Chapter

A General Overview of Sweet Sorghum Genomics

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Abstract

Sorghum is one of the main cereal crops, its consumption is large, since it provides grain, fiber and biofuel. Likewise, its genome, with only 10 diploid chromosomes, makes it an attractive model for research and genetic improvement. Sorghum is the most studied C4 plant of its genus; several lines have been developed under three main characteristics: grain, forage and sugar biomass. Compared to other crops, sweet sorghum possesses high levels of highly fermentable sugars in the stem. Also, it has the ability of producing high production yields in marginal lands. These characteristics make it and attractive crop for the generation of biofuels. Molecular markers associated to several resistances and tolerances to biotic and abiotic factors have been described in literature. These allow the development of high-density linkage maps, which, along with the rising availability of sorghum genomes, will accelerate the identification of markers and the integration of the complete genome sequence. This will facilitate the selection of traits related to biofuels and the marker-assisted genetic improvement. Most of the information presented in this review is focused in *Sorghum bicolor* (L.) Moench. However, from the bioenergetics perspective, it is limited to sweet sorghum, which represents a promising opportunity for further studies.

Keywords: genetic differences, trait sequencing, biofuel, post-transcriptional regulation

1. Introduction

Sorghum (*Sorghum bicolor*) was originated in Africa; however, it is of high agricultural and economic importance in México. It is a multipurpose crop, since it serves as food, fiber and fuel. In addition, it can be grown in a wide range of agroecosystems, especially those of fragile conditions. It is also known as the "camel" of the crops, since to complete a productive cycle it requires less amount of water compared to other cereals of agricultural relevance. It has a remarkable ability to produce under low water availability regimes, as well as in other adverse abiotic conditions. Regarding the global production, it occupies the fifth place among the grains of economic importance [1]. Every year, sorghum production has been stabilized in 60 million tons in a producing area of 44 million hectares. México has

the third place worldwide with 4.5 million tons produced in 2018 [2]. From this, 62% corresponds to grain production, while 38% to forage.

Sweet sorghum is a natural variant of the common grain sorghum, with high content of sugars in the stem. It is frequently regarded as a "smart crop", since it can produce food as well as fuel. Currently, there are no records of commercial production of sweet sorghum in México, since it is a crop of recent introduction in the country. The first appearance of this crop in México was in the INIFAP Campo Experimental Río Bravo in the year of 2006, with the varieties Dale, Topper 76–6, Theis and M-81E [3]. Since then, the INIFAP genetic improvement program for sorghum, has searched for varieties which can adapt to the arid and semi-arid regions of the northeast or the country. These conditions are met by the variety RB Cañero, which has been tested in different environments, where it has shown a remarkable development and a high potential to produce bioethanol sugars [4].

Despite sweet sorghum is still a new crop in Mexico, the productive potential and the area of exploitation are highly promising. In 2010, INIFAP estimated the area available in México for sweet sorghum production, being the northeast region suitable with more than 4.38 million hectares [3]. The latter, pictures México as a country with great expectative of self-sufficiency in the generation of its own biofuel. The use of biofuels, being a cleaner and more efficient way of producing energy, would help to reduce pollution and greenhouse gases, which destabilize climate [5]. Pursuing this goal, biotechnology is an effective tool to develop methods which can optimize the bioethanol production from sweet sorghum. This review presents some of the biotechnological advances, as well as the current state of the genetic and molecular studies in sorghum, related to new routes to achieve an efficient generation of biofuels from this crop. This knowledge makes clear the necessity for effort and economic investment on this field, to reach self-sufficiency in the generation of energy sources in the country.

2. Sorghum as a research model

Sorghum possesses a small genome, which makes it attractive as a model organism for studies focused on understanding the structure, function and evolution of the cereals' genome. Sorghum is a tropical crop with a typical C4 photosynthesis, which uses a complex specialized biochemical and morphological system for carbon assimilation when it is exposed to elevated temperatures. This is a unique feature among the species of the same family it belongs to.

