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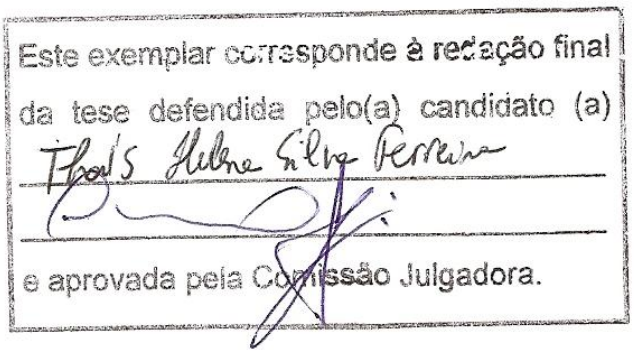
Thaís Helena Silva Ferreira

"IDENTIFICAÇÃO E CARACTERIZAÇÃO DE miRNAs ENVOLVIDOS NA RESPOSTA AO ESTRESSE HÍDRICO EM CANA-DE-AÇÚCAR (*Saccharum* spp)."



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Orientador: Prof. Dr. Marcelo Menossi Teixeira

Coorientadora: Dra. Agustina Gentile

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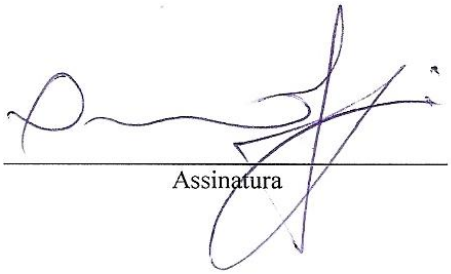

Assinatura

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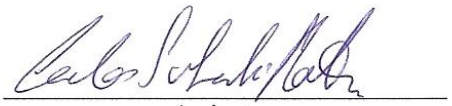

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Prof. Dr. Rogério Margis

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Aos meus pais e ao meu irmão com
muito amor por todo carinho,
dedicação e confiança.

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Índice

Resumo

A cana-de-açúcar é uma das mais importantes espécies vegetais cultiváveis do mundo, sendo o Brasil o maior produtor. A seca é um dos principais estresses que reduzem a produtividade da cana-de-açúcar e a produção de variedades tolerantes não só representa ganhos econômicos como contribui para a sustentabilidade do setor de bioenergia. A base genética da tolerância à seca ainda é pouco conhecida. Uma nova forma de regulação mediada por micro RNAs (miRNA) foi recentemente descrita como um componente importante e decisivo no desenvolvimento vegetal e na modulação da resistência aos mais diversos estresses. Nesse contexto, o objetivo desse trabalho foi identificar miRNAs expressos durante o estresse hídrico e correlacioná-los com a tolerância à seca em cana-de-açúcar. Para tal foram avaliados os perfis de expressão de miRNAs em dois cultivares de cana contrastantes quanto à tolerância à seca, mantidos em condições de irrigação normal, sob déficit hídrico (dois e quatro dias de estresse) e após recuperação por irrigação. Os dados foram obtidos através do sequenciamento de bibliotecas de miRNAs e a confirmação foi realizada por qRT-PCR. A comparação dos microtranscritomas dos cultivares RB867515 (mais tolerante à seca) e RB855536 (mais sensível à seca) permitiu a identificação de 7 miRNAs diferencialmente expressos em resposta à seca. Cinco miRNAs tiveram sua expressão confirmada através de ensaios de RT-qPCR. Também foram preditos, através de análises *in silico*, precursores e alvos para esses miRNAs. Aparentemente, muitos desses alvos desempenham papéis diversos na

tolerância à seca. Esses resultados contribuíram para a descoberta de novos miRNAs em cana-de-açúcar e forneceram maior entendimento sobre a complexa rede de regulação gênica envolvida na resposta ao estresse hídrico.

Abstract

Drought stress is a major abiotic stress factor that reduces significantly sugarcane yields. Sugarcane (*Saccharum* spp.) is one of the most important crop plants in the world and the molecular processes that mediate plant response to water stress are barely known. Although several microRNA mediating post-transcriptional regulation during water stress were described in other species, the role of the sugarcane microtranscriptome during drought stress is not known so far. The objective of this work was to identify miRNAs differentially expressed under drought stress and to correlate this expression with the tolerance of two cultivars contrasting for drought tolerance. The sugarcane cultivars RB867515 (higher drought tolerance) and RB855536 (lower drought tolerance) were cultivated in greenhouse for three months and then submitted to drought for 2 and 4 days. By using small RNA deep-sequencing we were able to identify 18 conserved miRNAs families, of which 12 families represent new sugarcane miRNA families. From the total miRNAs identified, 7 were differentially expressed under drought. Six of those were differentially expressed in two days and 5 miRNAs in four days of stress. Five miRNAs had their expression validated by RT-qPCR. The precursors and targets of the differentially expressed miRNAs were predicted using an *in silico* approach. Many of those targets probably play important roles in drought tolerance. These findings contribute significantly to increase the number of identified miRNAs in sugarcane and also to uncover the complex regulation network that is activated by drought stress.

Introdução

Cana-de-açúcar

A cana-de- açúcar é uma planta monocotiledônea, alógama e perene. Pertence à família Poaceae e ao gênero *Saccharum.* É uma das mais importantes plantas cultiváveis do planeta e as espécies parentais dos cultivares comerciais possuem dois centros de origem: Nova Guiné e norte da Índia (Stevenson, 1965; Watson et al., 1985).

Os cultivares comerciais são híbridos e seu genoma é constituído por duas a quatro espécies de *Saccharum*. Poliplóides e aneuplóides, os híbridos possuem de 100 a 120 cromossomos num total de 10 Gbp de DNA (D'Hont A. et al., 2001).

Devido à alta complexidade do genoma da cana, os esforços para sua caracterização estão baseados em descobertas de genes através de ESTs (*Expression Sequence Tags*). Coleções de ESTs têm sido desenvolvidas por vários grupos no mundo todo, como na África (Carson and Botha, 2000), na Austrália (Casu et al., 2003), nos Estados Unidos (Ma et al., 2004), tendo sido realizado no Brasil o estudo mais completo (Vettore et al., 2003).

O Brasil é o maior produtor mundial de cana-de-açúcar, seguido pela Índia e pela Austrália (FAO, 2009). A área total cultivada pelo setor sucroalcooleiro no Brasil foi de 8.368,4 mil hectares em 2011 (Conab, 2011). O Estado de São Paulo é o maior produtor com 52,2% (4.370 mil hectares), seguido por Minas Gerais com 8,87% (742,65 mil

hectares), Goiás com 8,1% (678,42 mil hectares), Paraná com 7,3% (611,44 mil hectares), Mato Grosso do Sul com 5,70% (480,86 mil hectares), Alagoas com 5,45% (463,65 mil hectares) e Pernambuco com 3,89% (326,11 mil hectares) (Conab, 2011).

A previsão do total de cana moída na safra 2011/12 é de aproximadamente 571 milhões de toneladas. Deste total, 47,3% serão destinadas à produção de açúcar (estimada em 36,9 milhões de toneladas) e 50,3% destinadas à produção de etanol, sendo 9.069,3 bilhões de litros de etanol anidro e 13.788,3 bilhões de litros de etanol hidratado (Conab, 2011).

A lavoura de cana-de-açúcar continua em expansão no Brasil. Em 2011, as áreas em produção tiveram aumento considerável, sendo mais significativo nos Estados de: Minas Gerais, Mato Grosso do Sul, Goiás e Mato Grosso. O total da área de expansão em todo país deve ficar em 697 mil hectares, aproximadamente 8,3% a mais do total já utilizado (Conab, 2011). Porém, a produtividade média brasileira sofreu um declínio de 11,8% em relação à safra de 2010/11, estimada em 68.289 kg/ha. O principal causador da queda da produção foi o clima, principalmente a escassez de chuva nas regiões Centro-Oeste e Sudeste durante os meses de abril a outubro de 2010. A falta de chuvas no período indicado não permitiu o pleno desenvolvimento dos canaviais, provocou atraso no início da moagem da safra 2011/12 e o período de entre safra ficou maior (Conab, 2011).

A disponibilidade de água doce no mundo reduziu mais de seis vezes entre 1900 e 1995, mais do que o dobro da razão de crescimento populacional, e a agricultura, principalmente a irrigação, é responsável por 70% do uso de água (Parris and Kates, 2003).

Como a seca é um dos principais estresses abióticos que atingem a cultura da cana-de-açúcar e com a escassez de água prevista para o futuro, o sustento da indústria canavieira no Brasil e no mundo dependerá do desenvolvimento de variedades de cana-de-açúcar mais tolerantes ao estresse hídrico.

Cana-de-açúcar e estresse hídrico

A cana-de-açúcar possui o metabolismo fotossintético do tipo C4 e é considerada altamente eficiente na conversão de energia radiante em energia química. As características dos cultivares influenciam a eficiência fotossintética da cana, além das variações climáticas que prevalecem durante o desenvolvimento da cultura, como mudanças na intensidade e qualidade de luz, na concentração de CO_2, na temperatura e na disponibilidade de água e de nutrientes (Rodrigues, 1995).

Uma situação de déficit hídrico durante a fase vegetativa destas plantas (desenvolvimento inicial da parte aérea e desenvolvimento tardio de folhas) diminui a biomassa e consequentemente o crescimento de colmos, reduzindo drasticamente a produtividade. Porém, na fase de maturação os efeitos da seca moderada podem ser benéficos, já que a pequena redução na taxa de fotossíntese é compensada pelo maior direcionamento de sacarose a ser acumulada nos colmos em relação ao seu uso para crescimento de biomassa (Inman-Bamber and Smith, 2005; Rodrigues, 1995).

O déficit hídrico ocorre quando a taxa de transpiração excede a absorção de água. Em nível celular, pode ocorrer maior concentração de solutos, mudanças no volume da célula e forma da membrana, interrupção de gradientes de potencial de água, perda de turgor, ruptura da integridade da membrana e desnaturação de proteínas. A capacidade

da planta em responder e sobreviver ao déficit hídrico celular depende de mecanismos da planta inteira, da espécie, da duração e severidade da perda de água, do estágio de desenvolvimento, do tipo de órgãos e do tipo celular. Respostas ao déficit hídrico podem ocorrer dentro de alguns segundos (como uma mudança no estado de fosforilação de uma proteína) ou dentro de minutos e horas (como mudança na expressão gênica) (Bray, 1997).

É sabido que existem variações nas respostas dos genótipos de cana em relação ao estresse por seca. Dentre as alterações morfológicas mais comuns, destacam-se: a inibição da germinação dos toletes, da germinação da raiz e da formação de novas folhas no palmito, a redução da elongação de folhas e de colmos, o enrolamento das folhas, o fechamento estomático (Inman-Bamber and Smith, 2005) e o crescimento do sistema radicular (Smith et al., 2005). Adicionalmente ao fechamento estomático, em condições mais severas ocorre também inibição de enzimas e metabólitos essenciais para a fotossíntese.

Os processos moleculares que levam a planta a apresentar resposta ao estresse hídrico não são totalmente compreendidos. Muitas pesquisas sugerem o envolvimento de microRNAs em processos celulares ligados à resposta ao estresse por seca (Kantar et al., 2011; Liu et al., 2008; Sunkar and Zhu, 2004; Zhang et al., 2011; Zhang et al., 2008b; Zhao et al., 2007a). Portanto, os miRNAs são candidatos a mediadores da resposta metabólica ao estresse por seca.

miRNAs

MicroRNAs (miRNAs) são sequências endógenas de RNA com aproximadamente 22 nucleotídeos que desempenham um papel chave na regulação da expressão de genes em animais e plantas (Bartel, 2009; Carthew and Sontheimer, 2009). Compreendem uma das mais abundantes classes de moléculas regulatórias em organismos multicelulares (Bartel, 2004). Em animais, os miRNAs estão envolvidos no controle da atividade de aproximadamente 50% dos genes codificantes de proteínas e estudos funcionais indicam que participam da regulação de quase todos os processos celulares investigados até agora (Krol et al., 2010).

Reportado pela primeira vez em 1993, quando Victor Ambros e Rosalind Lee descobriram que *lin-4*, um gene conhecido por controlar o tempo de desenvolvimento larval de *Caenorhabditis elegans*, não codificava uma proteína, mas sim um par de pequenos RNAs, um deles com aproximadamente 22 nucleotídeos e o outro com 61 nucleotídeos. O maior deles seria o precursor do menor e era capaz de se dobrar formando um *loop* (Lee et al., 1993).

Naquele mesmo ano, verificou-se que esses pequenos RNAs eram reversamente complementares à região 3'UTR do gene *lin-14* e que, dessa forma, mediavam a repressão desse gene, diminuindo a quantidade da proteína LIN-14, sem mudanças no nível do mRNA (Wightman et al., 1993). Isso deu origem ao modelo de que pequenos RNAs endógenos regulam a expressão de outros genes através da repressão da tradução. Assim, esse RNA de 22 nucleotídeos foi o primeiro membro da abundante classe de pequenos RNAs reguladores chamada de microRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).

