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ROBERTA LANE DE OLIVEIRA SILVA

IDENTIFICAÇÃO DE GENES EXPRESSOS EM CANA-DE-AÇÚCAR EM CONDIÇÕES DE ESTRESSE HÍDRICO ATRAVÉS DA TÉCNICA DE SUPERSAGE

Recife

2014

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia Da Universidade Federal De Pernambuco, como requisito parcial para a obtenção do título de DOUTORA em Biotecnologia.

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Orientadora: Profª Drª Ana Maria Benko Iseppon

Coorientador: Profº Dr. Ederson Akio Kido

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RESUMO

O presente trabalho teve como objetivo analisar o perfil de transcrição de genótipos de cana-de-açúcar tolerantes e sensíveis ao déficit hídrico, utilizando a técnica de SuperSAGE. Para tanto, genótipos selecionados a partir do programa de melhoramento do Centro de Tecnologia Canavieira (CTC), com performances contrastantes sob déficit hídrico foram submetidos à supressão de rega por 24 horas e constituíram o material vegetal avaliado. Foram geradas quatro bibliotecas SuperSAGE, as quais incluíram 8.787.315 *tags* (26 pb) que, após a exclusão dos *singlets*, permitiram a identificação de 205.975 *unitags*. As bibliotecas mais representativas, considerando o número de *tags*, foram TC (tolerante controle; 2.516.454 *tags*) e SD (sensível estressada; 2.133.587 *tags*), enquanto TD (tolerante estressada; 750.226 *tags*) e SC (sensível controle; 762.492 marcas) foram menos abundantes. Para a anotação tag-gene, foram realizados alinhamentos contra ESTs de gramíneas de diferentes bancos de dados públicos, os quais foram posteriormente categorizados em termos de Ontologia Gênica (GO) usando a ferramenta Blast2GO. A partir das análises estatísticas considerando $P \leq 0,05$ (teste Audic-Claverie) foi possível detectar as *tags* diferencialmente expressas, dentre as quais 213 foram identificadas como superexpressas. Desse total, 68 unitags possuíam anotação ou termo GO e 145 permaneceram sem anotação. Com o intuito de eleger um grupo com as melhores *tags* para validação via RT-qPCR foram selecionados candidatos que apresentaram respostas exclusivas, diferencialmente significativas e divergentes entre os *bulks* de genótipos tolerantes e sensíveis quando comparadas as condições de estresse e controle. Adicionalmente, dez genes de referência foram selecionados para avaliação em algoritmos estatísticos (GeNorm, NormFinder and BestKeeper), dos quais seis (Histona, Ubiquitina, SAMDC, 25S rRNA, α-tubulina e GAPDH) foram considerados apropriados para normalizar os dados de expressão de raízes de genótipos de cana-de-açúcar sob seca, sendo Histona e α-tubulina relatados pela primeira vez para a espécie. Análises adicionais permitiram identificar representantes das quatro subfamílias de aquaporinas descritas em vegetais superiores, apresentando 42 isoformas distintas. Ao menos 10 potenciais alvos distintos de isoformas de aquaporinas e suas respectivas *unitags* foram considerados promissores para estudos futuros, dos quais dois (*Ss*PIP1-1 e *So*PIP1-3/PIP1-4) foram validados via RT-qPCR e apresentaram potencial para desenvolvimento de marcadores moleculares para uso no melhoramento genético.

Palavras-chave: Déficit hídrico. Perfil transcricional. RT-qPCR. Genes de referência. Aquaporinas.

ABSTRACT

The present study aimed to analyze the comparative transcriptional profiling of tolerant and sensitive sugarcane genotypes to water deficit, using the SuperSAGE technology. To this end, accessions with contrasting performances under water deficit (drought sensitive and tolerant) were selected from the breeding program of the Center for Sugarcane Technology (CTC) and submitted to water deficit (irrigation suspension of 24 h) and used to generate four SuperSAGE libraries, producing 8,787,315 tags (26 bp) which, after singlets exclusion, allowed the identification of 205,975 unitags. Considering the number of tags the most representative libraries were TC (tolerant control: 2,516,454) and SD (sensitive after stress: 2,133,587), whereas TD (tolerant after stress: 750,226) and SC (sensitive control: 762,492) presented a lower number of transcripts. For tag-gene annotation alignments were carried out against ESTs from grass species available in different public data banks used for a posterior Gene Ontology (GO) categorization with Blast2GO. Statistical evaluation (Audic-Claverie test at $P \leq 0.05$) allowed the identification of differentially expressed tags from which 213 were overexpressed. From these, 68 unitags presented GO terms and 145 had no functional annotation. Aiming to elect the most promising tags for RT-qPCR validation, candidate sequences were selected among those that presented exclusive responses and significant differential expression comparing bulks of tolerant and sensible genotypes under stress and their respective non stressed controls. Additionally ten reference genes were selected for evaluation with statistical algorithms (GeNorm, NormFinder and BestKeeper), whereas six (Histone, Ubiquitin, SAMDC, 25S rRNA, α-tubulin e GAPDH) of them were considered appropriate to normalize expression data of sugarcane root tissues under drought, being Histone and α-tubulin genes reported for the first time for sugarcane studies. Further analysis allowed the identification of representatives of the four aquaporin subfamilies previously described in higher plants, with 42 distinct isoforms. At least 10 potential targets among the identified isoforms were considered promising for future studies, from which two (SsPIP1-1 and SoPIP1-3/PIP1-4) were validated via RT-qPCR, representing potential candidates for molecular marker development and use in breeding programs.

Key words: Water deficit. Transcriptional profiling. RT-qPCR. Reference genes. Aquaporins.

LISTA DE ILUSTRAÇÕES

REVISÃO DE LITERATURA

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE ABREVIATURAS E SIGLAS

ABA	Ácido abscísico
BLAST	Ferramenta básica de busca de alinhamento local
BLASTN	BLAST de nucleotídeos
BLASTX	BLAST de proteínas
cDNA	DNA complementar
CONAB	Companhia Nacional de Abastecimento
Ct	Ciclo Limiar
CTC	Centro de Tecnologia Canavieira
DNA	Ácido desoxirribonucléico
EST	Etiquetas de sequências expressas
Fa	*Festuca arundinacea*
FC	Modulação da expressão
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*
GO	Ontologia gênica
HT-SuperSAGE	Técnica de SuperSAGE combinada às tecnologias de sequenciamento de alta performance
Hv	*Hordeum vulgare*
IAC	Instituto Agronômico de Campinas
LEA	Abundantes no final da embriogênese
LongSAGE	Técnica de Análise Serial da Expressão Gênica que gera *tags* de 21 nucleotídeos
MIP	Principais proteínas intrínsecas
NIP	Proteínas de 26 kDa intrínsecas ao nódulo
Os	*Oryza sativa*
PCR	Reação em cadeia da polimerase
PIP	Proteínas intrínsecas à membrana plasmática
Primer	Oligonucleotídeo iniciador em técnicas de amplificação de ácido nucléico
Pv	*Panicum virgatum*
RNA	Ácido ribonucleico
RNAi	RNA de interferência
RNA-Seq	Sequenciamento direto de RNA de alta performance

mRNA	RNA mensageiro
NaCl	Cloreto de sódio
RIDESA	Rede Interuniversitária para o Desenvolvimento do Setor Sucroenergético
RT-qPCR	Reação quantitativa da transcrição reversa em cadeia da polimerase
So	*Saccharum officinarum*
SAGE	Análise Serial da Expressão Gênica
SuperSAGE	Técnica de Análise Serial da Expressão Gênica que gera *tags* de 26 nucleotídeos
SC	*Bulk* de genótipos sensíveis ao estresse sob condições controle
SD	*Bulk* de genótipos sensíveis ao estresse sob condições de estresse (supressão de rega de 24 h)
SIP	Pequenas proteínas intrínsecas básicas
Ss	*Saccharum* spp.
SSH	Hibridização Subtrativa Supressiva
Ta	*Triticum aestivum*
Tags	Curta sequência de DNA (até 26 pb) obtida através das técnicas de SAGE, LongSAGE e SuperSAGE
TC	*Bulk* de genótipos tolerantes ao estresse sob condições controle
TD	*Bulk* de genótipos tolerantes ao estresse sob condições de estresse (supressão de rega de 24 h)
TIP	Proteínas intrínsecas ao tonoplasto
UNICA	União das Indústrias da Cana-de-Açúcar
Unitags	*Tags* únicas
Zm	*Zea mays*

SUMÁRIO

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1 Introdução

A cana-de-açúcar (*Saccharum* spp.) é uma espécie vegetal de origem tropical/subtropical, sendo considerada uma das principais culturas agrícolas do Brasil, fornecendo matéria-prima para a produção de açúcar, álcool, alimentação animal, entre outros. Desde os tempos do Brasil colônia até os dias atuais, a cultura de cana-de-açúcar tem sido uma grande fonte de riquezas para a economia do país, representando importante caráter social através da geração de empregos diretos e indiretos, como também gerando divisas através da exportação do açúcar e álcool. Além disso, a atual demanda por recursos energéticos renováveis substitutos do petróleo faz da cana-de-açúcar uma cultura em total expansão no Brasil.

Com o cenário das mudanças climáticas no sentido de aumentos gradativos de temperatura e seca, bem como a maior aridez em muitas áreas do globo terrestre, pesquisas relacionadas aos mecanismos de resposta das plantas frente ao déficit hídrico têm ganhado grande importância, uma vez que representa um dos fatores ambientais mais limitantes para a cultura da cana-de-açúcar, afetando diretamente o seu crescimento e desenvolvimento. Como forma de se adaptarem a esse tipo de estresse, as plantas alteram seu metabolismo por meio da ativação de diferentes mecanismos de resistência.

Um melhor entendimento dos efeitos da seca nas plantas torna-se vital, principalmente no que se refere à melhoria das práticas de manejo agrícola, assim como para garantir sucesso em programas de melhoramento genético vegetal. Consequentemente, a geração de genótipos de cana-de-açúcar com maior tolerância à seca permitirá ampliar o número de variedades disponíveis para plantio, proporcionando ganhos de produtividade, além de maior lastro de adaptação. Nesse sentido, a engenharia genética se apresenta como uma ferramenta viável para auxiliar o desenvolvimento de variedades de cana-de-açúcar tolerantes à seca. A utilização de ferramentas biotecnológicas para análise genética da cana-de-açúcar submetida a estresse ambiental poderá ampliar os conhecimentos existentes sobre seu genoma e elucidar a estrutura de alguns genes, contribuindo para identificar genes representativos sob tal condição de estresse.

Entre as abordagens atuais, a técnica de SuperSAGE apresenta-se como uma das ferramentas mais eficientes na análise da expressão gênica, por ser de arquitetura aberta, apresentando grande especificidade. O estudo da expressão diferencial via SuperSAGE representa um caráter inovador, comparativamente a outras estratégias utilizadas em estudos de transcriptoma desenvolvidos no Brasil. Com esta metodologia é possível eleger um número

significativo de genes diferencialmente expressos sob condições de estresse, os quais poderão apresentar diversas possibilidades de aplicação no melhoramento de várias culturas de valor socioeconômico no Brasil. Além disso, as informações geradas poderão auxiliar também na compreensão dos mecanismos moleculares envolvidos na resposta do estresse salino.

O presente trabalho teve como objetivo analisar o perfil de transcrição de genótipos contrastantes (tolerantes e sensíveis ao déficit hídrico) de cana-de-açúcar submetidos à supressão de rega por 24 h, utilizando a técnica de SuperSAGE, visando identificar genes diferencialmente expressos relacionados à resposta à seca, bem como possíveis marcadores moleculares funcionais para aplicação em programas de melhoramento genético da cultura.

2 Revisão de literatura

2.1 A cultura da cana-de-açúcar

A cana-de-açúcar é uma planta alógama, pertencente à família Poaceae e ao gênero *Saccharum*, originária do Sudoeste Asiático, na região central de Nova Guiné e da Indonésia. Essa espécie possui um dos mais complexos genomas vegetais, apresentando um variável nível de ploidia, devido à sua origem multiespécie. Atualmente, as variedades cultivadas são resultado de hibridações interespecíficas envolvendo *Saccharum officinarum* L. com *S. espontaneum* L., *S. barberi*, *S. sinense* ou *S. robustum* (INGELBRECHT, IRVINE e MIRKOV, 1999; DILLON et al., 2007; SOUZA e LORENZI, 2008). Mundialmente, a cana-de-açúcar está entre as dez mais importantes culturas, contribuindo com mais de 50% de todo o açúcar produzido (INGELBRECHT, IRVINE e MIRKOV, 1999; MAPA, 2014). O Brasil é o maior produtor mundial de cana-de-açúcar, apresentando uma produção de aproximadamente 659,85 milhões de toneladas (safra de 2013/2014, 12% superior à safra de 2012/2013), distribuída em 8.810,79 milhões de hectares (3,8% ou 325,8 mil hectares, em relação à safra anterior), com uma produtividade média de 74.89 kg/ha (CONAB, 2013).

A cana-de-açúcar apresenta relevante caráter social através da geração de empregos diretos e indiretos nas indústrias de açúcar e álcool de todo o país. Além disso, gera divisas através da exportação desses produtos, apresentando um aproveitamento racional da biomassa vegetal para a indústria alcoolquímica e para alimentação animal, entre outros (SACILOTO, 2003). Os principais produtos gerados são o açúcar e o etanol. Além desses, outros produtos também são gerados após seu processamento, como o álcool hidratado, a aguardente, a garapa, o bagaço e a vinhaça (THIAGO e VIEIRA, 2002; CAPUTO et al., 2008).

Devido a suas múltiplas utilidades, a cana-de-açúcar, seus produtos e subprodutos têm recebido grande atenção, com destaque para a produção de etanol, que representa uma importante fonte de biocombustível renovável. Os dados apresentados no último levantamento da safra 2013/2014 corroboram a essas perspectivas, pois o Brasil conseguiu produzir cerca de 27,76 bilhões de litros/ano de etanol, apresentando um incremento de 4,02 bilhões de litros (alta de 16,49%) em relação à última safra (CONAB, 2013). Segundo projeções do Ministério da Agricultura, Pecuária e Abastecimento - MAPA, a produção de etanol no Brasil em 2019 está estimada em 58,8 bilhões de litros, com um consumo interno de 50 bilhões de litros e exportações de U$8,8 bilhões (MAPA, 2014). Em relação à produção de açúcar, o Brasil é o primeiro produtor mundial, apresentando uma produção de aproximadamente 38,81 milhões

de toneladas na safra de 2013/2014. Desse total, 71,69% deverá ser produzido na região Sudeste, 10,46% na região Nordeste, 9,79% na região Centro-Oeste e 7,92% na região Sul (CONAB, 2013).

Em função da posição de destaque que ocupa na economia mundial, a cana-de-açúcar está constantemente inserida em programas de melhoramento que visam geração de cultivares cada vez mais adaptadas às condições ambientais adversas, resistentes a pragas e patógenos e apresentando maior teor de sacarose (CIDADE et al., 2006). No entanto, as várias etapas exigidas no processo do melhoramento convencional tornam-o muitas vezes inviável, uma vez que pode levar até 15 anos até o lançamento de uma cultivar. Esse fato, aliado à complexidade de seu genoma, torna a cana-de-açúcar uma excelente candidata ao melhoramento por meio da engenharia genética (INGELBRECHT, IRVINE e MIRKOV, 1999; KIMBENG e COX, 2003).

2.2 Déficit hídrico

Estresses abióticos são responsáveis por desencadear uma série de respostas nas plantas, que podem ser percebidas através de modificações morfológicas, fisiológicas, moleculares e metabólicas, a fim de tolerar estes estresses (BENKO-ISEPPON et al., 2011). Além disso, as plantas podem apresentar respostas funcionais distintas aos diferentes tipos de estresse, podendo se ajustar a condições sub-ótimas ou desenvolver mecanismos para superá-los (LAMBERS, CHAPIN e PONS, 1998; LARCHER 2000). A ocorrência de déficit hídrico em plantas cultivadas afeta o crescimento e o desenvolvimento das culturas em todo o mundo. De forma geral, os mecanismos que podem ajudar a planta a suportar a deficiência de água são: redução da atividade fotossintética, fechamento estomático, redução da área foliar, crescimento das raízes, maior eficiência do uso da água, produção de compostos específicos e ajuste osmótico. No entanto, as respostas fisiológicas a condições de déficit hídrico podem variar de acordo com a espécie, cultivar, tempo de exposição, fatores edáficos, entre outros (HOLMBERG e BÜLOW, 1998; LECOEUR e SINCLAIR, 2006).

Ao nível da parte aérea da planta, como resposta usual ao déficit hídrico, ocorre a diminuição no crescimento, a qual está diretamente associada a alterações no metabolismo de carbono e nitrogênio. O fechamento estomático, por sua vez, é considerado como a primeira linha de defesa contra o dessecamento, ocorrendo antes mesmo de haver diminuição no conteúdo de água da folha (YORDANOV, VELIKOVA e TSONEV, 2000). Esse mecanismo de defesa tem como principal objetivo inibir a cavitação e uma provável falha no sistema de

condução de água, uma vez que os estômatos respondem espontaneamente ao aumento da tensão de água no xilema de forma a evitar perdas de água pela transpiração (KRAMER e BOYER, 1995; ZWIENIECKI e HOLBROOK, 2000; COCHARD et al., 2002).