Like in other crops, sorghum is compared to other Poaceae of agricultural relevance such as maize (*Zea mays*), rice (*Oryza sativa*), sugar cane (*Saccharum officinarum*) or wheat (*Triticum aestivum*). Sorghum has ten diploid chromosomes and has a low degree of genetic duplication, which if compared to maize, makes it an outstanding model to develop functional genomics studies. On the other hand, maize developed a duplication of its entire genome since its evolutionary divergence from sorghum, changing the chromosome number from n = 5 to n = 10, being one of the sub genomes very similar to sorghum than to any other cereal [6]. However, evidence shown in NCBI database indicate that homologous genes between these species vary mainly in gene size and protein coding [7]. In the case of rice, it possesses a bigger genome than sorghum and also more genetic duplication. However, some comparisons prove that between sorghum and rice homologous gene exist, but with differences that could be attributed to the particularities of each species. Some reports indicate that sorghum and maize split from a common ancestor 12 million years ago [6], whereas the rice lineage is about 42 million years old compared to these two crops [7]. Lastly, sugar cage, possibly the most important sugar

producing crop worldwide, could have share ancestors with sorghum approximately 5 million years ago [8], since they conserve similar genes [9], and even it is viable to generate offspring from intergeneric crosses. Sugar cane contains at least two genomic duplications [9].

Sorghum represents an excellent model for research, since linkage mapping methodologies have been successfully implemented on it and possesses a wide mating system by self-pollination, which tends to preserve the association relationships for longer time periods compared to the self-pollination of cereals like maize, which facilitates the development of pure lines. Also, its genome sequence is available in several databases [10].

3. Differences between sweet sorghum and grain sorghum

Sweet sorghum plant produces sugars which can be directly fermented, together with its ability to produce high biomass volumes under adverse conditions, this crop is consider ideal for the generation of bioethanol of first and second generation. Also, its cultivation is suitable for marginal lands, avoiding competence for land with other food crops [11–13]. However, the genetics underlying these traits have been barely studied. The genetic differences between sweet and grain sorghum consist on a series of variations in the sequence and alterations of the genetic structure. The variations at sequence level are usually identified by single-nucleotide polymorphisms (SNPs), association sequences, genetic diversity and domestication [14–16].

S. bicolor has three subspecies: arundinaceum, bicolor y drummondii. All the feasible varieties belong to bicolor, which is divided into 5 races: bicolor, caudatum, durra, guinea y kafir. Sweet sorghum differs phenotypically from grain sorghum in several aspects: juicy stem rich in sugars, higher plant length, higher biomass production and less amount of grains in the spike [11]. These differences cannot be only explained by the genetic variation among these two varieties, which suggest DNA methylation and other epigenetic mechanisms are key factors to describe them [17].

	Trait	**Grain sorghum**	**Sweet Sorghum**
Phenotypic	Height (cm)	97.6	230–281
	Biomass (g plant^{-1})	67	605–1096
	Spike (dry weight, g) (g plant^{-1})	24.2	60–80
	Roots (dry weight, g) (g plant^{-1})	15.1	68–88
	Stem (dry weight, g) (g plant^{-1})	27	164
Genotypic	SNPs	85,041(14,782 genes)	
Line BTx623 (grain)	Indels	16,781 (7,977 genes)	
vs.	SVs	1,847 (2,071 genes)	
Line Keller (sweet)	Protein functional divergence	563(SNP), 287(Indels), 69 (SV)	

Grain sorghum data obtained from Assefa et al., *[20] and Wang* et al., *[21]. Sweet sorghum data obtained from Ekefre* et al., *[22]. Genotypic differences data obtained from Jiang* et al., *[17].*

Table 1.
Main differences between grain and sweet sorghum.

Sweet sorghum has been found on different races [18], which challenges its origin, selection and genetics. This also suggest high genetic variability between sweet and grain sorghum, which could be exploited for genetic improvement of sweet sorghum. Currently the BTx623 grain sorghum genome sequence is available [10], which provides a genomic base for comparative studies of the genome. Regardless this achievement, it is still difficult to access the information related to the hidden variability among genomes of the same species. Zheng et al., [19] studied the resequencing of the two sweet and one grain sorghum genomes, with the aim of identify polymorphism patterns of the sequences and structural variations, using BTx623 as a reference genome. This study allowed the identification of great differences in the number of SNPs, indels, copy number variations and structural variations (SV) among these genomes. The comparison of this genetic variation defined potential genomic regions and metabolic pathways associated to sweet sorghum and traits such as sugar production. **Table 1** presents phenotypic and genotypic differences between grain and sweet sorghum.

