DEVELOPMENT AND CHARACTERISATION OF MICROSATELLITE MARKERS FOR PUCCINIA MELANOCEPHALA, CAUSAL AGENT OF SUGARCANE BROWN RUST

By

R.F. PEIXOTO JUNIOR¹, A.V.O. FIGUEIRA¹, M.G.A. LANDELL², D.S. NUNES², L.R. PINTO², A. SANGUINO², I.A. ANJOS² and S. CRESTE²

¹CENA/USP, Piracicaba, SP, Brazil
²Cento de Cana/IAC, Ribeirão Preto, SP Brazil

rfpeixoto@live.com

KEYWORDS: Sugarcane Disease, Molecular Marker, Genetic Diversity.

Abstract

BROWN RUST (caused by the fungus Puccinia melanocephala H. & P. Sydow) is a major sugarcane disease, responsible for large yield losses. Knowledge of the population structure of P. melanocephala is required for the development of sustainable control strategies, including resistant cultivars. Here, we describe the development of a library enriched for microsatellite loci to identify polymorphic loci for P. melanocephala isolates. Initially, 44 field-collected sugarcane rust isolates were classified based on morphological structures of urediniospores, from which 34 were identified as P. melanocephala and 10 as Puccinia kuehnii, causal agent of sugarcane orange rust. From 384 clones sequenced, 52.2% of the clones generating 135 microsatellite sequences were identified. Of the microsatellite loci identified, the most frequent motif observed was dinucleotide, representing 46.6% of the total. Trinucleotide repeat motifs, accounted for 19.2% while tetranucleotide (3%), pentanucleotide (0.8%) and hexanucleotide (0.8%) motifs were also observed. From the enriched library, 16 microsatellite loci produced scorable products, but only 10 were polymorphic for the isolates tested. All ten isolates identified as P. kuehnii showed the same pattern of alleles found for P. melanocephala. The method used to enrich the library was efficient in isolating microsatellite loci for P. melanocephala.

Introduction

Sugarcane brown rust, caused by the basidiomycete Puccinia melanocephala H. & P. Sydow, is a major sugarcane disease, responsible for large yield losses worldwide. In countries where sugarcane is widely cultivated, and subject to large P. melanocephala populations and edapho-climatic conditions, it is important to know the range of the genetic diversity of this pathogen. Such information is of extreme importance for breeding programs to avoid releasing cultivars described as resistant, which, in the presence of more aggressive variants of the pathogen, may become susceptible. Thus, understanding the fungus population structure and its genetic diversity, together with processes that may affect its diversity, are important for developing disease control strategies as well as resistant cultivars.

DNA markers have been widely adopted to analyse the dynamic of plant pathogen populations due to their high levels of precision and accuracy (Milgroom and Peever, 2003). Simple sequence repeats (SSR) or microsatellites are sequences relatively abundant and are evenly distributed in eukaryote genomes (Weising et al., 1995).
Noteworthy, fungal genomes appear to contain fewer SSR sequences than other eukaryotes and, when available, the loci exhibit less polymorphism, partly due to genome specificities (Dutech et al., 2007). However, when polymorphic loci are available, they can be highly useful for genome mapping, genetic diversity and population genetic studies, which warrant the search and development of such genetic markers. Therefore, the objectives of this work were to identify polymorphic microsatellites loci from a \textit{P. melanocephala} enriched genomic library.

**Material and methods**

**Sampling and identification of rust isolates**

Sugarcane leaf segments exhibiting typical rust symptoms were collected from four Brazilian States (São Paulo, Minas Gerais, Goiás and Alagoas). All leaf samples were examined under a microscope to differentiate brown rust (\textit{P. melanocephala}) from orange rust (\textit{P. kuehnii}) (Dixon et al., 2010; Virtudazo et al., 2001a).

**Construction of microsatellite-enriched genomic library**

Urediniospores from \textit{P. melanocephala} isolated from cultivar RB 835486 were used to extract genomic DNA. DNA was extracted using the method described by Aljanabi et al. (1999). The microsatellite-enriched genomic library was developed based on the methods described by Creste et al. (2006). Briefly, \textit{RsaI} digested DNA was bonded with specific adapters, enriched by hybridisation with a biotin-labelled microsatellite containing oligonucleotide, followed by enrichment using streptavidin-magnetic beads, amplification and cloning into pGEM-T prior to sequencing. Primers were developed using Primer3 (Rozen and Skaletsky, 2000) and quality of the primers was checked by Netprimer (http://www.premierbiosoft.com/netprimer/index.html).

**Microsatellite loci analysis**

DNA of isolates used for analysis of microsatellite loci were obtained from a suspension of urediniospores from a single pustule as described by Virtudazo et al. (2001b). Amplifications were performed on a MyCycle (Bio-Rad; Hercules, CA, USA) thermocycler programmed for one cycle at 95°C for 3 min, followed by 10 cycles of 40 s at 94°C, and 40 s with decreasing annealing temperatures, ranging from 60°C to 50°C ramping 1 degree each cycle, followed by 30 cycles of 40 s at 50°C. All cycles ended with an extension step at 72°C for 60 s. Allelic frequencies were obtained from visual analysis of 6% polyacrylamide gels.

**Results**

A total of 44 samples were collected and analysed. From the 44 samples analysed by microscopy, 34 were identified as \textit{P. melanocephala}, whereas 10 were confirmed as \textit{P. kuehnii}, the causal agent of sugarcane orange rust. Due to the recent introduction of the orange rust (\textit{P. kuehnii}) into Brazil (Barbasso, 2010), the collected material was carefully and rigorously analysed through microscopy to identify possible mixtures between the two rust species.

From 384 clones sequenced, 52.2% of the clones were identified containing sequences with microsatellites, from which 135 microsatellite sequences were generated. From the microsatellite loci identified, the most frequent dinucleotide motif observed was GT/TG (20.7%), followed by CA/AC (15.5%), GA/AG (5.2%), TA/AT (3.0%) and CT/TC (2.2%). Trinucleotide repeat motifs accounted for 19.2% of the total SSR loci. Tetranucleotide (TAAT and TTTA; 1.5%), pentanucleotide (TTGGG; 0.8%) and hexanucleotide (ACACGC; 0.8%) motifs were also observed. Only four sequences showed microsatellite motifs interrupted with non-repeated motifs. Mononucleotides were identified with only A- or T-repeat motifs ranging from 8 to 15 times, but these were not included for primer design since mononucleotide microsatellites tend to show low polymorphism (Tóth et al., 2000).

A set of 21 pairs of primers was developed from the enriched library. The remaining 114 sequences were not suitable for primer development, since some loci were at the edge of the sequences; or encompassed small size fragments; or were of low quality sequences that impaired.
primer design. From the 21 microsatellite loci tested using the *P. melanocephala* isolates, only 16 showed clear single-product amplification, with regard to the expected fragment size, and of which 10 loci were polymorphic (Table 1). The remaining loci were monomorphic for the isolates tested, amplifying fragments with the expected size. All ten isolates identified as *P. kuehnii* were analysed with the 10 loci developed for *P. melanocephala*. Both monomorphic and polymorphic loci showed the same pattern of alleles found for *P. melanocephala*, and no exclusive allele was detected for both species.

**Table 1**—Polymorphic microsatellite loci.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Motifs</th>
<th>Ta (°C)</th>
<th>Alleles</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPmlAC101</td>
<td>(GT)8</td>
<td>55</td>
<td>5</td>
<td>232–248</td>
</tr>
<tr>
<td>mPmlAC103</td>
<td>(TC)17</td>
<td>55</td>
<td>4</td>
<td>185–198</td>
</tr>
<tr>
<td>mPmlAC105</td>
<td>(AG)9</td>
<td>53</td>
<td>2</td>
<td>188,246</td>
</tr>
<tr>
<td>mPmlAC107</td>
<td>(AC)14</td>
<td>55</td>
<td>4</td>
<td>246–252</td>
</tr>
<tr>
<td>mPmlAC108</td>
<td>(TC)18</td>
<td>60</td>
<td>2</td>
<td>155,161</td>
</tr>
<tr>
<td>mPmlAC111</td>
<td>(AT)8</td>
<td>60</td>
<td>5</td>
<td>220–249</td>
</tr>
<tr>
<td>mPmlAC112</td>
<td>(AG)17</td>
<td>57</td>
<td>2</td>
<td>225,246</td>
</tr>
<tr>
<td>mPmlAC114</td>
<td>(TG)29</td>
<td>60</td>
<td>2</td>
<td>178,210</td>
</tr>
<tr>
<td>mPmlAC116</td>
<td>(AAT)6</td>
<td>56</td>
<td>2</td>
<td>136,138</td>
</tr>
<tr>
<td>mPmlAC120</td>
<td>(AGA)6</td>
<td>55</td>
<td>2</td>
<td>162,176</td>
</tr>
</tbody>
</table>

**Discussion**

In spite of the fact that fungal genomes have low frequency of microsatellites when compared to other eukaryotes (Morgante *et al.*, 2002), the enriched genomic library of *P. melanocephala* successfully allowed the identification of polymorphic loci (52.2%). Dutech *et al.* (2007) developed 16 microsatellite-enriched libraries for various species of fungi, obtaining variable yield for the enrichment of microsatellite sequences (82.3–17.8%). The most abundant dinucleotide motives for *P. melanocephala* were GT/TG, CA/AC and GA/AG. These repeats were expected due to the probes used. The fact that the dinucleotide GT/TG was the most frequent one in the library suggests that this motive may be more abundant in the *P. melanocephala* genome. Despite advances on methodologies for the development of microsatellite makers, there is still a lack of studies with fungi, compared to other phyla (Dutech *et al.*, 2007).

Transferability of *P. melanocephala* microsatellite loci may be observed for *P. kuehnii*. Such result indicates the possibility of transferability for *P. kuehnii* of the microsatellite developed specifically for *P. melanocephala*. A study carried out by Wang *et al.* (2010) showed that 8 out of 21 polymorphic EST-microsatellite loci developed for *Puccinia triticina* (wheat leaf rust) were transferable for *Puccinia coronata*, causal agent of oat leaf rust and *Puccinia graminis* (causal agent of wheat stem rust). Transferability of three microsatellite loci described for *P. coronata* and one locus described for flax rust (*Melampsora lini*) was reported for *Hemileia vastatrix* (coffee rust), where only two of them were polymorphic (Cristancho and Escobar, 2008). The interspecific transferability of microsatellite loci represents an important alternative to the development of new enriched libraries, therefore avoiding the high cost of its development. In conclusion, morphological characterisation by microscopy enabled a reliable and clear differentiation among *P. melanocephala* and *P. kuehnii*. The method used to enrich the library was efficient in isolating microsatellite loci for *P. melanocephala* and *P. kuehnii*.

**REFERENCES**


FROM THE TOP TO THE BOTTOM: HOW IS LIGNIN BIOSYNTHESIS REGULATED IN SUGARCANE?

By

MICHAEL DOS S. BRITO¹,²*, PAULA M. NOBILE¹,², SILVANA CRESTE², ALEXANDRA BOTCHER¹, EDWARD J. STRINI⁴, LAERTI ROQUE¹, VANESSA O. RODRIGUES², ADRIANA B. SANTOS¹, RENATO VICENTINI³, MARCOS G.A. LANDELL², IVAN A ANJOS² and PAULO MAZZAFERA¹*

¹Departamento de Biologia Vegetal, Instituto de Biologia, CP 6109, Universidade Estadual de Campinas, 13083-970, Campinas, SP, Brazil
²Centro Avançado da Pesquisa Tecnológica do Agronegócio de Cana, CP 206, 14001-970, Ribeirão Preto, SP, Brazil
³Centro de Biologia Molecular e Engenharia Genética, CEBMEG - Universidade Estadual de Campinas, 13083-875, Campinas, SP, Brazil
⁴Laboratório de Biologia Molecular de Plantas, FFCLRP/USP, 14040-901 Ribeirão Preto, SP, Brazil

*Corresponding authors: M.S. Brito (msantosbrito@yahoo.com.br)
P. Mazzafera (pmazza@unicamp.br)

KEYWORDS: Sugarcane, Lignin, Transcription Factors.

Abstract

CELL WALL RECALCITRANCE is the main bottleneck to produce lignocellulosic ethanol, due to lignin. Transcription factors (TFs) have been suggested as better targets for cell wall modification instead of lignin biosynthesis-related genes. We studied two contrasting sugarcane genotypes for lignin content/composition (thioacidolysis, acid-soluble and insoluble Klason, S/G ratio by GC/MS and TFs gene expression. Sugarcane culm was sampled in immature, intermediary and mature internodes and in the pith and rind, from the top to the bottom of the stalk. Lignin increased from the top to the base and the highest content was found in the rind. S/G ratio was also different in these tissues, indicating a suitable model to study lignin composition/deposition in sugarcane. qPCR analyses of 13 TFs showed that all of them were positively correlated with lignin content, showing that all these TFs are necessary to modulate lignin biosynthesis. A Pearson correlation analysis indicated a possible interaction between ScMYB58 and ScF5H (enzyme related to S monomer synthesis). These findings suggest that different lignin deposition between rind and pith might be under transcriptional regulation. Complementary, we cloned a promoter of F5H that together with our culm cDNA library will be used in Yeast-One-Hybrid (Y1H) assay, aiming to reveal new TFs involved with lignin transcriptional regulation.

Introduction

Lignin is one of the most abundant plant polymers, directly associated with cell wall recalcitrance in the conversion of cellulose to ethanol (Chen and Dixon, 2007). Lignin is basically composed of three compounds known as monolignols: p-coumaryl, coniferyl, sinapyl and giving rise to the three subunits of lignin predominantly found in nature, respectively, p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Li et al., 2010).

Thus, genes participating in the synthesis of monolignols have been identified and characterised in recent years in different organisms, aiming not only at the reduced amount of
lignin, but also a change in its composition. Recent studies have expanded our knowledge about the process of lignification and genes related to transport of monolignols across the plasma membrane (Liu et al., 2011), besides studies related to the regulation of lignin biosynthesis, mainly performed by transcription factors (TFs) (Grotewold, 2008; Gray et al., 2012; Handakumbura and Hazen, 2012). We know so far that two families of TFs participate in the regulation of lignin as well as the whole plant secondary wall biosynthesis. They are NAC and MYB. NAC (NAM, ATAF, CUC2) are TFs that trigger all regulation of lignin synthesis and secondary wall in different tissues and are known as NAC-master switches (Zhong et al., 2008; Demura and Fukuda, 2007).

Besides the NACs, another family of TFs, MYB (MYB master switches), are directly related to the control of secondary wall synthesis. Members of this family, according to their regulatory targets, may regulate other TFs involved with lignin biosynthesis. These TFs are known as MYBs-master switches, such as previously characterised MYB46 and MYB83 (McCarthy et al., 2009).

There are also other MYB TFs that directly regulate genes encoding enzymes of lignin biosynthesis. These MYBs have, as a main feature, a domain that binds in a specific region of the promoter (AC-rich region) of its target genes (Raes et al., 2003).

Despite all this knowledge and sketches made in different plant models, not much is known about these regulatory mechanisms of lignin biosynthesis in sugarcane yet. Thus, our goal was to study sugarcane varieties contrasting to the lignin content as well as identify and characterise TFs involved in lignin biosynthesis in sugarcane.

Material and methods

Plant material

Two sugarcane (Saccharum spp. hybrids) varieties were used in the study, IACSP04-529 and IACSP04-683. Both varieties originated from the sugarcane breeding program of the Centro de Cana - IAC/Apta, at Ribeirão Preto, São Paulo, Brazil. They were among the canes with the highest and the lowest lignin content. The sugarcane samples were separated in rind and pith in three different categories, the first made with young (1, 2, and 3), the second with intermediary (5, 6, and 7), and the third with mature (15, 16, and 17) internodes.

qPCR, Statistical and Correlation analysis

Orthologous sequences of TFs previously characterised as involved with lignin biosynthesis were searched in two databases: SUCEST and from our own RNAseq database of sugarcane culm, and PCR primers were designed. qPCR assays were carried out in triplicate with three biological samples. The PCR efficiency (>90% and <110%) was estimated by LinReg Software (Ramakers et al., 2003) for all primers used. GAPDH was previously validated as a better reference gene for this experimental condition.