A partir de então, com a evolução das técnicas de clonagem, sequenciamento de genomas e das análises de bioinformática, milhares de miRNAs foram descobertos, bem como o envolvimento destes na regulação de diversos processos celulares em animais, plantas e, inclusive, em organismos unicelulares (Bartel, 2004; Zhao et al., 2007b). Além de seus papéis fundamentais no desenvolvimento (Flynt and Lai, 2008), os miRNAs estão envolvidos em uma vasta gama de funções biológicas, como no controle hormonal, na resposta imunológica e na adaptação a uma variedade de estresses bióticos e abióticos (Pedersen and David, 2008; Sunkar et al., 2007).

Biogênese dos miRNAs

A biogênese de miRNAs em plantas é muito similar à encontrada em animais. A maioria dos genes mir são transcritos pela *RNA polimerase II* (*Pol II*), possuindo *CAP 5'* e cauda *poliA* (Lee et al., 2004). Os transcritos primários, chamados de pri-miRNAs, tipicamente formam uma estrutura com dobras imperfeitas, que é processada, por pelo menos duas etapas de clivagens mediadas por *RNase III-endonucleases.* Nos animais, a primeira clivagem é realizada pela proteína *Drosha* no núcleo e a segunda pela proteína *Dicer* no citoplasma, enquanto que, nas plantas, as duas clivagens são realizadas por enzimas *Dicer-like* no núcleo. O produto final das duas clivagens é uma fita dupla de RNA de aproximadamente 21-22 nucleotídeos (miRNA/miRNA*) e o produto intermediário das clivagens é conhecido como pré-miRNA, apresentando estrutura características de *hairpin* (Bartel, 2004; Kim, 2005). Nas plantas, os pré-miRNAs são mais variáveis no tamanho e maiores; a maioria dos miRNAs possuem 21 nucleotídeos e há uma preferência pela base U na posição 5' do miRNA maduro (Reinhart et al., 2002).

Uma vez no citoplasma, o duplex miRNA:miRNA* é separado por *helicases* e o miRNA maduro é incorporado a um complexo enzimático denominado *RISC* (Complexo de silenciamento induzido por RNA), enquanto que o miRNA* é degradado (Bartel, 2004). Em animais, a escolha do miRNA maduro é dependente da estabilidade da porção 5' do duplex: a que apresentar menor força no pareamento entre as bases será incorporada pelo complexo (Khvorova et al., 2003; Tomari et al., 2004). O complexo *RISC/miRNA* irá promover o silenciamento de um gene alvo específico que contém uma região complementar a sequência do miRNA, assim como nas etapas dos fenômenos conhecidos por PTGS (silenciamento gênico pós-transcricional) em plantas e por RNAi em animais (Bartel, 2004; Chapman and Carrington, 2007). O complexo *RISC*, em eucariotos, possui invariavelmente um membro da família das proteínas *ARGONAUTA (AGO)*, que possui um domínio de ligação a sRNAs (pequenos RNAs), chamado *PAZ* e um domínio catalítico que confere atividade endonucleolítica, chamado *PIWI* (Chapman and Carrington, 2007).

O silenciamento gênico pós-transcricional pode ocorrer através de dois mecanismos: clivagem do mRNA ou repressão da tradução. O mecanismo é, na maioria dos casos em animais, dependente da complementariedade entre a sequência do miRNA e do mRNA, de forma que ocorrerá repressão da tradução se não houver complementariedade suficiente para que ocorra a clivagem, devido ao pareamento incorreto e a pequenas alças (Bartel, 2004).

A biogênese dos miRNAs é fundamental para diferenciá-los dos demais sRNAs e para entender os mecanismos de ação na regulação da expressão dos genes alvos, principalmente em plantas, que possuem uma complexa população de pequenos RNAs,

sendo miRNAs geralmente a minoria. Dessa forma a identificação e caracterização é dependente das etapas de sua síntese, sendo importantes a estrutura do pri-miRNA, do pré-miRNA, do duplex miRNA/miRNA* e do miRNA maduro (Meyers et al., 2008).

miRNAs em plantas

Os miRNAs em plantas foram encontrados pela primeira vez em *Arabidopsis thaliana*, dez anos depois da descoberta em animais, em um experimento no qual 16 miRNAs foram clonados e posteriormente confirmados por *Northern blot* (Reinhart et al., 2002). Assim como em animais, quase todos os processos biológicos em plantas envolvem a participação de um ou mais miRNAs (Voinnet, 2009).

Com a descoberta dessa classe de pequenos RNAs em animais e plantas somada às diferenças na sua biogênese ao fato de não terem sido encontrados miRNAs conservados entre esses reinos, foi proposto por Bartel et al. (2004) que o surgimento dos miRNAs ocorreu de forma independente nessas duas linhagens multicelulares depois do seu último ancestral comum (provavelmente um organismo unicelular). Isso reforça a importância dessa classe de pequenos RNAs na formação do padrão multicelular.

Genes mir de animais são encontrados principalmente em íntrons e éxons e geralmente formam *clusters* no genoma (Kim, 2005). Já em plantas, a maioria dos genes mir são intergênicos e raramente encontrados em conjunto, com exceção à soja (Zhang et al., 2008a).

Em *Arabidopsis*, os miRNAs são altamente complementares às ORFs (*Open Reading Frames*) dos mRNAs-alvos (Rhoades et al., 2002). Um maior grau de pareamento foi encontrado entre os nucleotídeos 9 a 11, sugerindo a existência de um sítio de clivagem,

comprovado por técnicas como 5’ RACE (Llave et al., 2002). Mais tarde, em estudos nos quais os produtos protéicos dos genes alvo foram mensurados, a quantidade dos mRNA-alvo e das proteína foi discrepante, sugerindo que miRNAs de plantas também podem inibir a produção de proteína a partir de seus alvos (Gandikota et al., 2007). Diferentemente do que acontece em animais, a escolha do mecanismo de inibição não é dependente da posição (ORF, 5’ou 3’ UTR) e nem do grau do pareamento entre miRNA e mRNA-alvo, sendo que ambos possuem a mesma frenquência (Brodersen et al., 2008; Palatnik et al., 2007). Dessa forma, a regulação de genes alvos só por repressão ou só por clivagem é rara e é dependente de proteínas repressoras que interagem com AGO em tecidos particuares (Voinnet, 2009).

O advento de técnicas de sequenciamento de última geração e de novas estratégias e ferramentas de bioinformática (Yang and Li, 2011) aplicadas na identificação e quantificação de pequenos RNAs facilitou a caracterização de miRNAs não conservados (espécie específicos) e expressos em ocasiões singulares em plantas, como por exemplo, sob condições de estresse ambiental (Fahlgren et al., 2007; Zhu et al., 2008). A análise do padrão de expressão dos miRNAs em plantas revelaram inúmeros miRNAs tecido ou estágio específicos e que muitos são induzidos por estímulos externos, demostrando a ação peculiar dos miRNAs nos diferentes estágios do desenvolvimento, em diferentes tecidos e após diversos tratamentos (Meng et al., 2011).

miRNAs e estresse hídrico

Por serem organismos sésseis, as plantas sofrem muito com as mudanças ambientais, que, quando extremas, podem causar danos celulares irreversíveis, podendo

levar à morte. Para evitar os danos causados pelo estresse, as plantas desenvolveram respostas adaptativas bem coordenadas que operam no nível transcricional, pós-transcricional, traducional e pós-traducional (Sunkar, 2010). Análises do transcritoma e proteoma de plantas submetidas a diferentes estresses ambientais revelaram variações nos níveis de centenas, até mesmo milhares de mRNAs e proteínas, sugerindo a indução e a repressão de genes e a regulação da produção, estabilidade e degradação de proteínas (Bartels and Sunkar, 2005; Hasegawa et al., 2000; Zhu, 2002). Sabe-se que os miRNAs fazem parte das redes regulatórias de respostas a estresse em plantas (Sunkar, 2010) e podem ser induzidos ou reprimidos de acordo com estímulos ambientais.

A estrutura dos promotores dos genes mir é similar à dos promotores de genes codificadores de proteínas e possuem elementos cis para o controle da transcrição, como, por exemplo, sítios de início de transcrição, TATA Box e de ligação de fatores de transcrição (Zhou et al., 2007). Um exemplo é a regulação da via envolvida na homeostase do fósforo. Quando há diminuição do fósforo disponível, o fator de transcrição PHR1 (*PHOSPHATE STARVATION RESPONSE1*) é induzido e leva ao aumento da expressão do miR399 (Bari et al., 2006).

Foi recentemente demonstrado que o nível de expressão de miRNAs também varia em resposta ao estresse por seca (Li et al., 2008; Sunkar and Zhu, 2004; Trindade et al., 2010; Zhao et al., 2009). Em *Arabidopsi*s, uma série de miRNAs (miR157, miR167, miR168, miR171, miR319, miR393, miR396, miR397 e miR408) foram induzidos por seca (Liu et al., 2008; Sunkar and Zhu, 2004). Similarmente, trabalhos com arroz, pópulus e alfafa também mostraram a indução ou repressão de miRNAs por falta de água (Lu et al., 2008; Trindade

et al., 2010; Zhao et al., 2007a). Em tomate, a superexpressão do miR169 conferiu tolerância ao estresse hídrico (Zhang et al., 2011).

Em cana-de-açúcar foram identificados 34 miRNAs, que estão depositados no miRBase (http://www.mirbase.org), banco de dados de miRNAs. Porém, nenhum trabalho foi realizado visando encontrar quais miRNAs estão envolvidos na resposta à seca. Até o momento só foram realizados trabalhos não envolvendo miRNAs, como por exemplo utilizando macroarranjos de DNA com coleção de ESTs (http://sucest-fun.org/) para a identificação de genes envolvidos em diversos processos (De Araujo et al., 2005; De Rosa et al., 2005; Drummond et al., 2005; Nogueira et al., 2003), para a catalogação dos componentes das vias de transdução de sinal (Papini-Terzi et al., 2005) e para análise da expressão diferencial das vias de transdução de sinais frente a diferentes estresses bióticos e abióticos (Rocha et al., 2007; Schlögl et al., 2008).

Com relação à seca, o transcritoma de cana foi avaliado em plantas expostas a ácido abscísico, metil jasmonato, herbivoria, interação com bactéria fixadora de nitrogênio, deficiência de fosfato e seca. Dentre todos os tratamentos avaliados, a seca foi o que causou maiores alterações no transcritoma da cana, com 93 genes diferencialmente expressos em um cultivar sensível à seca submetido à suspensão de rega em casa de vegetação (Rocha et al., 2007). Esse dado dá margem à suposição de que poderiam ser identificados muitos miRNAs envolvidos na regulação destes genes de resposta à seca. A identificação desses miRNAs pode contribuir para ampliar o conhecimento da regulação molecular da resposta à seca em cana.

Hipótese

Existem microRNAs que estão envolvidos na resposta molecular à seca em cana-de-açúcar, variando seu perfil de expressão de acordo com o genótipo (tolerante ou sensível à seca). Os alvos desses miRNAs estão envolvidos em uma ou mais vias de resposta ao estresse por seca.

Objetivos

O objetivo geral deste projeto foi identificar miRNAs de cana-de-açúcar responsáveis pela regulação da resposta fisiológica da planta sob déficit hídrico, de forma a poder identificar possíveis correlações entre a expressão dos miRNAs e os diferentes níveis de tolerância a seca observado em variedades de cana.

Os objetivos específicos foram:

- Identificar miRNAs cuja expressão seja modulada pelo déficit hídrico em cana-de-açúcar;

- Identificar *in silico* os potenciais alvos dos miRNA diferencialmente expressos sob seca;

Apresentação do trabalho

O presente trabalho identificou miRNAs diferencialmente expressos sob condições de estresse hídrico em cana-de-açúcar através do sequenciamento de pequenos RNAs, caracterizou a expressão desses miRNAs através da análise da quantidade de transcritos por sequenciamento e por PCR em tempo real e relacionou esses miRNAs a possíveis funções envolvidas na tolerância à seca através da predição computacional dos respectivos alvos.

Capítulo I

Microtranscriptomes associated with drought stress responses in the bioenergy crop sugarcane (*Saccharum* spp.)

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Abstract

Sugarcane (*Saccharum* spp.) is one of the most important crop plants in the world. Drought stress is a major abiotic stress factor that significantly reduces sugarcane yields, but the molecular processes that mediate plant responses to water stress remain largely unknown. Although several microRNAs that mediate post-transcriptional regulation during water stress have been described in other species, the role of the sugarcane microtranscriptome during drought stress has not been studied. The objective of this work was to identify sugarcane miRNAs that are differentially expressed under drought stress and to correlate this expression with the behavior of two sugarcane cultivars with different drought tolerances. The sugarcane cultivars RB867515 (high drought tolerance) and RB855536 (low drought tolerance) were cultivated in a greenhouse for three months and then submitted to drought for 2, 4, 6 or 8 days. Using small RNA deep sequencing, we were able to identify 18 miRNA families, including 12 new sugarcane miRNA families. Among all of the miRNAs thus identified, seven were differentially expressed during drought. Six of these miRNAs were differentially expressed at two days of stress, and five miRNAs were differentially expressed at four days. The expression levels of five miRNAs (ssp-miR164, ssp-miR394, ssp-miR397, ssp-miR399a and miR528) were validated by RT-qPCR (reverse transcription followed by quantitative PCR). The precursors and targets of the differentially expressed miRNAs were predicted using an *in silico* approach; many of these targets may play important roles in drought tolerance. These findings constitute a significant increase in the number of identified miRNAs in sugarcane and contribute to the elucidation of the complex regulatory network that is activated by drought stress.

Keywords: Sugarcane, miRNAs, drought, small deep sequencing, RT-qPCR.