4. Sorghum genetic mapping

Building a linkage map is the fundamental step required for a detailed study of genetic improvement of crops by marker-assisted selection. Mapping of sorghum genome based on DNA markers started in the 90's, and nowadays there are several genetic maps available. It is important to mention that sorghum, particularly *S. bicolor*, possesses 10 chromosomes and has been classified as a diploid (2n = 2x = 20) [23]. However, it has been assumed that sorghum has a tetraploid origin, due to the large number of complementary gene *loci* and to some studies on meiotic mating among chromosomes as in *S. halepense*, which is 2n = 4x = 40. Other studies with fluorescence *in situ* hybridization (FISH) have reached the same conclusion [24]). Nonetheless, [25] used the same FISH technique and other structural genomic resources, including genomic clones with large inserts in artificial bacterial chromosomes (BACs), and identified the 10 chromosomes simultaneously. Years after, Paterson *et al.,* [7] found identities and homologies among the linkage groups in metaphase state and this determined *S. bicolor* diploidy (2n = 20) as well as the genome length of 730 Megabases (Mb).

The first genetic maps built where based on DNA analogy tests based on corn genome Binelli *et al*., [26]; Whitkus *et al*., [27]; Melake-Berhan *et al*., [28]; Pereira *et al*., [27] After, maps were built from genomic DNA analysis Chittenden *et al*., [29]; Ragab *et al*., [30]; Xu *et al*., [31]. Other published map was based on tests done in sugar cane and maize [32]. All these maps were built using restriction fragments length polymorphisms (RFLPs) and the majority of them used F_2 populations, while Dufour *et al*., [32] used two populations of recombinant inbred lines (RILs). This last map was extended by Boivin *et al.,* [33] with the addition of a great number of RFLPs and AFLPs (Amplified Fragment Length Polymorphisms). On the other hand, [34] built a sorghum map using a RIL population and a variety of tests which include sorghum genomic DNA, corn and sugar cane DNA and cDNA, versus tests of other cereals, and 8 simple sequence repeat (SSRs) microsatellite *loci*. Subudhi and Nguyen [35] completed the alignment of the 10 linkage groups using RFLPs on a RIL population and completing the maps of de Chittenden *et al*. [29], Ragab *et al*. [30, 31] of corn and other cereals.

Kong et al. [36] mapped a RIL population with 31 SSR polymorphic *loci* obtained from 51 clones isolated from a *S. bicolor* genetic library, which was provided with four oligomers di- and trinucleotides radioactively labeled. Haussmann *et al*. [37] mapped molecular markers related to resistance of the hemiparasite

Striga hermonthica in two recombinant populations (RIP-1, −2) of $F_{3:5}$ lines. RIP-1 and RIP-2 maps covered 1,498 cM and 1,599 cM respectively with 157 markers distributed in among the linkage groups.

Apart from these linkage maps, integrated maps have also been built. An integrated linkage map of SSRs and AFLPs from sorghum was reported by Kong *et al*. [36] using different sorghum lines. SSR *loci* were designed from clones isolated from two sorghum BAC libraries. The linkage map covered 1,406 cM and consisted in 147 SSR *loci* and 323 RFLP *loci*. Klein *et al*. [36] constructed an integrated physical and genetic map of sorghum genome (750 Mb) from PCR methods for the creation of BAC libraries and the localization of BAC clones in sorghum genetic maps. Also, Menz *et al*. [38] built a genetic map using AFLPs. The 1713 cM of the map covered 2,926 *loci* distributed among the 10 linkage groups, where 2,454 were AFLPs, 136 were SSRs previously mapped in sorghum and 203 were cDNAs and genomic clones coming from rice, barley, oat and maize. Another reported map was the one from [39], which consisted in 2,512 *loci* spaced in intervals of 0.4 cM on average, and it was based in 2,050 RFLPs, including 865 heterology tests from sugar cane, maize, rice, *Pennisetum setaceum* and *Arabidopsis thaliana*.

