht et al. 1999). Table 5.1 lists the various reports on genetic transformation of sugarcane. One of the reasons behind the failure in sugarcane transformation is explant choice; that is, sugarcane meristematic explants don't have the capacity of expressing the transferred gene (Zucchi et al. 2002). Therefore, subsequently leaf sheath and apical meristematic region were frequently used as explants for efficient transformation (Ali et al. 2004; Solangi et al. 2016). Acetosyringone (200 μM) was used as an optimal concentration for sugarcane during preculture of explants, infection medium and cocultivation medium. *Vir* gene

Table 5.1 Summary of significant reports on sugarcane genetic transformation

Gene	Transformation technique	Explants	Author
Chimeric	Polyethylene glycol-mediated transfer	Protoplasts	Chen et al. (1987)
Chloramphenicol acetyl transferase, *ß-glucuronidase*	Electroporation	Protoplasts	Rathus and Birch (1992)
Neomycin phosphotransferase	Microprojectile bombardment	Embryogenic callus	Bower and Birch (1992)
ß-*Glucuronidase*	Electroporation	Embryogenic callus	Arencibia et al. (1995)
Luciferase, *ß-glucuronidase*	Microprojectile bombardment	Embryogenic callus	Bower et al. (1996)
Green fluorescent protein	Microprojectile bombardment	Embryogenic callus	Elliott et al. (1999)
ß-Glucuronidase	Electroporation	Embryogenic callus	Seema et al. (2001)
Herbicide-resistant sugarcane	*Agrobacterium*-mediated genetic transformation	Axillary buds	Manickavasagam et al. (2004)
ß-Glucuronidase	Particle bombardment	Embryogenic callus	Kaur et al. (2007)
Resveratrol synthase	Microprojectile bombardment	Embryogenic callus	Xu et al. (2008)
*Cry1Aa*3	*Agrobacterium*-mediated transformation	Apical meristem and young leaf discs	Kalunke et al. (2009)
Aprotinin	Particle bombardment	Apical meristem and young leaf discs	Christy et al. (2009)
Cry1Ab, aprotinin	*Agrobacterium* and particle bombardment	Apical meristem and young leaf discs	Arvinth et al. (2010)
Green fluorescent protein	Particle bombardment	Basal part of the leaf roll	Vyver (2010)
Luciferase	*Agrobacterium* and particle bombardment	Embryogenic callus	Jackson et al. (2013)
Yellow fluorescent protein, *ß-glucuronidase*	Particle bombardment	Leaf roll discs, protoplast	Gao et al. (2013)
Neomycin phosphotransferase, green fluorescent protein-ß-glucuronidase fusion	*Agrobacterium tumefaciens*-mediated *in planta* seed transformation	Seed fluff	Mayavan et al. (2013)
Glyphosate tolerant	Microprojectile bombardment	Embryogenic callus	Nasir et al. (2014)

(continued)

Table 5.1 (continued)

Gene	Transformation technique	Explants	Author
Cyano fluorescent protein, green fluorescent protein	*Agrobacterium*-mediated genetic transformation	Immature leaf whorl	Dong et al. (2014a, b)
Arabidopsis vacuolar H + −pyrophosphatase	*Agrobacterium*-mediated genetic transformation	Calli	Kumar et al. (2014)
ß-Glucuronidase gene(gus A)	*Agrobacterium*-mediated transformation	Sugarcane setts	Mayavan et al. (2015)
Cry1Ac	Microprojectile bombardment	Embryonic callus	Gao et al. (2016)

induction was reported to be comparatively low even after the addition of acetosyringone in infection and cocultivation media due to pH conditions (Sood et al. 2011). Depending upon the acetosyringone concentration used in plant transformation, integration/expression efficiency changes in the host system (Hiei et al. 1994; Ishida et al. 1996; Rashid et al. 1996; Cheng et al. 1997; Hiei et al. 1997; Tingay et al. 1997; Zhao et al. 2000; Nandakumar et al. 2004). Karami (2008) reported that target gene, explants, *Agrobacterium* strain (GV3101, AGL0, AGL1, NT1 (pKPSF2), EHA105, MP90 and LBA4404) and bacterial infection duration (dark and light incubation) together play a vital role in enhancing the sugarcane transformation. *Agrobacterium* culture used for the plant transformation must be checked at 600 nm and OD should be ranging from 0.6 to 0.8 (Arvinth et al. 2010).

Cocultivation plays a major role for infection of *Agrobacterium* strain into explants; usually it is 3 days of cocultivation; however, based on the explants, duration should be standardized to avoid higher possibilities of plant suppression in regeneration and multiple copies of gene transfer (Cheng et al. 1996; Naureby et al. 1997; Sunilkumar and Rathore 2001). Transgene cell divisions are affected by antibiotic concentration supplemented in cocultivation and selection media, whereas widely used concentration ranges from 250 to 300 mg/L (Zhao et al. 2001; Karami 2008; Grzebelus and Skop 2014). Binary/super-binary vectors (Komori et al. 2007; Lee and Gelvin 2008; Anderson and Birch 2012) comprising Ti region, antibiotic selection, and *vir* gene region are suitable for genetic transformation. Further to ameliorate transformation efficiency, factors like promoter region, gene and NOS terminator that are a specific response to monocot expression should be preferred. pCAMBIA, pBin19, and pBI vectors are commercially available and commonly used for transformation (Bevan 1984; Dafny-Yelin and Tzfira 2007; Lutz et al. 2007; Lee and Gelvin 2008; Que et al. 2014).

5.4 Biolistic/Particle Bombardment Method

Biolistic/particle bombardment/gene gun/direct DNA delivery are different names used for transferring naked DNA into a host system by direct physical forces. First particle gun was developed by Sanford and colleagues in 1987. *CAT* (chloramphenicol acetyl transferase) gene was first used to detect transient gene activity in onion epidermal cells. In 1980s first transgenic plants were produced in soybean and tobacco expressing neomycin phosphotransferase II (*NptII*) (Christou et al. 1988) and β-*glucuronidase* gene (*GUS*) (Klein et al. 1988).

In sugarcane, first bombardment technique was developed from embryogenic calli in Australia (Bower and Birch 1992) using *npt-II* under strong monocot-specific promoter. Soon after *GUS* (Franks and Birch 1991), *Cry1A* gene (Arencibia et al. 1997) was transferred and produced stable transgenic sugarcane plant (Chowdhury and Vasil 1992). Gallo-Meagher and Irvine (1993) reported about the effects of a promoter with *GUS* gene in different parts of sugarcane tissue. Co-transformation and transformation were reported using bombardment technique for producing herbicide-resistant, disease-resistant, virus-resistant, drought- and salinity-tolerant stable transgenic plants (Gallo-Meagher and Irvine 1993; Snyman et al. 1998; Ingelbrecht et al. 1999; Nutt et al. 1999; Leibbrandt and Snyman 2001; Joyce et al. 2010; Khamrit et al. 2012).

Bombardment is a successful method when compared to other techniques for producing stable and transient transgenic sugarcane plants (Guo et al. 2014). Embryogenic callus (Falco et al. 1996), meristematic tissue (Gambley et al. 1993), and leaf disks were used as explants for bombardment technique to produce transgenic *S. officinarum* plants. Major factors affecting particle bombardment are nature of explants, DNA concentration and quality, gold/tungsten particle size, pressure level and a distance between the bombardment and target tissue. Embryogenic calli were produced using modified MS medium provided that the concentration of 2,4-D, BAP and kinetin is maintained (Heinz and Mee 1969; Naz et al. 2008). Tungsten or gold particles play a crucial role in direct gene transfer in sugarcane (Kaur et al. 2007). Certain measures and care should be taken for efficient transformation as mentioned below:

1. Lower DNA concentrations reduce the multiple copies of transgene integration.
2. Higher possibilities of truncated DNA into host cell may be produced due to high pressure.
3. Size and concentration of tungsten or gold particle should be chosen depending upon the explants.

After bombardment, callus should be cocultivated for 3 days. Further selection and regeneration of explants should be carried out as described in *Agrobacterium*-mediated transformation.

5.5 *In Planta* Transformation

In 2010s, *in planta* transformation techniques were standardized for sugarcane seeds and setts (Mayavan et al. 2013, 2015). *In planta* transformation so far reported in rice, wheat, *Arabidopsis* etc. has reduced the time period for producing transgenic plant by avoiding somaclonal variation and producing direct regeneration of transgenic plants. Due to the complex structure of sugarcane genome, the transformation made using *Agrobacterium*-mediated transformation and particle bombardment undergoes several selections in order to avoid escapism/false positive of transgenic. Gibberellic acid helps to break dormancy during sugarcane transformation, whereas the variation in concentration leads to transient expression. Measures should be taken care for exposure and concentration range to produce good stable transgenic plants. However, in *in planta* method vegetative propagation is suitable, which transgene in next generation plant will be maintained as in mother plant.

5.6 Electroporation

The first technique applied for producing transgenic sugarcane is electroporation with CAT gene using protoplast as explants. Important factors determining the success of this technique are electric pulse duration, DNA concentration, explant cell level, buffer system and heat shock (Rathus and Birch 1992). Molina et al. (1993) and Arencibia et al. (1992) reported electroporation through meristematic tissue to produce transgenic sugarcane. As in particle bombardment, DNA concentration should be limited to produce an efficient transformation. Electric pulse ranging from 6.5 to 10.4 KV/cm has been used for penetration of DNA into protoplast. An important factor that affects after electroporation is regeneration of transformed plants. The technique has failed due to lack of a well-established nutrient medium. Further, the well-established nutrient medium was standardized to regenerate the transformed plants (Seema et al. 2001 and Singh et al. 2013).

5.7 Conclusion

Sugarcane is a highly productive and profitable grass that provides valuable products such as sugar, ethanol, biomass for energy production, cattle feed and raw material for paper and other industries. Present-day sugarcane crop is developed through conventional breeding methods but due to increasing demand from both domestic and industrial sides it is time consuming to develop an improved variety. The traditional breeding approach is time consuming and cumbersome as it is difficult to select a specific trait from a whole genome of plant. Quick and better alternative is a transgenic approach as you can select a specific trait to be downregulated

or upregulated and time can also be reduced. Nevertheless, there are many bottlenecks related to transformation in sugarcane such as genotype, explant, cocultivation time, selectable markers used, and regeneration time and have to be standardized for developing transgenic sugarcane with desired traits.

References

Ali S, Hassan SW, Razi-ud-Din S, Shah SZR (2004) Micropropagation of sugarcane through bud culture. Sarhad J. Agric 20:79–82

Anderson DJ, Birch RG (2012) Minimal handling and super-binary vectors facilitate efficient, *Agrobacterium*-mediated transformation of sugarcane (*Saccharum* spp. hybrid). Trop Plant Biol 5:183–192

Arencibia AD, Carmona ER (2006) Sugarcane (*Saccharum* spp.) Methods Mol Biol 344:227–235

Arencibia A, Molina P, Gutierrez C, Fuentes A, Greenidge V, Menendez E, de la Riva G, Selman-Housein G (1992) Regeneration of transgenic sugarcane (*Saccharum officinarum* L.) plants from intact meristematic tissue transformed by electroporation. Biotecnol Apl 9:156–165

Arencibia A, Molina PR, de la Riva G, Selman-Housein G (1995) Production of transgenic sugarcane *(Saccharum officinarum* L.) plants by intact cell electroporation. Plant Cell Rep 14:305–309

Arencibia A, Vazquez R, Prieto D, Tellez P, Carmona E, Coego A, Hernandez L, de la Riva G, Selman-Housein G (1997) Transgenic sugarcane plants resistant to stem-borer attack. Mol Breed 3:247–255

Arencibia AD, Carmona ER, Tellez P, Chan MT, SM Y, Trujillo LE, Oramas P (1998) An efficient protocol for sugarcane (*Saccharum* spp. L.) transformation mediated by *Agrobacterium tumefaciens*. Transgenic Res 7:1–10

Arvinth S, Arun S, Selvakesavan RK, Srikanth J, Mukunthan N, Kumar PA, Premachandran MN, Subramonian N (2010) Genetic transformation and pyramiding of aprotinin-expressing sugarcane with C*ry1Ab* for shoot borer (*Chiloinfuscatellus*) resistance. Plant Cell Rep 29:383–395

Asad S, Amoozegar MA, Pourbabaee AA, Sarbolouki MN, Dastgheib SM (2007) Decolorization of textile dyes by newly isolated halophilic and halotolerant bacteria. Bioresour Technol 98:2082–2088

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12:8711–8721

Bower R, Birch RG (1992) Transgenic sugarcane plants via microprojectile bombardment. Plant J 2:409–416

Bower R, Elliott AR, Potier BAM, Birch RG (1996) High-efficiency, microprojectile-mediated co-transformation of sugarcane, using visible or selectable markers. Mol Breed 2:239–249

Cheavegatti-Gianotto A, de Abreu HMC, Arruda P, Bespalhok-Filho JC, Burnquist WL, Creste S et al (2011) Sugarcane (*Saccharum* X *officinarum*): a reference study for the regulation of genetically modified cultivars in Brazil. Trop Plant Biol 4:62–89

Chen WH, Gartland KMA, Davey MR, Sotak R, Gartland JS, Mulligan BJ, Power JB, Cocking EC (1987) Transformation of sugarcane protoplasts by direct uptake of a selectable chimeric gene. Plant Cell Rep 6:297–301

Cheng M, Jarret RL, Li Z, Xing A, Demski JW (1996) Production of fertile transgenic peanut (*Arachishypogaea* L.) plants using *Agrobacterium tumefaciens*. Plant Cell Rep 15:653–657

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol 115:971–980

Chowdhury MKU, Vasil IK (1992) Stably transformed herbicide resistant callus of sugarcane via microprojectile bombardment of cell suspension cultures and electroporation of protoplasts. Plant Cell 11:494–498

Christou P, McCabe DE, Swain WF (1988) Stable transformation of soybean callus by DNA-coated gold particles. Plant Physiol 87:671–674

Christy LA, Arvinth S, Saravanakumar M, Kanchana M, Mukunthan N, Srikanth J, George T, Subramonian N (2009) Engineering sugarcane cultivars with bovine pancreatic trypsin inhibitor (aprotinin) gene for protection against top borer (*Scripophaga excerptalis* Walker). Plant Cell Rep 28:175–184

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium* mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Dafny-Yelin M, Tzfira T (2007) Delivery of multiple transgenes to plant cells. Plant Physiol 145:1118–1128

De La Riva GA, Gonzalez-Cabrera J, Vazquez-Padron R, Ayra-Pardo C (1998) *Agrobacterium tumefaciens*: a natural tool for plant transformation. Electron J Biotechnol 1:24–25

Dong S, Delucca P, Geijskes RJ, Ke J, Mayo K, Mai P, Sainz M, Caffall K et al (2014a) Advances in *Agrobacterium*-mediated sugarcane transformation and stable transgene expression. Sugar Tech 16:366–371

Dong H, Zhao Y, Wang Y, Li H (2014b) Recombinant proteins expressed in lettuce. Indian J Biotechnol 13:427–436

Elliott AR, Campbell JA, Dugdale B, Brettell RIS, Grof CPL (1999) Green-fluorescent protein facilitates rapid *in vivo* detection of genetically transformed plant cells. Plant Cell Rep 18:707–714

Enriquez-Obregon GA, Vazquez-Padron RI, Prieto-Samsonov DL, Perez M, Selman-Housein G (1997) Genetic transformation of sugarcane by *Agrobacterium tumefaciens* using antioxidants compounds. Biotecnol Apl 14:169–174

Enriquez-Obregon GA, Vazquez-padron RI, Prieto-sansonov DL, de la Riva GA, Selman-Housein G (1998) Herbicide resistant sugarcane (*Saccharum officinarum* L.) plants by *Agrobacterium*-mediated transformation. Planta 206:20–27

Falco MC, Neto AT, Mendes BMJ, Arias FJZ (1996) Isolation and cultivation of protoplasts. Bras Fisiol Veg 8:175–179

Franks T, Birch RG (1991) Gene transfer into intact sugarcane cells using microprojectile bombardment. Funct Plant Biol 18:471–480

Gallo-Meagher M, Irvine JE (1993) Effects of tissue type and promoter strength on transient GUS expression in sugarcane following particle bombardment. Plant Cell Rep 12:666–670

Gallo-Meagher M, Irvine JE (1996) Herbicide resistant sugarcane containing the bar gene. Crop Sci 36:1367–1374

Gambley RL, Ford R, Smith GR (1993) Microprojectile transformation of sugarcane meristems and regeneration of shoots expressing *β-glucuronidase*. Plant Cell Rep 12:343–346

Gao SJ, Damaj MB, Park JW, Beyene G, Buenrostro-Nava MT, Molina J, Wang X, Ciomperlik JJ, Manabayeva SA, Alvarado VY, Rathore KS, Scholthof HB, Mirkov TE (2013) Enhanced transgene expression in sugarcane by co-expression of virus-encoded RNA silencing suppressors. PloS One 14:e66046

Gao S, Yang Y, Wang C, Guo J, Zhou D, Wu Q, Su Y, Xu L, Que Y (2016) Transgenic sugarcane with a *Cry1Ac* gene exhibited better phenotypic traits and enhanced resistance against sugarcane borer. PLoS One 11:e0153929

Geijskes RJ, Wang L, Lakshmanan P, Mckeon MG, Berding N, Swain RS et al (2003) Smartsett™ seedlings: tissue cultured seed plants for the Australian sugar industry. Sugarcane Int 25:13–17

Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol Mol Biol Rev 67:16–37

Grzebelus E, Skop L (2014) Effect of β-lactam antibiotics on plant regeneration in carrot protoplast cultures. In Vitro Cell Dev Biol Plant 50:568–575

Guo JL, Ling H, Wu QB, Xu LP, Que YX (2014) The choice of reference genes for assessing gene expression in sugarcane under salinity and drought stresses. Sci Rep 4:7042

Hauptmann RM, Vasil V, Ozias-Skins P, Tabaeizadeh Z, Rogers SG, Fraley RT, Horsch RB, Vasil IK (1988) Evaluation of selectable markers for obtaining stable transformation in the *Gramineae*. Plant Physiol 86:602–606

Heinz DJ, Mee GW (1969) Plant differentiation from callus tissue of *Saccharum* species. Crop Sci 9:346–348

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol Biol 35:205–218

Hille J, Verheggen F, Roelvink P, Franssen H, van Kammen A, Zabel P (1986) Bleomycin resistance: a new dominant selectable marker for plant cell transformation. Plant Mol Biol 7:171–176

Ingelbrecht IL, Irvine JE, Mirkov TE (1999) Post transcriptional gene silencing in transgenic sugarcane. Dissection of homology-dependent virus resistance in a monocot that has a complex polyploid genome. Plant Physiol 119:1187–1198

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat Biotechnol 14:745–750

Jackson MA, Anderson DJ, Birch RG (2013) Comparison of *Agrobacterium* and particle bombardment using whole plasmid or minimal cassette for production of high-expressing, low-copy transgenic plants. Transgenic Res 22:143–151

Jain M, Chengalrayan K, Abouzid A, Gallo M (2007) Prospecting the utility of a PMI/mannose selection system for the recovery of transgenic sugarcane (*Saccharum* spp. hybrid) plants. Plant Cell Rep 26:581–590

Joyce P, Kuwahata M, Turner N, Lakshmanan P (2010) Selection system and co cultivation medium are important determinants of *Agrobacterium*-mediated transformation of sugarcane. Plant Cell Rep 29:173–183

Joyce P, Hermann S, Connell A, Dinh Q, Shumbe L, Lakshmanan P (2014) Field performance of transgenic sugarcane produced using *Agrobacterium* and biolistics methods. Plant Biotechnol J 12:411–424

Kalunke RM, Kolge AM, Babu KH, Prasad DT (2009) *Agrobacterium* mediated transformation of sugarcane for borer resistance using *Cry1Aa*3 gene and one-step regeneration of transgenic plants. Sugar Tech 11:355–359

Karami O (2008) Factors affecting *Agrobacterium*-mediated transformation of plants. Transgenic Plant J 2:127–137

Kaur A, Gill MS, Gill R, Gosal SS (2007) Standardization of different parameters for ‘particle gun’ mediated genetic transformation of sugarcane (*Saccharum officinarum* L.) Indian J Biotechnol 6:31–34

Khamrit R, Jaisil P, Bunnag S (2012) Callus induction, regeneration and transformation of sugarcane (*Saccharum officinarum* L.) with chitinase gene using particle bombardment. Afr J Biotechnol 11:6612–6618

Klein TM, Harper EC, Svab Z, Sanford JC, Fromm ME, Maliga P (1988) Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process. Proc Natl Acad Sci 85:8502–8505

Komori T, Imayama T, Kato N, Ishida Y, Ueki J, Komari T (2007) Current status of binary vectors and super-binary vectors. Plant Physiol 145:1155–1160

Kumar T, Uzma KMR, Abbas Z, Ali GM (2014) Genetic improvement of sugarcane for drought and salinity stress tolerance using Arabidopsis vacuolar pyrophosphatase (AVP1) gene. Mol Biotechnol 56:199–209

Lakshmanan P, Geijskes RJ, Aitken KS, Grof CLP, Bonnett GD, Smith GR (2005) Sugarcane biotechnology: the challenges and opportunities. In Vitro Cell Dev Biol Plant 41:345–363

Lee LY, Gelvin SB (2008) T-DNA binary vectors and systems. Plant Physiol 146:325–332

Leibbrandt NB, Snyman SJ (2001) Initial field testing of transgenic glufosinate-ammonium resistant sugarcane. S Afr Sugar Technol Proc 75:105–108

Lutz KA, Azhagiri AK, Tungsuchat-Huang T, Maliga P (2007) A guide to choosing vectors for transformation of the plastid genome of higher plants. Plant Physiol 145:1201–1210

Manfroi E, Yamazaki-Lau E, Grando MF, Roesler EA (2015) Acetosyringone, pH and temperature effects on transient genetic transformation of immature embryos of Brazilian wheat genotypes by *Agrobacterium tumefaciens*. Genet Mol Biol 38:470–476

Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, Kasthurirengan S (2004) *Agrobacterium*-mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharum* species hybrids) using axillary buds. Plant Cell Rep 23:134–143

Mayavan S, Subramanyam K, Arun M, Rajesh M, Dev GK, Sivanandhan G, Jaganath B, Manickavasagam M, Selvaraj N, Ganapathi A (2013) *Agrobacterium tumefaciens*-mediated *in planta* seed transformation strategy in sugarcane. Plant Cell Rep 32:1557–1574

Mayavan S, Subramanyam K, Jaganath B, Sathish D, Manickavasagam M, Ganapathi A (2015) *Agrobacterium*-mediated *in planta* genetic transformation of sugarcane setts. Plant Cell Rep 34:1835–1848

Mohan C (2016) Genome editing in sugarcane: challenges ahead. Front Plant Sci 7:1542

Molina P, Menendez E, Arencibia A, Fuentes A, Alfonso J, Coll Y, Greenidge V, Perez S, Selman-Housein G (1993) Transient gene expression in sugarcane protoplasts after electroporation and polyethylene glycol treatment. Biotecnol Apl 10:171–175

Nandakumar R, Chen L, Rogers SMD (2004) Factors affecting the *Agrobacterium*-mediated transient transformation of the wetland monocot, *Typhalatifolia*. Plant Cell Tissue Organ Cult 79:31–38

Nasir IA, Tabassum B, Qamar Z, Javed MA, Tariq M, Farooq AM, Butt SJ, Qayyum A, Husnain T (2014) Herbicide-tolerant sugarcane (*Saccharum officinarum* L.) plants: an unconventional method of weed removal. Turk J Biol 38:439–449

Naureby B, Billing K, Wyndaele R (1997) Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentration suitable for elimination of *Agrobacterium tumefaciens*. Plant Sci 123:169–177

Naz S, Ali A, Iqbal J (2008) Phenolic content in vitro cultures of chick pea (Cicerarietinum L.) during callogenesis and organogenesis. Pak J Bot 40:2525–2539

Nutt KA, Allsopp PG, McGhie TK, Shepherd KM, Joyce PA (1999) Transgenic sugarcane with increased resistance to canegrubs. In: Conference of the Australian Society of Sugar Cane Technologists Proceedings, vol 21. pp 171–176

Que Q, Elumalai S, Li X, Zhong H, Nalapalli S, Schweiner M, Fei X, Nuccio M, Kelliher T, Gu W, Chen Z (2014) Maize transformation technology development for commercial event generation. Front Plant Sci 5:379

Rajeevkumar S, Anunanthini P, Ramalingam S (2015) Epigenetic silencing in transgenic plants. Front Plant Sci 6:693

Rashid H, Yoki S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by *Agrobacterium* in *indica* rice. Plant Cell Rep 15:727–730

Rathus C, Birch RG (1992) Stable transformation of callus from electroporated sugarcane protoplasts. Plant Sci 82:81–89

Sanford JC, Klein TM, Wolf ED, Allen N (1987) Delivery of substances into cells and tissues using a particle bombardment process. Part Sci Technol 5:27–37

Seema G, Pande HP, Lal J, Madan VK (2001) Plantlet regeneration of sugarcane varieties and transient GUS expression in calli by electroporation. Sugar Tech 3:27–33

Shrawat AK, Lorz H (2006) *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers. Plant Biotechnol J 4:575–603

Singh RK, Kumar P, Tiwari NN, Rastogi J, Singh SP (2013) Current status of sugarcane transgenic: an overview. Adv Genet Eng 2:2

Snyman SJ, Meyer GM, Carson DL, Botha FC (1996) Establishment of embryogenic callus and transient gene expression in selected sugarcane varieties. S Afr J Bot 62:29–37

Snyman SJ, Leibbrandt NB, Botha FC (1998) Buster resistant sugarcane. S Afr Sugar Technol Proc 72:140–142

Solangi SK, Qureshi ST, Khan IA, Raza S (2016) Establishment of *in vitro* callus in sugarcane (*Saccharum officinarum* L.) varieties influenced by different auxins. Afr J Biotechnol 15:1541–1550

Song ZY, Tian JL, WZ F, Li L, LH L, Zhou L, Shan Z, Tang GX, Shou HX (2013) Screening Chinese soybean genotypes for *Agrobacterium*-mediated genetic transformation suitability. J Zhejiang Univ Sci B 14:289–298

Sood P, Bhattacharya A, Sood A (2011) Problems and possibilities of monocot transformation. Biol Plant 55:1–15

Sunilkumar G, Rathore KS (2001) Transgenic cotton: factors influencing *Agrobacterium*-mediated transformation and regeneration. Mol Breed 8:37–52

Taylor PW, Ko HL, Adkins SW, Rathus C, Birch RG (1992) Establishment of embryogenic callus and high protoplast yielding suspension cultures of sugarcane (*Saccharum* spp. hybrids). Plant Cell Tissue Organ Cult 28:69–78

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. Plant J 11:1369–1376

Tzfira T, Citovsky V (2006) *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. Curr Opin Biotechnol 17:147–154

Vacher C, Kossler TM, Hochberg ME, Arthur E (2011) Weis impact of inter-specific hybridization between crops and weedy relatives on the evolution of flowering time in weedy phenotypes. PLoS One 6:e14649

Vyver CVD (2010) Genetic transformation of the euploid *Saccharum officinarum* via direct and indirect embryogenesis. Sugar Tech 12:21–25

Weng LX, Deng HH, Xu JL, Li Q, Zhang YQ, Jiang ZD, Li QW, Chen JW, Zhang LH (2011) Transgenic sugarcane plants expressing high levels of modified *Cry1Ac* provide effective control against stem borers in field trials. Transgenic Res 20:759–772

Xu LP, Que YX, Xu JS, Fang SR, Zhang MQ, Chen YQ, Chen RK (2008) Establishment of genetic transformation system and obtaining transgenic sugarcane (var. badila) transformed with *RS* gene. Sugar Tech 10:128–132

Zhao ZY, Cai T, Tagliani L, Miller M, Wang N, Pang H, Rudert M, Schroeder S, Hondred D, Seltzer J, Pierce D (2000) *Agrobacterium*-mediated sorghum transformation. Plant Mol Biol 44:789–798

Zhao ZY, Gu W, Cai T, Tagliani L, Hondred D, Bond D, Schroeder S, Rudert M, Pierce D (2001) High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. Mol Breed 8:323–333

Zucchi MI, Arizona H, Morais VA, Fungaro MHP, Vieira MLC (2002) Genetic instability of sugarcane plants derived from meristem cultures. Genet Mol Biol 25:91–96

Chapter 6
Novel Potential Candidate Promoters and Advanced Strategies for Sugarcane Transformation

Chakravarthi Mohan, Vanessa K. Schneider, and Flavio Henrique-Silva

Abstract Plant transformation technology offers unique prospects to transfer a wide spectrum of functionally relevant genes in plants. Expression of genes is regulated by a number of factors among which promoter strength, specificity, and *cis*- and *trans*-acting elements play a critical role. The choice of promoter is a key determinant for the levels and specificities of gene expression. In sugarcane, the maize ubiquitin promoter has been the workhorse promoter for decades. The availability of limited promoters for sugarcane transformation is critical in sugarcane crop improvement through genetic engineering. However, recent advancements in biotechnology have provided greater insights into promoter validation from wild and commercially cultivated sugarcane, which is evident from an array of different promoters reported. This review describes the various promoters isolated from sugarcane and its wild relatives that would benefit future genetic engineering studies in sugarcane. In addition, the challenges ahead and improved strategies for sugarcane transformation are discussed.

Keywords Enhanced expression • Promoter • Silencing • Sugarcane • Transformation • Transgene

6.1 Introduction

Sugarcane (*Saccharum* spp. hybrid) belonging to Poaceae family is an economically important food and energy crop grown worldwide. Large genome size, polyploidy, low fertility, complex environmental interactions, slow breeding advances, and nobilization hinder the breeding for this crop. In addition, several issues like

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C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_6

low cane and sugar yields; susceptibility to abiotic stresses such as drought, cold, and salinity; and biotic stresses such as pest insects and fungal diseases are the major constraints in sugarcane cultivation (Tiwari et al. 2010). Transgenic technology provides an effective tool for sugarcane crop improvement. Both biolistic and *Agrobacterium*-mediated transformation methods have been well established and are widely used to develop transgenic sugarcane. Several factors have to be considered in the development of transgenic sugarcane among which the choice of promoter plays a crucial role.

Promoters are the regulatory sequences present upstream of the genes and are involved in the regulation of the gene expression. In recent years, an array of promoters from plant and viral origin have been characterized and extensively used in transgene expression in plants. They can be broadly grouped into constitutive, spatiotemporal, and inducible promoters based on their activity. The CaMV 35S promoter has been well described and widely used for constitutive transgene expression (Potenza et al. 2004). However, in monocots, the CaMV 35S promoter confers lower levels of transgene expression. The maize ubiquitin promoter (M-ubi1) is the promoter of choice for sugarcane transformation and has been widely used for over two decades. Sugarcane ubiquitin promoters (Ubi4 and Ubi9) when expressed in sugarcane have led to posttranscriptional gene silencing (PTGS) (Wei et al. 2003). The promoters used currently for the development of transgenic sugarcane are limited in number and very few provide tissue-specific expression. Limited tissue-specific promoters have been characterized so far for sugarcane transformation (Damaj et al. 2010). Hence, there is a need for identification of more promoters for specific applications, and from unrelated sources, which would be of great value for future genetic engineering studies in sugarcane. This review discusses the various advancements that took place in the past decade pertaining to sugarcane transformation and promoter validation which will benefit researchers aiming to develop transgenic sugarcane with desirable traits.

6.2 Plant Promoters: Structure and Function

Promoters are defined as regions upstream of a gene's coding region and are involved in the regulation of the frequency of transcription. They usually contain specific DNA sequences and regulatory elements and are the key regulators of transcription, also called as molecular switches. Promoters can be broadly classified into constitutive, tissue specific, and inducible based on their activity. Another type of promoters which are currently of importance is the synthetic promoters that combine the available core promoters with different motifs and are designed for specific expression. A typical plant promoter is composed of a transcription start site (TSS), the core promoter region, the proximal region (or upstream regulatory elements), and the distal regulatory region (or long-range regulatory elements). Plant promoters and their *cis*-acting regulatory elements have been reviewed

extensively (Hernandez-Garcia and Finer 2014; Porto et al. 2014; Grunennvaldt et al. 2015; Shah et al. 2015; Naqvi et al. 2016; Biłas et al. 2016) and hence are not focused in this review.

6.3 Promoters for Enhanced Transgene Expression in Sugarcane

In sugarcane, the maize ubiquitin (Zmubi1) promoter is being used worldwide for more than two decades for genetic engineering. However, it has failed to drive sustained transgene expression throughout the sugarcane growth cycle (Wang et al. 2005). Promoters of viral origin such as sugarcane bacilliform virus promoter (Braithwaite et al. 2004), banana streak virus promoter (Schenk et al. 1999), and CaMV35S:Zmubi1 tandem promoter (Groenewald and Botha 2008) conferred enhanced expression in mature canes. Some of the other promoters used were the enhanced maize ubiquitin promoter and maize carboxylase promoter (Kinkema et al. 2014a, b) which conferred enhanced transgene expression than the Zmubi1 promoter (five and fourfold). The advent of novel tools for promoter discovery, next-generation sequencing boom, and advanced bioinformatics techniques have led to isolation of new promoters from sugarcane and its wild relatives that could drive enhanced transgene expression than the routine promoters. Table 6.1 lists the different promoters characterized from sugarcane and its wild relatives that would enable researchers to develop GM sugarcane with enhanced transgene expression. Yet, the number of promoters is very limited when compared to other plant species. Mudge et al. (2013) characterized three promoters from sugarcane which conferred preferential transgene expression in mature stems and thus have practical application in sucrose-targeted metabolic engineering.

6.4 Challenges Ahead in Sugarcane Biotechnology

Sugarcane is affected by several biotic and biotic stresses which lead to losses in productivity. Using conventional breeding practices, the release of a new variety typically takes 12–15 years after rigorous testing of performance, sugar content, agronomic traits, and genetic stability (Gazaffi et al. 2010). With the advent of transgenic technology, considerable progress has been made in the recent years. Biolistic and *Agrobacterium* methods of transformation, though routinely used, have several disadvantages. Biolistic bombardment method usually generates multiple transgene integration sites. *Agrobacterium*-mediated transformation is rather a time-consuming laborious process, has low transformation efficiency (Joyce et al. 2010), has high variability between experiments, and is genotype dependent (Anderson and Birch 2012). In fact, the time taken from DNA delivery till whole-plant regeneration is longer than any other crop plants.

Table 6.1 Potential candidate promoters from sugarcane and its wild relatives with enhanced transgene expression

Type	Promoter	Source	Gene and function	Strategy for promoter isolation	Inference	References
Constitutive	ScMybR1	Sugarcane	Myb transcription factor gene	BAC library screening and PCR	Identified and validated 8 alleles; 3 found expressive	Mudge et al. (2009)
	EaUbiD7	*Erianthus arundinaceus*	Ubiquitin	RAGE PCR	Deletion ubiD7 drove higher *GUS* expression in sugarcane	Chakravarthi et al. (2015)
Inducible	EaPR10	*E. arundinaceus*	PR-10 gene	RAGE PCR	Induced upon wounding; also constitutive	Chakravarthi et al. (2016)
	ScMybAS1	Sugarcane	Myb transcription factor gene	cDNA library and PCR	Induced upon exposure to abiotic stress and hormones	Prabu and Prasad (2012)
	SoCIN1	Sugarcane	Cell wall invertase	RACE PCR	Induced expression	Niu et al. (2015)
	Sc-ERS	Sugarcane	Ethylene receptor gene	TAIL PCR	Induced upon circadian rhythm, hormone, drought, and light	Li et al. (2013)
	EaMybAS1	*E. arundinaceus*	Myb transcription factor gene	PCR	Revealed enhanced stress tolerance in transgenic tobacco	Kharte et al. (2016)
	alcA	Sugarcane	Ethanol-inducible gene switch	NA	Identified an effective ethanol-inducible promoter	Kinkema et al. (2014b)

Type	Promoter	Source	Gene and function	Strategy for promoter isolation	Inference	References
Stem specific	Sc-c22a	Sugarcane	Dirigent-like protein	Inverse PCR	Conferred mature culm-specific expression	Abraha (2005)
	ScLSG	Sugarcane	Loading stem gene	BAC library screening and PCR	9 alleles of LSG identified and validated	Moyle and Birch (2013)
	ShDIR16	Sugarcane	Dirigent protein	BAC library screening and PCR	Both conferred stem-regulated expression; induced expression in leaf and root upon hormone exposure	Damaj et al. (2010)
	ShOMT		O-methyl transferase			

Another major challenge in sugarcane transformation is the transgene inactivation/silencing problem. Several promoters failed to drive transgene expression in mature canes despite showing activity in callus (Wei et al. 2003). Moreover both transcriptional and posttranscriptional gene silencing has been reported in sugarcane (Ingelbrecht et al. 1999). Mudge et al. (2009) characterized eight distinct promoters of MYB family of which three were expressive. Interestingly, their results illustrated that multiple copies of promoter do not trigger silencing and polyploids may have intrinsic silencing mechanisms that are yet to be deciphered. Birch et al. (2010) reported that silencing in sugarcane is 5′-sequence specific, independent of copy number, developmentally regulated, and posttranscriptional in T_0 transgenic lines. Transgenes fused with strong tissue-specific promoters may alleviate the silencing problem.