INTRODUCTION

miRNAs are a class of small, non-coding RNAs, approximately 21 nucleotides in length, that are endogenous to both plants and animals [1,2] and function to regulate gene expression by sequence-based interaction with target mRNAs [3,4]. In plants, each primary miRNA is transcribed by the RNA polymerase II enzyme [5] and forms an imperfect foldback structure that is processed into a stem-loop precursor (pre-miRNA) by Dicer-like 1 (DCL1), a nuclear RNase III-like enzyme. The pre-miRNA is then cleaved into a double-stranded RNA duplex, called miRNA/miRNA*, by the same enzyme [3,6,7]. One of the strands, called the mature miRNA, is incorporated into the RNA-induced silencing complex (RISC), whereas the other strand, called miRNA*, is usually degraded. The incorporated mature miRNA guides the RISC to a target mRNA by base pairing, leading to mRNA cleavage or translational repression [3,4,7].

The first plant miRNAs were described in *Arabidopsis thaliana* [7]. Since then, several miRNAs have been identified in plants and are known to be involved in developmental and physiological processes such as flowering, leaf and flower differentiation and the auxin response [2,8]. Recently, the levels of a number of miRNAs have been demonstrated to be sensitive to abiotic or biotic stresses [9,10,11,12,13,14,15], with evidence that several play roles in drought stress [9,16]. To date, only 34 sugarcane miRNAs are known (http://www.mirbase.org). Considering that drought stress significantly reduces sugarcane yields [17], the identification of sugarcane miRNAs that

respond to this stress will help to elucidate the molecular basis of drought stress tolerance in this important biofuel crop.

Deep sequencing strategies have revolutionized the discovery of small RNAs, constituting the most effective method for plant miRNA detection [18] and providing tools for the discovery of additional species-specific or low-expression miRNAs [19,20]. Because miRNAs are conserved across species, bioinformatics strategies [21,22] based on expressed sequence tags (ESTs) from sugarcane and genomic survey sequences (GSSs) from sorghum were used in this study, and an emphasis was placed on homology and structural similarity. In this work, our goal was to identify and characterize miRNAs that may be regulated by water deficit in sugarcane.

RESULTS

Water stress in sugarcane plants

Two sugarcane cultivars differing in their tolerance to drought stress, RB867515 (higher tolerance, HT) and RB855536 (lower tolerance, LT), were grown in the greenhouse for three months. Water was withheld for 2, 4, 6 or 8 days, and stress symptoms, such as leaf rolling and senescence, appeared early on the second day. By the sixth day, almost all of the stressed plants were severely affected (Supporting Information, FS1 and FS2). Based on these results, we decided to evaluate the plants that were stressed at two and four days.

To characterize the degree of stress at the molecular level, we performed RT-qPCR (Reverse transcription followed by quantitative PCR) amplification of a sugarcane gene encoding a dehydrin (Fig. 1). The sugarcane assembled sequence (SAS) SCQGLR1085F11.g,

corresponding to a member of the dehydrin family, is induced by drought in sugarcane [23], and several homologues have been reported to be modulated by this stress in other species [24,25,26,27]. This gene was induced after two and four days of stress (Fig. 1) in both sugarcane varieties. However, the cultivar with lower tolerance displayed greater induction at both experimental time points.

Analysis of *Saccharum* spp. Solexa sequencing

To identify the miRNAs involved in drought stress in sugarcane, eight small RNA libraries from mature leaves were sequenced using the Solexa technology. These libraries were representative of plants stressed for two and four days and control plants from both sugarcane cultivars. A total of 90 million reads were obtained, ranging from 8 to 15 million reads per library (Table 1), similar to the available sequencing data for citrus, sorghum and maize [19,28,29]. All Solexa reads were aligned against the GenBank (http://www.ncbi.nlm.nih.gov/) and RFam (http://rfam.sanger.ac.uk/) databases to sort the reads into categories (Table 1). All of the sequences were then regarded as miRNAs for further analysis.

The size distribution of all of the sequences is presented in Fig. 2. The majority of the reads were 21 to 24 nt in length for all libraries, with 21 nt being the most represented class of redundant species, followed by 24 nt (Fig. 2). However, sequences of 24 nt were the most represented class of non-redundant species (Fig. 3). In the 21 nt reads, the first base position was composed of a U or an A in approximately 80% of the reads in all libraries (Fig. 4), with equal numbers of each nucleotide.

Identification of new potential miRNAs in *Saccharum* spp.

To identify sugarcane miRNA candidates within our sequenced set, unique small RNA species were aligned against the *Sorghum bicolor* genome to identify loci corresponding to putative miRNA precursors. A total of 21 miRNAs were detected, and 17 of them correspond to 12 novel miRNAs families in sugarcane: miR160, miR164, miR166, miR171, miR172, miR319, miR390, miR393, miR394, miR397, miR399 and miR529 (Table 2). The sugarcane miRNAs were named based on their homology to sorghum miRNAs.

Precursors were also identified among the sugarcane ESTs (Table 3 and Fig. 5) derived from six genomic loci because six different SASs were found to correspond to six different miRNA genes (Table 3) and their RNA sequences have the intramolecular capacity to fold into hairpin structures (Fig. 5). Five precursor sequences were from the SUCEST (http://sucest-fun.org/) database, whereas only one was found in the SoGI (http://compbio.dfci.harvard.edu/) database (Table 3). Two distinct SASs were found for the ssp-miR167 precursor. The MFEs (minimum free energy) ranged from -145 to -338 kcal/mol, and the G/C content ranged from 42 to 49% (Table 3).

Analysis of sugarcane miRNAs modulated by drought stress

Among all of the miRNAs identified, seven were differentially expressed during drought (Fig. 6), of which six were differentially expressed after two days of stress (Table 4) and five were differentially expressed after four days of stress (Table 5). The ssp-miR164 miRNA was differentially expressed only at two days of stress. Additionally, three miRNAs (ssp-miR164, ssp-miR397 and ssp-miR528) were up-regulated in the RB867515 (higher tolerance, HT) cultivar, and none were down-regulated by drought. In the RB855536 (lower tolerance, LT) cultivar, four miRNAs (ssp-miR164, ssp-miR394, ssp-

miR399c and ssp-miR1432) were down-regulated, and only one (ssp-miR397) was up-regulated by drought. Only ssp-miR397 displayed the same pattern in both cultivars, as it was induced after two days of water stress (Table 4).

ssp-miR393 was only differentially expressed at four days of stress. Four miRNAs (ssp-miR394, ssp-miR397, ssp-miR399a and ssp-miR528) were down-regulated by drought in the HT cultivar. Two other miRNAs (ssp-miR399a and ssp-miR528) were down-regulated, and one (ssp-miR393) was up-regulated in the LT cultivar. Only miRNAs ssp-miR399a and ssp-miR528 were down-regulated in both cultivars (Table 5).

Four miRNAs (ssp-miR394, ssp-miR397, ssp-miR399a and miR528) were differentially expressed at both time points during the stress period, and only one miRNAs (ssp-miR399a) presented similar expression profiles in the LT cultivar (Table 4 and 5).

Stem-loop RT-qPCR [30,31] was used to validate the expression of all seven differentially expressed miRNAs during drought according to the sequencing data. The expression patterns of five miRNAs (ssp-miR164, ssp-miR394, ssp-miR397, ssp-miR399a and ssp-miR528) were validated by this approach. Among the 15 differentially expressed profiles observed with the sequencing approach (Fig. 6), six (40.0%) displayed the same expression profile using RT-qPCR (Fig. 7). The RT-qPCR analysis confirmed two out of eight (25.0%) profiles after two days of stress (Fig. 7A) and four out of seven (57.1%) after four days (Fig 7B). In the HT cultivar, six out of seven (85.7%) profiles were confirmed (Fig 7A and B), whereas in the LT cultivar, zero out of eight (0.0%) profiles were confirmed. In all of the RT-qPCR profiles, the miRNA levels of the rehydrated plants demonstrated a tendency to return to the control levels (Fig. 7A and B).

Prediction of miRNA targets

The mature sequences of the seven miRNAs modulated by drought were used to search for their targets in sugarcane (Table 6 and Supporting information, TS1). All of these miRNAs had putative targets in the SUCEST database (Table 6), several of which encode transcription factors (ssp-miR164, ssp-miR394, ssp-miR528 and ssp-miR1432), growth or development regulators (ssp-miR393), proteins associated with floral development (ssp-miR394) and several phosphatases, kinases, and oxidases (ssp-miR397, ssp-miR399a, ssp-miR528 and ssp-miR1432), among others (TS1). The targets had homologous proteins in several plant species, especially maize, rice and *Brachypodium*, suggesting that the pathways where these targets act may be conserved.

DISCUSSION

The sugarcane microtranscriptome

Although the miRNAs of several plant species have been recently studied, neither miRNA sequencing identification nor an analysis of differential miRNA expression in response to drought stress has been performed in sugarcane. We identified 21 sugarcane miRNAs comprising 18 families using deep sequencing, including 12 families described here for the first time in sugarcane (Table 2). Because the sugarcane genome has not yet been sequenced, *Sorghum bicolor* was chosen as a reference organism because of the high level of identity between the genes in both species [32,33,34]. One of the strategies for the characterization of miRNAs is to examine the phylogenetic conservation of their sequences [35], which permitted the identification and classification of the miRNAs from eight sugarcane libraries. The identification of these miRNAs was accomplished by

precursor sequence folding into genuine hairpin structures of the *S. bicolor* sequences where the reads matched, and their classification was based on homology to *S. bicolor* miRNAs in the miRBase database.

In this study, only six putative precursors were found in the sugarcane EST databases, representing five miRNA families (Fig. 5 and Table 3). Because such precursors have short lifetimes in plants due to rapid processing by Dicer-like 1 in the nucleus [3,36], EST databases do not typically have sufficient coverage to facilitate the discovery of a great number of precursors. For example, in *Brachypodium distachyon*, a model organism for grass species, only 0.05% of the ESTs and 0.012% of the GSSs contain potential miRNAs [37]. Of the five families found in sugarcane, four have been previously described, and the secondary structures of the pre-miRNAs have been evaluated using an *in silico* approach [38]. The sugarcane precursor for ssp-miR166 is herein described for the first time.

The majority of the small RNAs in sugarcane are 21 or 24 nt in length, as expected in plants (Fig. 2) [4]. The 24 nt species are the most abundant in the non-redundant pool of short reads in sugarcane (Fig. 3), and similar distributions have been reported in studies with several other organisms [18,39,40]. This distribution is likely because 24 nt RNAs are siRNAs and unlike miRNAs, a longer double strand of RNA can result in several different small sequences that act in post-transcriptional gene silencing (PTGS).

It has been reported that uracil appears to be dominant as the first nucleotide at the 5´ ends of mature miRNAs [41]. In sugarcane, the first positions of the mature sequences were equally composed of U and A (Fig.4). The 5´end nucleotide of a small RNA strand determines the identity of at least several of the recruited AGO proteins [42]. Thus,

the 5´end nucleotide has important implications for miRNA function because AGO1 preferentially associates with 21 nt RNAs with a U at the 5´ end, resulting in PTGS activity. In contrast, AGO2 seems to preferentially associate with 21 nt RNAs that have an A at the 5´ end, abolishing their silencing activity and rendering the miRNA functionally inert [43].

miRNAs associated with drought response in sugarcane

To identify miRNAs that are differentially expressed during drought, eight libraries from sugarcane leaf were analyzed: four from RB867515, a cultivar with high tolerance to drought, and four from RB855536, which has a lower drought tolerance. A total of seven miRNAs were differentially expressed during drought (Fig. 6). Six of these were differentially expressed at two days (Table 4), and five miRNAs were differentially expressed at four days of stress (Table 5). For five of these seven miRNAs, the differential expression profiles obtained by sequencing displayed the same expression profiles by RT-qPCR (Fig. 7): ssp-miR164, ssp-miR394, ssp-miR397, ssp-miR399a and ssp-miR528. The lack of correlation observed for the other two miRNAs is likely due to cross-amplification of miRNA variants or to the presence of very similar miRNAs in the RT-qPCR samples.

The putative targets of the miRNAs modulated by drought provide interesting insights into the defenses activated by sugarcane. For instance, a NAC transcription factor was found among the targets of ssp-miR164 (Table 6), in agreement with previous studies [44,45]. It is known that miR164 regulates the expression of five NAM/ATAF/CUC (NAC) domains in *Arabidopsis*. This class of plant-specific transcription factors has important roles in development, growth and stress responses, such as cold, drought and pathogen stresses [46,47,48,49,50]. In one study, overexpressing a member of the NAC gene family

resulted in enhanced salt tolerance in tobacco [51]. In addition, NAC is responsible for transmitting auxin signals [52]. Another ssp-miR164 target was an MDR-like ABC transporter that also participates in auxin transport [53]. In this study, ssp-miR164 was differentially expressed in response to drought after only two days, suggesting that this miRNA acts in the early stage of the stress response. Considering that the targets of this miRNA presumably help sugarcane plants to withstand drought stress, we would expect that ssp-miR164 targets are repressed in both cultivars. This was the case for the LT cultivar (Fig. 6 and 7), suggesting that both NAC and the MDR-like ABC transporter, and possibly auxin, play roles in the drought response. However, in the HT cultivar, ssp-miR164 was induced by drought. These data indicate that the two analyzed cultivars differ in their activation of molecular mechanisms in response to drought and that the target genes may play a more relevant role in the LT cultivar.