Relative quantification (RQ) was determined comparing the transcriptional expression between tagged genes and the reference gene GAPDH by the formula: $2^{-\Delta\Delta Ct}$. Statistical analysis of qPCR data were carried out using REST tool (Pfaffl et al., 2002). Expression data, together with metabolic data, were used in a Pearson correlation analysis aiming to identify possible relation among TFs expression and metabolic content.

Results and discussion

Our plant material (genotypes IACSP04529 and IACSP04-683) was previously characterised with respect to the amount and composition of lignin. For this, analysis by Thioglicol and Klason was conducted in these two varieties. It was observed that genotype IACSP04-529 had a higher lignin content than IACSP04-683 and the amount of lignin accumulated in the culm increased from the top to the bottom of the stem.

Complementarily, we evaluated, using gas chromatography – mass spectrometry (GC-MS), the composition of lignin. In both genotypes, S monolignol was predominantly produced, and the ratio S / G was higher in the pith when compared to the rind. In addition, we chose 13 sugarcane
genes similar to TFs previously characterised in other organisms, to be validated by qPCR. Initially, we carried out phylogenetic analysis to identify the probable orthologs of these genes.

After identifying candidate genes in sugarcane, primers were designed to be used in qPCR assay. In general, all genes were better expressed in the genotype IACSP04-529, with the higher lignin content, suggesting that all TFs tested here could participate in the regulation of lignin biosynthesis. Among the candidate genes evaluated, there was one with similarity to AtMYB58/63, here named ScMYB58. It was shown in other species that this TF was able to bind to the promoter of lignin biosynthesis genes, except for the gene encoding F5H (ferulate-5-hydroxylase) (Zhou et al., 2009). However, a Pearson correlation analysis showed a strong correlation between the expression of ScMYB58 gene and the deposition of type S-monolignol.

Moreover, co-expression between ScMYB58 and F5H genes was observed. So, it is plausible to suggest that, contrary to other plant models, ScMYB58 may act in the regulation of F5H in sugarcane. Differences in patterns of regulation have been shown when comparing orthologs in different plants (Shen et al., 2012). In order to identify the TFs that regulate F5H gene expression in sugarcane, we have cloned a region of 1000 kb upstream the F5H gene and constructed a culm cDNA library. Both the F5H gene promoter and the cDNA library will be used in a Yeast One Hybrid (Y1H) approach.

Conclusion

The data obtained in this study is unprecedented in sugarcane. Together with the TFs expression data, the Y1H assay will help us to identify the expression regulators of F5H. This set of data would eventually assist in the design of new varieties of sugarcane suitable for the production of lignocellulosic ethanol.

Acknowledgments:

This work was supported by grants from Fundação de Amparo a Pesquisa no Estado de São Paulo – Brazil (FAPESP no. 08/58035-6 and 2010/11476-8).

REFERENCES


PRODUCTION OF BIODEGRADABLE PLASTICS WITH CONTROLLED MONOMER COMPOSITION

By

Laboratory of Bioproducts, Institute of Biomedical Sciences – University of São Paulo
¹mailto:rafinharc15@hotmail.com, ²mailto:lukneif@usp.br

KEYWORDS: Burkholderia sacchari, poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), Aeromonas sp., Operon pha.

Abstract
Polyhydroxyalkanoates (PHA) are polyesters accumulated by bacteria as granular reserves of carbon. Burkholderia sacchari is a bacterial species isolated from soil able to accumulate high amounts of PHA. Poly-3-hydroxybutyrate (P3HB) is the most studied PHA, but its use as a biodegradable plastic is limited by its mechanical properties. The improvement of these properties is accomplished by synthesising copolymers. Poly-3-hydroxybutyrate-co-3-hydroxyhexanoate (P3HB-co-3HHx) has received special attention since it presents properties similar to that of low density polyethylene (LDPE). The aim of the present study was to evaluate the potential of B. sacchari LFM101 and its recombinants to produce P3HB-co-3HHx with controlled molar fractions of 3HHx units. Wild type strain B. sacchari LFM101 produced P3HB-co-3HHx from glucose and hexanoic acid (3HHx molar fraction of 0.66 mol%). A pha mutant of B. sacchari was used to construct recombinant strains harbouring genes from pha operon of Aeromonas sp. The highest molar fraction of 3HHx was obtained expressing the entire operon phaPCJ (22.24 mol%). In conclusion, it is possible to produce P3HB-co-3HHx using B. sacchari strains with a large range of 3HHx molar fraction (0–22 mol%), allowing the production of co-polymers for different applications.

Introduction
Polyhydroxyalkanoates (PHA) are accumulated by several bacteria as granules when cultivated under limitation of an essential nutrient and simultaneous excess of the carbon source (Steinbüchel and Lütke-Eversloh, 2003; Silva et al., 2007; Park et al., 2011).

Despite the fact that the primary pathway for PHA biosynthesis has been described in different bacteria, the supply of the appropriate metabolite intermediates for PHA biosynthesis requires a variety of different metabolic pathways. These metabolite intermediates can be supplied by the catabolic pathways of carbohydrates (e.g., Enteroff–Doudoroff, ED, and Embden–Meyerhof–Parnas, EMP), by the fatty acids β-oxidation, and de novo fatty acids synthesis (Sudesh et al., 2000; Tsuge, 2002). PHA can be composed either by short-chain length hydroxyacid monomers (HA_{MCL} = 4–5 carbon atoms) or by medium-chain length hydroxyacid monomers (HA_{SCL} = 6–14 carbons).

The production of PHA with improved properties has been limited partially because the specificity of PHA synthase (Rehm, 2010) also limits the incorporation of HA_{MCL} intermediates to HA_{SCL} in bacteria able to accumulate high amounts of PHA, namely 80% of cell dry weight (CDW). The co-polymer, poly-3-hydroxybutyrate-co-3-hydroxyhexanoate, P3HB-co-3HHx, has a
wide range of applications, mostly on the medical area (Chen and Wu, 2005; Bian et al., 2009; Chen, 2009) and is known for its similar mechanical properties to low density polyethylene (Doi et al., 1995; Sudesh et al., 2000).

The production of P3HB-co-3HHx has been studied using wild type (Lee et al., 2000) or recombinant \textit{Aeromonas} (Lu et al., 2004a; Tian et al., 2005; Qiu et al., 2006; Jian et al., 2010). The genes responsible for the production of PHA in \textit{Aeromonas} are organised in the operon \textit{phaPCJ} (Fukui et al., 1998; Sudesh et al., 2000). \textit{Aeromonas} strains have shown the ability to incorporate up to 20 mol\% of 3HHx to the PHA, however producing low amounts of PHA (Matsuda, 2009).

\textit{Burkholderia sacchari} has demonstrated the ability to accumulate high amounts of P3HB (80\% CDW) and to produce P3HB-co-3HHx with a maximum 3HHx content of 2 mol\% (Mendonça, 2010). Thus, the importance of the present work consists in the development of \textit{B. sacchari} recombinant strains with higher ability to produce P3HB-co-3HHx to generate PHA with controlled molar fractions of 3HHx.

\textbf{Materials and methods}

Recombinant \textit{B. sacchari} strains (Table 1) were constructed by inserting genes from \textit{Aeromonas} (\textit{A. hydrophila} and \textit{Aeromonas} TSM81) (Matsuda, 2009). The genes were inserted into the vector pBBR1MCS-2 (Kovach et al., 1995) applying conventional molecular cloning procedures (Sambrook and Russell, 2001).

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Bacteria} & \textbf{Characteristics} & \textbf{Reference} \\
\hline
\textit{B. sacchari} LFM101 & Wild type: Suc\(^+\), PHA\(^+\), Hx\(^+\), Kan\(^\#\), Amp\(^\circ\), Te\(^\circ\) & Brämer et al., 2001 \\
LFM936 & LFM101 with pBBR1MCS-2, Km\(^\circ\). & Mendonça, 2010 \\
LFM344 & UV mutant (PHA\(^-\)). & Oliveira et al., 1998 \\
LFM1034 & LFM344 with pBBR1MCS-2::phaPCJ from \textit{Aeromonas} sp. (TSM81) & Present work \\
LFM1035 & LFM344 with pBBR1MCS-2::phaC from \textit{A. hydrophila} ATCC 7966 & Present work \\
LFM1036 & \textit{B. sacchari} LFM101 with pBBR1MCS-2::phaJ from \textit{A. hydrophila} ATCC 7966 & Present work \\
LFM1037 & \textit{B. sacchari} LFM344 with pBBR1MCS-2::phaCJ from \textit{A. hydrophila} ATCC 7966 & Present work \\
LFM1038 & \textit{B. sacchari} LFM344 with pBBR1MCS-2::phaPC from \textit{A. hydrophila} ATCC 7966 & Present work \\
LFM1039 & \textit{B. sacchari} LFM344 with pBBR1MCS-2 & Present work \\
\hline
\end{tabular}
\caption{Bacterial strains.}
\end{table}

\*LFM: Laboratório de Fisiologia de Microrganismos, ICB-USP; Suc\(^+\): growth in sucrose; PHA\(^+\): PHA accumulation; Kans: sensibility to kanamicin; Amps: sensibility to ampicilin; Tcs: sensibility to tetraciclin; Kanr: resistance to kanamicin.

Strains were cultivated in 50 mL mineral (Gomez, 2000) medium limited in nitrogen, in 250 mL flasks, agitated at 150 r/min at 30 \(^\circ\)C for 72 h. Glucose and hexanoic acid were added as carbon sources, at zero and 24 h, respectively. PHA content and composition were determined by gas chromatography as described by (Gomez, 2000).

Bioreactor experiment employed a glucose:hexanoic acid ratio equal to 140:45 g/L with a feed rate of 4.95 mL/h under conditions previously described (Gomez et al., 1996)

\textbf{Results and discussion}

The vector itself had no considerable effect on PHA accumulation as proven by the evaluation of the recombinant LFM1039. This PHA negative mutant did not recover the production
capacity of PHA. All the *B. sacchari* recombinants harbouring *Aeromonas* genes, were able to accumulate PHA in comparable amounts to the wild type strain (Figure 1).

Recombinant LFM1034, harbouring the operon from *Aeromonas sp.* TSM81, showed the highest increase on 3HHx content (14.09±0.34 mol%). The expression of the operon genes allowed the targeting of a greater quantity of β-oxidation intermediates for the synthesis of PHA. This increase was easily justified by the presence of PHA synthase from *Aeromonas*, an enzyme with higher affinity for MCL monomers (Fukui and Doi, 1998; Fukui et al., 1999; Fukui et al., 2002; Lu et al., 2003). Similar results were obtained by Park et al. (2001) and Lu et al. (2004b) for recombinant *E. coli* with lauric acid as the carbon source.

Cloning of the *phaC* gene from *A. hydrophila* did not result in the formation of the desired copolymer, despite restoring the ability of accumulating PHA. Recombinants with *phaJ* and *phaPC* did not reveal significant change in PHA compared to wild type strain (0.72±0.11 mol% and 1.88±0.34 mol%, respectively).

On the other hand, simultaneous expression of *phaJ* and *phaC* increased 3HHx content (7.84±0.29 mol%). Therefore, it can be concluded that the presence of the complex *phaCJ* is necessary to increase directing 6 carbons intermediates of β-oxidation pathway for synthesis of PHA. Similar results were obtained for recombinant strains of *E. coli* (Fukui et al., 1999; Lu et al., 2003) and for *A. hydrophila* when *phaCJ* was overexpressed (Han et al., 2004).

Bioreactor experiment with strain LFM1034 in mineral medium produced 15.95 g cells per litre, with 70.84% of PHA and 22.24 mol% 3HHx. Similar experiments with the wild type strain yielded cells at14.40 g/L, with 74% of PHA and 0.66 mol% 3HHx. These results reinforced previous investigations that the presence of genes of *Aeromonas* significantly promotes the formation of P3HB-co-3HHx.

**Conclusion**

Recombinant and wild type strains studied in this work allowed the production of P3HB-co-3HHx, controlling the 3HHx contents from 0.6 to 22 mol%, a high value compared to what has been reported in the literature. Therefore, with this adopted strategy, one can produce biodegradable polymers with controlled monomer fractions appropriated for specific applications.
REFERENCES


PRODUCTION OF PLASTIC FILMS BY BACTERIA FROM CARBOHYDRATES

By

L.G. CESPEDES, M.K. TACIRO, L.F. SILVA, R.R. TAVARES,
T.T. MENDONÇA and J.G.C. GOMEZ
Institute of Biomedical Sciences – University of São Paulo
lucas.cespedes@usp.br

KEYWORDS: Polyhydroxyalkanoates, Aeromonas, Pseudomonas, PHA Synthase, Carbohydrates.

Abstract

POLYHYDROXYALKANOATES (PHA) are polyesters accumulated by bacteria as intracellular granules from renewable carbon sources. These bio-based polymers have properties similar to industrial plastics which attract great industrial interest especially since they are biodegradable. An undesirable property of one of these polyesters, poly-3-hydroxybutyrate (P3HB), is its low tensile strength. In this study we modify P3HB to improve this property by engineering the polymer to contain a portion of medium-chain-length monomers (C6-C14) in P3HB chain. The result is increased tensile strength of plastic films which are more flexible. In this work, mutants of Pseudomonas sp. unable to produce PHA were used as host strains for PHA synthesis genes from Aeromonas sp (phaPCJ) through the broad host range plasmid pBBR1MCS-2. Three recombinants of Pseudomonas were obtained that produced different compositions of the copolymer P3HB-co-3HHx-co-3HO-co-3HD depending on growth conditions and on gene expression. The most notable recombinant, LFM 461 phaPC, accumulates PHA at up to 39% of the cell dry weight in mineral salts medium containing glucose as the sole carbon source. These recombinants were constructed based on the ability of Pseudomonas to supply monomers of medium-chain-length 3HA (C6-C14) and the high affinity of PHA synthase (enzyme responsible for PHA production from monomers) from Aeromonas to 3HB and 3HHx. This work opens the possibility of polymer biosynthesis for the production of plastic films from carbohydrates in biorefineries.

Introduction

Polyhydroxyalkanoates (PHA) are a family of thermoplastic, biodegradable and biocompatible polymers which are accumulated by a wide range of bacteria as intracellular granules. The monomer composition in the polymer chain determines polymer characteristics such as melting temperature, crystallinity, and rupture strength.

These monomers can be classified as short-chain-length (HA_{SCL}) when monomers are C3-C5 and medium-chain-length (HA_{MCL}) when they are C5-C12. The copolymers of HA_{SCL} and HA_{MCL} have been studied in order to develop new applications for these materials. Although more than 150 monomers have been found as PHA constituents in nature, copolymers combining HA_{SCL} and HA_{MCL} are naturally produced only by a few bacteria (e.g. Aeromonas) and in low amounts when plant oils or fatty acids are supplied; thus, the carbon sources which could be used are restricted.

This can be attributed to the PHA synthase specificity and to the availability of HA_{SCL} and HA_{MCL} in the bacterial intracellular metabolic pool. However, the tools of modern molecular biology allow us to manipulate and engineer bacteria to give them the ability to produce these biopolymers with a range of new properties to obtain features not observed in natural strains.
Pseudomonas sp. LFM 461, a strain deficient in PHA accumulation due to a UV mutation on its PHA synthase gene (phaC), was used as the host for genes related to PHA biosynthesis in Aeromonas sp. (operon phaPCJ). It was expected that the resulting recombinant would produce P3HB-co-HAMCL since PHA synthases of Aeromonas sp. have greater specificity for HAMCL when compared to other class I PHA synthases. On the other hand, LFM 461 has metabolic pathways able to provide monomers for both HASCL and HAMCL.

Methodology

Primers from Lu et al. (2004) and others were re-designed based on Seshadri et al. (2006) and used to amplify phaP, phaC, and phaJ genes from Aeromonas hydrophyla ATCC 7966 and Aeromonas sp. TSM 81 (Matsuda, 2009) in different combinations. Restriction sites for appropriate cloning in the plasmid pBBR1MCS-2 were designed into the 5’ ends.

The amplicons cloned in pBBR1MCS-2 were introduced in E. coli XL-1 Blue (Stratagene) by chemical transformation (Sambrook and Russell, 2001). The recombinant clones were selected in LBK (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl and 50 mg/L kanamycin). For confirmation of successful cloning, blue/white selection using IPTG/XGal as well as PCR screening were employed.

Positive clones were confirmed by plasmid digestion and sequencing of the inserted DNA. Plasmids harbouring the appropriate genes were recovered and introduced into E. coli S17-1 by chemical transformation. The plasmids were transferred from E. coli S17-1 to Pseudomonas sp. LFM461 by conjugation.