Sugarcane genome is about 10 Gb size with homologous genes ranging from 8 to 12 copies (Souza et al. 2011) and the monoploid genome size being 750–930 Mb (D'Hont and Glaszmann 2001). Currently there is a lack of whole genomic data in sugarcane. The major factors that make the whole-genome sequencing of sugarcane difficult are (1) polyploidy—80% of sugarcane genome is inherited from *S. officinarum* and 10% from *S. spontaneum*; (2) high level of recombination—more than 10% of sugarcane genome is mosaic and unknown; (3) heterozygosity—leads to variations that deter genome assembly; and (4) repeats—high number of repetitive sequences present throughout the genome. Moreover, sugarcane lacks diploid progenitors that aid in a faster and easier genome assembly (Garcia et al. 2013) unlike banana (D'hont et al. 2012). In addition, it is difficult to employ shot-gun sequencing such as Illumina which generates shorter reads. Thus, the large and complex genome, high ploidy levels, and high content of repetitive DNA make sugarcane an unusually recalcitrant crop species for both forward and reverse genetic studies.

6.5 Improved Strategies for Sugarcane Transformation

Several researchers are striving hard to overcome the challenges in sugarcane transformation which has led to a breakthrough with an array of advanced techniques, modified protocols, and strategies for efficient sugarcane transformation. This section of the review discusses some of the significant studies which will have a greater impact on sugarcane transgenic research in the near future. Joyce et al. (2010) optimized different parameters for *Agrobacterium* transformation in sugarcane and observed that selection and cocultivation systems were critical factors that affected sugarcane transformation. Jackson et al. (2013) compared both the methods using whole plasmids and minimal cassettes and observed that both the procedures were high expressing and yielded single-gene insertions at a reasonable transformation efficiency (TE).

Taparia et al. (2012a, b) used minimal expression cassettes for biolistic gene transfer and with reduced plasmid concentration and achieved simple transgene integration and stable transgene expression. They also described a rapid transformation procedure that only needs 3 months from culture initiation to potting of transgenic sugarcane. Use of minimal cassettes has shown to be effective since

they are devoid of prokaryotic backbone sequences that may contribute to recombination or induce methylation, thereby leading to transgene silencing.

Anderson and Birch (2012) studied several parameters that are critical for transformation of sugarcane variety Q117. They reported that the key factors influencing transformation efficiency in *Agrobacterium* method were minimal handling of callus during cocultivation and the use of a super-binary vector in AGL *Agrobacterium* strain which led to the highest transformation efficiency reported so far for *Agrobacterium*-mediated transformation in sugarcane. Recently, Mayavan et al. (2015) have developed a rapid, efficient, and genotype-independent *in planta* transformation protocol using sugarcane setts as explants. They have claimed a maximum of 32.6% TE which is so far the highest TE in sugarcane. Their group had earlier developed a seed-based transformation protocol which also proved to be efficient to develop transgenic sugarcane in a shorter duration (Mayavan et al. 2013).

Dong et al. (2014) developed a robust protocol that could be applied on a larger industrial scale for sugarcane improvement through genetic engineering. This protocol employs desiccation during cocultivation that leads to higher TE and has also been tested in several varieties and in several laboratories proving its versatility. In addition, the transgenes were stable across multiple generations and growing seasons that further proves the great utility of the protocol. Sandhu et al. (2016) have recently reported single-step direct transgenic plant regeneration from agro-infected spindle leaf roll segments of sugarcane with a very short period of 8 weeks since it avoids the callusing phase. Stable integration was observed in the transgenics making the protocol reliable for sugarcane transformation.

Jackson et al. (2014) presented a set of rules to achieve sustained transgene expression and validated them in sugarcane. They used the following methods independently or in combination—removal of rare codons, removal of RNA instability sequences, blocking of putative endogenous sRNA-binding sites, and randomization of non-rare codons. This technique can be applied in sugarcane effectively to alleviate transgene silencing. Recently, Lowe et al. (2016) reported an efficient monocot transformation strategy wherein they over-expressed the maize morphogenic regulators *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) genes in previously non-transformable maize inbred lines and achieved high transformation frequencies. They also successfully employed this approach to enhance transformation frequency in sorghum, sugarcane, and rice.

Other notable advances worth mentioning are (1) development of synthetic reporter genes in order to alleviate silencing effects to validate promoter expression in sugarcane (Chou and Moyle 2014); (2) use of alternate monocot models such as *Setaria viridis* that yields higher transformation efficiency, has shorter duration, and contains a similar cell wall composition to that of sugarcane. Hence it can be used as an alternate model plant for sugarcane-applied research for stress resistance, improved biomass, and bioethanol production (Martins et al. 2015); (3) use of novel promoters that drive higher levels of transgene expression than the routine promoters and exploiting codon-optimized target genes specific for sugarcane to enhance transgene expression (Kinkema et al. 2014a); (4) application of RNAi technology to

develop improved sugarcane for desired traits (Gan et al. 2010; Gao et al. 2013; Jung et al. 2012); (5) use of a combinatorial approach wherein multiple promoters/enhancers/terminators/5′UTRs are employed to achieve higher transgene expression (unpublished data); and (6) use of systems biology and metabolic modeling approach to unravel gene regulatory networks underlying key mechanisms such as sucrose synthesis and accumulation.

6.6 Conclusion and Future Perspectives

Sugarcane biotechnology has advanced rapidly over the years and transgenic lines for various biotic and abiotic stresses have been developed and are being tested in laboratories worldwide. Commercial testing of transgenic sugarcane has already been approved in Indonesia and is in pipeline in several other countries. Several recombinant proteins have already been produced using sugarcane as a bio-factory. With the advances in transgenic technology, genome sequencing tools, and systems biology coupled with bioinformatics, it is now feasible to manipulate the metabolic pathways in sugarcane, thereby enhancing the crop productivity and increased sugar content. Although obstacles including transgene inactivation, lack of whole genome, and long duration for transformation are certainly a hindrance, genetic engineering combined with the novel advanced strategies would undoubtedly be instrumental in helping the sugarcane industries develop into a stronger bio-economy.

References

Abraha TG (2005) Isolation and characterization of a culm-specific promoter element from sugarcane. MSc Dissertation, Department of Botany and Zoology, Institute of Biotechnology, Stellenbosch University, South Africa

Anderson DJ, Birch RG (2012) Minimal handling and super-binary vectors facilitate efficient, *Agrobacterium*-mediated, transformation of sugarcane (*Saccharum* spp. hybrid). Trop Plant Biol 5(2):183–192

Biłas R, Szafran K, Hnatuszko-Konka K, Kononowicz AK (2016) Cis-regulatory elements used to control gene expression in plants. Plant Cell Tiss Organ Cult 127:269–287. doi:10.1007/s11240-016-1057-7

Birch RG, Bower RS, Elliott AR (2010) Highly efficient, 5′-sequence-specific transgene silencing in a complex polyploid. Trop Plant Biol 3(2):88–97

Braithwaite KS, Geijskes RJ, Smith GR (2004) A variable region of the sugarcane bacilliform virus (SCBV) genome can be used to generate promoters for transgene expression in sugarcane. Plant Cell Rep 23:319–326

Chakravarthi M, Philip A, Subramonian N (2015) Truncated ubiquitin 5′ regulatory region from *Erianthus arundinaceus* drives enhanced transgene expression in heterologous systems. Mol Biotechnol 57(9):820–835

Chakravarthi M, Syamaladevi DP, Harunipriya P, Augustine SM, Subramonian N (2016) A novel PR10 promoter from Erianthus arundinaceus directs high constitutive transgene expression and is enhanced upon wounding in heterologous plant systems. Mol Biol Rep 43(1):17–30

Chou TC, Moyle RL (2014) Synthetic versions of firefly luciferase and Renilla luciferase reporter genes that resist transgene silencing in sugarcane. BMC Plant Biol 14(1):92

D'Hont A, Glaszmann JC (2001) Sugarcane genome analysis with molecular markers, a first decade of research. Proc Int Soc Sug Cane Tech 24:556–559

D'hont A, Denoeud F, Aury JM, Baurens FC, Carreel F et al (2012) The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. Nature 488:213–217

Damaj MB, Kumpatla SP, Emani C, Beremand PD, Reddy AS, Rathore KS, Buenrostro-Nava MT, Curtis IS, Thomas TL, Mirkov TE (2010) Sugarcane DIRIGENT and O-METHYLTRANSFERASE promoters confer stem-regulated gene expression in diverse monocots. Planta 231(6):1439–1458

Dong S, Delucca P, Geijskes RJ, Ke J, Mayo K, Mai P, Sainz M, Caffall K, Moser T, Yarnall M, Setliff K (2014) Advances in *Agrobacterium*-mediated sugarcane transformation and stable transgene expression. Sugar Tech 16(4):366–371

Gan D, Zhang J, Jiang H, Jiang T, Zhu S, Cheng B (2010) Bacterially expressed dsRNA protects maize against SCMV infection. Plant Cell Rep 29(11):1261–1268

Gao SJ, Damaj MB, Park JW, Beyene G, Buenrostro-Nava MT, Molina J, Wang X, Ciomperlik JJ, Manabayeva SA, Alvarado VY, Rathore KS (2013) Enhanced transgene expression in sugarcane by co-expression of virus-encoded RNA silencing suppressors. PLoS One 8(6):e66046

Garcia AA, Mollinari M, Marconi TG, Serang OR, Silva RR et al (2013) SNP genotyping allows an in depth characterisation of the genome of sugarcane and other complex autopolyploids. Sci Rep 3:3399

Gazaffi R, Oliveira KM, Souza AP, Garcia AAF (2010) The importance of the germplasm in developing agro-energetic profile sugarcane cultivars. In: Cortez LAB (ed) Sugar cane bioethanol: R&D for productivity and sustainability. Blucher, São Paulo, pp 333–343. ISBN: 9788521205302

Groenewald JH, Botha FC (2008) Down-regulation of pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP) activity in sugarcane enhances sucrose accumulation in immature internodes. Trans Res 17(1):85–92

Grunennvaldt RL, Goldbach JD, Grhardt IR, Quoirin M (2015) Promoters used in genetic transformation of plants. Res J Biol Sci 10(1–2):1–9

Hernandez-Garcia CM, Finer JJ (2014) Identification and validation of promoters and *cis*-acting regulatory elements. Plant Sci 217:109–119

Ingelbrecht IL, Irvine JE, Mirkov TE (1999) Posttranscriptional gene silencing in transgenic sugarcane dissection of homology dependent virus resistance in a monocot that has a complex polyploid genome. Plant Physiol 119:1187–1197

Jackson MA, Anderson DJ, Birch RG (2013) Comparison of *Agrobacterium* and particle bombardment using whole plasmid or minimal cassette for production of high-expressing low-copy transgenic plants. Trans Res 22(1):143–151

Jackson MA, Sternes PR, Mudge SR, Graham MW, Birch RG (2014) Design rules for efficient transgene expression in plants. Plant Biotechnol J 12(7):925–933

Joyce P, Kuwahata M, Turner N, Lakshmanan P (2010) Selection system and co-cultivation medium are important determinants of *Agrobacterium*-mediated transformation of sugarcane. Plant Cell Rep 29(2):173–183

Jung JH, Fouad WM, Vermerris W, Gallo M, Altpeter F (2012) RNAi suppression of lignin biosynthesis in sugarcane reduces recalcitrance for biofuel production from lignocellulosic biomass. Plant Biotechnol J 10(9):1067–1076

Kharte SB, Watharkar AD, Shingote PR, Chandrashekharan S, Pagariya MC, Kawar PG, Govindwar SP (2016) Functional characterization and expression study of sugarcane MYB transcription factor gene PEaMYBAS1 promoter from *Erianthus arundinaceus* that confers abiotic stress tolerance in tobacco. RSC Adv 6(23):19576–19586

Kinkema M, Geijskes J, Palupe A, Shand K, Coleman HD, Brinin A, Williams B, Sainz M, Dale JL (2014a) Improved molecular tools for sugar cane biotechnology. Plant Mol Biol 84(4–5):497–508

Kinkema M, Geijskes RJ, Shand K, Coleman HD, De Lucca PC, Palupe A et al (2014b) An improved chemically inducible gene switch that functions in the monocotyledonous plant sugar cane. Plant Mol Biol 84(4–5):443–454

Li HJ, Qi NJ, Hui Z, Tao YL, Rui LY, Qin WA (2013) Cloning and sequence analysis of promoter in sugarcane ethylene receptor gene (Sc-ERS). J South Agric 44(5):722–729

Lowe K, Wu E, Wang N, Hoerster G, Hastings C, Cho MJ et al (2016) Morphogenic regulators baby boom and Wuschel improve monocot transformation. Plant Cell 28(9):1998–2015

Martins PK, Ribeiro AP, da Cunha BADB, Kobayashi AK, Molinari HBC (2015) A simple and highly efficient *Agrobacterium*-mediated transformation protocol for *Setaria viridis*. Biotechnol Rep 6:41–44

Mayavan S, Subramanyam K, Arun M, Rajesh M, Dev GK, Sivanandhan G, Jaganath B, Manickavasagam M, Selvaraj N, Ganapathi A (2013) Agrobacterium tumefaciens-mediated in planta seed transformation strategy in sugarcane. Plant Cell Rep 32(10):1557–1574

Mayavan S, Subramanyam K, Jaganath B, Sathish D, Manickavasagam M, Ganapathi A (2015) Agrobacterium-mediated in planta genetic transformation of sugarcane setts. Plant Cell Rep 34(10):1835–1848

Moyle RL, Birch RG (2013) Sugarcane loading stem gene promoters drive transgene expression preferentially in the stem. Plant Mol Biol 82(1–2):51–58

Mudge SR, Osabe K, Casu RE, Bonnett GD, Manners JM, Birch RG (2009) Efficient silencing of reporter transgenes coupled to known functional promoters in sugarcane a highly polyploid crop species. Planta 229(3):549–558

Mudge SR, Basnayake SW, Moyle RL, Osabe K, Graham MW, Morgan TE, Birch RG (2013) Mature-stem expression of a silencing-resistant sucrose isomerase gene drives isomaltulose accumulation to high levels in sugarcane. Plant Biotechnol J 11(4):502–509

Naqvi RZ, Mubeen H, Raza S (2016) Role of plant promoters and their cis regulatory elements in gene expression regulation. Eur J Pharm Med Res 3(1):347–352

Niu JQ, Wang AQ, Huang JL, Yang LT, Li YR (2015) Isolation characterization and promoter analysis of cell wall invertase gene SoCIN1 from sugarcane (*Saccharum* spp). Sugar Tech 17(1):65–76

Porto MS, Pinheiro MPN, Batista VGL, dos Santos RC, de Albuquerque Melo Filho P, de Lima LM (2014) Plant promoters: an approach of structure and function. Mol Biotechnol 56(1):38–49

Potenza C, Aleman L, Sengupta-Gopalan C (2004) Targeting transgene expression in research agricultural and environmental applications: promoters used in plant transformation. In Vitro Cell Dev Biol Plant 40(1):1–22

Prabu G, Prasad DT (2012) Functional characterization of sugarcane MYB transcription factor gene promoter (PScMYBAS1) in response to abiotic stresses and hormones. Plant Cell Rep 31(4):661–669

Sandhu JS, Kaur M, Kaur A, Kalia A (2016) Single step direct transgenic plant regeneration from adventive embryos of agro-infected sugarcane (*Saccharum* spp.) spindle leaf roll segments with assured genetic fidelity. Plant Cell Tissue Org Cult 125(1):149–162

Schenk PM, Sagi L, Remans T, Dietzgen RG, Bernard MJ, Graham MW, Manners JM (1999) A promoter from sugarcane bacilliform badnavirus drives transgene expression in banana and other monocot and dicot plants. Plant Mol Biol 39:1221–1230

Shah SH, Jan SA, Ahmad N, Khan SU, Kumar T, Iqbal A, Nasir F (2015) Use of different promoters in transgenic plant development: current challenges and future perspectives. Am Eurasian J Agric Environ Sci 15:664–675

Souza GM, Berges H, Bocs S, Casu R, D'Hont A, Ferreira JE et al (2011) The sugarcane genome challenge: strategies for sequencing a highly complex genome. Trop Plant Biol 4:145–156

Taparia Y, Gallo M, Altpeter F (2012a) Comparison of direct and indirect embryogenesis protocols, biolistic gene transfer and selection parameters for efficient genetic transformation of sugarcane. Plant Cell Tissue Organ Cult 111(2):131–141

Taparia Y, Fouad WM, Gallo M, Altpeter F (2012b) Rapid production of transgenic sugarcane with the introduction of simple loci following biolistic transfer of a minimal expression cassette and direct embryogenesis. In Vitro Cell Dev Biol Plant 48(1):15–22

Tiwari AK, Bharti YP, Tripathi S, Mishra N, Lal M, Rao GP, Sharma PK, Sharma ML (2010) Biotechnological approaches to improve sugarcane crop with special reference to disease resistance. Acta Phytopath et Entom Hung 45(2):235–249
Wang ML, Goldstein C, Su W, Moore PH, Albert HH (2005) Production of biologically active GM-CSF in sugarcane: a secure biofactory. Trans Res 14(2):167–178
Wei H, Wang ML, Moore PH, Albert HH (2003) Comparative expression analysis of two sugarcane polyubiquitin promoters and flanking sequences in transgenic plants. J Plant Physiol 160(10):1241–1251

Chapter 7
Sugarcane: An Efficient Platform for Molecular Farming

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Abstract Production of recombinant proteins in plants is referred to as molecular farming. Plant-based production of pharmaceutical and nonpharmaceutical products is gaining momentum around the world. Many plant species have become a promising alternative over the traditional expression systems to produce a variety of valuable or high-value bioltableogical molecules of pharmaceutical and nonpharmaceutical products. Plants are preferred as a platform for production of recombinant proteins because of the low costs and greater scalability of plant production systems without incurring the high costs associated with downstream processing and purification. Of the plant systems, sugarcane represents an ideal candidate for biofactory applications due to its large biomass, rapid growth rate, efficient carbon fixation pathway, a well-developed storage tissue system, minimal transgene dispersal due to vegetative method of propagation, high quantity of extractable juice with very low protein content (0.01–0.02%), and a well-established downstream processing technology. The unique aspect of sugarcane is the extraction of large juice volume (700 mL) by crushing 1 kg of cane. Therefore, sugarcane is possibly an efficient platform for molecular farming.

Keywords Biofactory • Biomolecules • Juice • r-Protein • Sugarcane • Targeting

7.1 Introduction

The improvement of agricultural crop plants relied largely on the conventional breeding programs to increase the productivity, alter the quality characteristics, or impart resistance to biotic or abiotic stresses. However, with the advancement of molecular biology techniques it has become possible to introduce entirely new characteristics efficiently through insertion of the genes coding for the desired

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C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_7

characteristics directly into the genome of the plant. Also, these techniques have made possible to introduce non-native genes from varied sources, thus tailoring the plant to produce entirely new products for innovative applications. There is an increasing demand over the years for production of recombinant proteins but the extraction and purification from their natural sources are difficult and expensive. The proteins should be produced in high amount and easily purified without any damage to the structural and functional properties. The main criteria for the selection of an expression system include cost, yield, product quality, production timescale, scale-up capacity, contamination risks, and storage. Traditionally microorganisms, plants, and animals have been used as expression systems for recombinant proteins. Bacterial expression system is the pioneer system in which first therapeutic protein, somatostatin, was successfully expressed (Itakura et al. 1977). Recombinant DNA technology was later improved and adapted to yeast (Curry et al. 1988), animal (Simons et al. 1987; Gordon et al. 1987), and plant cells (Barta et al. 1986) allowing the production of a wide range of recombinant proteins.

The advent of genetic engineering and the development of heterologous plant expression system have paved the way of using plants as biofactories for the production of commercially valuable products. Plants are modified to produce a wide range of heterologous proteins including pharmaceutical and industrial proteins, through recombinant DNA technology (Faye and Gomord 2010; Obembe et al. 2011; Wilken and Nikolov 2012). Since the first report on the application of plant genetic engineering for the production of novel protein molecules in 1986, there was a rapid development of this technology (Curtiss 1999; Curtiss and Cardineau 1990). Plants have provided humans with useful molecules for many centuries, but only in the past 20 years it has become possible to use plants for the production of heterologous proteins by means of genetic engineering (Kusnadi et al. 1997). The genes coding for the protein of interest are expressed in crops, targeted to a specific plant tissue which can be cultivated in large scale with ease and subsequently can be extracted and purified. There is an increasing demand for the production of recombinant proteins in biomedical research, industrial production, and academic investigation. Using plants as expression system provides advantages like lower production costs, large-scale production of safe and biologically active proteins (Giddings 2001), ability to perform most of the posttranslational modifications (Giddings et al. 2000; Gomord and Faye 2004), absolutely free of animal pathogens (Lienard et al. 2007), easy scale-up, better purification technology, and easier storage and transportation without refrigeration (in seed form). Hence, plants are considered as more efficient biofactories with the highest economic benefit compared to transgenic animals, animal cells, yeast, fungi, and bacteria for the production of high-value molecules.

As green bioreactors, plants offer a variety of advantages such as nearly unlimited scalability, from small-scale trials in growth chambers to large open-field mass production, and all at relatively inexpensive cost. Plants have become a promising alternative over the traditional expression systems to produce a variety of valuable biological molecules ranging from medicinal applications such as vaccines to materials like biodegradable plastics with industrial uses (Twyman et al. 2005) and

nonpharmaceutical products (Tschofen et al. 2016). Plants can produce sufficiently high yields of proteins than bacterial or yeast fermentation systems and at 0.1% of the cost of mammalian cell cultures (Twyman et al. 2003). In addition plants have an advantage over other protein expression systems, such as bacteria, for the production of antibodies and other complex proteins because they are able to make, fold, and correctly assemble proteins consisting of multiple subunits. As an example, secretory immunoglobulin A (sIgA) which consists of four linked proteins is successfully produced in tobacco plants (Goldstein and Thomas 2004). The comparison of recombinant protein production in plants, yeast, and mammalian systems (Ma et al. 2003) is given in Table 7.1.

7.2 Types of Valuable and High-Value Biomolecules

In recent years, several important products such as human biopharmaceuticals, recombinant antibodies, recombinant subunit vaccines, nutritional supplements, biodegradable plastics, and many other nonpharmaceuticals have been produced in plants with high success (Miao et al. 2008; Tschofen et al. 2016). The first pharmaceutically relevant protein made in plants was human growth hormone, which was expressed in transgenic tobacco in 1986 (Barta et al. 1986). The first antibody was also expressed in tobacco in 1989, which proved that plants could assemble complex functional glycoproteins with several subunits (Hiatt et al. 1989). Since then, other important vaccine candidates and therapeutic proteins have been produced in transgenic plants and are in different stages of clinical trials (Ma et al. 2003). Some important recombinant products produced in plant systems are given in Tables 7.2, 7.3, and 7.4.

For efficient production of recombinant products, the selection of the host plant plays an important role (Sharma and Sharma 2009). Apart from this, the life cycle of the host, biomass yield, containment, scale-up costs, form of recombinant protein, and ease of downstream processing are the deciding factors. The site of protein localization in the plant cell is another important criterion which decides the correct protein folding and its yield. Various plant organs (leaves, roots, seeds) and plant cell compartments (endoplasmic reticulum, vacuole, chloroplast, oil bodies) are being tested as sites for recombinant protein accumulation (Goldstein and Thomas 2004).

However, the synthesis of the majority of proteins of a eukaryotic cell occurs in the cytosol, and from there proteins are migrated to reach their final destination. These proteins thus contain the information necessary to be transported to the correct target compartment. Targeting to the cell secretory pathway, in particular, has been proposed to improve the stability and yield of several proteins (Ma et al. 2003; Yoshida et al. 2004; Vitale and Pedrazzini 2005). In the absence of any specific targeting signals, a protein entering the endomembrane system will follow the default secretory pathway and will be secreted to the cell exterior.

Table 7.1 Comparison of recombinant protein productions in different systems (Ma et al. 2003; Twyman et al. 2003)

System	Cost	Production time	Scale-up capacity	Product quality	Glycosylation	Contamination risks	Storage cost
Bacteria	Low	Short	High	Low	None	Endotoxins	Medium
Yeast	Medium	Medium	High	Medium	Incorrect	Low risk	Medium
Mammalian cell culture	High	Long	Very low	Very high	Correct	Virus, prions, oncogenic DNA	High
Transgenic animals	High	Very long	Low	Very high	Correct	Virus, prions, oncogenic DNA	High
Plant cell culture	Medium	Medium	Medium	High	Minor differences	Low risk	Medium
Transgenic plants	**Low**	**Long**	**Very high**	**High**	**Minor differences**	**Low risk**	**Very low**

Table 7.2 Transgenic plant-based products available in the market (modified from Sharma and Sharma 2009; Horn et al. 2004)

Product	Plant system	Company name	Commercial name
Aprotinin	Corn, tobacco	Prodigene	AproliZean
β-glucuronidase	Corn	Prodigene	GUS
Trypsin	Corn	Prodigene	TrypZean™
Recombinant human lactoferrin	Corn, rice	Meristem Therapeutics	Lacromin™
Recombinant human lysozyme	Rice	Ventria Biosciences	Lysobac™
Recombinant lipase	Corn	Meristem Therapeutics	Merispase™
Avidin	Corn	Prodigene	Avidin
Recombinant human intrinsic factor	*Arabidopsis*	Cobento Biotech AS	Coban
Collagen	Corn	Prodigene, Medicago	–

Table 7.3 Plant-derived pharmaceutical proteins for commercialization for the treatment of human diseases (Ma et al. 2003)

Product	Class	Indication	Company/ organization	Crop	Status
Various single-chain Fv	Antibody	Non-Hodgkin's lymphoma	Large Scale Biology	Tobacco	Phase I
CaroRx	Antibody	Dental caries	Planet Biotechnology Inc.	Transgenic tobacco	Phase II
E. coli heat-labile toxin	Vaccine	Diarrhea	Prodigene Inc. Arntzen group	Transgenic maize	Phase I
Gastric lipase	Therapeutic enzyme	Cystic fibrosis, pancreatitis	Meristem Therapeutics Arntzen group	Transgenic potato	Phase I
Hepatitis B virus surface	Vaccine	Hepatitis B	Thomas Jefferson University/Polish	Transgenic lettuce	Phase II
Human intrinsic factor	Dietary	Vitamin B12 deficiency	Cobento Biotech AS	Transgenic *Arabidopsis*	Phase II
Lactoferrin	Dietary	Gastrointestinal infections	Meristem Therapeutics	Transgenic maize	Phase I
Norwalk virus capsid protein	Vaccine	Norwalk virus infection	Arntzen group (Tacket et al. 2000)	Transgenic potato	Phase I
Rabies glycoprotein	Vaccine	Rabies	Yusibov et al. (2002)	Viral vectors in spinach	Phase I

Table 7.4 Commercial development of nonpharmaceutical proteins produced in plants (Tschofen et al. 2016)

Product	Company	Application	Plant species	Development stage	Country	Processing degree	Advantage
Trypsin, avidin, endo-1,4-β-D-glucanase	ProdiGene/Sigma-Aldrich	Technical reagents	Maize seeds	Commercialized	USA	Purified	Cost, animal-free
Cellobiohydrolase I	Infinite Enzymes/ Sigma-Aldrich	Technical reagent	Maize seeds	Commercialized	USA	Purified	Cost, integrated production
Growth factors, cytokines, thioredoxin, TIMP-2	Agrenvec	Research reagents	Tobacco leaves, transient	Commercialized	Spain	Purified	Cost, animal-free
Growth factors, cytokines	ORF Genetics	Research reagent	Barley seeds	Commercialized	Iceland	Purified	Cost, animal-free
Epithelial growth factor	Sif Cosmetics	Cosmetics	Barley seeds	Commercialized	Iceland	Purified	Cost, animal-free
Albumin, lactoferrin, lysozyme, transferrin, insulin	Ventria Bioscience/ InVitria	Research reagents	Rice seeds	Commercialized	USA	Purified	Cost, animal-free
Aprotinin	Kentucky Bio-Processing	Research reagent	Tobacco leaves, transient	Commercialized	USA	Purified	Cost
Collagen	CollPlant	Research reagent, tissue culture, health applications	Transgenic tobacco	Commercialized	Israel	Purified	Cost, animal-free
Trypsin, enterokinase, growth factors, cytokines	Natural Bio-Materials	Research reagents, cosmetic ingredients	Rice cell suspension	Commercialized	South Korea	Purified	Cost, animal-free

Antibody	Center for Genetic Engineering and Biotechnology	Purification of a hepatitis B vaccine	Transgenic tobacco	Commercial application	Cuba	Purified	Cost
α-Amylase	Syngenta	Bioethanol production	Maize seeds	Commercialized	USA	Biomass extract	Cost, integrated production
Phytase	Origin Agritech	Feed	Maize seeds	Commercialization pending	China	Delivered in biomass	Increased mineral availability, integrated production
Growth factors	NexGen	Tissue culture reagent	Tobacco leaves, transient	Commercialized	South Korea	Purified	Cost, animal-free

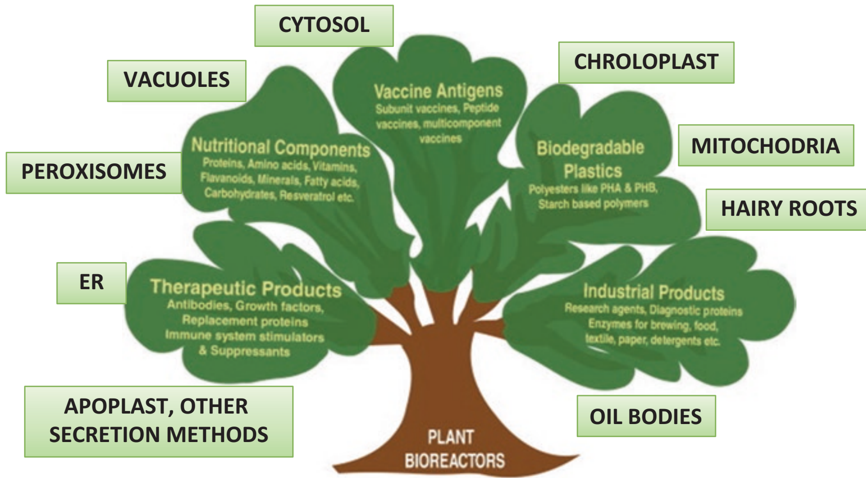

Fig. 7.1 Recombinant products and their localization sites in the plant (Sharma and Sharma 2009)

7.3 Importance of Subcellular Targeting of Proteins in Plants

Organelle-specific protein targeting, protein sequestration, or targeting to a specific cell compartments has also been readily recognized as a key factor determining the overall stability and yield of recombinant proteins in plants (Wandelt et al. 1992; Schouten et al. 1996; Gomord et al. 1997). Targeting signals can be used to intentionally retain recombinant proteins within distinct compartments of the cell to protect them from proteolytic degradation, preserve their integrity, and increase their accumulation levels (Seon et al. 2002). Several subcellular compartments have been considered as possible destinations for recombinant proteins in plant cells, endoplasmic reticulum, chloroplast, and different subcompartments of the cell secretory pathway (Ma et al. 2003; Daniell 2006; Goulet and Michaud 2006). Some recombinant products and their localization sites in the plant were shown in Fig. 7.1.

7.3.1 Targeting to the Vacuole

Plant vacuoles not only maintain the cell turgor but also store proteins and secondary metabolites. There are two distinct types of vacuole in plant cells: lytic (or vegetative) vacuoles, which have an acidic environment rich in hydrolytic enzymes, and protein storage vacuoles, which show a slightly acidic or neutral pH well adapted to

protein storage (Robinson et al. 2005). Signal sequences that are responsible for targeting the protein to the vacuoles have been identified (Brown et al. 2003; Jolliffe et al. 2004; Matsuoka and Neuhaus 1999) but no consensus protein sorting signal has been optimized so far. High accumulation levels have been reported for a number of recombinant proteins targeted to the vacuole including synthetic analogue of spider dragline silk protein DP1B in *Arabidopsis thaliana* (Yang et al. 2005), *E. coli* heat-labile enterotoxin B in tobacco (Streatfield and Howard 2003), toxic biotin-binding proteins avidin and streptavidin in tobacco (Murray et al. 2002), *Aspergillus niger* phytase in maize (Arcalis et al. 2004), and a thermo stable glucanase of bacterial origin in barley (Howard and Donnelly 2004). In contrast to protein-storing vacuoles, vacuoles of vegetative tissues like leaves have higher hydrolytic activity and recombinant protein targeted would degrade. Therefore, mechanisms or the signals required to store recombinant proteins in the vacuoles need further exploration. The impact of subcellular targeting on recombinant protein yield in transgenic plant systems is given in Table 7.5.

In general, lytic vacuoles are not considered as a suitable destination for most of the recombinant proteins *in planta*, owing to their high proteolytic content (Goulet and Michaud 2006). By contrast, protein storage vacuoles present a milder acidic environment compatible with protein accumulation (Stoger et al. 2005), especially in seeds, where they are most abundant (Park et al. 2004). But this situation is not the case for all the proteins; the recombinant proteins which are highly stable to acidic conditions can withstand in lytic vacuole. Therefore, it is possible that this subcellular compartment has a dominant effect on the localization of foreign proteins expressed in the secretory pathway (Vitale and Pedrazzini 2005). Compounds like hydrolases, esterases, nucleases, and peroxidases are involved in degradation and recycling of proteins by maintaining ion homeostasis (Marty 1999).

Among the different role of vacuoles, some physical and chemical functions are fundamental for cell viability. These roles include mechanical support by turgor maintenance, which is also involved in cell growth. The vacuole is also implicated in autophagy, an autodigestion of cell material involved in turnover of several cellular compounds. Moreover, they can fuse to form a unique large central compartment or convert between the several kinds of vacuoles of plant cells (Bethke et al. 1998; He et al. 2007). Finally, the vacuole is also involved in programmed cell death which is an active process involved in the selective elimination of certain cells upon chemical and physical stresses (Gietl and Schmid 2001; Greenwood et al. 2005).

Plant cells can have up to three different vacuoles with different functions in a single cell: the lytic vacuole (LV), the vegetative storage (neutral), and the protein storage vacuole (PSV) (Hoh et al. 1995; Paris et al. 1996; Sansebastiano et al. 2001). Tonoplast intrinsic proteins (TIPs, a family of aquaporins) have been used as maker proteins for different types of vacuoles (Paris et al. 1996; Jiang et al. 2000).