Recently, a high genetic density map was published by Ji *et al.* [40], where specific length amplified fragment markers (SLAFs) were utilized. This map was based on a F_2 population of 130 individuals originated from a cross between a grain sorghum variety, J204, and a sweet sorghum variety, Keter. Massive sequencing was used to cover the 52,928 SLAFs from the 43 million reads generated. From these markers, 12% appeared to be polymorphic and from 2,246 of these SLAFs a linkage map was built, covering the 10 chromosomes. The total length was 2,158 cM, which is 50% more compared to the previous maps available, which were constructed using RFLPs.

Another method used is the comparative genome mapping. This particular method is interesting for geneticists and evolutionary biologists to elucidate the mechanisms determining chromosome's evolution. Comparative genome mapping provides a powerful technique to study the way and the time where chromosomal evolution occurs [23]. This approach involves the use of molecular markers, such as RFLPs, to map the genomes of two species for a group of markers in common (*loci*). Even it is an expensive and intensive duty, this method can determine the reach and the nature of the chromosomes recombination in incompatible species crosses. The finding of small chromosomal regions which retain a similar gene order in sorghum and in two dicotyledon species (*Arabidopsis* y *Gossypium hirsutum*), suggest that comparative mapping can reach a major evolutive distance compared to what has been reached until now.

Among the *Andropogoneae* grass tribe, comparative mapping facilitates the understanding of sorghum genetics. At this point, several research groups have established a relationship between sorghum and maize genomes [27, 28, 32, 41, 42]. The high degree of conservation of the genes order between these two crops has limited the identification of chromosomal rearrangements between them. Apart from being compared with maize, sorghum has also been compared to rice, where certain apparent collinearity was also found.

Until 2015, more than 850 *loci* associated to traits relevant to biofuels production were identified in sorghum. These are traits regarding plant architecture (roots, leaves and stem), flowering time, and conversion rate of biomass into biofuels. These quantitative trait *loci* (QTLs) related to biofuels generation have been found in different mapped populations, which suggest the plasticity of these traits in different environments. This makes the genes located in these QTL regions could be potential targets to improve sweet sorghum yield for biomass and biofuels production [43].

Regardless of the multiple QTLs already reported, very few studies have been done with the aim of genetically improving these traits. In one of these, a quantitative gene (dw3), orthologous to branchytic 2 (br2) from corn, was cloned with the intention of reducing plant height. This gene is a P-glycoprotein which modules auxin transport in maize stems [44]. Another group of researchers cloned and sequenced, from the cultivar dulce Rio, homologous genes of the sucrose transporter proteins (SUTs), which were compared to the published sequence of BTX623 grain sorghum variety. It was possible to identify six SUTs in BTx623, along with nine differences in the amino acids sequence of SbSUT5 between the two varieties. Two of the five remaining SUTs exhibited unique variations in the amino acids sequences of SbSUT1 and SbSUT2, whereas the rest shared identical sequences. It was also proven that in a mutant of *Saccharomyces* (SEY6210), uncapable of growing with sucrose as the only available carbon source, sorghum SUTs are capable of transporting sucrose [45]. This showcases the relatively low knowledge of the genes underlying the traits associated to biofuels generation in sweet sorghum and bolsters the potential of sweet sorghum breeding to produce biofuel through the exploitation of its genetic resources.

5. Genome sequencing and sorghum functional genomics

Massive sequencing of the line BTx623 is nowadays completed and approximately 10.5 million of reads (8X coverage) have been deposited in the NCBI database. In the preliminary assembly, more than 97% of the genes codifying for proteins (Expressed Sequence Tag, EST) in sorghum were found in 250 large contigs. The majority was able to be joined, ordered and oriented using genetic and physical maps to reconstruct the full chromosomes. The preliminary alignment assembly for the sorghum sequence was based on methyl-filtrated sequences. Also, the assembly for sorghum, maize, sugar cane transcripts, as well as *Arabidopsis* and rice proteomes, confirmed the correct assembly of the bases and local structure. This allowed the approximate prediction of 30,000 to 50,000 *loci* which code for proteins. The conserve genetic synteny with rice is evident, as expected from the comparisons obtained from the maps [10].