A redução na atividade fotossintética ocasionada pela diminuição na assimilação do CO_2 e senescência das folhas são também indicadores importantes do efeito do déficit hídrico em uma cultura (FAVER *et al*., 2006). Para Begg e Turner (1995) os efeitos causados pelo déficit hídrico nos tecidos mais jovens de plantas são maiores que nos tecidos adultos; porém, quando se interrompe o estresse, o desenvolvimento é recuperado apenas nas folhas mais jovens. Corroborando a essa ideia, Pires, Arruda e Sakai (2008) afirmaram que as plantas apresentam maior suscetibilidade ao déficit hídrico quando estão em período de rápido desenvolvimento, apresentando grande área foliar, e dessa forma, necessitando de maior quantidade de água para a realização das trocas gasosas com a atmosfera. Na cana-de-açúcar, especificamente, a suscetibilidade à deficiência hídrica se dá principalmente quando as plantas estão na fase de alongamento dos colmos, causando sérios prejuízos à produção de fitomassa e ao rendimento de sacarose (SILVA e COSTA, 2004; INMAN-BAMBER e SMITH, 2005). Entretanto, Ramesh (2000) afirma que a alta demanda hídrica ocorre, nesta espécie, nos períodos de perfilhamento e de rápido crescimento (entre 60 e 150 dias após o plantio), os quais correspondem a sua fase de formação.

As respostas fisiológicas específicas ao déficit hídrico representam na verdade combinações de eventos moleculares que são ativados ou desativados pela percepção do estresse (BRAY, 1993). As respostas moleculares, por sua vez, envolvem a ativação de genes específicos, assim como catabolismo e anabolismo de proteínas (HOLMBERG e BÜLOW, 1998). Em situações de déficit hídrico, proteínas de proteção ao estresse, como as do grupo LEA ("*late abundant embriogenesis*"), chaperonas (ZHU, 2001), proteínas formadoras de canais de água (aquaporinas), transportadoras, proteinases e enzimas de detoxificação são produzidas. Além dessas, outras proteínas destacam-se por sua função regulatória, como as quinases, fosfatases, fatores de transcrição, enzimas que participam do metabolismo de fosfolipídios e da biossíntese do ABA (SHINOZAKI e YAMAGUCHI-SHINOZAKI, 2007; ASHRAF, 2010).

Diversos estudos têm sido direcionados para o entendimento dos mecanismos fisiológicos e genéticos relacionados à tolerância à seca, visando a identificação de genes fundamentais para a determinação destas características. O conhecimento de tais genes tem crescido cada vez mais em importância para a biotecnologia, dada à escassez de água que vem se agravando progressivamente em termos globais (BENKO-ISEPPON et al., 2011). Ensaios

sobre respostas específicas ao estresse hídrico foram publicados aplicando tecnologias de genômica funcional (RODRIGUES; LAIA; ZINGARETTI, 2009; WANG et al., 2011; LEMBKE et al., 2012). Estas avaliações têm fornecido informações importantes a respeito das estratégias moleculares e bioquímicas no estudo da tolerância à seca em várias culturas e espécies modelo.

2.3 Cana-de-açúcar e seca: perspectivas e uso da ciência na busca de soluções

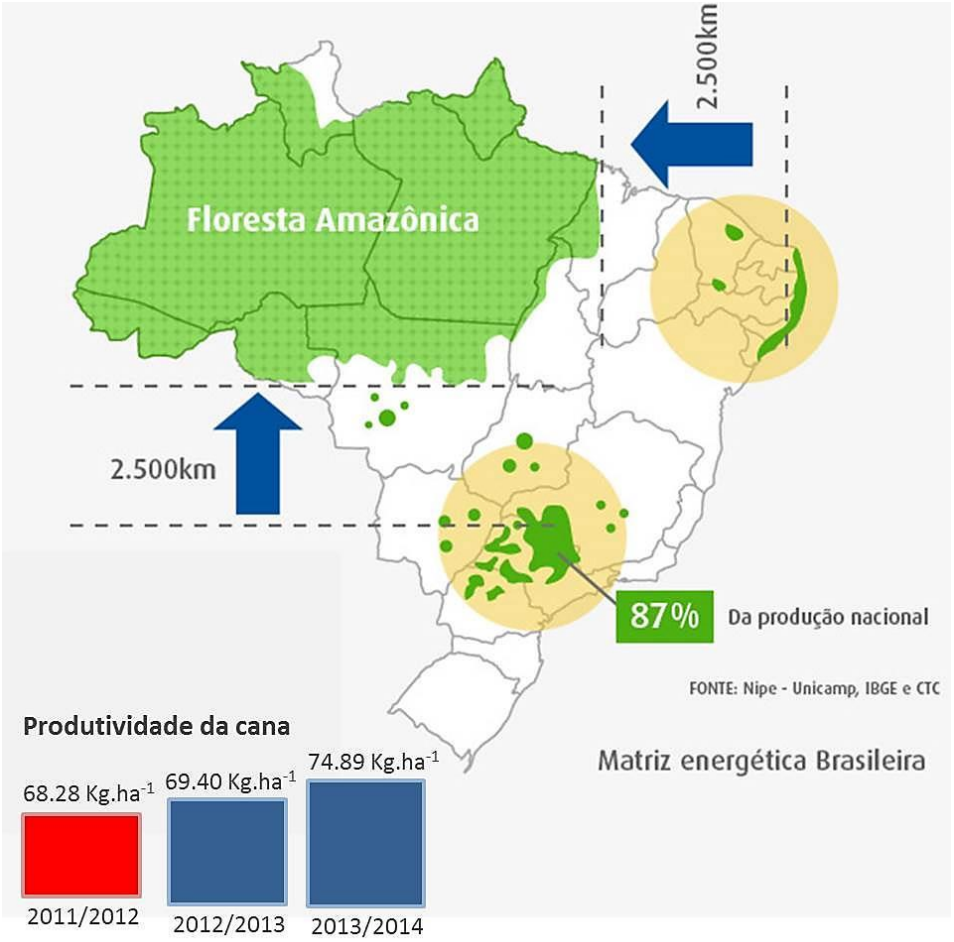
Fatores abióticos como seca, salinidade, frio e alagamento, afetam negativamente o crescimento das plantas, levando a alterações fisiológicas e metabólicas, problemas de germinação e até mesmo a morte da planta sob condições extremas (SMITH e BHAVEL, 2007). Entre os fatores mencionados, a seca se destaca, sendo a ameaça mais séria tendo em vista a escassez de água doce em muitas regiões do mundo, trazendo sérias limitações para a agricultura (JURY e VAUX, 2005).

Um terço da população mundial vive em áreas com escassez de água, este fato é especialmente grave quando se consideram outros fatores adversos, como os altos níveis de CO_2 atmosféricos, cenários de mudanças climáticas e previsões de aquecimento global, todos contribuindo significativamente para a frequência e severidade da seca no futuro (VALLIYODAN e NGUYEN, 2008). Para Hazell e Wood (2008), a mudança climática afetará diferentes localidades de diferentes maneiras, tornando a agricultura mais difícil em algumas regiões, como em muitas áreas propensas à seca na África e Américas. Tais previsões também reforçam que os sistemas agrícolas têm grande capacidade para se adaptar às mudanças climáticas, desde que se destinem esforços em pesquisas para identificar as melhores formas de adaptação a essa condição.

No Brasil, dentre todos os fatores bióticos e abióticos, o estresse hídrico é o que mais frequentemente influencia de forma negativa a cultura da cana-de-açúcar, sendo considerado um fator de extrema importância quando se trata do aumento da produtividade desta cultura (LAWLOR, 2002; TAIZ e ZEIGER, 2004). Em 2011, as condições climáticas influenciaram na queda da produtividade da cana-de-açúcar, em consequência das chuvas escassas e do longo período de estiagem, afetando severamente toda a região Sudeste e Centro-Oeste (regiões mais produtoras do país; Figura 1) e o final da colheita de 2012 da região Nordeste, acarretando numa perda de 8,3% no rendimento da safra anterior (CONAB, 2012). Apenas no estado de São Paulo, detentor de aproximadamente 60% da produção brasileira, as perdas atingiram cerca de 10% devido a períodos de seca, como relatado na safra de 2010 (UNICA,

2011).

Figura 1 - Mapa da produção da cana-de-açúcar no Brasil



Fonte: Adaptado de UNICA (2011).

Diversos programas de melhoramento genético para a cana-de-açúcar são desenvolvidos em diferentes países, tanto por instituições públicas e privadas, como também por cooperativas formadas por produtores. No Brasil, os principais programas de melhoramento para essa cultura têm sido executados pela Rede Interuniversitária para o Desenvolvimento do Setor Sucroenergético (RIDESA), Centro de Tecnologia Canavieira (CTC), Instituto Agronômico de Campinas (IAC), Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) e Monsanto, e envolvem cruzamentos e desenvolvimento de novas variedades, demandando altos investimentos e produzindo resultados em longo prazo (SOUZA e SLUYS, 2010). Apesar do sucesso de produção no que tange a variedades de cana-de-açúcar advindas de programas de melhoramento genético convencional, o uso da

engenharia genética se apresenta como uma excelente oportunidade para o desenvolvimento de uma agricultura mais eficiente, ambientalmente adequada e sustentável, e para obtenção de novas variedades com características que não são obtidas pelo melhoramento convencional.

A identificação de genes responsáveis por características de interesse agronômico através da utilização de técnicas de biologia molecular representa uma alternativa para aplicação no melhoramento da cana-de-açúcar, os quais poderão ser utilizados para produção de plantas transgênicas (silenciamento ou superexpressão) e desenvolvimento de marcadores funcionais para uso em seleção assistida, o que proporcionará a obtenção de variedades superiores, reduzindo significativamente as perdas na agricultura, e fornecendo maior produtividade e tolerância a condições de estresses bióticos e abióticos (NOGUEIRA, 2004; SOUZA e SLUYS, 2010). Uma vez que os mecanismos de defesa envolvidos no processo de tolerância à seca se apresentam similares entre as espécies vegetais, elucidar a forma como esses eventos interagem é essencial para o entendimento das vias metabólicas relacionadas à tolerância à seca, o que facilitará o desenvolvimento de variedades adaptadas a esse tipo de estresse (LAN et al., 2005).

2.4 Transcriptômica vegetal

A resposta das plantas a estresses abióticos envolve muitos genes e mecanismos moleculares e/ou bioquímicos complexos, de forma que múltiplas vias de transdução de sinais podem ser ativadas para regular a expressão gênica sob tais estresses (CHINNUSAMY, SCHUMAKER e ZHU, 2004). Na era pós-genômica, análises abrangentes utilizando as tecnologias da genômica funcional como transcriptômica, proteômica e metabolômica, aumentaram a compreensão das complexas redes regulatórias associadas à adaptação e tolerância aos diversos tipos de estresses (URANO, KURIHARA e SHINOZAKI, 2010, YIN e STRUIK, 2010). A partir da infinidade de dados advindos da genômica funcional, foi possível esclarecer importantes aspectos relativos à modulação de redes regulatórias e vias metabólicas relacionadas às respostas da planta a estresses.

Análises globais da expressão gênica, ou estudos de transcriptômica, têm contribuído bastante para o esclarecimento de processos biológicos complexos, elucidando de forma clara caminhos metabólicos e de sinalização, que são base para as respostas ambientais e do desenvolvimento (EXPÓSITO-RODRÍGUEZ et al., 2008). Por meio dessas análises, é possível avaliar um conjunto de transcritos de um organismo em uma determinada situação

celular, identificar genes candidatos e suas funções e fornecer informações sobre possíveis regiões regulatórias (MIR, 2005; MOCHIDA e SHINOZAKI, 2010).

As metodologias utilizadas para análise da expressão gênica baseiam-se em: (I) hibridização de fitas de nucleotídeos complementares a sequências alvos imobilizadas, as quais dependem do conhecimento da sequência gênica; (II) sequenciamento e contagem de transcritos, que por sua vez, não necessitam do conhecimento prévio da sequência (TERAUCHI et al., 2008). Entre os métodos de hibridização destaca-se o de microarranjo de DNA (SCHENA, 1995), o qual monitora mudanças na expressão gênica baseados na hibridização de moléculas mRNA com sua sequência codante disposta em um arranjo, podendo avaliar a expressão de milhares de transcritos simultaneamente (MEYERS et al., 2004). Essa técnica, porém, apresenta algumas limitações, as quais, em sua grande maioria, resultam do princípio da hibridização, que é o ponto central desta tecnologia. De forma geral, essas limitações incluem baixa sensibilidade e especificidade, além da incapacidade para analisar e descobrir novos genes, apesar do seu alto desempenho e ampla utilização (SHIMKETS, 2004; WANG et al., 2009).

Em relação às técnicas baseadas em sequenciamento, os principais representantes são as bibliotecas de EST (*Expressed Sequence Tag*; ADAMS et al., 1991), a técnica de SAGE (*Serial Analysis of Gene Expression*; VELCULESCU et al., 1995) e suas versões derivadas LongSAGE (*Long Serial Analysis of Gene Expression*; SAHA et al., 1995) e SuperSAGE (*Super Serial Analysis of Gene Expression*; MATSUMURA et al., 2003) e mais recentemente o *RNA sequencing* (RNA-Seq) (MORTAZAVI et al., 2008).

Os ESTs são formados por sequências contendo centenas de pares de bases de comprimento, as quais são derivadas de um único sequenciamento de insertos de clones de cDNA selecionados aleatoriamente (ADAMS et al., 1991). As bibliotecas de EST representam uma alternativa relativamente rápida e barata para a descoberta de novos genes (BOURDON et al., 2002). Entre as suas utilizações estão: confirmação de regiões codificantes em sequências genômicas (ADAMS et al., 1991); construção de mapas genômicos (PATERSON et al., 2000) e desenvolvimento de chips de DNA (SCHENA, 1995). Adicionalmente, o uso de EST e de tecnologias de alto rendimento podem resultar na identificação de porções significativas de conteúdo genético de um organismo, servindo como base para iniciar projetos de sequenciamento de genomas (VAN DER HOEVEN et al., 2002). Portanto, apesar de apresentar algumas restrições, esse método é uma importante alternativa para compreender e adquirir novas informações acerca do cenário molecular de diversos

organismos, principalmente quando utilizado em conjunto a outras técnicas mais representativas.

O método de SAGE baseia-se no isolamento de pequenas sequências de cDNA (denominadas *tags*), obtidas a partir de moléculas individuais de mRNAs, e sua posterior concatenação em longas moléculas de DNA. As moléculas de DNA são então clonadas e sequenciadas, permitindo assim a análise qualitativa e quantitativa dos transcritos celulares (VELCULESCU et al., 1995). A grande limitação desse método refere-se à extensão de sua *tag*, 15 pb, muito curta para a identificação do gene correspondente. Para contornar esse problema, foi desenvolvido a técnica nomeada SuperSAGE, na qual as *tags* são maiores (26 pb) e utiliza o tipo III da enzima de restrição EcoP15I (Figura 2). Como as *tags* longas são altamente específicas, podem ser anotadas e associadas a um gene correspondente, garantindo assim, uma análise exata da expressão gênica (KAHL et al., 2006).

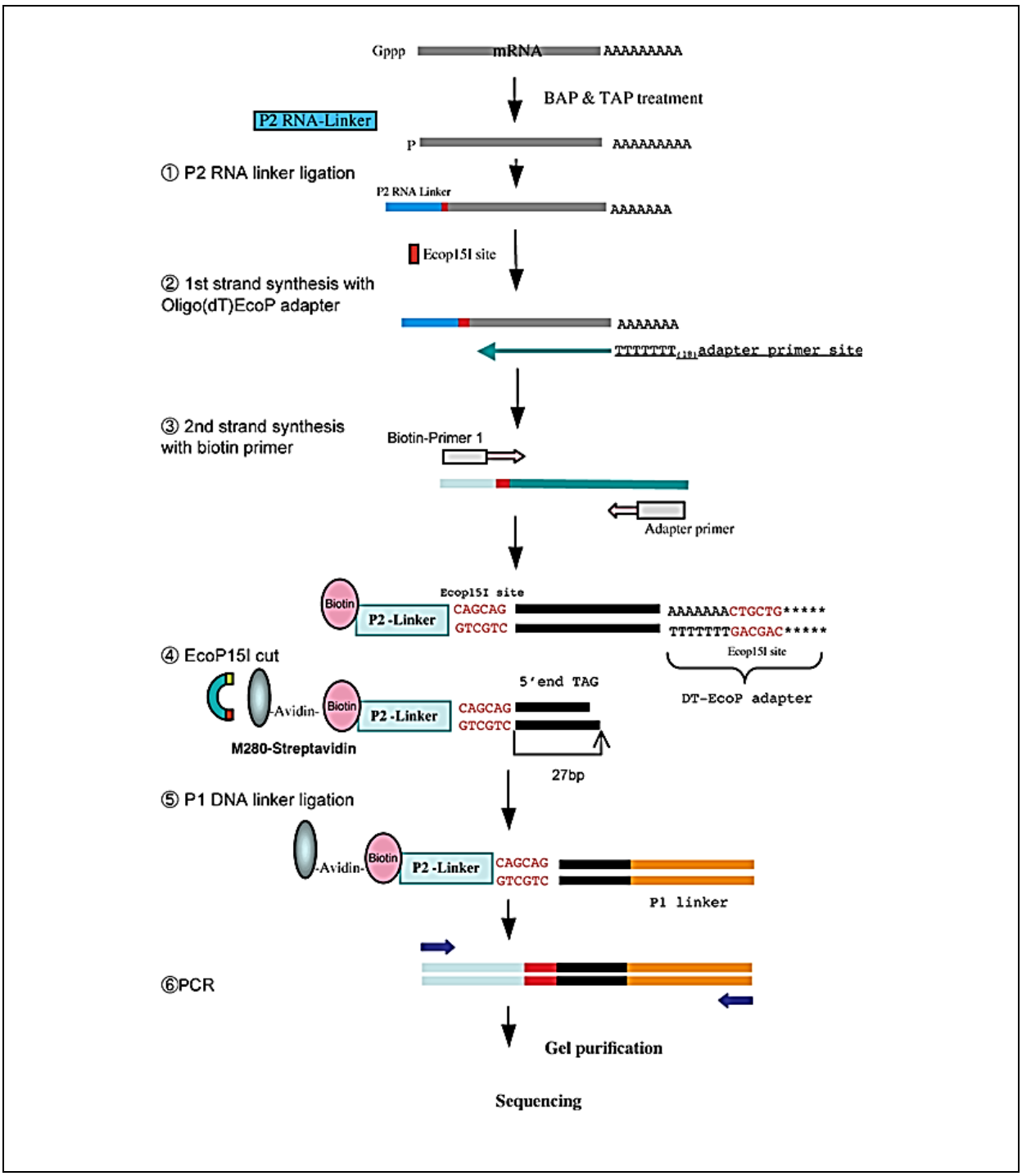
A técnica SuperSAGE apresenta-se como uma das mais modernas ferramentas de genômica funcional para uso em qualquer eucarioto, permitindo a identificação dos genes transcritos em determinada situação, através da análise e contagem de sequências diagnósticas específicas para cada mRNA individual. Além disso, possibilita análises qualitativas e quantitativas de padrões de transcrição de um ou mais genomas de eucariotos a partir da mudança da frequência de, teoricamente, todo mRNA possuidor de cauda poliA (MATSUMURA et al., 2003). Outra vantagem do método diz respeito à arquitetura aberta, ao contrário das plataformas de microarranjos (limitadas à amostragem existente no chip), onde qualquer transcrito (conhecido ou desconhecido) pode ser identificado e exatamente quantificado.