Table 7.5 Impact of subcellular targeting on recombinant protein yield in transgenic plant systems (Benchabane et al. 2008)

Yield (fold increase of r-proteins with that of control)									
Protein	Transformed species	Plant organ	C	ER	V	A	P	N	Reference
ScFv anticutinase	*N. tobacum*	Leaf	0	100		1			Schouten et al. (1996)
Antioxazolone	*N. tobacum*	Leaf		0–20		1			Fiedler et al. (1997)
ScFv anti-oxazolone	*Solanum tuberosum*	Tuber				1			Artsaenko et al. (1998)
Dihydroflavonol 4-reductase	*Petunia hybrida*	Petal	1	2, 30					De Jaeger et al. (1999)
BiscFv 2429	*N. tobacum*	BY-2 cells	low	10		1			Fischer et al. (1999)
FAb MAK33	*Arabidopsis thaliana*	Leaf/seed		1		1			Peeters et al. (2001)
scFv anti-carcinoembryonic	*N. tobacum*	Leaf		25		1			Stoger et al. (2002)
Carcinoembryonic	*N. tobacum*	Leaf		2–6		1			Vaquero et al. (2002)
Ab 14D9 κ chain	*N. tobacum*	Leaf		8		1			Petruccelli et al. (2006)
Aprotinin	*Saccharum* sp.	Stem			Many				Palaniswamy et al. (2016)
GUS	*Saccharum* sp.	Stem			Many				Palaniswamy et al. (2016)
GFP	*Saccharum* sp.	Stem			Many				Palaniswamy et al. (2016)
Vaccines									
Escherichia coli heat-labile enterotoxin B	*Zea mays*	Seed	1	100	20,000	3300	7	21	Streatfield and Howard (2003)
Hepatitis B surface antigen	*N. tobacum*	BY-2 cells	1	1.4		1.8			Sojikul et al. (2003)
Pollen allergens	*Oryza sativa*	Seed	0	4–6		1			Takagi et al. (2005)
Medical proteins									

Yield (fold increase of r-proteins with that of control)									
Protein	Transformed species	Plant organ	C	ER	V	A	P	N	Reference
Human epidermal growth factor	*N. tobacum*	Leaf	1			10,000			Wirth et al. (2004)
Human growth hormone	*N. benthamiana*	Leaf	1			1000	10		Gils et al. (2005)

C cytoplasm, *ER* endoplasmic reticulum, *V* vacuole, *N* nucleus, *A* apoplast, P peroxisomes

7.4 Different Plant Species as a Platform for Molecular Farming

Plants provide a promising platform for the production of recombinant proteins, offering advantages over conventional fermentation systems that employ bacteria, yeast, and mammalian cells in terms of scalability (agricultural scale because no specific facilities are required), safety (no contamination with mammalian pathogens such as viruses and prions), and cost-effectiveness (Sharma and Sharma 2009; Tiwari et al. 2009; Twyman et al. 2003). Plant production systems can be divided into those using stable transgenic plants generated by nuclear genome and plastid genome transformation and plant virus-based or *Agrobacterium*-based transient expression platforms. Each of the production systems has advantages or disadvantages. When recombinant proteins are produced by transient expression system, robust yields of recombinant products can be obtained within a few weeks, but they have to be extracted and purified for use because tobacco is mainly used as production host. Plastid-based expression system also gives rise to high-level expressions of recombinant proteins without gene silencing and position effect due to the site-specific homologous recombination and multicopy number of genome. Furthermore, there is little possibility of pollen-mediated gene contamination due to the maternal transgene inheritance, but there is no posttranslational modification of products such as glycosylation (Daniell et al. 2002).

The range of plant species amenable to transformation is growing at a phenomenal rate and it is unclear at present which species are optimal for molecular farming. Many factors need to be taken into consideration (Schillberg et al. 2003). The factors that are taken into consideration are the total biomass yield and the storage and distribution of the product. Various production platforms have been developed for molecular farming in plants which include leafy crops (alfalfa, lettuce, Arabidopsis, spinach, tobacco), cereals and legumes (barley, maize, pea, pigeon pea, rice, wheat), fruits and vegetables (banana, carrot, potato, tomato, carrots), oil-yielding plants (false flax, flax, rape, safflower, soybean, white clover, white mustard), and sugar crops (sugar beet and sugarcane) (Twyman et al. 2003, 2005).

Tobacco has well-developed technology for gene transfer and expression, high biomass yield, potential for rapid scale-up owing to prolific seed production, and availability of large-scale infrastructure for processing. The demerits of this system include degradation of protein through proteolysis, presence of toxic alkaloids, and interference of transgene with normal plant metabolism.

Cereals lack the phenolic substances, thereby increasing the efficiency of downstream processing (Ma et al. 2003). Legumes, such as alfalfa and soybean, and cereal crops, such as corn and rice, have been considered as ideal candidates for protein production because the protein can be targeted to accumulate in the seed and the seed can be harvested and stored for an extended amount of time. Alfalfa and soybean produce lower amounts of leaf biomass than tobacco but have the advan-

tage of using atmospheric nitrogen through nitrogen fixation, thereby reducing the need for chemical inputs.

Fruits and vegetables: The main benefit of fruits, vegetables, and leafy salad crops is that they can be consumed raw or partially processed, which makes them particularly suitable for the production of recombinant subunit vaccines, food additives, and antibodies (Ma et al. 2003).

Perennial grass: Perennial grasses like sugarcane provide a "secure" platform for production of recombinant proteins. Sucrose, the food commodity derived from sugarcane, is sold as a refined crystal that is essentially free of protein, rather than a whole fruit or vegetable. Hence sugarcane producing a pharmaceutical protein and nonpharmaceutical products are not mixed into the food supply; the food product (refined sucrose) would remain unaffected.

7.5 Sugarcane as a Platform for Biofarming

Sugarcane, a monocot C_4 plant, is a candidate crop to be exploited as a platform for molecular farming due to its huge biomass production, the availability of a large above-ground storage tissue in the form of parenchyma cells with a large storage space in the form of a vacuole that stores sugar, and a canopy which constitutes 20% of the total biomass (Welbaum and Meinzer 1990). Diagrammatic representation of partition of sugarcane biomass is shown in Fig. 7.2. Sugarcane is vegetatively propagated and many commercial canes are either nonflowering or fail to produce viable sexual seeds. This precludes the transgenes being dispersed through pollen to other cultivars or related species. In addition, a robust transgenic technology is in place for sugarcane with a rapid clonal multiplication protocol (Arencibia et al. 1998; Manickavasagam et al. 2004; Lakshmanan et al. 2005; Arvinth et al. 2010). Sugarcane is one of the largest producers of biomass among the crop plants. Sugarcane has many significant advantages for transgene product containment that makes it a "safe" platform for the production of high-value molecules. Moreover, sucrose or jaggery, the economic products from sugarcane, is produced from sugarcane juice after subjecting it to very high temperature, during which most of the proteins would get degraded. Sucrose is a refined crystal that is essentially free of proteins, unlike a transgenic fruit or vegetable. These facts point towards the safety of transgenic sugarcane in an environmental perspective. The most attractive aspect of sugarcane is that one can get up to 700 mL of juice by crushing 1 kg of cane. Sugarcane juice is stored in the vacuoles which occupies about 80–85% of the parenchymatous storage cells in the stem. Sugar is stored in lytic vacuoles. For the production of any high-value molecules these should be targeted to the vacuole so as to extract and purify the protein of interest from the juice.

Considerable progress has been achieved in *in planta* production of novel compounds. Compounds traditionally synthesized from petrochemicals have been produced in field crops like maize and sorghum (McQualter et al. 2005). McQualter

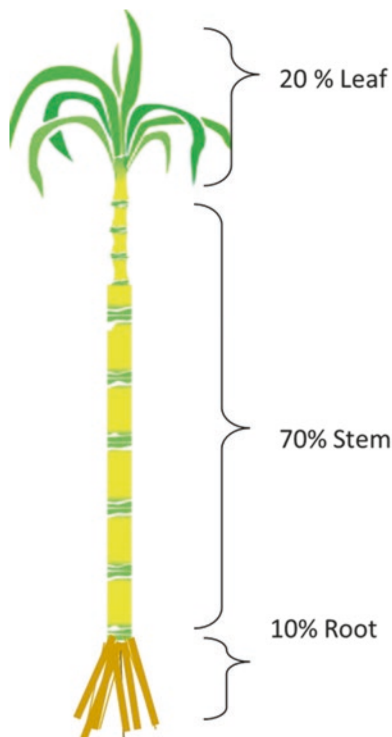

Fig. 7.2 Diagrammatic representation of partition of different parts of the sugarcane biomass

et al. (2005) have reported the production of p-hydroxybenzoic acid (pHBA) in sugarcane, a major monomer used in the manufacture of liquid crystal polymers (LCPs) through metabolic engineering of two independent pathways namely shikimic and phenylpropanoid pathways. Transgenics expressing the chloroplast-targeted version of chorismate pyruvate-lyase (CPL) derived from *Escherichia coli* catalyzed the synthesis of pHBA from chorismate, an intermediate in shikimate pathway. In another set of transgenics, the enzyme 4-hydroxycinnamoyl-coA hydratase/lyase (HCHL) hydrated and catalyzed the retro-aldol cleavage of 4-hydroxycinnamoyl-coA thioesters, an intermediate in the phenyl propanoid pathway, for the production (pHBA). A comparison of these two approaches has shown that the transgenics expressing HCHL, which produced a maximum of 7.3% and 1.5% of glucose conjugates of pHBA in the leaf and stem, respectively, was more efficient in terms of pHBA production than that of the transgenics with CPL. In general it was observed that the accumulation of transgene products in sugarcane culm was less than that in the leaves when constitutive promoters were used. This may be because with the constitutive promoters transgene products are generally accumulated in cytosol and the parenchyma cells, which constitutes the major cell type in the culm which is largely filled with the vacuole with a thin layer of cytosol. Perhaps one way of addressing this problem is the use of strong stem-specific

promoters, or wherever possible to use a signal peptide to secrete the transgene product to the apoplastic space or to direct it to vacuole.

Attempts were also made for the production of polyhydroxybutyrate (PHB), a bacterial polyhydroxyalkanate polymer with a side chain, in transgenic sugarcane through metabolic engineering mediated through successive action of three enzymes—ketothiolase (PHAA), acetacetyl-reductase (PHAB), and PHB synthase (PHAC)—on acetyl-coenzyme A (acety-coA), as an alternative to the current production through bacterial fermentation (Petrasovits et al. 2007). A comparative analysis on the pattern of PHA accumulation in cytosol, mitochondria, and plastids has shown that the leaves had the maximum (1.88% dry leaf weight) followed by traces in the cytosol of the cells in culms and practically no accumulation in the mitochondria. As a continuation, the same group has also studied the spatiotemporal accumulation of PHA in the plastid-targeted transgenic sugarcane in a limited glasshouse trial and reported the existence of a vertical gradient in the concentration—lowest in the youngest leaves and highest in the oldest leaves. However, the maximum yield achieved was only (0.79%) 11.9 g in 1.51 kg dry weight of leaf, which is very low for an economic production of the polymer.

An attempt has been made for the field production of the human cytokine granulocyte macrophage colony-stimulating factor (GM-CSF) in transgenic sugarcane that is otherwise produced by multiple cell types throughout the human body in low concentration. GM-CSF is required for the division of the human bone marrow cells. This glycoprotein with varying molecular weight (14.5–28 kDa) has clinical applications for the treatment of neutropenia and aplastic anemia and has also been administered to patients in bone marrow transplantation to reduce infection risk by increasing the response of neutrophils (Shin et al. 2003). Sugarcane has been transformed with constructs with the gene coding for GM-CSF driven by maize ubi1 or SCubi9. The gene was tagged with or without a C-terminal HDEL for ER retention and 6× histidine tag for the purification of the extracted protein. The maximum level of protein accumulation achieved in around 200 transgenics was 0.02% of total soluble protein. The study has shown that ER retention signals improved the accumulation of the protein though there was no significant difference between the two promoters used. Interestingly use of 6X His tag has shown a twofold decrease in GM-CSF accumulation. There was gradual decrease in the recombinant protein in the sugarcane juice from the top younger internodes to older internodes at the bottom of the culm. Bioassay with the extracts from the transgenic sugarcane has demonstrated the biological activity of the sugarcane-produced GM-CSF (Wang et al. 2005).

Sugarcane has an advantage over other plants in the production of certain compounds that can be obtained from the sucrose synthesis pathway through metabolic engineering (Chang et al. 2007). An attempt has been made to manipulate the sucrose synthesis pathway to produce sorbitol, a six-carbon sugar alcohol by converting glucose-6-phosphate to sorbitol through the action of sorbitol-6-phosphate dehydrogenase (S6PHD) gene (mds6pdh) derived from *Malus domestica*, and thus sorbitol-producing "sorbitol cane" was developed. A gradient in sorbitol accumulation was observed from the tip to the base of the leaves. The necrotic zones at the tip

had the highest concentration of 38.5 mg/g dry weight and the bleached region towards the middle of the leaf with 3.7 mg/g dry weight and the healthy green zones with 0.9 mg/g dry weight. However, sorbitol was not detectable in the stalk in the 11-month-old crop except in the tenth internode and the sucrose remained unchanged. Sorbitol production in these canes has led to a yield penalty with the reduction in stalk yield, stalk length, number of internodes, and total biomass compared to that of the untransformed control plants, probably due to the sorbitol-mediated stress (Chang et al. 2007).

Jackson et al. (2007) have isolated and characterized a vacuolar targeting motif from the N-terminus (NTPP) of a legumain homologue from sugarcane that was found to be highly conserved across legumain homologues known to target to the vacuoles. The efficacy of this motif for targeting the protein to lytic vacuole of sugarcane parenchymatous cells was demonstrated through transformation studied with vectors containing this motif fused to GFP as the reporter protein. Also it has been shown that only a five-amino-acid sequence (IRLPS) is required to target the protein to the vacuole. This sequence shared some common features with legumain of other species. However, the highly acidic environment and the proteolytic activity of different enzymes present in the lytic vacuoles of sugarcane inhibited the visualization the GFP, but the treatment with Concanamycin A probably inhibited specifically the vacuolar H^+ATPase and thus prevented the quenching of GFP fluorescence either through neutralizing the acidity or reducing the proteolytic activity. Based on these results Jackson et al. (2007) have suggested that the proteolytic environment and the acidic nature of sugarcane vacuole need to be considered for future use of sugarcane vacuoles of the stem parenchyma for biofarming applications.

Attempt has been made to over-express His-tagged cystatin, a protease inhibitor in sugarcane, and it was extracted from the transgenic leaves. Cystatin was subsequently purified through nickel affinity column and resulted in an estimated yield of 400 g/ha (Ribeiro et al. 2008). Barros et al. (2013) purified the recombinant lysozyme from transgenic sugarcane stalks using two extraction steps of cross-flow filtration and hydrophobic interaction chromatography which resulted in 50% purity and 69% of recovery of lysozyme. These two recombinant proteins were not targeted to any particular organelle. Targeting of recombinant proteins to the lytic vacuoles of sugarcane may be an alternative for enhancing the yield of the proteins severalfold due to the large size of vacuoles in sugarcane stem parenchyma cells.

The experiments so far conducted for the production of heterologous proteins or biomolecules in sugarcane indicate the possibility to use sugarcane as a platform for biofarming. However, all attempts to produce recombinant proteins in sugarcane have resulted in very low yields (0.02% of total soluble protein) (Wang et al. 2005) when compared with what has been achieved in rice (1.3%) and tobacco (2%) (Jackson et al. 2010). However, the results of the studies so far conducted on the use of sugarcane as a platform for the production of heterologous molecules point towards the fact that targeting to vacuolar compartments was more effective than their accumulation in cytoplasm, endoplasmic reticulum, or apoplasm. This is

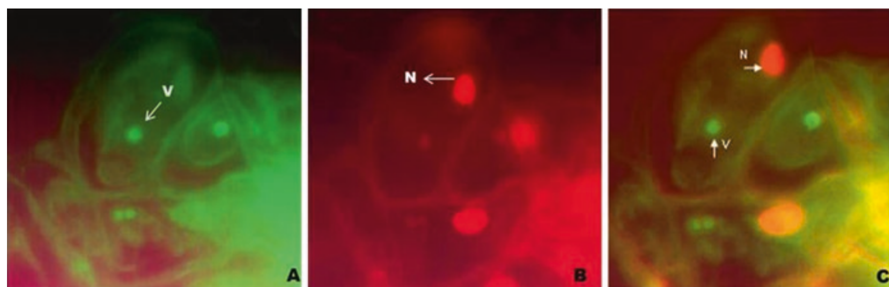

Fig. 7.3 Fluorescent microscopic image of sugarcane calli transformed with GFP gene fused with 78 bp vacuolar targeting determinant (**a**) V-vacuole with GFP fluorescence. (**b**) N-propidium iodide-stained nucleus, (**c**) Merged images of (**a**) and (**b**) (Palaniswamy et al. 2016)

particularly important when the expressed molecule is phyotoxic. Expression of avidin in sugarcane has shown that with its high affinity to co-vitamin biotin, the normal plant growth has been affected when it was accumulated in cytoplasm, apoplast, or endoplasmic reticulum, but not when it was accumulated in lytic vacuole, through which cellular biotin pool was separated from avidin. However, the proteolytic environment in the lytic vacuole resulted in site-specific proteolysis of the recombinant protein (Jackson et al. 2010).

More recently, with 78-bp-long putative vacuolar targeting sequence from the N-terminal domain of unknown function (DUF) in *Triticum aestivum* 6-SFT (sucrose: fructan 6-fructosyl transferase) was fused with gene coding for the green fluorescent protein (GFP) under strong constitutive promoter (Port ubi882) and targeted to vacuoles of sugarcane stem cells (Figs. 7.3 and 7.4). In addition, the study also generated sugarcane transgenics with His-tagged β-glucuronidase (GUS) and aprotinin targeted to the lytic vacuole (Fig. 7.5), and these two proteins were isolated and purified from the transgenic sugarcane and compared with commercially available protein samples. These experiments demonstrated that the novel vacuolar targeting determinant could localize recombinant proteins (r-proteins) to the vacuole in high concentrations and such targeted r-proteins can be purified from the juice with a few simple steps (Palaniswamy et al. 2016).

Till date, substantial efforts have been directed toward sugarcane as a biofabric for high-value products. While these achievements are commendable, a greater understanding of the sugarcane genome, cell, and whole-plant biology will accelerate the implementation of commercially significant biotechnology outcomes (Lakshmanan et al. 2005; Ming et al. 2006). The rapid progress in molecular biology and emerging biotechnology innovations will play significant roles in future sugarcane crop improvement programs and will offer many new opportunities to develop it as a new-generation industrial crop and a sustainable biofactory (Gomez-Merino et al. 2014). The possible diagrammatic representation of different steps involved in r-protein production in sugarcane is shown in Fig. 7.6.

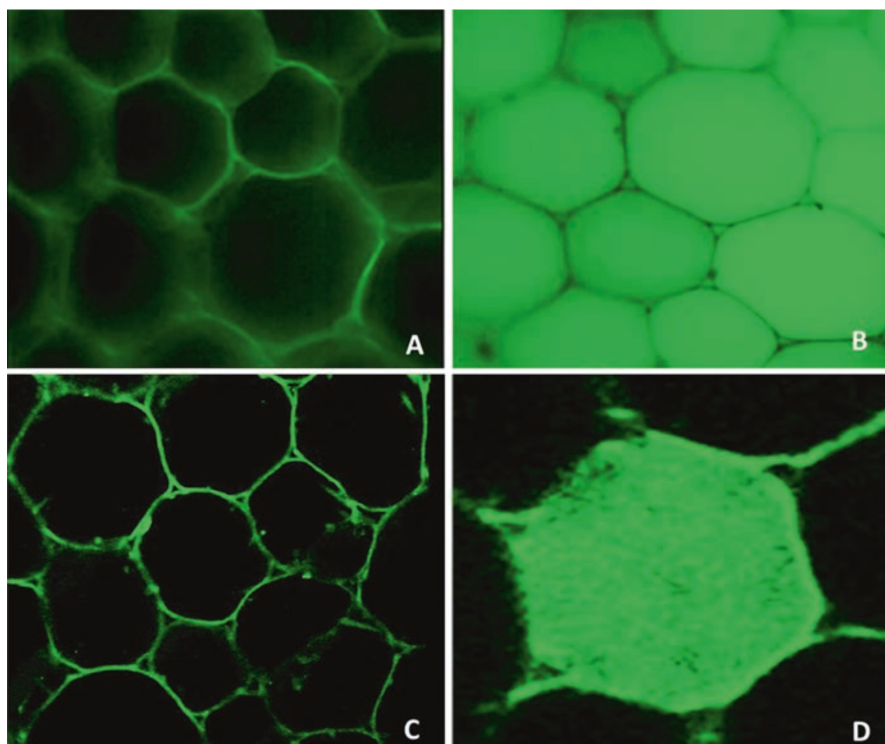

Fig. 7.4 Fluorescent microscopic images (**a**, **b**) and confocal microscopic images (**c**, **d**) of mature sugarcane stem parenchymal cells. (**a**) Untransformed sugarcane. (**b**) Localization of GFP in vacuoles of transgenic sugarcane. (**c**) Untransformed sugarcane. (**d**) Localization of GFP in vacuoles of transgenic sugarcane (Palaniswamy et al. 2016)

7.6 Conclusion

Sugarcane is one of the most productive crops among cultivated plats. It is also an important crop for food and energy production. Another unique feature of sugarcane is that it accumulates high levels of sucrose in its stems, an economy part of the crop. Sucrose is stored in the vacuoles and this vacuolar compartment occupies a large proportion of the stage parenchyma cells in sugarcane stem. With the advent and advancement in biotechnology, sugarcane vacuoles have been regarded as an ideal site for the production of and (or) storage of commercially valuable pharmaceuticals, nonpharmaceuticals, and industrial products. Worldwide, there are several significant efforts under way to develop sugarcane as a biofactory system. The results of different experiments showed that targeting of heterologous molecules to vacuolar compartments is more effective than their accumulation in other parts of the cell. And also production of heterologous products targeted to the vacuole helps to extract and purity the high-value molecules from the juice easily with negligible amount of

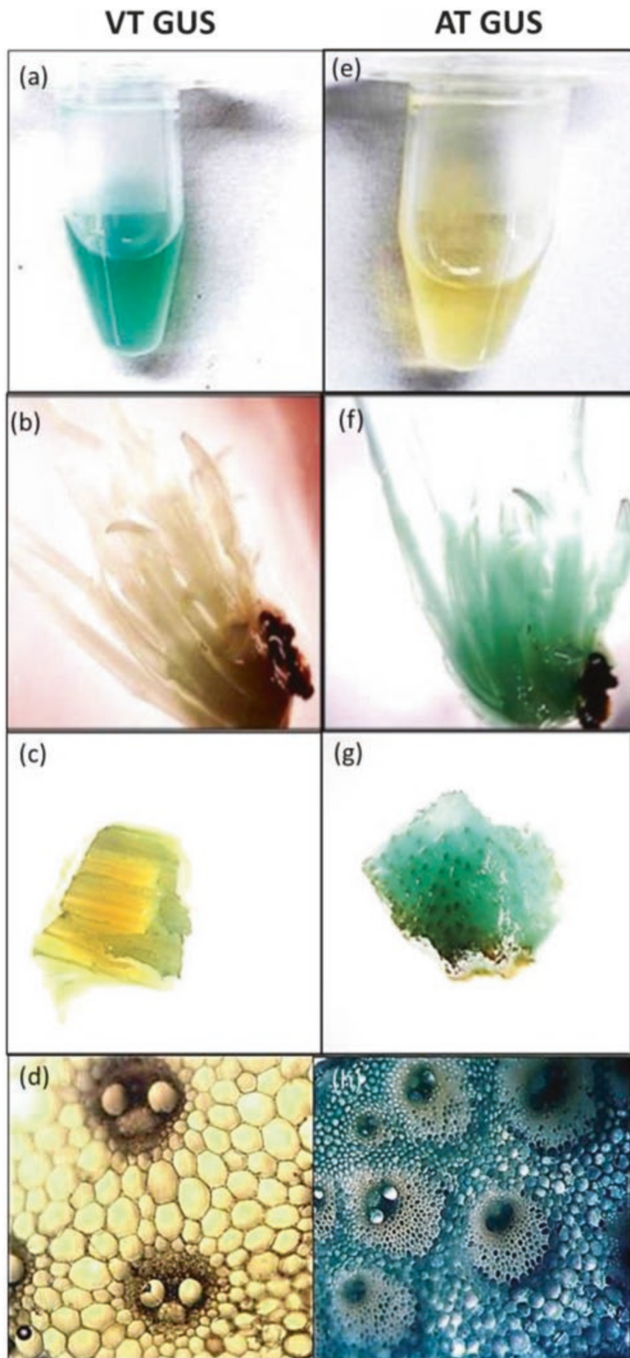

Fig. 7.5 Histochemical GUS staining. (**a**–**e**) show GUS activity in sugarcane transgenics. (**a**) Juice, (**b**) plantlets, (**c**) stem, (**d**) cross section of the stem, (**e**) enlarged vascular bundles. (**f**–**j**) show GUS activity in transformed sugarcane. (**f**) Juice, (**g**) plantlets, (**h**) stem, (**i**) cross section of the stem, (**j**) enlarged vascular bundles. *Px* protoxylem, *Mx* metaxylem, *Py* parenchyma cells, *P* phloem (Palaniswamy et al. 2016)

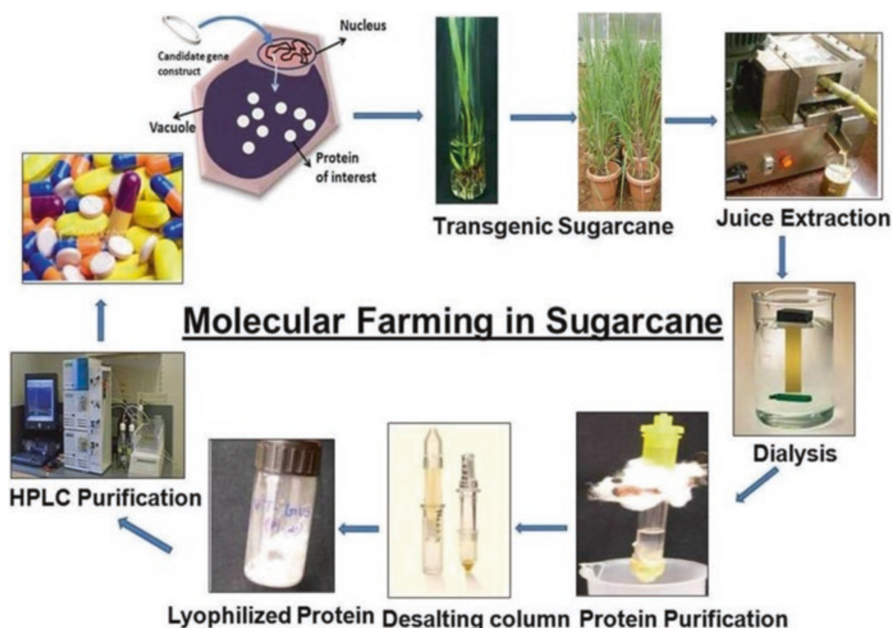

Fig. 7.6 Diagrammatic representation of different steps involved in r-protein production in sugarcane

proteins. This makes sugarcane a better platform for production of commercially important recognizant proteins. However, high yield of recombinant proteins in sugarcane depends on properties of protein, targeting to the vacuoles as lytic vacuoles of sugarcane rich in proteolytic enzymes, and easy downstream processing.

References

Arcalis E, Marcel S, Altmann F, Kolarich D, Drakakaki G, Fischer R, Christou P, Stoger E (2004) Unexpected deposition patterns of recombinant proteins in post-endoplasmic reticulum compartments of wheat endosperm. Plant Physiol 136(3):3457–3466

Arencibia AD, Carmona ER, Téllez P, Chan MT, SM Y, Trujillo LE, Oramas P (1998) An efficient protocol for sugarcane (Saccharum spp. L.) transformation mediated by *Agrobacterium tumefaciens*. Transgenic Res 7:213–222

Artsaenko O, Kettig B, Fiedler U, Conrad U, During K (1998) Potato tubers as a biofactory for recombinant antibodies. Mol Breed 4:313–319

Arvinth S, Arun S, Selvakesavan RK, Srikanth J, Mukunthan N, Ananda Kumar P, Premachandran MN, Subramonian N (2010) Genetic transformation and pyramiding of aprotinin-expressing sugarcane with cry1Ab for shoot borer (*Chilo infuscatellus*) resistance. Plant Cell Rep 29:383–395

Barros GO, Ballen MA, Woodard SL, Wilken LR, White SG, Damaj MB, Mirkov TE, Nikolov ZL (2013) Recovery of bovine lysozyme from transgenic sugarcane stalks: extraction, membrane filtration, and purification. Bioprocess Biosyst Eng 36(10):1407–1416

Barta A, Sommergruber K, Thompson D, Hartmuth K, Matzke MA, Matzke AJ (1986) The expression of a nopaline synthase—human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue. Plant Mol Biol 6(5):347–357
Benchabane M, Goulet C, Rivard D, Faye L, Gomord V, Michaud D (2008) Preventing unintended proteolysis in plant protein biofactories. Plant Biotechnol J 6:633–648
Bethke PC, Swanson SJ, Hillmer S, Jones RL (1998) From storage compartment to lytic organelle: the metamorphosis of the aleurone protein storage vacuole. Ann Bot 82(4):399–412
Brown JC, Jolliffe NA, Frigerio L, Roberts LM (2003) Sequence-specific, Golgi-dependent vacuolar targeting of castor bean 2S albumin. Plant J 36(5):711–719
Chang MC, Eachus RA, Trieu W, Ro DK, Keasling JD (2007) Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. Nat Chem Biol 3(5):274–277
Curry C, Gilkes N, O'Neill G, Miller RC, Skipper N (1988) Expression and secretion of a cellulomonas fimi exoglucanase in *Saccharomyces cerevisiae*. Appl Environ Microbiol 54(2):476–484
Curtiss R (1999) Genetically modified plants for use as oral immunogens. Mucosal Immunol Update 7(1):10–12
Curtiss R, Cardineau GA (1990) Oral immunization by transgenic plants. World Patent Application WO 90/02484. WIPO, Washington University (assignee)
Daniell H (2006) Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. Biotechnol J 1(10):1071–1079
Daniell H, Khan MS, Allison L (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends Plant Sci 7(2):84–91
De Jaeger G, Buys E, Eeckhout D, De Wilde C, Jacobs A, Kapila J et al (1999) High level accumulation of single-chain variable fragments in the cytosol of transgenic *Petunia hybrida*. Eur J Biochem 259(1–2):426–434
Faye L, Gomord V (2010) Success stories in molecular farming-a brief overview. Plant Biotechnol J 8(5):525–528
Fiedler U, Phillips J, Artsaenko O, Conrad U (1997) Optimization of scFv antibody production in transgenic plants. Immunotechnology 3:205–216
Fischer R, Schumann D, Zimmermann S, Drossard J, Sack M, Schillberg S (1999) Expression and characterization of bispecific single-chain Fv fragments produced in transgenic plants. Eur J Biochem 262:810–816
Giddings G (2001) Transgenic plants as protein factories. Curr Opin Biotechnol 12(5):450–454
Giddings G, Allison G, Brooks D, Carter A (2000) Transgenic plants as factories for biopharmaceuticals. Nat Biotechnol 18(11):1151–1155
Gietl C, Schmid M (2001) Ricinosomes: an organelle for developmentally regulated programmed cell death in senescing plant tissues. Naturwissenschaften 88(2):49–58
Gils M, Kandzia R, Marillonnet S, Klimyuk V, Gleba Y (2005) High-yield production of authentic human growth hormone using a plant virus-based expression system. Plant Biotechnol J 3(6):613–620
Goldstein DA, Thomas JA (2004) Biopharmaceuticals derived from genetically modified plants. QJM 97(11):705–716
Gomez-Merino FC, Trejo-Tellez LI, Senties-Herrera HE (2014) Sugarcane as a novel biofactory: potentialities and challenges. In: Guevara-Gonzalez R, Torres-Pacheco I (eds) Biosystems engineering: biofactories for food production in the century XXI. Springer, Cham, pp 129–149
Gomord V, Faye L (2004) Post translational modification of therapeutic proteins in plants. Curr Opin Plant Biol 7(2):171–181
Gomord V, Denmat LA, Fitchette-Laine AC, Satiat-Jeunemaitre B, Hawes C, Faye L (1997) The C-terminal HDEL sequence is sufficient for retention of secretory proteins in the endoplasmic reticulum (ER) but promotes vacuolar targeting of proteins that escape the ER. Plant J 11(2):313–325
Gordon K, Lee E, Vitale JA, Smith AE, Westphal H, Hennighausen L (1987) Production of human tissue plasminogen activator in transgenic mouse milk. Nat Biotechnol 11:1183–1187

Goulet C, Michaud D (2006) Degradation and stabilization of recombinant proteins in plants. In: Taxeira da Silva J (ed) Floriculture, ornamental and plant biotechnology, vol IV. Global Science Books, London, pp 35–40

Greenwood JS, Helm M, Gietl C (2005) Ricinosomes and endosperm transfer cell structure in programmed cell death of the nucellus during Ricinus seed development. Proc Natl Acad Sci U S A 102(6):2238–2243

He F, Huang F, Wilson KA, Tan-Wilson A (2007) Protein storage vacuole acidification as a control of storage protein mobilization in soybeans. J Exp Bot 58(5):1059–1070

Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342(6245):76–78

Hoh B, Hinz G, Jeong BK, Robinson DG (1995) Protein storage vacuoles form *de novo* during pea cotyledon development. J Cell Sci 108(Pt 1):299–310

Horn ME, Woodard SL, Howard JA (2004) Plant molecular farming: systems and products. Plant Cell Rep 22(10):711–720

Howard JA, Donnelly KC (2004) A quantitative safety assessment model for transgenic protein products produced in agricultural crops. J Agric Environ Ethics 17:545–558

Itakura K, Hirose T, Crea R, Riggs AD, Heyneker HL, Bolivar F, Boyer HW (1977) Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. Science 198(4321):1056–1063

Jackson MA, Rae AL, Casu RE, Grof CPL, Bonnet GD, Maclean DJ (2007) A bioinformatic approach to the identification of a conserved domain in of a sugarcane legumain that directs GFP to the lytic vacuole. Funct Plant Biol 34:633–644

Jackson MA, Nutt KA, Hassall R, Rae AL (2010) Comparative efficiency of subcellular targeting signals for expression of a toxic protein in sugarcane. Funct Plant Biol 37(8):785–793

Jiang L, Phillips TE, Rogers SW, Rogers JC (2000) Biogenesis of the protein storage vacuole crystalloid. J Cell Biol 150(4):755–770

Jolliffe NA, Brown JC, Neumann U, Vicre M, Bachi A, Hawes C, Ceriotti A, Roberts LM, Frigerio L (2004) Transport of ricin and 2S albumin precursors to the storage vacuoles of *Ricinus communis* endosperm involves the Golgi and VSR-like receptors. Plant J 39(6):821–833

Kusnadi AR, Nikolov ZL, Howard JA (1997) Production of recombinant proteins in transgenic plants: practical considerations. Biotechnol Bioeng 56(5):473–484

Lakshmanan P, Geijskes RJ, Aitken KS, Grof CLP, Bonnett GD, Smith GR (2005) Sugarcane biotechnology: the challenges and opportunities. In Vitro Cell Dev Biol Plant 41:345–363

Lienard D, Sourrouille C, Gomord V, Faye L (2007) Pharming and transgenic plants. Biotechnol Annu Rev 13:115–147

Ma JKC, Drake PMW, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794–805

Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, Kasthurirengan S (2004) *Agrobacterium*-mediated genetic transformation and development of herbicide-resistant sugarcane (Saccharum species hybrids) using axillary buds. Plant Cell Rep 23:134–143

Marty F (1999) Plant vacuoles. Plant Cell 11(4):587–600

Matsuoka K, Neuhaus J (1999) Cis-elements of protein transport to the plant vacuoles. J Exp Bot 50:165–174

McQualter RB, Chong BF, Meyer K, Van Dyk DE, O'Shea MG, Walton NJ, Viitanen PV, Brumbley SM (2005) Initial evaluation of sugarcane as a production platform for p-hydroxybenzoic acid. Plant Biotechnol J 3(1):29–41

Miao Y, Ding Y, Sun QY, ZF X, Jiang L (2008) Plant bioreactors for pharmaceuticals. Biotechnol Genet Eng Rev 25:363–380

Ming R, Moore PH, KK W, D'Hont A, Glaszmann JC, Tew TL, Mirkov TE, da Silva J, Jifon J, Rai M, Schnell RJ, Brumbley SM, Lakshmanan P, Comstock JC, Paterson AH (2006) Sugarcane improvement through breeding and biotechnology. In: Janick J (ed) Plant breeding reviews, vol 27. Wiley, New Jersey, pp 15–118

Murray C, Sutherland PW, Phung MM, Lester MT, Marshall RK, Christeller JT (2002) Expression of biotin-binding proteins, avidin and streptavidin, in plant tissues using plant vacuolar targeting sequences. Transgenic Res 11(2):199–214

Obembe OO, Popoola JO, Leelavathi S, Reddy SV (2011) Advances in plant molecular farming. Biotechnol Adv 29(2):210–222