PHA production assays of LFM 461 recombinant strains were carried out in shaker-flasks. Each recombinant clone was cultured in LBK (24 h, 30°C). These cultures were used to inoculate mineral medium (Ramsay et al., 1990) containing 15 g/L of glucose as the sole carbon source and 50 mg/L of kanamycin and incubated (72 h at 30°C, 150 r/min). Cell dry weight (CDW), pH, PHA content and composition were measured at the end.

Results and discussion

Three genetic constructions were expressed in Pseudomonas sp. LFM461: A. hydrophyla phaPC (phaPC_A.h.), A. hydrophyla phaCJ (phaCJ_A.h.), Aeromonas sp. TSM 81 phaPCJ (phaPCJ_TSM81). The results of PHA production assays are shown in Table 1. Pseudomonas sp. LFM461 hosting only the vector (pBBR1MCS-2) as well as the vector harbouring the PHA synthase gene from Ralstonia eutropha phaC (phaC_R.e.) were used as controls. The recombinant strain hosting only pBBR1MCS-2 did not produce PHA as expected. The expression of phaC_R.e. led to accumulation of PHA corresponding to 25–30% of cell dry weight. Besides 3-hydroxybutyrate (3HB), 3-hydroxyhexanoate (3HHx) and 3-hydroxyoctanoate (3HO) were also detected, as previously observed by Gomes (2010).

The expression of the entire Aeromonas sp. TSM81 PHA operon (phaPCJ_TSM81) led to PHA accumulation corresponding to 25–30% of CDW. The polymer composition revealed the higher affinity of PHA synthase from Aeromonas for HAMCL which represented about 23 mol % regardless of the addition of Isopropylthio-β-galactoside (IPTG).

It was expected that addition of IPTG (Lac inducer) would increase the gene expression since the Lac promoter is located upstream of the PHA biosynthesis genes. Interestingly 3-hydroxydecanoate (3HD) was also detected as a constituent of PHA produced by this recombinant strain.

LFM 461 phaCJ_A.h. strain achieved similar results compared to LFM461 phaPCJ_TSM81 (about 20.2% of PHA containing 26 mol % of HAMCL).

Finally, the strain LFM 461 phaPC_A.h. showed the best results reaching 3.68 g/L of CDW containing 38.9% of PHA. The PHA composition of LFM 461 phaPC_A.h. strain was singular, with 52.0 mol % of 3HB, 27.0 mol % of 3HHx, 6.5 of 3HO and 14.6 mol % of 3HD.
Table 1—PHA production from glucose by *Pseudomonas* sp. LFM461 recombinant strains harbouring PHA synthesis genes from *Aeromonas* sp.

<table>
<thead>
<tr>
<th>Recombinant strains</th>
<th>CDW (g/L)</th>
<th>pH</th>
<th>PHA composition (mol%)</th>
<th>PHA (%CDW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3HB</td>
<td>3HHx</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>Avg</td>
<td>2.20</td>
<td>4.76</td>
<td>–</td>
</tr>
<tr>
<td>LFM461 pBBR1MCS-2</td>
<td>Var</td>
<td>+0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>Avg</td>
<td>2.43</td>
<td>4.80</td>
<td>–</td>
</tr>
<tr>
<td>LFM461 pBBR1MCS-2</td>
<td>Var</td>
<td>+0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>Avg</td>
<td>2.93</td>
<td>5.03</td>
<td>94.71</td>
</tr>
<tr>
<td>LFM461 pBBR1MCS-2::phaC-Re.e.</td>
<td>Var</td>
<td>+0.01</td>
<td></td>
<td>+0.59</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>Avg</td>
<td>3.24</td>
<td>5.24</td>
<td>95.85</td>
</tr>
<tr>
<td>LFM461 pBBR1MCS-2::phaC-Re.e.</td>
<td>Var</td>
<td>+0.00</td>
<td></td>
<td>+0.05</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>Avg</td>
<td>2.83</td>
<td>5.89</td>
<td>76.53</td>
</tr>
<tr>
<td>LFM461 pBBR1MCS-2::phaPCjTSM81</td>
<td>Var</td>
<td>+0.02</td>
<td></td>
<td>+1.02</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>Avg</td>
<td>2.98</td>
<td>6.05</td>
<td>77.20</td>
</tr>
<tr>
<td>LFM461* pBBR1MCS-2::phaPCjTSM81</td>
<td>Var</td>
<td>+0.00</td>
<td></td>
<td>+4.59</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>Avg</td>
<td>3.68</td>
<td>6.44</td>
<td>52.00</td>
</tr>
<tr>
<td>LFM461 pBBR1MCS-2::phaCpa.h.</td>
<td>Var</td>
<td>+0.02</td>
<td></td>
<td>+2.39</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>Avg</td>
<td>3.13</td>
<td>6.40</td>
<td>73.36</td>
</tr>
<tr>
<td>LFM461 pBBR1MCS-2::phaCpa.h.</td>
<td>Var</td>
<td>+0.01</td>
<td></td>
<td>+1.20</td>
</tr>
</tbody>
</table>

Average of results for biological triplicates and analytical duplicates. Avg – average; Var – variance. CDW – Cell dry weight; 3HB- 3-hydroxybutyrate; 3HHx- 3-hydroxyhexanoate; 3HO- 3-hydroxyoctanoate; 3HD- 3-hydroxydecanoate; PHA- Polyhydroxyalkanoate.

To evaluate PHA production in the bioreactor, an assay with LFM 461 *phaPCjTSM81* strain was carried out. Kinetics of carbon source consumption as well as residual biomass and product formation were evaluated (Figure 1).

After 10 h of culture, a solution containing glucose (330 g/L) and ammonium sulfate (23 g/L) was fed in a controlled manner: 0.20 mL/min for 12 h, 0.15 mL/min for 4 h and 0.11 mL/min in the last 11 h of the assay.

After 41 h of culture, LFM 461 *phaPCjTSM81* reached a total biomass (X) of 7.51 g/L with a PHA content of 20.70%. A lag phase of about 2 h was observed followed by an exponential growth phase of about 8 h with 0.32/h of specific growth rate. After exhaustion of nitrogen in the culture medium (10 h), PHA started to accumulate (Figure 1).

PHA analysis of the bioreactor cultures revealed a different composition from that obtained in shaken-flask experiments. A high amount of HA_{MCL} was detected in the beginning of the accumulation phase followed by a reduction at the end.

At the end of the assay, the PHA composition was: 50.7% 3HB, 37.0% 3HHx, 3.3% 3HO and 9.0% 3HD. Polymer accumulation was observed even after 41 h of growth, despite the increasing nitrogen available at the end of the assay.
Conclusions

The genes from operon phaPCJ from two *Aeromonas* strains were cloned and expressed in *Pseudomonas* sp. LFM 461.

The recombinant strains produced PHA with different compositions depending on culture conditions.

Strain LFM 461 *phaPCJ* gave variable PHA composition throughout cultivation in the bioreactor.

This study opens the possibility to produce P3HB-co-3HAc using carbohydrates as the sole carbon source.

REFERENCES


APPLICATION OF CHLOROPLAST DNA MARKERS (cpSSRS) IN WILD ACCESSIONS OF SUGARCANE AND ERIANTHUS

By

M.N.G. MELLONI¹, L.R. PINTO¹, M.A. XAVIER¹, S. CRESTE¹, L.A. CARLINI-GARCIA³ and M.G. LANDELL¹

¹IAC/Centro de Cana-de-Açúcar, C.P. 206 – CEP 14001-970 – RIBEIRÃO PRETO, SP, Brasil
²Universidade Estadual Paulista, Campus Jaboticabal, Depto Ciências Exatas, pesquisador bolsista CNPq
³Agência Paulista de Tecnologia dos Agronegócios, Departamento de Descentralização do Desenvolvimento, Pólo Centro Sul

natmell@ig.com.br

KEYWORDS: Polymorphism, Genetic Similarity, Sugarcane.

Abstract

CHLOROPLAST DNA DERIVED markers can provide important information regarding phylogenetic relationships among different species. In this work, we used five chloroplast DNA (cpSSRs) primers in a population of 83 individuals of the IAC sugarcane germplasm collection. The statistical analysis generated a genetic similarity matrix ranging from 0.95 to 0. There was a low polymorphism among members of Saccharum and Erianthus and the 5 primers were not sufficient to detect large differences between the accessions. The dendrogram clustered the accessions in 3 groups. The first one was formed by S. spontaneum, S. barberi and S. officinarum accessions, the second one only by S. officinarum accessions and the third one by S. spontaneum, S. barberi, S. officinarum, S. robustum and Erianthus accessions. For a better understanding of the variability and interrelationships among wild sugarcane accessions, another type of markers with higher levels of polymorphism should be used to complement the cpSSRs.

Introduction

The chloroplast genome of plants is haploid, non-recombinant, uniparental maternal inheritance in most angiosperms and varies slightly among species showing uniformity in weight, structure and genes (Shaw et al. 2007; Olmstread and Palmer, 1994).

Based on these features, studies related with cpDNA have been conducted in different areas of biology, but mainly in molecular phylogeny, plant systematics and phylogeographic studies of genetic variability among and within populations (Hanza, 2010).

The present work aims to study the genetic variability among sugarcane accessions available at the IAC germplasm collection through chroloplast microsatellite (cpSSRs) markers.

Material and methods

Eighty-three accessions of the genus Saccharum and Erianthus were characterised with 5 cpSSRs primer pairs (Sac-3, Sac-10, Sac-13, Sac-17 and Sac-23) obtained from Cesare et al. (2010). The accessions evaluated were: 75/09 (Erianthus), Chunee (S. barberi), IM76229 (S. robustum), IN8488 (S. spontaneum), Krakatau (S. spontaneum), Zwart Manila, Zopilota, IJ76560, Maneira, Chin (S. barberi), IJ76293 (S. robustum), IN8485 (S. spontaneum), SES205A (S. spontaneum), IJ76317, NG5750 (S. officinarum (hybrid)), NG57213, Gandacheni (S. barberi), 57NG12 (S. robustum), US5714105 (S. robustum), IN8482 (S. spontaneum), US851008
(S. spontaneum), NG2121, IN84105, IJ76418, Red, Caiana fita, Sac.off.8276, IJ76325, Pitu, Badila, IN8446, White transparent, IS76116, Caiana manteiga, Sac.of.8284, Cana blanca, Caiana listrada, Ajax, Badila de Java, MZ151, Crioula raiada, IJ76566, Formosa, Cana alho, IS76155 (S. spontaneum), Manall, Creoula, NG57221, Sac.off.8272, Sabura, Louisana Stripes, NG7718, IJ76313, Flor de Cuba, Caiana verdadeira, IN84126, Brava de Perico, Caiana riscada, Fiji62, Caiana roxa, Sac.off8280, NG7792, Mali, HJ5741, Muntok Java, Fiji19, Green German, Caram red, Black Borneo, Lam76427, Midas, Demos, Mundau, Cinca77318, D152, Yasawa, Endor, Zeus, Q117, Ragnar, Mali, MALI, IJ 76-326, NG576 (S. officinarum (hybrid)) and Cayana.

The PCR reactions and amplification conditions were described by Cesare et al. (2010). The samples were separated by electrophoresis on 5% polyacrylamide gel and silver staining according to Creste et al. (2001).

The markers were genotyped based on their presence (1) and absence (0). The estimation of the genetic relationships among all the accessions was based on the dissimilarity (D) estimated as the complement of the similarity (S) calculated with the Jaccard coefficient (D=1-S). The dendrogram was constructed by Neighbour Joining method using the Darwin 5 program (Perrier and Jacquemoud-Collet, 2006).

Results and discussion

The five cpSSRs produced a total of 23 markers which detected low polymorphism among the accessions. The genetic dissimilarity ranged from 0 to 95% with an average of 25%. This may be due to the low number of primers evaluated and the heterologous nature of the primers as they were developed for Miscanthus.

In fact, a high polymorphism was reported by Cesare et al. (2012) for these primers in five species of Miscanthus while a low polymorphism were observed for accessions of S. officinarum and S. spontaneum.

Takahashi et al. (2004) pointed out that the sugarcane chloroplast genome has a very close relationship among species, and is closely related suggesting a recent rapid evolution of the genus Saccharum with the five Saccharum species showing no or very little intraspecific variation.

According to the dendrogram (Figure 1), many S. officinarum accessions exhibited great genetic similarity which agrees with Nair et al. (1998), and that affirmed that the species has a low genetic diversity due to prolonged directional selection for sucrose content added to the continued vegetative propagation of selected native accessions in New Guinea and Indonesian habitats.

The dendrogram clustered the 83 accessions (Figure 1) in 3 groups. The first one was formed by S. spontaneum, S. barberi and S. officinarum accessions, the second one only by S. officinarum accessions and the third one by S. spontaneum, S. barberi, S. officinarum, S. robustum and Erianthus accessions.

The individuals of S. spontaneum are grouped in the third group, with the exception of two accessions (IN8482 and US851008) which are hybrids of S. spontaneum.

Studies with chloroplast sequences revealed that S. robustum accessions are more related to S. officinarum accessions (Takahashi et al., 2004). This close relationship is due to the fact that S. robustum is considered the immediate progenitor of S. officinarum (Nair et al., 1998). In our work with cpSSRs, S. robustum accessions were more related to S. spontaneum than S. officinarum.

Three alleles (2 alleles from Sac-10 and 1 allele from Sac-13) were exclusive of the Erianthus accession. Similar results were reported by Nair et al. (1998) with RAPD in which distinct markers were observed in Erianthus. In fact, the genus Erianthus is well differentiated from the genus Saccharum (Grivet et al., 2004), but it can contribute substantially to sugarcane improvement, in introgression programs.

The information generated in this study allied to the one obtained by other types of molecular markers may allow a better understanding of the variability among wild sugarcane accessions.
Fig. 1—Dendrogram of ‘Saccharum Complex’ accessions generated by cpSSR markers.
REFERENCES


MOLECULAR CHARACTERISATION OF ‘SACCHARUM COMPLEX’ ACCESSIONS FOR GENETIC INTROGRESSION

By

M.N.G. MELLONI\textsuperscript{1,2}, L.R. PINTO\textsuperscript{1}, M.L.G. MELLONI\textsuperscript{1,2}, L.A. CARLINI-GARCIA\textsuperscript{3}, S. CRESTE\textsuperscript{3}, M.A. XAVIER\textsuperscript{1} and M.G. LANDELL\textsuperscript{1}

\textsuperscript{1}IAC/Centro de Cana-de-Açúcar, C.P. 206 – CEP 14001-970 – RIBEIRÃO PRETO, SP, Brasil
\textsuperscript{2}Universidade Estadual Paulista, Campus Jaboticabal, Depto Ciências Exatas, pesquisador bolsista CNPq
\textsuperscript{3}Agência Paulista de Tecnologia dos Agronegócios, Departamento de Descentralização do Desenvolvimento, Pólo Centro Sul

natmell@ig.com.br

KEYWORDS: Molecular Markers, Polymorphism, Genetic Similarity, Sugarcane, Germplasm Collection.

Abstract

The incorporation of wild germplasm as sources of new genes related to fibre and ratooning, among other traits, is one of the strategies used to promote significant increases in biomass. The present study aimed to characterise the level of polymorphism and the genetic similarity of 81 accessions of the ‘Saccharum Complex’ from the Campinas Agronomic Institute (IAC) germplasm collection using microsatellites (SSRs). The 30 SSRs tested generated 427 markers. The greatest genetic diversity (0.865) was observed between 75I109 (Erianthus) and NG57221 (S. officinarum), while the lowest genetic diversity (0.177) was found between the noble canes: Caiana Manteiga and Crioula Raiada. The analysis of a dendrogram produced showed three large groups: one with only S. officinarum accessions, another with Erianthus, S. robustum, S. barberi, S. officinarum and S. spontaneum accessions and a third one with S. officinarum and S. spontaneum accessions. The information generated with the molecular markers will allow to avoid duplicate accessions and also promote a better knowledge of the genetic variability among the accessions, particularly those that arose in Brazil after the first sugarcane introductions for which little or no molecular information is available in the literature.

Introduction

Sugarcane is an important economic crop which generates several products and incomes. Currently, sugarcane breeding programs have been challenged to develop new sugarcane varieties with high biomass as a source of electricity and second generation ethanol.