The spatial structure of the genes in sorghum is represented by approximately 125,000 ESTs, which have been grouped in 22,000 unigenes, representing more than the 20 diverse libraries of different genotypes [46]. Around 50,000 methyl-filtrated reads, which provide an estimated coverage of 1X [47] have been assembled into contigs. Another representative strategy is the cloning and direct sequencing (Cot-Base cloning), which was used in sorghum in 2001 for the first time [48]. This method offers the potential to cover and increase this coverage more than could be achieved with ESTs and methyl-filtrated reads as demonstrated in maize.

The progress in transcriptomes' characterization has been parallel to the identification of differential genes expressing in response to biotic and abiotic factors, as well as to damage caused by insects, dehydration, high salt concentration, abscisic acid [49], methyl-jasmonate, salicylic acid and amino cyclopropane carboxylic acid [50].

6. Post-transcriptional regulation by miRNAs in sorghum

The micro-RNAs (miRNAs) are small RNA molecules of approximately 21 nucleotides, which play an important role in the post-transcriptional genetic regulation inhibiting the translation of the messenger RNAs (mRNAs) by blocking

translation machinery or by excision of the mRNAs [51]. In plants, the majority of miRNAs promote the degradation of mRNA targets by perfect or almost perfect mating of the complimentary RNA strands [52]. miRNAs intervene in a variety of biological processes, such as development and identity of organs, metabolism and stress responses [53]. A substantial number of miRNAs has been identified in different plants, and recently the number of studies in sorghum has been increasing with respect to the identification of miRNAs and their target genes.

Recently, Katiyar et al. [54] showed the importance of studying miRNAs and other RNA molecules using RNA sequencing from the libraries created from genotypes of a variety tolerant to drought (M35–1) and one susceptible. These varieties were cultivated in controlled conditions as well as in drought stress. After sequencing the RNA profiles generated, it was possible to identify 96 miRNAs regulated by the stressed caused by drought conditions. This represents new perspectives for the genetic engineering regarding the potential of miRNAs to improve drought resistance as well as other types of abiotic stresses.

Following the same research line, in 2016, Hamza et al., used 8 deregulated miRNAs by abiotic stress in 11 elite varieties of sorghum under low water availability and drought [55]. This study showed that the miRNAs miR396, miR393, miR397-5p, miR166, miR167 and miR168 have a significative deregulation, being sbi-miR396 and sbi-miRNA398 the ones with higher overexpression for all the genotypes. This same research group has studied the effects of drought and salinity in the miRNAs profiles generated in *S. bicolor* [56]; these results confirm that the miRNAs expression patterns are related to the dose of stress the plants are subjected; however, every miRNA responded in a unique way in every of the six genotypes.

Other important trait to improve sweet sorghum is sugar accumulation, which has been already studied by Yu et al. [57], who propose mir-271 as a specific miRNA of the Rio sweet sorghum variety, related to cellulose synthesis and sugar accumulation. A full detailed list with most of the relevant miRNAs for the genetic improvement of sorghum in biofuels production was published by Dhaka et al. [58].

7. Transformation and reverse genetic in sorghum

Methods for sorghum transformation have been available since the beginning of the 90's, initially by protoplasts [59] and cell culture [60], and subsequently *in planta* [61, 62], using *Agrobacterium* and protocols based in microprojectiles which are now available and with substantially improved efficiencies [63–69]. Sorghum is a crop hard to transform, since it is a recalcitrant genus for tissue culture and the transformation protocols reported are scarce and not very reproducible. In the particular case of sweet sorghum, [70] proposed a transformation system based on optimizing tissue culture conditions using embrionary callus with a regeneration of 90% in 12 weeks. Also, hygromycin resistance selection conferred by the Ubi-*hpt* transgene was performed, followed by particle bombardment. This method proved to be highly reproducible with an efficiency of transformation of 0.09% in every embryo. In 2012, Liu and Godwin, published a method with a better transformation efficiency in *S. bicolor*, in which using pure line embryos (IEs) Tx430, reaching an efficiency of 20.7% in the three independent experiments [71]. The protocol, which involves the use of microprojectiles and transgenes regulated under the *ubi1* constitutive promoter, improves the conditions of the media culture for embryos, as well as the parameters for transformation with microprojectiles. In this experiment, the transgenes were inherited by the T1 generation.