Palaniswamy H, Syamaladevi DP, Mohan C, Philip A, Petchiyappan A, Narayanan S (2016) Vacuolar targeting of r-proteins in sugarcane leads to higher levels of purifiable commercially equivalent recombinant proteins in cane juice. Plant Biotechnol J 14:791–807

Paris N, Stanley CM, Jones RL, Rogers JC (1996) Plant cells contain two functionally distinct vacuolar compartments. Cell 85(4):563–572

Park M, Kim SJ, Vitale A, Hwang I (2004) Identification of the protein storage vacuole and protein targeting to the vacuole in leaf cells of three plant species. Plant Physiol 134(2):625–639

Peeters K, De Wilde C, Depicker A (2001) Highly efficient targeting and accumulation of a F(ab) fragment within the secretory pathway and apoplast of *Arabidopsis thaliana*. Eur J Biochem 268(15):4251–4260

Petrasovits LA, Purnell MP, Nielsen LK, Brumbley SM (2007) Production of polyhydroxybutyrate in sugarcane. Plant Biotechnol J 5(1):162–172

Petruccelli S, Otegui MS, Lareu F, Tran Dinh O, Fitchette AC et al (2006) A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and sorted to protein storage vacuoles in seeds. Plant Biotechnol J 4(5):511–527

Ribeiro CW, Soares-Costa A, Falco MC, Chabregas SM, Ulian EC et al (2008) Production of a His-tagged canecystatin in transgenic sugarcane and subsequent purification. Biotechnol Prog 24(5):1060–1066

Robinson DG, Oliviusson P, Hinz G (2005) Protein sorting to the storage vacuoles of plants: a critical appraisal. Traffic 6(8):615–625

Sansebastiano GPD, Paris N, Marc-Martin S, Neuhaus JM (2001) Regeneration of a lytic central vacuole and of neutral peripheral vacuoles can be visualized by green fluorescent proteins targeted to either type of vacuoles. Plant Physiol 126:178–186

Schillberg S, Fischer R, Emans N (2003) 'Molecular farming' of antibodies in plants. Naturwissenschaften 90(4):145–155

Schouten A, Roosien J, van Engelen FA, de Jong GA, Borst-Vrenssen AW et al (1996) The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. Plant Mol Biol 30(4):781–793

Seon JH, Szarka JS, Moloney MM (2002) A unique strategy for recovering recombinant proteins from molecular farming: affinity capture on engineered oilbodies. J Plant Biotechnol 4(3):95–101

Sharma AK, Sharma MK (2009) Plants as bioreactors: recent developments and emerging opportunities. Biotechnol Adv 27(6):811–832

Shin Y-J, Shin-Young Hong S-Y, Kwon T-H, Jang Y-S, Yang M-S (2003) High level of expression of recombinant human granulocytemacrophage colony stimulating factor in transgenic rice cell suspension culture. Biotechnol Bioeng 82(7):778–783

Simons JP, McClenaghan M, Clark AJ (1987) Alteration of the quality of milk by expression of sheep beta-lactoglobulin in transgenic mice. Nature 328(6130):530–532

Sojikul P, Buehner N, Mason HS (2003) A plant signal peptide-hepatitis B surface antigen fusion protein with enhanced stability and immunogenicity expressed in plant cells. Proc Natl Acad Sci U S A 100(5):2209–2214

Stoger E, Sack M, Fischer R, Christou P (2002) Plantibodies: applications, advantages and bottlenecks. Curr Opin Biotechnol 13(2):161–166

Stoger E, Ma JK, Fischer R, Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotechnol 16(2):167–173

Streatfield SJ, Howard JA (2003) Plant-based vaccines. Int J Parasitol 33(5–6):479–493

Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM, Arntzen CJ (2000) Human immune responses to a novel norwalk virus vaccine delivered in transgenic potatoes. J Infect Dis 182(1):302–305

Takagi H, Hiroi T, Yang L, Tada Y, Yuki Y, Takamura K, Ishimitsu R, Kawauchi H, Kiyono H, Takaiwa F (2005) A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. Proc Natl Acad Sci U S A 102(48):17525–17530

Tiwari S, Verma PC, Singh PK, Tuli R (2009) Plants as bioreactors for the production of vaccine antigens. Biotechnol Adv 27(4):449–467

Tschofen M, Knopp D, Hood E, Stoger E (2016) Plant molecular farming: much more than medicines. Annu Rev Anal Chem 9:271–294

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21(12):570–578

Twyman RM, Schillberg S, Fischer R (2005) Transgenic plants in the biopharmaceutical market. Expert Opin Emerg Drugs 10(1):185–218

Vaquero C, Sack M, Schuster F, Finnern R, Drossard J, Schumann D, Reimann A, Fischer R (2002) A carcinoembryonic antigen-specific diabody produced in tobacco. FASEB J 16(3):408–410

Vitale A, Pedrazzini E (2005) Recombinant pharmaceuticals from plants: the plant endomembrane system as bioreactor. Mol Interv 5(4):216–225

Wandelt CI, Khan MR, Craig S, Schroeder HE, Spencer D, Higgins TJ (1992) Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. Plant J 2(2):181–192

Wang ML, Goldstein C, Su W, Moore PH, Albert HH (2005) Production of biologically active GM-CSF in sugarcane: a secure biofactory. Transgenic Res 14(2):167–178

Welbaum GE, Meinzer FC (1990) Compartmentation of solutes and water in developing sugarcane stalk tissue. Plant Physiol 93(3):1147–1153

Wilken LR, Nikolov ZL (2012) Recovery and purification of plant-made recombinant proteins. Biotechnol Adv 30(2):419–433

Wirth S, Calamante G, Mentaberry A, Bussmann L, Lattanzi M, Baranao L, Bravo-Almonacid F (2004) Expression of active human epidermal growth factor (hEGF) in tobacco plants by integrative and non-integrative systems. Mol Breed 13:23–35

Yang J, Barr LA, Fahnestock SR, Liu ZB (2005) High yield recombinant silk-like protein production in transgenic plants through protein targeting. Transgenic Res 14(3):313–324

Yoshida K, Matsui T, Shinmyo A (2004) The plant vesicular transport engineering for production of useful recombinant proteins. J Mol Catal B Enzym 28:167–171

Yusibov V (2002) Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine. Vaccine 20(25–26):3155–3164

Chapter 8
Biotechnological Interventions for Improving Sucrose Accumulation in Sugarcane

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Abstract Sugarcane is a C_4 grass grown in tropical and subtropical regions, cultivated in 22 million hectares in more than 100 countries (FAOSTAT 2008, http://faostatfaoorg/defaultaspx). Major sugar requirement of the world is met by sugarcane. It contributes almost 75% of total sugar produced from all the sugar crops. Sugarcane is also used for generation of biofuel and bagasse as lignocellulosic raw material for paper industries. Sucrose content and cane weight are the key traits determining the income of sugarcane farmers and industries. In general, sugarcane varieties in cultivation are capable to accumulate higher sucrose in the stems to levels more than 50% of the stem dry weight. Ability of sugarcane to produce and store higher concentration of sucrose in the mature internodes has made the crop more suitable for commercial sucrose extraction. In this chapter, we reviewed the work carried out by various sugarcane researchers around the world on sugarcane biotechnology in relation to sucrose enhancement using various molecular approaches and technologies.

Keywords Stem • Sucrose • Sucrose synthase • Sucrose phosphatase • Targeting

8.1 Introduction

Most of the world's sugar is produced from sugarcane. It contributes almost 70% of total sugar produced from all the sugar crops. Sugarcane is a C4 grass that can accumulate sucrose in the stems to levels exceeding 25% of the fresh weight (FAOSTAT 2008, http://faostatfaoorg/defaultaspx). Sucrose metabolism is the most unique and complex mechanism in sugarcane due to compartmentalization between source and

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© Springer International Publishing AG 2017
C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_8

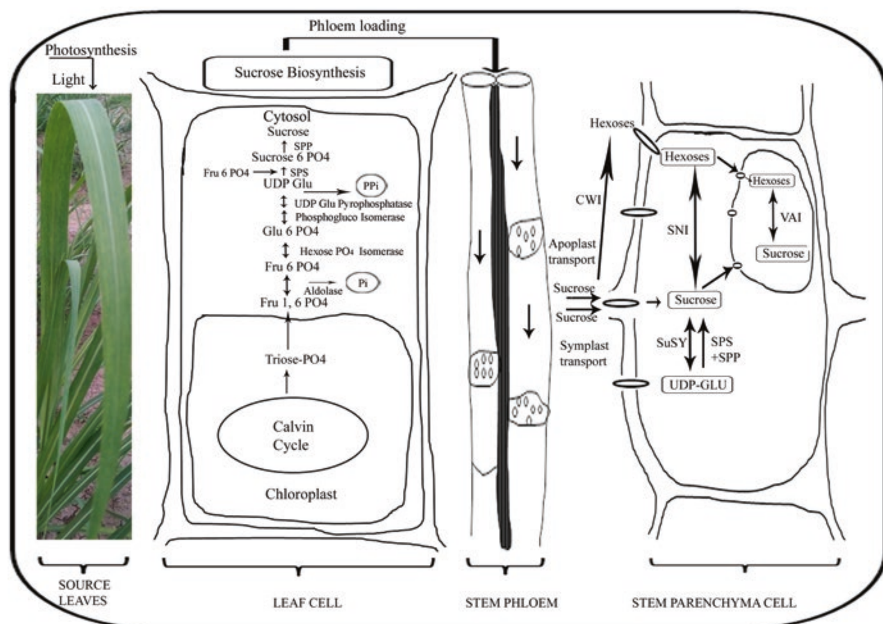

Fig. 8.1 Overview of sucrose metabolism in sugarcane. *SPP* sucrose phosphate phosphatase, *SPS* sucrose phosphate synthase, *CWI* cell wall invertase, *SAI* soluble acid invertase, *VAI* vacuolar acid invertase, *SuSY* sucrose synthase

sink. Sucrose biosynthesized in the leaves (source) is transported through phloem and stored in the stem parenchyma cells (sink). Stem can be able to store the sucrose up to 650 mM (Welbaum and Meinzer 1990) or 18% of stem fresh weight in the commercial sugarcane varieties (Inman-Bamber et al. 2011). As sugarcane attains maturity, carbon pool in the cell is diverted towards biosynthesis of osmotically active "sucrose" (Whittaker and Botha 1997). Another unique feature of sugarcane is that sucrose storage occurs in the stalk (culm) parenchyma cells (Uys et al. 2007). During growth period, sucrose synthesized in the leaves is translocated via phloem to stem internodes. There is correlation between leaf photosynthetic activity and stem sucrose content in sugarcane. As plant gets matured, the leaf photosynthetic activity decreases significantly, as stem sucrose content increases (McCormick et al. 2008, 2009). This indicates the possible regulation of sucrose accumulation in the sink in relation to source capacity (Watt et al. 2005).

Sugarcane sucrose metabolism regulated at four levels, viz., sucrose biosynthesis, transport, accumulation, and degradation. There are number of factors involved at each level of regulation. An overview of sucrose metabolism in sugarcane is shown in Fig. 8.1. Sucrose is biosynthesized in both photosynthetic and storage cells by sequential action of sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP). The SPS catalyzes the biosynthesis of sucrose phosphate (sucrose-P) from fructose-6-phosphate (Fru-6-P) and UDP-glucose (UDP-Glu). This nonreversible reaction occurs due to rapid conversion of sucrose-P to sucrose by SPP (Botha and Black 2000).

8.2 Sucrose Metabolism in Sugarcane

In plants, sucrose and starch metabolism are inter-regulated (Fig. 8.1). Sucrose biosynthesis occurs in cytosol whereas starch synthesis in chloroplast. The triose phosphate formed in the chloroplast from Calvin cycle is transported to cytosol through inorganic phosphate-triose phosphate translocator and serves as substrate to aldolase for biosynthesis of fructose 1,6-bisphosphate. Fructose 1,6-bisphosphate is catalyzed by fructose 1,6-bisphosphatase leads to formation of fructose 6-phosphate. Thus formed fructose 6-phosphate is catalyzed by hexose phosphate isomerase to form glucose 6-phosphate. Subsequently UDP-glucose is synthesized by succeeding reactions by phosphoglucomutase and UDP-glucose pyrophosphorylase. Thus formed UDP-glucose condensed with fructose 6-phosphate to form sucrose 6-phosphate through catalytic action of sucrose phosphate synthase or more preferentially called as sucrose 6-phosphate synthase. In the final step, phosphate group cleaved from sucrose 6-phosphate by sucrose 6-phosphate phosphatase leads to formation of free sucrose. Sucrose synthase (SuSy) is another enzyme that catalyzes the synthesis of sucrose in reversible reaction. However, the equilibrium of SuSy activity is towards degradation of sucrose rather than synthesis. Hence, it is considered as an important enzyme required for maintaining high carbon pools for energy metabolism during plant growth (Goldner et al. 1991; Schafer et al. 2004). SuSy activity is high in young internodes of sugarcane stems, negatively correlated with sucrose levels and positively correlated with hexose levels (Verma et al. 2011).

Sucrose synthesized in the leaves is transported through phloem to the storage cells in the stem, possibly through symplastic and apoplastic system (Rae et al. 2005a, b) and mainly the symplast in mature internodes (Patrick 1997; Grof and Campbell, 2001; Patrick et al. 2013). Therefore, distribution and storage of sugars between the apoplast, cytosol, and vacuole is a very important feature of sucrose accumulation in sugarcane stem parenchyma cells. Sucrose is unloaded from phloem into the apoplast and stored in compartment (vacuoles) of parenchyma cells through two paths. In the first path, sucrose is transported directly into parenchyma cells by sucrose transporters of the plasma membrane, then into the vacuole. This happens mostly under low turgor conditions. In the second path, sucrose in the apoplast is hydrolyzed by apoplastic/cell wall acid invertase (CWI) into glucose and fructose and transported by hexose carriers. Sucrose is resynthesized in cytoplasm prior to vacuolar storage.

Sucrose transporters of the plasma membrane play an important role in loading and unloading of sucrose in phloem, and also in transportation of sucrose in apoplastic and symplastic compartments (Riesmeier et al. 1994; Burkle et al. 1998; Braun and Slewinski 2009; Chen et al. 2012). Sugarcane stem transcripts analysis revealed higher levels of sugar transporters compared to sugar metabolizing enzymes in maturing culms (Carson et al. 2002; Casu et al. 2003). Novel sucrose transporter gene "ShSUT1" transcripts were reported to be abundant in both source leaves and sink stems (Rae et al. 2005a, b). The functional role of ShSUT1 in sucrose transport was confirmed by the presence of high number of transcripts at the periphery of the vascular parenchyma and bundle sheath cells, instead of in the phloem. This indicates

two different possible roles of ShSUT1. Eventually, this may contribute to a biochemical barrier that inhibits sucrose apoplastic back-flow out of tissues and also aid retrieval of sucrose released to the apoplast (Rae et al. 2005a, b, 2009). Thus ShSUT1 protein plays an important role in the partitioning of sucrose between vascular tissue and storage sites in sugarcane stem parenchyma cells (Reinders et al. 2006).

Apart from sucrose synthesis and transport, storage of sucrose in vacuole, cytosol, and apoplast is a critical function under the regulatory action of invertases [EC 3.2.1.26; β-fructosidase], a family of enzymes that hydrolyze sucrose into glucose and fructose (Moore 1995). Sucrose stored in sugarcane internodes is cleaved into glucose and fructose by invertase (Hatch and Glasziou 1963). In sugarcane, sucrose is unloaded from the phloem and passes through three distinct cellular compartments: apoplastic space (cell wall), metabolic compartment (cytoplasm), and storage compartment or vacuole (Sacher et al. 1963). Each compartment contains a characteristic invertase isoform: acid invertase located in the apoplastic space (cell wall invertase, CWI), vacuolar acid invertase (VAI) or soluble acid invertase (SAI) located in the vacuole, and a neutral invertase (NI) located in cytoplasm. SAI activities are usually high in rapidly growing tissues, such as root apices and immature stem internodes. SAI is more active in immature internodes that accumulate the least sucrose and minimally active in maturing internodes that accumulate high sucrose (Ma et al. 2000). In general, SAI activity decline with ageing coupled with rapid rise in sucrose/reducing sugar ratio indicated better sink strength (Batta et al. 2008). High activity of CWI enzyme is found to be associated with low level sucrose accumulation phenotype of a sugarcane genotype (Batta et al. 2002).

8.3 Genome Biology of Sugarcane

Understanding the genomic structure and biology of sugarcane is prerequisite to any researcher working in this crop. The genus *Saccharum* belongs to the *Poaceae* family that consists of different species, namely *S. officinarum*, *S. spontaneum, S. barberi, S. robustum*, *and S. edule.* In the early nineteenth century, sugarcane breeders in Java and India carried out crosses between *S. officinarum* and *S. spontaneum* to develop high sucrose, disease and pest resistant sugarcane cultivars (Grivet and Arruda 2001). Modern sugarcane cultivars are developed from crosses of early interspecific genotypes with repeated intercrossing and selection. They are polyploidy aneuploid hybrids with unequal contribution from *S. officinarum* (80–90%) and *S. spontaneum* (10–20%) parental genomes and a small percentage of recombinant chromosomes (Piperidis et al. 2010; D'Hont 2005). Recent sugarcane hybrids record ploidy level of 10 or more and have a much larger total genome size (10,000 Mb and $2n = 115$) as compared to maize (5500 Mb, $2n = 20$), *Sorghum* (1600 Mb, $2n = 20$), or rice (860 Mb, $2n = 24$) reflecting the high polyploidy level of sugarcane cultivars (D'Hont and Glaszmann 2001).

The closest relative of sugarcane is *Sorghum*, and hence *Sorghum* genomic resources are widely used as reference genome for comparison with sugarcane.

Using modern bioinformatics tools, it has become very easy to annotate the gene sequences and their regulatory mechanism. The regulatory genes for sucrose biosynthesis and their pathways have been functionally characterized for the allelic variation, copy number, and expression pattern in modern sugarcane cultivars. However, the complex polyploidy nature of sugarcane cultivars limits the breeders in understanding genotype to phenotype allelic variation and dosage. There is big challenge ahead in elucidating the complete genome sequence of sugarcane due to its complex ploidy and aneuploidy nature.

8.4 Molecular Breeding for High Sucrose Yield

Increasing sucrose content through introgression of new genes is a major objective of sugarcane breeding programs. The key regulatory enzymes like sucrose phosphate synthase, sucrose synthase, and invertases have been used as biochemical marker to develop high sucrose sugarcane cultivars in the breeding programs. The crossing between *S. officinarum* genotype and sugarcane hybrids with subsequent backcrosses is the most common practice in sugarcane breeding to develop high sucrose hybrids. Further measurement of SPS, SuSy, and invertase enzyme activities in the progenies is the precise way to confirm the high sucrose sugarcane hybrids. The enzymes sucrose-phosphate synthase (SPS), three isoforms of invertase, and sucrose synthase were used as biochemical markers and evaluated their activities in four high and four low CCS clones from an initial cross between a *S. officinarum* and the commercial cultivar Q165. Later, SPS and the two soluble isoforms of invertase were measured in clones derived from a backcross of one of the progenies to another commercial cultivar Mida (Grof et al. 2007). Enzyme activities were measured in tissue from internodes taken from four different positions down the stem profile. SPS was significantly higher in the upper internodes (one to three) of high CCS clones as compared with low CCS clones in both populations, suggesting that this enzyme may have a key role in establishing metabolic and developmental processes, necessary for high sugar accumulation during stem growth and maturation (Grof et al. 2007). Advancement DNA marker technology allowed the breeders to develop and identify the marker linked to the important traits in plants. Expressed sequence tags (ESTs) derived from RFLP marker associated with sucrose synthase gene is identified and used to identify QTL for sucrose content in sugarcane (Da Silva and Bressiani 2005).

8.5 Sugarcane Transcriptome and Gene Expression Profiling in Relation to Sucrose Metabolism

Genes associated with sucrose content have been characterized in sugarcane at transcriptional level. Traditional approaches like northern blotting, RT-PCR, quantitative PCR, and micro arrays have been extensively used to study the gene expression

pattern in sugarcane. Differential expression pattern of sucrose phosphate synthase (SPS), sucrose synthase (SuSy), and invertase enzymes between mature and immature internodes have been observed in high and low sucrose sugarcane genotypes (Verma et al. 2011; Prathima et al. 2011; Chandra et al. 2015). From these studies, it is clear that expression of SPS is very high in mature internodes and positively correlated with sucrose content whereas SuSy and invertases are high in immature internodes and negatively correlated with sucrose content. cDNA microarrays were also employed in sugarcane to identify differentially expressed transcripts for sucrose content (Papini-Terzi et al. 2005).

Most recently NGS technologies have been applied in sugarcane (Wu et al. 2013; Cardoso-Silva et al. 2014) for identification of differentially expressed pathogen responsive genes for smut disease caused by *Sporisorium scitaminea* by using Solexa technology (Wu et al. 2013). De novo assembly and transcriptome annotation of six sugarcane genotypes involved in bi-parental crosses were performed with the Illumina RNA-seq platform to generate a dataset for future genetic and genomic studies (Cardoso-Silva et al. 2014). The transcriptome of a high-sucrose sugarcane variety, GT35, was sequenced using high-throughput Solexa technology and identified many unigenes involved in various metabolic pathways (Huang et al. 2016). Tissue specific transcriptome analysis of mature sugarcane stalks revealed the spatial deployment of pathways responsible for sucrose accumulation and fiber synthesis within the stalk (Casu et al. 2015). Expression profiling of storage parenchyma, vascular bundles, and rind dissected from a maturing stalk internode of sugarcane has identified ten cellulose synthase subunit genes and several sugar transporters with significant differences in the expression of their corresponding transcripts. The sugar transporter genes ShPST2a, ShPST2b, and ShSUT4 were significantly upregulated in storage parenchyma while ShSUT1 was upregulated in vascular bundles. Specific group of genes involved in sucrose accumulation and cell wall synthesis provides the new information on the mechanism sucrose transport and fiber synthesis in sugarcane (Casu et al. 2015).

8.6 Genetic Engineering of Sugarcane for Enhanced Sucrose Accumulation

Genetic engineering has revolutionized the modern field of biotechnology. Many attempts have been made to modify sugarcane plant for improving overall sugar yield through genetic engineering (Table 8.1). Sucrose enhancement through transgenic approach necessitates complete understanding of mechanism of sucrose accumulation process and its regulation in sugarcane (Watt et al. 2005). Sucrose phosphate synthase is the principal regulatory enzyme of sucrose metabolism. Although SPS activity correlates with sucrose content in diverse sugarcane genotypes, overexpression of SPS alone in transgenic sugarcane plants has not led to improved sucrose yields (Vickers et al. 2005; Grof et al. 2007). Apart from SPS, invertase has been a

Table 8.1 Metabolic engineering and genetic manipulation for increasing sugar yield in sugarcane

Source	Gene/enzyme	Approach	Sugar accumulate	References
Pantoea dispersa UQ68	Sucrose isomerase (SI)	SI gene overexpression in vacuoles	Isomaltulose	Wu and Birch (2007) Basnayake et al. (2012)
Malus domestica	Sorbitol-6-phosphate dehydrogenase gene (*mds6pdh*)	*Mds6pdh* gene overexpression	Sorbitol	Chong et al. (2007)
Grifola frondosa	Trehalose synthase	Trehalose synthase gene overexpression	Trehalose	Zhang et al. (2006)
Pseudomonas mesoacidophila MX-45	Trehalulose synthase	Trehalulose synthase gene overexpression	Trehalulose	Hamerli and Birch (2011)
Sugarcane	Neutral invertase (*NI*)	*NI* gene: Downregulation of endogenous gene by antisense repression	Sucrose	Rossouw et al. (2010)
Sugarcane	Proton translocating vacuolar pyro phosphatase (*VPPase*)	*VPPase* gene: Downregulation of endogenous gene by antisense repression	Sucrose	Swart (2007); South African Patent No:2007/02680
Sugarcane	Pyrophosphate fructose 6-phosphate1-phosphotransferase (*PFP*)	*PFP* gene: Downregulation of endogenous gene by antisense repression	Sucrose	Groenewald and Botha (2007) and van der Merwe et al. (2010)
Sugarcane	Soluble acid invertase (*SAI*)	*SAI* gene: Downregulation of endogenous gene by antisense repression	Sucrose	Botha et al. (2001) and Ma et al. (2000)
Yeast	Soluble acid invertase (*SAI*)	*SAI* gene: Apoplasmic overexpression of yeast invertase gene (*SUC2*)	Sucrose	Ma et al. (2000)
Sugarcane	UDP-glucose dehydrogenase (*UDP-Glc DH*)	*UDP-Glc DH* gene: Downregulation of endogenous gene by antisense and RNAi repression	Sucrose	Bekker (2007); South African Patent No:2006/07743

main target for molecular manipulation in a number of plants, including *Arabidopsis* (von Schaewen et al. 1990), tobacco (von Schaewen et al. 1990; Sonnewald et al. 1991), tomato (Ohyama et al. 1995), potato (Bussis et al. 1997), and carrot (Tang et al. 1999). Many efforts have been made to control invertase enzymes (both SAI and SNI) activity in sugarcane through transgenesis and despite having successful transgenic events, there was no significant increase in sucrose accumulation (Botha et al. 2001; Rossouw et al. 2007; Ma et al. 2000). Although suppression of the soluble acid invertase also increased sucrose content in sugarcane in suspension cell culture (Ma et al. 2000), a similar response was not evident for the overall sucrose content of mature, transgenic sugarcane plants (Botha et al. 2001). This might be due to the regulatory feedback mechanism between culm (sink) and leaf (source) during sucrose accumulation in sugarcane (McCormick et al. 2006, 2009; Chandra et al. 2011). The balance between soluble acid invertase and SPS activities influences the sucrose accumulation in sugarcane internodes, favoring sucrose storage when SPS predominates whereas there was a critical threshold of SAI activity above which high concentrations of sucrose did not accumulate (Zhu et al. 1997). Downregulation of neutral invertase activity to the tune of 40% in transgenic lines has increased both sucrose and hexoses content (Rossouw et al. 2010). Specifically, sucrose content increased by 25% and 14% in the immature and mature culms, respectively, but this benefit was outweighed by a severe reduction in plant vigor (Rossouw et al. 2010). The reduced neutral invertase in these stems appeared to be compensated by an increase in SuSy activity (Rossouw et al. 2010). Cell wall invertase is also important for providing hexoses for growing tissue involved in sucrose loading through apoplastic route (Moore 1995). Increases in cell wall invertase activity are associated with higher sucrose content in sugarcane (Lingle, 1989). High cell wall invertase activity in high-sugar genotypes may operate by enhancing sucrose unloading into the internode tissue (Chandra et al. 2012).

8.7 Conclusion

With the rapid increase in population, the demand of sugar is also increasing. One of the most significant works in sugarcane transgenics was the transformation of a bacterial sucrose isomerase (SI) gene to convert sucrose to isomaltulose, a metabolite not synthesized in higher plants. Transgenic sugarcane lines expressing bacterial SI which were targeted to the vacuole increased the total sugar content in mature sugarcane stem to 50% (Wu and Birch 2007; Wu and Birch 2010). This has doubled the total sugar content in sugarcane. A similar effect was also achieved by overexpressing a fructosyl-transferase gene from the *Cynara scolymus* in transgenic sugarcane. Resulting plants converted 78% of culm sucrose to fructans, which led to 63% greater total sugar content (Nell 2007). These studies showed that additional metabolic sinks for sucrose could increase sink capacity, and lead to expected enhancement of photosynthesis and overall sugar accumulation (Koch 1996, 2004).

With the advent of biotechnological strategies and novel sequencing technologies, the possibility of enhancing sugar accumulation has been broadened. A system biology approach combined with metabolic engineering would be of great significance in the near future for sugarcane crop improvement.

References

Basnayake SWV, Morgan TC, Wu L, Birch RG (2012) Field performance of transgenic sugarcane expressing isomaltulose synthase. Plant Biotechnol J 10:217–225

Batta SK, Kaur S, Mann APS (2002) Sucrose accumulation and maturity behavior in sugarcane is related to invertase activities under subtropical conditions. Int Sugar J 104:10–13

Batta SK, Pant NC, Thind KS, Uppal SK (2008) Sucrose accumulation and expression of enzyme activities in early and mid-late maturing sugarcane genotypes. Sugar Tech 10(4):319–326

Bekker JPL (2007) Genetic manipulation of the cell wall composition of sugarcane. PhD Thesis, University of Stellenbosch, Stellenbosch

Botha FC, Black KG (2000) Sucrose phosphate synthase and sucrose synthase activity during maturation of internodal tissue in sugarcane. Aust J Plant Physiol 27:81–85. doi:10.1071/PP99098

Botha FC, Sawyer BJB, Birch RG (2001) Sucrose metabolism in the culm of transgenic sugarcane with reduced soluble acid invertase activity. Proc Int Soc Sugar Cane Technol 24:588–591

Braun DM, Slewinski TL (2009) Genetic control of carbon partitioning in grasses: roles of sucrose transporters and tie-dyed loci in phloem loading. Plant Physiol 149:71–81. doi:10.1104/pp.108.129049

Burkle L, Hibberd JM, Quick WP, Kühn C, Hirner B, Frommer WB (1998) The $H^{\pm}$ sucrose cotransporter NtSUT1 is essential for sugar export from tobacco leaves. Plant Physiol 118:59–68. doi:10.1104/pp.118.1.59

Bussis D, Heineke D, Sonnewald U, Willmitzer L, Raschke K, Heldt HW (1997) Solute accumulation and decreased photosynthesis in leaves of potato plants expressing yeast-derived invertase either in the apoplast vacuole or cytosol. Planta 206:126–136

Cardoso-Silva CB, Costa EA, Mancini MC, Balsalobre TWA, Canesin LEC, Pinto LR, Carneiro MS, Garcia AAF, Souza AP, Vicentini R (2014) *De novo* assembly and transcriptome analysis of contrasting sugarcane varieties. PLoS One 9:e88462

Carson D, Huckett B, Botha F (2002) Differential gene expression in sugarcane leaf and intermodal tissues of varying maturity. S Afr J Bot 68:434–442

Casu RE, Grof CPL, Rae AL, McIntyre CL, Dimmock CM, Manners JM (2003) Identification of a novel sugar transporter homologue strongly expressed in maturing stem vascular tissues of sugarcane by expressed sequence tag and microarray analysis. Plant Mol Biol 52:371–386. doi:10.1023/A:1023957214644

Casu RE, Rae AL, Nielsen JM, Perroux JM, Bonnett GD, Manners JM (2015) Tissue-specific transcriptome analysis within the maturing sugarcane stalk reveals spatial regulation in the expression of cellulose synthase and sucrose transporter gene families. Plant Mol Biol 89:607–628

Chandra A, Jain R, Rai RK, Solomon S (2011) Revisiting the source–sink paradigm in sugarcane. Curr Sci 100:978–980

Chandra A, Jain R, Solomon S (2012) Complexities of invertases controlling sucrose accumulation and retention in sugarcane. Curr Sci 102:857–866

Chandra A, Verma PK, Islam MN, Grisham MP, Jain R, Sharma A, Roopendra K, Singh K, Singh P, Verma I, Solomon S, Adams W (2015) Expression analysis of genes associated with sucrose accumulation in sugarcane (spp. hybrids) varieties differing in content and time of peak sucrose storage. Plant Biol 17(3):608–617

Chen L, Qu X, Hou BH, Sosso D, Osorio S, Fernie AR, Frommer WB (2012) Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. Science 335:207–211. doi:10.1126/science.1213351

Chong BF, Bonnett GD, Glassop D, O'Shea MG, Brumbley SM (2007) Growth and metabolism in sugarcane are altered by the creation of a new hexose-phosphate sink. Plant Biotechnol J 5:240–253

D'Hont A (2005) Unraveling the genome structure of polyploids using FISH and GISH: examples of sugarcane and banana. Cytogenet Genome Res 109:27–33

D'Hont A, Glaszmann JC (2001) Sugarcane genome analysis with molecular markers: a first decade of research. In: International Society of Sugar Cane Technologists. Proceedings of the XXIV Congress. pp 556–559

Da Silva JA, Bressiani JA (2005) Sucrose synthase molecular marker associated with sugar content in elite sugarcane progeny. Genet Mol Biol 28(2):294–298

FAOSTAT (2008) http://faostatfaoorg/defaultaspx

Goldner W, Thom M, Maretzki A (1991) Sucrose metabolism in sugarcane cell suspension cultures. Plant Sci 73:143–147. doi:10.1016/0168-9452(91)90021-Y

Grivet L, Arruda P (2001) Sugarcane genomics: depicting the complex genome of an important tropical crop. Curr Opin Plant Biol 5:122–127

Groenewald JH, Botha FC (2007) Down-regulation of pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP) activity in sugarcane enhances sucrose accumulation in immature internodes. Transgenic Res 17:85–92

Grof CPL, Campbell JA (2001) Sugarcane sucrose metabolism: scope for molecular manipulation. Aust J Plant Physiol 28:1–12

Grof CPL, Albertson PL, Bursle J, Perroux JM, Bonnett GD, Manners JM (2007) Sucrosephosphate synthase a biochemical marker of high sucrose accumulation in sugarcane. Crop Sci 47:1530–1539. doi:10.2135/cropsci2006.12.0825

Hamerli D, Birch RG (2011) Transgenic expression of trehalulose synthase results in high concentrations of the sucrose isomer trehalulose in mature stems of field-grown sugarcane. Plant Biotechnol J 9:32–37

Hatch MD, Glasziou KT (1963) Sugar accumulation cycle in sugarcane II Relationship of invertase activity to sugar content and growth rate in storage tissue of plants grown in controlled environments. Plant Physiol 38:344–348

Huang DL, Gao Y, Gui Y, Chen Z, Qin C, Wang M, Liao Q, Yang L, Li Y (2016) Transcriptome of high-sucrose sugarcane variety GT35. Sugar Tech 18(5):520–528

Inman-Bamber G, Jackson P, Bonnett G, Morgan T (2011) Have we reached peak CCS? In: Bruce RC (ed) 33rd Annual Conference of the Australian Society of Sugar Cane Technologists. Curran Associates Inc, Red Hook, pp: 1–9

Koch KE (1996) Carbohydrate modulated gene expression in plants. Ann Rev Plant Physiol Plant Mol Biol 47:509–540. doi:10.1146/annurev.arplant.47.1.509

Koch KE (2004) Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr Opin Plant Biol 7:235–246. doi:10.1016/j.pbi.2004.03.014

Lingle SE (1989) Evidence for the uptake of sucrose intact into sugarcane internodes. Plant Physiol 90(1):6–8

Ma H, Albert HH, Paull R, Moore PH (2000) Metabolic engineering of invertase activities in different sub-cellular compartments affects sucrose accumulation in sugarcane cells. Aust J Plant Physiol 27:1021–1030

McCormick AJ, Cramer MD, Watt DA (2008) Regulation of photosynthesis by sugars in sugarcane leaves. J Plant Physiol 165:1817–1829. doi:10.1016/j.jplph.2008.01.008

McCormick AJ, Cramer MD, Watt DA (2009) Supply and demand: sink regulation of sugar accumulation in sugarcane. J Exp Bot 60:357–364. doi:10.1093/jxb/ern310

van der Merwe M, Groenewald JH, Stitt M, Kossmann J, Botha F (2010) Downregulation of pyrophosphate-fructose-6-phosphate 1-phosphotransferase activity in sugarcane culms enhances sucrose accumulation due to elevated hexose-phosphate levels. Planta 231:595–608. doi:10.1007/s00425-009-1069-1

Moore PH (1995) Temporal and spatial regulation of sucrose metabolism in the sugarcane stem. Aust J Plant Physiol 22:661–679

Nell JS (2007) Genetic manipulation of sucrose-storing tissue to produce alternative products. PhD thesis, University of Stellenbosch, Stellenbosch. http://scholar.sun.ac.za/handle/10019.1/1359

Ohyama A, Ito H, Sato T, Nishimura S, Imai T, Hirai M (1995) Supression of acidic invertase activity by antisense RNA modifies the sugar composition of tomato fruit. Plant Cell Physiol 36:369–376

Papini-Terzi FS, Rocha FR, Vencio RZ, Oliveira KC et al (2005) Transcription profiling of signal transduction related genes in sugarcane tissues. DNA Res 12:27–38

Patrick JE (1997) Phloem unloading: sieve element unloading and post-sieve element transport. Ann Rev Plant Physiol Plant Mol Biol 48:191–222. doi:10.1146/annurev.arplant.48.1.191