Significant increase in biomass can be achieved through the use of wild sugarcane germplasm in introgression programs, specially S. spontaneum and S. robustum.

Therefore, the molecular characterisation of sugarcane germplasm collections can contribute to the effective management of these collections by avoiding duplicate accessions and also allow a better understanding of the genetic variability of the species to be used in the introgression programs (Creste et al., 2010).

The present study aimed to characterise, through the use of microsatellite markers, accessions from ‘Saccharum Complex’ of the Campinas Agronomic Institute.
Materials and methods

Eighty-one accessions were characterised with 30 microsatellite primer pairs (SSRs) (list not provided). The 81 accessions were: 751109 (Erianthus), Chunee (S. barberi), IM76229 (S. robustum), IN84888 (S. spontaneum), Krakatau (S. spontaneum), Zwart Manila, Zopilota, IJ76560, Maneira, Chin (S. barberi), IJ76293 (S. robustum), IN8485 (S. spontaneum), SES205A (S. spontaneum), IJ76317, NG5750 (S. officinarum (hybrid)), NG57213, Gandacheni (S. barberi), 57NG12 (S. robustum), US5714105 (S. robustum), IN8482 (S. spontaneum), US851008 (S. spontaneum), NG2121, IN84105, IJ76418Red, Caiana fita, Sac.of.8276, IJ76325, Pitu, Badila, IN8446, White transparent, IS76116, Caiana manteiga, Sac.of.8284, Cana blanca, Caiana listrada, Ajax, Badila de Java, MZ151, Crioula raiada, IJ76566, Formosa, Cana alho, IS76155 (S. spontaneum), ManaII, Creoula, NG57221, Sac.of.8272, Sabura, Lousiana Stripes, NG7718, IJ76313, Flor de Cuba, Caiana verdadeira, IN84126, Brava de Perico, Caiana riscada, Fiji62, Caiana roxa, Sac.of.8280, NG7792, HJ7541, Muntok Java, Fiji19, Green German, Ceram red, Black Borneo, Lam76427, Midas, Demos, Mundau, Cinca77318, D152, Yasawa, Endor, Zeus, Q117, Ragnar, Mali, MALI and Cayana.

DNA extraction was performed according to Al Janabi et al. (1999) and PCR reactions according to Oliveira et al. (2009). The amplification products were separated by electrophoresis on 5% (55 W constant) denaturing polyacrylamide gel on a Licor 4300 DNA Analyser for the SSR primer pairs labelled with IR700 or IR800 fluorescent dyes, whereas, for the unlabelled primer pairs, the amplification products were revealed by silver staining (Creste et al. 2001).

The markers were genotyped based on their presence (1) and absence (0). The estimation of the genetic relationships among all the accessions was based on the dissimilarity (D) estimated as the complement of the similarity (S) calculated with the Jaccard coefficient (D=1-S).

The dendrogram (Figure 1) was constructed by Neighbour Joining method using the Darwin 5 program (Perrier and Jacquemoud-Collet, 2006).

Fig. 1—Dendrogram of the 81 accessions of the ‘Saccharum Complex’.
Results and discussion

The 30 primer pairs generated 427 markers, which were used to estimate genetic dissimilarity. The greatest genetic diversity (0.865) was observed between 75II09 (Erianthus) and NG57221 (S. officinarum). The Erianthus genus is an important member of the 'Saccharum Complex'. They are used in sugarcane breeding due to their good adaptability and resistance to pests and diseases (Nair and Praneetha, 2005).

Six markers exclusive to the Erianthus accession were observed and evaluated. The analysis of the dendrogram (Figure 1) showed three large groups: one formed only by S. officinarum accessions (Group 1), another formed by Erianthus, S. robustum, S. barberi, S. officinarum and S. spontaneum accessions (Group 2) and a third formed by S. officinarum and S. spontaneum accessions (Group 3).

The accessions IJ76293 and IM76229 showed a low dissimilarity (0.098), suggesting that they may be the same genotype (duplicate or mislabelled accessions). Two accessions labelled as Mali had a low dissimilarity score (0.07), confirming them both as Mali. Crioula Raiada and Caiana Manteiga showed a low dissimilarity between them (0.177).

Crioula and Caiana were the first sugarcanes introduced to Brazil (Figueiredo, 2008). Therefore, it is likely that these two accessions (Crioula Raiada and Caiana manteiga) may have a progenitor in common. It is possible that natural hybridisation occurred between Caiana and Crioula followed by local selection to attractive types and smooth sugarcane types by native people giving rise to Crioula Raiada and Caiana Manteiga.

Studies with molecular markers are extremely important for crops with unknown ancestors and with no accurate or even non-existing pedigree records (Farooq and Azam, 2002).

The present work contributes to the improvement of sugarcane as it will allow the effective management of the IAC sugarcane germplasm collection and increase knowledge of the genetic variability among the accessions, particularly those that arose in Brazil after the first sugarcane introductions and for which little or no molecular information is available in the literature.

REFERENCES


LINKAGE MAPPING OF SUGARCANE VARIETY CP 67412 TO IDENTIFY MARKERS LINKED TO EARLY RIPENING

By

Y. PARMESSUR1, G. BADALOO1, R. MING2
and A. DOOKUN-SAUMTALLY1

1Mauritius Sugarcane Industry Research Institute,
Mauritius Cane Industry Authority, Réduit, Mauritius
2Department of Plant Biology,
University of Illinois at Urbana-Champaign, Urbana, IL 61801-3873, USA

Yogesh.parmessur@msiri.mu

KEYWORDS: Restricted Site Associated DNA Sequencing (Radseq), Illumina Hiseq 2000 Sequencer, QTL Mapping.

Abstract

In Mauritius where sugarcane is generally grown as a 12 month crop, the harvest period starts in mid-June/early July to end in November/mid-December. The sucrose accumulation pattern varies between varieties during the maturing period and four categories namely; precocious, early, middle and late are reported. In order to benefit from the improved sugar yield throughout the harvest period, it is recommended that varieties of the different categories are cultivated. With these objectives, the MSIRI is looking for early ripening, high sucrose genotypes in its breeding program. In order to meet these objectives, molecular tagging of QTLs for these traits are being studied based on the construction of a genetic linkage map of an early ripening genotype followed by QTL mapping for the trait. Ten parental clones of either early ripening/high sucrose x late ripening/low sucrose involved in the production of crosses of seven genetic combinations were screened with six microsatellite primers. Ninety four markers were scored and the genetic distance (GD) among the parents was calculated using NTSys software. Based on their GD and the ripening pattern, parents CP 67412 (precocious/high sucrose) and M 245/76 (late/low sucrose) were selected to produce 477 progeny. The population was planted in the field for bulking material for replicated trials. A set of 600 sorghum EST SSRs was used to screen the two parents and more than two thirds were found polymorphic and were selected for linkage map construction of both varieties. With the development of next generation sequencing, a novel approach, the Restricted Site Associated DNA Sequencing (RADSeq), is being applied for linkage mapping of parent variety CP 67412.

Introduction

In Mauritius, the sugarcane harvest period starts in mid-June/July and ends in November/December. At the beginning of the harvest period, sucrose content of cane stalks is relatively low and it generally increases towards the end of the season. The development of high sucrose and early-ripening varieties is an important objective of research especially for the early part of the milling season where sugar yields are sub-optimal as a result of low sucrose content. Demand for high sucrose and early-ripening varieties is expected to accrue in view of the centralisation of milling operations and changes on date of harvest being brought earlier.

Since the genetics and physiological processes leading to early ripening of sugarcane is poorly understood, identification of parental lines able to produce early ripening progenies remains
challenging. Furthermore, field selection for early ripening is highly labour and resource intensive. Marker assisted selection is thus being sought for implementation in the sugarcane breeding program in order to reduce total dependency on field selection. This paper hereby describes the progress achieved in the identification of molecular markers linked to early ripening of sugarcane.

**Material and methods**

**DNA extraction**

Total genomic DNA was extracted from sugarcane using the CTAB extraction method as described by Aljanabi et al. (1999). Instead of meristematic tissue, leaf blade (1.0 g), finely ground in liquid nitrogen, was used. DNA was checked on agarose gel for its quality and its concentration was measured using a biophotometer. DNA was diluted to 100 ng/µL and stored at 4 °C for further use.

**Genetic diversity of putative ‘mapping parents’**

Parental clones used for early ripening/high sucrose × late ripening/low sucrose crosses were tested for the genetic distance (GD) using SSR profiling. Six SSR primers were used to genotype ten parents used in seven such available crosses.

The following reaction conditions were used; 1X reaction buffer, 0.4 µM dNTPs, 1 unit Taq polymerase 0.4 µM fluorescently labelled forward primer, 0.4 µM reverse primer and 50 ng DNA template in 20 µL reaction volumes. PCR conditions for sorghum EST-SSR were as follows; an initial denaturation at 94 °C for 3 min followed by 32 cycles at 94 °C for 30 s, annealing at 50 °C and extension at 72 °C each for 45 s and a final extension at 72 °C for 5 min. PCR products were denatured in deionised formamide for 5 min and quenched on ice.

PCR products were run on an automated ABI 310 Genetic Analyser. Electropherograms were analysed using Genotyper v3.1 and Genescan v2.0 software. Alleles were manually scored and analysed using the GD matrix constructed using the SAHN clustering from the NTSys software.

**SSR primer screening of mapping parents**

Six hundred sorghum EST-SSR primers were screened on DNA of parents CP 67412 and M 245/76 for polymorphism. PCR reactions and cycling conditions were similar to above except that the reverse primer was labelled with 33P and the forward primer was not labelled.

Amplified products were run for 2 h on 6% polyacrylamide gels, pre-warmed at about 60 °C. Gels were blotted on filter paper, vacuum dried and exposed to X-ray films. Depending on the intensity of the 33P signal, films were developed 2–5 days after exposure.

**RADSeq genotyping of mapping population.**

The protocol used for sugarcane RADSeq is a modification of the method described by Pfender et al. (2011). DNA from the mapping parents and progeny population (260) was digested with enzymes MseI and NsiI. Digested fragments were ligated with Illumina adaptors also known as Barcodes and Index. Ligated fragments were amplified, size selected after excision from agarose gel and equally pooled into three sequencing libraries. The libraries were sequenced using the Illumina HiSeq 2000 Sequencer. Sequence data were retrieved from each individual and aligned against the *Sorghum bicolor* sequence genome. SNP between the mapping parents and the mapping population was scored for segregating SNPs.

**Results and discussion**

A prerequisite for bi-parental linkage mapping is to use parents with maximum GD and with extreme divergence in the trait under study. This is useful in capturing maximum polymorphic markers between the mapping parents to be used in linkage map construction, whereas diverging traits are useful in capturing maximum frequency of Quantitative Trait Loci (QTLs).

Among the seven crosses tested, the CP 67412 X M 245/76 cross showed the highest GD. This is also the only cross where one of the parents was tagged as precocious ripening due its very early sucrose accumulation, making it the ideal cross for this QTL mapping study.
A population of more than 477 individuals was therefore established from seeds derived from this cross in the field for further studies.

Sorghum EST-SSR (600) was selected for linkage mapping since their design was based on their even in silico coverage of the rice genome. Sorghum EST-SSR has the added advantage of being gene-based and one of the closest genus to Saccharum sp., both derived from a common ancestor about 8 million years ago (Wang et al., 2010).

This makes sorghum EST-SSR markers ideal for comparative mapping between the two genera. Screening for polymorphism between the two mapping parents revealed more than two thirds polymorphic primers i.e present in CP 67412 and absent in M 245/76 with an average of 1.8 polymorphic markers. Depending on their frequency and in silico distribution across the sorghum genome, a selection of sorghum EST-SSR will thus be mapped on the two sugarcane parental lines.

Over the past two decades, molecular markers have evolved from the tedious low throughput restriction fragment length polymorphism (RFLP) to the latest high throughput, next generation sequence-based genotyping platform. Here we investigated the application of RADSeq as a novel approach to the genotyping of sugarcane. Up to 192 million reads were obtained from one sequencing lane. Sequence reads per individual did show a normal distribution with the majority producing 1.6 to 2 million reads.

Preliminary results enabled identification of at least 10 000 SNP markers between the two parents. This figure is based on an algorithm whereby there is an initial alignment of the parental sequence to a reference sorghum genome and identification of SNP markers, hence is based on conserved sequences. This figure is likely to increase considerably when the reference genome sequence of the Saccharum sp monoploid genome is available and/or if the alignment is based solely between the mapping parents.

Increasing template concentration of the mapping parents by threefold as compared to the mapping population individuals equally produced a threefold increase in sequence reads and an equivalent increase in SNP marker detection. These demonstrate that the potential of RADSeq in SNP marker detection is yet to be disclosed with some additional optimisation.

With the large size and complex genome of sugarcane, the major challenge to the application of RADSeq remains the reduced representation of the sugarcane genome to a size small enough for maximum coverage without compromising on the high SNP marker throughput. However, with the falling cost of next generation sequencing, re-sequencing provides a viable solution to this limitation.

The high throughput, low cost of RADSeq thus clearly provides a promising solution to the molecular mapping of sugarcane and will be further applied to this work.

Acknowledgements

The authors extend their acknowledgements to the ACP Secretariat and the European Commission for providing the necessary financial assistance to conduct this work, which forms part of the ACP Sugar Research Program funded by the EU.

REFERENCES


A PROFILE OF LIGNIN AND RELATED GENE EXPRESSION
IN THE STEM OF TWO SUGARCANE VARIETIES

By

ALEXANDRA BOTTCHER1, IGOR CESARINO1, ADRIANA B. SANTOS1, RENATO VICENTINI2, JULYANA CRISTINA M.S. MOURA, JULIANA L. MAYER1, PAULA M. NOBILE1,3, SANDRA MARIA C. GUERREIRO1, SILVANA CRESTE3, IVAN A. ANJOS3, MARCOS G.A. LANDELL3 and PAULO MAZZAFERA1,*

1Dept Plant Biology-IB, State University of Campinas, CP 6109, 13083-970, Campinas-SP, Brazil
2CBMEG, State University of Campinas, Campinas-SP, Brazil
3Centre of Sugarcane, Institute of Agronomy at Campinas, Ribeirão Preto-SP, Brazil

* pmazza@unicamp.br

KEYWORDS: Sugarcane, Lignin, Gene Expression.

Abstract

A SYSTEMATIC STUDY of the genes involved in lignin biosynthesis was carried out in two varieties contrasting in lignin content (IACSP04-529 – high/IACSP04-683 – low) and these data were integrated with physiological, biochemical, histological and transcriptional (RNAseq) data. A stem profile analysis showed that lignin content is low in the top internodes, reaching a maximum in intermediary internodes and remaining constant in the older ones. Searching the SUCEST sugarcane database allowed the identification of 4 orthologous genes for the enzyme PAL, 5 for C4H, 3 for 4CL, 2 for HCT, 2 for C3H, 4 for CcoAOMT, 2 for CCR, 2 for COMT, 1 for F5H and 4 for CAD, which when analysed by RT-qPCR showed a complex differential expression considering the two varieties and the different parts of the stem, (rind and pith of young, intermediary and old internodes). Differences were also found when comparing the pith and the rind, which also differed in lignin amount and composition. In conclusion, our results showed that lignin biosynthesis varied in a single sugarcane stem in different ways, indicating a complex control.

Introduction

Lignocellulose refers to plant biomass composed of the polymers cellulose, hemicellulose, and lignin. Lignocellulose presents a highly complex nature due to its molecular structure and heterogeneity, where cellulose is arranged in a tight network of microfibrils embedded in a matrix of hemicellulosic polysaccharides that are covalently cross-linked with the heterogenic and complex structure of lignin (Vega-Sanchez and Ronald, 2010).

The recalcitrant nature of this mixture is one of the major obstacles to convert plant cell wall polysaccharides into fermentable sugars for biofuels production also known as second generation ethanol (Vanholme et al., 2010). In tropical countries, sugarcane bagasse is the most plentiful lignocellulosic material.

Currently, the bagasse is used for generation of heat and power for sugar processing into ethanol and also to produce electricity that is sold into the grid (Carroll and Somerville, 2009). Therefore, the production of second-generation ethanol can be advantageous for the sugarcane industry, once the sugarcane bagasse is promptly available after sucrose extraction for ethanol/sugar production.