Essa metodologia apresenta diferentes possibilidades de aplicação, no caso de plantas, para definição de genes em sequências genômicas; detecção de antisense e de transcritos oriundos de processamento alternativo; análises de funções gênicas pelo silenciamento gênico; seleção de plantas em bancos de germoplasma; detecção do padrão de expressão de indivíduos; identificação do potencial agronômico de uma planta e análise de processos regulatórios ou metabólicos em organismos não modelo (para os quais sequências genômicas não estão disponíveis) (KAHL et al., 2006). Esta técnica destaca-se também pela sua eficiência na geração de perfis de transcrição, principalmente com a sua associação com plataformas de sequenciamento de alto desempenho [Pirossequenciamento (454 Roche®), Solexa (Illumina®) e SOLiD (Applied Biosystems®)].

A SuperSAGE vem sendo aplicada com sucesso em cana-de-açúcar (KIDO et al., 2012) e em outras espécies vegetais, tais como, arroz (MATSUMURA et al., 2003), banana

(COEMANS et al., 2005), pimenta (HAMADA et al., 2008), grão-de-bico (MOLINA et al., 2008; 2011), tabaco (GILARDONI et al., 2010) e feijão caupi (KIDO et al., 2011). A aplicação dessa metodologia tem um enorme potencial no melhoramento genético de plantas auxiliando no processo de seleção de genes candidatos, identificados pela citada técnica.

Figura 2 - Esquema de construção da biblioteca HT-SuperSAGE utilizando a plataforma de sequenciamento SOLiD (Applied Biosystems®)



Fonte: Hashimoto et al., (2009).

Outra abordagem recentemente desenvolvida, o sequenciamento direto de RNA de alta performance (RNA-Seq), analisa perfis transcricionais utilizando tecnologias de *deep-sequencing*. Atualmente, este é um dos métodos mais utilizados para análises globais de expressão gênica, pois fornece alta reprodutibilidade, ampla visão da extensão e da complexidade dos transcriptomas eucariotos, além de fornecer uma medida muito mais exata dos níveis de transcritos e suas isoformas quando comparado a outros métodos (WANG et al., 2009).

Vários projetos com o objetivo de estudar transcriptomas de diversas espécies vêm sendo desenvolvidos no mundo. Em cana-de-açúcar, estudos envolvendo genômica funcional foram realizados utilizando técnicas de hibridização e sequenciamento, como SSH [Hibridização Subtrativa Supressiva] (PATADE, RAI, e SUPRASANNA, 2011), micro / macroarranjos (RODRIGUES; LAIA; ZINGARETTI, 2009; LEMBKE et al., 2012), e EST (MENOSSI et al., 2008), avaliando o desempenho sob seca e salinidade. Adicionalmente, com os custos do sequenciamento em larga escala reduzidos, a combinação de técnicas de transcriptômica às novas plataformas de sequenciamento têm sido favorecida e estudos mais aprimorados envolvendo expressão gênica diferencial em cana-de-açúcar tem sido desenvolvidos (KIDO et al., 2011; SILVA et al., 2011; KIDO et al., 2012).

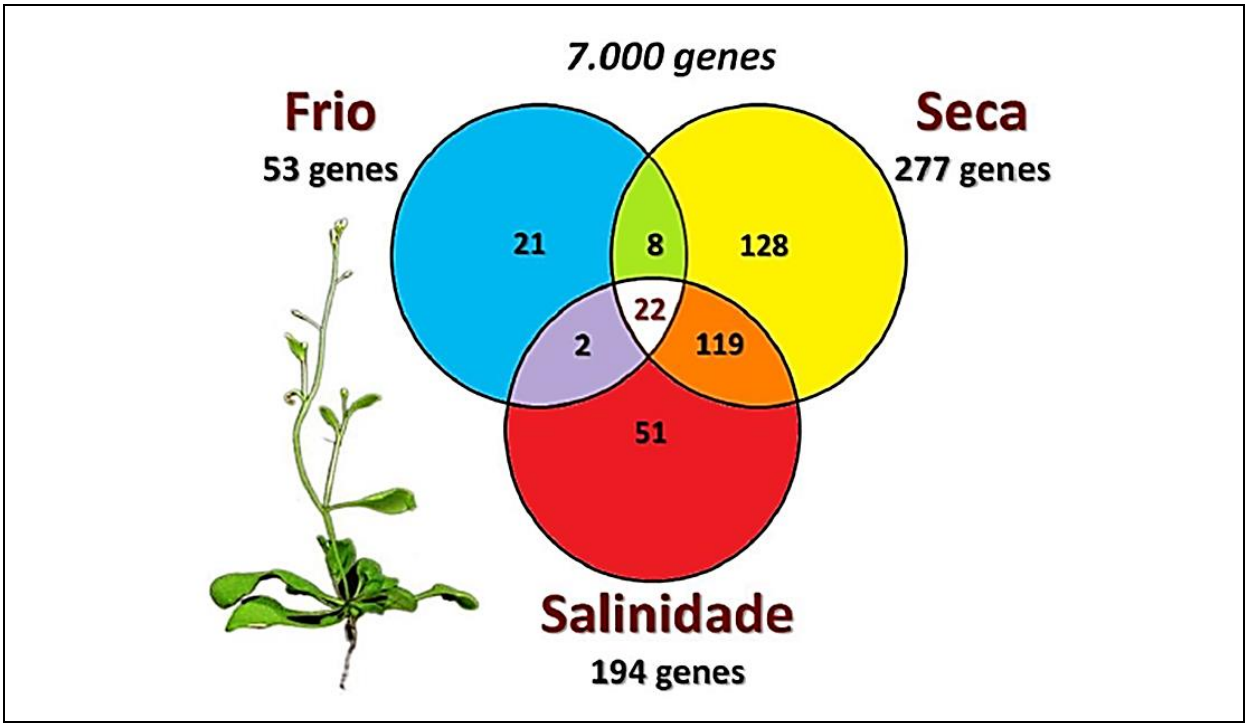
2.5 Orquestração da resposta a estresses abióticos

Por muitos anos se discutiu com base em evidências fisiológicas quais os possíveis fatores induzidos após o aparecimento de seca, salinidade, mudanças de temperatura, etc., havendo controvérsias quanto ao número de genes associados a estes estresses e à velocidade que estes podiam ser induzidos após a percepção do estresse. Em nível de genoma e transcriptoma, as primeiras evidências surgiram a partir dos estudos com a planta modelo *Arabidopsis thaliana*, pertencente à família das Brassicaceae (à qual pertence a couve, a mostarda, o rabanete e a canola, entre outras). Esta planta foi proposta como modelo devido a seu genoma pequeno (125 megabases), tamanho diminuto (em uma área equivalente a uma folha de papel A4 é possível crescer até 50 indivíduos), curto tempo de geração (~2,5 semanas), produzindo um número significativo de descendentes a cada geração (MEINKE et al., 1998). Esta planta encontra-se distribuída em vários países da Europa, ocorrendo inclusive em áreas secas e extremamente frias, como a Sibéria. Trata-se da planta mais detalhadamente estudada até o momento, sendo que além do sequenciamento completo de seu genoma, existe um extenso número de estudos de expressão diferencial e programas envolvendo a geração de

mutantes para o entendimento dos processos envolvendo seus estimados 25.498 genes de 11.000 famílias (THE ARABIDOPSIS GENOME INICIATIVE, 2000).

Estudos visando monitorar a expressão de 7.000 genes de indivíduos de *A. thaliana* submetidos em experimentos separados à seca, a altos níveis de salinidade e ao frio revelaram expressão aumentada (em mais de cinco vezes comparativamente aos controles não estressados) de 277, 194 e 53 genes, respectivamente, havendo um grande número de genes compartilhados entre diferentes tipos de estresse (Figura 3). Além disso, a indução da ativação destes genes após os estímulos citados ocorria muito rapidamente, com a maioria dos genes recrutados apenas no intervalo de duas horas após o estresse (SEKI et al., 2002). É interessante notar que 22 genes tinham sua expressão aumentada em todas as três situações de estresse abiótico, enquanto seca e salinidade compartilharam 119 genes induzidos (Figura 3). Na definição da resistência e/ou da susceptibilidade aos estresses citados, torna-se importante não apenas observar <u>*quais*</u> genes são ativados, mas <u>*a velocidade*</u> com que determinados fatores são transcritos, observando-se acentuadas diferenças entre indivíduos resistentes e suscetíveis, sob este aspecto. Entre os três grupos de estresses, 11 genes imediatamente induzíveis na fase pós-estresse (entre poucos minutos até 2 horas após o tratamento) foram observados, revelando-se como possíveis fontes gênicas de resistência a estresses abióticos (SEKI et al., 2002).

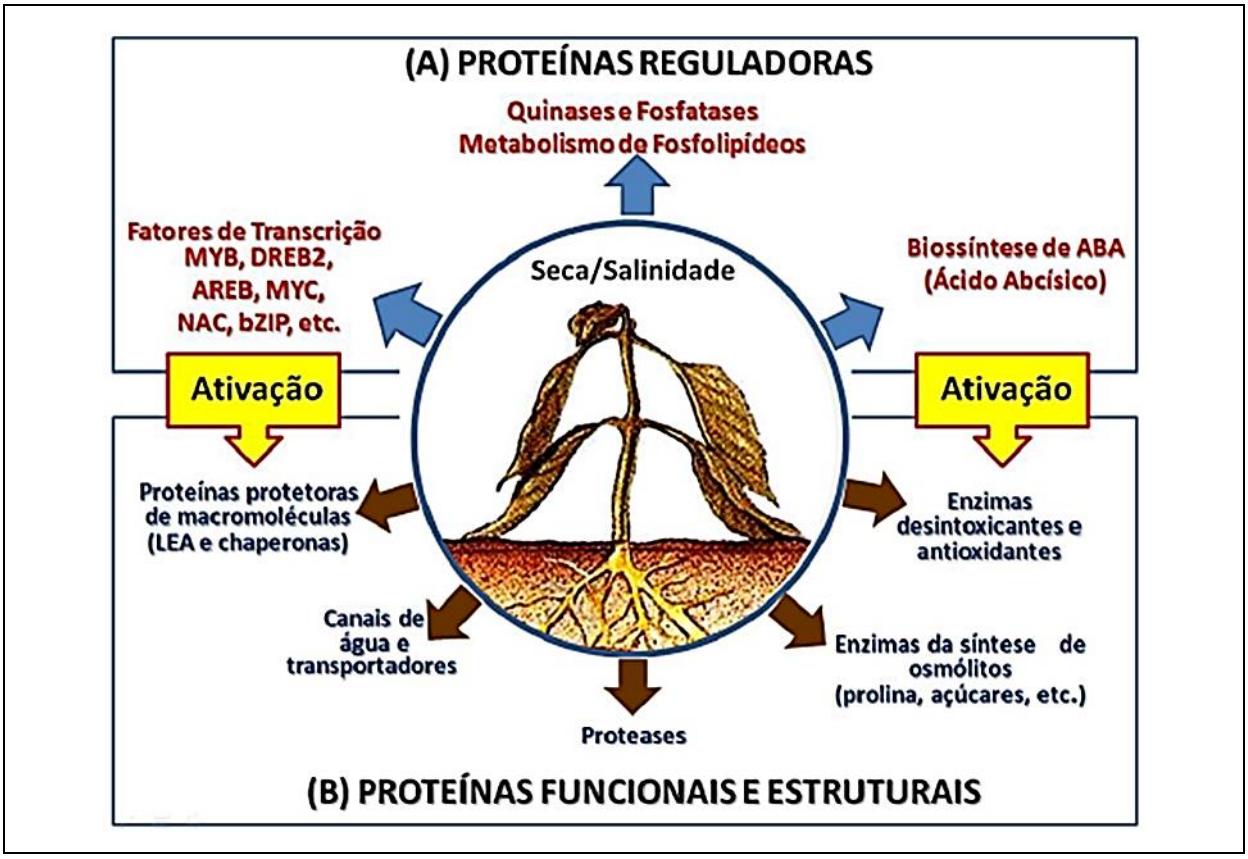
Figura 3 – Transcriptoma de *Arabidopsis thaliana* sob estresses abióticos



Fonte: Adaptado de Seki (2002).

Estudos como o de Seki et al. (2002) revelaram que os mecanismos genéticos de percepção que estão sendo relacionados em plantas às respostas aos estresses hídrico e salino incluem diversos genes, os quais têm sido tradicionalmente divididos em duas categorias: (I) os envolvidos na cascata de sinalização e no controle transcricional, como MyC, quinases MAP e quinase SOS2, fosfolipases e fatores transcricionais, como HSF, CBF/DREB, bHLH, bZIP, ERF (Figura 4A); (II) aqueles que funcionam diretamente na proteção de membranas e proteínas, como proteínas de choque térmico e chaperonas; proteínas LEA, osmoprotetores e removedores de radicais livres; e aqueles envolvidos na captura e transporte de água e íons, como as aquaporinas e os trocadores e transportadores de íons (Figura 4B). Tais categorias têm sido enfatizadas em análises com vários organismos (BLUMWALD, AHARON e APSE 2000; SEKI et al., 2002; BENKO-ISEPPON et al., 2005; SHINOZAKI e YAMAGUCHI-SHINOZAKI, 2007; GOLDACK, KING e YANG, 2011; HUANG et al., 2012), confirmando a complexidade da adaptação a estresses abióticos como os aqui tratados.

Figura 4 - Principais categorias de genes envolvidos na resposta de angiospermas a estresses abióticos como seca e salinidade



Fonte: Adaptado de Shinozaki e Yamaguchi-Shinozaki (2000).

Considera-se que as reações dos vegetais ao estresse hídrico e salino são complexas especialmente porque envolvem muitos genes com efeitos aditivos, tornando complexa a real transferência de tolerância a estes estresses para plantas de importância econômica. Devido à complexidade destas respostas moleculares, vários projetos têm investido esforços usando técnicas biotecnológicas via transgenia, super-expressando diferentes genes entre estes das categorias listadas na Figura 4.

2.5.1 Proteínas reguladoras

A diversidade genética e de mecanismos associados às proteínas reguladoras e seus genes codificantes é significativa, merecendo extensas considerações. Dessa forma, os principais representantes e seus mecanismos associados serão comentados a seguir, destacando seu potencial para o melhoramento, com ênfase para os três grupos indicados na Figura 4A.

Fatores de transcrição

A regulação da expressão gênica é um processo essencial, sendo relativamente conservado desde bactérias, até humanos e vegetais, passando por mecanismos complexos no decurso da evolução e atuando sobre a expressão gênica em nível de transcrição de RNA mensageiro a partir do DNA, a qual é regulada através de diversos processos, dentre os quais se destaca a regulação por fatores de transcrição (TF) (GROTEWOLD e AUER, 2010).

Praticamente todos os processos biológicos são diretamente regulados ou influenciados por TFs. Estes fatores regulam a expressão de genes codificantes de proteínas contendo um domínio de ligação ao DNA (DBD, *DNA Binding Domain*), que reconhece uma sequência de DNA específica. Tais proteínas são classificadas com base na estrutura de seus DBD, embora existam alguns TFs sem DBD, os quais interagem com uma proteína DBD para formar um complexo transcricional (GROTEWOLD e AUER, 2010; WANG et al., 2010). Um número significativo de TFs tem sido identificado em angiospermas, capazes de mediar o controle de expressão de dezenas ou centenas de genes-alvo em cascatas de transdução de sinais (WELLMER e RIECHMANN, 2005; VANDEPOELE et al., 2009). O genoma de Arabidopsis, por exemplo, apresenta pelo menos 1.554 TFS, correspondentes a cerca de 6% dos genes que a planta codifica – uma quantidade superior a, por exemplo, os 4,7% existentes na mosca de frutas *Drosophila melanogaster* (RIECHMANN et al., 2000; VANDEPOELE et

al., 2009) – indicando que a regulação da transcrição desempenha um papel mais importante nas plantas do que em animais (MITSUDA e OHME-TAKAGI, 2009).