It is known that miR393 is commonly up-regulated during drought stress in *Arabidopsis*, *Oryza sativa*, M*edicago truncatula* and *Pinguicula vulgaris* [54]. In this work, the ssp-miR393 miRNA was also up-regulated under drought stress in the LT cultivar after four days (Fig 6). The predicted target of ssp-miR393 encodes a UDP-glycosyltransferase (Table 6), which catalyzes the glycosylation of various acceptor molecules, including flavonoids, playing roles in the storage of secondary metabolites and in plant defenses against stresses [55]. A loss-of-function mutation in the *UGT71C1* gene enhanced resistance to oxidative stress in *Arabidopsis*, suggesting that glycosylation may affect the levels of secondary metabolites such as flavonoids by decreasing their scavenging activity for reactive oxygen species (ROS) [56]. Because this activity is related to drought tolerance

[57,58], a decrease in the level of this protein should increase the ability of sugarcane plants to counteract the deleterious effects of ROS generated during drought stress.

ssp-miR394 was down-regulated under drought stress in the two sugarcane cultivars (Fig. 6), indicating that this miRNA is important in the drought stress response independently of the plant genotype. The predicted target of ssp-miR394 is the gene encoding a glyceraldehyde-3-phosphate dehydrogenase (GAPDH); see Table 6. GAPDH catalyzes the oxidation of D-glyceraldehyde-3-P (D-G3P) to 3-phosphoglycerate (3-PGA) with the generation of NADPH in the sixth step of glycolysis [59]. Due to the increased need for available ATP and $NADH_2$ under drought conditions, glycolysis is usually enhanced. This hypothesis is supported by the fact that rice plants exposed to drought display increased levels of *OsGAPDH* transcripts [60]. In this context, a decrease in the ssp-miR394 level would increase the GAPDH level and consequently the ATP content.

We also found that the target of ssp-miR397 is a gene encoding a laccase (Table 6), in agreement with previous studies [61]. Laccases are glycoproteins with roles in lignin synthesis [62] and iron acquisition [63]. It has been proposed that laccases are involved in cell wall modifications, such as lignification, acting to reduce cell wall extensibility and elongation [64,65]. In maize [66] and tomato [67], increases in laccase transcripts were reported at high concentrations of NaCl. Under drought stress, ssp-miR397 was up-regulated in both cultivars on the second day (Fig. 6), suggesting a reduction in laccase expression levels. However, on the fourth day of drought stress, this miRNA was down-regulated in the HT cultivar (Fig. 6), likely leading to increased laccase levels, which would be reflected as a decrease in cell elongation due to lignin accumulation in the cell wall in

response to stress. The complex expression pattern observed between the two cultivars indicates that lignification may act in different ways during drought stress.

miR399 has been described as a negative regulator of inorganic phosphate concentration because it targets a pyrophosphatase [68]. In this study, we also found an inorganic pyrophosphatase 2-like protein to be a target of ssp-miR399a (Table 6). Overexpression of *AVP1 (Arabidopsis* vacuolar pyrophosphatase gene) results in increased drought and salt tolerance in transgenic *Arabidopsis*, tomato, rice and cotton plants [69,70,71,72]. An increased proton pump activity by the vacuolar pyrophosphatase is the molecular explanation for drought resistance in these plants. This activity leads to a lower water potential in the plant vacuole and increases the activity of secondary transporters, preventing ion accumulation in the cytoplasm [72]. The down-regulation of ssp-miR399a under drought stress in both sugarcane cultivars likely leads to increased pyrophosphatase levels. These data suggest that pyrophosphatases also play a role in sugarcane responses to drought stress and that this role is at least partially controlled by the microtranscriptome.

ssp-miR528 targets a gene encoding a UBX domain-containing protein (Table 6). UBX domains have been identified in several proteins with diverse functions in eukaryotes. Among the 15 UBX-containing proteins encoded by the *Arabidopsis* genome [73], PUX1 was shown to play important roles in plant growth and development. Loss-of-function mutant *pux1* plants display accelerated growth in various plant organs, including roots and inflorescence shoots [74]. In this work, ssp-miR528 was down-regulated under

drought stress in both cultivars on the fourth day (Fig. 6), suggesting that this miRNA may be involved in the reduction of growth under this stress.

The expression levels of ssp-miR397, ssp-miR399a and ssp-miR528 were quite variable in the control plants (Fig. 6), and this variability is likely due to other environmental conditions and/or intrinsic changes, such as developmental influences.

A gene encoding a bZIP transcription factor was determined to be an ssp-miR1432 target (Table 6). Interestingly, several bZIPs are known to play important roles in conferring stress tolerance to plants [75] by regulating the expression of genes that are involved in mechanisms that are essential for stress adaptation, such as cytoplasmic ion homeostasis and osmotic adjustment [76]. A triple knockout in the genes encoding the bZIPs AREB1, AREB2, and ABF3 in *Arabidopsis* displayed increased tolerance to ABA and reduced drought tolerance [77]. ssp-miR1432 was down-regulated by drought at similar levels at all time points in both cultivars (Fig. 6), suggesting that the sugarcane bZIP activates genes that help sugarcane plants to overcome drought stress. However, because the expression patterns observed in both cultivars are similar, this transcription factor alone may not be responsible for the different levels of drought tolerance in RB867515 and RB855536.

It is notable that the expression patterns of the majority of the miRNAs did not display clear correlations with the differences in drought tolerance observed in the two sugarcane cultivars. Nevertheless, it is clear that the microtranscriptomes differ considerably between the two cultivars, suggesting that the miRNAs with differential expression do not fully explain the drought higher tolerance observed in RB867515.

In summary, our results provide insight into the sugarcane microtranscriptome, highlighting the regulatory network triggered by drought stress in an important bioenergy crop. We found that transcription factors, kinases, phosphatases and chaperones may be targets of the miRNAs modulated by drought in sugarcane. Further work with transgenic sugarcane overexpressing or silencing the miRNAs or their targets will increase our knowledge of the molecular mechanism of drought stress response and tolerance in sugarcane.

MATERIALS AND METHODS

Plant samples

Sugarcane cultivars RB867515 (high tolerance to drought) and RB855536 (low tolerance to drought) were obtained from RIDESA (Rede Universitária para o Desenvolvimento do Setor Sucroalcooleiro). Plants were grown in a greenhouse at the Federal University of Alagoas (Alagoas, Brazil, 9°45'32" S, 36°13'09" W) under normal irrigation for three months. To develop drought stress, water was withheld from the test plants. The rehydrated plants received normal watering for two days after the period without irrigation. The control plants received normal watering throughout the experiment. Tissue was collected from Leaf +1 (the highest unfolded leaf with a visible dewlap) in quadruplicate after 2, 4, 6 and 8 days from irrigated, non-irrigated (drought-stressed) and rehydrated plants. The samples were quick-frozen and maintained at -80°C until RNA extraction using a miRVana miRNA isolation kit (Life Technologies, USA) according to the manufacturer's protocol. A pool of two replicates was used for Solexa sequencing, whereas four individual plants were used for RT-qPCR validation.

Small RNA Sequencing

RNAs from 16 to 27 nt long were selected by polyacrylamide gel electrophoresis, ligated with adaptors at both ends and the products used for cDNA synthesis at BGI (Beijing Genomic Institute, Tai Po, Hong Kong). The sequencing was performed using the Solexa platform at BGI.

Bioinformatics analysis

To identify the sequences corresponding to true mature miRNAs, we predicted their precursors (pre-miRNAs). Reads were preprocessed by removing adapters and discarding reads <19 nt and 24 nt. The miRDeep-P program [21] was used to map the reads to the reference sequences (*Sorghum bicolor* genomic sequence and sugarcane ESTs). For a given mapped read, a 250 bp window was used from which reference sequences were extracted to predict RNA secondary structure [78]. The miRDeep core algorithm with a plant-specific scoring system based on known characteristics of plant miRNA genes was used to determine the secondary structure of the extracted reference sequences [35]. RNA sequences were classified as miRNA precursor candidates based on the following parameters: the folding of the RNA sequence into a characteristic stem-loop hairpin secondary structure, the position of the mature miRNA in one arm of the hairpin structure (allowing a maximum of six mismatches with the miRNA* sequence in the opposite arm), negative MFE values for the predicted secondary structures, and a G/C content in the 30- 70% range [38], using the RNAfold [79]. After the normalization of the number of reads in each library, the expression of each miRNA was calculated [80].

The targets of the miRNA were predicted using psRNATarget (http://plantgrn.noble.org/psRNATarget/) by searching for target genes based on both complementarity scoring and secondary structure analyses [81]. The BlastX algorithm [82] was used to find the hits in the Sugarcane Assembled Sequences (SAS) against the NCBI database to identify the coding strands because several SASs corresponded to the minus strand. Only hits complementary to the coding strand of the sugarcane mRNAs were selected.

Stem-loop reverse transcription and qPCR validation

RT-loop primers (loop-RT), sequence-specific forward PCR primers (loop-FW) and universal reverse primers were designed following Chen et al., 2005 [30] for reverse transcription and PCR amplification of sugarcane miRNAs (Table 7). Reverse transcriptase reactions and cDNA amplification were performed as described by Varkonyi-Gasic et al., 2007 [31]. The miRNA and reference gene reverse transcriptase reactions were conducted under the same reaction condition.

qPCR validation

To validate and quantitate the expression levels of the miRNAs in sugarcane leaf tissues, qPCR was performed using SYBR Green PCR Master Mix (Life Technologies, USA) on a 7500 Real-Time PCR System (Life technologies, USA). Each PCR reaction (18 µL) included 2 µL cDNA, 10 µL SYBR Green Master Mix (1X), 1 µL sequence-specific forward primer (10 µM), 1 µL universal reverse primer (10 µM) and 4 µL water. The sugarcane polyubiquitin gene [83] was used as a reference (Table 7). The reactions were performed at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute,

with a final dissociation curve analysis. All reactions were run with four biological replicates, each in triplicate. The real-time PCR data analysis was performed by considering the reaction efficiencies to calculate the fold changes in miRNA levels using a web-based QPCR system [84].

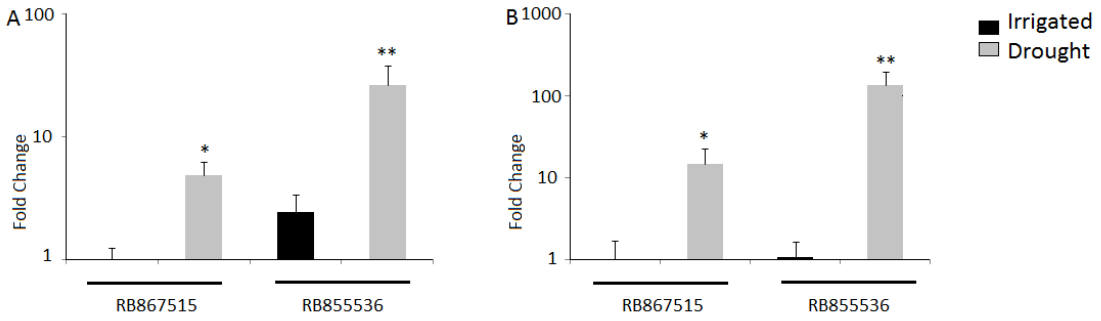


Figure 1. Real-time PCR of a sugarcane gene encoding a dehydrin (SCQGLR1085F11.g). RB867515 (higher drought tolerance) and RB855536 (lower drought tolerance) plants were irrigated (black bars) or subjected to drought stress by withholding irrigation (gray bars) for two (A) and four (B) days. Error bars represent the standard error (n=4). * $p<0.07$ and ** $p<0.05$. Statistics was calculated between irrigated and drought treatments in each cultivar using the permutation mean test.

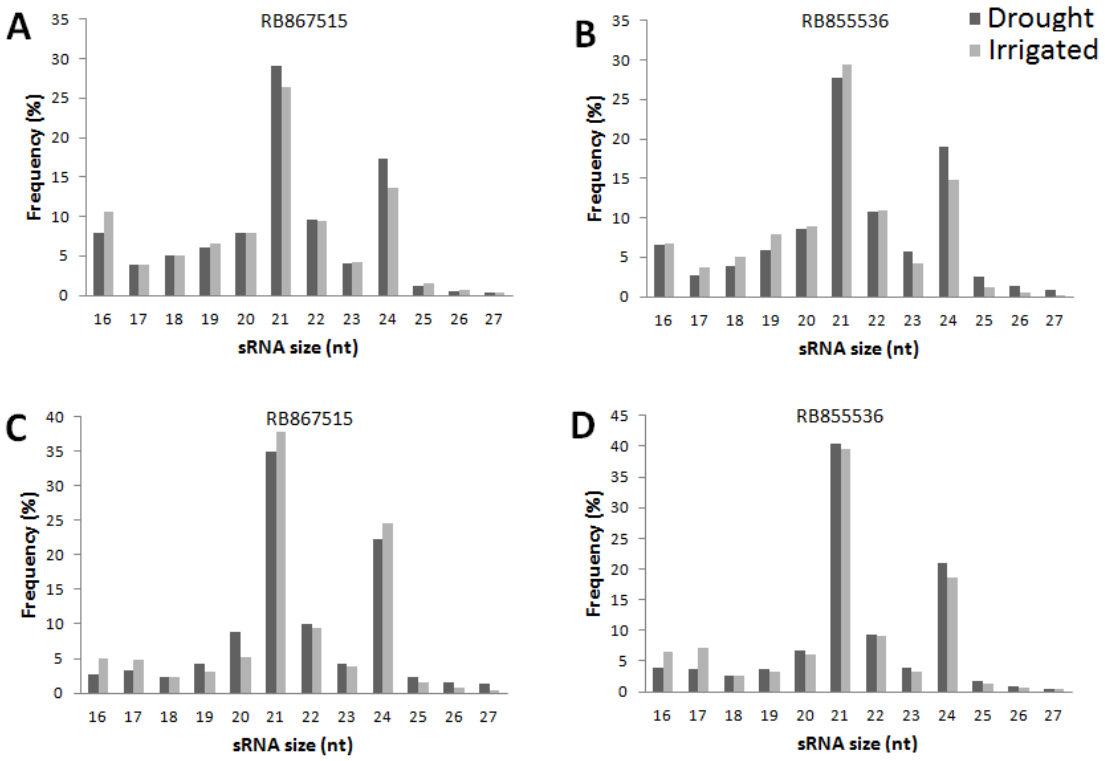


Figure 2. Size distribution of small RNA (sRNA) redundant sequences in two sugarcane cultivars. Cultivars RB867515 (A and C) and RB855536 (B and D) were irrigated (gray bars) or drought-stressed (black bars) for two (A and B) and four (C and D) days. RB867515 is the higher drought tolerant genotype, and RB855536 is the lower drought tolerant genotype.