Information on lignin metabolism in sugarcane is very scarce. In an attempt to broaden our knowledge in sugarcane, we performed a systematic study of the genes involved in lignin biosynthesis in two varieties contrasting in lignin content (IACSP04-529 – high/IACSP04-683 –...
We also integrated these data with physiological, biochemical, histological and transcriptional (RNAseq) data. This allowed us to trace a detailed signature of lignin and phenolics components in the sugarcane stem.

**Materials and methods**

Varieties IACSP04-529 and IACSP04-683 were grown at the Centre of Sugarcane, IAC/Apta, at Ribeirão Preto, São Paulo State, Brazil. The material harvested for analysis was from the first ratoon crop. The stems were separated in young internodes (internodes 1, 2, and 3), intermediary internodes (internodes 5, 6, and 7), and mature internodes (internodes 15, 16, and 17).

Each pool of internodes was separated into central (pith) and peripheral (rind) regions. Lignin analysis, Syringyl/Guaiacyl ratio, histochemical analysis, and gene expression studies were carried out according to established protocols reported in the literature. Genes were selected by analysing the SUCEST database (http://sucest-fun.org).

**Results and discussion**

A stem profile analysis using the Klason method showed that lignin content is low in the top internodes, reaching a maximum in intermediary internodes and remaining constant in the older ones. Histochemical observations using phloroglucinol-HCl confirmed these chemical analyses, and Maüle's reagent showed more intense staining for S units in IACSP04-683.

Using gas chromatography-mass spectrometry (GC-MS), we observed a higher S/G ratio in the rind of variety IACSP04-683 internodes when analysed separately from the pith.

Searching the SUCEST sugarcane database, we obtained 4 orthologous genes for the enzyme PAL, 5 for C4H, 3 for 4CL, 2 for HCT, 2 for C3H, 4 for CcoAOMT, 2 for CCR, 2 for COMT, 1 for F5H and 4 for CAD (see Figure 1 for genes in the lignin biosynthesis pathway).

Fig. 1—Lignin biosynthetic pathway and the enzymes involved.
RT-qPCR analysis showed a complex differential expression considering the two varieties and the different parts of the stem, which were analysed (rind and pith of young, intermediary and old internodes). Interestingly, different expression profiles were found for some phenylpropanoid genes when comparing pith and rind, which also differed in lignin amount and composition (Figure 2).
Fig. 2—Expression pattern of genes of the lignin biosynthesis pathway. PAL = phenylalanine ammonia-lyase; C4H = cinnamate 4-hydroxylase; C3H = p-coumarate 3-hydroxylase; CoAOMT = caffeoyl-CoA O-methyltransferase; CCR = cinnamyl-CoA reductase; ferulate 5-hydroxylase; COMT = Caffeic acid O-methyltransferase; CAD = cinnamyl alcohol dehydrogenase; 4Cl = 4-coumarate:coenzyme A ligase; HCT = p-hydroxycinnamoyl-CoA:quinone shikimate p-hydroxycinnamoyltransferase.

YP= young pith, IP= intermediate pith, MP= mature pith
YR= young rind, IR= intermediate rind, MR= mature rind

Noteworthy, S branch-specific genes (i.e. $F5H$ and $COMT$) were up-regulated with maturity and normally presented higher relative expression in the pith, which is in agreement with lignin results showing an increased S/G ratio in intermediary and mature pith when compared to young tissues. Conversely, the same genes were down-regulated in the rind samples, in which the maturation process favoured the deposition of G-units.

The lower transcript levels for some family members may be related with expressions in limited tissues or cell types, or their expression may be regulated by different environmental conditions. In conclusion, our results showed that lignin biosynthesis varied in a single sugarcane stem in different ways, indicating a complex control.

Acknowledgements
This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (grant BIOEN 2008/58035-6), and CAPES-Brazil and CNPq-Brazil (fellowships)

REFERENCES
SUGARCANE GENES CONFERRING TOLERANCE TO DROUGHT IN TRANSGENIC TOBACCO PLANTS

By

M. MENOSSI1, C.B. SOUZA1, A.A. HOSHINO1, G.C. BUENO1, E.R.B. CHAGAS1 and G.M. SOUZA2

1Universidade Estadual de Campinas
2Universidade de São Paulo
menossi@unicamp.br

KEYWORDS: Sugarcane, Drought Tolerance, Transgenic, Photosynthesis.

Abstract

Drought is a major stress affecting sugarcane productivity. Transcriptomic analyses have shown that drought modulates the expression of several genes, encoding proteins with a wide array of functions. To gain further insights on the role of the genes modulated by drought, we have used transgenic tobacco plants. We have overexpressed genes encoding proteins with similarity to a subunit of ATP synthase, a subunit of a cytochrome b6f and a protein with unknown function. Transgenic plants were submitted to drought stress during 2–3 weeks and compared to wild type plants. After rewatering, a higher fraction of surviving plants was observed among the transgenic plants. Chlorophyll content, photosynthesis and stomatal conductance were less affected by drought in the transgenic plants. Our data indicate that increased tolerance to drought can be achieved by the manipulation of several aspects of sugarcane metabolism. These genes are useful targets aiming at the production of sugarcane plants with increased tolerance to drought.

Introduction

Drought is the major abiotic stress affecting crops worldwide. Losses in sugarcane productivity, in the range of 5–15%, are common in many areas in Brazil due to drought stress, even in regions not considered as drought-prone. Different sugarcane cultivars display various levels of drought tolerance and the genetic basis of these differences is not known.

In a previous work (Rocha et al., 2007), we found 93 genes that had their expression levels modulated by drought in sugarcane plants. A wide array of gene functions was encoded by these genes, including transcription, phosphatases, fatty acid desaturases and CO2 assimilation.

Among the genes encoding proteins involved in CO2 assimilation, we found a gene encoding a protein with similarity with of the PetC subunit of the cytochrome b6f and another one encoding a homolog of a b-subunit of the ATP synthase.

The cytochrome b6f is involved in the electron transfer from photosystem I to photosystem II, creating a proton gradient that drives the ATP synthesis by ATP synthases in the chloroplast. Here, we show that overexpression of these two sugarcane genes can protect transgenic tobacco plants from drought.

Methods

Plant material

Tobacco (Nicotiana tabacum, var. SR1) wild-type seeds were germinated on Murashige and Skoog (MS) medium with 0.9% (w/v) agar. For tobacco transformation, leaf disks from plants with 4–7 leaves were used.
Gene constructs

The genes encoding the PetC subunit of the cytochrome b6f and a b-subunit of an ATP synthase were cloned using PCR. To this end, gene specific primers were used to amplify the coding regions from sugarcane cDNA clones obtained from the Brazilian Clone Collection Centre (Jaboticabal, Brazil). Each coding region was cloned in pRT104 (Topfer et al., 1987), under the control of the 35S promoter pRT-PetC and pRT-ATP. The expression cassettes from these vectors were excised using HindIII and cloned in pCAMBIA2301 (Cambia, Australia), producing constructs pCAMBIA2301-PetC and pCAMBIA2301-ATP.

Tobacco transformation

Leaf disks from wild-type tobacco plants were transformed with Agrobacterium tumefaciens LBA4404 containing either pCAMBIA2301-PetC or pCAMBIA2301-ATP. Transgenic cells were selected on media containing kanamycin.

Drought tolerance assays

Wild-type plants and T2 homozygous seeds from plants transformed with pCAMBIA2301-PetC and pCAMBIA2301-ATP were germinated as described before and grown for 2 months in pots containing 300 g of soil (Flores e Folhagens brand from Biomix, Brazil). Plants were watered or withheld from water, and gas exchange parameters were measured after 15 days using an Infrared Gas Analyser (IRGA, LCpro+; ADC Bioscientific, UK). Thereafter, plants from both groups were irrigated with water and the number of surviving plants was scored. For salt stress tolerance assay, leaf disks were incubated in liquid MS media containing varying concentrations of NaCl and leaf damage was scored visually. Three independent transgenic events were used for each gene construct.

Results

Plants overexpressing the PetC subunit of the cytochrome b6f

Tobacco plants overexpressing the PetC subunit had between 80 and 100% survival rates, while all wild type plants died. After 8 days of water withholding, transgenic plants were able to maintain photosynthetic rates between 2 and 4 times higher than wild-type plants. Stomatal conductance and transpiration also were higher in transgenic plants during water stress.

Leaf disks from wild-type and transgenic were subjected to salt stress as described above. Chlorophyll levels were quantified spectrometrically. At 0 and 200 mM NaCl, chlorophyll content in PetC plants was two times higher than in wild-type plants. At 400 NaCl, this protective effect was also observed.

Plants overexpressing the b-subunit of ATP synthase

In plants overexpressing the ATP synthase gene between 66.6 and 88.3% of the transgenic plants survived up to 15 days without watering, while only 16.6% of the wild type plants survived.

Leaf disks from wild-type and transgenic plants were incubated in MS media containing 0, 200, 300 and 400 mM NaCl for 5 days. We found that at 200 mM NaCl, while most of the leaf disks from wild-type plants presented chlorosis with areas with brown tissues, most transgenic plants remained almost unaffected. This protective effect was not observed at higher NaCl concentrations.

Conclusions

We observed that the overexpression of either the PetC subunit of the cytochrome b6f or the b-subunit of the ATP synthase had an impact on drought tolerance in transgenic tobacco plants. In the mesophilic cyanobacterium Synechocystis PCC 6803, reduced levels of PetC increased photo damage caused by high light intensity (Tsunoyama et al., 2009). Similarly, reduced ATP synthase levels in transgenic tobacco plants also increased the stress caused by light (Rott et al., 2011). These authors also found that acidification of the thylacoid lumen due to stress can inhibit photosynthesis, with deleterious effects on plant development.
Our data indicate that increased levels of both PetC and the b subunit of the ATP synthase have the potential to overcome at least in part the effects of drought stress in plants. These findings open new options for the production of transgenic sugarcane with increased tolerance to drought.

REFERENCES


MicroRNAs MODULATED BY DROUGHT STRESS IN THE BIOENERGY CROP SUGARCANE (SACCHARUM SPP.)

By

L.I. DIAS¹, A. GENTILE¹, T.H. FERREIRA¹, R.S. MATTOS¹, MONALISA S. CARNEIRO², GLAUCIA M. SOUZA³, TERCÍLIO CALSA JR.⁴, REJANE M. NOGUEIRA⁵, ENDRES⁶ and M. MENOSI³¹*

¹Universidade Estadual de Campinas, Campinas, Brazil  
²Universidade Federal de São Carlos, Araras, Brazil  
³Universidade de São Paulo, Brazil  
⁴Universidade Federal de Pernambuco, Brazil  
⁵Universidade Federal Rural de Pernambuco, Brazil  
⁶Universidade Federal de Alagoas

* menossi@unicamp.br

KEYWORDS: Sugarcane, miRNAs, Drought, Small Deep Sequencing, Field Condition.

Abstract

SUGARCANE (SACCHARUM SPP.) IS one of the most significant crops in the world. Drought stress is one of the main abiotic stress factors that reduce sugarcane yields considerably. However, which genes mediate plant responses to water stress remains largely unknown in several crop species. Even though several microRNAs have been studied during water stress in other species, the role of the microRNAs in sugarcane during drought stress has not been analysed. The aim of this work was to identify sugarcane miRNAs that are modulated by drought stress. Four sugarcane cultivars: RB867515, RB92579 (higher drought tolerance), RB855536 and RB72454 (lower drought tolerance) were grown under field conditions for eleven months under irrigation or rainfed. Several physiological parameters indicated that rainfed plants were subjected to drought stress. Using deep sequencing of small RNAs, we identified 18 miRNA families, from which 11 miRNAs were differentially expressed during drought stress. The targets of the miRNA modulated by drought were predicted using an in silico approach; many of these targets codified transcription factors that may play important roles in drought tolerance. These findings contribute to the elucidation of the complex regulatory network that is triggered by drought stress in sugarcane.

Introduction

miRNAs are a class of small, non-coding RNAs, approximately with 21 nucleotides in extent, that were described in both plants and animals (Bartel, 2009; Carthew and Sontheimer, 2009) and regulate gene expression by sequence-specific interaction with target mRNAs (Bartel, 2004; Chapman and Carrington, 2007). The first miRNA in plants was described in Arabidopsis thaliana (Reinhart et al., 2002) and, from that moment, an increasing number of miRNAs have been identified in several plants.

Many miRNAs were described to be implicated in developmental and physiological processes such as flowering, leaf and flower differentiation and also in hormone response (Carthew and Sontheimer, 2009; Sunkar et al., 2005). Some works described the expression of miRNAs that has been found to be sensitive under abiotic and biotic stresses (Kantar et al., 2011; Liu et al., 2008;
Sunkar and Zhu, 2004; Zhang et al., 2008; Zhang et al., 2011; Zhao et al., 2007; Kulcheski et al., 2011), demonstrating that several play roles in drought stress (Kantar et al., 2011; Zhou et al., 2010). Up to now, 34 sugarcane miRNAs are deposited in the miRBase database (http://www.mirbase.org).

Because drought stress considerably reduces sugarcane yields (Inman-Bamber and Smith, 2005), the sugarcane miRNAs identification responding to drought stress will assist in the elucidation of the molecular basis of this stress tolerance in this important bioenergy crop.

In the present work, our purpose was to identify and characterise sugarcane miRNAs that may be regulated by water deficit.

**Methods**

**Plant material**

Four sugarcane cultivars RB867515, RB92579 (high tolerance to drought), RB855536 and RB72454 (low tolerance to drought) were cultivated in the field under normal irrigation or drought (rainfed) conditions for eleven months.

Control plants received normal irrigation throughout the experiment. Leaf +1 tissue from irrigated and stressed plants was collected in quadruplicate and then the RNA was extracted. A pool of two replicates was employed for Solexa sequencing.

**RNA Sequencing**

All the sequencing was done using the Solexa platform at BGI (Beijing Genomic Institute, Tai Po, Hong Kong.

**Bioinformatics analysis**

For mapping the reads to the reference sequences (Sorghum bicolor genomic sequence and sugarcane ESTs), the miRDeep-P program (Yang and Li, 2011) was used. After the normalisation of the number of reads in each library, the expression of each miRNA was calculated using the Audic-Claverie method (Audic and Claverie, 1997).

The targets of the miRNA were predicted using psRNATarget (Dai and Zhao, 2011). The BlastX algorithm (Altschul et al., 1997) was used to find the hits in the Sugarcane Assembled Sequences (SAS) against the NCBI database to identify the coding strands.

**Results**

**Analysis of the microtranscriptome in sugarcane**

A total of 154 million reads were obtained, ranging from 15 to 22 million reads per library (data not shown). The reads were aligned against the GenBank and RFam databases to classify the reads into categories. Most of the reads were 21 to 24 nt in length for all libraries, with 21 nt being the most redundant species.

**Identification of miRNAs in Saccharum spp.**

To identify sugarcane miRNA candidates inside our sequenced set, unique small RNA species were aligned against the genome of Sorghum bicolor to identify loci corresponding to presumed miRNA precursors.

We were able to detect 30 mature miRNAs corresponding to 18 miRNAs families in sugarcane. The sugarcane miRNAs were named based on their homology to the miRNAs already described in sorghum.

**miRNAs modulated by drought stress**

Among all of the miRNAs found in this study, 11 were differentially expressed under drought (Table 1). Among them, several miRNAs displayed a very complex expression profile, varying their expression depending on the cultivar.

Three of them were repressed in all cultivars (ssp-miR164, ssp-miR166-seq1 and ssp-miR171-seq2), while only one was induced in all cultivars (ssp-miR156-seq2).
Table 1—Expression profiles of eleven sugarcane miRNAs modulated by drought stress.