After, Tien-Do *et al*. [72] developed a fast and efficient system for sorghum transformation using binary vectors and the AGL1 *Agrobacterium* strain instead of

microprojectiles. With the public genotype P898012 and the *bar* gene as a selective marker, callus regeneration time was reduced to 7–12 weeks, producing 18 plants per callus. This experiment achieved a frequency of 14%, where 40–50% of the transformation events possessed a single copy of integrated T-DNA with a segregation of mendelian 3: 1 estimated by Southern blot. An example of the utility of genetic transformation of sweet sorghum is presented by Zhu *et al.* [73], where using *Agrobacterium* and induce early flowering, the gene Bt cry1Ah was introduced. BT or Cry proteins are produced naturally in aggregates or crystals by *Bacillus thuringiensis.* There proteins are specific for the digestive system of different insects. Protein Cry1Ah, confers resistance to *Ostrinia furnacalis.* In this study, the generated plants, after being selected for herbicide resistance to confirm transformation, showed a high resistance at T0. Apart from the resistance tests, transgene expression was confirmed by RT-PCR and the presence of BT proteins produced by the plant by Western blot and ELISA.

One of the main arguments against the use of transgenics is the use of selection markers such as herbicide tolerance and the fact that they stay inside the genome of the transformed plant. The main issue is the possibility of the cultivar's pollen to pollinate related weeds and therefore the resistance is inherited to undesired plants. Against this problematic, there are several efforts to generate marker-free transgenics. An example is presented by Lu *et al.* [74], where at the cost of reducing the selection pressure, they manage to obtain marker-free transgenics.

In other hand, sorghum offers the opportunity to complete what has been previously described in the reverse genetics of rice and maize, providing to the genetic and familiar studies, those genes which are hard to manipulate in these crops. This allows the directed functional analysis to specify the genes in sorghum related to traits such as hydric stress and the production of certain sugars by genetic association. To accelerate the specific direct identification of genes, mutant lines using ethyl methane sulfonate (EMS) have been created. For the genotype BTx623 there are 1,600 M3 annotations, individual pedigrees, which was characterized [75]. Currently, each of the inspected M3 lines is distinguishable from the original stock and some have multiple mutant phenotypes. The additional M2 mutants are available for the scientific community for the production of thousands of M3 additional lines.

Until few years ago, even with the genome sequencing technology for the elite line BTx623, the genetic sources and sorghum germplasm where limited, making hard the functional validation of the sequenced genes. In 2016, 4,600 M4 mutant pedigrees where created by EMS mutagenizing of BTx623 seed, which were later transformed in lines by single-seed descendant method [76]. The sequencing of 256 mutant lines revealed more than 1.8 million of induced canonical mutations, affecting 95% of the sorghum genome.

8. Perspectives

The studies here presented represent an introduction to the current state of sorghum genomics. Regardless these advances have contributed relevant achievements to what it is known about genetic diversity of this species, it is still necessary to develop further studies, which its aim is focused in sweet sorghum. However, the knowledge acquired in grain sorghum and other related species, constitute an important molecular base to continue developing research studies which allow to know sweet sorghum and its unreported genomic regions. In them could lie the key for the increased production of sugars, lignin and other traits of interest such as tolerance to new plague's appearances such as yellow aphids and/or diseases. It is also necessary

to develop genetic maps which allow the localization of genetic codifying regions to certain traits of agronomic interest. Regarding molecular studies in Mexico, there are no reports of genetic maps or genomics performed in sweet sorghum. This represents an opportunity to develop research lines which allow to generate the country's own sweet sorghum genotypes carrying tolerances to adverse biotic and abiotic conditions predominant in the country. This would allow the growth on its production and of its sub products, focusing in alternative environment friendly energy sources.

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