No que tange à resposta à seca, alguns fatores merecem especial menção. DREB (*Dehydration Responsive Element Binding*) e ERF (*Ethylene-Responsive Factor*) compreendem duas importantes famílias que integram a superfamília AP2/EREBP (*Ethylene-Responsive-Element-Binding Protein*) de fatores de transcrição, a qual é exclusiva de plantas. Membros deste grupo têm sido reconhecidos por mais de uma década por seu papel na tolerância ao estresse hídrico através de vias metabólicas ABA-dependentes e independentes e de sua regulação estresse-responsiva que envolve mais de 100 genes-alvo. No entanto, estudos com plantas transgênicas indicam que sua expressão precisa de uma sintonia fina, uma vez que a superexpressão constitutiva da via DREB/CBF levou a graves defeitos de desenvolvimento dos transformantes, embora acompanhada por uma maior tolerância ao sal, à seca, e ao frio (KASUGA et al., 1999; SINGH, FOLEY e OÑATE-SÁNCHEZ, 2002; SHINOZAKI, YAMAGUCHI-SHINOZAKI e SEKI, 2003).

MYB compreende outra família de TFs que merece menção, estando presente em todos os eucariotos. Em animais e fungos observa-se um número restrito de genes que codificam para estes fatores, os quais apresentam domínios conservados com três repetições (R1, R2 e R3), enquanto em plantas há uma grande variabilidade de fatores, na maioria com duas repetições R2R3 imperfeitas. Por exemplo, em Arabidopsis foram reconhecidos 85 genes MYB da classe R2R3, enquanto 42 foram reconhecidos em cana-de-açúcar e 71 em eucalipto (ROMERO et al., 1998; SOARES-CAVALCANTI et al., 2009). Proteínas deste grupo são transcricionalmente induzidas em resposta a ABA, à salinidade, à seca, e ao frio, influenciando também a expansão celular e a deposição de cutícula, o que relaciona a resposta ao estresse abiótico também com modificações na estrutura da parede celular, entre outras (COMINELLI et al., 2008; LIPPOLD et al., 2009; GOLDACK, KING e YANG, 2011).

Quinases e fosfatases

Quinases e fosfatases estão entre as mais importantes enzimas envolvidas na reação de transferência de fosfato a partir de moléculas de alta energia do doador (por exemplo, ATP) para substratos específicos (proteínas e lipídios), controlando diferentes vias bioquímicas (MANNING et al., 2002) e compreendendo um dos principais mecanismos bioquímicos na sinalização celular. Estima-se que 30% das proteínas em um organismo vivo sejam frequentemente fosforiladas em resíduos de serina e treonina (COHEN, 2000). Por exemplo, observou-se que Arabidopsis codifica cerca de 1.000 genes pertencentes à superfamília das

quinases, bem como cerca de 300 genes que codificam fosfatases (THE ARABIDOPSIS GENOME INICIATIVE, 2000). A atividade das quinases é regulada por mecanismos tais como: (I) fosforilação (cis-fosforilação/autofosforilação), (II) por proteínas ativadoras ou inibidoras, e (III) por proteínas de ligação no caso moléculas pequenas. Membros da família quinase estão envolvidos nas vias de sinalização de muitas plantas na resposta a estresses abióticos e bióticos (CHEN, CHORY, FANKHAUSER, 2004). Por exemplo, em arroz, a expressão de muitos CDPKs (*calcium-dependent protein kinases*) é induzida por estímulos abióticos. Por exemplo, a expressão do gene *OsCPK7* em arroz transgênico leva a um aumento da sobrevida após estresse por frio, enquanto a expressão de *OsCPK13* confere maior tolerância à salinidade e à seca (SAIJO et al., 2000; ABBASI et al., 2004). Em feijão-caupi, a análise da modulação da expressão destas proteínas após estresses abiótico (salinidade) e biótico (virose) indicaram a indução de 100 diferentes candidatos a quinases, contra 69 reprimidos nas diferentes situações analisadas (KIDO et al., 2011).

Biossíntese de ABA

Em plantas, o hormônio ácido abscísico (ABA) é um produto da clivagem de carotenóides e desempenha um importante papel na resposta a estresses como seca e salinidade, atuando na percepção de sinal de desidratação e como mediador da cascata de sinais ativadora de outros genes responsivos a estes estresses, com ênfase para fatores de transcrição. Sob estresse hídrico, a planta induz acúmulo de ABA, presumivelmente a partir da clivagem do 9-cis-epoxycarotenoide (9-cis-violaxanthina e 9-cis-neoxanthina) para xanthoxina, pela enzima 9-cis-epoxycarotenoide dioxigenase. Considera-se que o ABA desencadeie vários mecanismos de defesa a estresses abióticos, bem como a alguns estresses bióticos (um tipo de "resposta cruzada" denominada "*crosstalk*" em inglês), considerando-se seu importante papel na ativação de fatores de transcrição, incluindo-se os fatores DREB já citados, bem como fatores bZip, MYB e MYC. Além disso, este hormônio tem sido associado à maturação e ao desenvolvimento de sementes e ao processo de fechamento dos estômatos pós-percepção de estresse hídrico (YAMAGUCHI-SHINOZAKI e SHINOZAKI, 2006). A mencionada resposta cruzada tem atraído a atenção de vários pesquisadores, uma vez que genes responsáveis por resistência a patógenos em plantas também são capazes de ativar vias associadas à tolerância a estresses abióticos, como tem sido observado em vários trabalhos recentes na área de transcriptômica (KIDO et al., 2011; SOARES-CAVALCANTI et al., 2012; WANDERLEY-NOGUEIRA et al., 2012a,b).

2.5.2 Proteínas funcionais e estruturais

Esta categoria envolve diferentes tipos de proteínas, indicadas na Figura 4B, ativadas após a percepção de sinais pelos fatores indicados na Figura 4A, sendo brevemente comentadas a seguir.

Proteínas protetoras de macromoléculas (LEA e chaperonas)

Chaperonas são proteínas capazes de dobrar ou modelar outras proteínas, auxiliando no dobramento de cadeias polipeptídicas nascentes, no redobramento de proteínas desnaturadas, além de prevenir a agregação de partes a proteínas com superfícies hidrofóbicas expostas. Chaperonas moleculares estão também relacionadas a mecanismos de resposta a estresses, sendo algumas identificadas como HSPs (*Heat-Shock Proteins*), dentre as quais se destacam as HSP70, as chaperoninas, as HSP90, as HSP100 e as sHSP (small HSP). As diferentes classes de chaperonas e de HSPs representam um papel na proteção de proteínas contra o estresse (WANG et al., 2004a).

Por sua vez, proteínas LEA (*Late Embryogenesis Abundant*) como o próprio nome sugere, são acumuladas em sementes durante a fase de maturação, momento em que uma tolerância à dessecação é demandada. Após sua descrição, estudos adicionais revelaram que as proteínas LEA também se acumulam em tecidos vegetativos durante períodos de seca, indicando um papel também na proteção contra a dessecação. São proteínas hidrofílicas, apresentando um alto conteúdo de glicina, sugerindo-se que tenham uma função na manutenção estrutural de outras proteínas, vesículas, endomembranas e no sequestro de íons, como o cálcio, na retenção de água, além de função como chaperonas moleculares (WANG et al., 2004a; PORCEL et al., 2005).

Canais de água e transportadores

Para sua sobrevivência, as plantas dependem da aquisição, transporte e transpiração de água. O mecanismo de passagem da água pelas membranas celulares tem sido foco de diversos estudos. Entre os transportadores de membrana, destacam-se os chamados canais de água ou aquaporinas, membros de uma família de pequenas proteínas transmembranas (24-30 kDa), que compreendem como subgrupos principais as proteínas de membrana plasmática (PIP), proteínas de membrana de tonoplasto (TIP), proteínas de membrana de nódulos (NIP) e pequenas proteínas básicas intrínsecas de membranas (SIP) (MAESHIMA e ISHIKAWA, 2008). Tais proteínas apresentam seis segmentos transmembrana e geralmente formam

tetrâmeros protéicos, onde cada monômero forma um poro simples seletivo para o transporte de água e de outros pequenos solutos, permitindo o controle diferencial da osmorregulação entre estágios de desenvolvimento, tecidos e órgãos (LUDEWIG e DYNOWSKI, 2009). Tais proteínas são importantes para a manutenção da osmolaridade em vegetais, com expressão regulada pela salinidade nos primeiros instantes após o estresse, indicando possibilidades promissoras para sua aplicação na biotecnologia vegetal.

Enzimas desintoxicantes e antioxidantes

O acúmulo de solutos compatíveis também pode proteger as plantas contra danos, através da eliminação de espécies reativas de oxigênio (ROS, *Reactive Oxigen Species*), permitindo a manutenção da estrutura e do funcionamento de proteínas. Na maioria dos organismos aeróbicos, há uma necessidade de efetivamente eliminar as ROS geradas como resultado de pressões ambientais. Dependendo de sua natureza, ROS podem ser altamente tóxicas e demandam rápida desintoxicação. A fim de controlar os níveis de ROS e proteger as células dos danos oxidativos, plantas desenvolveram um complexo sistema antioxidante, que inclui várias enzimas e metabólitos não enzimáticos (VRANOVÁ, INZÉ e VAN BREUSEGEM, 2002). Entre as enzimas envolvidas na proteção oxidativa destacam-se as glutationas peroxidases, as superóxido dismutases, as peroxidases de ascorbato e as glutationa redutases.

Por exemplo, plantas transgênicas de Arabidopsis expressando o dobro de uma superóxido dismutase (*Mn-SOD*) apresentaram notável crescimento, mesmo após tratamento com 150 mM de NaCl, enquanto as plantas não transformadas secaram gradualmente (WANG et al., 2004b).

Proteases

Proteases desempenham papéis multi-facetados, praticamente em todos os aspectos da fisiologia e desenvolvimento vegetal, incluindo crescimento, desenvolvimento, senescência e morte celular programada, bem como na acumulação e mobilização das proteínas de armazenamento (por exemplo, em sementes). Além disso, estão envolvidas em vias de sinalização e na resposta a estresses bióticos e abióticos (GRUDKOWSKA e ZAGDAŃSKA, 2004). Especialmente as proteinases de cisteínas são importantes, uma vez que a degradação de proteínas danificadas ou desnaturadas sob estresse está intimamente associada à síntese de novas proteínas, sendo os aminoácidos reutilizados para tal síntese ou para ajustar as células osmoticamente à limitação de água (VINCENT e BREVIN, 2000). Por exemplo, observou-se

que em resposta ao déficit hídrico, proteinases de cisteína foram induzidas em plantas aclimatadas e não aclimatadas de trigo (ZAGDAŃSKA e WISNIEWSKI, 1996).

Osmoprotetores

Osmoprotetores são pequenos solutos utilizados pelas células de diversos organismos e tecidos em situações de carência de água para manter o volume da célula (YANCEY, 2001), podendo desempenhar outros papéis associados à tolerância a estresses hídricos e osmóticos, atuando como proteínas estabilizantes, com ação antioxidante. Compreendem açúcares, principalmente frutose e sacarose, alcoóis de açúcar (como o mio-inositol), açúcares complexos (como trealose e frutanos) e metabólitos (como glycina-betaina, prolina e ectoína) (YANCEY, 2005). O acúmulo de solutos compatíveis em resposta ao estresse osmótico é um processo onipresente em organismos tão diversos como bactérias, plantas e animais (BOHNERT e JENSEN, 1996). Osmoprotetores se acumulam principalmente no citosol, nos cloroplastos, bem como em outros compartimentos citoplasmáticos (RONTEIN, BASSET e HANSON, 2002), protegendo as plantas de formas diferentes, incluindo: defesa por ajuste osmótico (ajudando as células a manter seu turgor e seu estado hidratado); estabilização de proteínas e enzimas; indução de proteínas de estresse e aceleração dos sistemas de limpeza de espécies reativas de oxigênio (BOHNERT e JENSEN 1996; ASHRAF e FOOLAD, 2007). Em plantas que naturalmente acumulam osmoprotetores, seus níveis são aumentados sob estresse, desempenhando importante papel no estabelecimento de mecanismos de tolerância (SHINOZAKI e YAMAGUCHI-SHINOZAKI, 2007).

Estudos analisando a diversidade e abundância destes compostos entre ESTs (*Expressed Sequence Tags*) de eucalipto (SANTOS et al., 2009) e cana-de-açúcar (SANTOS et al., 2011) avaliando as cinco categorias (prolinas, glycinas-betainas, mio-inositol, trehalose e cisteínas) revelaram, respectivamente, 56 e 51 membros com distribuição na maioria das bibliotecas genômicas, no caso da cana com ênfase em bibliotecas sob estresse (cultivo de calos e infectadas com *Herbaspirilum rubrisubalbicans*). Uma recente análise de expressão diferencial de osmoprotetores em cana-de-açúcar (SILVA et al., 2011) usando a técnica de SuperSAGE e sequenciamento de alta performance revelou vários candidatos induzidos em bibliotecas submetidas a estresse por seca, com diferenças significativas entre indivíduos tolerantes e sensíveis a este tipo de estresse.

3 Objetivos

3.1 Geral

- Analisar o perfil de transcrição de diferentes genótipos de cana-de-açúcar submetidos a déficit hídrico por 24 h, utilizando a técnica HT-SuperSAGE, visando à identificação de marcadores moleculares funcionais para aplicação no melhoramento genético da cultura.

3.2. Específicos

- Identificar, a partir de bibliotecas HT-SuperSAGE, *tags* diferencialmente transcritas, utilizando-se de métodos estatísticos ($p < 0,05$);
- Anotar *tags* HT-SuperSAGE, obtidas de diferentes bibliotecas, por meio de ferramentas de bioinformática, utilizando diversos bancos de dados públicos e/ou de acesso restrito;
- Identificar genes candidatos que possam estar envolvidos em vias metabólicas relacionadas ao déficit hídrico;
- Validar a expressão diferencial de genes candidatos via RT-qPCR;
- Identificar genes normalizadores apropriados para estudos de expressão gênica em cana-de-açúcar sob déficit hídrico.

4 Resultados

Artigo 1 - New Insights in the Sugarcane Transcriptome Responding to Drought Stress as Revealed by SuperSAGE

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In the scope of the present work four SuperSAGE libraries have been generated, using bulked root tissues from four drought-tolerant accessions as compared with four bulked sensitive genotypes, aiming to generate a panel of differentially expressed stress-responsive genes. Both groups were submitted to 24 hours of water deficit stress. The SuperSAGE libraries produced 8,787,315 tags (26 bp) that, after exclusion of singlets, allowed the identification of 205,975 unitags. Most relevant BlastN matches comprised 567,420 tags, regarding 75,404 unitags with 164,860 different ESTs. To optimize the annotation efficiency, the Gene Ontology (GO) categorization was carried out for 186,191 ESTs (BlastN against Uniprot-SwissProt), permitting the categorization of 118,208 ESTs (63.5%). In an attempt to elect a group of the best tags to be validated by RTqPCR, the GO categorization of the tag-related ESTs allowed the *in silico* identification of 213 upregulated unitags responding basically to abiotic stresses, from which 145 presented no hits after BlastN analysis, probably concerning new genes still uncovered in previous studies. The present report analyzes the sugarcane transcriptome under drought stress, using a combination of high-throughput transcriptome profiling by SuperSAGE with the Solexa sequencing technology, allowing the identification of potential target genes during the stress response.

Introduction

Sugarcane (*Saccharum* spp.) is an outstanding crop throughout the tropical regions of the world [1]. It represents an important food and bioenergy source, being

cultivated in many tropical and subtropical countries [2], and covering more than 23 million hectares worldwide, with a production of 1.6 billion metric tons of crushable stems [3]. This crop is responsible for almost two-thirds of the global sugar production [1]. Brazil, the world's largest sugarcane producer, processed and generated in 2008 about 31 million tons of sugar [4]. In contrast to most plants, sugarcane stores sucrose – rather than polymeric compounds such as starch, proteins or lipids – as the primary carbon and energy reserve [1]. Hence, sugarcane byproducts have received greater attention, due to their multiple uses, with the ethanol generation being highlighted, as an important renewable biofuel source [5]. Moreover, the bagasse of sugarcane has been largely used for energy cogeneration at distilleries, production of animal feed and also for paper production [6]. Nevertheless, similarly to other meaningful agronomical crops, sugarcane cultivation faces considerable losses due to inappropriate or unfavorable edaphoclimatic conditions.

Abiotic stresses are among the main causes of major crops worldwide productivity losses [7], causing negative impacts on crop adaptation and productivity. In this scenario, drought figures as the most significant stress and is considered an extremely important factor when it comes of losses in the productivity of sugarcane [8]. Several plant biotechnology programs have been initiated aiming to increase drought stress tolerance in crop plants using genetic engineering and traditional breeding [9]. Although breeding activities have provided significant progress for the understanding of the physiological and molecular responses of plants to water deficit, there is still a large gap between yields in optimal and stress conditions [10]. For this purpose, case sensitive methods are demanded, not only to discover new genes associated to those stress conditions, but also to effectively detect differentially expressed genes on a drought tolerant variety. The identification and expression profile of such responsive genes may be helpful to unravel the basic mechanism of stress tolerance [11]. In this sense, previous works uncovered genes associated to important roles in stress perception, signal transduction and transcriptional regulatory networks in cellular responses, useful for the improvement of stress tolerance in plants by gene transfer [12, 13].