<table>
<thead>
<tr>
<th>miRNA name</th>
<th>Mature sequence</th>
<th>HT1I/HT1D</th>
<th>LT1I/LT1D</th>
<th>HT2I/HT2D</th>
<th>LT2I/LT2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssp-miR156</td>
<td>ugacagaagagagugagcac</td>
<td>1.39</td>
<td>-1.60</td>
<td>1.03</td>
<td>1.24</td>
</tr>
<tr>
<td>ssp-miR156-seq2</td>
<td>ugacagaagagagcgacgac</td>
<td>1.07</td>
<td>1.31</td>
<td>1.48</td>
<td>1.28</td>
</tr>
<tr>
<td>ssp-miR160-seq1</td>
<td>ugcuguacacccuauagcca</td>
<td>1.21</td>
<td>1.45</td>
<td>-1.61</td>
<td>2.63</td>
</tr>
<tr>
<td>ssp-miR164</td>
<td>uggagaacagggcagagccguc</td>
<td>-1.38</td>
<td>-1.28</td>
<td>-1.61</td>
<td>-</td>
</tr>
<tr>
<td>ssp-miR166-seq1</td>
<td>ugggaccggcucaauuuccc</td>
<td>-1.08</td>
<td>-1.21</td>
<td>-1.29</td>
<td>-1.51</td>
</tr>
<tr>
<td>ssp-miR167b</td>
<td>ugaacagccaccaugaucugca</td>
<td>1.37</td>
<td>1.29</td>
<td>-1.11</td>
<td>1.23</td>
</tr>
<tr>
<td>Ssp-miR168a</td>
<td>ugcuugggucagaugggagc</td>
<td>1.06</td>
<td>-1.06</td>
<td>1.22</td>
<td>-1.12</td>
</tr>
<tr>
<td>ssp-miR171-seq2</td>
<td>ugaugacggcugccauaucuc</td>
<td>0.00</td>
<td>-1.36</td>
<td>1.85</td>
<td>-1.08</td>
</tr>
<tr>
<td>ssp-miR393</td>
<td>cuccaaaggggauacauugca</td>
<td>1.22</td>
<td>1.28</td>
<td>-1.19</td>
<td>1.32</td>
</tr>
<tr>
<td>Ssp-miR528</td>
<td>uggaggggcaugcagagaggag</td>
<td>-1.25</td>
<td>-3.82</td>
<td>1.50</td>
<td>-4.79</td>
</tr>
<tr>
<td>ssp-miR5564</td>
<td>ugcacguucuacaggaauugc</td>
<td>1.26</td>
<td>1.29</td>
<td>1.60</td>
<td>-1.22</td>
</tr>
</tbody>
</table>


**miRNA targets search**

To search for the miRNA targets in sugarcane, four mature miRNA sequences modulated by drought were used (Table 2). All of these miRNAs had potential targets in the SUCEST database, most of them encoding transcription factors (ssp-miR164, ssp-miR171 seq 2 and ssp-miR528) and one putative growth/development regulator (ssp-miR393).

Table 2—Target prediction of four miRNAs modulated by drought stress.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target Acc.</th>
<th>Expectation</th>
<th>UPE</th>
<th>Mature miRNA</th>
<th>Target beginning</th>
<th>Target end</th>
<th>Target description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssp-miR164</td>
<td>SCEPRT2048G05.g</td>
<td>1.0</td>
<td>20.052</td>
<td>20</td>
<td>699</td>
<td>718</td>
<td>NAC transcription factor (<em>Hordeum vulgare</em>)</td>
</tr>
<tr>
<td>ssp-miR171-seq2</td>
<td>SCJFAD1013C10.g</td>
<td>0.5</td>
<td>23.909</td>
<td>21</td>
<td>574</td>
<td>594</td>
<td>Sc1 protein (<em>Oryza sativa japonica</em> group)</td>
</tr>
<tr>
<td>ssp-miR393</td>
<td>TC120009</td>
<td>1.0</td>
<td>20.653</td>
<td>19</td>
<td>302</td>
<td>320</td>
<td>TIR1 protein – Auxin responsive factor (<em>Populus tomentosa</em>)</td>
</tr>
<tr>
<td>ssp-miR528</td>
<td>SCCCCL1002D10.b</td>
<td>2.5</td>
<td>10.325</td>
<td>21</td>
<td>133</td>
<td>153</td>
<td>Pyruvate dehydrogenase α E1 subunit (<em>Zea mays</em>)</td>
</tr>
</tbody>
</table>

Acc.: Access numbers on SUCEST or SoGI databases; Expectation: designed value for the mature miRNA and target alignment, ranging from 0 (perfect alignment) to 5; UPE: energy required to open the target secondary structure and the recognising site (less energy means better accessibility to the target); mature miRNA: length of mature miRNA (nucleotide); Target beginning: position of the base where the annealing with miRNA begins; Target end: position of the base where the annealing with miRNA ends; Target description: target description according to the BLAST search on GenBank database, including the name of the organism with the best hit.

**Conclusions**

It is worth noting that the expression patterns of most of the miRNAs did not correspond with the differences in drought tolerance perceived in the two sugarcane cultivars.

281
Our results provide insight into the sugarcane microRNAs, highlighting the regulatory network triggered by drought stress in a significant crop. Many of the targets found in this study, like transcription factors, may be targets of the miRNAs modulated by drought in sugarcane. More work with transgenic sugarcane modulating the miRNAs or their targets will increase our understanding on the molecular mechanisms associated with drought response in sugarcane.

REFERENCES


CHANGES IN MICROTUBULE CYTOSKELETON DURING MITOSIS IN STEM APEX CELLS OF SUGARCANE

By

SU-LI LI¹, ZHI-GANG LI¹, XIN-CHENG ZHANG¹, LI LIN¹, LI-TAO YANG¹* and YANG-RUI LI¹.²*

Agricultural College, Guangxi University/ State Key Laboratory of Conservation and Utilization of Subtropical Agro-Bioresources, Nanning 530005, China; ²Sugarcane Research Center, Chinese Academy of Agricultural Sciences/Sugarcane Research Institute, Guangxi Academy of Agricultural Science/Guangxi Key Laboratory of Sugarcane Genetic Improvement/ Guangxi Crop Genetic Improvement and Biotechnology Laboratory, Nanning 530007, China

*Corresponding author: Li-Tao Yang: *liyr@gxu.edu.cn;
Yang-Rui Li: liyr@gxaas.net

KEYWORDS: Sugarcane (Saccharum officinarum L.), Microtubule Cytoskeleton, Indirect Immunofluorescence Microscopy, Mitosis, Primary Thickening Meristem.

Abstract

THE MICROTUBULE CYTOSKELETON of the stem apex of sugarcane (Saccharum officinarum L., cv. YT86/368) was investigated using improved cryosectioning and an indirect immunofluorescence labelling technique. The results showed that there were four basic types of microtubule arrays, namely; cortical microtubule, preprophase microtubule band, spindle microtubule and phragmoplast microtubule. In addition, many different transitional microtubule arrays were also observed, constituting the typical microtubule cycle in dividing cells of plants. The cortical microtubule arrays were mainly oriented transversely to the cell elongation axis in elongating cells of young leaves in the stem apex. Microtubule arrays were mainly longitudinal and oblique cortical to the axis in the slowly elongating cells of young leaves, although all kinds of cortical microtubule arrays such as transverse, longitudinal, oblique and random microtubules were observed in the primary thickening primordium meristem of sugarcane stem apex. Double preprophase microtubule bands were found in a few preprophase cells, whose functions are still unknown. The dynamic state and change process of microtubule cycle during mitosis observed in this study provides a basic reference for investigating the mechanism of cell division and differentiation in the sugarcane stem apex.

Introduction

Microtubules are one of main cytoskeleton members in eukaryote cells and play a very important role in the life process of plants, such as the growth and development (Cyr, 1994; Chafey, 1999; Tian et al., 2004).

Several experiments have shown that microtubules are involved in the morphogenesis of plants (Sakaguchi et al., 1988; Abe et al., 1995a; 1995b; Holdaway et al., 1995). Cortical microtubules possibly participate in the arrangement of cellulose microfibril in cell walls, preprophase microtubule bands foreshow the position of the cell division plate, phragmoplast microtubules participate in the formation of the cell plate, and spindle microtubules play a role in the normal arrangement and equal separation of chromosomes (Gu et al., 1997). The immunofluorescence research of plant microtubule cytoskeleton mainly focuses on pollen grain (tube), megaspore, root tip or hypocotyl, and the main method adopted is the isolating single cell
method initiated by Wick et al. (1981). There are comparatively few studies of microtubules at large tissue or organ levels, due to lack of convenient and reliable techniques (He and You, 2004).

As yet, investigation of the microtubule cytoskeleton during mitosis in the stem apex of monocotyledonous plants has not been reported to our knowledge. In this study, microtubule cytoskeleton in the stem apex of sugarcane was studied by using improved cryosectioning and an indirect immunofluorescence labelling technique.

The data obtained provide new experimental evidence for the universality and regularity of periodic change of plant microtubule cytoskeleton, as well as the theoretical basis on microtubule dynamics for the thickening mechanism of sugarcane stem.

Materials and methods

Stem apices of sugarcane cultivar YT86/368 (Saccharum spp. hybrid) in early elongation stage were used in this study. The young leaves surrounding the growth tip were stripped off under stereo-anatomical lens and only the third to the sixth young leaves and the leaf primordium were retained.

The sugarcane stem apex was shaped into tissue blocks (4×1×4 mm), including the primary thickening meristem and maturation zone with the leaf primordium, growth cone and elongation zone. The tissue blocks were placed into 9% (v/v) formaldehyde solution (50 mM Pipes, pH 7.0) and evacuated for 15–20 min.

The solution was then agitated until the tissue blocks sank. The tissue blocks were fixed for 4–24 h at room temperature, and then subjected to three to five 30 min washes under vacuum with 10% dimethyl sulfoxide (50 mM PBS, pH 7.0). The materials were preserved in the final washing liquor for 2–3 h before sectioning on a cryomicrotome at –25~–35°C.

The section thickness was 15–20 µm, depending on the size of material cells. The typical vertical slices in the mid stem apex were adhered to glass slides with poly-L-lysine (Sigma P8920), then preserved in 50 mM PBS (pH 7.0).

The antigenicity of microtubules was retained for one week when the materials were maintained at 4 °C. The slices were washed for 30 min with detergent (2% (v/v) Nonidet P-40) so as to increase the cell membrane permeability, after which they were treated for 5 min with a mixed solution containing 1% cellulase and 0.5% pectase.

The tissue samples were then subjected to three 20 min washes followed by a 30 min treatment 2% Nonidet P-40 solution and three 20 min washes with PBS. The slices were incubated for one hour with anti-α Tubulin antibody (Sigma, 1:200, diluted in PBS containing 2% bovine serum albumin) at room temperature, washed three times for 30 min each with PBS. Thereafter, the slices were incubated for one hour with Rabbit anti-mouse IgG/FITC at room temperature, washed for 1–2 h with PBS, with 4–5 changes of the PBS washing solution.

The plant slices, placed on the glass slides, were covered with mounting medium containing 50% glycerol and 0.1% p-phenylenediamine (PBS used as solvent, pH 8.5) and preserved for 1–2 week(s) at 4 °C in the dark. The slides were observed with an Olympus BX51 fluorescence microscope (Japan), with blue light excitation light and oil immersion objective lens (100×). Images were obtained from the stained slices by using the image acquisition system (Olympus digital camera DP70), and saved in JPG format and further edited with Photoshop 7.0, printed using a colour high-resolution printer.

Results and discussion

Cortical microtubule array in interphase cells

Laskowaki (1990) observed the microtubule arrangement in different internodes of peas and in different position cells of pea internodes, and found that microtubule arrangement was in transverse in rapidly elongating cells, but microtubule arrangement was in oblique or longitudinal in non-elongating cells. The latter change in arrangement follows the initiation of growth rate decline.
In the present study, in the interphase cells of sugarcane stem apex, the fluorescence that represents fibrillous or bunchy beneath cytoplasm membrane, namely cortical microtubules, observed with the fluorescence microscope, were found arranged around the cell axis (Plate A). The arrangement of cortical microtubules from different growth phase cells was obvious. Transverse cortical microtubules were mainly distributed in the rapidly elongating young leaves (Plate A), and longitudinal cortical microtubules were distributed in the slowly expanding young leaves (Plate B).

There were transverse, longitudinal, oblique and random cortical microtubules in the primary thickening meristem of young leaf bases (Plates C-F). The cortical microtubules generally appeared after plasmodieresis and disappeared when preprophase microtubule bands appeared in dividing cells. In the young leaf base, transverse, longitudinal, oblique and random cortical microtubules were distributed in the primary thickening meristem. Different types of cortical microtubules were observed in the same area of cells, possibly related to the function of the primary thickening of sugarcane meristem (Abe et al., 1995a, 1995b).

**Preprophase microtubule band array**

When the cell division moved from interphase to preprophase, fibrillous cortical microtubules disappeared in interphase cells as observed under the fluorescence microscope. Intense fluorescence bands were observed clearly in the mid-part of some cells and in the envelope of perikaryon, and these bands were typical preprophase microtubule band arrays (Plates G-H). Although the cell shape varied in different areas, the structures of preprophase microtubule bands were essentially similar, with strong fluorescence bands, all revolving around the nuclear centre. Preprophase microtubule bands were also made up of many parallel arrangement fibrils, as for cortical microtubules.

Preprophase microtubule band formed a light band because of very close arrangement; meanwhile, no microtubules were distributed in other areas except for the nuclear centre. In the formation process of preprophase microtubule bands, the fluorescence appeared in the envelope of the nucleus (Plates G-H).

Then the preprophase microtubule bands began to be depolymerised or loosened, and the fluorescence became weaker and darkened gradually, and moved towards the two poles. Thereafter, they extended towards the cell centre, and finally formed a spiry structure (Plate I). The fibrils from the two poles continued to extend towards the cell centre, when preprophase microtubule bands already disappeared completely (Plate J). The fibrils from the two poles continued to extend towards the cell centre (Plate K), when spindle microtubules began to appear.

In the cell cycle of the stem apex, there were many kinds of microtubules in the development and transformation processes of preprophase microtubule bands (Plates G-J). In a few preprophase cells, double preprophase microtubule bands revolved around the nucleus (Plates G1-G2). No abnormal mitotic cells appeared when double preprophase microtubule bands appeared; the function of such specific microtubule bands is unclear yet.

**Spindle microtubule array**

In the telophase of preprophase microtubule bands, spindle microtubule arrays were observed (Plate K). Along with cell division, the spindle fibrils from the two poles continued to extend towards the cell centre (Plates K-N). They were arranged symmetrically on both sides of the equatorial plate, without fluorescence in the middle of cells, where chromosomes were just arranged, and this is typical of spindle microtubule array (Plate O).

Spindle microtubule arrays of different division states were observed in different areas of the stem apex (Plates M-P). Spindle microtubules pulled the chromosomes on the equatorial plate towards the two poles, when the spindle fibrils began to shorten and anaphase spindle microtubules appear (Plate P). Anaphase spindle microtubules continued to pull chromosomes towards the two poles, telophase spindle microtubules were formed when the fluorescence intensity and length of spindle microtubules decreased heavily.
Telophase spindle microtubules pulled the chromosomes towards the two poles, when a large area of low fluorescence appeared on the equatorial plate, that is, the preprophase phragmoplast microtubules began to be formed (Plates Q-T). Meanwhile, part of telophase spindle microtubules remained in the two poles.

**Phragmoplast microtubule array**

In the telophase of spindle microtubules, spindle microtubules which presented galericulate structures were oppositely distributed in the two poles, when spindle microtubules were getting less, and low fluorescence bands appear in the middle of cells, that is, transitional microtubules appeared from telophase spindle microtubules to phragmoplast microtubules (Plates R-U). Then the telophase spindle microtubules gradually decreased and finally disappeared (Plates U-V), and the fluorescence bands in the middle of cells formed prophase phragmoplast microtubule arrays (Plate V). The prophase phragmoplast microtubules arranged according to spindle direction, gradually shorten towards the direction of the equatorial plate where the fluorescence gradually increases.

At the same time, a line of narrow zone without fluorescence appeared in the middle of phragmoplast microtubules, and the dark line was just the position of the cell plate, showing that typical phragmoplast microtubule arrays already formed (Plate W). In the whole process of cytoplasmic division, the width of phragmoplast microtubules diminishes and the length got longer.

After cytoplasmic division, cortical microtubule arrays appeared again in the interphase of the daughter cells (Plate X). The immunofluorescence labelling technique presented a very good advantage for studying phragmoplast microtubules, because the spatial structure of phragmoplast microtubules can be observed with a light microscope.

**REFERENCES**


Explanation of Plates I, II and III

Photographs of stem apex cells of *Saccharum officinarum* L., cv. YT86/368 based on improved cryosectioning coupled with indirect immunofluorescence labelling technique, showing the microtubule cytoskeleton.