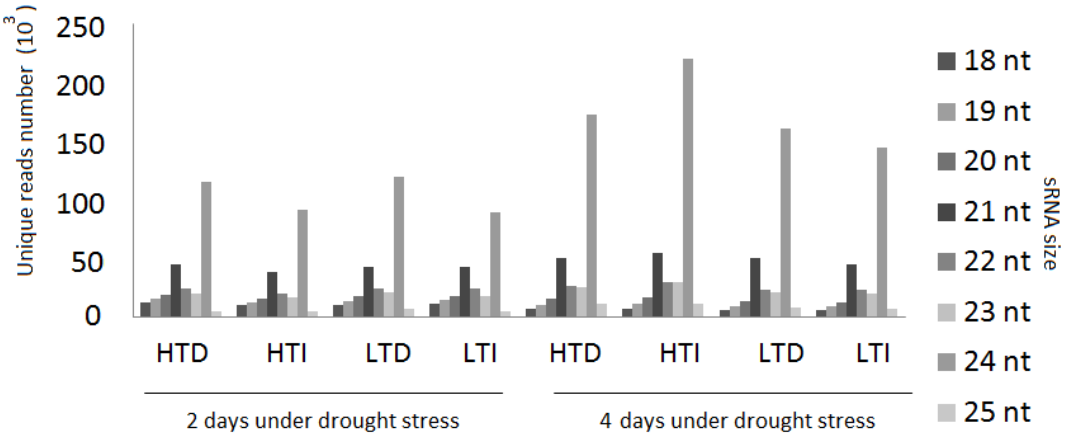


Figure 3. Size distribution of small RNA (sRNA) non-redundant sequences in two sugarcane cultivars. HTD: RB867515 (higher tolerance cultivar) plants without watering. HTI: RB867515 plants under irrigated conditions. LTD: RB855536 (lower tolerance cultivar) plants without watering. LTI: RB855536 plants under irrigated conditions.

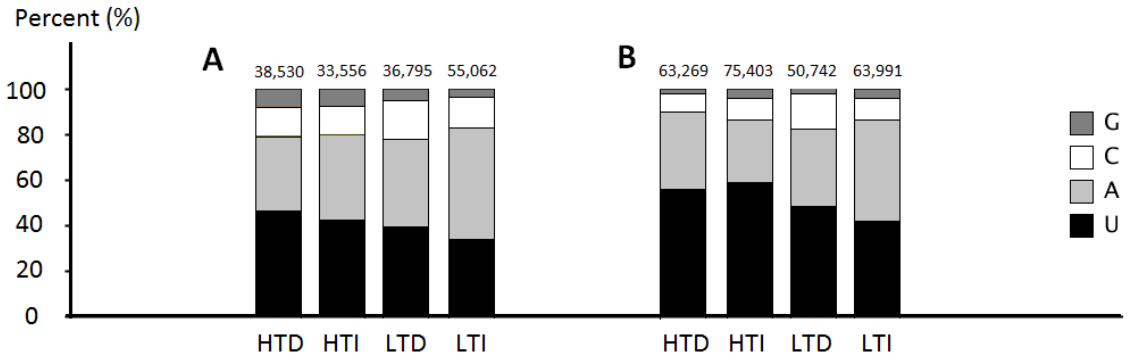


Figure 4. Composition of nucleotides in the first base positions of all 21 nt sequences. A: Control and stressed plants after two days of drought conditions. B: Control and stressed plants after four days of drought conditions. HTD: RB867515 (higher tolerance cultivar) plants without watering. HTI: RB867515 (higher tolerance cultivar) plants under irrigated conditions. LTD: RB855536 (lower tolerance cultivar) plants without watering. LTI: RB855536 (lower tolerance cultivar) plants under irrigated conditions. The total numbers of 21 nt reads in each library are shown at the tops of the bars.

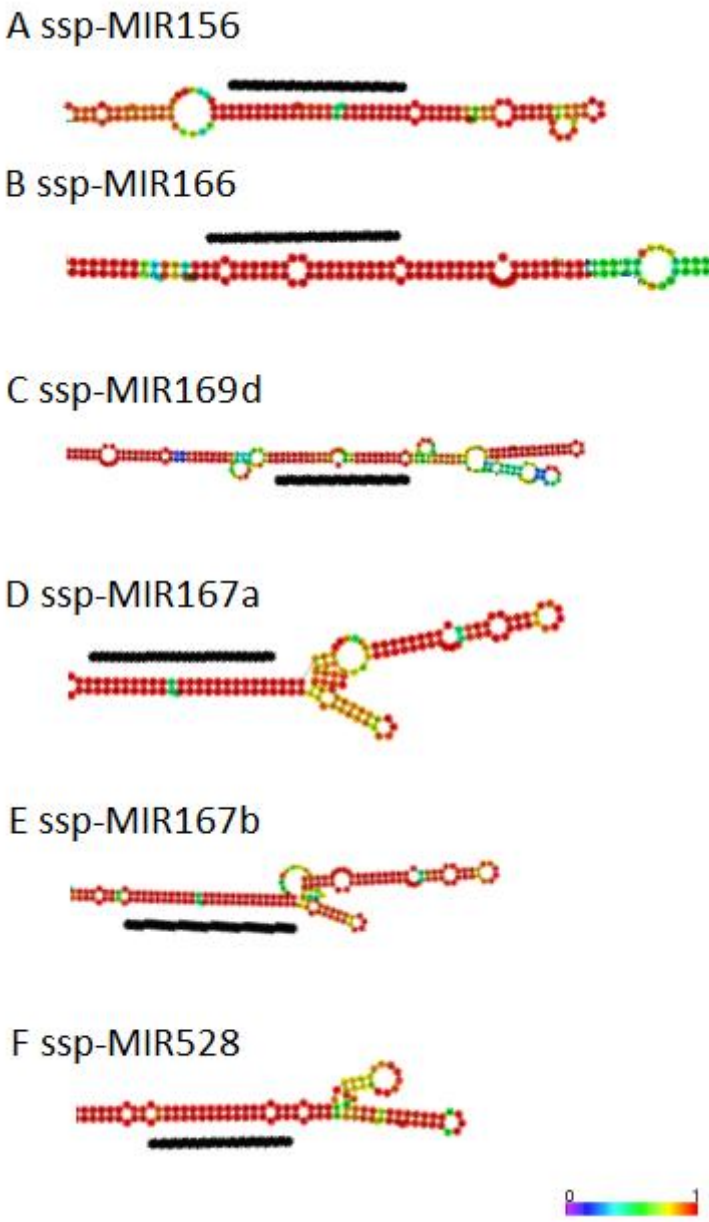


Figure 5. Primary transcripts containing the predicted stem-loop structures of the precursors of the sugarcane miRNAs. The mature miRNAs identified in the sugarcane sRNA library are highlighted in black. The sizes of the precursors may be slightly longer than represented. The colors represent the probabilities for sequence alignment. Red is the highest probability of alignment (1), and purple is the lowest probability of alignment (0).

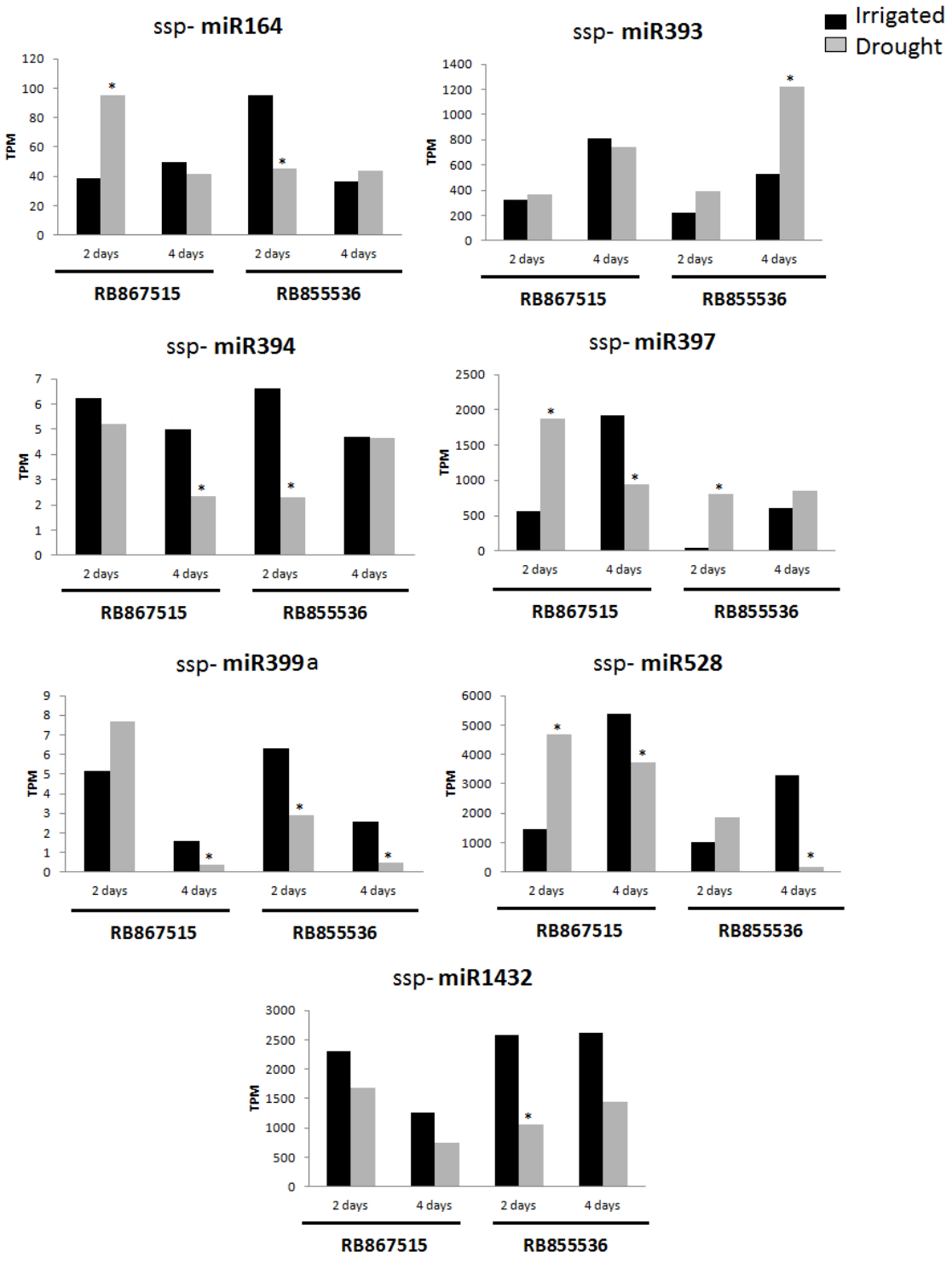


Figure 6. Expression profiles of seven sugarcane miRNAs modulated by drought stress. The values are expressed as the number of transcripts per million (TPM) for irrigated plants (control, black bars) and drought-stressed plants (gray bars). RB867515 (higher drought tolerance) and RB855536 (lower drought tolerance) plants were evaluated after two and four days of stress, as indicated below each graphic. * $p<0.05$ and fold change > 2.0. Statistics was calculated between irrigated and drought treatments in each cultivar.