Molecular approaches concerning drought and salinity performance in sugarcane were carried out using techniques based on molecular hybridization such as *Suppression Subtractive Hybridization* (SSH) [11] and micro/macro-arrays [14]. In general, the main limitations of these methods are their low sensibility and specificity [15]. Among the methodologies for transcriptomic analysis, the SuperSAGE [16] approach represents one of the most recent and informative methods [17], especially with its association to the high-performance sequencing platforms [pyrosequencer (454 Roche®), Solexa (Illumina®) and SOLiD (Applied Biosystems®)]. SuperSAGE regards an evolution of the traditional *Serial Analysis of Gene Expression* [18] generating longer (26 bp) tags and thus allowing a most reliable annotation analysis. Since, it is an open architecture method (i.e., allowing the discovery of new genes), it presents the potential to provide a global and quantitative gene expression analysis, based on the study of the entire transcriptome produced in a given time and tissue, under a given stimulus. Additionally, SuperSAGE permits a simultaneous analysis of two interacting eukaryotic organisms, full-length cDNAs amplification using tags as primers, potential use of tags via RNA interference (RNAi) in gene function studies, identification of antisense and rare transcripts, and identification of transcripts with alternative splicing [19]. Besides, this method has been recently associated to the next generation sequencing technologies, allowing a less expensive and faster covering of the analyzed transcriptomes, permitting a deep insight of the modulated responses under different physiological conditions. The association of SuperSAGE with the rapid advances in high throughput sequencing opened the possibility of performing genome-wide transcriptome studies in non model organisms. Additionally, this technique has been successfully applied in plant species such as rice [16], banana [20], chickpea [21, 22] chili pepper [23], tobacco [24], and tropical crops (cowpea, soybean, sugarcane; [25]). In the present work we profit from the high resolution power of SuperSAGE coupled to the Illumina® sequencing to characterize the transcriptome of drought-stressed sugarcane roots after 24 hours of submission to this stress, aiming to elect a best group of tags to be validated by RTqPCR.

Methodology

Identification of drought-tolerant and sensitive sugarcane accession. For the selection of the drought-tolerant and sensitive accessions used in the present evaluation, a previous assay was carried out in order to identify contrasting genotypes for these features. For this purpose 20 commercial sugarcane varieties (CTC 1 to 15, SP83-2847, SP83-5073, CT94-3116, SP90-1638 and SP90-3414) from CTC (Sugarcane Technology Center, Piracicaba, Brazil) were evaluated. Among these, the four above mentioned varieties were used as a standard for the interpretation of results, including two varieties (SP83 and SP83-2847-5073) identified as drought-tolerant and other two (SP90 and SP90-1638-3414) indicated as drought-sensitive based on field empirical observations performed by specialized technicians during several years in sugarcane commercial fields.

For this assay, mini-cuttings from the 20 varieties above were planted in 50 L pots containing inert substrate (Plantmax®) in order to allow the slow increase of water deficit by removing irrigation. Tests were performed with six months old plants under greenhouse conditions and the treatments included plant permanently irrigated (without stress), suppression of irrigation for three days (72 hours stress), suppression of irrigation for 10 days (240 hours stress), and suppression of irrigation for 20 days (480 hours stress). Physiological measurements applied in all treatments included chlorophyll content using a SPAD-507.B Chlorophyll Meter; analysis of chlorophyll fluorescence ratio between variable and maximum chlorophyll-*a* (Fv/Fm); estimation of chlorophyll content with a fluorometer, and determining the relative water content. For the parameters of chlorophyll-*a* fluorescence and chlorophyll content, three measurements were taken from three plants from each treatment. Data analysis was performed by comparing the percentage change considering the parameters mentioned above. After this assay, four drought-tolerant and four sensitive accessions could be selected according to the parameters used, revealing a gradient of water stress tolerance among the varieties analyzed. Considering the classification of the standard varieties identified previously as drought-tolerant (SP83-2847 and SP83-5073) and drought-sensitive (SP90-1638 and

SP90-3414) and also considering the measurements taken after stress under glasshouse conditions (results from Chabregas et al. *in preparation*; not presented in this work) four varieties were considered as drought-tolerant (CTC15, CTC6, SP83-2847 and SP83-5073) and another four as drought-sensitive (CTC9, CTC13, SP90-3414 and SP90-1638).

Drought stress application and the SuperSAGE libraries. Plants of each selected accession were grown under glasshouse conditions in 40 L pots, in randomized experimental design (comprising six repetitions) under daily irrigation until the age of three months. After that, part of the material was submitted to drought by interruption of irrigation during 24 hours. Roots of both, stressed and non stressed plants were collected and frozen in liquid N_2, being maintained in a deep freezer until total RNA extraction using Trizol® (Invitrogen). The extracted samples were quantified by spectrophotometry, digested with DNAse and purified with aid of the RNeasy Mini kit® (Qiagen). The samples were quantified again by spectrophotometry, allowing the composition of the bulks using equimolar amounts of poli-A^+ messenger RNA, for all treatments. Four libraries have been generated: **TD** (bulk of four tolerant accessions under stress); **TC** (bulk of four tolerant genotypes without stress, as tolerant negative control); **SD** (bulk of four sensitive materials after stress) and **SC** (bulk of non-stressed sensitive accessions, as sensitive negative control). The procedures for SuperSAGE library generation followed Matsumura *et al.* [26], including the attachment of library specific adaptors carried out by GenXPro GmbH (Frankfurt am Main, Germany) allowing the identification of library specific reads after SOLEXA sequencing.

Statistical analysis and tag-gene annotation. The 26-bp tags were extracted from each library. Singlets (reads appearing only once) were excluded from the present evaluation. Statistical tests were applied to the remaining tags (Audic Test, Claverie; $p \leq 0.05$) with aid of the DiscoverySpace 4.1 software [27] regarding the four contrasting treatments [**T**: (TD *vs* TC); **S**: (SD *vs* SC); **D**: (TD *vs* SD); **C**: (TC *vs* SC)]. The tests allowed the identification of the total number of expressed unitags (or tag

species) for each situation and contrast, as well as the differentially expressed tags, including up- (UR) and downregulated (DR) tags. The tag-gene annotation was performed by independent evaluations via BlastN [28] against different EST databases: NCBI: [*i*] dbEST including only *Saccharum* ESTs; [*ii*] Gene Index (including *Arabidopsis thaliana*, AtGI 15.0 and Poaceae species: *S. officinarum*, SOGI 3.0; *Sorghum bicolor*, SBGI 9.0; *Zea mays*, ZMGI 19.0; *Panicum virgatum*, PAVIGI 1.0; *Oryza sativa*, OsGI 18.0; *Triticum aestivum*, TAGI 12.0; *Hordeum vulgare*, HVGI 11.0; *Festuca arendinaceae*, FAGI 3.0; *Secare cereale*, RYEGI 4.0), and [*iii*] KEGG (including *A. thaliana* and Fabaceae ESTs)]. Valid BlastN alignments were considered when the following parameters were observed: score from 42 to 52; integrity of the CATG sequence at the 5' end; plus/plus alignments. Inferences about the modulation of a specific tag (Fold Change; FC) were carried out considering the ratio of the observed frequencies of a given library in relation to the other.

Gene ontology of SuperSAGE hits. Matching ESTs to the analyzed tags were categorized via GO using the software Blast2GO [29] after BlastX alignment against the Uniprot-SwissProt protein database (e-value $\leq e^{-10}$). ESTs related to the GO subcategories concerning abiotic stress response (to water deprivation, GO:0009414; to heat/cold, GO:0009408/GO:0009409; to osmotic stress, GO:0006970, to oxidative stress, GO:0006979, to abscisic acid stimulus, GO:0009737; to jasmonic acid stimulus, GO:0009753) were identified, as well as UR tags related to these classes. Sets of UR tags considering the different contrasting situations (T, S, D and C) were annotated, generating Venn diagrams, aiming the visualization of specific or shared tags considering the different treatments.

Results and Discussion

Qualitative and quantitative analysis of the SuperSAGE libraries. The four SuperSAGE libraries produced 8,787,315 tags, from which 1,862,064 (21.2%) regarded singlets (tags sequenced only once), and were excluded from this evaluation. The most representative libraries considering the number of tags were TC (drought-tolerant

control; 2,516,454 tags) and SD (drought-sensitive under stress; 2,133,587 tags), while the less representative were TD (drought-tolerant under stress; 750,226 tags) and SC (drought-sensitive control; 762,492 tags). The coverage of the transcriptome by the tags was estimated considering the total number of tags per genotype (3,266,680 for the tolerant bulk and 2,896,079 for the sensitive bulk) in relation to the number of expected transcripts per cell. The total number of average-sized transcripts is estimated to range from 100,000 [30] to 500,000 [31] per cell in higher plants. Considering the high value (500,000), the coverage provided by the tags in relation to the sugarcane transcriptome was 6.5 times higher for the tolerant bulk and 5.8 for the sensitive bulk, i.e., the number of expected single copy transcripts per cell should be represented by their tags in the absolute frequencies of around six in each library. Taking the less represented libraries (TD and SC) in account, the coverage of the transcriptome regarded 1.5 times higher for both, tolerant and sensitive bulks. Considering this value, we established the $n < 2$ frequency as cut-off threshold, allowing the exclusion of singlet tags. Coverage of this magnitude allowed a comprehensive evaluation of a given transcriptome, also including rare transcripts expressed during the response to the evaluated stress.

Taking all valid tags ($n \geq 2$) into account, a total of 205,975 unitags remained for evaluation. In a recent approach, Yamaguchi *et al*. [32] observed similar amounts (≈ 190,000 unitags) in the roots of *Solanum torvum* under heavy metal stress ($CdCl_2$ 0.1 μM). The high number of unitags, here observed, shows the diversity of transcripts (and expressed genes), possibly also reflecting the allopolyploid nature of sugarcane, since tags diverging in a single nucleotide were considered to be distinct unitags. It has been speculated that, in some cases, unitags could be the result of artifacts generated by the amplification process during library construction [33] or incomplete digestion of the synthesized cDNA by the *Nla*III enzyme [34], and also by PCR amplifications associated to innate features of the sequencing technology [32]. In order to minimize error sources, some precautions were taken during library development in this study, including double digestion of the total RNA extracted with DNAse, double digestion with the *Nla*III enzyme, and exclusion of singlet tags. An additional way to minimize potential errors would be the exclusion of unitags

related to other similar sister-tags, grouping them to other most frequent, so called mother-tags. On the other hand, this procedure would eliminate transcripts bearing important SNPs (Single Nucleotide Polymorphisms). Still, another possibility would be to establish a minimum frequency (n) of a given tag to be considered valid. In the present work only canonical tags were accepted, with complete adapter sequences (removed by *in silico* procedures) bearing the full CATG restriction site and with n > 2. A more stringent value (n > 10) was adopted by Yamaguchi *et al*. [32], to reduce the number of unitags per library (from 300,000 to 450,000) for each 33 thousand tags, in an attempt to reach the number of expected genes for model species as rice (32,000 genes) and *A. thaliana* (26,000 genes). However, this procedure impairs the identification of rare and alternative transcripts that possibly play important roles in the cell metabolism.

Statistical analysis considering $p \leq 0.05$ (Audic–Claverie test) among libraries permitted the identification of differentially expressed tags including up (UR) or downregulated (DR) tags for the four contrasting situations (**T**; **S**; **D**; **C**), as shown in Table 1.

TABLE 1: Total number of differentially expressed (DE; $p \leq 0.05$) up or downregulated tags observed in different contrasting SuperSAGE root libraries from sugarcane under drought stress (24 hours without irrigation) as compared with negative control (irrigated materials).

Contrasting	Upregulated	Downregulated	DE	Total
T (TD x TC)	12,179	12,482	24,661	152,049
S (SD x SC)	12,085	16,339	28,424	141,946
D (TD x SD)	15,591	12,269	27,860	148,657
C (TC x SC)	12,961	16,342	29,303	148,631

TD: bulk of tolerant genotypes under stress; TC: bulk of tolerant genotypes without stress (control); SD: bulk of sensitive genotypes under stress; SC: bulk of sensitive genotypes without stress (control).

Primary annotation of SuperSAGE tags. Relevant BlastN alignments comprised 567,420 tags (75,404 unitags with 164,860 different ESTs). Details about the results obtained after alignment to different databases are not itemized here, since this is not the aim of the present evaluation. Despite of that, the choice of the databases and the adopted criteria allowed: a) the identification of ESTs related to most tags, preferentially

concerning sequences from sugarcane or taxonomic related species sequences; b) annotation of a considerable number of tags considering a minimum alignment of 21 bp (similar to a LongSAGE tag); c) identification of tags with perfect alignments (100% identity) or with a maximum of a single mismatch among tag and EST, important for future development of primers; d) avoidance of plus/minus alignments, minimizing false NATs (natural antisense transcripts).

The strategy of considering the alignments without the election of a best hit allowed the maximization of annotation chances, since no alignment was disregarded in the acceptable score range. Thus, alignments with annotated ESTs could be more informative than similar alignments with a slightly superior score in relation to non-annotated ESTs. Moreover, tags aligned with distinct ESTs could be analyzed, minimizing the chance of a wrong choice that could compromise the validation of the expression results, especially considering that they are used as targets for RTqPCR primer design. In this context, seeking the maximization of the annotation procedures, the use of the Gene Index database for tag identity annotation was carried out trying to circumvent at least two limitations, when compared with the partial dbEST bank additionally used: (a) no need of clusterization concerning ESTs deposited at dbEST, since the Gene Index project provides tentative clusters (TC); (b) best functional annotation, with the Uniref100 (Uniprot) bank as reference. Thus, in view of the posterior need of primer design for RTqPCR and data validation of SuperSAGE tags, alignments with tolerance of a maximum of a single mismatch (TSM) tag-hit represented up to one third (186,191 or 32.8%) of the data, indicating high identity among 26 bp tags and similar ESTs, since a minimum of 21 bp alignment (size of a LongSAGE tag) was considered relevant. Almost all valid alignments (471,672 or 83.12%) regarded *Saccharum* spp. (partial dbEST) and *S. officinarum* (Gene Index SOGI), as expected. TSM alignments restricted to these databases comprised 163,742 tags. Considering TSM alignments with sequences of the SOGI only, from 26,884 ESTs, 73.0% presented informative gene descriptions and/or their functions, allowing the identification of molecular targets and gene-feature association. Despite of the higher number of TSM matches concerning alignments with dbEST sequences (136,858); the EST annotation was not informative

for most contemplated ESTs (97.0%). To overcome this deficiency, the Gene Ontology categorization proved to bring a valuable contribution.

Functional categorization of SuperSAGE tags. BlastX evaluations (e-value ≤ e^{-10}) of the 186,191 ESTs (diverse databases and TSM alignments) against the peptide Uniprot-SwissProt bank allowed the characterization of 118,208 ESTs (63.5%) that presented at least one GO reference. From this categorization the Biological Process (BP) subcategories in response to abiotic stress were considered more informative to evaluate the sugarcane response to drought conditions. The first interesting indicators were UR tags associated to EST in the BP subcategories responding to: water deprivation (GO:0009414), heat (GO:0009408), cold (GO:0009409), osmotic stress (GO:0006970), oxidative stress (GO:0006979), abscisic acid stimulus (GO:0009737) and jasmonic acid stimulus (GO:0009753). By the analysis of the UR tags observed in the above mentioned subcategories (Table 2) it was possible to generate the Venn diagrams presented in Figure 1, where Figure 1 (a) represents the UR tags evaluated in the contrasting situations **T** (TD *vs* TC; 20 tags) and **D** (TD *vs* SD; 25 tags), both important for future gene validation. The first case (**T**) related to tags from the tolerant bulk induced after water deficit when compared with the bulk control; the second group refers to induced tags from both bulks submitted to drought stress (tolerant *vs* sensitive), with higher expression (UR) in the tolerant bulk. The first group exhibited 17 non annotated tags and only three identified genes (encoding 18S ribosomal RNA, membrane integral protein and viviparous-14). The second group included 17 tags without annotation and other eight bearing descriptions (18S ribosomal RNA gene (two tags); ABA responsive element binding factor 2; Auxin-induced protein; DRF-like transcription factor DRFL2a; ERF/AP2 domain containing transcription factor; GST; RAPB protein), discussed latter in this manuscript. Additionally, 11 tags are worth mentioning, since they were UR in both tolerant (**T**) and sensitive (**S**) comparisons after stress (Figure 1(a)), when compared to the expression of the respective controls. Despite of being not genotype-dependent, these tags may influence positively in the plant adaptation process under drought stress. Such results and other for similar subcategories are presented in

Table 2. This table comprises the total number of UR tags induced in the tolerant bulk under stress, highlighting the exclusive (**T** comparison) or differentially expressed tags in comparison to the sensitive bulk (**D** comparison), bringing interesting candidates for validation via RTqPCR. Since the same tags may be involved in different stresses, the identified tags (exclusive in T and common in the comparisons **T** e **D**; Table 2) may not be exclusive of a given condition or response. Thus, the total number of UR tags (alone or in combination) in response to water deprivation (W), heat/cold (H), osmotic stress (Os) and oxidative stress (Ox) are presented in Figure 2(a). Likewise, the number of tags induced in response to osmotic and oxidative stress is presented in Figure 2(b), while the tags responsive to hormonal stimuli (abscisic and jasmonic acids) is showed in the Figure 2(c), and a Venn diagram showing all the categories is presented in the Figure 2(d).