A: Transverse cortical microtubule in the rapidly elongating young leaves.
B: Longitudinal cortical microtubule in the slowly swelling young leaves.
C: Transverse cortical microtubule in the primary thickening meristem.
D: Longitudinal cortical microtubule in the primary thickening meristem.
E: Oblique cortical microtubule in the primary thickening meristem.
F: Random or network cortical microtubule in the primary thickening meristem.

G–H: show all kinds of preprophase microtubule band 
G and big arrow in H show the fluorescence in the envelope of nucleus(G1–G2: Show the abnormal PPB, the arrows show the double bands).

I: Show the PPB is becoming less and low fluorescence, while fluorescence is moving towards the two poles.
J: Show the PPB is disappearing and fluorescence in the two poles is becoming lighter.

K–N: show various transitional microtubule arrays from the PPB to spindle, which indicates microtubules are extending from the poles to the centres of the nucleus, and the microtubule fibrils are becoming clearer and visible.

O: Show typical spindle microtubule arrays, the arrow shows the site of chromosomes.
P: spindle microtubule arrays are moving from the cell plate to the two poles, and the fluorescence is becoming weaker.

Q–U: Show spindle microtubule arrays are becoming less, preprophase phragmoplast microtubules appear.
U–V: Preprophase phragmoplast microtubules are becoming narrower and longer, spindle microtubules are almost disappearing.

W: Typical phragmoplast microtubules already formed, the arrow shows the site in which the cell plate will form.
X–Z: The redeveloped cortical microtubule arrays, X and Z show transverse cortical microtubule arrays, Y shows oblique cortical microtubule arrays.
EXPRESSION OF *UIDA* GENE DIRECTED BY TWO FRAGMENTS OF SUCROSE SYNTHASE GENE PROMOTER IN SUGARCANE EMBRYOGENIC CALLUS

By

J.D. CORTES, J.J. RIASCOS and J. LÓPEZ

Colombian Sugarcane Research Center, CENICAÑA, Cali, Colombia

jlopez@cenicana.org

KEYWORDS: Sucrose Synthase, Ubiquitin, Promoter, Gus, Sugarcane.

Abstract

Promoters are composed of short regulatory nucleotide sequences called *cis*-elements, which bind transcription factors and facilitate transcription initiation. Research on plant genetic transformation often requires the use of different types of promoters for the purpose of increasing the expression of the transgenes and currently there are no promoters from sugarcane with stable transgene expression over time. Therefore, the goal of this study was to isolate a promoter from the sugarcane genome and evaluate its functionality by transient expression of the reporter gene *uidA* (*Gus*). Three different fragments were amplified from the complete sequence of the sucrose synthase (SuSy) gene promoter with sizes: 1282 bp (FgS2), 1303 bp (FgS2), 2000 bp (FgS1). Maize ubiquitin and CaMV35S promoters were used as controls. Three genetic constructs, pC-FgS2, pC-FgS3, pC-Ubi, were generated after replacement of the CaMV35S promoter in the pCambia 1305.2 commercial vector for each of the three promoter fragments. Sequence analysis of the control promoters and promoter fragments showed that the Ubiquitin promoter had the highest amount of *cis*-regulatory elements. The most common categories of regulatory *cis*-elements, in both the SuSy gene promoter and Ubiquitin, were ‘response to light’ and ‘typical *cis*-element’. The highest level of Gus activity, measured as the number of blue spots in embryogenic sugarcane callus, was observed with the Ubiquitin promoter (406 blue spots) followed by CaMV35S (46 blue spots) and the SuSy fragment FgS3 (4 blue spots). The results of this study are important because they contribute to the understanding of the function and possible applicability of sugarcane promoters in genetic transformation.

Introduction

Promoters are DNA sequences that interact with various transcription factors and RNA polymerase II. They are important in plant genetic transformation because they allow transcription and subsequent expression of target genes (of agronomic importance) and marker selection genes. Promoters are categorised into constitutive, tissue and/or organ-specific, inducible or synthetic, and therefore they provide different possibilities for transgene expression studies. Different types of promoters have been isolated in sugarcane. They include the promoter of the transcription factor ScMYBAS1 (Prabu and Prasad, 2011), the constitutive promoters Ubi4 and Ubi9 (Wei *et al.*, 2003) and promoters of different enzymes such as the small subunit of the Rubisco and the UDP-glucose dehydrogenase (Tang *et al.*, 1996; Van der Merwe *et al.*, 2003).

However, in sugarcane there are difficulties associated with loss of transgene expression due to post-transcriptional silencing and the redundant nature of the sugarcane polyploid genome (Mudge *et al.*, 2009). Another concern arises with intellectual property issues associated with commercial promoters, which makes necessary the identification of new promoters in sugarcane.
Material and methods

Transformation vectors

From the Gene Bank sequence AY118266, corresponding to the sugarcane sucrose synthase gene, three fragments were amplified (FgS1, and FgS3 FgS2) from the region corresponding to the promoter.

Subsequently, they were cloned into the vector pCR 2.1 (Invitrogen®). The maize ubiquitin and CaMV35S promoters were used as controls. The Ubiquitin promoter was amplified from a plasmid kindly provided by Dr Erick Mirkov at the Texas A&M University and was also cloned into the vector pCR 2.1.

Next, the CaMV35S promoter, which drives the expression of the gene uidA (GUS) in the commercial vector pCambia 1305.2, was replaced by two of the three fragments amplified from SuSy gene promoter (FgS2, FgS3) and by the Ubiquitin promoter.

Finally, a total of four constructs were used for calli transformation: pC-FgS2, pC-FgS3, pC-Ubi and pCambia 1305.2.

Calli transformation and evaluation of transient expression

A total of 140 sugarcane embryogenic calli obtained from the variety CC 84-75, were transformed with the four constructs according to the following parameters: 6 cm between the embryogenic callus and mesh retainer, helium pulse at 1350 psi, 1 μm gold micro-particle size and 5 μg of DNA.

Transient expression of the reporter gene (GUS) in each transformed callus was performed by counting the blue spots after treatment with the X-gluc substrate.

Statistical analysis and bioinformatics

The experiment was organised in a completely randomised design and the software SAS (version 9.3) (SAS, 2009) was used for statistical analysis.

Analysis of the regulatory cis-elements from the promoter fragments was performed using the software PlantCare (Lescot et al., 2002).

Results and discussion

Promoter analysis

Bioinformatic analysis of the five promoter fragments (FgS1, FgS2, FgS3, Ubiquitin, and 35S) identified a total of 43 types of regulatory cis-elements. The Ubiquitin promoter and FgS3 showed the greatest number of regulatory cis-elements with a total of 32 and 25 respectively.

The 43 different types of regulatory cis-elements were classified into eight categories according to their related function.

These categories were ‘Hormone’, ‘Light sensitive’, ‘Anoxia’, ‘Tissue specific’, ‘Temperature stress and defence’, ‘Cell cycle’ and ‘Typical cis-element’. Categories ‘Light sensitive’ and ‘Typical cis-element’ were the most represented in the SuSy and Ubiquitin promoters.

The category ‘typical cis-element’ had the highest representation in the CaMV35S promoter (Figure 1).

The promoter fragments differ by at least one cis-element, associated with at least one of the eight categories.

The cis-element fragments in FgS1, FgS2, FgS3 were found associated with the expression pattern reported for the sucrose synthase enzyme (Baud et al., 2004), such as anoxia (ARE and GC-motif), low temperature and wounds (Box-W1, LTR, HSE, CGTCA-motif, TGACG-motif), endosperm and seed (CCGTCC-box, RY-element, Skn-1 motif).
**GUS expression in sugarcane embryogenic callus**

After biolistic genetic transformation and *GUS* expression, histochemical staining blue spots were counted. An average of 406 blue spots was obtained when the pC-Ubi construct was used, 46 using pC-35S construct and 4 using pC-FgS3. Significant difference was achieved (P < 0.005) between the promoter Ubiquitin – CaMV35S, Ubiquitin – FgS3 and CaMV35S – FgS3.

The highest amount of blue spots obtained using the Ubiquitin promoter could be related to the great amount of *cis*-element within the category ‘Typical *cis*-element’ (86 *cis*-element), like TATA-box and CAAT-box (Figure 2), which allow the formation of a better pre-initiation complex and binding to the RNA polymerase II.

However, the relationship between promoter length and the number of regulatory *cis* elements is not always related to the efficient induction of expression. The above statement is supported by the results obtained with the CaMV35S and FgS3 promoter.

It was observed that the FgS3 promoter was a greater length and showed an increased number of *cis* regulatory elements in the category ‘Typical *cis* element’, similar to those found for the Ubiquitin promoter, but lower compared with the CaMV35S promoter (Figure 2). However, the number of blue spots obtained by the FgS3 promoter was lower.

The above analysis suggests that the CaMV35S promoter was more efficient than FgS3, which may be related to the way in which the number and the types of the different *cis* regulatory elements are distributed along the promoter.

**Conclusions and future consideration**

The Ubiquitin promoter showed the highest amount of *cis*-elements found in all tested promoter fragments as well as the highest blue spots number, followed by the CaMV35S promoter and lastly by the fragment FgS3 (1303 bp) of SuSy gene promoter.

The categories that showed the highest number of *cis*-element of SuSy gene promoter and Ubiquitin were: ‘light sensitive’ and ‘typical *cis*-element’.
Fig. 2—Diagram with cis-element categories present in the (a) Ubiquitin (b) SuSy and (c) CaMV35S promoters.
The GUS expression obtained with FgS3 promoter fragment needs to be evaluated at the level of tissue specific and continue with the research isolation of different promoters that could be used in sugarcane transformation.

Acknowledgements

The authors are thankful to Hugo Harley Jaimes and Paola Andrea Mosquera, Biotechnology Laboratory at Cenicaña, Colombia, for the technical advice in genetic construct generation and biolistic genetic transformation.

REFERENCES


ASSESSING GENETIC DIVERSITY IN SUGARCANE CLONES USING ISSR MOLECULAR MARKERS

By

CAROLINA HORTÊNCIO MALHEIROS¹, MÁRCIA HELENA SCABORA², REGINALDO CARVALHO³, DANIELA TIAGO SILVA CAMPOS¹* and ANTÔNIO MARCOS IAIA¹

¹Universidade Federal de Mato Grosso, ²SENAI-MT, ³Universidade Federal Rural de Pernambuco

*camposdts@yahoo.com.br

KEYWORDS: CTAB, Genetic Diversity, Polymerase Chain Reaction, Polymorphism.

Abstract

SUGARCANE SHOWS HIGH genetic variability and, therefore, new molecular methods to distinguish between cultivars are important for use in breeding programs, with the goal of producing high-yielding cultivars that are adapted to different climatic regimes. The objective of this preliminary study was to assess the genetic diversity among sugarcane (Saccharum hybrids) clones using ISSR-PCR to identify cultivars best adapted to the characteristics of the state of Mato Grosso, Brazil. Genomic DNA was extracted from the young leaves of six sugarcane clones using the CTAB method and amplified using 22 ISSR primers. Of the 22 primers tested, 17 showed optimal PCR product bands (ranging from 200 to 2000 bp) for each of the six sugarcane cultivars, indicating that ISSR markers can be used to analyse genetic diversity in this crop. This work is part of an ongoing project by RIDESA UFMT (Campus Cuiabá-MT). Further work will examine whether ISSR-PCR can be used to select cultivars with desirable characteristics for Mato Grosso state.

Introduction

Sugarcane production is expanding in Brazil, with an increase of 5.5% reported in the state of Mato Grosso (CONAB, 2012). This rise is attributed to production technologies and new varieties annually obtained in breeding programs existing in Brazil. An ideal variety should have a high production and low degree of fluctuation in performance when grown under adverse environmental conditions (Kumar et al., 2004). Molecular markers have been widely used for the germplasm characterisation of crop varieties, including sugarcane. These phylogenetic analyses aimed mainly to study the relationships and interspecific genetic diversity.

The study presented herein consisted of identifying dominant markers ISSR (Inter Simple Sequence Repeat) (Zietkiewicz et al., 1994), in order to avoid the problem of null alleles associated with microsatellite markers. ISSR does not require prior information of DNA sequences of the target species and produces fragments with high reproducibility when compared to other marker systems based on non-specific PCR (Wolfe and Liston, 1998). Therefore, the objective of the first phase of this study was to estimate the genetic diversity using PCR-ISSR in sugarcane clones.

Materials and methods

The six clones of sugarcane used in this study were provided by the Inter-University Network Development Sector Ethanol – RIDESA and from a commercial program at the Agronomic Institute of Campinas – IAC (Table 1). All six clones have important agronomic traits, which are promising materials for the state of Mato Grosso.
Table 1—Sugarcane clones used in the assessment of genetic diversity based on ISSR markers.

<table>
<thead>
<tr>
<th>Order</th>
<th>Cultivar</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – clone 269</td>
<td>RB 72454</td>
<td>RIDEASA</td>
</tr>
<tr>
<td>2 – clone 250</td>
<td>RB 931564</td>
<td>RIDEASA</td>
</tr>
<tr>
<td>3 – clone 251</td>
<td>IAC 82-3092</td>
<td>IAC</td>
</tr>
<tr>
<td>4 – clone 246</td>
<td>RB 845210</td>
<td>RIDEASA</td>
</tr>
<tr>
<td>5 – clone 194</td>
<td>RB 991513</td>
<td>RIDEASA</td>
</tr>
<tr>
<td>6 – clone 205</td>
<td>RB 991532</td>
<td>RIDEASA</td>
</tr>
</tbody>
</table>

Total genomic DNA was isolated from young sugarcane leaves using the CTAB method (hexadecyltrimethylammonium bromide) following the method modified by Doyle and Doyle (1990). It necessitated the selective precipitation of polysaccharides, which in some plant species can interfere with the process of DNA quantification. Quantification and quality of DNA were evaluated by electrophoresis in 2% agarose gel (w/v) in 1X TBE buffer (Tris borate, 0.09 M; EDTA, 0.002 M) at 80 V for 40 min. DNA quantification was performed using known DNA amounts of phage λ.

The amplification reactions for ISSR marker contained 15 ng genomic DNA, 1.5 µL 10x PCR Buffer (-MgCl₂), 2.0 µL of 25 mM MgCl₂, 1.0 µL of 10 mM dNTP, 0.5 µL of 5U/µL Taq DNA polymerase and 1.0 µL of primer (5 pmoles) and the final volume adjusted to 20 µL with MilliQ water.

The amplification reaction was performed in a thermocycler. The reaction included an initial step of denaturation of 15 minutes at 95°C, followed by 30 cycles, each consisting a denaturing step at 94°C for 30 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 2 minutes, and the PCR reaction terminated with a final extension of 7 minutes at 72°C.

The products of PCR reaction were separated on a 2% agarose gel in 1X TBE buffer and visualised using a photo documentation system after staining with 0.5 mg/mL ethidium bromide. The molecular weight marker 100 bp DNA ladder was included in one lane of the gel. The 22 ISSR primers tested are listed in Table 2.

Table 2—Primers used and ISSR nucleotide sequence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBC 807</td>
<td>AGA GAG AGA GAG AGA GT</td>
</tr>
<tr>
<td>UBC 810</td>
<td>AGA GAG AGA GAG AGA AT</td>
</tr>
<tr>
<td>UBC 811</td>
<td>AGA GAG AGA GAG AGA AC</td>
</tr>
<tr>
<td>UBC 812</td>
<td>GAG AGA GAG AGA GAG AA</td>
</tr>
<tr>
<td>UBC 815</td>
<td>CTC TTC TCT CTC TCT CTG</td>
</tr>
<tr>
<td>UBC 823</td>
<td>TCT CTC TCT CTC TCT CC</td>
</tr>
<tr>
<td>UBC 828</td>
<td>TGT GTG TGT GTG TGT GA</td>
</tr>
<tr>
<td>UBC 830</td>
<td>TGT GTG TGT GTG GTG GG</td>
</tr>
<tr>
<td>UBC 831</td>
<td>ATA TAT ATA TAT ATA TYA</td>
</tr>
<tr>
<td>UBC 834</td>
<td>AGA GAG AGA GAG AGA GYT</td>
</tr>
<tr>
<td>UBC 835</td>
<td>AGA GAG AGA GAG AGA GYC</td>
</tr>
<tr>
<td>UBC 836</td>
<td>AGA GAG AGA GAG AGA GYA</td>
</tr>
<tr>
<td>UBC 840</td>
<td>GAG AGA GAG AGA GAG AYT</td>
</tr>
<tr>
<td>UBC 841</td>
<td>GAG AGA GAG AGA GAG GYC</td>
</tr>
<tr>
<td>UBC 843</td>
<td>CTC TCT CTC TCT CTC TRA</td>
</tr>
<tr>
<td>UBC 844</td>
<td>CTC TCT CTC TCT CTC TRC</td>
</tr>
<tr>
<td>UBC 845</td>
<td>CTC TCT CTC TCT CTC TRG</td>
</tr>
<tr>
<td>UBC 846</td>
<td>CAC ACA CAC ACA CAC ART</td>
</tr>
<tr>
<td>UBC 848</td>
<td>CAC ACA CAC ACA CAC ARG</td>
</tr>
<tr>
<td>UBC 851</td>
<td>GTG TGT GTG TGT GTG TYG</td>
</tr>
<tr>
<td>UBC 855</td>
<td>ACA CAC ACA CAC ACA CYT</td>
</tr>
<tr>
<td>UBC 857</td>
<td>ACA CAC ACA CAC ACA CYG</td>
</tr>
</tbody>
</table>
Results and discussion

17 out of 22 ISSR primers tested gave amplification product of good quality. Among these 17 primers, there were 7 amplified monomorphic loci and 10 amplified polymorphic bands. The amplicons ranged between 200 to 2000 bp. In the near future, other ISSR primers will be tested and other varieties also screened, since ISSR markers are promising for accessing genetic diversity and identification of DNA fingerprinting and to identify cultivar-specific markers for sugar cane (Almeida et al., 2009).