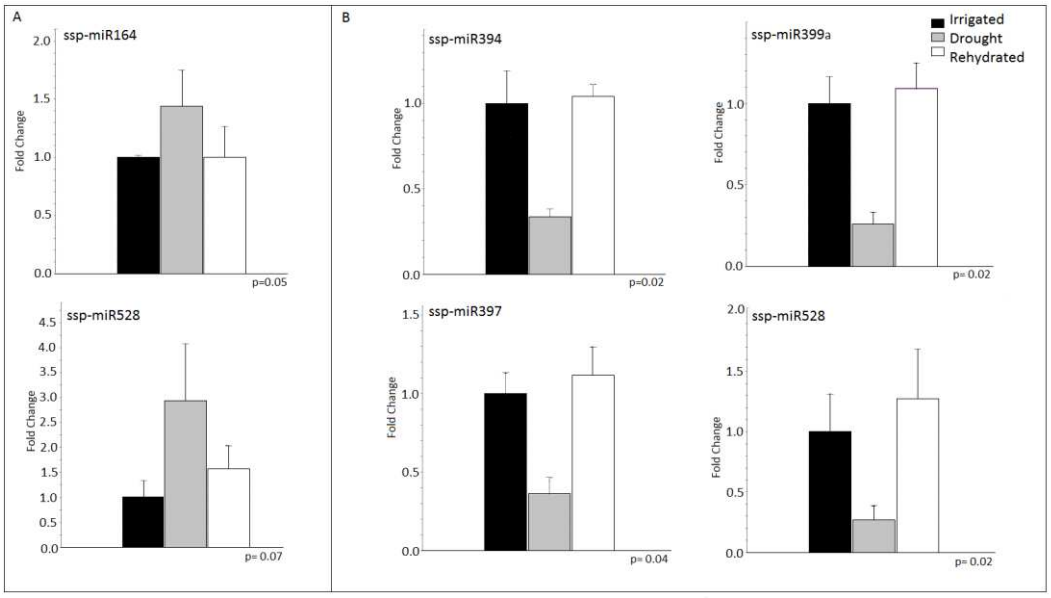


Figure 7. The RT-qPCR expression profiles of five sugarcane miRNAs modulated by drought stress. Only the confirmed profiles are shown. The values are expressed as fold changes relative to the irrigated control for each gene. The bars represent the average of the irrigated plants (control, black bars), drought-stressed plants (gray bars) and rehydrated plants (white bars) for RB867515 (higher drought tolerance) after two (A) and four (B) days of stress. Error bars represent the standard error (n=4). The p-values are shown in each chart. Statistics was calculated between irrigated and drought treatments using the permutation mean test.

Table 1. Small RNA deep-sequencing data from sugarcane leaves. HTD: RB867515 (higher drought tolerance) plants under drought stress (without watering). HTI: RB867515 plants under irrigation. LTD: RB855536 (lower drought tolerance) under drought stress. LTI: RB855536 (lower drought tolerance) plants under irrigation. 2: two days of treatment. 4: four days of treatment.

Category	HTD2				HTD4			
	Unique RNAs	Percent (%)	Total RNAs	Percent (%)	Unique RNAs	Percent (%)	Total RNAs	Percent (%)
antisense exon	12,762	0.50	108,151	1.22	15,312	0.47	86,674	0.65
sense exon	76,415	2.98	651,383	7.34	139,293	4.24	814,958	6.10
miRNA	23,950	0.94	1,375,593	15.49	26,926	0.82	3,467,791	25.95
rRNA	58,355	2.28	925,787	10.43	63,945	1.95	866,844	6.49
siRNA	59,384	2.32	370,579	4.17	61,333	1.87	552,249	4.13
snRNA	2,094	0.08	7,456	0.08	4,056	0.12	39,774	0.30
snoRNA	780	0.03	1,516	0.02	1,487	0.05	4,854	0.04
tRNA	13,254	0.52	549,361	6.19	19,375	0.59	1,399,409	10.47
unannotated	2,313,810	90.35	4,888,685	55.06	2,951,339	89.90	6,128,310	45.87
total small RNAs	2,560,804		8,878,511		3,283,066		13,360,863	
Category	HTI2				HTI4			
	Unique RNAs	Percent (%)	Total RNAs	Percent (%)	Unique RNAs	Percent (%)	Total RNAs	Percent (%)
antisense exon	10,400	0.49	102,953	1.21	16,899	0.44	131,528	0.87
sense exon	77,102	3.66	831,951	9.78	76,252	2.00	628,631	4.18
miRNA	19,404	0.92	1,310,300	15.40	32,423	0.85	4,305,127	28.62
rRNA	65,322	3.10	1,214,350	14.28	61,239	1.60	861,743	5.73
siRNA	50,116	2.38	286,130	3.36	88,861	2.33	763,503	5.08
snRNA	1,904	0.09	6,942	0.08	2,382	0.06	7,433	0.05
snoRNA	878	0.04	1,897	0.02	809	0.02	1,766	0.01
tRNA	14,825	0.70	788,156	9.27	13,975	0.37	520,672	3.46
unannotated	1,867,650	88.61	3,963,113	46.59	3,528,379	92.34	7,819,918	51.99
total small RNAs	2,107,601		8,505,792		3,821,219		15,040,321	
Category	LTD2				LTD4			
	Unique RNAs	Percent (%)	Total RNAs	Percent (%)	Unique RNAs	Percent (%)	Total RNAs	Percent (%)
antisense exon	13,525	0.52	130,805	1.30	17,226	0.59	154,743	1.17
sense exon	96,298	3.72	1,044,692	10.38	76,303	2.62	735,969	5.58
miRNA	22,271	0.86	1,501,828	14.93	22,744	0.78	3,483,293	26.42
rRNA	77,634	3.00	1,521,502	15.12	60,765	2.09	926,250	7.03
siRNA	58,478	2.26	399,760	3.97	63,817	2.19	587,248	4.45
snRNA	2,755	0.11	11,421	0.11	2,464	0.08	8,252	0.06
snoRNA	1,179	0.05	2,660	0.03	757	0.03	2,020	0.02
tRNA	17,741	0.69	797,948	7.93	14,831	0.51	727,495	5.52
unannotated	2,298,296	88.80	4,649,978	46.22	2,652,688	91.11	6,557,000	49.74
total small RNAs	2,588,177		10,060,594		2,911,595		13,182,270	
Category	LTI2				LTI4			
	Unique RNAs	Percent (%)	Total RNAs	Percent (%)	Unique RNAs	Percent (%)	Total RNAs	Percent (%)
antisense exon	14,157	0.64	131,400	1.40	14,757	0.55	161,302	1.33
sense exon	81,911	3.72	792,014	8.44	67,798	2.52	506,603	4.17
miRNA	20,041	0.91	1,515,240	16.15	23,936	0.89	3,682,698	30.32
rRNA	65,089	2.96	1,150,319	12.26	54,672	2.03	676,834	5.57
siRNA	63,279	2.87	469,568	5.00	68,251	2.53	664,751	5.47
snRNA	2,849	0.13	13,901	0.15	2,362	0.09	8,234	0.07
snoRNA	1,180	0.05	3,190	0.03	973	0.04	2,283	0.02
tRNA	14,087	0.64	736,610	7.85	13,576	0.50	415,579	3.42
unannotated	1,939,448	88.08	4,571,452	48.72	2,447,018	90.85	6,028,355	49.63
total small RNAs	2,202,041		9,383,694		2,693,343		12,146,639	

Table 2. Sugarcane miRNAs identified by Solexa sequencing. miRNAs found in the leaves of two sugarcane cultivars (RB867515 and RB855536). Two mismatches were allowed against sorghum mature miRNAs.

miRNA family	miRNA	mature sequence	Sorghum precursor	Already described in sugarcane
miR1432	ssp-miR1432	TCAGGAAAGATGACACCAA	sbi-MIR1432	yes
miR156	ssp-miR156	TTGACAGAAGAGAGTGAGCAC	sbi-MIR156b sbi-MIR156c sbi-MIR156d sbi-MIR156e sbi-MIR156g sbi-MIR156h sbi-MIR156i	yes
miR160	ssp-miR160a	TGCCTGGCTCCCTGTATGCCA	sbi-MIR160c	no
miR164	ssp-miR164	TGGAGAAGCAGGGCACGTGCA	sbi-MIR164b	no
miR166	ssp-miR166a	TCGGACCAGGCTTCATTCCCC	sbi-MIR166a sbi-MIR166b sbi-MIR166c sbi-MIR166d sbi-MIR166h sbi-MIR166j	no
miR167	ssp-miR167b	TGAAGCTGCCAGCATGATCTGA	sbi-MIR167c sbi-MIR167d sbi-MIR167e sbi-MIR167g sbi-MIR167h sbi-MIR167i	yes
miR169	ssp-miR169c	CTAGCCAAGAATGACTTGCCT	sbi-MIR169f	no
	ssp-miR169d	CAGCCAAGGATGACTTGCCGA	sbi-MIR169a	no
miR171	ssp-miR171a	TTGAGCCGCGTCAATATCTCC	sbi-MIR171h	no
	ssp-miR171b	TGATTGAGCCGTGCCAATATC	sbi-MIR171g sbi-MIR171i sbi-MIR171k	no
	ssp-miR171c	TGAGCCGAACCAATATCACTC	sbi-MIR171c sbi-MIR171f	no
miR172	ssp-miR172	AGAATCTTGATGATGCTGCAT	sbi-MIR172a sbi-MIR172d sbi-MIR172e	no
miR319	ssp-miR319	TTTGGATTGAAGGGTGCT	sbi-MIR319b	no
miR390	ssp-miR390	AAGCTCAGGAGGGATAGCGCC	sbi-MIR390	no
miR393	ssp-miR393	CTCCAAAGGGATCGCATTGAT	sbi-MIR393b	no
miR394	ssp-miR394	TTGGCATTCTGTCCACCTCC	sbi-MIR394a	no
miR396	ssp-miR396b	TCCACAGGCTTTCTTGAACTG	sbi-MIR396d	no
miR397	ssp-miR397	TTGACTGCAGCGTTGATGAGC	sbi-MIR397	no
miR399	ssp-miR399a	TGCCAAAGGAGAGTTGCCCTG	sbi-MIR399a sbi-MIR399f sbi-MIR399h sbi-MIR399i sbi-MIR399j	no
miR528	ssp-miR528	TGGAAGGGGCATGCAGAGGAG	sbi-MIR528	yes
miR529	ssp-miR529	AGAAGAGAGAGAGTACAGCCT	sbi-MIR529	no

Table 3. Bioinformatics prediction of sugarcane miRNAs precursors. The corresponding precursor clusters in SOGI and SAS in the SUCEST databases are indicated in the Cluster column. MFE: minimum free folding energy. G/C contents and the mature miRNA sequences are also shown.

Precursor name	Cluster	MFE (Kcal/mol)	G/C (%)	Mature miRNA sequence (5' - 3')
ssp-MIR156	SCSBAD1086B12	-155	43	UUGACAGAAGAGAGTGAGCAC
ssp-MIR166	TC144774	-145,32	45	UCGGACCAGGCUUCAUUCCCC
ssp-MIR167a	SCMCSD2060C04	-156,92	46	UGAAGCUGCCAGCAUGAUCUGA
ssp-MIR167b	SCSFSD1065B12	-225,44	42	UGAAGCUGCCAGCAUGAUCUGA
ssp-MIR169d	SCJLRZ1019E10	-338,87	48	CAGCCAAGGATGACTTGCCGA
ssp-MIR528	SCUTSD1026H02	-204,5	49	UGGAAGGGGCAUGCAGAGGAG

Table 4. Sugarcane miRNAs differentially expressed after two days of drought stress. Nt: number of nucleotides of the mature miRNA. HTD: RB867515 (higher drought tolerance) plants under drought stress (without watering). HTI: RB867515 plants under irrigation. LTD: RB855536 (lower drought tolerance) under drought stress. LTI: RB855536 (lower drought tolerance) plants under irrigation. A red background indicates down-regulated miRNAs ($p<0.05$), and a green background indicates miRNAs ($p<0.05$) that are up-regulated during drought (minimum fold change > 2.00).

Family	miR name	Mature sequences	Nt	HTD (TPM)	HTI(TPM)	LTD(TPM)	LTI (TPM)	HTD/HTI	LTD/LTI
miR164	ssp-miR164	TGGAGAAGCAGGGCACGTGCA	21	95.07	38.57	45.43	95.28	2.46	-2.10
miR394	ssp-miR394	TTGGCATTCTGTCCACCTCC	20	5.19	6.24	2.30	6.62	-1.20	-2.88
miR397	ssp-miR397	TTGACTGCAGCGTTGATGAGC	21	1873.63	562.10	799.76	42.42	3.33	18.85
miR399	ssp-miR399a	TGCCAAAGGAGAGTTGCCC	19	7.67	5.18	2.89	6.30	1.48	-2.18
miR528	ssp-miR528	TGGAAGGGGCATGCAGAGGAG	21	4674.10	1445.14	1874.15	1025.19	3.23	1.83
miR1432	ssp-miR1432	TCAGGAAAGATGACACCAA	19	1687.91	2300.44	1053.92	2583.96	-1.36	-2.45

up-regulated under drought
down regulated under drought

Table 5. Sugarcane miRNAs differentially expressed after four days of drought stress. Nt: number of nucleotides of the mature miRNA. HTD: RB867515 (higher drought tolerance) plants under drought stress (without watering). HTI: RB867515 plants under irrigation. LTD: RB855536 (lower drought tolerance) under drought stress. LTI: RB855536 (lower drought tolerance) plants under irrigation. A red background indicates down-regulated miRNAs ($p<0.05$), and a green background indicates miRNAs ($p<0.05$) that are up-regulated during drought (minimum fold change > 2.00).