Patrick JW, Botha FC, Birch RG (2013) Metabolic engineering of sugars and simple sugar derivatives in plants. Plant Biotechnol J 11:142–156. doi:10.1111/pbi.12002

Piperidis G, Piperidis N, D'Hont A (2010) Molecular cytogenetic investigation of chromosome composition and transmission in sugarcane. Mol Gen Genomics 284:65–73

Prathima PT, Suresha GS, Selvi A (2011) Expression profiling of key genes involved in sucrose metabolism from different Saccharum sps and 'Co' Hybrids. J Sugarcane Res 1(2):35–42

Rae AL, Grof CPL, Casu RE, Bonnett GD (2005a) Sucrose accumulation in the sugarcane stem: pathways and control points for transport and compartmentation. Field Crops Res 92:159–168. doi:10.1016/j.fcr.2005.01.027

Rae AL, Perroux JM, Grof CPL (2005b) Sucrose partitioning between vascular bundles and storage parenchyma in the sugarcane stem: a potential role for the ShSUT1 sucrose transporter. Planta 220:817–825. doi:10.1007/s00425-004-1399-y

Rae AL, Jackson MA, Nguyen CH, Bonnett GD (2009) Functional specialisation of vacuoles in sugarcane leaf and stem. Trop. Plant Biol 2:13–22. doi:10.1007/s12042-008-9019-9

Reinders A, Sivitz AB, Hsi A, Grof CPL, Perroux JM, Ward JM (2006) Sugarcane ShSUT1: analysis of sucrose transport activity and inhibition by sucralose. Plant Cell Environ 29:1871–1880. doi:10.1111/j.1365-3040.2006.01563.x

Riesmeier JW, Willmitzer L, Frommer WB (1994) Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. EMBO J 13:1–7

Rossouw D, Bosch S, Kossmann J, Botha FC, Groenewald JH (2007) Down-regulation of neutral invertase activity in transgenic sugarcane cell suspension cultures leads to a reduction in respiration and growth and an increase in sucrose accumulation. Funct Plant Biol 34:490–498

Rossouw D, Kossmann J, Botha FC, Groenewald JH (2010) Reduced neutral invertase activity in the culm tissues of transgenic sugarcane plants results in a decrease in respiration and sucrose cycling and an increase in the sucrose to hexose ratio. Funct Plant Biol 37:22–31. doi:10.1071/FP08210

Sacher JA, Hatch MD, Glasziou KT (1963) Sugar accumulation cycle in sugarcane III physical and metabolic aspects in immature storage tissues. Plant Physiol 38:348–354

von Schaewen A, Stitt M, Schmidt R, Sonnewald U, Willmitzer L (1990) Expression of yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly growth influences growth and phenotype of transgenic tobacco plants. EMBO J 9:3033–3044

Schafer EW, Rohwer JM, Botha FC (2004) Protein level expression and localization of sucrose synthase in sugarcane culm. Physiol Plant 121:187–195. doi:10.1111/j.0031-9317.2004.00316.x

Sonnewald U, Brauer M, von Schaewen A, Stitt M, Willmitzer L (1991) Transgenic tobacco plants expressing yeast derived invertase in the cytosol vacuole or the apoplast: a powerful tool for studying sucrose metabolism and sink/source interactions. Plant J 1:95–106

Swart JC (2007) The characterization of vacuolar pyrophosphate activity in sugarcane. MSc dissertation, University of Stellenbosch, Stellenbosch

Tang GQ, Luscher M, Sturm A (1999) Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. Plant Cell 11:177–189

Uys L, Botha FC, Hofmeyr JHS, Rohwer JM (2007) Kinetic model of sucrose accumulation in maturing sugarcane culm tissue. Phytochemistry 68:2375–2392. doi:10.1016/j.phytochem.2007.04.023

Verma AK, Upadhyay SK, Verma PC, Solomon S, Singh SB (2011) Functional analysis of sucrose phosphate synthase (SPS) and sucrose synthase (SS) in sugarcane (Saccharum) cultivars. Plant Biol 13:325–332. doi:10.1111/j.1438-8677.2010.00379.x

Vickers JE, Grof CPL, Bonnett GD, Jackson PA, Morgan TE (2005) Effects of tissue culture biolistic transformation and introduction of PPO and SPS gene constructs on performance of sugarcane clones in the field. Aust J Agric Res 56:57–68. doi:10.1071/AR04159

Watt DA, McCormick AJ, Govender C, Carson DL, Cramer MD, Huckett BI, Botha FC (2005) Increasing the utility of genomics in unraveling sucrose accumulation. Field Crops Res 92:149–158. doi:10.1016/j.fcr.2005.01.012

Welbaum GE, Meinzer FC (1990) Compartmentation of solutes and water in developing sugarcane stalk tissue. Plant Physiol 93:1147–1153. doi:10.1104/pp.93.3.1147

Whittaker A, Botha FC (1997) Carbon partitioning during sucrose accumulation in sugarcane internodal tissue. Plant Physiol 115:1651–1659

Wu L, Birch RG (2007) Doubled sugar content in sugarcane plants modified to produce a sucrose isomer. Plant Biotechnol J 5:109–117. doi:10.1111/j.1467-7652.2006.00224.x

Wu L, Birch RG (2010) Physiological basis for enhanced sucrose accumulation in an engineered sugarcane cell line. Funct Plant Biol 37:1161–1174. doi:10.1071/FP10055

Wu Q, Xu L, Guo J, Su Y, Que Y (2013) Transcriptome profile analysis of sugarcane responses to *Sporisorium scitaminea* infection using Solexa sequencing technology. Biomed Res Int 2013:298920

Zhu YJ, Komor E, Moore PH (1997) Sucrose accumulation in the sugarcane stem is regulated by the difference between the activities of soluble acid invertase and sucrose phosphate synthase. Plant Physiol 115:609–616. doi:10.1104/pp.115.2.609

Zhang SZ, Yang BP, Feng CL, Chen RK, Luo JP, Cai WW, Liu FH (2006) Expression of the Grifola frondosa trehalose synthase gene and improvement of drought-tolerance in sugarcane (Saccharum officinarum L.) J Integr Plant Biol 48(4):453–459

Chapter 9
Sugarcane as a Potential Biofuel Crop

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Abstract Sugarcane (*Saccharum* spp.) belonging to family Poaceae is a tropical perennial grass used widely for sugar production. Research scientists have discovered sugarcane as an alternative biofuel source to conventional petroleum fuels that lead to global warming. The sugars extracted from sugarcane can be easily fermented to produce ethanol. In addition, the bagasse (biomass remaining after the juice is extracted from the stalks) can be further used by sugar mills to generate steam and electricity. The current total global production of renewable fuels is 50 billion liters a year, and sugarcane alone accounts for about 40%, thus becoming a major contributor for biofuel production. The tremendous success of sugarcane industry to produce ethanol as biofuel in Brazil has also enhanced the interest in other parts of the world. With conventional technologies, sugarcane can yield several products from fiber to chemicals. But with the help of genetic recombination, sugarcane would roll to produce the novel biofuels more efficiently. Research scientists have identified the key enzymes that can hasten the process of ethanol production more powerfully. There is tremendous potential of sugarcane as a biofactory which can uplift both socioeconomic status of a country and sustainability of natural resources. Now, it's time to augment weightage to produce biofuels in developing

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C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_9

countries like India which would initiate rural development, create more job opportunities, and also save foreign exchange to great extent.

Keywords Biofuel • Ethanol • Genetic recombination • Socioeconomic • Sugarcane

9.1 Introduction

Globally with rapid increase in population along with increased demand of the fossil fuels, a time will come which will reveal the truth of the extinction of these renewable sources. The world population is estimated to increase from 6.7 billion to 8 billion by 2030 (USCB 2016). On the other hand, global oil production is expected to decline from 25 billion barrels to 5 billion barrels by 2050 (Campbell and Laherree 1998). But with a new discovery of use of biofuel, now the demand of this biofuel has increased to many folds. Sugarcane is one of the main source for biofuel that would totally replace the fossil fuel in coming years and would not only contribute to maintenance of ecological balance but also would strengthen the industry and contribute to energy source diversification worldwide (Ericlam et al. 2009). Use of biofuel has positive impacts as it eliminates lead compounds from petrol as well as reduction of poisonous emission of harmful gases (Goldemberg et al. 2008). There is also the reduction of CO_2 emissions as sugarcane ethanol requires only a small amount of fossil fuels for its production, belonging to a renewable source of fuel energy. Out of total global production of renewable fuels of 50 billion liters a year, about 40% of it comes from sugarcane that is mostly produced by Brazil. Brazil tops the annual production with 73,93,000 metric tons while India ranks 2nd with 341,200 metric tons and third comes China with annual production of 125,500 metric tons. Bioethanol production from sugarcane and starch-rich feedstocks such as corn and potato is considered as the first-generation process and it has already been developed. Sugarcane (*Saccharum* spp.) is now considered as the most productive first-generation energy crop. The success of the Brazilian sugarcane industry in ethanol production has increased interest in producing sugarcane for ethanol throughout the world. The sugars extracted from sugarcane can be easily fermented to produce ethanol. In addition to this, the bagasse (biomass remaining after the juice is extracted from the stalks) is used by sugar mills to generate steam and electricity (Salassi and Breaux 2006; Mahmood-ul-Hassan et al. 2015). Brazil, which is considered as leading producer of sugarcane (Orellana and Neto 2006), has revealed that in last 20 years, the amount of sugarcane harvested and processed has almost tripled to meet the demand for sugarcane ethanol and bioelectricity within the country. As compared to other agricultural activities, sugarcane occupies only petite quantity of land, but yet, this small portion has been able to swap almost 42% of its gasoline needs with sugarcane biofuel (ethanol). In 2015/16, Brazilian ethanol production reached 30.23 billion liters (8 gallons) (Barros 2015). Brazil has been considered as a commendable model in developing and commercializing use of biofuels in its proposition to minimize enslavement on foreign oil alone with decreasing hydrocarbon air pollution and maintaining

ecological balance. There is a positive correlation between increase in atmospheric greenhouse gases such as carbon dioxide with that of petroleum use and contributes global warming has created a vital need to develop and optimize "green fuels" that will have carbon neutral or even carbon negative capabilities (Graham-Rowe 2008) and sugarcane-based ethanol is the answer. Thus we can say that there is tremendous prospective of sugarcane biofactory which can boost both socioeconomic conditions of a country and sustainability of natural resources. Now it's the phase to produce biofuels in the developing countries like India which would strengthen rural progress, create greater job facilities, and also save foreign trade to great point.

9.2 Biology of Sugarcane

Sugarcane belonging to the genus *Saccharum* L., of the tribe Andropogoneae in the grass family (Poaceae) (Hodkinson et al. 2002) is a tropical perennial grass. Although sugarcane performs best in tropical and subtropical environments with temperatures between 70 and 90 °F, it is highly sensitive to cold, and yields are reduced in areas that experience frequent frost and below freezing temperatures. Commercial sugarcane varieties are complex hybrids of *Saccharum officinarum* and other *Saccharum* spp. like *S. spontaneum, S. robustum, S. officinarum, S. barberi, S. sinense*, *and S. edule*. This hybridization results in a wide range of physical characteristics, pests and disease tolerance, fiber and sucrose content, and cold tolerance. The height of the mature hybrid is about 16 ft. Likewise; stalk diameters can range from pencil-thin to up to 2 in. The inflorescence, or tassel, of sugarcane is a red- to white-colored, open-branched panicle. Sugarcane is clonally propagated by means of "seed-cane" which is a section of a mature cane stalk with buds or "eyes" located at the nodes. Sugarcane is harvested after 9–14 months of growth in Florida, but in other countries it is harvested 10–12 months after growth. Once an established sugarcane crop has been harvested, it ratoons annually from underground buds on basal portions of old stalks (Sandhu et al. 2016) and typically four rations can be used (Bull 2002). Sugarcane is having stout jointed fibrous stalks that are rich in the sugar sucrose accumulating in the stalk internodes. Sucrose is extracted and purified and fermented to produce ethanol which can be used as biofuel.

9.3 Sugarcane Biofuel Production

9.3.1 First-Generation Biofuel

The first-generation biofuel plants utilize either sugars or starch and sugar-based biofuel are predominately produced in Brazil from sugarcanes. Globally, 21 million m^3 ethanol is produced from sugarcane while 60 million m^3 ethanol is produced from corn and grains (REN21 2012). The foremost step is the liquidification of the

sugar extracted from the sugarcane. This is followed by the hydrolysis or saccharification that releases the sugars (glucose) monomers into the solution. During the subsequent fermentation with yeast (*Saccharomyces cerevisiae*) the sugar monomers are converted into ethanol and carbon dioxide. An ethanol concentration of 10% (w/v) is obtained at the end of the fermentation. The fermented liquid is then distilled to separate and purify the ethanol, which is then dehydrated to concentrations above 99.7% applicable for fuel (NSAI 2014). In the bottom of the distillation column, the sillage consisting of about 10% total solids (which includes residual substrate, yeast, and by-products). Some of the solid particles are removed from liquid via centrifugation by a decanter and the remaining thin silage is sent to an evaporator. The centrifugation cake and the resulting syrup from the evaporation are normally mixed to produce distillers dried grains and soluble (DDGS) which is used as protein source for animal feed (Taherzadeh et al. 2013). The first-generation bioethanol production is shown in the flowchart in Fig. 9.1.

9.3.2 Second-Generation Biofuel

Second-generation ethanol utilizes different types of lignocellulosic materials as substrate. Here, the energy balance for production from cellulosic materials is predicted to be superior to the present methods from sugarcane (Larson 2006). Currently, only negligible amounts of second-generation biofuel are produced around the world and are not commercially feasible. In Norway, one company named Borregaard is considered to be the largest producer of second-generation biofuel production with annual production of 20,000 m^3 (Rodsrud et al. 2012). The production of bioethanol from lignocelluloses is followed by few important steps, i.e., milling, thermophysical pretreatment hydrolysis, fermentation, distillation, and product separation/processing. It involves pretreatment and hydrolysis of the lignocellulosic material where steam explosion is followed by an alkaline delignification step. In the steam explosion, 70% of the hemicellulose is hydrolyzed into pentoses, with small cellulose losses and no lignin solubilization (Ojeda et al. 2011). The pretreated solids are separated from the obtained pentoses liquor using a filter. Pentoses are either fermented into ethanol or biodigested (producing biogas for the cogeneration system). In some cases, pretreatment is followed by an alkaline delignification step, where most of the lignin is removed from the pretreated material decreasing its inhibitory effects on enzymes in the enzymatic hydrolysis step (Rocha et al. 2012). The solid fraction obtained after filtration of the material is sent to enzymatic hydrolysis. The hydrolyzed liquor produced in the enzymatic hydrolysis, rich in glucose, is separated from the unreacted solids, i.e., residual cell lignin, which are used as fuels in the cogeneration system. In the integrated process, the hydrolyzed liquor is mixed with sugarcane juice; thus, concentration, fermentation, distillation, and dehydration operations are shared between both processes (Fig. 9.2).

Yeasts are specially used for the conversion of sugars into ethanol (mostly *Saccharomyces* spp.) to convert glucose into ethanol. C-5 sugars like xylose are

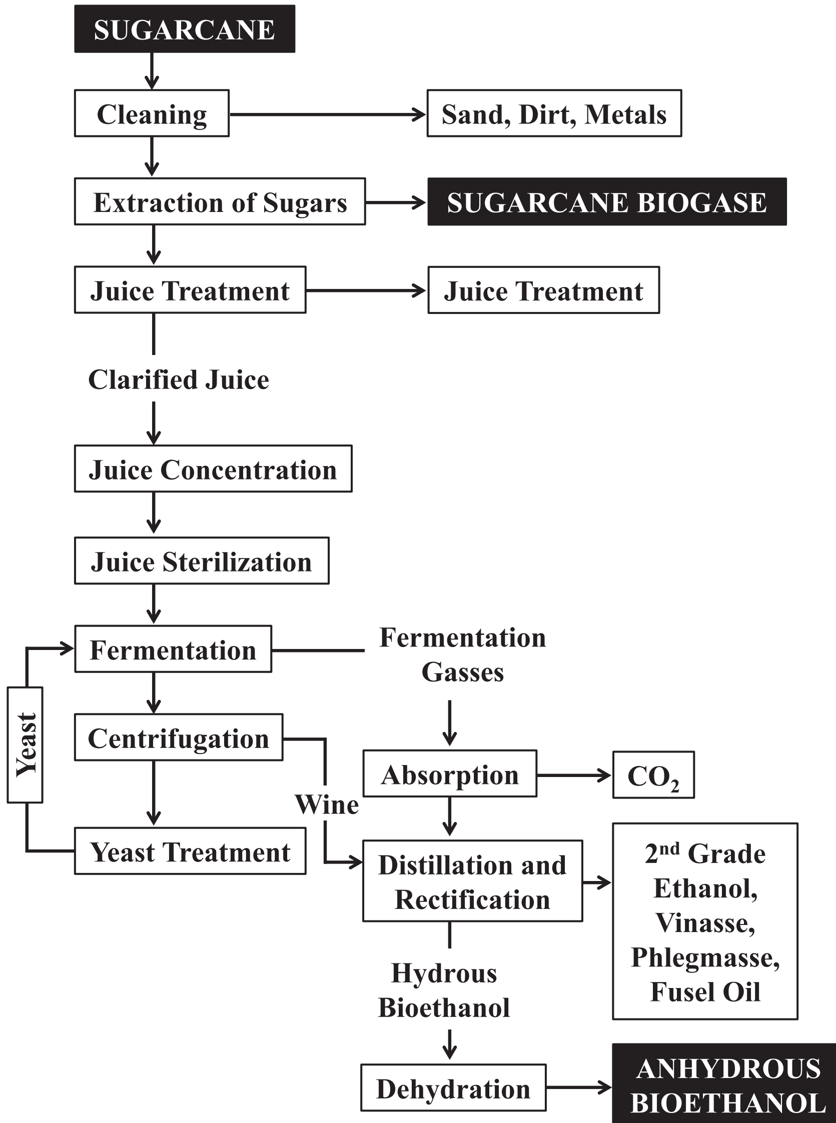

Fig. 9.1 Flowchart for the bioethanol production process from sugarcane (Source: Dias et al. 2011)

converted into ethanol at low rates by very few yeast (*Pichia* spp.) strains. Research has been carried out to undergo either to adapt yeasts for the use of both C-5 and C-6 sugars or to modify *Saccharomyces* genetically to obtain yeast that produces ethanol simultaneously from C-5 and C-6 sugars. Lignocellulosic biomass is the most promising feedstock for the production of fuel bioethanol. Large-scale production of bioethanol from lignocellulose containing materials has still not been implemented commercially in many places.

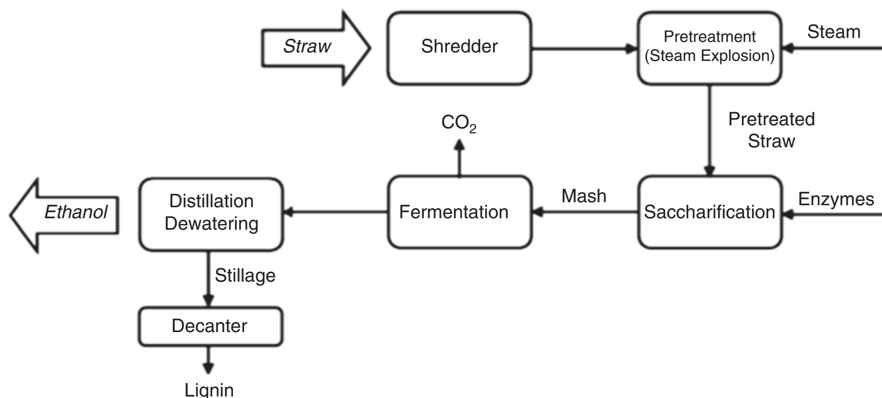

Fig. 9.2 Flow chart showing bioethanol production from lignocelluloses (Kahr et al. 2012)

9.4 Technological Improvement in Sugarcane Biofuel

Although ethanol is being successfully produced from the sugarcane, the produce is not up to the mark. There is a lot of scope for increasing production of novel biofuel (ethanol) and more efficiently in many ways. There are certain biological problems in the pathways of sucrose synthesis, translocation pathways, cell wall composition, and lignin synthesis and that needed to be more focused and carefully understood with the help of biotechnology approaches so that we can improve regulation of these and other pathways (Ericlam et al. 2009).

A big challenge is how to reduce cost of pretreatment and enzyme and how to enlarge the technologies for maximum efficiency of conversion of sugarcane biomass into biofuels. So, it is possible through genetic approaches including genetic modification, molecular biology, and plant breeding efforts to improve sugarcane cultivars with high cellulose, biomass yields, less lignin, fiber content, and maximum conversion of the biomass to biofuels in addition to improve pretreatment process and enzyme hydrolysis process (Hoang et al. 2015). However, despite these limitations, significant progress has been made towards genetically modified microorganisms that will digest the cellulose organic waste and xylose utilizing yeast strains have been developed for ethanol production (Paula 2016).

9.4.1 Importance of Genetic Engineering for Improvement of Efficacy of Biofuel

For efficient production of biofuels from plant materials, it requires proper processes that would initiate the biochemical makeup of the starting materials. Microbes are commonly used in industrial processing of crop materials to produce biofuels. The biological processes of these microbes which involve breakdown of cellulose

and other molecules to sugar, fermentation of sugar to yield ethanol or butanol, etc. are involved in the stepwise process of converting plant materials to biofuels. Most of the plants, with the exception of sugarcane or sugar beet, do not store considerable amount of sugar. Starch is produced in many plants as a storage form of carbon and energy. Maize and cassava produce large amounts of starch in seeds (maize) or roots (cassava). Starch is composed of long chains of sugar molecules, which can be hydrolyzed to simple sugars using microbial enzymes. Sugar produced in this way can then be fermented to ethanol. Here lie the opportunities of genetically engineered organisms producing starch hydrolyzing enzymes. Research is also being directed at identifying biochemical approaches to metabolize lignin and hemicelluloses present in plants so that usable products might be produced from these molecules as well. A much broader range of sugars can be fermented by organisms such as *Saccharomyces*, *E. coli*, *Xymomonas*, and *Pichia*, all of which have shown promising results for use in fermentation. These organisms produce enzymes that ferment a broader array of five and six carbon sugars. Since the microorganisms used for fermentation cannot survive at ethanol levels greater than 10–15%, distillation must be used to remove the remaining water and achieve high concentrations of ethanol. The use of genetic engineering to increase the tolerance to ethanol of the organisms used in fermentation is an active field of research. The possibility to “engineer a single organism to secrete all the necessary enzymes and utilize all the available sugars in a process referred to as integrated bioprocessing” represents a goal that many recognize as achievable (Somerville 2007).

Secondly, there are other relevant problems like sucrose synthesis and translocation pathways, cell wall composition and pathways for lignin synthesis that are needed to be more thoroughly understood. Thus the traditional plant physiology studies combined with molecular techniques are to be developed for a better understanding of plant development and gene expression before taking any biotechnological interventions to improve regulation of these and other pathways.

Many scientists have carried out the different interacting processes involved in accumulation of sucrose in sugar-storing stems of sugarcane. They have identified the key role of enzymes in this process through genetic engineering. Several genetic modification was done in sugarcane for boosting of the sucrose yield; for example, Groenewald and Botha (2008) identified a particular enzyme that raised the amount of sucrose in young stems of the genetically modified sugarcane plants and discussed metabolic engineering of sugars and its derivatives in plants (Patrick et al. 2013).

The prospect for genetic modification (GM) has large impact on devoted energy crops like sugarcane which started its pace from 2015 and continues till 2025 in the whole world. There is need for lot of technical challenges in using cellulose and lignin for biofuels production which have been already discussed above that can be solved with genetic modification. The most important impact of biotechnology on biofuels in the next 5 years will be on microorganisms involved in the processing of biomass to biofuels. Development and improvement of enzymes used for digesting cellulose, hemicellulose, and lignin into sugars and other simpler components are essential. Improvements in the efficiency and yield of fermentation will also continue till the time speculated.

9.5 Benefits of Ethanol as Biofuel

Ethanol is a comparatively low-cost alternative fuel. Sugarcane ethanol is an alcohol-based fuel produced by the fermentation of sugarcane juice and molasses. It is clean, affordable, and low carbon biofuel; sugarcane ethanol has emerged as a leading renewable fuel for the transportation sector. Ethanol is used in mainly two ways, namely, blended with gasoline and as pure ethanol. The ethanol is better than petroleum because it reduces air pollution and harmful emissions by adding oxygen to gasoline. Ethanol-fueled vehicles produce lower carbon monoxide and carbon dioxide emissions, and lower levels of hydrocarbon and oxides of nitrogen emissions. It always burns with a smokeless blue flame that is invisible in normal light (Sukesh et al. 2010). It is high octane fuel that helps prevent engine knocking and generates more power in higher compression engines. Moreover ethanol reduces global dependence on oil.

Below are some few valid reasons why to use biofuel instead of gasoline. They are:

9.5.1 Balance in Energy Due to Ethanol Production

Ethanol production from sugarcane has become an attractive replacement for gasoline in context that it is basically a renewable source of fuel which supplies electricity in surplus (Goldemberg et al. 2014). This is responsible for the low carbon emissions in the country Brazil (most of the carbon dioxide emission of the country), 75% of all national emissions, is due to Amazonia Forest deforestation (MCTI 2004). But for second-generation processes, the energy balance for production from cellulosic materials is estimated to be much better than the present methods from sugarcane (Larson 2006).

9.5.2 Environmental Aspects

As the amount of alcohol in gasoline increased, lead additives were reduced and they were completely eliminated by 1991. Brazil was then one of the first countries in the world to eliminate lead entirely from gasoline. The aromatic hydrocarbons (such as benzene), which are mainly harmful, were also eliminated and the sulfur content was reduced to many fold. In pure ethanol cars, sulfur emissions were eliminated. The simple addition of alcohol instead of lead in commercial gasoline has dropped the total carbon monoxide, hydrocarbons, and sulfur transport-related emissions by significant numbers. Due to the ethanol blend, lead ambient concentrations in Saa Paulo Metropolitan Region were reported to be dropped from 1.4 mg/m^3 in 1978 to less than 0.10 mg/m^3 in 1991, according to CETESB (the Environmental Company of Sao Paulo State), far below the air quality standard of

1.5 mg/m^3 (Coelho and Goldemberg 2004). Also, ethanol hydrocarbon exhaust emissions are less toxic than those of gasoline, since they present as lower atmospheric reactivity.

9.5.3 Social Aspects

Regarding socioeconomics aspects of the agribusiness, the most vital point is on the subject of job and income creation for a very wide range of workface capacity building programs, with the flexibility to support local characteristics using different technologies on the farm. Biomass and biofuels trade contribute to rural development, allowing additional income and job creation for developing countries, contributing to the sustainability of natural resources, collaborating with GHGes emission reduction in a cost-effective way, and thus diversifying the world's fuel needs.

9.6 Government Interventions

The global production of biofuels has almost tripled since 2005 in Brazil. This rapid increase in production in industrialized countries has been due to the fact that it reduces both their dependency on imported fossil fuel products and carbon emissions. For many developing countries like India, this trend presents new trade opportunities, as it increases the rural employment opportunities and also decreases the dependency of oil import from foreign. However, this poses a number of governance challenges. There have been reports of emerging and developing economies in certain developing countries (Brazil, Mexico, Indonesia, Malaysia, Zambia, and Ghana). All these happened due to the active role of government in developing a viable domestic biofuel industry and then exploring the effectiveness of national governance systems in managing the potential externalities of biofuel sector expansion.

A case study has been reported here for Brazil and Indonesia to depict how government has played a major role in expansion of biofuel production in the countries.

9.6.1 Government Role in Market Development in Brazil

One of the oldest and most competitive biofuel sectors compared to other parts of the worlds is Brazil having sugarcane-derived ethanol production since 1970s. Almost one-third of total global production has been accounted from Brazil, thus making it the second largest biofuel producer in the world. Government initiation

for the need to biofuel started with the country's exposure to high oil prices during the 1973 oil crisis. With a well-established sugarcane sector, which at that time was under pressure from low world sugar prices, diversification into ethanol production also created an opportune market outlet for sugarcane. Brazilian government imposed the phased implementation of mandatory blending requirements, which now stand at 20–25%, and offered discount for ethanol fuels at the fuel pump. This created a guaranteed domestic market in the country. In addition, ethanol producers were eligible for several other incentives, including concessionary credit lines, price and offtake guarantees, and tax breaks. Moreover, research and development by public institutions was critical to sector innovation, especially with regard to agronomic and biotechnological improvements. Although the cost of production in the early stage was more but technological advances and gains from economies of scale brought down the cost of production to large extent. With pure ethanol typically selling at between 60 and 70% of the price of gasoline, producer subsidies and pricing interventions are no longer necessary in the country (Goldemberg et al. 2004a, b).

9.6.2 Government Role in Market Development in Indonesia and Malaysia

After Brazil, other countries started to give commitment on domestic biofuel sector after examining the profits of it. Domestic consumption of biodiesel was seen as offering two key benefits: it would support the creation of another, more profitable, market for palm oil products, and it could contribute to alleviating the burgeoning federal cost of fuel subsidies. The rise in oil prices between 2005 and 2008, in particular, put biofuels firmly on the political agenda in many countries. Both Malaysia and Indonesia, for instance, adopted biofuel policies and laws during this period. Like Brazil, both countries are well positioned to exploit a well-established feedstock sector. Both countries heavily subsidize the end-price of transportation fuels; they have sought to ease this burden through the blending of biofuels, particularly biodiesel (Chin 2011; Caroko et al. 2011). The effect of high oil prices was especially detrimental to Indonesia, which, in contrast to Malaysia, is now a net oil importer. When oil prices peaked in 2008, fuel subsidies in Indonesia constituted almost one-third of total government spending (Dillon et al. 2008). In response to these pressures, both countries announced ambitious blending targets and established dedicated government agencies to oversee development of the biofuel sector. In response to this apparent government commitment and renewed global interest in biofuels, many sugarcane and palm oil sector actors in both countries made considerable investments in their biodiesel production capacities. Total production capacity in 2010 was estimated at 2.6 billion L for Malaysia and almost 4 billion L for Indonesia (Adnan 2010; Van Gelder and German 2011). Despite this early enthusiasm amongst both private and public sectors, current production remains well under

installed capacities, with Malaysia producing only 222 million L and Indonesia 104 million L of biodiesel in 2009 (Hoh 2010; Baskoro 2010). In Indonesia, the government has since introduced consumer subsidies over and above the existing fuel subsidy, and is providing various producer incentives to encourage domestic biodiesel production and prevent price inflation at the pump.

9.6.3 Government Role in Market Development in India

India is one of the rapid growing economies in the world. The development agencies focus primarily on economic growth, equity, and well-being of human. Energy is a critical input for socioeconomic development. The energy plan of each and every country aims at efficiency and security and also to provide environment-friendly approaches and optimal use of primary resources for energy generation. Although fossil fuels plays a dominant role in the energy scenario in our country in the next few decades but this conventional fossil fuel resources are limited, nonrenewable, and polluting and, therefore, need to be used wisely. On the other hand, renewable energy resources are indigenous, nonpolluting, and virtually inexhaustible. India is endowed with abundant renewable energy resources. Sugarcane is a good source of renewable biofuel which is nonpolluting in nature. The petro-based oil meets about 95% of the requirement for transportation fuels, and the demand has been steadily rising. The domestic crude oil is able to meet only about 23% of the demand, while the rest is meeting from imported crude for which India has to spent large bulk of money. Thus from the security point of view, alternative fuels like sugarcane biofuel need to be developed in order to curb pollution. Biofuels are environment-friendly fuels and their utilization would address global concerns about suppression of carbon emissions. In the context of the International perspectives and National imperatives, it is the endeavor of this Policy to facilitate and bring about optimal development and utilization of indigenous biomass feedstocks for production of biofuels. Thus our government policy has tried to accelerate the development and promotion of the cultivation, production, and use of biofuels to meet the increasing demand and substitute for petrol and diesel for transport and other applications which will not only throw in to energy security but also climate change mitigation, apart from creating new employment opportunities and leading to environmentally sustainable development. The scope of the Policy encompasses bioethanol, biodiesel, and other biofuels, as listed below:

(a) ***Bioethanol:*** Ethanol produced from biomass such as sugar containing materials, like sugar cane, sugar beet, sweet sorghum, etc.; starch containing materials such as corn, cassava, algae, etc.; and cellulosic materials such as bagasse, wood waste, agricultural and forestry residues.
(b) ***Biodiesel:*** A methyl or ethyl ester of fatty acids produced from vegetable oils, both edible and nonedible, or animal fat of diesel quality.

In India, bioethanol is produced mainly from molasses, a by-product of the sugar industry. Cultivators, farmers, landless laborers, etc. would be encouraged to undertake plantations that provide the feedstock for biodiesel and bioethanol. Corporates would be enabled to undertake plantations through contract farming by involving farmers, cooperatives, Self Help Groups, etc. in consultation with Panchayats, where necessary. Such cultivation or plantation would be supported through a Minimum Support Price for the nonedible oil seeds used to produce biodiesel. Ethanol is mainly being produced in the country at present from molasses, which is a by-product of the sugar industry. 5% blending of ethanol with gasoline has already been taken up by the Oil Marketing Companies (OMCs) in 20 States and 4 Union Territories. 10% mandatory blending of ethanol with gasoline has become effective from October 2008 in the states of India. In order to boost up the availability of ethanol and reduce over supply of sugar, the sugar industry has been permitted to produce ethanol directly from sugarcane juice. Financial incentives, including subsidies and grants were being formulated by different government bodies. National Biofuel Fund has been considered for providing financial incentives for producing sugarcane biofuel. International scientific and technical cooperation in the area of biofuel production, conversion, and utilization will be established in accordance with national priorities and socioeconomic development strategies and goals. Both bilateral and multi-lateral cooperation programs for sharing of technologies and funding projects would be urbanized, and participation in international partnerships, whenever necessary, will also be taken into account (MNERGOI 2016).

9.7 Summary and Conclusion

Use of biofuel leads to breeze the gap between food, fodder, and fuel security**.** Biofuels have become agreeable to most of the developing countries because of their potentiality to stimulate economic development in rural areas and lessen poverty by creating employment opportunities and increased income level in the agricultural sector. Biofuel production is labor intensive and thus a good initiator of rural employment. In addition, the production of biofuels requires investment in roads and other forms of rural and transport infrastructure which will have a "host in" effect by encouraging other investments in this section (Hausman 2007, 2012). Many developing countries have therefore begun to explore biofuel policies of their own feasible ways. China, for example, has announced a biofuel fusing target of 15% for all kinds of transportation by fuels by 2020 (Dong 2007). India too has implemented a 5% ethanol blend mandate for gasoline fuel, scheduled to be increased to 20% by 2017. By expanding sugarcane ethanol production, the Government of India hopes to increase domestic food and energy security, accelerate rural development, and reduce carbon emissions (GOI 2016). India's interest in improving its energy security stems from its rapidly growing dependence on foreign oil. India's economy is growing at a rate of 7% per year, making it the second fastest

growing economy in the world. The country is projected to become the third largest consumer of transportation fuel in 2020, after the USA and China (Kiuru 2002).

In 2008 India imported 128.15 million metric tons of crude oil valued at $75.7 billion, constituting 75% of its total petroleum consumption for that year. By 2025, it will be importing 90% of its petroleum (Parimal et al. 2010). India's increasing dependence on foreign energy sources will make the country increasingly vulnerable to external price shocks and supply distortions. Another reason for India to take an interest in a domestic biofuels industry is its potential to accelerate rural development. As in most developing countries, the majority of India's labor force works in the agricultural sector, therefore in India there is particularly high potential for biofuels to raise incomes, provide employment, and contribute to rural development. This combined with India's aforementioned concerns over energy security has led the Government of India recently to develop a keen interest in encouraging the expansion of a domestic biofuels industry. In 2003, they launched the first phase of their biofuels program in which 5% blending of ethanol in gasoline was mandated in certain areas of nine major sugarcane growing states and four union territories. In 2009 the Indian sugar industry estimated that 680 million liters of ethanol would be needed to meet just a 5% blend but only 585 million liters of ethanol were produced that year (Bureau 2009). In December of 2009 the Government of India set an official target of at least 20% blending of ethanol with gasoline by 2017 (IANS 2009).