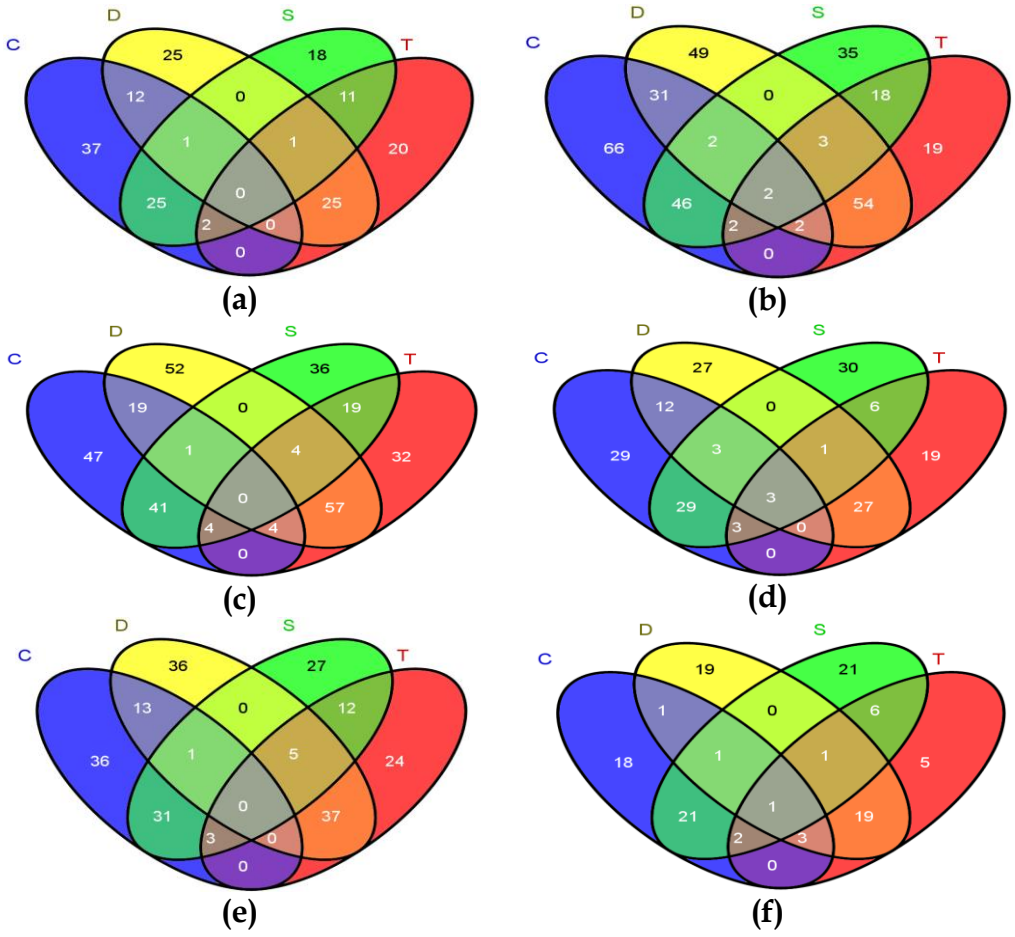

FIGURE 1: Venn diagrams with numbers of differentially upregulated (UR) tags from sugarcane roots ($p \leq 0.05$) under drought stress, considering different comparisons between

SuperSAGE libraries [contrasts: T (TD *vs* TC); S (SD *vs* SC); D (TD *vs* SD); C (TC *vs* SC)]. UR tags associated with gene ontology (GO) response to: a) water deprivation; b) heat/cold; c) osmotic stress; d) oxidative stress; e) abscisic acid stimulus; f) jasmonic acid stimulus. Libraries: TD (drought tolerant bulk under stress); TC (tolerant bulk control); SD (drought sensitive bulk under stress); SC (sensitive bulk control).

TABLE 2: Total number of sugarcane upregulated (UR) root tags observed on contrasting SuperSAGE libraries when associated with ESTs classified by Gene Ontology (GO) in the subcategories related to abiotic stress response.

Response against	**GO categories**	**Exclusive UR tags**	**Common UR tags after comparison**	
		T	**T&D**	**T&S**
Water deprivation	0009414	20	25	11
Heat and cold	0009408; 0009409	19	54	18
Osmotic stress	0006970	32	57	19
Oxidative stress	0006979	19	27	6
Abscisic acid stimulus	0009737	24	37	12
Jasmonic acid stimulus	0009753	5	19	6

EST: expressed sequence tag; contrast of libraries [**T** (TD *vs* TC); **D** (TD *vs* SD); **S** (SD *vs* SC)]; Libraries [TD: drought-tolerant bulk under stress; TC: tolerant bulk control; SD: drought-sensitive bulk under stress; SC: sensitive bulk control].

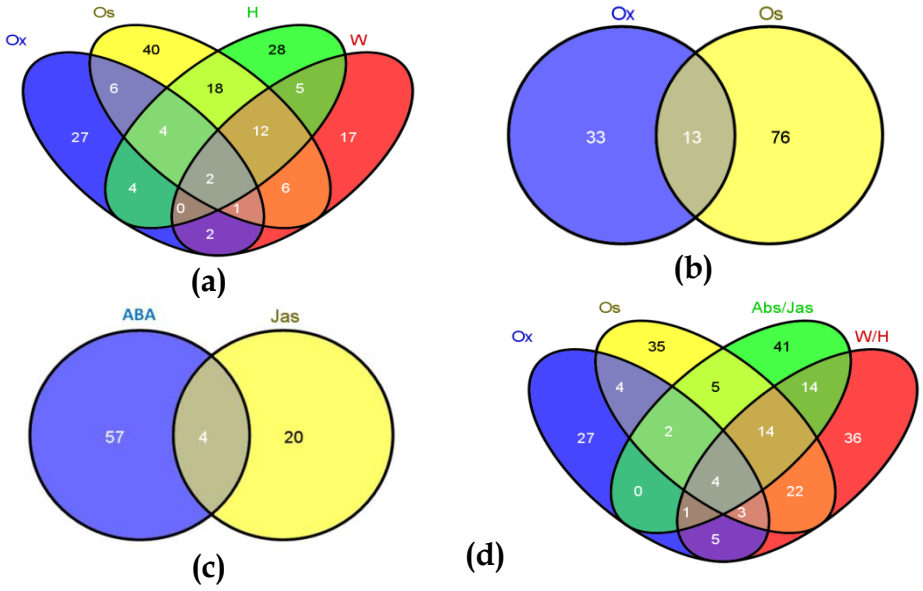

FIGURE 2: Venn diagrams with numbers of SuperSAGE tags overexpressed ($p \leq 0.05$) in sugarcane roots under drought stress, considering different tag sets related to gene ontology (GO) subcategories associated in response to: W (water deprivation), H (heat/cold), Os (osmotic stress), Ox (oxidative stress), ABA (abscisic acid stimulus), Jas (jasmonic acid stimulus).

In relation to the 213 UR tags, including the exclusive ones from the **T** contrast and those presented in both **T&D** contrasts (Table 2), the gene-function annotation together with the GO descriptions were available for 68 of them, while 145 tags remained unknown candidates. The annotation of these 68 UR tags and respective GO subcategories, as well as the fold change (FC) for both most relevant contrasts (**T** and **D**), are listed in Table 3. Some of them will be further addressed.

TABLE 3: Upregulated SuperSAGE tags associated via Gene Ontology (GO) to abiotic stress, with fold change for **T** (FC_T = TD/TC) and **D** (FC_D = TD/SD) comparisons for tag frequencies in the sugarcane roots libraries, as well as the annotation of the best aligned EST.

Tag	GO	FC_T	FC_D	Annotation
SD159390	AJ	5.6	ns	50S ribosomal prot. L5, chloroplast
SD191288	AJ	2.9	2.3	AP2 domain transcription factor EREBP
SD122727	AJ	2.2	2.7	Bet v I allergen-like
SD75453	AJ	2.8	2.8	Chromatin-remodeling factor CHD3
SD123546	AJ	1.6	ns	Chromatin-remodeling factor CHD3
SD9608	AJ	2.4	3.3	Initiator-binding prot.; ibp
SD15756	AJ	1.9	1.6	OSK1; SNF1-related prot. Kinase
SD108270	AJ	6.4	2.6	ZIM motif family prot.
SD258836	AJ	3.1	ns	ZIM motif family prot.
SD108269	AJ	2.9	2.1	ZIM motif family prot.
SD133809	AJ	2.8	2.6	ZIM motif family prot.
SD196399	AJ/Os	3.2	3.2	P18; Nucleoside diphosphate kinase I; NDK1
SD169158	AJ/Os/Ox	3.2	ns	Peptidyl-prolyl cis-trans isomerase
SD252082	AJ/WH	3.6	3.6	Auxin-induced prot.
SD237930	AJ/WH/Os	2.4	ns	18S ribosomal RNA gene
SD282917	AJ/WH/Os	1.5	1.6	ABA responsive element binding factor 2
SD237939	AJ/WH/Os	6.8	6.8	Branched-chain-amino-acid aminotransf.
SD238059	AJ/WH/Os	2.4	2.4	Branched-chain-amino-acid aminotransf.
SD140270	AJ/WH/Os	3.2	ns	Viviparous-14
SD237936	AJ/WH/Os/Ox	2.8	2.8	Ribosomal prot. L28e domain cont. prot.
SD178862	AJ/WH/Os/Ox	2.8	2.8	18S ribosomal RNA gene
SD203616	WH	1.3	ns	RAP2-like prot.
SD246714	WH	2.8	2.8	CoA-thioester hydrolase CHY1
SD286424	WH	1.4	2.6	ERF/AP2 domain cont. transcription factor
SD279457	WH	2.8	ns	Mitochondrial uncoupling prot. 2
SD107875	WH	3.7	5.2	Nucleic acid binding
SD191687	WH	2.1	6.9	RAPB prot.
SD147607	WH	5.6	ns	Salt tolerance prot.
SD109060	WH	1.4	1.3	Transposable element Mu1 sequence
SD199146	Os	1.1	1.1	Alpha tubulin-4[a]
SD54073	Os	2.3	6.8	Calreticulin-like prot.
SD102228	Os	6.8	6.8	Endo-1,4-beta-glucanase Cel1
SD80163	Os	4.7	ns	Endo-1,4-beta-glucanase Cel1
SD13344	Os	1.9	ns	Eukaryotic translation if 2 alpha sub family

Table 3: Continued

Tag	GO	FC_T	FC_D	Annotation
SD182876	Os	4.4	ns	Phosphopantetheine adenylyl transf. dephospho CoA kinase
SD129463	Os	3.0	ns	Serine/threonine-prot. kinase SAPK1
SD87319	Os	2.4	1.6	Serine/threonine-prot. kinase SAPK6
SD270381	Ox	6.5	4.5	Allene oxide synthase
SD272257	Ox	2.4	ns	Allene oxide synthase
SD63148	Ox	2.0	2.5	Allene oxide synthase
SD113907	Ox	2.4	2.4	Brassinosteroid biosynthesis-like prot.
SD219102	Ox	3.2	ns	Glutathione peroxidase
SD213044	Ox	2.2	2.4	Na+/H+ antiporter
SD54454	Ox	2.0	3.1	Nicotianamine aminotransferase A
SD125582	Ox	3.8	1.9	Nicotinate phosphoribosyltransferase-like
SD122742	Ox	3.2	ns	Nucleotide repair prot.
SD102844	Ox	1.6	ns	Peroxidase precursor
SD17103	Ox	6.4	ns	Tyrosine/nicotianamine aminotransf. family
SD17107	Ox	2.5	ns	Tyrosine/nicotianamine aminotransf. family
SD17108	Ox	1.8	ns	Tyrosine/nicotianamine aminotransf. family
SD151691	WH/Os	1.8	1.6	DRF-like transcription factor DRFL2a
SD9805	WH/Os	25.0	25.0	Glycine-rich RNA binding prot.
SD9802	WH/Os	14.7	14.7	Glycine-rich RNA binding prot.
SD9806	WH/Os	13.1	13.1	Glycine-rich RNA binding prot.
SD9767	WH/Os	2.8	2.8	Glycine-rich RNA binding prot.
SD9803	WH/Os	2.4	2.4	Glycine-rich RNA binding prot.
SD9800	WH/Os	2.4	2.4	Glycine-rich RNA binding prot.
SD9801	WH/Os	1.1	ns	Glycine-rich RNA binding prot.
SD108120	WH/Os	6.0	6.0	Glycine-rich RNA-binding prot.
SD108115	WH/Os	1.3	1.3	Glycine-rich RNA-binding prot. 2; GRP2
SD264077	WH/Os	3.6	ns	Membrane integral prot.
SD92627	WH/Ox	5.7	2.4	Glutathione transferase III
SD243418	WH/Ox	4.0	ns	Serine hydroxymethyltransferase
SD179937	WH/Ox	3.8	2.5	Serine hydroxymethyltransferase
SD21923	WH/Ox	1.3	1.7	Whitefly-induced gp91-phox
SD184083	Os/Ox	3.2	3.2	Delta-1-pyrroline-5-carboxylate dehydrog.
SD8088	Os/Ox	3.2	ns	MutT domain prot.-like
SD251703	Os/Ox	7.1	ns	P5cs; delta 1-pyrroline-5-carboxylate synth.

Libraries [TD: drought-tolerant bulk under stress; TC: tolerant bulk control; SD: drought-sensitive bulk under stress]; ns: fold change of tag not significant ($p \leq 0.05$). WH: response to water deprivation and to heat/cold; Os: response to osmotic stress, Ox: response to oxidative stress; AJ: response to abscisic acid stimulus and to jasmonic acid stimulus; ns: not significant at $P< 0.05$.

Response to hormone stimulus. Response to hormonal stimulus, such as jasmonic (JA) and abscisic acid (ABA), together with other plant hormones, as salicylic acid (SA) and ethylene (ET), form a complex network that plays major roles in disease resistance and response to abiotic stresses, including drought [35, 36]. In our study,

21 potential hormone-responsive tags were identified (Table 3) and some of them are thereafter discussed.

a) ZIM motif family protein. Accordingly to the DATF (Database of Arabidopsis Transcription Factors; http://datf.cbi.pku.edu.cn/index.php), this short motif is associated to a panel of plant transcription factors and JA signaling, that is among the most important defense-related signals in plants, acting under environmental stresses, such as UV radiation, osmotic shock, heat and drought [37]. Examining a jasmonate-insensitive 3 (jai3-1) mutant gene, Chini *et al*. [38] identified a novel family of jasmonate-regulated nuclear targets of SCFCOI1, named JAZ (jasmonate ZIM-domain) proteins repressing JA signaling and targeted by the E3-ubiquitin ligase SCFCOI1 for proteasome degradation. The overexpression of this hormone activated a damping mechanism concerning the JA signaling cascades after stress initiation [39]. In our evaluation, five UR tags were hormone-related, with one candidate (SD108270) presenting expressive fold change in both contrasts (FC_T = 6.4 and FC_D = 2.6; Table 3).

b) Chromatin-remodeling factor. CHD3 has been implicated in the repression of transcription [40]. Association of these proteins to drought-responsive genes was related during Arabidopsis seed germination process by regulating the ABA dependent and GA (gibberellic acid) dependent responses, modulating the plant reaction to mild osmotic stresses and limiting the expression levels of transcription factors, preventing a maladapted growth arrest. In other words, it refines the pace of seed germination in response to ABA and maintains embryonic characters silent in response to GA [41]. Our results indicate a differential expression of CHD3 also in roots of adult sugarcane plants undergoing water deficit, with two UR tags (SD75453 and SD123546) with FC values of 1.6 (SD123546; FC_T) and 2.8 (SD75453; FC_T = FC_D; Table 3).

c) AP2/EREBP. It is a large family of plant transcriptional regulators that plays key roles in the development and environmental stress response pathways. Transcription

factors encoded by AP2/EREBP genes contain the highly conserved AP2/ERF DNA binding domain [42] constituting a plant supergene family [43] subdivided into five subfamilies according to the number of AP2/ERF motifs [44]. The AP2/EREBP subgroup induced by biotic and abiotic stresses was identified by Sharoni *et al.* [45]. Among the upregulated genes, 52 were induced in response to diverse abiotic stress, such as cold, drought and salt. Lin *et al*. [46] working with a full-length cDNA *OsEBP2* (ethylene-responsive-element binding protein2) in japonica rice leaves infected by blast fungus *Magnaporthe grisea*, observed that *OsEBP2* responded transiently to the treatments with methyl jasmonate (MeJA), ABA and ethophen (ethylene generator). In our analysis, an UR tag was annotated as *APETALA 2/ethylene response element binding protein* (AP2/EREBP) showing expressive modulation (FC_T = 2.9 and FC_D = 2.3; Table 3). Additionally, one UR tag (SD286424; FC_T = 1.4 and FC_D = 2.6; Table 3) annotated as *AP2 /ERF domain containing transcription factor* was associated to our WH group (response to water deprivation + response to heat/cold; Table 3), indicating an important candidate for validation, since the overexpression of an ERF transcription factor GmERF3 from soybean in tobacco plants raised the tolerance to salinity (up to 400 mM, NaCl) and drought [47] in transgenic plants.