Family	miR name	Mature sequences	Nt	HTD(TPM)	HTI(TPM)	LTD(TPM)	LTI(TPM)	HTD/HTI	LTD/LTI
miR393	ssp-miR393	CTCCAAAGGGATCGCATTGAT	21	743.15	808.97	1220.51	582.06	-1.09	2.10
miR394	ssp-miR394	TTGGCATTCTGTCCACCTCC	20	2.33	5.00	4.64	4.70	-2.14	-1.01
miR397	ssp-miR397	TTGACTGCAGCGTTGATGAGC	21	940.44	1926.63	858.82	602.07	-2.05	1.43
miR399	ssp-miR399a	TGCCAAAGGAGAGTTGCCC	19	0.38	1.61	0.47	2.56	-4.18	-5.51
miR528	ssp-miR528	TGGAAGGGGCATGCAGAGGAG	21	3717.14	5397.83	182.68	3293.76	-1.45	-18.03

up-regulated under drought
down regulated under drought

Table 6. Target prediction of the miRNAs that are differentially expressed in drought-stressed sugarcane plants. Target Acc: accession number in the SUCEST or SoGI databases; Expectation: value assigned to the alignment of the mature miRNA and the target, ranging from 0 (perfect alignment) to 5; UPE: the energy required to open the secondary structure of the target at the recognition site (less energy means better accessibility to the target); Mature miRNA: miRNA mature size (in nucleotides); Target start: the base position where the annealing with the miRNA starts; Target end: the base position where the annealing with the miRNA ends; Inhibition: the type of regulation by the miRNA; and Target description: description of the target according to a BLAST search in the GenBank database, including the name of the organism presenting the best hit.

miRNA	Target Acc.	Expectation	UPE	mature miRNA	Target start	Target end	miRNA aligned fragment	Target aligned fragment	Inhibition	Target description
ssp-miR164	SCEPRT2048G05.g	1.0	20.052	20	699	718	UGGAGAAGCAGGGCACGUGC	GCAGGUGCCCUGCUUCUCCA	Cleavage	NAC transcription factor [Hordeum vulgare subsp. vulgare]
	SCCCAM1001A03.g	2.5	20.361	19	1774	1792	UGGAGAAGCAGGGCACGUG	CACGUGUCCACCUUCUCCA	Translation	MDR-like ABC transporter [Oryza sativa Japonica Group]
ssp-miR394	SCQGAM2027G09.g	3.0	19.627	19	759	777	UUGGCAUUCUGUCCACCUC	CAGGUGGGCAGGGUGCUAA	Cleavage	glyceraldehyde-3-phosphate dehydrogenase [Triticum aestivum]
ssp-miR528	SCJFRT2058D11.g	2.5	22.413	20	101	120	UGGAAGGGGCAUGCAGAGGA	UUCUCGGCAUGCCCCUUCUG	Cleavage	UBX domain-containing protein [Oryza brachyantha]
ssp-miR397	SCQSAD1056B07.g	2.5	22.911	20	64	83	UUGACUGCAGCGUUGAUGAG	UUCAUCAACGCCGCACUCAA	Translation	laccase-23-like [Brachypodium distachyon]
ssp-miR1432	TC134052	3.0	17.32	19	630	648	UCAGGAAAGAUGACACCAA	UUGGUGUUUUCUUCCCUGA	Translation	ABRE-binding factor BZ-1 [Zea mays]
	SCSFFL4085D03.g	3.0	15.088	19	624	642	UCAGGAAAGAUGACACCAA	UUGGUGUUUUCUUCCCUGA	Translation	bZIP transcription factor1 [Zea mays]
ssp-miR393	SCUTHR1064E02.g	3.0	23.95	19	69	87	CUCCAAAGGGAUCGCAUUG	UAAUGCACUUCCUUUGGAG	Cleavage	UDP-glycosyltransferase [Zea mays]
ssp-miR399a	SCACHR1037A06.g	1.5	19.642	19	463	481	UGCCAAAGGAGAGUUGCCC	GGGUAGUUCUCCUUUGGCA	Cleavage	inorganic pyrophosphatase 2-like [Brachypodium distachyon]

Table 7. Primers used in the reverse transcription and real-time PCR analyses of sugarcane miRNAs expression. RT: primer loop for reverse transcription; FW: forward primer for real-time PCR; reverse universal: reverse primer for real-time PCR; PUB: polyubiquitin gene primer; and Nt: number of nucleotides. The numbers in each primer name indicate the corresponding number of the sugarcane miRNA.

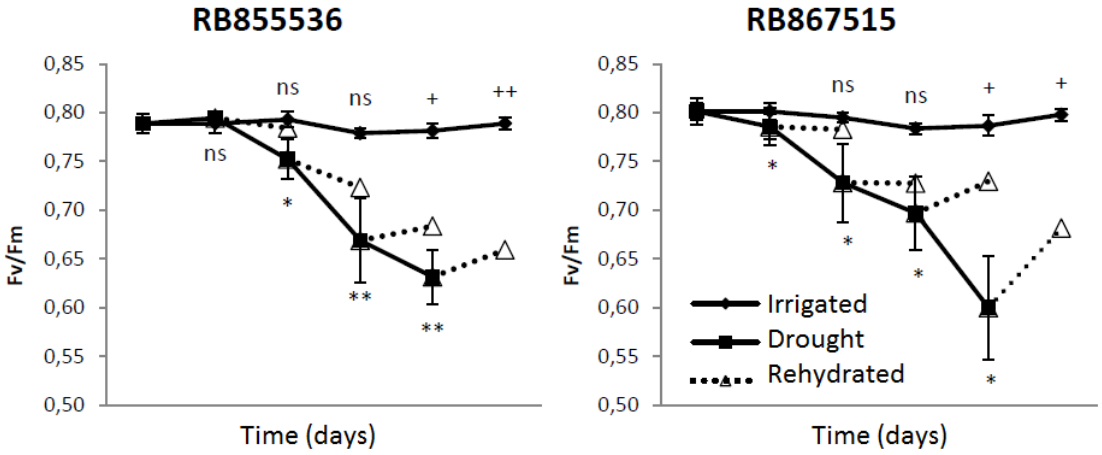
Primer name	Sequence (5'-3')	Nt	Tm (°C)
loop-RT164	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT GCA CG	50	99
loop-FW164	GCG TGG AGA AGC AGG GCA	18	61
loop-RT393	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA TCA AT	50	99
loop-FW393	GGC TCC AAA GGG ATC GCA TTG AT	23	59.9
loop-RT394	GTC GTA TCC AGT GCAGGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GAG GT	50	99
loop-FW394	GCC TTG GCA TTC TGT CC	17	60.2
loop-RT397	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG CTC AT	50	99
loop-FW397	TTG ACT GCA GCG TTG ATG AGC	21	58.3
loop-RT399a	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GGC AA	50	99
loop-FW399a	TGC CAA AGG AGA GTT GCC C	19	58.1
loop-RT528	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TCC TC	50	99
loop-FW528	CGT GGA AGG GGC ATG CA	17	59
loop-RT1432	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT TGG TG	50	99
loop-FW1432	GGC GTC AGG AAA GAT GAC ACC AA	23	59.5
PUB-FW	CCG GTC CTT TAA ACC AAC TCA GT	23	57
PUB-RV	CCC TCT GGT GTA CCT CCA TTT G	22	57.3
Dehydrin_FW	GGC GGA AGG AGG AAG AAG G	19	64.5
Dehydrin_Rv	CCG TGG CGT GCT GGT TGT C	19	66
reverse universal	GTG CAG GGT CCG AGG T	16	58.4

Supporting Information

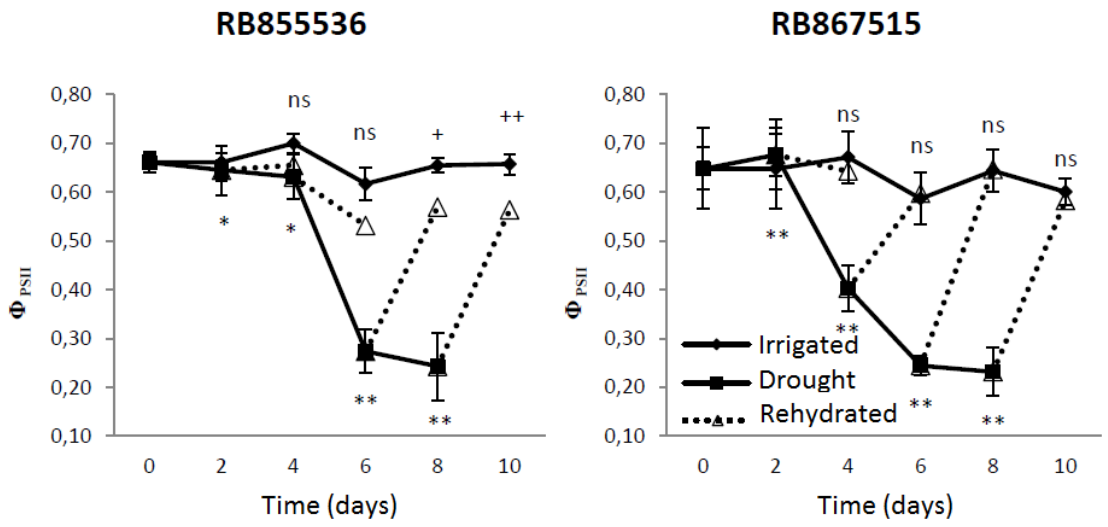
Sugarcane plants were adapted in the dark for 20 min, and a portable fluorometer (Opti-Sciences model OS1-FL, Hudson, USA) was used to measure the minimum fluorescence yield (F_0), maximum fluorescence yield (Fm) and the variable fluorescence (Fv). The Fv/Fm ratio, indicating the light-induced chlorophyll fluorescence, represents the Photosystem II activity and was used to evaluate the damage caused by drought to the photosynthetic efficiency of the sugarcane plants.

When chlorophyll fluorescence was evaluated in the control, water-stressed and rehydrated plants (see Materials and Methods), photoinhibition increased over the period of drought stress, leading to strong reductions in Fv/Fm in both cultivars (FS1). The HT cultivar RB867515, when rehydrated, displayed a higher capacity to recover the Fv/Fm ratio, as expected.

The fluorescence quantum yield, ΦPSII, is an indicator of the light used in photochemistry by the chlorophyll associated with the PSII. This parameter was also negatively affected in both cultivars during drought stress (FS2). LT plants were able to recover only a fraction of their original ΦPSII levels upon rehydration, whereas ΦPSII in the HT plants fully recovered to the levels observed in the control plants.



FS1. Quantum potential efficiency (Fv/Fm) in sugarcane plants. The sugarcane cultivars RB855536 (LT - lower drought tolerance) and RB867515 (HT- higher drought tolerance) were maintained under irrigation, without irrigation and without irrigation and then rehydrated, as indicated. * indicates differences between irrigated and drought-stressed plants; + indicates differences between irrigated and rehydrated plants.** and ++ indicate $p<0.01$, and * and + indicate $p<0.05$ using the t-test. ns - not significant.



FS2. **Fluorescence quantum yield (ΦPSII) in sugarcane plants**. The sugarcane cultivars RB855536 (LT - lower drought tolerance) and RB867515 (HT- higher drought tolerance) were maintained under irrigation, without irrigation and without irrigation and then rehydrated, as indicated. * indicates differences between irrigated and drought-stressed plants; + indicates differences between irrigated and rehydrated plants. ** and ++ indicate $p<0.01$, and * and + indicate $p<0.05$ using the t-test. ns - not significant.

TS 1. Target prediction of the miRNAs differentially expressed in drought-stressed sugarcane plants. (All bioinformatics data without selection.) Target Acc: accession number in the SUCEST or SoGI databases; Expectation: value assigned to the alignment of the mature miRNA and the target, ranging from 0 (perfect alignment) to 5; UPE: the energy required to open the secondary structure of the target at the site recognition (less energy means better accessibility to the target); Mature miRNA: miRNA mature size (in nucleotides); Target start: the base position where the annealing with the miRNA starts; Target end: the base position where the annealing with the miRNA ends; Inhibition: the type of regulation by the miRNA; and Target description: description of the target according to a BLAST search in the GenBank database, including the name of the organism presenting the best hit.