Some important advances have been made by researchers for plant genetic engineering for biofuel production, but still there is dire need to develop technologies for elite sugarcane varieties having high sucrose content, less lignin, and easy to conversion process for biofuel production. There are some specific challenges related to the cost of microbial cellulose enzyme because their production is still expensive. To solve these problems, research is being focused on the targeting of these enzymes to multiple subcellular locations in order to increase levels of enzyme production and produce enzymes with higher biological activities. Genetic engineering play an important role in deconstruct plant cell wall polysaccharides, to suppress lignin biosynthesis enzymes, increase the level of sugars and plant biomass that have decreasing the overall biofuel production cost.

References

Adnan H (2010) Malaysia's biodiesel industry at a standstill. http://www.thestar.com.my/business/business-news/2010/09/06/malaysias-biodiesel-industry-at-a-standstill/. Accessed 22 Nov 2016

Barros S (2015) Brazil biofuels annual: biofuels-ethanol and biodiesel. BRI5006 Gain Report Global Agricultural information Network USDA Foreign Agricultural Service. http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Biofuels%20Annual_Sao%20Paulo%20ATO_Brazil_8-4-2015.pdf. Accessed 22 Nov 2016

Baskoro FM (2010) Renewable energy producers to get tax breaks. The Jakarta Globe, 16 May. http://www.thejakartaglobe.com/business/renewableenergy-producers-to-get-tax-breaks/375280 Accessed 28 Sept 2010

Bull TA (2002) The sugarcane plant. In: Hogarth M, Allsopp P (eds) Manual of cane growing. Bureau of Sugar Experimental Stations, Indooroopilly, Australia, pp 71–83

Bureau ET (2009) OMCs and sugar industry agree on reworking purchase agreement. The Economic Times. http://economictimes.indiatimes.com/markets/commodities/omcs-sugar-industry-agree-on-reworking-purchase-agreement/articleshow/5389052.cms. Accessed 22 Nov 2016

Campbell CJ, Laherree JH (1998) The end of cheap oil. In: Preventing the next oil crunch. Special Report. Scientific American Inc. p 77–83

Caroko W, Komarudin H, Obidzinski K, Gunarso P (2011) Policy and institutional frameworks for the development of palm oil–based biodiesel in Indonesia. Working Paper 62, CIFOR, Bogor, Indonesia

Chin M (2011) Biofuels in Malaysia: an analysis of the legal and institutional framework. Working Paper 64, CIFOR, Bogor, Indonesia

Coelho ST, Goldemberg J (2004) Alternative transportation fuels: contemporary case studies. http://energyprofessionalsymposium.com/?p=22124. Accessed 22 Nov 2016

Dias MOS, Modesto M, Ensinus AV, Nebra SA, Filho RM, Rossell CE (2011) Improving bioethanol production from sugarcane: evaluation of distillation thermal integration and cogeneration systems. Energy 36(6):3691–3703

Dillon HS, Laan T, Dillon HS (2008) Biofuels—at what cost? Government support for ethanol and biodiesel in Indonesia. Global Subsidies Initiative (GSI) of the International Institute for Sustainable Development (IISD), Geneva, p 55

Dong F (2007) Food security and biofuels development: the case of China. Briefing Paper 07-BP 52. Center for Agricultural and Rural Development Iowa State University, Ames, p 16. http://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=1001&context=card_briefingpapers. Accessed 22 Nov 2016

Ericlam SJ, Silvaz JD, Lawton M, Bonos S, Calvino M, Carrer H, Marcio C et al (2009) Improving sugarcane for biofuel: engineering for an even better feedstock. GCB Bioenergy 1(3):251–255

GOI (Government of India) (2016) Report of the Committee on Development of bio-fuel. Planning Commission Government of India, New Delhi. p 130. http://planningcommission.nic.in/reports/genrep/cmtt_bio.pdf. Accessed 22 Nov 2016

Goldemberg J, Coelho ST, Nastari PM, Lucon O (2004a) Ethanol learning curve—the Brazilian experience. Biomass Bioenergy 26(3):301–304

Goldemberg J, Coelho ST, Lucona O (2004b) How adequate policies can push renewables. Energy Policy 32:1141–1146

Goldemberg J, Coelho ST, Guardabassi P (2008) The sustainability of ethanol production from sugarcane. Energy Policy 36(6):2086–2097

Graham-Rowe D (2008) Electricity without carbon. Nature 454:816–823

Groenewald JH, Botha FC (2008) Down-regulation of pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP) activity in sugarcane enhances sucrose accumulation in immature internodes. Transgenic Res 17:85–92

Hausman R (2007) Biofuel can match oil production. Financial Times. https://www.ftcom/content/ad770a0c-8c7d-11dc-b887-0000779fd2ac. Accessed 22 Nov 2016

Hausman C (2012) Biofuels and land use change: sugarcane and soybean Acerage response in Brazil. Environ Resour Econ 51(2):163–187

Hoang NV, Furtado A, Botha FC, Simmons BA, Henry RJ (2015) Potential for genetic improvement of sugarcane as a source of biomass for biofuels. Front Bioeng Biotechnol 3(182):1–15

Hodkinson TR, Chase MW, Liedo MD, Salamin N, Renvoiz SA (2002) Phylogenetics of Miscanthus Saccharaum and related genera (Saccharinae Andropogoneae Poaceae) based on DNA sequences from ITS nuclear ribosomal DNA and plastid trnL intron and trnl-F intergenic spacers. J Plant Res 115:381–392

Hoh R (2010) Malaysia: Biofuels Annual Report 2010. GAIN Report No MY0008. United States Department of Agriculture Foreign Agricultural Service, Kuala Lumpur, Malaysia. https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Biofuels%20Annual_Kuala%20Lumpur_Malaysia_7-16-2010.pdf. Accessed 22 Nov 2016

IANS (India's Largest Independent News Service) (2009) India approves new policy to promote biofuels The Economic Times. http://www.thaindian.com/newsportal/business/india-approves-new-policy-to-promote-biofuels_100294317.html. Accessed 22 Nov 2016

Kahr H, Jagar A, Lanzerstorfer C (2012) Bioethanol production from steam explosion pretreated straw. In: Lima MAP, Natalense APP (eds) Bioethanol. InTech Pub, Rijeka, pp 153–172. doi:10.5772/21008

Kiuru L (2002) Worldwide fuel quality trends: focus on Asia better air quality in Asian and Pacific Rim Cities. Paper presented at Hong Kong Convention and Exhibition Centre (HKCEC), December 16–18

Larson ED (2006) A review of life-cycle analysis studies on liquid biofuels systems for the transport sector. Energy Sustain Dev 10(2):109–126

Mahmood-ul-Hassan M, Suthar V, Rafique E, Ahmad R, Yasin M (2015) Kinetics of cadmium chromium and lead sorption onto chemically modified sugarcane bagasse and wheat straw. Environ Monit Assess 187:470

MCTI (Ministry of Science Technology and Innovation) (2004) Brazil National Communication to the UNFCCC. http://www.mct.gov.br/index.php/content/view/4004.html. Accessed on 22 Nov 2016

MNERGOI (2016) National policy on biofuels. Ministry of New & Renewable Energy. Government of India New Delhi, India. pp 1–18. http://mnre.gov.in/file-manager/UserFiles/biofuel_policy.pdf. Accessed 22 Nov 2016

NSAI (National Standards Authority of Ireland) (2014) Automotive fuels—ethanol as a blending component for petrol-requirements and test methods. Standard Number: BSEN 15376:2014. http://shop.bsigroup.com/ProductDetail/?pid=000000000030275106. Accessed 22 Nov 2016

Ojeda K, Avila O, Suarez J, Kafarov V (2011) Evaluation of technological alternatives for process integration of sugarcane bagasse for sustainable biofuels production—part 1. Chem Eng Res Des 89(3):270–279

Orellana C, Neto RB (2006) Brazil and Japan give fuel to ethanol market. Nat Biotechnol 24:232. doi:10.1038/nbt0306-232

Parimal R, Saxena A, Gupta S (2010) Energy security an indian perspective: the way forward. In: 8th Biennial International Conference & Exposition on Petroleum Geophysics Hyderabad India. http://www.spgindia.org/2010/137.pdf. Accessed on 22 Nov 2016

Patrick JW, Botha FC, Birch RG (2013) Metabolic engineering of sugars and sugar derivatives in plants. Plant Biotechnol J 11:142–156

Paula ND (2016) Opportunities and challenges of new technological paths in the ethanol market. Espacios 37:1–20

REN21 (2012) Renewables Global Status Report REN21. Secretariat, Paris, France. http://www.ren21.net/Portals/0/documents/Resources/GSR2012_low%20res_FINAL.pdf. Accessed on 22 Nov 2016

Rocha GJM, Goncalves AR, Oliveira BR, Olivares EG, Rossell CEV (2012) Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production. Ind Crop Prod 35(1):274–279

Rodsrud G, Lersch M, Sjode A (2012) History and future of world's most advanced biorefinary in operation. Biomass Bioenergy 46:46–59

Salassi ME, Breaux JB (2006) Allocation of sugarcane planting costs in 2006. Staff Report No 2006-01. Louisiana State University-Ag Center Research & Extension. http://www.lsuagcenter.com/NR/rdonlyres/84909F41-4DFD-4D57-A291-E6DA0929504B/20942/AllocationofSugarcanePlantingCostsin2006.pdf. Accessed 22 Nov 2016

Sandhu HS, Singh MP, Gilbert RA, Oredo DC (2016) Sugarcane botany: a brief. EDIS publication SS-AGR-234. pp 1–5. http://edis.ifas.ufl.edu/pdffiles/SC/SC03400.pdf. Accessed 22 Nov 2016

Somerville C (2007) Biofuels. Curr Biol 17(4):1–5

Sukesh K, Joe MM, Sivakumar PK (2010) An introduction to industrial microbiology. S Chand Publishing, New Delhi, India, p 307

Taherzadeh MJ, Lennartsson PR, Teichert O, Nordholm H (2013) Bioethanol production processes. In: Babu V, Thapliyal A, Patel GK (eds) Biofuels production. Scrivener Publishing, Beverly, pp 211–253

USCB (United States Census Bureau) (2016) World population clock. http://www.census.gov. Accessed 22 Nov 2016

Van Gelder JW, German L (2011) Biofuel finance global trends in biofuel finance in forest-rich countries of Asia, Africa and Latin America and implications for governance. http://www.cifor.org/publications/pdf_files/infobrief/3340-infobrief.pdf. Accessed 22 Nov 2016

Chapter 10
Plastome Engineering: Yesterday, Today, and Tomorrow

Sarma Rajeev Kumar, Pushpanathan Anunanthini, and Ramalingam Sathishkumar

Abstract Plant transformation has made significant strides in last two decades with main focus on developing stress-tolerant crops and pharmaceutically important compounds for therapeutic purpose. There are many success stories describing the production of therapeutic proteins in large scale that are targeted to either nuclear or plastid genomes. The plastid genome (plastome) represents an attractive target for genetic engineering in crop plants. Transgenes integrated to plastome have several advantages like high expression levels, genes can be stacked in operons and genes integrated to plastome do not exhibit silencing mechanism. An additional advantage lies in the maternal inheritance of plastids in most plant species, which addresses the biosafety concerns related to transgenic plants. The plastid engineering usually results in alteration of several thousand plastid genome copies in a cell. In this chapter, the evolution of this technology with respect to the current state-of-the-art methods and the advantage of this technology over nuclear transformation are discussed. The recent advancement in plastome engineering and novel tools/methods developed to overcome potential limitations of chloroplast transformation are discussed in this chapter. Finally, future application of chloroplast engineering with a perspective for sugarcane plastome engineering is also briefed.

Keywords Chloroplast • Homoplasmy • Plastome • Sugarcane • Transplastomic plants

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© Springer International Publishing AG 2017

C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_10

10.1 Introduction

A range of different expression platforms have been used for overexpression or heterologous production of recombinant proteins with pharmaceutical, industrial, and agricultural applications (Demain and Vaishnav 2009). From simple bacterium, *Escherichia coli* to *Saccharomyces cerevisiae* and Chinese Hamster Ovary (CHO) cells have been engineered for heterologous production of foreign proteins and novel metabolites (Rader 2008). Each system has its own advantage and demerits (Demain and Vaishnav 2009; Fernandez-Robledo and Vasta 2010). Plants have been used as an economic and easily scalable production platform for the expression of recombinant proteins, enzymes, and valuable metabolites. Plants can efficiently produce complex foreign proteins and process them post-translationally like in animals. Further, they are considered under category GRAS (Generally Recognized as Safe) organisms and can also be exploited for oral and mucosal delivery of vaccines (Demain and Vaishnav 2009; Karg and Kallio 2009). Moreover transgenic plants for therapeutic protein production avoid risk of contamination with animal pathogens including prions (Yao et al. 2015). Plants can be transformed stably, inserting foreign genes in either nuclear or plastid genome (Scotti et al. 2012). Plastid transformation results in accumulation of foreign proteins in the same organelles, whereas those targeted to nucleus are synthesized in the cytosol, and later directed to different subcellular compartments based on the signal peptide. The choice of localization of proteins in subcellular compartment depends on the nature of recombinant protein, downstream application, etc. (Daniell et al. 2009a, b; Lau and Sun 2009). Despite the above advantages, biocontainment of foreign genes and the level and stability of protein in transgenic hosts are the main concerns related to the use of plants as biofactories (Scotti et al. 2012).

10.2 Advantages of Plastid Transformation

Plant cells have three genome containing compartments, nucleus, mitochondria, and plastids. The plastid genome (plastome) is semi-autonomous replicating unit with a small circular double-stranded DNA having own transcription-translation machinery. The genome size varies from 120 to 220 kb encoding more than 120 genes. The plastome can be engineered by genetic transformation and this possibility has stirred enormous interest among plant biotechnologists (Bock 2014).

Plastome engineering emerged as an alternative platform to nuclear transformation for the expression of foreign proteins. There are several advantages associated with targeting transgenes into the plastid genome rather than the nuclear genome. The high number of plastids per cell and high copy number of plastome per plastid offer the possibility of expressing foreign genes to extremely high levels that is not possible with the nuclear genome (Oey et al. 2009). Typical plant cell contains approximately 100 chloroplasts, each with about 100 identical genomes; a single

gene is represented perhaps 10,000 times in a cell. As transgene integration to plastome is by homologous recombination phenomena, plastome engineering is highly precise as compared to nuclear genome that follows non-homologous recombination (Cerutti et al. 1992). Hence, plastid transformation vectors are designed to contain homologous flanking sequences on either side of the transgenes to facilitate site-specific integration (Kumar and Daniell 2004). Another major advantage of plastome engineering is that genetic machinery in plastids is devoid of position effect, gene silencing, and other epigenetic mechanisms that interfere with stable transgene expression (Ruf et al. 2007; Bock 2014). It has been reported that silencing was not observed despite accumulation of transcripts to more than 150-fold higher than nuclear targeted expression (Lee et al. 2003) and the accumulation of foreign proteins was 46% of total soluble protein (De Cosa et al. 2001). The feasibility of stacking of genes in synthetic operons or as gene clusters represents another greatest attraction of plastome engineering technology that is very useful for production of novel secondary metabolites (Fuentes et al. 2016). Initial attempts to express bacterial operons in plastids were not successful. This has resulted in strategies including reengineering of operons by altering untranslated regions (UTRs) and intercistronic spacers, codon optimization, and incorporation of intercistronic processing elements for efficient expression from plastome. Finally, plastid transformation has received significant attention mainly because of transgene containment. Plastids are maternally inherited in most crops and are therefore not spread through pollen (Daniell 2002; Hagemann 2004). Plastid genetic engineering is therefore particularly suitable for the use of plants as biofactories for the large-scale production of different proteins. Infinite number of recombinant proteins including antigens, antibodies, and commercially important enzymes has been successfully expressed using plastid transformation (Table 10.1).

10.3 Plastome Engineering

The major breakthrough that made plastid transformation possible was the invention and use of gene gun that can be used to bombard living cells with accelerated DNA coated with tungsten or gold particles. Transformation of the chloroplast genome was first accomplished in unicellular algae *Chlamydomonas reinhardtii*, harboring a single chloroplast that occupies approximately half the cell volume and contains close to 80 identical copies of the plastid genome (Boynton et al. 1988). In the beginning, plastid transformation was thought to be challenging in higher plants as a typical leaf mesophyll contain more than 2000 copies of the plastid genome with more than 100 chloroplasts. However, after the initial success with *Chlamydomonas*, chloroplast transformation was also achieved in model system tobacco (Svab et al. 1990; Svab and Maliga 1993). For more than two decades, these two organisms have remained the model system for plastid transformation. Although a few agronomically important crop species have been engineered, progress in developing plastid transformation technology for many genetic model species and

Table 10.1 List of therapeutic proteins and commercially important enzymes produced by plastome engineering

Sl. No	Transgenic host	Recombinant protein expressed	Source	References
Therapeutic proteins				
01	Tobacco	Multi epitopic protein (gp120 and gp41)	HIV	Rosales-Mendoza et al. (2009)
02	Lettuce	E protein of DENV domain I and II	Dengue virus	Maldaner et al. (2013)
03	*Chlamydomonas reinhardtii*	Surface protein (Pfs25) and (Pfs28)	*Plasmodium falciparum*	Gregory et al. (2012)
04	Tobacco	VP6	Rota virus	Inka Borchers et al. (2012)
05	Tobacco/lettuce	Proinsulin	Human proinsulin A, B, C peptides	Boyhan and Daniell (2011)
06	Tobacco	Anthrax protective antigen	*Bacillus anthracis*	Gorantala et al. (2011)
07	Lettuce	Thioredoxin 1	Human	Lim et al. (2011)
08	Tobacco/lettuce	Apical membrane antigen-1	*Plasmodium falciparum*	Davoodi-Semiromi et al. (2010)
09	Tobacco	Immunogenic 2 L21 peptide	Canine parvovirus	Ortigosa et al. (2010)
10	Tobacco	Coagulation factor IX	Human	Verma et al. (2010a, b)
11	Tobacco	Insulin like growth factor-1	Human	Daniell et al. (2009a, b)
12	Tobacco	E7-CP	Human Papiloma virus-16	Morgenfeld et al. (2009)
13	Tobacco	Alpha1-antitrypsin	Human	Nadai et al. (2009)
14	Tobacco	Immunogenic fusion protein F1-V	*Yersinia pestis*	Arlen et al. (2008)
15	Tobacco	Aprotinin	Bovine	Tissot et al. (2008)
Commercially important enzymes				
16	Tobacco	Endoglucanase and exoglucanase	*Clostridium thermocellum*	Verma et al. (2010a, b)
17	Tobacco	Lipase	*Mycobacterium tuberculosis*	Verma et al. (2010a, b)
18	Tobacco	Xylanase	*Trichoderma reesei*	Verma et al. (2010a, b)
19	Tobacco	Choline monooxygenase	*Beta vulgaris*	Zhang et al. (2008)
20	Tobacco	Chitinase	*Brassica juncea*	Guan et al. (2008)
21	Tobacco	β-Glucosidase	*Thermobifida fusca*	Gray et al. (2011)
22	Tomato	Lycopene β-cyclase	*Narcissus pseudonarcissus*	Apel and Bock (2009)
23	Tobacco	Cellulase	*Thermobifida fusca*	Petersen and Bock (2011)

(continued)

Table 10.1 (continued)

Sl. No	Transgenic host	Recombinant protein expressed	Source	References
24	Tobacco	(hemi) cellulolytic enzymes	Thermophilic or hyperthermophilic bacteria	Castiglia et al. (2016)
25	Tobacco	Polyhydroxybutyrate	*Bacillus megaterium*	Bohmert-Tatarev et al. (2011)
26	Tobacco	Agglutinin	*Pinellia ternata*	Jin et al. (2012)
27	Tobacco	β-Mannanase	*T. reesei*	Agrawal et al. (2011)
28	Tobacco	Cutinase	*Fusarium solani*	Verma et al. (2013)
29	Tobacco	Swollenin	*T. reesei*	Verma et al. (2013)
30	Tobacco	Protease inhibitor	Sweet potato	Chen et al. (2014)

agriculturally relevant crops like cereals and monocots is slow and is still in its infancy (Maliga and Bock 2011; Bock 2014). In last few years, plastid transformation have opened new dimensions for using plants as a production platform due to several advantages offered by transplastomic plants compared to conventional transgenic plants (Bock and Warzecha 2010; Cardi et al. 2010).

The integration of foreign DNA in plastome occurs exclusively only via homologous recombination (Bock et al. 1994). Recently, Valkov et al. (2011) reported the extent of similarity between the plastid genome sequences involved in homologous recombination is crucial to ensure high transformation efficiency. Generally, plastid transformation vectors harbor plastid sequences involved in homologous recombination (flanking sequences), selectable marker gene (chimeric *aadA* gene that confers resistance to spectinomycin and streptomycin) and foreign gene to be expressed in the cassette. Selection of recombination sites for integration of expression cassette in plastome is one of the important parameters for higher expression of transgenes as different insertion sites are reported to have different levels of expression (Waheed et al. 2015). The location of insertion sites should be in actively transcribing region and within the inverted repeat region of plastome (Verma et al. 2008). Insertion site can also have some negative effects on expression of foreign proteins (Waheed et al. 2015). Several plastid sequences have been used as transgene insertion sites; however, most commonly used vectors contain either trnV-3′/rps12 and trnI/trnA regions located in the inverted repeats (IR) (Zoubenko et al. 1994; Daniell et al. 1998) or trnfM/trnG region in the large single copy region (LSC) (Ruf et al. 2001). Daniell et al. 2016 reported that IR regions are found in duplicate in most plastid genomes; hence transgenes inserted within the IR region (instead LSC regions) should have double the copy number of transgenes. Also, integration of the cassette into one copy of the IR facilitates integration into the duplicate copy. The extent of sequence divergence is reported to influence the frequency of recombination, transformation efficiency, and functionality of recombinant coding sequences. Due to sequence variability in different regions, incorporation of proper flanking sequence in vectors will determine the efficiency of protein expression in plastids.

Nevertheless, it is technically challenging to develop species-specific vectors for different plants to be transformed due to lack of availability of complete chloroplast genome sequence in many plants. In addition, the expression cassettes usually consist of a 5′-regulatory region, including a strong promoter and a translational control region, and a 3′-regulatory region with a terminator sequence that corresponds to the mRNA 3′-UTR. The presence of 5′UTR is crucial to obtain a notable increase in protein yield (Scotti et al. 2009).

Plastid expression cassettes can be delivered to the plastome either by the biolistic approach (commonly used technique) or by the polyethylene glycol (PEG) treatment of protoplasts (Cardi et al. 2010). Protoplast isolation is tricky and cumbersome process for many plant species and lack of efficient regeneration method from protoplasts is the main bottleneck associated with protoplast mediated transformation. Even though transformation by biolistic method is expensive, it is the most widely used choice for chloroplast transformation (Verma et al. 2008; Bock 2014). As plastids are polyploid in nature, the primary transformants always are heteroplasmic with mixed population of non-transformed and transformed plastomes in plastids. Hence, several rounds of selection and regeneration are required to achieve homoplasmic lines (Verma et al. 2008; Ahmad and Mukhtar 2013). Homoplasmy is a condition in which all plastomes in the plants are transformed, and to achieve homoplasmy, two or more extra regeneration cycle under selection pressure is necessary. Young leaves with immature chloroplasts having low plastome copy number are the best source for transformation, and homoplasmy can be quickly attained in these explants.

Recently, using reverse genetic approach, minimum gene set of a plastome that is functional under heterotrophic conditions has been designed using synthetic biology approach (Scharff and Bock 2014). Similarly, a minimum plastid genome for tobacco plants has been designed and its synthesis and assembly is currently underway (Scharff and Bock 2014). The feasibility of transforming plastids with an entire genome is reported in *C. reinhardtii* (O'Neill et al. 2012). Due to sequence homology between the transformed genome and the endogenous plastid genome, complete genome replacement was not achieved. Homologous recombination between the two genome resulted in mosaic plastid genomes composed of endogenous and exogenous pieces (O'Neill et al. 2012).

10.4 Novel Approaches in Plastome Engineering

The development of a tissue culture-independent plastome engineering technology would make this technology accessible to a much wider range of crops. Tungsuchat-Huang and Maliga (2012) reported the manipulation of tobacco plastid genome in greenhouse-grown plants by site-specific recombination employing phage-derived recombinase targeted to chloroplasts. A recombinase gene was delivered by injecting *Agrobacterium* to axillary buds of soil-grown tobacco plants. The lateral shoots formed from the injected site exhibited marker-free plastid genomes and,

interestingly, 7% of the plants transmitted the trait (marker-free plastid genome) to progeny. Although this attempt was not completely independent of tissue culture, complete culture-independent method for primary manipulation of the plastid genome (similar to transient *Agrobacterium* transformation using vacuum infiltration or virus-induced gene silencing method) is still far and will be difficult to achieve in near future.

Gottschamel et al. (2013) reported used of Gateway system to simplify the plastid vector construction and also to improve the vector design for incorporating desirable sites. It was the first study that reported use of Gateway-based recombination system for plastid vector construction. Vafaee et al. (2014) developed modular cloning for designing and assembly of complete plastid transformation vector. The authors described three-level assembly process, including vector fostering gene expression and formation of griffithsin, a potential viral entry inhibitor and HIV prophylactic in chloroplasts of tobacco. Similar strategy could be used for efficient application of synthetic biology approaches for plastome engineering.

As mentioned previously, plastid transformation method is highly restricted to relatively few species (Maliga and Bock 2011). Efficient methods for plastome engineering in cereals and many monocots are still lacking and even closely related species or different cultivars or genotypes of the same species respond differentially to plastid transformation, and hence this makes the established method challenging (McCabe et al. 2008). Sigeno et al. (2009) reported transfer of engineered plastids from tobacco, an easy-to-transform species, to *Petunia* (unrelated and recalcitrant species) by protoplast fusion. Similar strategies have been employed for transfer of transgenic plastome to unrelated species (Kuchuk et al. 2006; Ovcharenko et al. 2011). Nevertheless, the procedure is highly laborious, time-consuming, and still applicable only to a limited number of plant species. Stegemann and Bock (2009) reported that plastids can migrate between cells in grafted plants. This innate ability was later exploited by different research groups and showed that transgenic plastome can be exchanged in grafted species. Although this is tissue culture-independent method, its applicability is restricted only to the closely related species. The use of synthetic DNA and artificially synthesized plastid transformation vector including the flanking regions for integration of transgenes in plastome is another breakthrough in plastid engineering.

10.5 New Tools and Major Challenges in Transplastomic Technology

One of the main advantages of plastid transformation is the high level of foreign protein accumulation, which in some cases reached more than 50% of the total soluble protein in leaves (Oey et al. 2009; Ruhlman et al. 2010). Unfortunately, it is also worth to mention here that significant number of proteins whose expression in plastid was not preferred resulted in poor expression (Bock 2014). Stability of

foreign protein and or improper protein folding is key factor limiting accumulation in many cases. It was later found that N-terminus amino acid in the protein determines the stability of proteins expressed in plastids (Apel et al. 2010). Engineering of the N-terminus sequence of unstable proteins or fusing them to the N-terminus of stable protein alleviated the problems associated with protein stability in plastids (Elghabi et al. 2011; De Marchis et al. 2012). Unfortunately, not much is known about internal determinants of protein stability and folding of proteins expressed in the plastids. This is one of the major challenges that need to be dissected.

Post-translational modifications are one of the main points that should be considered when expressing eukaryotic genes in plants. Cysteine disulfide bridges are reported to influence the stability of the plastid-made foreign proteins (Tissot et al. 2008; De Marchis et al. 2011). De Marchis et al. (2011) expressed fusion protein zeolin (a chimeric protein with bean phaseolin fused to truncated maize γ-zein) with its native signal peptide and two mutated forms, one without signal peptide and other without Cys residues. The signal peptide of phaseolin targeted zeolin to the thylakoid membranes and accumulated as trimers. Further, they were able to form disulfide bonds using plastid machinery. Recombinant proteins including interferons and insulin formed disulfide bridges and were functional when expressed in chloroplasts (Arlen et al. 2007; Boyhan and Daniell 2011). Interestingly, there are reports of formation of viral like particles (VLPs) in chloroplasts, Human papilloma virus L1 protein formed VLPs in tobacco and Dengue 3 premembrane and envelope polyprotein formed VLPs in lettuce (Fernández-San Millán et al. 2008; Kanagaraj et al. 2011). Different post-translational modifications like protein lipidation, multimerization, N-terminal methionine excision are known to function in plastids (Hennig et al. 2007; Tissot et al. 2008; Rigano et al. 2009). In general, proteins are not glycosylated in plastids; rather they are modified in endoplasmic reticulum and later transported as glycoproteins to plastids. Hence, proteins requiring glycosylation cannot be expressed in plastids. This is a major drawback as many of the therapeutic proteins including antibodies are glycated. The absence of protein glycosylation is also useful in some cases like expression of human alpha1-antitrypsin (α1AT), where the absence of glycosylation could be considered an advantage (Nadai et al. 2009).

Due to prokaryotic nature of the plastid gene expression machinery (Bock 2014), it is possible to stack multiple transgenes as in operons to co-express them from a single promoter resulting in a polycistronic mRNA. As many of the genes related to secondary metabolic pathway exist as clusters and are co-regulated; transgene stacking is useful for engineering of metabolic pathways. There are also several cases of poor expression of transgenes in an operon model in plastome (Bock 2013). This is mainly because many of the polycistronic transcripts undergo post-translational cleavage resulting in monocistronic transcripts in plastids. To some extent, the above problem was overcome by including sequences that mediates intercistronic processing elements into stable monocistronic mRNAs (Lu et al. 2013). These sequences act as a valuable tool for synthetic operon design and enhanced the probability of successful operon expression in transgenic tobacco and tomato plastomes (Lu et al. 2013). Lately, several novel metabolic pathways have

been introduced to plants by plastid transformation including biosynthesis of artemisinin (Fuentes et al. 2016), improving tocopherol biosynthesis in tobacco and lettuce (Lu et al. 2013; Yabuta et al. 2013), enhancing triterpenoid precursor squalene (Pasoreck et al. 2016).

The constitutive high expression of transgenes in plastids can interfere with plant development, inducing phenotypic alterations and reduced growth (Zhou et al. 2008; Rigano et al. 2009; Scotti et al. 2009). This could be due to enzymatic activities of foreign protein that might interfere with endogenous metabolic processes in the plastid, non-targeted interactions of foreign protein with plastid membranes or due to high metabolic burden imposed by extremely high levels of recombinant protein. An ideal strategy to deal with this situation would be to make transgene expression inducible. An ethanol-inducible T7 RNA polymerase was targeted to plastid, and transgenes with T7 promoter was transcribed only when nuclear gene was induced (Lössl et al. 2005). However, design of an efficient system in plastid that offers both tight control and high induction upon activation is still challenging. Two additional approaches were reported to induce the expression of plastid genes, the first one exploits the bacterial lac repressor and lac operator system, where transgene expression is induced by spraying or leaf infiltration with IPTG (Muhlbauer and Koop 2005). The second one is an engineered "riboswitch" that functions as translational regulator of transgene expression in transgenic plastids in response to the application of its ligand theophillin (Verhounig et al. 2010). Although the concept represents a very simple approach to regulate the introduced gene, the efficiency of switching is very low and modulation of gene expression after addition or on removal of theophillin is poor (Jin and Daniell 2015). This technique requires further tuning for regulating foreign gene expression in plants.

Since plastids are active only in green tissues, low expression levels of transgenes in non-green plant parts like fruits, tubers, and seeds is another drawback with plastome engineering. Therefore, low transgene expression in non-green tissue is a major limitation. Transplastomic tomato expressing p24-Nef accumulated fusion protein only in green tomato whereas they failed to accumulate in ripe tomato fruits (Zhou et al. 2008). Very little information is known about the genes and regulators involved in plastome expression in non-green tissues. Transcriptome and transplastome analysis of tomato fruit and potato tuber revealed that almost all plastid genes were highly downregulated at RNA and protein levels in these non-green storage organs (Kahlau and Bock 2008; Valkov et al. 2009). Interestingly, transcripts abundance of very few genes were high; however, they were poorly translated. Alternatively, few genes exhibited low transcript levels but exhibited strong ribosome association implicating active translation. The above finding resulted in a novel strategy in which chimeric constructs were made with promoter from a gene showing high mRNA accumulation in fruits or tubers with 5′UTR from mRNA showing strong polysome association. Transgenic plastome expressing foreign gene under the chimeric construct resulted in significant increase in foreign protein accumulation in non-green tissues (Caroca et al. 2012; Zhang et al. 2012). Strategies or further modifications based on above methods could open new avenues for metabolic engineering in plastids of non-green tissues.

The biolistic transformation method predominantly used for plastid engineering involves relatively harsh mechanical treatments of foreign DNA. Transformation of plastid genome with very large DNA molecules is less efficient, which is attributed to mechanical shearing during DNA purification and coating. Although, damaged DNA molecules are repaired by endogenous DNA repair mechanism, how efficiently plastids perform repair of fragmented DNAs is still not known. Hence, development of more gentle protocols for particle coating and DNA delivery into plastids would be highly desirable for efficient plastome engineering and expression of foreign proteins in plastids.

10.6 Plastome Engineering in Sugarcane

Chloroplast transformation in sugarcane offers several advantages compared to nuclear transformation strategy. First and foremost, it leads to precise gene integration into plastomes of sugarcane through homologous recombination and avoids position effects or gene silencing. Secondly, multiple genes can be expressed as a single transcriptional unit that can lead to enhanced accumulation of recombinant proteins. This strategy also avoids pollen outflow as plastids are maternally inherited. Finally, using this method, one can generate selectable marker-free transgenic sugarcane.

As discussed in other chapters of this book, sugarcane is one of the most productive species that converts the solar energy into carbohydrates more efficiently than any other crop plant. The plants possess the unusual ability to store sugars in vacuoles. The high biomass production and ease of cultivation enable sugarcane as a biofactory. Researchers have considered sugarcane as a potential biofactory for generating high-value products lately. *Agrobacterium* and biolistic mediated transformation met with varying degrees of success in sugarcane. Genetic engineering of sugarcane varieties for producing economically important metabolites, novel proteins, nutraceuticals, biopolymers, and therapeutic proteins is concrete and will be a promising approach to launch sugarcane biofactories in near future.

Although plastid engineering is still in its infancy in monocots like rice (Lee et al. 2006) and wheat (Cui et al. 2011), there are no reports for sugarcane. This might be achieved in near future as the chloroplast genome of sugarcane has been completely sequenced. The genome information could be used for designing recombination-based transformation vectors. One of the major hurdles in developing plastid transformation in sugarcane is low regeneration efficiency from non-green embryonic cells, containing undifferentiated plastids. Another impediment in developing homoplasmic lines is extremely low level of marker gene expression in non-green plastids in embryogenic cells due to low genome copy number and low rates of protein synthesis. Use of promoters and UTRs active in non-green tissues could help to overcome this limitation. The rRNA operon in plastids possesses two promoters, one for plastid-encoded RNA polymerase and other for nuclear-encoded plastid RNA polymerase. Use of rRNA-based promoters to drive transgene expression in plastids can recognize both polymerases and could result in enhanced accumulation of for-

eign protein leading to development of stable homoplasmic lines of sugarcane. As described above, *aadA* is the marker used for selection of plastid transformants. Most of the monocots including sugarcane are naturally resistant to spectinomycin but are sensitive to streptomycin. However, another problem associated with using streptomycin is that it might prolong the selection of resistant shoots and subsequently hinder the recovery of homoplasmic lines. Plastome engineering using dual selection system was obtained in sugarcane and heteroplasmic shoots were recovered on streptomycin and kanamycin as selection agents (Mustafa 2011).

Attempts to develop transplastomic lines in sugarcane have been unsuccessful so far. Researchers from Vasantdada Sugar Institute (VSI), Pune, used leaf discs as explants and transformed CoC671 variety with two chloroplast transformation vectors. However, they failed to obtain any positive transplastomic lines (Singh et al. 2013). Chloroplast genomes of sugarcane have been sequenced (Asano et al. 2004). Recently, a comparative study of the chloroplast genomes of *Miscanthus*, *Erianthus*, and sugarcane was reported (Tsuruta et al. 2017). Knowledge of the complete chloroplast genome, proper vector design with sugarcane-specific flanking regions, and efficient transformation and regeneration strategies will certainly aid to generate transplastomic sugarcane lines in the near future.