Response to water deprivation, oxidative and osmotic stress. In our analysis, 47 potential stress-responsive UR tags with acceptable annotation were identified (Table 3) and some of them deserve special mentioning when considering their GO categorization and the fold change data.

a) Glycine-rich RNA binding protein (GRP) superfamily. This superfamily, characterized by the presence of a glycine-rich domain arranged in (Gly)n-X repeats, was recently reviewed by Mangeon *et al.* [48] that highlighted the diversity in structure, expression pattern and sub-cellular localization, suggesting that these proteins perform different functions in plants, such as processing, transport, localization, stability and translation of mRNA molecules. This supposition is consistent with literature data regarding GRPs and biotic and abiotic stresses [49, 50]. Wang *et al.* [50]

analyzing the transcriptome of *Malus prunifolia* (an apple relative with strong drought tolerance) identified a GRP (*MpGR-RBP1*) expressed in roots and leaves, that plays a role in the response to plant dehydration. Among the most representative tags found to be water-deprivation responsive in our analysis, nine tags with FC ranging near 1.1 up to 25.0 (both FC_T and FC_D; Table 3) in roots, showed to be upregulated in the drought-tolerant bulk under stress when compared to non-stressed control (TD *vs* TC) or in relation to the drought-sensitive bulk also under stress (TD *vs* SD).

b) CoA-thioester hydrolase (CHY1; synonym: β-hydroxyisobutyryl-CoA hydrolase). In our analysis, one UR tag of this class showing FC of 2.8 (FC_T = FC_D; Table 3) was identified. This peroxissomal metabolic enzyme is needed for valine catabolism and fatty acid b-oxidation. Analyzing freezing sensitive Arabidopsis mutants (*chy1-10*) after cold acclimation, Dong *et al*. [51] observed that the disruption of CHY1 function leads to an excess of methylacrylyl-CoA, causing accumulation of *Reactive Oxygen Species* (ROS), electrolyte leakage, impairing cold-induced gene expression. Additionally, methylacrylyl–CoA may be sequestered in the peroxisome leading to localized changes in this sub cellular region and influencing peroxisome-derived signals after cold-induction. Potential alterations in auxin response or homeostasis in the *chy1* mutant may contribute to the impaired cold stress tolerance of the mutant, since peroxisome-defective mutants showed resistance to the inhibitory effects of exogenous IBA, analogous to the IAA molecule (a hormone that inhibits the root elongation and promotes lateral root formation).

c) Glutathione transferase (GST; EC 2.5.1.18). GSTs encode an ancient, heterogeneous and widely distributed protein group in living organisms catalyzing a variety of reactions [52], including hormonal metabolism, vacuolar sequestration of anthocyanin, tyrosine metabolism, hydroxyperoxide detoxification and regulation of apoptosis [52, 53]. In our study, one UR tag (SD92627) associated to GST showed a significant expression modulation (FC_T = 5.7 and FC_D = 2.4; Table 3). GST expression is induced by a wide variety of stresses, as oxidative stress [54], xenobiotic-type of

stresses [55], and dehydration [56]. Expression of *TaGSTU1B (Triticum aestivum)* was induced by drought stress in four genotypes investigated, but high transcript amounts were detected only in drought-tolerant genotypes [57]. George *et al.* [58] reported the subcellular localization and the ability of GST from *Prosopis juliflora* (*PjGSTU1*), a drought-tolerant woody Fabaceae species, to confer drought tolerance in transgenic tobacco. Ji *et al*. [59] working with tobacco plants over-expressing a GST gene from *Glycine soja* showed six fold higher GST activity enhanced dehydration tolerance than wild-type plants.

d) Serine hydroxymethyltransferase (SHMT; EC 2.1.2.1). The SHMT genic family comprises five genes in *A. thaliana* [60] bearing both cytosolic and mitochondrial isoforms in eukaryotes [61] with activity associated to the Serine and Glycine metabolism (EMBL, 2010). In our evaluation two SHMT candidates [SD243418 (FC_T = 4.0; FC_D = ns); SD179937 (FC_T = 3.8; FC_D = 2.5); Table 3] were identified. According to Moreno *et al*. [62], Arabidopsis SHMT1 functions in the photorespiratory pathway and influences resistance to biotic and abiotic stresses. The Arabidopsis SHMT1 mutant (*shmt1-1*) showed enhanced susceptibility to pathogens, as well as to abiotic stresses (50 mM NaCl and high light intensity). The reduced activity in *shmt1-1* mutant appears to hinder the ability of the plant to cope with any kind of additional stress, compromising the cellular mechanisms during oxidative stress. In proteome analysis [63], ten out of twelve drought responsive proteins identified from rice leaf sheaths, were upregulated including a SHMT. The authors suggested that SHMT was induced for protection from oxidative degradation under drought stress.

e) Peptidyl-prolyl cis-trans isomerase (PPIase). Also known as rotamases or immunophilins (cyclophilins included), is an enzyme superfamily with catalytic function, facilitating metabolism regulation through a chaperone or a cis-trans isomerization of proline residues during protein folding [64, 65]. An UR tag (SD169158) showing a FC_T = 3.2 (Table 3) concerns a potential PPIase. In plants PPIases have been associated with the response to adverse environmental conditions. Using contrasting genotypes of *Sorghum bicolor* under water deficit, Sharma and Sing

[64] observed a significant increase in leaf- and root-PPIase activity in the drought-tolerant cultivar. Similarly, various rice PPIases were differentially expressed under water deficit and salinity (200 mM NaCl) stresses [65]. Also a correlation with plant hormones was pointed out by Godoy *et al*. [66] working with cyclophilins (CyPs) of *Solanum tuberosum*. CyPs are ubiquitous proteins with an intrinsic enzymatic activity of PPIase that catalyzes the rotation of X-Pro peptide bonds. *StCyP* mRNA accumulation was stimulated by the application of abscisic acid (ABA) and methyl jasmonate (MeJA) in potato tubers. The accumulation of *StCyP* transcripts was also detected when the potato tubers were exposed to heat-shock treatment.

f) Viviparous14:. It is a key enzyme involved in the biosynthesis of the phytohormone abscisic acid [67], represented in our analysis by the SD140270 tag with FC_T of 3.2 (Table 3). Viviparous genes are encoded in the process of plant vivipary, also reported as early germination. Of the 15 genes described so far for maize, 12 control specific steps in ABA biosynthesis [68, 69], with *vip14* (viviparous-14) associated to the control of final steps of ABA synthesis, encoding a 9-cis-epoxycarotenoid dioxygenase 1 (NCED1) enzyme that catalyzes the cleavage of the C40 neoxanthin chain into the C15 ABA skeleton xanthoxin [70]. Maize mutants for the *nced1* gene have strongly reduced kernel ABA content [71] while in Arabidopsis, NCED1 over expression conferred a significant increase in ABA accumulation in the plant and also in drought tolerance [72]. Wang *et al*. [73] demonstrated that the expression of *AhNCED1* gene in peanut plants was significantly upregulated by dehydration and high salinity (250 $mmol.L^{-1}$ NaCl).

g) Branched-chain amino acid transaminase. BCATs are enzymes that play a crucial role in the metabolic pathway of BCAAs (branched-chain amino acids that include leucine, isoleucine and valine) by catalyzing the last step of synthesis and the initial step of degradation of these amino acids [74]. Plants contain a small family of *bcat* genes, which have been characterized in *Solanum tuberosum* (potato), *Hordeum vulgaris* and *A. thaliana* [75, 76]. Malatrasi *et al*. [77] evaluated the role of these enzymes in the drought tolerance process. In this study the transcriptional levels of

Hvbcat-1, in *H. vulgaris*, increased seven fold (results obtained by double-checking with RTqPCR) after progressive drought stress (up to 14 days of water deprivation). Physiologically the authors associated the overregulation to the activation of the BCAAs catabolism, since this is the first enzyme in the branched-chain amino acid (BCAA) catabolic pathway. In high concentrations these amino acids are toxic to the cells; therefore activation of their catabolism may play an important role as detoxification mechanism. In our analysis, two UR tags annotated as BACTs were identified exhibiting an expressive modulation of the FC, mainly for the SD237939 tag (FC of 6.8 for both FC_T and FC_D; Table 3), while the other tag (SD238059) showed a FC of 2.4 ($FC_T = FC_D$).

h) Allene oxide synthase. AOS is the first enzyme in the pathway leading to the biosynthesis of JA (Jasmonic acid), catalyzing the production of unstable allene epoxides that cyclize to form cyclopentenone acids, the precursors for JA [78]. Three tags of this category were identified (SD270381, SD272257, SD63148) being upregulated in most comparisons (Table 3). For example the SD270381 tag presented high FC values in both T and D comparisons. The overexpression of AOSs has been observed also in other drought assays, as reported by Ozturk *et al*. [79] and Talamè *et al*. [80] with barley (*H. vulgare*) and peanut (*Arachis hypogaea*) [81].

i) Na+/H+ antiporter. Membrane proteins involved in the Na+ and H+ transport of both eukaryotes and prokaryotes act in the homeostasis maintenance of such ions [82]. In our analysis the SD213044 tag, annotated as potential Na+/H+ antiporter was overexpressed in both analyzed contrasting situations (Table 3). Assays evaluating those proteins under salinity stress showed that these salt-responsive genes may be able to activate the expression of drought-related genes in the tolerance acquisition [83]. Thus, ions are stored in vacuoles, acting as osmolytes, decreasing the hydric potential of the cell. Evaluations with transgenic plants overexpressing those genes, including *Petunia hybrida* [83], *A. thaliana* [84] and *A. hypogaea* [85], conferred higher tolerance to dehydration under drought and salinity.

j) Glutathione peroxidase (EC 1.11.1.9). In the present approach an UR GPX candidate (SD219102) was overexpressed FC_T de 3.2 (Table 3). These enzymes are known as cell protectors against oxidative damage generated by reactive oxygen species [86]. They present a very broad distribution in the cell, occurring in several subcellular compartments [87]. Miao *et al.* [88] suggested that ATGPX3 might play dual and distinctive roles in H_2O_2 homeostasis, acting as a general scavenger and relaying the H_2O_2 signal, and also as an oxidative ABA signal transducer during drought stress signaling. Their differential regulation during biotic and abiotic stresses was reported by Navrot *et al*. [87], indicating their importance for plant breeding.

l) Serine-threonine kinase SAPK1 (also known as JNK). Belongs to the MAPK family [89], including important proteins active in the osmosensory signal transduction pathways in cells exposed to osmotic stress [90]. A wheat candidate (*W55a*) with about 90% homology to rice SAPK1 was evaluated by Xu *et al*. [91]. Transgenic Arabidopsis plants overexpressing W55a exhibited higher tolerance to drought, being also upregulated by salt, exogenous abscisic acid, salicylic acid, ethylene and methyl jasmonate. In addition, W55a transcripts were abundant in leaves, but not in roots or stems, under environmental stresses. Expression of *SAPK* members analyzed by RNA gel blot hybridization with samples of leaves (blades and sheath), roots and treatments with ABA (50 µM), NaCl (150 mM), or mannitol (600 mM) showed that *SAPK1* was upregulated by all three treatments in both roots and leaves, although the effect of ABA was weaker than those of the other two treatments. *SAPK6* was weakly upregulated by all treatments in the blades and the sheaths, and weakly by ABA or NaCl but strongly by mannitol treatment in the roots [92]. Overexpressed candidates analyzed here (SD129463; FC_T = 3.0; Table 3) included a SAPK1 as well as a second tag matching SAPK6 (SD87319; FC_T = 2.4 and FC_D = 1.6; Table 3), both in roots, indicating their activation also in this tissue.

m) Delta-1-pyrroline-5-carboxylate synthetase. P5CS is an enzyme that catalyzes the initiation of the proline biosynthesis in plants [93]. The excessive production of this amino acid would increase the osmotolerance in plants [94]. Rice plants transformed

with the P5CS gene underwent 10 days of irrigation withdrawal with higher growth rates, when compared to the control group [94]. Effects of salt in transgenic tobacco transformed with *P5CS* gene revealed the overexpression of P5CS after 24 - 48 h exposure to NaCl (300 mM), when compared with non-transgenic plants under the same stress [95]. Transgenic lines of petunia [96] and tobacco [97] with enhanced accumulation of proline showed also high drought tolerance. Transcripts involved in amino acid metabolism, such as *P5CS*, *OAT* and *AS*, were also induced more than 10-fold during the identification of drought-responsive genes during sucrose accumulation and water deficit in sugarcane [98]. In our study, an UR tag (SD251703) showing an expressive induction (FC_T 7.1) was annotated as a potential *P5CS* candidate (Table 3).

Concluding Remarks

The present report is the first to analyze contrasting sugarcane accessions under drought stress with a combination of the high-throughput transcriptome profiling SuperSAGE technology coupled with a next-generation sequencing platform. This approach allowed the identification of many potential target candidates in the drought stress response. The adopted methodology of annotation and GO categorization revealed the success of the work in accessing genes from very different pathways, ranging from those controlling the perception and first reaction against the stress (as transcription factors) to those known as classic genes of the osmotic stress (as *P5CS*). The number of induced tags (213) with GO categorization and high modulation is surprising, especially considering the short time (24 h) after drought stress application. Besides, a high number of important gene candidates with no hits (145) – probably completely new to the research community – will demand additional efforts for the recognition of their function. Validation procedures as well as transient expression assays are planned for future works, aiming to collaborate with breeding and biotechnological approaches for the benefit of the sugarcane culture, especially facing the scenario of future climate changes.

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Artigo 2 - Expression Analysis of Sugarcane Aquaporin Genes under Water Deficit

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The present work is a pioneer study specifically addressing the aquaporin transcripts in sugarcane transcriptomes. Representatives of the four aquaporin subfamilies (PIP, TIP, SIP, and NIP), already described for higher plants, were identified. Forty-two distinct aquaporin isoforms were expressed in four HT-SuperSAGE libraries from sugarcane roots of drought-tolerant and -sensitive genotypes, respectively. At least 10 different potential aquaporin isoform targets and their respective unitags were considered to be promising for future studies and especially for the development of molecular markers for plant breeding. From those 10 isoforms, four (*So*PIP2-4, *So*PIP2-6, *Os*PIP2-4, and *S*sPIP1-1) showed distinct responses towards drought, with divergent expressions between the bulks from tolerant and sensitive genotypes, when they were compared under normal and stress conditions. Two targets (*S*sPIP1-1 and *So*PIP1-3/PIP1-4) were selected for validation via RT-qPCR and their expression patterns as detected by HT-SuperSAGE were confirmed. The employed validation strategy revealed that different genotypes share the same tolerant or sensitive phenotype, respectively, but may use different routes for stress acclimation, indicating the aquaporin transcription in sugarcane to be potentially genotype-specific.

Introduction

Sugarcane (*Saccharum* spp.) is a valuable crop once it accumulates high levels of sucrose in the stems [1, 2]. In 2011, the twenty largest sugarcane producers generated about 1.7 billion tons of sucrose worldwide, valued about 52.5 billion dollars [3]. However, abiotic stresses can reduce the potential yield of these cultivated plants by 70%, with drought being the most dangerous one [4]. Water deficit, and its influence onto a variable number of morphological and functional characters in

plants, eventually becomes one of the main obstacles to sustainable agricultural production worldwide [5].

The reduction of the water content in a plant cell provokes a complex network of molecular responses, involving stress perception, signal transmission in a transduction cascade and physiological, cellular, and morphological changes [6], including stomatal closure, suppression of cell growth and photosynthesis, and activation of cellular respiration. Plants under drought still respond to it and adapt by accumulating specific osmolytes and proteins for stress tolerance [7].

Genes expressed during drought can be classified into two functional groups. The first group encodes proteins that increase plant tolerance to stress, such as water channels proteins (aquaporins), proteases, and detoxification enzymes, all having a protective function. To this group belong enzymes catalyzing the biosynthesis of osmolytes, like derivatives of amino acids, sugars and various LEA (Late-Embryogenesis-Abundant) proteins. The second group of genes encodes various proteins, such as transcription factors, kinases, phosphatases, and enzymes involved in regulatory pathways, as phospholipid metabolism and ABA biosynthesis [7]. The aquaporins or MIPs (Major Intrinsic Proteins) are proteins assembling into water channels of cell membrane and facilitate osmosis for rapid bidirectional transport of water [8]. Besides, these proteins are also involved in many plant metabolic processes, including acquisition of nutrients, cell growth, carbon fixation, cell signaling, and various stress responses [9, 10]. The aquaporins also allow permeation of small molecules such as glycerol [11], urea [12] CO2 [13], ammonia [14], boric acid [15], H2O2 [16], and even arsenic [17]. According to the phylogenetic analysis of Johanson and Gustavsson [18], plant aquaporins are classified into four main subfamilies, widely distributed among higher plants: PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), SIPs (small basic intrinsic proteins), and NIPs (26 kDa intrinsic proteins). The aquaporins are presently and extensively studied, since their importance spans from animal [19] and human physiology [20] to osmoadaptation of microorganisms [21] and vegetables [22, 23]. The transcripts encoding sugarcane aquaporins have only marginally been described, despite their significant physiological influence and participation in several

processes during plant growth and acclimation against biotic and abiotic stresses [24, 25].

The present study is a first attempt to derive expression markers (functional molecular markers) from HT-SuperSAGE transcriptional profiles in contrasting sugarcane genotypes, in particular addressing specific sugarcane aqua- porins, with the aim of better understanding the molecular processes occurring during drought response of the plant. HT-SuperSAGE, among all the genome-wide transcriptome profiling techniques was chosen for its efficiency to generate highly reliable transcription profiles. The increase in the size of the tag to 26 bp, the characteristic of SuperSAGE, drastically improves the annotation of the tag to the corresponding gene [28], allowing to establish genome-wide gene expression profiles of two or more species in one sample (e.g., host-parasite interactions [29, 30]). Besides, SuperSAGE combined high-throughput next-generation sequencing [31, 32], designated DeepSuperSAGE or HT-SuperSAGE, provides even more informations (three to four orders of magnitude) at relatively low cost compared to traditional Sanger sequencing.

Methodology

Unitags Annotation, GO Categorization of ESTs, and Aquaporin Isoforms Identification. Bioinformatics analyses covered the 8,787,315 tags (26 bp) described by Kido et al. [33] from four root HT-SuperSAGE libraries [SD24T (the bulk of the tolerant genotypes CTC6, CTC15, SP83-2847, SP83-5073, under stress (24 h of continuous dehydration), totalizing 2,542,552 tags); SDTC (the tolerant bulk with daily irrigation, comprising 1,909,543 tags); SD24S (the sensitive bulk of stressed genotypes CTC9, CTC13, SP90-3414 and SP90- 1638, with 2,170,998 tags) and SDSC (the bulk of sensitive genotypes without stress, covering 2,164,222 tags)]. After singlet exclusion (tags sequenced only once per library), the unique tags (unitags) were classified as up- (UR) or down-regulated (DR), based on the Audic and Claverie test ($p< 0.05$; [34]), using the DiscoverySpace 4.0 software [35]. The unitag frequencies normalized to a million per library allowed the evaluation of the unitag expression modulation by fold change values (FC) comparing two frequencies. The unitags were aligned by BLASTn with

expressed sequence tags (ESTs) from nine public databases, comprising sugarcane ESTs from NCBI (http://www.ncbi.nlm.nih.gov/nucest), and grass ESTs (Poaceae family) from Gene Index (http:z/compbio.dfci.harvard.edu/tgi/plant.html), including *Saccharum officinarum* (SoGI 3.0), *Sorghum bicolor* (SbGI 9.0), *Zea mays* (ZmGI 19.0), *Oryza sativa* (OsGI 18.0), *Panicum virgatum* (PaviGI 1.0), *Triticum aestivum* (TaGI 12.0), *Hordeum vulgare* (HvGI 11.0), and *Festuca arundinacea* (FaGI 3.0). Only BLASTn alignments (*e value* < 0.0001) with scores 42 to 52 (100% identity), plus/plus orientation and a preserved 5CATG were accepted, and the best tag-hit was selected prioritizing sugarcane sequences or sequences from closely related species with adequate annotation. ESTs anchoring unitags were then categorized via Gene Ontology (GO; http://www.geneontology.org/GO.doc.shtml), using the Blast2GO tool [36].