Molecular markers are abundant in plant genomes and are not influenced by environmental factors. Molecular markers, whether or not PCR-based on the amplification, have been widely used in plant breeding programs for the characterisation of populations and construction of linkage maps (Ferreira and Grattapaglia, 1998).

Conclusion

This work is ongoing but our preliminary results show that sugarcane has a high genetic variability, which it is important for use in breeding programs, justifying the continuation of the work carried out by RIDESA in UFMT – Campus Cuiaba-MT.

REFERENCES


FUNCTIONAL CHARACTERISATION FROM A SEQUENCE IDENTIFIED IN SUBTRACTIVE cDNA LIBRARIES USING CONTRASTING SUGARCANE CULTIVARS FOR FLOWERING PROCESS UNDER EQUATORIAL CONDITIONS

By

F.S. LEITE, A.L, MEDEIROS, A.F. UCHOA and K.C. SCORTECCI

Departamento de Biologia Celular e Genética, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Brazil

kaescort@yahoo.com

KEYWORDS: Early-Flowering Cultivar, Shoot Apical Meristem, Transgenic Plants, Interatomic Tools.

Abstract

EARLY FLOWERING IS a serious problem for sugarcane production as it may reduce the production up to 60%. The identification of genes and proteins involved in the flowering process will produce tools to improve genotypes that are more suitable for different climatic regions. Then, in order to identify differentially expressed genes and proteins in the shoot apical meristem (SAM) of early-flowering and late-flowering cultivars grown in the equatorial region, subtractive cDNAs and proteomics (2D gels) were used. Previous results from subtractive cDNA libraries identified 9 potential cDNAs, and proteomic tools identified 19 proteins expressed in the SAM. The aim of this study was to characterise the sequence that had homology to SHAGGY-LIKE Kinase, which was identified in the subtractive cDNA libraries. Overexpression constructs were produced and tobacco plants were transformed. The transgenic plants T0 and T1 showed an alteration in gynoecium morphology. An interatomic tool was also used to identify possible patterns in this protein and the result suggested that SHAGGY-LIKE Kinase protein may interact with ELF3, XTR6, BIN2, ATAF1, BEL1, and other proteins that are directly and indirectly associated with flowering in the Arabidopsis plant model. Our data proposed a connection of these sequences identified with flowering pathways.

Introduction

Sugarcane (Saccharum spp.) is of great importance to Brazil as its juice is converted into sugar and ethanol. Brazil is responsible for 40% of the world production (Scortecci et al., 2011). Flowering is an important step in the plant life cycle and it has been well-characterised in the Arabidopsis plant model. However, in the rice model, it has been shown that monocots have additional pathways and gene regulation may also change (Colasanti and Coneva, 2009; Higgins et al., 2010).

It has been shown in the rice model and in sugar beet that there are two copies of the florigen or FT gene that are regulated differently (Jarillo and Pineiro, 2011; Tsuji et al., 2010). However, for sugarcane, this step has an impact on production as the sucrose accumulated in the stem is drained to develop the flower and seeds.

There are many factors that are related to sugarcane flowering such as high temperature, photoperiod, plant maturity, and poor mineral nutrition (Araldi et al., 2010). Sugarcane cultivars are classified according to their flowering response: early-flowering or late-flowering (Figure 1).

Despite the importance of sugarcane in the world, the flowering process is not well-understood. Due to this lack of knowledge, we are using differential molecular tools to identify...
genes/proteins that may be related to the flowering process in sugarcane. We constructed four subtractive cDNA libraries using contrasting sugarcane cultivars (early- and late-flowering) in order to identify messages expressed in the SAM. They identified nine cDNAs differently expressed in the shoot apical meristem (SAM).

Furthermore, using proteomic (2D) gels, Duarte et al. (Pers. Comm.) identified 19 proteins that may also be related to the sugarcane flowering process. Here, we present the functional characterisation of one sequence previously identified. The functional characterisation was done by overexpression constructs and tobacco transformation as well as using interatomic tools for \textit{in silico} analysis. Our results indicate that this gene may have a role in the sugarcane flowering pathway.

Results and discussion

Sequence identification

The subtractive cDNA libraries identified nine cDNAs, one of which had homology to SHAGGY-Like Kinase. Comparing early- and late-flowering cultivars, we previously proposed that this sequence may play a role as an inhibitor. For this reason, we chose this sequence for further functional characterisation.

Functional characterisation for \textit{SHAGGY}-Like Kinase

SHAGGY-like kinases correspond to a gene family involved in a different biological mechanism (Saidi \textit{et al.}, 2012). In \textit{Arabidopsis}, two sequences were related to the flowering process: \textit{AtSK11} and \textit{AtSK12} (Dornelas \textit{et al.}, 2000). Then, in order to characterise the sugarcane function, the complete cDNA was amplified by PCR. This was under the control of the CaMV35S strong promoter in the sense orientation (Figure 2).
The Agrobacterium system was used to transform tobacco leaf disc (T0). These plants were regenerated and grown in the presence of the kanamycin for transgenic plant selection. Thereafter, the plants (T0) were transferred to soil and the phenotype was analysed and seeds were collected (T1).

These T1 seeds were germinated and the plant phenotype was characterised considering plant development: plant height, leaf area, and leaf distance. No changes were observed when the transgenic plants were compared to the wild-type tobacco plants (Figure 3).

However, when the flower structures were analysed, it was observed that the transgenic plants had an increase in gynoecium structure (Figure 4).

Dornelas et al. (2000) have observed a similar phenotype but with AtSK12 mutants. Homozygous seeds are being obtained in order to further analyse the plant phenotypes.

Fig. 2—Overexpression construction. Schematic representation of CaMV35S strong promoter, SHAGGY cDNA, restriction sites and T-DNA sequence from pZP211 binary vector.

Fig. 3—Plant development for 35S:SHAGGY/S overexpression. (A) Average height (cm), (B) leaf area (cm²), and (C) distance between leaves (cm). The values represent the average with standard deviation.
Fig. 4—Flower morphologic changes in transgenic plants with overexpression cassette – 35S::SHAGGY/S. (A) wild type inflorescence (WT); (B) 35S::SHAGGY/S inflorescence; (C) and (D) WT flower compared with transgenic flower 35S::SHAGGY/S.

Interatomic analysis

An in silico tool was also used to analyse the possible relation between the SHAGGY-like kinase protein and the flowering process. As the interatomic tool was available only for the Arabidopsis plant model, the AtSK12 protein from Arabidopsis, which had homology to the identified sugarcane sequence, was used.

The resulting interaction map is presented in Figure 5, where the network obtained is formed by different proteins such as BIN2, XTR6, IAA6, ATAF1, ANAC102, ELF3, BEL1, and others. The BIN2 protein is related to the Brassisteroid pathway and flowering pathway (Yan et al., 2009). Kyaersgaard et al. (2011) showed that ATAF1 is a transcription factor (TF) that is related to senescence and expressed in the SAM.

The other TF identified in this interaction map was ANAC102 and it is also associated with a different biological process. ELF3 has been shown to be connected to the circadian rhythm and flowering pathway (Thines and Harmon, 2010).

Furthermore, BEL1 is also expressed in the SAM and it has been associated with SAM identity. Then, the possible interactions with the SHAGGY in Arabidopsis support our data that this sequence in sugarcane might be associated with the flowering process in the SAM.

In conclusion, the results presented here along with the previous results correspond to the first attempt to identify and characterise genes/proteins present in the SAM that may be associated with the flowering difference between sugarcane cultivars.

The results obtained with SHAGGY-like kinase suggest a potential marker for flowering; however, further experiments are needed in order to understand the exact way that this sequence interacts in the flowering pathway.
The results from this study may be useful in using these genes/proteins as molecular markers in plant breeding to look for late or non-flowering cultivars, and as markers to determine the period during which chemical or nutritional treatments may be applied in the field to prevent flowering.

Fig. 5—SHAGGY-Like kinase protein interaction map. Schematic representation of the interaction map obtained using the AtSK32 protein from the Arabidopsis genome, which was homologous to the sugarcane sequence.

Acknowledgments

This research was supported by the Brazilian Council for Research and Development (CNPq 552722/2007-3); F.S.L. and A.L.M. obtained a scholarship from CAPES.
REFERENCES


CLONING OF SMALL HEAT-SHOCK PROTEIN (HSP) GENE FROM SUGARCANE AND ANALYSIS OF ITS EXPRESSION UNDER DROUGHT STRESS

By

PAN-XIA LIANG 1,2,3,4, YANG-RUI LI 1,2,4*, LI-TAO YANG1,4, XING HUANG, HU CHEN, CHANG-NING LI, FU SUN and LI-HANG QIU

1State Key Laboratory of Subtropical Agro-Bioresources Conservation and Utilisation, Guangxi University, Nanning 530004, China
2Guangxi Crop Genetic Improvement and Biotechnology Lab, Nanning 530007, China
3Resources and Environment Research Institute, Guangxi Academy of Agricultural Sciences, Nanning 530007, China
4Sugarcane Research Center, Chinese Academy of Agricultural Sciences/Key Laboratory of Sugarcane Biotechnology and Genetic Improvement (Guangxi), Ministry of Agriculture/Guangxi Key Laboratory of Sugarcane Genetic Improvement, Nanning 530007, China

*Corresponding author: Yang-Rui Li, liyr@gxaas.net

KEYWORDS: Sugarcane, Silicon, Drought, HSP, Clone, Gene Expression.

Abstract

It has been reported that small heat-shock proteins are related to tolerance to adverse conditions such as drought in some plant species. The effects of silicon addition on differential protein expressions in sugarcane under water stress caused by 200 g/L PEG6000 treatment were studied. The results showed that the expression of heat-shock protein (HSP) was up-regulated by the PEG6000 treatment. The HSP transcript obtained by RT-PCR was 659 bp long, contained an open reading frame of 459 bp, and encoded a putative protein of 152 amino acids. Comparison of the amino acid sequence of the isolated HSP with HSP from 14 other species revealed identity levels between 69–96%. The deduced amino acids sequence contained a typical HSP domain that was very conserved. The results of quantitative real-time PCR analysis showed that the mRNA of HSP decreased before 40 h after PEG stress treatment and then significantly increased up to 90 h after PEG stress treatment, and Si addition significantly enhanced the gene expression. These results suggest that HSP might be involved in drought tolerance in sugarcane and that its expression is enhanced by Si application.

Introduction

Small heat shock protein (sHSP) widely exists in prokaryotes and eukaryotes. Sanmiya et al. (2004) confirmed that HSP is highly related to high temperature stress. More recent studies have provided evidence that low temperature, oxidative stress and drought stress can also induce sHSP expression. It means sHSP and abiotic stress have a certain relationship that causes the plant to produce shock protein in abiotic stress.

At present, HSP has been already found in soybean, corn, tobacco, broad bean and many other kinds of plants. However, we have not seen reports on the cloning of cane HSP gene and the relationship between sugarcane HSP and drought stress. Our previous studies found that Si improved the drought resistance of sugarcane plants.

In the present study, the full length HSP gene from sugarcane was cloned using RT-PCR. Likewise, the expression of this cDNA was evaluated using quantitative real-time PCR under...
drought stress, providing a reference to illustrate the role of HSP genes in water stress and drought resistance in sugarcane enhanced by Si application.

Materials and methods

The yang plants of sugarcane varieties ROC22 and ROC16 were cultured in 1/2 Hoagland solution. Three treatments, No PEG Control, PEG-6000 (200 g/L) and PEG-6000 (200 g/L) + Si (1.7 mM), were used when the plants had 5–6 leaves. Samplings were done at 0, 18, 40, 63, 96 and 144 hours after treatment.

Primers to amplify full-length HSP:

sHSP F: 5’- ATGTCGCT(C/G)GTGAG(G/T)CGCAGCA (A/G) CG -3'
sHSP R: 5’-GGCCACCGCTGACATAGTAC-3'

Amplification parameters: 95°C – 5 min; 35 cycles [95°C – 40 s, 58°C – 45 s, 72°C – 2 min]; and 72°C – 10 min.

Bioinformatic analysis of sHSP: software of BioXM2.6, Clustalx, DNAMAN, WoLF PSORT, Motif Scan, SMART, SOSUI signal, ExPASy Proteomics Serve

Real-time PCR: HSPQF: 5’- AGCATCCAGATCTCCGTTG -3'
HSPQR: 5’- GACATACCAAGACGAGATACC -3'
25S F: 5’- GCAGCCAAGCGTTCCATAGC -3'
25S R: 5’- CCAGCTACGTTCCCTATTG -3'

Amplification parameters: 95°C 10 min; 40 cycles [95°C 15s, 60°C 1 min] 72°C–30 s.

Results

1. The HSP transcript obtained by RT-PCR was 659 bp long, contained an open reading frame of 459 bp, and encoded a putative protein of 152 amino acids. The full transcript contained a 200 bp 3’ non-coding region, of which 19 bp corresponded to a poly A tail (Figure 1).

2. The relative protein molecular weight of sHSP clone was 17.15 kD, the PI was 5.83. Its sub-cellular localisation was found in the cytoplasm and contained no signal peptide. The amino acid sequence predicts that this is a soluble protein. The hydrophobic value was from –0.567 to 2.1. The sHSP had no transmembrane area. In addition, the sHSP sequence had no glycosylation sites, but six serine and 2 threonine phosphorylation sites.

3. The analysis on function domain of sHSP protein showed that amino acids 33–36 correspond to a casein kinase phosphorylation site, amino acids 1–4 to a bacterial

Fig. 1—Nucleotide and predicted amino acid sequence of sHSP.
immune globulin sample structure domain, amino acids 48–137 is a typical HSP 20 function domain, amino acids 42–251 is a CS function domain, and amino acids 37–83 is a MVP repetitive sequence.

4. The sHSP gene of sugarcane had 79–96% identity of nucleotide sequence from other species. Among them, its lowest identity is with rice, 79%, and the highest homology is with sorghum, 96%, and with corn, 93%. The amino acid sequences of sHSP gene and other species showed the homology between 69% and 96%, the lowest is with soybean, 69%, the highest is with sorghum, 96%, and then with corn, 89%.

5. Before 40 h after PEG stress treatment, the expression level of sHSP gene showed a small decrease. However, after 40 h, the expression level of sHSP increased significantly in the PEG treatment, it increased by 4.18 and 13.56 times after 63 h and 96 h, respectively, compared to the control. A decreased expression was observed after 96 h under drought stress. PEG + Si treatment showed the maximum value at 144 h, which corresponds to an increase of 44.64 times compared to the control (Figure 2).

Fig. 2—Expression of sHSP gene in sugarcane under water stress detected by quantitative real time PCR.

**Conclusion**

The sugarcane sHSP gene is 659 bp in length. The homology analysis shows that it is highly conservative in evolution. The expression of sHSP gene in sugarcane increased significantly under drought stress. Si improved the expressions of sHSP gene which could be helpful to improve the drought resistance of sugarcane.

**REFERENCE**