10.7 Concluding Remarks and Future Perspectives

There is an increasing number of proof-of-concept reports employing progress made in plastome engineering in the past few years with respect to high level protein expression and gene stacking. The last few years have witnessed close to 100 different recombinant proteins produced in transgenic plastids. The outcome from these studies holds great promise for the commercialization of the technology in near future. Further, the list of plants used for plastome engineering is also increasing. It can be expected that plastid transformation approach will be applied to more plant species including monocots like sugarcane for wider applications. This technology could lead to new paradigm shift and it is expected to contribute for the development of sustainable production of high-value proteins and metabolites in plastids. Nevertheless, social and biosafety issues should be considered while developing plant-based production platforms.

References

Agrawal P, Verma D, Daniell H (2011) Expression of Trichoderma reesei β-mannanase in tobacco chloroplasts and its utilization in lignocellulosic woody biomass hydrolysis. PLoS One 6:e29302

Ahmad N, Mukhtar Z (2013) Green factories: plastids for the production of foreign proteins at high levels. Gene Ther Mol Biol 15:14–29

Apel W, Bock R (2009) Enhancement of carotenoid biosynthesis in transplastomic tomatoes by induced lycopene-to-provitamin A conversion. Plant Physiol 151:59–66

Apel W, Schulze WX, Bock R (2010) Identification of protein stability determinants in chloroplasts. Plant J 63:636–650

Arlen PA, Falconer R, Cherukumilli S, Cole A, Cole AM, Oishi KK, Daniell H (2007) Field production and functional evaluation of chloroplast-derived interferon-alpha2b. Plant Biotechnol J 5:511–525

Arlen PA, Singleton M, Adamovicz JJ, Ding Y, Davoodi-Semiromi A, Daniell H (2008) Effective plague vaccination via oral delivery of plant cells expressing F1-V antigens in chloroplasts. Infect Immun 76:3640–3650

Asano T, Tsudzuki T, Takahashi S, Shimada H, Kadowaki KI (2004) Complete nucleotide sequence of the sugarcane (*Saccharum officinarum*) chloroplast genome: a comparative analysis of four monocot chloroplast genomes. DNA Res 11(2):93–99

Bock R (2013) Strategies for metabolic pathway engineering with multiple transgenes. Plant Mol Biol 83:21–31

Bock R (2014) Genetic engineering of the chloroplast: novel tools and new applications. Curr Opin Biotechnol 26:7–13

Bock R, Warzecha H (2010) Solar-powered factories for new vaccines and antibiotics. Trends Biotechnol 28:246–252

Bock R, Kossel H, Maliga P (1994) Introduction of a heterologous editing site into the tobacco plastid genome: the lack of RNA editing leads to a mutant phenotype. EMBO J 13:4623–4628

Bohmert-Tatarev K, McAvoy S, Daughtry S, Peoples OP, Snell KD (2011) High levels of bioplastic are produced in fertile transplastomic tobacco plants engineered with a synthetic operon for the production of polyhydroxybutyrate. Plant Physiol 155:1690–1708

Boyhan D, Daniell H (2011) Low-cost production of proinsulin in tobacco and lettuce chloroplasts for injectable or oral delivery of functional insulin and C-peptide. Plant Biotechnol J 9:585–598

Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark KB et al (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240:1534–1538

Cardi T, Lenzi P, Maliga P (2010) Chloroplasts as expression platforms for plant-produced vaccines. Expert Rev Vaccines 9:893–911

Caroca R, Howell KA, Hasse C, Ruf S, Bock R (2012) Design of chimeric expression elements that confer high-level gene activity in chromoplasts. Plant J 73:368–379

Castiglia D, Sannino L, Marcolongo L, Ionata E, Tamburino R, De Stradis A, Cobucci-Ponzano B, Moracci M, La Cara F, Scotti N (2016) High-level expression of thermostable cellulolytic enzymes in tobacco transplastomic plants and their use in hydrolysis of an industrially pretreated *Arundo donax* L biomass. Biotechnol Biofuels 9:154. doi:10.1186/s13068-016-0569-z

Cerutti H, Osman M, Grandoni P, Jagendorf AT (1992) A homolog of *Escherichia coli* RecA protein in plastids of higher plants. Proc Natl Acad Sci U S A 89:8068–8072

Chen PJ, Senthilkumar R, Jane WN, He Y, Tian Z, Yeh KW (2014) Transplastomic *Nicotiana benthamiana* plants expressing multiple defence genes encoding protease inhibitors and chitinase display broad-spectrum resistance against insects, pathogens and abiotic stresses. Plant Biotechnol J 12:503–515

Cui C, Song F, Tan Y, Zhou X, Zhao W, Ma F, Liu Y, Hussain J, Wang Y, Yang G, He G (2011) Stable chloroplast transformation of immature scutella and inflorescences in wheat (*Triticum aestivum* L). Acta Biochim Biophys Sin 43:284–291

Daniell H (2002) Molecular strategies for gene containment in transgenic crops. Nat Biotechnol 20:581–586

Daniell H, Datta R, Varma S, Gray S, Lee SB (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nat Biotechnol 16:345–348

Daniell H, Ruiz G, Denes B, Sandberg L, Langridge W (2009a) Optimization of codon composition and regulatory elements for expression of human insulin like growth factor-1 in transgenic chloroplasts and evaluation of structural identity and function. BMC Biotechnol 9:33

Daniell H, Singh ND, Mason H, Streatfield SJ (2009b) Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci 14:669–679
Daniell H, Lin CS, Yu M, Chang WJ (2016) Chloroplast genomes: diversity, evolution, and applications in genetic engineering. Genome Biol 17:134. doi:10.1186/s13059-016-1004-2
Davoodi-Semiromi A, Schreiber M, Nalapalli S, Verma D, Singh ND, Banks RK, Chakrabarti D, Daniell H (2010) Chloroplast-derived vaccine antigens confer dual immunity against cholera and malaria by oral or injectable delivery. Plant Biotechnol J 8:223–242
De Cosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nat Biotechnol 19:71–74
De Marchis F, Pompa A, Mannucci R, Morosinotto T, Bellucci M (2011) A plant secretory signal peptide targets plastome-encoded recombinant proteins to the thylakoid membrane. Plant Mol Biol 76:427–441
De Marchis F, Pompa A, Bellucci M (2012) Plastid proteostasis and heterologous protein accumulation in transplastomic plants. Plant Physiol 160:571–581
Demain AL, Vaishnav P (2009) Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv 27:297–306
Elghabi Z, Karcher D, Zhou F, Ruf S, Bock R (2011) Optimization of the expression of the HIV fusion inhibitor cyanovirin-N from the tobacco plastid genome. Plant Biotechnol J 9:599–608
Fernandez-Robledo JA, Vasta GR (2010) Production of recombinant proteins from protozoan parasites. Trends Parasitol 26:244–254
Fernández-San Millán A, Ortigosa SM, Hervás-Stubbs S, Corral-Martínez P, Seguí-Simarro JM, Gaétan J, Coursaget P, Veramendi J (2008) Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. Plant Biotechnol J 6:427–441
Fuentes P, Zhou F, Erban A, Karcher D, Kopka J, Bock R (2016) A new synthetic biology approach allows transfer of an entire metabolic pathway from a medicinal plant to a biomass crop. Elife 5:e13664
Gottschamel J, Waheed MT, Clarke JL, Lössl AG (2013) A novel chloroplast transformation vector compatible with the Gateway(®) recombination cloning technology. Transgenic Res 22:1273–1278
Gorantala J, Grover S, Goel D, Rahi A, Jayadev Magani SK, Chandra S, Bhatnagar R (2011) A plant based protective antigen [PA(dIV)] vaccine expressed in chloroplasts demonstrates protective immunity in mice against anthrax. Vaccine 29:4521–4533
Gray BN, Yang H, Ahner BA, Hanson MR (2011) An efficient downstream box fusion allows high-level accumulation of active bacterial beta-glucosidase in tobacco chloroplasts. Plant Mol Biol 76:345–355
Gregory JA, Li F, Tomosada LM, Cox CJ, Topol AB, Vinetz JM, Mayfield S (2012) Algae-produced Pfs25 elicits antibodies that inhibit malaria transmission. PLoS One 7:e37179
Guan Y, Ramalingam S, Nagegowda D, Taylor PW, Chye ML (2008) *Brassica juncea* chitinase BjCHI1 inhibits growth of fungal phytopathogens and agglutinates gram-negative bacteria. J Exp Bot 59:3475–3484
Hagemann R (2004) The sexual inheritance of plant organelles. In: Daniell H, Chase CD (eds) Molecular biology and biotechnology of plant organelles. Kluwer Academic Publishers, Dordrecht, pp 93–113
Hennig A, Bonfig K, Roitsch T, Warzecha H (2007) Expression of the recombinant bacterial outer surface protein A in tobacco chloroplasts leads to thylakoid localization and loss of photosynthesis. FEBS J 274:5749–5758
Inka Borchers AM, Gonzalez-Rabade N, Gray JC (2012) Increased accumulation and stability of rotavirus VP6 protein in tobacco chloroplasts following changes to the 5′ untranslated region and the 5′ end of the coding region. Plant Biotechnol J 10:422–434
Jin S, Daniell H (2015) The engineered chloroplast genome just got smarter. Trends Plant Sci 20:622–640

Jin S, Zhang X, Daniell H (2012) *Pinellia ternata* agglutinin expression in chloroplasts confers broad spectrum resistance against aphid, whitefly, Lepidopteran insects, bacterial and viral pathogens. Plant Biotechnol J 10:313–327

Kahlau S, Bock R (2008) Plastid transcriptomics and translatomics of tomato fruit development and chloroplast-to-chromoplast differentiation: chromoplast gene expression largely serves the production of a single protein. Plant Cell 20:856–874

Kanagaraj AP, Verma D, Daniell H (2011) Expression of dengue-3 premembrane and envelope polyprotein in lettuce chloroplasts. Plant Mol Biol 76:323–333

Karg SR, Kallio PT (2009) The production of biopharmaceuticals in plant systems. Biotechnol Adv 27:879–894

Kuchuk N, Sytnyk K, Vasylenko M, Shakhovsky A, Komarnytsky I, Kushnir S, Gleba Y (2006) Genetic transformation of plastids of different Solanaceae species using tobacco cells as organelle hosts. Theor Appl Genet 113:519–527

Kumar S, Daniell H (2004) Engineering the chloropalst genome for hyperexpression of human therapeutic proteins and vaccines antigens. Methods Mol Biol 267:365–383

Lau OS, Sun SS (2009) Plant seeds as bioreactors for recombinant protein production. Biotechnol Adv 27:1015–1022

Lee SB, Kwon HB, Kwon SJ, Park SC, Jeong MJ, Han SE, Daniell H (2003) Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. Mol Breed 11:1–13

Lee SM, Kang K, Chung H, Yoo SH, XM X, Lee SB, Cheong JJ, Daniell H, Kim M (2006) Plastid transformation in the monocotyledonous cereal crop, rice (*Oryza sativa*) and transmission of transgenes to their progeny. Mol Cells 21:401–410

Lim S, Ashida H, Watanabe R, Inai K, Kim YS, Mukougawa K, Fukuda H, Tomizawa K, Ushiyama K, Asao H, Tamoi M, Masutani H, Shigeoka S, Yodoi J, Yokota A (2011) Production of biologically active human thioredoxin 1 protein in lettuce chloroplasts. Plant Mol Biol 76:335–344

Lössl A, Bohmert K, Harloff H, Eibl C, Muhlbauer S, Koop HU (2005) Inducible trans-activation of plastid transgenes: expression of the R eutropha phb operon in transplastomic tobacco. Plant Cell Physiol 46:1462–1471

Lu Y, Rijzaani H, Karcher D, Ruf S, Bock R (2013) Efficient metabolic pathway engineering in transgenic tobacco and tomato plastids with synthetic multigene operons. Proc Natl Acad Sci U S A 110:E623–E632

Maldaner FR, Aragão FJ, dos Santos FB, Franco OL, da Rocha Queiroz Lima M, de Oliveira RR, Vasques RM, Nagata T (2013) Dengue virus tetra-epitope peptide expressed in lettuce chloroplasts for potential use in dengue diagnosis. Appl Microbiol Biotechnol 97:5721–5729

Maliga P, Bock R (2011) Plastid biotechnology: food, fuel, and medicine for the 21st century. Plant Physiol 155:1501–1510

McCabe MS, Klaas M, Gonzalez-Rabade N, Poage M, Badillo-Corona JA, Zhou F, Karcher D, Bock R, Gray JC, Dix PJ (2008) Plastid transformation of high-biomass tobacco variety Maryland Mammoth for production of human immunodeficiency virus type 1 (HIV-1) p24 antigen. Plant Biotechnol J 6:914–929

Morgenfeld M, Segretin ME, Wirth S, Lentz E, Zelada A, Mentarry A (2009) Potato virus X coat protein fusion to human papillomavirus 16 E7 oncoprotein enhance antigen stability and accumulation in tobacco chloroplast. Mol Biotechnol 43:243–249

Muhlbauer SK, Koop HU (2005) External control of transgene expression in tobacco plastids using the bacterial lacI repressor. Plant J 43:941–946

Mustafa G (2011) Development of plastid transformation in sugarcane, PhD Dissertation, Quaid-I-Azam University, Islamabad, Pakistan

Nadai M, Bally J, Vitel M, Job C, Tissot G, Botterman J, Dubald M (2009) High-level expression of active human alpha1-antitrypsin in transgenic tobacco chloroplasts. Transgenic Res 18:173–183

O'Neill BM, Mikkelson KL, Gutierrez NM, Cunningham JL, Wolff KL, Szyjka SJ, Yohn CB, Redding KE, Mendez MJ (2012) An exogenous chloroplast genome for complex sequence manipulation in algae. Nucleic Acids Res 40:2782–2792

Oey M, Lohse M, Kreikemeyer B, Bock R (2009) Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. Plant J 57:436–445

Ortigosa SM, Fernández-San Millán A, Veramendi J (2010) Stable production of peptide antigens in transgenic tobacco chloroplasts by fusion to the p53 tetramerisation domain. Transgenic Res 19:703–709

Ovcharenko O, Momot V, Cherep N, Sheludko Y, Komarnitsky I, Rudas V, Kuchuk N (2011) Transfer of transformed *Lesquerella fendleri* (Gray) Wats chloroplasts into *Orychophragmus violaceus* (L) OE Schulz by protoplast fusion. Plant Cell Tissue Org Cult 105:21–27

Pasoreck EK, Su J, Silverman IM, Gosai SJ, Gregory BD, Yuan JS, Daniell H (2016) Terpene metabolic engineering via nuclear or chloroplast genomes profoundly and globally impacts off-target pathways through metabolite signaling. Plant Biotechnol J 14:1862–1875

Petersen K, Bock R (2011) High-level expression of a suite of thermostable cell wall-degrading enzymes from the chloroplast genome. Plant Mol Biol 76:311–321

Rader RA (2008) Expression system for process and product improvement. BioProcess Int 6:4–9

Rigano MM, Manna C, Giulini A, Pedrazzini E, Capobianchi M, Castilletti C, Di Caro A, Ippolito G, Beggio P, De Giuli Morghen C, Monti L, Vitale A, Cardi T (2009) Transgenic chloroplasts are efficient sites for high-yield production of the vaccinia virus envelope protein A27L in plant cells dagger. Plant Biotechnol J 7:577–591

Rosales-Mendoza S, Alpuche-Solís AG, Soria-Guerra RE, Moreno-Fierros L, Martínez-González L, Herrera-Díaz A, Korban SS (2009) Expression of an *Escherichia coli* antigenic fusion protein comprising the heat labile toxin B subunit and the heat stable toxin, and its assembly as a functional oligomer in transplastomic tobacco plants. Plant J 57:45–54

Ruf S, Hermann M, Berger IJ, Carrer H, Bock R (2001) Stable genetic transformation of tomato plastids: foreign protein expression in fruit. Nat Biotechnol 19:870–875

Ruf S, Karcher D, Bock R (2007) Determining the transgene containment level provided by chloroplast transformation. Proc Natl Acad Sci U S A 104:6998–7002

Ruhlman T, Verma D, Samson N, Daniell H (2010) The role of heterologous chloroplast sequence elements in transgene integration and expression. Plant Physiol 152:2088–2104

Scharff LB, Bock R (2014) Synthetic biology in plastids. Plant J 78:783–798

Scotti N, Alagna F, Ferraiolo E, Formisano G, Sannino L, Buonaguro L, De Stradis A, Vitale A, Monti L, Grillo S, Buonaguro FM, Cardi T (2009) High-level expression of the HIV-1 Pr55gag polyprotein in transgenic tobacco chloroplasts. Planta 229:1109–1122

Scotti N, Rigano MM, Cardi T (2012) Production of foreign proteins using plastid transformation. Biotechnol Adv 30:387–397

Sigeno A, Hayashi S, Terachi T, Yamagishi H (2009) Introduction of transformed chloroplasts from tobacco into petunia by asymmetric cell fusion. Plant Cell Rep 28:1633–1640

Singh RK, Kumar P, Tiwari NN, Rastogi J, Singh SP (2013) Current status of sugarcane transgenic: an overview. Adv Genet Eng 2:112

Stegemann S, Bock R (2009) Exchange of genetic material between cells in plant tissue grafts. Science 324:649–651

Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene. Proc Natl Acad Sci U S A 90:913–917

Svab Z, Hajdukiewicz P, Maliga P (1990) Stable transformation of plastids in higher plants. Proc Natl Acad Sci U S A 87:8526–8530

Tissot G, Canard H, Nadai M, Martone A, Botterman J, Dubald M (2008) Translocation of aprotinin, a therapeutic protease inhibitor, into the thylakoid lumen of genetically engineered tobacco chloroplasts. Plant Biotechnol J 6:309–320

Tsuruta S-i, Ebina M, Kobayashi M, Takahashi W (2017) Complete chloroplast genomes of *Erianthus arundinaceus* and *Miscanthus sinensis*: comparative genomics and evolution of the Saccharum Complex. PLoS One 12(1):e0169992

Tungsuchat-Huang T, Maliga P (2012) Visual marker and *Agrobacterium*-delivered recombinase enable the manipulation of the plastid genome in greenhouse-grown tobacco plants. Plant J 70:717–725

Vafaee Y, Staniek A, Mancheno-Solano M, Warzecha H (2014) A modular cloning toolbox for the generation of chloroplast transformation vectors. PLoS One 9:e110222
Valkov VT, Scotti N, Kahlau S, Maclean D, Grillo S, Gray JC, Bock R, Cardi T (2009) Genome-wide analysis of plastid gene expression in potato leaf chloroplasts and tuber amyloplasts: transcriptional and posttranscriptional control. Plant Physiol 150:2030–2044
Valkov VT, Gargano D, Manna C, Formisano G, Dix PJ, Gray JC, Scotti N, Cardi T (2011) High efficiency plastid transformation in potato and regulation of transgene expression in leaves and tubers by alternative 5′ and 3′ regulatory sequences. Transgenic Res 20:137–151
Verhounig A, Karcher D, Bock R (2010) Inducible gene expression from the plastid genome by a synthetic riboswitch. Proc Natl Acad Sci U S A 107:6204–6209
Verma D, Samson NP, Koya V, Daniell H (2008) A protocol for expression of foreign genes in chloroplasts. Nat Protoc 3:739–758
Verma D, Moghimi B, LoDuca PA, Singh HD, Hoffman BE, Herzog RW, Daniell H (2010a) Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. Proc Natl Acad Sci U S A 107:7101–7106
Verma D, Kanagaraj A, Jin S, Singh ND, Kolattukudy PE, Daniell H (2010b) Chloroplast-derived enzyme cocktails hydrolyse lignocellulosic biomass and release fermentable sugars. Plant Biotechnol J 8:332–350
Verma D, Jin S, Kanagaraj A, Singh ND, Daniel J, Kolattukudy PE, Miller M, Daniell H (2013) Expression of fungal cutinase and swollenin in tobacco chloroplasts reveals novel enzyme functions and/or substrates. PLoS One 8:e57187
Waheed MT, Ismail H, Gottschamel J, Mirza B, Lössl AG (2015) Plastids: the green frontiers for vaccine production. Front Plant Sci 6:1005. doi:10.3389/fpls201501005
Yabuta Y, Tanaka H, Yoshimura S, Suzuki A, Tamoi M, Maruta T, Shigeoka S (2013) Improvement of vitamin E quality and quantity in tobacco and lettuce by chloroplast genetic engineering. Transgenic Res 22:391–402
Yao J, Weng Y, Dickey A, Wang KY (2015) Plants as factories for human pharmaceuticals: applications and challenges. Int J Mol Sci 16:28549–22865
Zhang J, Tan W, Yang XH, Zhang HX (2008) Plastid-expressed choline monooxygenase gene improves salt and drought tolerance through accumulation of glycine betaine in tobacco. Plant Cell Rep 27:1113–1124
Zhang J, Ruf S, Hasse C, Childs L, Scharff LB, Bock R (2012) Identification of cis-elements conferring high levels of gene expression in non-green plastids. Plant J 72:115–128
Zhou F, Badillo-Corona JA, Karcher D, Gonzalez-Rabade N, Piepenburg K, Borchers AM, Maloney AP, Kavanagh TA, Gray JC, Bock R (2008) High-level expression of human immunodeficiency virus antigens from the tobacco and tomato plastid genomes. Plant Biotechnol J 6:897–913
Zoubenko OV, Allison LA, Svab Z, Maliga P (1994) Efficient targeting of foreign genes into the tobacco plastid genome. Nucleic Acids Res 22:3819–3824

Chapter 11
CRISPR-Cas9 System as a Genome Editing Tool in Sugarcane

Sruthy Maria Augustine

Abstract The CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 (CRISPR-associated nuclease 9) system is a versatile tool for genetic engineering that uses cas9 to target the sequence-specific region and introduce a double-stranded break in the target area. This simple RNA-guided genome editing technology has become a revolutionary tool in biology and has many innovative applications in different fields. This technique helps to make precise genome modification in many different tissues and organisms. Development of genetically edited crops will assist sustainable productive agriculture for better feeding of the rapidly growing population in a changing climate. The emerging areas of research for the genome editing in plants include rewiring the regulatory signaling networks, and interrogating gene functions and sgRNA library for high-throughput loss-of-function screening. This chapter deals with the strengths and weaknesses of Cas9 nuclease-mediated genome editing in plants for development of designer crops like sugarcane. With this powerful and innovative technique, the genetically engineered non-GM plants will support the sustainable agriculture and maximize yield by combating abiotic and biotic stresses.

Keywords CRISPR-Cas9 • Designer crops • Genome editing • gRNA • Sugarcane • Targeted gene editing

11.1 Introduction

The targeted plant genome editing using sequence-specific nucleases opens a unique opportunity for crop improvement to meet the drastically increasing global food demands and to provide sustainable agriculture system (Liu et al. 2013). Usually, the crop improvement was done by conventional and mutation plant breeding techniques,

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C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6_11

which are now getting constrained by the decline of existing genetic variation of plants, hampering the production for future feeding (Chen and Gao 2014). There is an urgent need for efficient crop improvement strategies with novel genome editing techniques like CRISPR/Cas9 system, which can improve the current essential functions or make new valuable products (Zhang and Zhou 2014).

Genome editing is a type of genetic engineering with site-specific nucleases that allows genome engineering, reverse genetics, and targeted transgene integration experiments in an efficient and a precise manner. It involves the introduction of targeted DNA double-strand breaks (DSBs) using an engineered nuclease, stimulating cellular DNA repair mechanisms. Genome modification will occur depending on the repair pathway and the available template for repair. Two different types of repair pathways have been identified: nonhomologous end joining (NHEJ) and homologous recombination (HR). In most cases, NHEJ causes random insertions or deletions (indels) that result in a frameshift mutation if it occurs in the coding region of a gene results a gene knockout. In some cases, a template with homology regions to the sequence surrounding the double-stranded break is available; the DNA damage can be repaired by HR. This mechanism can be utilized to attain precise gene modifications or gene insertions. The most common double-stranded break repair mechanism is NHEJ in most of the organisms (Puchta 2005). The potential outcome of genome editing is given in Fig. 11.1.

Sugarcane (*Saccharum* spp. hybrid), a plant of the Poaceae family, is an important commercial cash crop and is the largest source of the world's sugar. Brazil leads the world in sugarcane production; India is the second largest producer and China the third largest producer (FAOSTAT 2015). Brazil uses sugarcane to produce sugar and ethanol for gasoline-ethanol blends (gasohol), a locally popular transportation fuel. In India, sugarcane is used to produce sugar, jaggery, and alcoholic beverages. Modern sugarcane cultivars are obtained from interspecific hybridization between *Saccharum officinarum* and *Saccharum spontaneum*, a wild relative of sugarcane, and result in a highly polyploid or aneuploid hybrid variety (Moore and Paterson 2013). From all over the world, there are many well-established protocols for sugarcane transformation. In Indonesia, the drought-tolerant transgenic sugarcane is approved for commercial cultivation (Parisi et al. 2016) and in India (Augustine et al. 2015a, b, c), and some other countries, it is under pipeline. The biggest challenges for the sugarcane transformation are transgene inactivation, low transformation efficiency, and time constraints (Hansom et al. 1999; Joyce et al. 2010).

The CRISPR-Cas (clustered regularly interspaced short palindromic repeat/Cas) system was initially identified in bacteria as an adaptive immune system that helps the bacteria in protecting itself against invading foreign DNA. This system consists of a CRISPR locus in the genome and a cas9 protein (Kim and Kim 2014). The CRISPR loci contain sequences for noncoding RNA elements called CRISPR RNA (crRNA) and sequences for small trans-encoded CRISPR RNA, i.e., trans-activating crRNA (tracrRNA). The two RNA sequences crRNA and tracrRNA form a complex and together are called as guide RNA (gRNA), and this determines the specificity of the cleavage of the target sequence in the DNA along with the protospacer adjacent motif (PAM), a 5′-NGG sequence (Barrangou 2013; Jinek et al. 2013). The double-stranded target DNA cleavage occurs at 3 bp upstream of the protospacer region. The Cas9 protein is an RNA-guided DNA endonuclease associated with CRISPR type II adaptive immune system, which will introduce the double-stranded breaks (DSBs) at the site

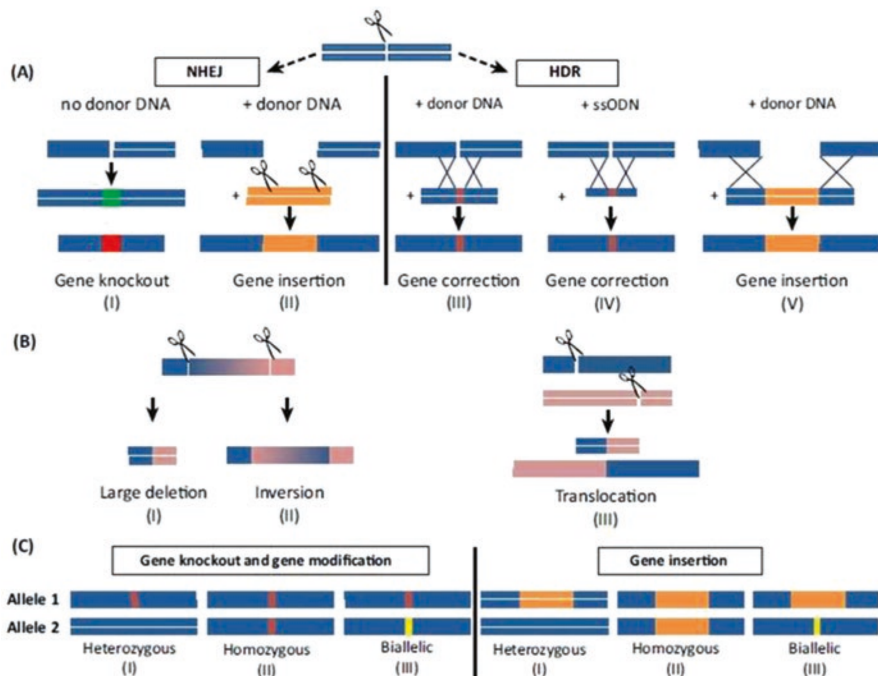

Fig. 11.1 The Potential outcomes of genome editing. Genome editing with designer nucleases can have different outcomes depending on the double-strand break (DSB) repair pathway and the nature of any donor DNA. (**a**) The major outcomes depending on the DSB repair pathway. In nonhomologous end joining (NHEJ) repair mechanism, in the absence of donor DNA (*I*), tends to generate short indels shown as insertions (*green*) or deletions (*red*) with all four types of nuclease. Both events tend to cause gene knockout. (*II*) If donor DNA is available, which is flanked by the same target sites present in the genomic locus, then the designer nuclease will generate compatible ends and this can result in the integration of the new sequence, often accompanied by small indels at the junctions. Homology-dependent repair (HDR) occurs if a donor DNA template is available carrying the desired mutation. Donor DNA carrying a subtle change such as a nucleotide substitution can be provided as either a duplex molecule (*III*) or a single-stranded oligo deoxyribonucleotide (ssODN) (*IV*) and both will lead to allele correction. Alternatively, the homology region may be used to flank a new sequence (*V*), and this will lead to gene insertion. (**b**) The possible outcomes when two DSBs are induced and repaired by NHEJ. If the DSBs are generated on a single chromosome the region between the two breaks can be deleted (*I*) or inverted (*II*). If the DSBs are induced on two different chromosomes, a chromosomal translocation can occur (*III*). (**c**) Shows the potential consequences in diploid plants—the results of gene editing can be (*I*) heterozygous (single allelic change), (*II*) homozygous (identical changes to both alleles), or (*III*) biallelic (different changes at each allele) depending on which repair pathway is in operation (adapted from Zhu et al. 2017)

when targeted by a guide RNA (Cong et al. 2013; Mali et al. 2013). In 2012, the molecular mechanism of the CRISPR has uncovered a vast area of applications as a potential component of genome editing termed as RNA-guided engineered nucleases (RGENs) that can be used as a sequence-specific nuclease for genome modifications. RGENs are composed of two major components: the cas9 nuclease and a single guide RNA (sgRNA). The sgRNA has a 20 bp sequence at the 5′ end that directs the cas9 to the

target site. Any DNA sequence in the form of N20-NGG can be targeted by changing the 20 bp sequence of the gRNA for genome editing applications (Sander and Joung 2014). Targeted genome editing is utilized for the creation of endogenous genetic modification like gene deletion or insertion by the introduction of nuclease-mediated DNA break using engineered nucleases. After the introduction of the double-stranded break in the target region, the recombination repair mechanism will do the modification. The discovery of CRISPR-cas9 leads to the elucidation of many fundamental processes in biology (Doudna and Charpentier 2014). Nowadays, many cas9 and gRNA variants are available that can be further used for the crop improvement strategies in plant biotechnology.

11.2 The cas9 Nuclease Variants

The cas9 endonuclease contains two different domains: a large globular recognition (REC)-specific functional domain and a smaller nuclease (NUC) domain. The NUC domain consists of two nuclease sites, i.e., RuvC and HNH, along with a PAM interacting site (Doudna and Charpentier 2014; Jinek et al. 2014). Recently, the cas9 nuclease mechanism was revealed by crystal structure studies. This gives an understanding about how cas9 can be engineered to generate variants with unique PAM specificities (Belhaj et al. 2013). Nowadays, there are many cas9 variants available; some of them are described here.

11.2.1 The Native cas9

The occurrence of the double-stranded break can be repaired by either NHEJ or HR repair mechanism (Wyman and Kanaar 2006; Shuman and Glickman 2007). HR repair mechanism can introduce specific point mutations like nucleotide substitution or insert a sequence from an exogenously supplied DNA template to the target locus through recombination (Jiang et al. 2013). NHEJ repair mechanism helps in the efficient introduction of insertion or deletion of nucleotides that can create a frameshift mutation in the genome (Cong et al. 2013). Until now the native cas9/sgRNA application has been demonstrated in many plant species including crop plants for gene knockouts or insertions as well as multiplex genome editing.

11.2.2 The cas9 Nickase

Cong et al. (2013) developed the cas9 nickase by introducing a mutation in native cas9 (D10A, aspartate-to-alanine substitution). The RuvC or HNH site with a cas9 nickase can make a nick in the genome instead of the double-stranded break. The nicks in the genome will be further repaired through homology-directed repair (HDR) mechanism. The cas9n has also been used in paired nickase system along with two different gRNA to increase the number of specifically identified nucleotides for target cleavage that will increase the specificity and reduce the off-target effects (Cong et al. 2013; Mali et al. 2013; Ran et al. 2013; Fauser et al. 2014) (Fig. 11.2a).

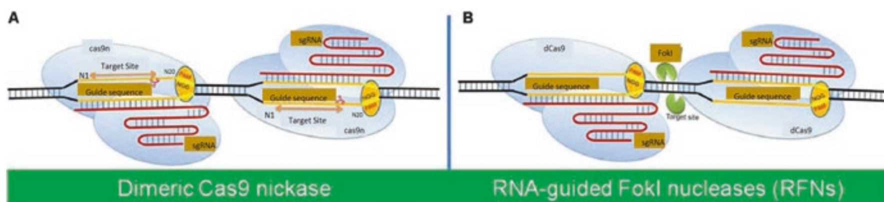

Fig. 11.2 The different variants of Cas9 system. (**a**) The Cas9 nickase (Cas9n) with a RuvC or HNH mutation creates a nick instead of a DSB at the target site. The dimeric Cas9n can be used for enhancing specificity and reducing off-target effects. (**b**) Dimeric RNA-guided FokI nucleases (RFNs) are the fusion of a catalytically inactive dCas9 protein with the FokI nuclease domain. Dimerization of two RFNs used for high genome editing frequencies and reduced off-target mutations (Khatodia et al. 2016)

11.2.3 The Inactive dCas9

The catalytically inactive nuclease-deficient cas9 (dCas9) has also been used for RNA-guided transcription instead of genome editing. The dCas9 can knock down the gene expression by interfering in the transcription. It can sometimes be fused with additional repressor peptides. This system has been used for many highly efficient and precise genome editing techniques like CRISPR interference (CRISPRi) and CRISPR activator (CRISPRa). The inactive cas9 can incorporate the gRNA and bind the target genome region (Xu et al. 2014). In CRISPRi the sgRNA and dCas9 provide a minimum system for gene-specific regulation in any organism (Qi et al. 2013).

11.2.4 Dimeric RNA-Guided FokI Nucleases (RFNs)

Dimeric RNA-guided FokI nucleases (RFNs) are a fusion of catalytically inactive nuclease-deficient dCas9 protein along with a FokI nuclease domain. For the efficient genome editing process, dimerization of two RFNs rather than co-localization is required. This as an advantage of RFNs compared to cas9 nickase for high genome editing frequencies and reduced off-target effects (Tsai et al. 2014; Bortesi and Fischer 2015). The cleavage strictly depends on the gRNAs with an accurate spacing and orientation that will reduce the probability of target site presence more than once in the genome (Tsai et al. 2014) (Fig. 11.2b).

11.3 Guide RNA Variants

Until now there are many improvements in the guide RNA of the cas9/sgRNA system that will provide an improvement in the native system for better application of the CRISPR-cas9 technology. Some of the gRNA variants are described here:

11.3.1 Truncated Guide RNAs (truRNA)

The sgRNA variants with shorter regions of the target sequence that is 17 nucleotides are known as truncated guide RNAs (truRNA). This offers a simple and efficient strategy to improve the cas9 nuclease specificities or paired nickases by reducing the off-target effects (Fu et al. 2014; Bortesi and Fischer 2015). The truncated RNAs can reduce some unwanted mutagenesis at some of the target sites without reducing the on-target efficiencies (Fu et al. 2014).

11.3.2 Ribozyme-gRNA-Ribozyme (RGR)

An artificial gene that can generate RNA molecule with ribozyme sequences is known as the ribozyme-gRNA-ribozyme (RGR). This can undergo a self-catalyzed cleavage to create the appropriate gRNA both in vitro and in vivo (Gao and Zhao 2014b). An additional advantage is that it can transcribe from any promoter and that allows tissue-specific genome modification and efficient detection of mutation (Fig. 11.3a).

11.3.3 Polycistronic tRNA-gRNA (PTG/Cas9)

An array of tandem tRNA-gRNA units and each gRNA accompanied with a target particular spacer for simultaneously targeting multiple sites is known as the polycistronic tRNA-gRNA (PTG/Cas9) (Xie et al. 2015). Through an endogenous tRNA processing system, the primary transcript of PTG is cleaved after accurate processing by the RNase P and RNase Z. This process releases numerous amounts of mature gRNAs in vivo from a synthetic polycistronic gene. The excised mature gRNAs regulate Cas9 to target the multiple sites that will significantly increase multiple genome editing in plants. Targeting one gene with two gRNAs with the help of PTG enhances the efficiency of the gene knockout in comparison to the single gRNA (Xie et al. 2015) (Fig. 11.3b).