miRNA	Target Acc.	Expectation	UPE	mature miRNA	Target start	Target end	miRNA aligned fragment	Target aligned fragment	Inhibition	Target_Desc.
ssp-miR164	SCEPRT2048G05.g	1.0	20.052	20	699	718	UGGAGAAGCAGGGCACGUGC	GCAGGUGCCCUGCUUCUCCA	Cleavage	NAC transcription factor [Hordeum vulgare subsp. vulgare], 70%
	SCCCCL3003H07.b	2.5	22.117	20	1156	1175	UGGAGAAGCAGGGCACGUGC	GCGAGUGCUCUGCUUUUCCA	Cleavage	lactoylglutathione lyase [Zea mays], 75%
	SCMCRZ3067E12.g	2.5	14.3	19	619	637	UGGAGAAGCAGGGCACGUG	GACAUGUCCUGCUUCUCCA	Cleavage	splicing factor 3B subunit 3-like [Brachypodium distachyon], 99%
	SCSGHR1072A03.g	2.0	17.053	21	596	616	UGGAGAAGCAGGGCACGUGCA	UGUACGUGAUCUGCUUCUCCA	Cleavage	PREDICTED: lisH domain and HEAT repeat-containing protein KIAA1468 homolog [Brachypodium distachyon] 72%
	SCRULB2063B10.g	2.5	19.622	21	281	301	UGGAGAAGCAGGGCACGUGCA	UCUACGUGCCCUUCUUCUCCU	Translation	exostosin-like [Zea mays] 92%
	SCUTAD1034F10.g	2.5	16.147	19	216	234	UGGAGAAGCAGGGCACGUG	UACGUGCUUAGCUUCUCCA	Translation	proline transport protein-like [Oryza sativa Japonica Group] 78%
	SCCCLR1C01A09.g	3.0	20.076	20	1091	1110	UGGAGAAGCAGGGCACGUGC	GCUUGUGCCCAGCUUCUUCA	Translation	putative pre-mRNA-splicing factor cwc-22 [Oryza sativa Japonica Group] 95%
	SCRLLR1016F07.g	3.0	22.083	20	1358	1377	UGGAGAAGCAGGGCACGUGC	GAAGGUGCUCUGCUUCUUCA	Cleavage	PREDICTED: alpha-1,4-galacturonosyltransferase 1-like [Brachypodium distachyo
	SCCCFL5094H09.g	3.0	21.571	21	297	317	UGGAGAAGCAGGGCACGUGCA	UCCACGUGCCCUACAUCUUCA	Translation	phosphatidylinositol transfer protein CSR1 [Zea mays] 92%; sec14 like protein [Oryza sativa] 74%
	SCCCAM1001A03.g	2.5	20.361	19	1774	1792	UGGAGAAGCAGGGCACGUG	CACGUGUCCACCUUCUCCA	Translation	MDR-like ABC transporter [Oryza sativa Japonica Group], 95%
ssp-miR394	SCEQRT1030A02.g	2.5	21.358	19	572	590	UUGGCAUUCUGUCCACCUC	GCGGAGGACAGGAUGCCAA	Cleavage	squamosa promoter-binding-like protein 11 [Zea mays], 92%
	SCUTLR1037A06.g	3.0	22.853	19	1222	1240	UUGGCAUUCUGUCCACCUC	GAGGUGGUCAGGAUGCUGG	Cleavage	NSP-Interacting Kinase 1-like [Brachypodium distachyon], 87%
	SCQGRT1038B02.g	3.0	19.415	20	320	339	UUGGCAUUCUGUCCACCUCC	GGAGGUGGAUGGAAUAUCAA	Cleavage	PREDICTED: scarecrow-like protein 9-like [Brachypodium distachyon] 55%
	SCQGAM2027G09.g	3.0	19.627	19	759	777	UUGGCAUUCUGUCCACCUC	CAGGUGGGCAGGGUGCUAA	Cleavage	glyceraldehyde-3-phosphate dehydrogenase [Triticum aestivum] 65%; GAPDH4 [x Doritaenopsis hybrid cultivar] 63%
	SCAGFL3023C11.g	3.0	22.227	19	149	167	UUGGCAUUCUGUCCACCUC	UAGGUGCACAGAAUGCCAC	Cleavage	GRF zinc finger family protein [Oryza sativa Japonica Group] 32%
	SCJFRT2056H02.g	3.0	19.993	19	286	304	UUGGCAUUCUGUCCACCUC	GAGGUGGUUACAAUGUCAA	Translation	RNA-directed RNA polymerase 6a [Hordeum vulgare], 91%
ssp-miR528	SCCCAM2004H11.g	3.0	23.853	21	73	93	UGGAAGGGGCAUGCAGAGGAG	CUCCUCUCCAUGCCCUAUCCA	Cleavage	amino acid permease YfnA-like [Brachypodium distachyon], 85%
	SCCCCL2001H04.b	2.0	21.591	19	1482	1500	UGGAAGGGGCAUGCAGAGG	UCUUUGCAUGCUCUUUCCA	Cleavage	alpha-trehalose-phosphate synthase [UDP-forming] 8-like [Brachypodium distachyon], 81%
	SCQGAM2110G03.g	3.0	14.851	21	463	483	UGGAAGGGGCAUGCAGAGGAG	CUCUUCUGCAAGCCUCUUCUG	Translation	tyrosyl-tRNA synthetase-like [Brachypodium distachyon], 87%
	SCJFRT2058D11.g	2.5	22.413	20	101	120	UGGAAGGGGCAUGCAGAGGA	UUCUCGGCAUGCCCCUUCUG	Cleavage	UBX domain-containing protein [Oryza brachyantha], 75%
	SCEQLR1029G04.g	3.0	22.356	20	701	720	UGGAAGGGGCAUGCAGAGGA	UCUUCUGUAUGCUCCUUUUG	Cleavage	putative transcription factor [Zea mays], 94%
	SCQGSB1144H02.g	2.5	24.172	19	579	598	UGGAAGGGGCAU-GCAGAGG	CCUCUGCUAUGCCCCUUUCA	Cleavage	primary amine oxidase-like [Brachypodium distachyon], 64%
	SCQSSB1056A02.b	3.0	13.047	19	527	545	UGGAAGGGGCAUGCAGAGG	AUUCUCCAUGCUCCUUCCA	Cleavage	lipoyl synthase 1, chloroplastic [Zea mays], 71%
	SCCCLR1077A10.g	2.5	7.141	19	1337	1355	UGGAAGGGGCAUGCAGAGG	GCACUGCAUGCCCCUUUCA	Cleavage	glutamyl-tRNA synthetase, cytoplasmic [Zea mays], 96%
	SCCCLR2C03G12.g	2.5	20.125	19	462	480	UGGAAGGGGCAUGCAGAGG	UCUCUGCUUGCCCUUUUCA	Cleavage	40S ribosomal protein S23 [Zea mays], 99%
ssp-miR397	SCQSAD1056B07.g	2.5	22.911	20	64	83	UUGACUGCAGCGUUGAUGAG	UUCAUCAACGCCGCACUCAA	Translation	laccase-23-like [Brachypodium distachyon], 85%
	SCQSLB2051G05.g	2.0	21.803	19	267	285	UUGACUGCAGCGUUGAUGA	UCCUCAAUGCUGUAGUCAA	Cleavage	mTERF family protein [Zea mays], 93%
	SCVPHR1096C12.g	2.5	18.506	19	376	394	UUGACUGCAGCGUUGAUGA	UUAUAAAUGCUGCAGUUAA	Cleavage	pseudouridylate synthase 7 homolog [Brachypodium distachyon], 76%
	SCSBFL5023G02.g	3.0	21.541	21	59	79	UUGACUGCAGCGUUGAUGAGC	GGUCAGCAACGCUGUAGUGAA	Cleavage	putative KOW domain-containing transcription factor [Oryza sativa Japonica Group], 63%
	SCEQRT2091D09.g	3.0	19.401	20	707	726	UUGACUGCAGCGUUGAUGAG	UUCUUCAGUGCUGCAGUUGA	Cleavage	beta 1,3-glycosyltransferase-like protein II [Oryza sativa], 90%
	SCVPLR1006E03.g	3.0	13.538	20	1018	1037	UUGACUGCAGCGUUGAUGAG	UUCAUCAACUUUGCAAUCAA	Translation	guanylate kinase-like [Brachypodium distachyon], 89%
miR1432	SCJFRT1009B10.g	3.0	14.779	19	407	425	UCAGGAAAGAUGACACCAA	CUGGUGUCAUUUUGCCUGA	Cleavage	glutaryl-CoA dehydrogenase [Zea mays], 77%
	SCCCRZ1C01C03.g	3.0	18.172	19	61	79	UCAGGAAAGAUGACACCAA	AUGGUGUCAUUUUUCCCGA	Cleavage	60S ribosomal export protein NMD3-like [Brachypodium distachyon], 92%
	SCQSRT2034G10.g	3.0	18.47	19	450	468	UCAGGAAAGAUGACACCAA	UAGGUGUUGUUUUUUCUUGA	Cleavage	protein kinase-like [Oryza sativa Japonica Group], 86%
	SCJFRZ1005H03.g	2.5	10.754	19	376	394	UCAGGAAAGAUGACACCAA	UUGUUCUCAUUUUUUCCUGA	Cleavage	ELMO domain-containing protein A-like [Brachypodium distachyon], 70%
	SCCCLR1C07G02.g	3.0	23.724	19	690	708	UCAGGAAAGAUGACACCAA	UUGGUGUCAUGUUUAUUGA	Translation	ras-related protein Rab-6A [Zea mays], 100%
	SCRFLR2038G05.g	3.0	8.11	19	1562	1580	UCAGGAAAGAUGACACCAA	UUGUUUUCAUCUUUCCUGC	Cleavage	putative ceramide glucosyltransferase [Oryza sativa], 79%
	SCEQRT1030B05.g	3.0	13.087	19	1309	1327	UCAGGAAAGAUGACACCAA	UUGGUUUCAUAUUUCCUGC	Translation	sodium-coupled neutral amino acid transporter 3-like [Brachypodium distachyon], 90%
	SCSFFL4085D03.g	3.0	15.088	19	624	642	UCAGGAAAGAUGACACCAA	UUGGUGUUUUCUUCCCUGA	Translation	bZIP transcription factor1 [Zea mays] 85%; G-box binding factor 1 [Eleusine coracana], 84%
	SCEQHR1080E10.g	3.0	18.09	19	747	765	UCAGGAAAGAUGACACCAA	CUAGUGUCAUCGUUCCUGA	Cleavage	membrane protein [Zea mays]
	SCRLSD1010A04.g	3.0	18.006	19	332	350	UCAGGAAAGAUGACACCAA	UUGGUGUUUUCAUUCUUGA	Translation	putative receptor-like protein kinase [Oryza sativa Japonica Group], 75%
	SCBGAM1093D09.g	3.0	21.099	19	975	993	UCAGGAAAGAUGACACCAA	UUGGUGUCGUGCUUCUUGA	Translation	putative OsLRK1(receptor-type protein kinase) [Oryza sativa Japonica], 92%
	SCVPRT2076H07.g	3.0	20.442	19	261	279	UCAGGAAAGAUGACACCAA	UUGGUGUGGUCAUUCCUGG	Cleavage	beta-glucosidase 31-like isoform 1 [Brachypodium distachyon], 78%
ssp-miR393	SCCCLR1C02D02.g	3.0	20.176	21	2658	2678	CUCCAAAGGGAUCGCAUUGAU	GUGAAUGUGAUGCUUUUGGAG	Translation	SWI/SNF related, matrix associated, actin dependent regulator of chromatin [Oryza sativa], 96%
	SCBGLR1117C04.g	3.0	18.893	21	1060	1080	CUCCAAAGGGAUCGCAUUGAU	AACAAUACGAUCCUUUUGGGC	Cleavage	aspartyl-tRNA synthetase, cytoplasmic-like [Brachypodium distachyon], 89%
	SCUTHR1064E02.g	3.0	23.95	19	69	87	CUCCAAAGGGAUCGCAUUG	UAAUGCACUUCCUUUGGAG	Cleavage	UDP-glycosyltransferase/ transferase, transferring glycosyl groups [Zea mays], 72%
	SCJFLR1074G04.g	3.0	21.736	21	95	115	CUCCAAAGGGAUCGCAUUGAU	ACCAAUUCGAUCUCCUUGGAG	Cleavage	plasminogen activator inhibitor 1 RNA-binding protein [Zea mays], 80%
ssp-miR399a	SCACHR1037A06.g	1.5	19.642	19	463	481	UGCCAAAGGAGAGUUGCCC	GGGUAGUUCUCCUUUGGCA	Cleavage	inorganic pyrophosphatase 2-like [Brachypodium distachyon], 71%

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Conclusões

I. O sequenciamento de pequenos RNAs permitiu a cobertura do microtranscriptoma de cana-de-açúcar, de forma que miRNAs já descritos *in silico* foram confirmados (ssp-miR1432, ssp-miR156, ssp-miR167, ssp-miR528) e novos miRNAs foram identificados em cana (ssp-miR160, ssp-miR164, ssp-miR166, ssp-miR169, ssp-miR171, ssp-miR172, ssp-miR319, ssp-miR390, ssp-miR393, ssp-miR394, ssp-miR396, ssp-miR397, ssp-miR399 e ssp-miR529).

II. Os miRNAs ssp-miR164, ssp-miR393, ssp-miR394, ssp-miR397, ssp-miR399a, ssp-miR528 e ssp-miR1432 possuem expressão modulada pelo estresse por seca em cana-de-açúcar, variando com o tempo de estresse e o com o genótipo.

III. A partir da identificação dos possíveis alvos dos miRNAs que possuem expressão modulada por seca, foram sugeridos pontos de controle exercidos pela rede regulatória envolvendo o microtranscritoma, destacando transcritos que codificam fatores de transcrição (ssp-miR164 e ssp-miR1432), fosfatases (ssp-miR394 e ssp-miR399), tranferase (ssp-miR399), glicoproteína (ssp-miR397) e proteínas portadoras do domínio UBX (ssp-miR528).

IV. O microtranscriptoma apresenta diferenças significativas entre os cultivares contrastantes quanto à tolerância à seca, porém, o padrão de expressão dos miRNAs juntamente com os prováveis genes alvos preditos não permitem a identificação de agentes causais capazes de explicar os fenótipos contrastantes.

V. Em resumo, esse trabalho forneceu informações sobre o microtranscriptoma de uma cultura importante para o setor bioenergético, destacando a rede regulatória desencadeada pelo estresse por seca. Trabalhos futuros, envolvendo superexpressão ou silenciamento dos miRNAs ou de seus alvos, irão aumentar o conhecimento sobre o mecanismo molecular da resposta e da tolerância ao estresse por seca em cana-de-açúcar.

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