11.4 The Crispr-cas9 System for Plant Genome Editing

At present, there are three classes of applications of the RNA-guided endonuclease, particularly in plants. In the first category, a double-stranded break generated by the Cas9 was repaired by nonhomologous end joining (NHEJ) repair method for the generation of indels that will be able to generate a frameshift mutation in the genome (Chen and Gao 2014; Saika et al. 2014). In the second category, a transgene or a small DNA repair template is introduced along with the Cas9 to repair the double-stranded break through homologous recombination (HR). This can be used for the creation of point mutation or transgene insertion, gene replacement, and gene stacking at target genome regions. The third category is the multiplex genome editing for targeting different target sites in the genome with multiple gRNAs along with the

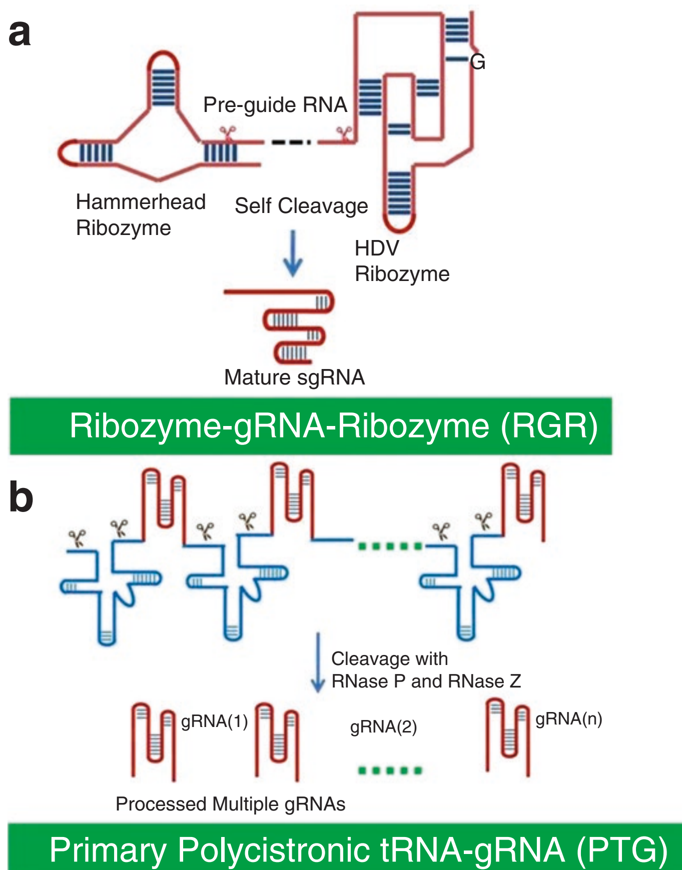

Fig. 11.3 The different variants of Cas9/sgRNA system of genome editing. (**a**) Ribozyme-gRNA-ribozyme (RGR) is an artificial gene, which generates self-catalyzed desired gRNA after transcribed from any promoter for tissue-specific genome editing. (**b**) Primary polycistronic tRNA-gRNA (PTG) is tandemly arrayed tRNA-gRNA units, which is cleaved by the endogenous tRNA-processing system for simultaneously targeting multiple sites (Khatodia et al. 2016)

Cas9 nucleases. Multiplex genome editing can be used for analyzing the function of gene family members with unnecessary functions and for analyzing the epistatic relationship in genetic pathways (Xing et al. 2014). The strategy of using plant genome editing using CRISPR-Cas9 is summarized in Fig. 11.4. Here is a brief description of the achievements of CRISPR-cas9-mediated genome editing in the plant genome.

Five reports were published in August 2013 discussing the first application of the CRISPR-Cas9 system in plant genome editing (Feng et al. 2013; Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013; Xie and Yang 2013). The first group demonstrated the system in the model species *Arabidopsis thaliana* and *Nicotiana benthamiana* as well as crop plants such as rice. Later the work was focused on monocots such as Sorghum (Jiang et al. 2013), wheat (Upadhyay et al. 2013; Wang et al. 2014), and maize (liang et al. 2014). These studies draw a clear picture about

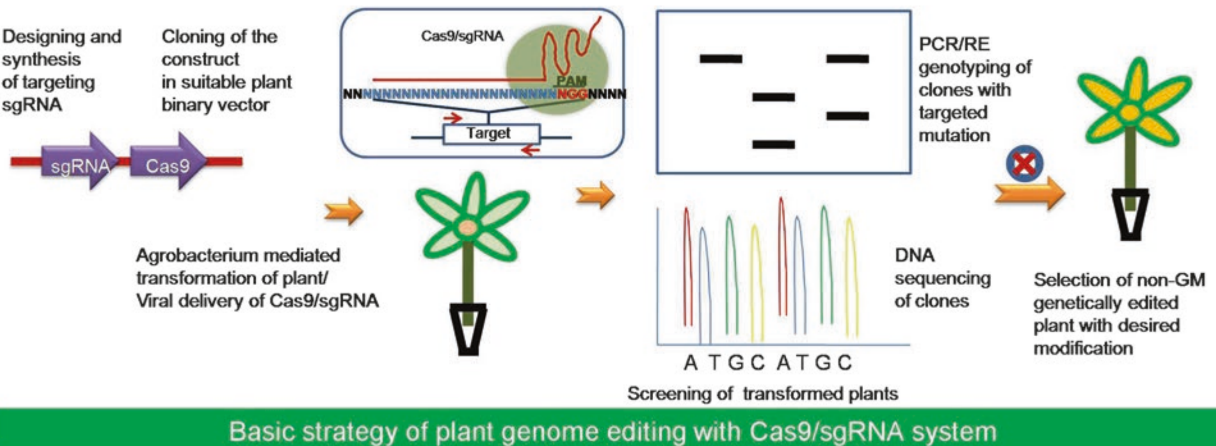

Fig. 11.4 Strategy of plant genome editing using Cas9/sgRNA system. Starting from the selection of the target gene, the available online resources have been utilized for designing and synthesis of sgRNA. The target sgRNA along with the suitable Cas9 variant has been cloned into a plant binary vector for transformation of the target plant species with *Agrobacterium* generally. After transformation the putative transformed plants would be selected for the presence of the Cas9 and sgRNA. Then screening of the plants with the desired mutation or editing would be done using PCR/RE genotyping and DNA sequencing (Khatodia et al. 2016)

the cleavage specificity, ability to create large deletions, mutation efficiency, and gRNAs that can express under the control of different promoters (Fauser et al. 2014; Feng et al. 2014; Gao et al. 2014; Jiang et al. 2013; Mao et al. 2013; Miao et al. 2013; Sugano et al. 2014; Upadhyay et al. 2013; Zhang et al. 2014; Zhou et al. 2014). Previous studies reported that single chimeric sgRNA is more efficient than separate crRNA and tracrRNA components in plants (Miao et al. 2013; Zhou et al. 2014). A 1.8 kb resistance cassette was introduced into the ADH1 locus *A. thaliana* through homologous recombination and was described in Schiml et al. (2014). Ron et al. (2014) showed that CRISPR/Cas9 system in tomato hairy roots through transformation using *Agrobacterium rhizogenes*. Sweet orange was the first fruit crop where genome editing was applied (Jia and Wang 2014).

Previous studies have shown that CRISPR-Cas9 system can generate homozygous mutations directly in the first generation of tomato and rice plants and showed high frequency of efficiency in these crops (Brooks et al. 2014; Shan et al. 2013; Zhang et al. 2014; Zhou et al. 2014). Studies also showed that mutations induced by the CRISPR-Cas9 are present in germ line and were able to segregate in the next generations without any more further modifications (Brooks et al. 2014; Fauser et al. 2014; Feng et al. 2014; Jia and Wang 2014; Schiml et al. 2014; Zhang et al. 2014; Zhou et al. 2014). A list of applications of CRISPR-Cas9 in plant genome editing through NHEJ and HR is reviewed in Bortesi and Fischer (2015).

CRISPR-cas9 system can create heritable and stable mutation in the genome, and can easily segregate from the Cas9/sgRNA construct to avoid any further mutations. This will allow for the development of homozygous transgene-free plants (Brooks et al. 2014; Fauser et al. 2014; Feng et al. 2014; Gao and Zhao 2014a; Jiang et al. 2014; Schiml et al. 2014; Zhang et al. 2014; Zhou et al. 2014). A transgene-free rice plant was successfully developed with desired gene mutation by segregating out the transgene with self-fertilization in the T1 generation (Xu et al. 2015). The CRISPR-cas9 cleavage efficiency at the target site is found higher in comparison with the TALENs and ZFNs (Gaj et al. 2013; Johnson et al. 2015). A toolkit was developed by Xing et al. (2014) for multiplex genome editing using CRISPR-cas9-based binary vector set and a gRNA vector set in plants. This toolkit will help to generate transient or stable transformants in a variety of plants, especially useful for multiplex plant genome editing (Xing et al. 2014). For an efficient genome editing technique, the only requirement is the successful delivery of the cas9 and gRNA into the host cell.

Gemini virus replicons (GVRs) can also be used for the transfer of Cas9/sgRNA into the plant system (Baltes et al. 2014). For further exploitation of CRISPR-cas9 technology virus-based delivery methods can be used in all plant parts with higher repair efficiencies with desired modifications along with no transformations (Baltes et al. 2014; Ali et al. 2015). As an example of the direct delivery using virus, two recent reports are available: tobacco rattle virus (TRV) (Ali et al. 2015) and cabbage leaf curl virus (CaLCvV) (Yin et al. 2015). They have demonstrated the possibility of different virus-mediated Cas9/sgRNA delivery for efficient plant genome editing.

11.5 Advantages of the CRISPR-Cas9 System

CRISPR-cas9 has become very popular in a very short period because of its simplicity, accessibility, cost, and versatility. This technique does not need any protein engineering steps which makes it a more convenient and straightforward method for testing multiple gRNAs in a target region. The target specificity can also be changed by changing the 20 base pair sequence in the gRNA. Some gRNAs can be produced by in vitro transcription by using two complementary annealed oligonucleotides (Cho et al. 2013). All these allow an inexpensive assembly of large gRNA libraries and the system is available for high-throughput functional genomics in a cost-effective way.

CRISPR-cas9 system is also able to cleave methylated DNA in human cells that are beyond the reach of other nucleases like ZFNs and TALENs (Hsu et al. 2013). Until now this aspect is not explored in the plant genome. According to Vanyushin and Ashapkin (2011) approximately 70% of CpG/CpNpG sites are methylated in plants. Therefore the CRISPR-Cas9 technology is more versatile for plant genome editing especially with the monocots that have high GC content in the genome, e.g., rice (Miao et al. 2013).

The major advantage of CRISPR-Cas9 technology over TALENs and ZFNs is ease of multiplexing. CRISPR-Cas9 system can introduce double-stranded break simultaneously at multiple sites and therefore can be able to edit different genes at the same time (Li et al. 2013; Mao et al. 2013). This strategy can be used to knock out redundant genes or parallel pathways and can also be used to introduce large genomic deletions or inversions by targeting two target sites on the same chromosome (Li et al. 2013; Upadhyay et al. 2013; Zhou et al. 2014). In CRISPR-Cas9, multiplexing requires only cas9 protein and multiple gRNAs, but ZFNs and TALENs required separate dimeric proteins specific for each protein.

There are many online resources available for selecting the gRNA sequences and predicting the specificity (Table 11.1). Also, another community is available for providing access to plasmids, e.g., Addgene.

11.6 The Specificity of CRISPR-Cas9

Previous studies reported a high frequency of off-target effects as a disadvantage of CRISPR-Cas9 technology (Cong et al. 2013; Fu et al. 2013; Hsu et al. 2013; Jiang et al. 2013; Mali et al. 2013; Pattanayak et al. 2013). Initially it was considered that a 20 bp nucleotide sequence in the gRNA is necessary to determine the specificity of the CRISPR-Cas9 technology, but later it was shown that an 8–12 bp sequence at the 3′ end (the seed sequence) is only needed for recognizing the target and for the cleavage (Cong et al. 2013; Jiang et al. 2013; Jinek et al. 2012). If there are multiple mismatches in the distal regions of the PAM site, that can be tolerated depending upon the total number of mismatches and arrangement (Fu et al. 2013; Hsu et al. 2013; Pattanayak et al. 2013). The off-target cleavage can also be induced if the DNA

Table 11.1 Available online resources for CRISPR-Cas9 system (Khatodia et al. 2016)

Name	Remarks	References
Addgene	Reagents and resources	https://www.addgene.org/crispr/
sgRNA Designer	Guide RNA design tool	http://broadinstitute.org/rnai/public/analysis-tools/sgrna-design
Cas9 Design	Guide RNA design tool	http://cas9.cbi.pku.edu.cn
CHOPCHOP	Target sites finding tool	https://chopchop.rc.fas.harvard.edu
CRISPR Design	Design and analysis of guide RNA	http://crispr.mit.edu
CRISPR Genome Analyzer	Genome editing experiment analysis platform	http://crispr-ga.net
CRISPR-PLANT	Genome-wide gRNA prediction tool in plants	http://genome.arizona.edu/crispr
CRISPRseek	Target-specific guide RNA design tool	http://bioconductor.org/packages/release/bioc/html/CRISPRseek.html
DNA 2.0 gRNA Design Tool	gRNA design tool	https://dna20.com/eCommerce/cas9/input
E-CRISP	Target site design tool	http://e-crisp-test.dkfz.de/E-CRISP
RGEN Tools	Potential off-target site prediction tool	http://rgenome.net/cas-offinder
sgRNAcas9	sgRNA design and potential off-target sites prediction tool	http://biootools.com
CRISPR MultiTargeter	Multiplex design tool	http://multicrispr.net/
CRISPR-P	Guide RNA design in plants	http://cbi.hzau.edu.cn/crispr/
AGEseq	Analysis of genome editing by sequencing	https://github.com/liangjiaoxue/AGEseq
Stupar Lab's CRISPR Design	Target site identifier	http://stuparcrispr.cfans.umn.edu/CRISPR/

sequence has an extra base (DNA bulge) or a missing base (gRNA bulge) is present at various locations along the corresponding gRNA sequence (Lin et al. 2014).

Nowadays there are several strategies adopted to reduce the off-target effects, and the most important one is the design of the gRNA. In the case of the ZFNs and TALENs, the target specificity is determined by the protein–DNA interaction, and that is unpredictable. But in the case of CRISPR-Cas9 technology, it follows a Watson–Crick base pairing, and that allows predicting the off-target effects more reliably by sequence analysis (Cho et al. 2014). Also, the gRNAs can be tested for off-target effects rapidly and inexpensively. Another way to control the specificity is through optimizing the nuclease expression because high concentrations of Cas9 and gRNA can increase the off-target effects (Fujii et al. 2013; Hsu et al. 2013; Pattanayak et al. 2013).

The use of the mutated Cas9, e.g., Cas9 nickase (Fig. 11.2a), can create single-strand nicks that will lead to a staggered double-stranded break. This strategy also

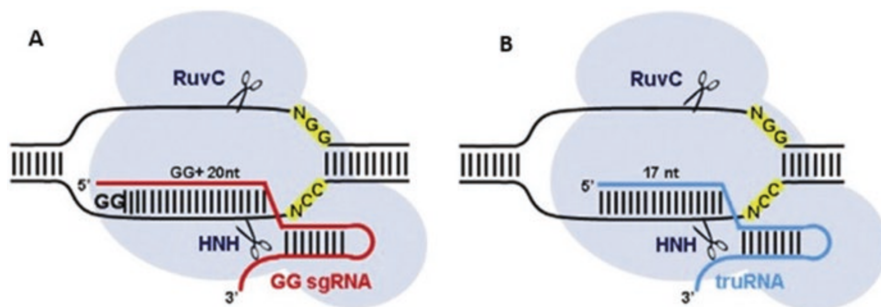

Fig. 11.5 Strategies for making truncated sgRNA. (**a**) Extending the gRNA by adding two guanidine residues at the 5′ end. (**b**) Shortening it to a truncated gRNA (truRNA) of 17 nt (Bortesi and Fischer 2015)

increases the number of base pairs that are individually recognized for the target cleavage and thereby reduces the occurrence of off-target cleavage. One limitation of this approach is that two equally efficient gRNAs are needed to generate an efficient nickase pair; not all the gRNAs are similar regarding activity (Cho et al. 2014). The disadvantage of this system is that each component of the paired nickase system remains active, and the nicks are repaired with high fidelity, but it is not possible to exclude the probability of forming additional off-target mutations. To overcome this problem, a fusion of catalytically inactive Cas9 and FokI nuclease is generated (Fig. 11.2b). This showed better efficiency like nickases and higher efficiency (>140-fold) compared to the wild-type enzymes (Guilinger et al. 2014; Tsai et al. 2014).

Another approach is by reducing the size of the gRNA or by adding two guanidine residues at the 5′ end of the gRNA were able to avoid the off-target effects compared to normal gRNAs (Fig. 11.5a, b). But the disadvantage is that it is less active on-target too (Cho et al. 2014). By combining the truncated gRNA and cas9 nickase in the system will potentially increase the specificity (Fu et al. 2014).

The off-target effects showed a different pattern in various cell types in the same species. For example based on the whole-genome sequencing data the human pluripotent cells showed less off-target effects compared to the cancer cell lines (Smith et al. 2014; Veres et al. 2014). Studies suggested that off-target effects are less in plants. Xie and Yang (2013) showed a 1.6% of off-target mutation rate in rice and it is five times lower than that of the on-target mutation. In case of the *Arabidopsis*, *N. benthamiana*, wheat, and sweet orange, no off-target mutation is reported (Feng et al. 2014; Jia and Wang 2014; Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013; Upadhyay et al. 2013; Zhou et al. 2014) even in whole-genome sequencing (Feng et al. 2014). Further work is needed to confirm the off-target effect in plants, and it is clear that the careful selection of gRNA should reduce the risk of off-target effects in the genome.

11.7 Advantage and Complexities in Sugarcane Genome Editing

Sugarcane is a highly polyploid crop with chromosomes ranging from 80 to 120 due to nobilization. Its genome size is about 10 Gb with homologous genes ranging from 8 to 12 copies (Souza et al. 2011) and the monoploid genome size is around 750–930 Mb (D'Hont and Glaszmann 2001). These complex genetics restrict conventional breeding in sugarcane. Nowadays, an array of protocols is available for developing genetically modified (GM) sugarcane. Facing challenges such as transgene inactivation, low transformation efficiency, and time restrictions prevent improvement of sugarcane through genetic engineering (Hansom et al. 1999; Joyce et al. 2010). Genetic engineering can be an insertion or a deletion or a replacement in the genome using engineered nucleases, and the genome editing is much easier with the CRISPR-Cas9. Recent studies reported that in sugarcane a targeted mutagenesis was introduced to modify the cell wall characteristics for increased production of lignocellulosic ethanol (Jung and Altpeter 2016).

CRISPR system offers the possibility to introduce double-stranded breaks at several sites in the genome; thereby it is possible to edit different genes simultaneously (Li et al. 2013; Mao et al. 2013). CRISPR approach also allows for stacking the genes and that have strong applications in molecular farming and metabolic engineering approaches. At present, the plants which are genome edited through CRISPR are not classified under the GM, and in future, there might be another different regulatory policy that will arise for the CRISPR-edited plants.

Recently, Mohan (2016) elaborated the major challenges that hinder genome editing in sugarcane. The primary requirement for genome editing is the genome sequence information for designing specific gRNAs to target specific genes. In case of sugarcane, the genome is not annotated yet. Therefore, it's hard to design gene-specific gRNAs. Another major problem in CRISPR technique is the off targets. Previous studies reported that CRISPR technology has fewer off-target effects in various plant species (Belhaj et al. 2013). In sugarcane, transgene silencing is another major drawback that hinders the crop development through genetic engineering. Both transcriptional and posttranscriptional transgene silencing effects are reported (Hansom et al. 1999).

The major step in the CRISPR technique is the analysis of the mutation, and in sugarcane, it is the biggest challenge due to the lack of high-throughput screening methods. CRISPR analysis tools include T7 assay/surveyor assay, PCR, restriction analysis followed by Sanger sequencing/NGS. However, using these methods for analyzing sugarcane genome mutation is the greatest challenge and needs to be further explored for finding a suitable analysis tool.

CRISPR-Cas9 technology : Genetically edited crops		
Types	Applications	Future possibilities
• Targeted gene Editing/replacement • Multiple gene editing • CRISPRi • Virus interference	• Gene transfer • Biotic/abiotic stress Tolerance • Transcriptional Modulation • Inducible Cas9 system	• Enhanced Photosynthesis • Receptor engineering • Root architecture • Haploid plants • Designer crops

Fig. 11.6 The types, applications, and future possibilities of CRISPR/Cas9 system for development of GE crops for crop improvement (adapted from Khatodia et al. 2016)

11.8 Conclusion and Outlook

For sustainable agriculture, crops can be generated through genome editing with enhanced pest resistance and nutritional value and that can withstand adverse environmental conditions, in near future. CRISPR-Cas9 technology can facilitate both forward and reverse genetics even in model species such as *Arabidopsis*. Genome editing technology can contribute significantly in developing new bioenergy crops. Moreover, it can be utilized to improve crops for better nutrition and food security. Here are some possibilities (Fig. 11.6) that can be utilized for crop improvement and plant biotechnology applications. Genome editing technology is now simplified by using direct delivery of cas9 and gRNA (Hiei et al. 2014; Khatodia and Khurana 2014; Nonaka and Ezura 2014, Liang et al. 2016; Mout et al. 2017). The generation of CRISPRi targeting the genes at a whole-genome level is also feasible for model plants (Heintze et al. 2013).

CRISPR technology can facilitate both forward and reverse genetics even in modal species such as Arabidopsis. The technique is becoming more efficient and precise with opportunities like the inducible cas9 expression and direct delivery of cas9 protein (Ramakrishna et al. 2014; Polstein and Gersbach 2015). These developments will help to avoid off-target effects by expressing cas9/gRNA only when it is required. The remarkable advantage of CRISPR technology is its accessibility and simplicity over other genome editing tools.

References

Ali Z, Abul-faraj A, Piatek M, Mahfouz MM (2015) Activity and specificity of TRV-mediated gene editing in plants. Plant Signal Behav 10:e1044191. doi:10.1080/15592324.2015.1044191

Augustine SM, Narayan JA, Syamaladevi DP, Appunu C, Chakravarthi M, Ravichandran V, Subramonian N (2015a) *Erianthus arundinaceus* HSP70 (EaHSP70) overexpression increases drought and salinity tolerance in sugarcane (*Saccharum* spp hybrid). Plant Sci 232:23–34

Augustine SM, Narayan JA, Syamaladevi DP, Appunu C, Chakravarthi M, Ravichandran V, Tuteja N, Subramonian N (2015b) Overexpression of EaDREB2 and pyramiding with the pea DNA Helicase gene PDH45 enhance drought and salinity tolerance in sugarcane (*Saccharum* spp hybrid). Plant Cell Rep 34:247–263

Augustine SM, Narayan JA, Syamaladevi DP, Appunu C, Chakravarthi M, Ravichandran V, Tuteja N, Subramonian N (2015c) Introduction of Pea DNA Helicase 45 into sugarcane (*Saccharum* spp hybrid) enhances cell membrane thermostability and abiotic stress tolerance. Mol Biotechnol 57:475–488

Baltes NJ, Gil-Humanes J, Cermak T, Atkins PA, Voytas DF (2014) DNA replicons for plant genome engineering. Plant Cell 26:151–163. doi:10.1105/tpc113119792

Barrangou R (2013) CRISPR-Cas systems and RNA-guided interference. Wiley Interdiscip Rev RNA 4:267–278. doi:10.1002/wrna1159

Belhaj K, Chaparro-Garcia A, Kamoun S, Nekrasov V (2013) Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods 9:39. doi:10.1186/1746-48 11-9-39

Bortesi L, Fischer R (2015) The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv 33:41–52. doi:10.1016/j.biotechadv.2014.12.006

Brooks C, Nekrasov V, Lippman ZB, Van EJ (2014) Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiol 166:1292–1297

Chen K, Gao C (2014) Targeted genome modification technologies and their applications in crop improvements. Plant Cell Rep 33:575–583. doi:10.1007/s00299-013-1539-6

Cho SW, Kim S, Kim JM, Kim JS (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol 31:230–232

Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S et al (2014) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Res 24:132–141

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N et al (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823

D'Hont A, Glaszmann JC (2001) Sugarcane genome analysis with molecular markers, a first decade of research. Proc Int Soc Sugar Cane Technol 24:556–559

Doudna JA, Charpentier E (2014) Genome editing the new frontier of genome engineering with CRISPR-Cas9. Science 346:1258096. doi:10.1126/science.1258096

FAOSTAT (2015) Food and Agriculture Organization of the United Nations Statistics Division. http://www.fao.org/statistics/en/

Fauser F, Schiml S, Puchta H (2014) Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. Plant J 79:348–359. doi:10.1111/tpj.12554

Feng Z, Zhang B, Ding W, Liu X, Yang DL, Wei P et al (2013) Efficient genome editing in plants using a CRISPR/Cas system. Cell Res 23:1229–1232

Feng Z, Mao Y, Xu N, Zhang B, Wie P, Yang DL et al (2014) Multi generation analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. Proc Natl Acad Sci U S A 111:4632–4637

Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK et al (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol 31:822–826

Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK (2014) Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nat Biotechnol 32:279–284

Fujii W, Kawasaki K, Sugiura K, Naito K (2013) Efficient generation of large-scale genome modified mice using gRNA and CAS9 endonuclease. Nucl Acids Res 41:e187

Gaj T, Gersbach CA, Barbas CF (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 31:397–405. doi:10.1016/jtibtech.2013.04.004

Gao Y, Zhao Y (2014a) Self-processing of ribozyme-flanked RNAs into guide RNAs in vitro and in vivo for CRISPR mediated genome editing. J Integr Plant Biol 56:343–349

Gao Y, Zhao Y (2014b) Specific and heritable gene editing in *Arabidopsis*. Proc Natl Acad Sci USA 111:4357–4358. doi:10.1073/pnas.1402295111

Gao J, Wang G, Ma S, Xie X, Wu X, Zhang X et al (2014) CRISPR/Cas9-mediated targeted mutagenesis in *Nicotiana tabacum*. Plant Mol Biol 10:1007

Guilinger JP, Thompson DB, Liu DR (2014) Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. Nat Biotechnol 32:577–582

Hansom S, Bower R, Zhang L, Potier B, Elliot A, Basnayake S et al (1999) Regulation of transgene expression in sugarcane. Proc Int Soc Sugar Cane Technol 23:278–290

Heintze J, Luft C, Ketteler R (2013) A CRISPR/Cas for high-throughput silencing. Front Genet 4:193. doi:10.3389/fgene.2013.00193

Hiei Y, Ishida Y, Komari T (2014) Progress of cereal transformation technology mediated by *Agrobacterium tumefaciens*. Front Plant Sci 5:628. doi:10.3389/fpls.2014.00628

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V et al (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 31:827–832

Jia H, Wang N (2014) Targeted genome editing of sweet orange using Cas9/sgRNA. PLoS One 9:e93806

Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. Nucleic Acids Res 41:e188. doi:10.1093/nar/gkt780

Jiang W, Yang B, Weeks DP (2014) Efficient CRISPR/Cas9-mediated gene editing in *Arabidopsis thaliana* and inheritance of modified genes in the T2 and T3 generations. PLoS One 9:e99225. doi:10.1371/journal.pone.0099225

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier EA (2012) Programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821

Jinek M, East A, Cheng A, Li S (2013) RNA-programmed genome editing in human cells. Elife 2:e00471. doi:10.7554/eLife.00471

Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E et al (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science 343:1247997. doi:10.1126/science.1247997

Johnson RA, Gurevich V, Filler S, Samach A, Levy A (2015) Comparative assessments of CRISPR-Cas nucleases cleavage efficiency in planta. Plant Mol Biol 87:143–156. doi:10.1007/s11103-014-0266-x

Joyce P, Kuwahata M, Turner N, Lakshmanan P (2010) Selection system and co-cultivation medium are important determinants of *Agrobacterium-mediated* transformation of sugarcane. Plant Cell Rep 29:173–183

Jung JH, Altpeter F (2016) TALEN mediated targeted mutagenesis of the caffeic acid O-methyltransferase in highly polyploid sugarcane improves cell wall composition for production of bioethanol. Plant Mol Biol 92:131–142

Khatodia S, Khurana SMP (2014) Trending: the Cas nuclease mediated genome editing technique. Biotech Today 4:46–49. doi:10.5958/2322-0996.2014.00019.2

Khatodia S, Bhatotia K, Passricha N, Khurana SMP, Tuteja N (2016) The CRISPR/Cas genome-editing tool: application in improvement of crops. Front Plant Sci 7:506. doi:10.3389/fpls.2016.00506

Kim H, Kim JS (2014) A guide to genome engineering with programmable nucleases. Nat Rev Genet 15:321–334. doi:10.1038/nrg3686

Li JF, Norville JE, Aach J, McCormack M, Zhang D, Bush J et al (2013) Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. Nat Biotechnol 31:688–691. doi:10.1038/nbt.2654

Liang Z, Zhang K, Chen K, Gao C (2014) Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. J Genet Genomics 41:63–68

Liang Z, Chen K, Li T, Zhang Y, Wang Y, Zhao Q, Liu J, Zhang H, Liu C, Ran Y, Gao C (2016) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nature Commun 8:14261. doi:10.1038/ncomms14261

Lin Y, Cradick TJ, Brown MT, Deshmukh H, Ranjan P, Sarode N et al (2014) CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. Nucleic Acids Res 42:7473–7485

Liu W, Yuan JS, Stewart CN (2013) Advanced genetic tools for plant biotechnology. Nat Rev Genet 14:781–793. doi:10.1038/nrg3626

Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S et al (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol 31:833–838. doi:10.1038/nbt.2675

Mao Y, Zhang H, Xu N, Zhang B, Gou F, Zhu JK (2013) Application of the CRISPR-Cas system for efficient genome engineering in plants. Mol Plant 6:2008–2011

Miao J, Guo D, Zhang J, Huang Q, Qin G, Zhang X et al (2013) Targeted mutagenesis in rice using CRISPR-Cas system. Cell Res 23:1233–1236

Mohan C (2016) Genome editing in sugarcane: challenges ahead. Front Plant Sci 7:1542. doi:10.3389/fpls.2016.01542

Moore PH, Paterson AH (2013) Sugarcane: the crop, the plant, and domestication. In: Sugarcane: physiology, biochemistry, and functional biology. Wiley, Chichester, pp 1–17. doi:10.1002/9781118771280.ch1

Mout R, Ray M, Yesilbag Tonga G, Lee YW, Tay T, Sasaki K, Rotello VM (2017) Direct cytosolic delivery of crispr/cas9-ribonucleoprotein for efficient gene editing. ACS Nano 11(3):2452–2458. doi:10.1021/acsnano.6b07600

Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S (2013) Targeted mutagenesis in the model plant Nicotiana Benthamiana using Cas9 RNA guided endonuclease. Nat Biotechnol 31:691–693

Nonaka S, Ezura H (2014) Plant-*Agrobacterium* interaction mediated by ethylene and super-*Agrobacterium* conferring efficient gene transfer. Front Plant Sci 5:681. doi:10.3389/fpls.2014.00681

Parisi C, Tillie P, Rodríguez-Cerezo E (2016) The global pipeline of GM crops out to 2020. Nat Biotechnol 34:32–36

Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA, Liu DR (2013) High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nat Biotechnol 31:839–843

Polstein LR, Gersbach CA (2015) A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. Nat Chem Biol 11:198–200. doi:10.1038/nchembio.1753

Puchta H (2005) The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. J Exp Bot 56:1–14

Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP et al (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152:1173–1183. doi:10.1016/j.cell.2013.02.022

Ramakrishna S, KwakuDad AB, Beloor J, Gopalappa R, Lee SK, Kim H (2014) Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. Genome Res 24:1020–1027. doi:10.1101/gr.171264.113

Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE et al (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154:1380–1389. doi:10.1016/j.cell.2013.08.021

Ron M, Kajala K, Pauluzzi G, Wang D, Reynoso MA, Zumstein K et al (2014) Hairy root transformation using *Agrobacterium rhizogenes* as a tool for exploring cell type-specific gene expression and function using tomato as a model. Plant Physiol 166:455–469

Saika H, Nishizawa-Yokoi A, Toki S (2014) The non-homologous end-joining pathway is involved in stable transformation in rice. Front Plant Sci 5:560

Sander JD, Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 32:347–355. doi:10.1038/nbt.2842

Schiml S, Fauser F, Puchta H (2014) The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. Plant J 80(6):1139–1150. doi:10.1111/tpj.12704

Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z et al (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol 31:686–688

Shuman S, Glickman MS (2007) Bacterial DNA repair by non-homologous end joining. Nat Rev Microbiol 5:852–861. doi:10.1038/nrmicro1768

Smith C, Gore A, Yan W, Abalde-Atristain L, Li Z, He C et al (2014) Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs. Cell Stem Cell 15:12–13

Souza GM, Berges H, Bocs S, Casu R, D'Hont A, Ferreira JE et al (2011) The sugarcane genome challenge: strategies for sequencing a highly complex genome. Trop Plant Biol 4:145–156

Sugano SS, Shirakawa M, Takagi J, Matsuda Y, Shimada T, Hara-Nishimura I et al (2014) CRISPR/Cas9-mediated targeted mutagenesis in the liverwort *Marchantia polymorpha* L. Plant Cell Physiol 55:475–481

Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, Reyon D et al (2014) Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. Nat Biotechnol 32:569–576. doi:10.1038/nbt.2908

Upadhyay SK, Kumar J, Alok A, Tuli R (2013) RNA-guided genome editing for target gene mutations in wheat. G3 3(12):2233–2238

Vanyushin BF, Ashapkin VV (2011) DNA methylation in higher plants: past, present and future. Biochim Biophys Acta 1809:360–368

Veres A, Gosis BS, Ding Q, Collins R, Ragavendran A, Brand H et al (2014) Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. Cell Stem Cell 15:27–30

Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C et al (2014) Simultaneous editing of three homoeo alleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol 32:947–951

Wyman C, Kanaar R (2006) DNA double-strand break repair: all's well that ends well. Annu Rev Genet 40:363–383. doi:10.1146/annurev.genet.40.110405.090451

Xie K, Yang Y (2013) RNA-guided genome editing in plants using a CRISPR-Cas system. Mol Plant 6:1975–1983

Xie K, Minkenberg B, Yang Y (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci USA 112:3570–3575. doi:10.1073/pnas.1420294112

Xing HL, Dong L, Wang ZP, Zhang HY, Han CY, Liu B et al (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol 14:327. doi:10.1186/s12870-014-0327-y

Xu T, Li Y, VanNostrand JD, He Z, Zhou J (2014) Cas9-based tools for targeted genome editing and transcriptional control. Appl Environ Microbiol 80:1544–1552. doi:10.1128/AEM.03786-13

Xu RF, Li H, Qin RY, Li J, Qiu CH, Yang YC et al (2015) Generation of inheritable and "transgene clean" targeted genome-modified rice in later generations using the CRISPR/Cas9system. Sci Rep 5:11491. doi:10.1038/srep11491

Yin K, Han T, Liu G, Chen T, Wang Y, Yu A et al (2015) A gemini virus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing. Sci Rep 5:14926. doi:10.1038/srep14926

Zhang L, Zhou Q (2014) CRISPR/Cas technology: a revolutionary approach for genome engineering. Sci China Life Sci 57:639–640. doi:10.1007/s11427-014-4670-x

Zhang H, Zhang J, Wie P, Zhang B, Gou F, Feng Z et al (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J 12:797–807

Zhou H, Liu B, Weeks DP, Spalding MH, Yang B (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res 42:10903–10914

Zhu C, Bortesi L, Baysal C, Twyman RM, Fischer R, Capell T, Schillberg S, Christou P (2017) Characteristics of genome editing mutations in cereal crops. Trends Plant Sci 22:38–52. doi:10.1016/j.tplants.2016.08.009

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C. Mohan (ed.), *Sugarcane Biotechnology: Challenges and Prospects*,
DOI 10.1007/978-3-319-58946-6

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