Potential ESTs from the MIP gene superfamily were identified using the keywords "aquaporin," "major intrinsic protein," "PIP," "TIP," "NIP," "SIP," "plasma membrane intrinsic protein" "tonoplast intrinsic protein," "nodulin-26- like intrinsic protein" and "small basic intrinsic protein" in the EST annotations, or "water transporter" in the GO terms. These ESTs were classified into the plant aquaporin subfamilies (TIP, NIP, SIP, PIP) and analyzed with the NCBI Conserved Domain Search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) in an effort to confirm their conserved domains. Also ESTs were BLASTx aligned with proteins sequences from the UniProtKB/Swiss-Prot database (http://www.uniprot.org/help/uniprotkb), trying to confirm the isoform identity by using curated sequences (*e*-value cutoff e^{-10}).

Comparative and Phylogenetic Analysis of the Putative Aquaporin Isoforms Based on Unitag Expressions. The predicted peptides from ESTs related to the tags after translation with the ORF finder tool (http://www.ncbi.nlm.nih.gov/ projects/gorf/) and BLASTp analysis were subsequently aligned via Clustal W [37]. A dendrogram of the aligned sequences was generated using the MEGA v.5.2.1 software [27], according to the following parameters: Neighbor Joining tree method, pairwise deletion option, and 1000 bootstrap replicates. For a better assignment of

the isoforms into the aquaporin subfamilies, 15 *A. thaliana* aquaporin protein sequences were included in addition to the predicted proteins. Also, two sequences served as outgroups, one from humans and one from *Yersinia pseudotuberculosis*. In addition to the phylogenic tree, a heat map was established based on fold changes of the unitags responding to the applied stress.

cDNA Synthesis, Primer Design, and RT-qPCR Analysis. The RNA of each genotype sample constituting the bulks (tolerant stressed, tolerant control, sensitive stressed, and sensitive control) was isolated from sugarcane roots using the RNAeasy Plus Micro Kit (Qiagen) and DNase treatment. The cDNA synthesis reaction was performed with the SuperScriptTM First Strand kit Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol, using 1 μg of RNA quantified by the Qubit fluorometer (Invitrogen). Primer pairs were designed from ESTs anchoring unitags, tolerating a maximum of one mismatch, and using the default parameters of the *Primer3* software [38] with some minor modifications [amplicon size: 70 to 200 bp; primer Tm: 40 to 60°C; GC content: 45 to 55%]. These ESTs anchored unitags differentially regulated in the tolerant and sensitive genotype bulks.

Prior to the validation of unitags by RT-qPCR, standard curves using a dilution series of the cDNA pool were made to calculate the gene-specific PCR efficiency and regression coefficient (R^2) for each gene (Table 6). The RT-qPCR amplifications were performed on the LineGene 9660 model (Bioer), using SYBR Green detection. Each reaction mixture comprised 1 μL of template cDNA (diluted 5-fold), 5 μL of *HotStart-IT SYBR Green qPCR Master Mix 2x* (USB), 0.05 μL of ROX, 1.95 μL of water, and 1μL primer (500 nM each) to a final volume of 10μL. The reactions were denatured at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 15 s in 96-well reaction plates, with the detection of the fluorescence signal at the end of each extension step. The dissociation curves were analyzed at 65–95°C for 20 min after 40 cycles. Three biological replicates and three technical replicates were used for RT-qPCR analysis. The relative quantification data were analyzed with the REST© v.2.0.13 software [26].

Results and Discussion

Aquaporin data-mining and categorization of EST anchoring unitags. The universe of 8,787,315 tags (26 bp) generated from the four HT-SuperSAGE libraries presented 205,975 unique tags (unitags) [33], from which 289 anchored in 484 putative aquaporin ESTs, distributed in nine different databases (details in Table 1), totaling 1,579 BLASTn results with scores of 42 to 52 (100% identity). This set of 484 ESTs anchoring unitags (Table 1) represented the basis for the analysis of transcript profiles based on the respective unitags.

The keyword ("aquaporin," "major intrinsic protein," "tonoplast intrinsic protein," "plasma membrane intrinsic protein," "small basic intrinsic protein," "nodulin-26-like intrinsic protein," "PIP," "TIP," "NIP," "SIP") searches in the EST annotation identified 1,347 ESTs, while the "water transport" GO expression identified 342 ESTs (Figure 1). The searches in the GO terms increased the aquaporin identifications by almost 15%, representing 230 alignments of the total (1,579; Figure 1).

The unitag annotation efficiency relied on the used EST database. As mentioned by Kido et al. [39], Gene Index is a good source for unitag annotation, as it displays adequate gene or protein function descriptions. In the present case, the SoGI (*Saccharum officinarum* L.) dataset representing 282,683 ESTs that resulted in 121,342 unique sequences [42,377 TC (Tentative Consensus clusters) plus 78,965 singletons] after clustering. This species took part in the sugarcane breeding programs [40, 41] performed around the world.

TABLE 1: EST databases used for unitag annotations from sugarcane HT-SuperSAGE libraries (tolerant/sensitive genotypes after 24 h of continuous dehydration or regular daily irrigation) and BLASTn nonredundant results.

Species	**Database**	**Annot.* / ESTs**	**PIP**	**TIP**	**NIP**	**SIP**	**Total BlastN Align.**	**Score 52**	**Align. (up to one mismatch)**	**Unitags**	**Unique ESTs+**	**Unitags With GO[1]**
Saccharum spp.	dbEST	24/277,266	0	0	0	0	154	41	105	43	34	23
Saccharum officinarum	SoGI	265/121,370	157	85	21	11	683	158	425	260	127	50
Sorghum bicolor	SbGI	68/46,043	34	23	5	6	74	11	63	19	7	74
Zea mays	ZmGI	347/315,134	142	154	35	19	49	7	29	13	15	20
Oryza sativa	OsGI	283/201,220	121	95	33	7	119	26	72	20	86	3
Panicum virgatum	PaviGI	147/85,244	58	45	28	6	174	31	116	22	57	0
Triticum aestivum	TaGI	542/222,152	138	128	32	14	253	20	197	23	123	5
Hordeum vulgare	HvGI	110/83,101	32	33	12	9	34	2	30	16	21	7
Festuca arundinacea	FaGI	27/30,244	5	1	1	1	39	10	27	17	14	5
Total		1,913/1,361,144	687	564	167	73	1,579	306	1,064	289[#]	484	45[#]

Aquaporin, tonoplast intrinsic protein and major intrinsic protein, membrane integral protein (PIP, TIP, NIP e SIP); [+]number of nonredundant ESTs (putative aquaporins) anchoring unitags, [1]"water transporter"; [#]number without redundancy among the nine databanks.

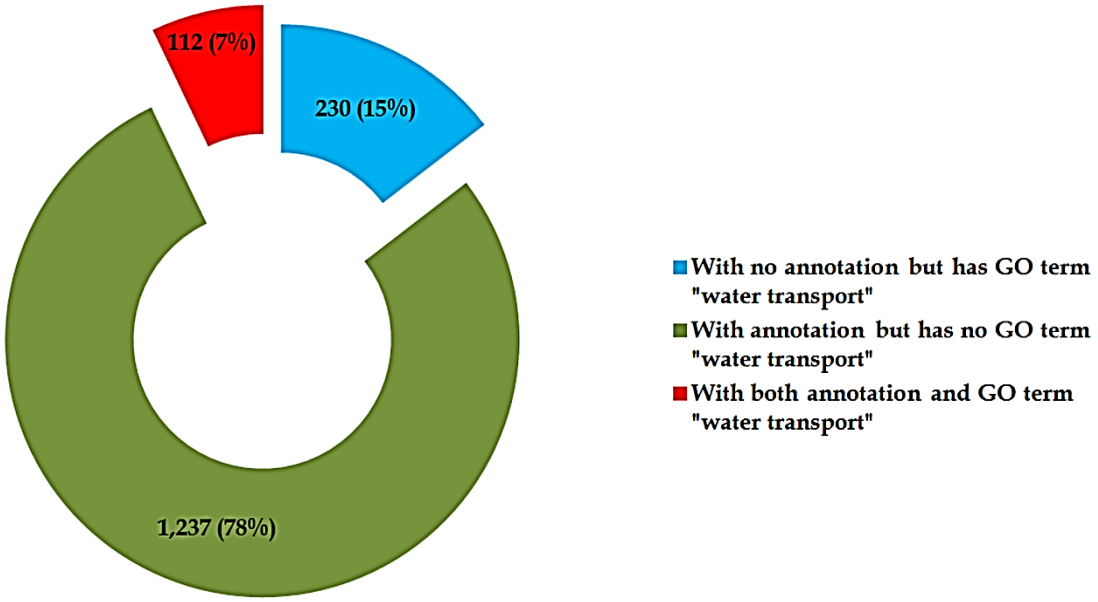

FIGURE 1: Percentage of sugarcane SuperSAGE unitags from plants (after 24 h of watering suppression or regular daily irrigation) identified as probable aquaporins after keyword searches in the ESTs ("aquaporin", "tonoplast intrinsic protein", "PIP", "TIP", "NIP", "SIP") or in the GO terms ("water transport"). Total of unitags: 1,579.

Almost all unitags related to the aquaporin annotations (260 of 289; Table 1) anchored in SoGI sequences, which exceeded those obtained with the partial dbEST dataset (http://www.ncbi.nlm.nih.gov/dbEST/dbEST summary. html) by almost six times. This dbEST is composed of 277,266 ESTs, mostly from Brazilian sugarcane hybrids (SUCEST- FAPESP project) [42] (Table 1). This may mainly be due to the sizes of the SoGI sequences, as most alignments occurred in the CTs. Nevertheless, the partial dbEST dataset was the second best source for mapping unitags, but its real annotation power was affected by the nonadequate descriptions of the cDNAs (many "unknown" hits). As Sorghum bicolor is the most closely related diploid of *S. officinarum* [2], this species could contribute to the identification of aquaporin isoforms. However, after redundancy exclusion, only 19 unitags anchored in seven unique ESTs based on the best hits (Table 1). This poor performance may be explained by the low number of ESTs available in the SbGI dataset (46,043), being the second smallest databank used (Table 1), in contrast with the high number of available sugarcane ESTs, reinforced by the high homology between sorghum and sugarcane.

The unitags proved to be highly specific for aquaporins. A total of 263 unitags (91% of 289) were associated with aquaporin isoforms (189 unitags anchored

in just a single EST from a unique database); 19 unitags (7%) were not isoform-specific but comprised the same subclass (PIP1 or PIP2) and only seven (2%) were not specific to any subclass.

Comparative Analysis of the Putative Aquaporin Isoforms Identified by the Unitags. Regarding the total of 484 annotated aquaporin ESTs anchoring unitags (Table 1), 470 of them (97.10%) aligned with aquaporin-proteins isoforms with a *e*value $< 10^{-20}$ (BLASTx), from the UniProtKB/Swiss-Prot database, a high-quality annotated databank. This fact confirmed the isoforms identities (data not shown). All of them represented 42 distinct isoforms belonging to the four subfamilies (PIP, TIP, SIP, and NIP) based on the ESTs annotations. The 42 isoforms and their respective number of unitags [considered UR, DR or n.s. (P<0.05)] in the two main HT-SuperSAGE libraries contrasts are shown in Table 2. According to this table, one unitag or more could be associated with a specific isoform. In some cases, two or more ESTs from one database present the same isoform annotation. The Gene Index databases used throughout this work minimized this situation due to the assembled TC (Tentative Consensus) clusters. Besides, unitags aligning more than one locus in the same EST could be resulted by partial *Nla*III digestions. In an attempt to avoid this situation, it was performed double digestions. Additionally, this event could be resulted by sister-tags anchoring one specific EST and isoform. In this case, tags showing a single base substitution (sister-tags) were considered as two different unitags. On the other hand, alternative transcripts could anchor varied unitags. Also, specific isoforms could be mapped in several loci (in the same or in different chromosomes). In addition, *Saccharum* hybrids show complex genomes, as a result of polyploidy and aneuploidy events [40, 41]. Therefore, this diversity of unitags (UR and DR) associated to aquaporin isoforms could allow identify biotechnologically interesting candidates.

TABLE 2: Putative sugarcane aquaporin isoforms (42) based on unitags of root dehydration (24 h) observed in the two main contrasts of HT-SuperSAGE libraries.

Anotação	nº of unitags	SD24T *vs*SDTC			SD24S *vs*SDSC		
		UR	DR	ns	UR	DR	ns
*Ss*PIP1-1	3	1	-	2	-	1	1
*So*PIP1-2	6	1	2	3	2	1	-
*Pv*PIP1-2	1	-	-	1	-	-	1
*So*PIP1-3/PIP1-4	64	3	24	26	7	7	25
*Os*PIP1-3/PIP1-4	3	1	-	2	-	1	-
*So*PIP1-5	9	1	2	2	3	-	2
*Pv*PIP1-5	2	-	-	2	-	-	1
*So*PIP2-1	62	2	14	23	4	24	19
*Zm*PIP2-1	7	-	2	4	1	-	4
*Os*PIP2-1	3	1	-	2	-	1	2
*Pv*PIP2-1	10	-	1	9	1	-	6
*Ta*PIP2-1	7	-	2	4	1	-	2
*Os*PIP2-2	3	-	-	2	-	-	3
*Pv*PIP2-2	9	-	2	6	1	-	4
*Os*PIP2-3	1	-	-	1	-	-	1
*Zm*PIP2-3	1	-	-	1	-	-	1
*So*PIP2-4	16	3	4	6	2	3	6
*Os*PIP2-4	3	-	2	-	1	2	-
*Pv*PIP2-4	8	-	4	2	1	-	5
*Fa*PIP2-4	10	-	3	7	3	-	3
*So*PIP2-5	3	1	1	-	-	-	2
*Ta*PIP2-5	5	-	1	4	1	-	2
*So*PIP2-6	5	2	-	2	2	-	2
*Ta*PIP2-6	4	-	1	3	1	-	2
*Ss*TIP1-1	40	4	9	14	6	5	18
*So*TIP1-1	28	3	7	10	6	7	11
*Sb*TIP1-1	2	1	-	1	-	1	1
*Zm*TIP1-1	1	-	-	1	-	-	-
*Hv*TIP1-1	1	1	-	-	-	1	-
*Ta*TIP1-1	5	-	1	4	-	1	4
*Ta*TIP1-2	1	-	1	-	-	-	1
*So*TIP2-2	28	2	9	9	1	8	11
*Hv*TIP2-2	2	1	-	1	-	-	1
*Pv*TIP2-2	2	-	-	2	-	-	2
*So*TIP2-3	21	5	5	10	3	3	5
*Pv*TIP2-3	3	-	-	3	-	-	3
*So*TIP4-2	1	-	-	1	-	-	1
*Pv*NIP1-1	2	-	1	-	-	1	1
*So*NIP1-2	1	-	-	1	-	-	1
*So*NIP3-1	17	1	2	12	-	1	10
*So*SIP1-1	3	1	-	2	1	-	1
*So*SIP1-2	15	3	5	2	2	1	10

Isoforms are preceded by the abbreviated species name (*Fa*: *Festuca arundinacea*; *Hv*: *Hordeum vulgare*; *Os*: *Oryza sativa*; *Pv*: *Panicum virgatum*; *Sb*: *Sorghum bicolor*; *So*: *Saccharum officinarum*; *Ss*: *Saccharum* spp.; *Ta*: *Triticum aestivum*; *Zm*: *Zea mays*). HT-SuperSAGE libraries: SD24T (bulk of tolerant genotypes under stress); SD24S (bulk of sensitive genotypes under stress); SDTC (control bulk of tolerant genotypes); SDSC (control bulk of sensitive genotypes). DR: down-regulated; UR: up-regulated. n.s.: not significant at $P< 0.05$.

From the 71 unique ESTs involved in perfect unitag-EST BlastN alignments (score 52; Table 1), 24 putative aquaporins showed ORFs with over 180 amino acids in size, and these sequences, together with MIP protein sequences from *Arabidopsis thaliana*, *Homo sapiens*, and *Yersinia pseudotuberculosis* were compared in a phenetic analysis. The resulting tree confirmed that putative aquaporins clearly divide into four major clusters, representing the PIP, TIP, SIP, and NIP subfamilies (Figure 2). This tree was consistent with a previous analysis of aquaporin phylogeny in higher plants [10, 18, 43, 44]. As expected, the human HsAPQ1 isoform grouped with the PIP subfamily, since the human APQ1 subfamily was recently recognized to be phylogenetically more similar to the PIP subfamily than to other plant subfamilies [45]. Also, YpGIpF grouped with the NIP aquaporin subfamily. The YpGIpF isoform belongs to a MIP family related to the bacterial GlpF protein glycerol uptake facilitator, classically associated with aquaglyceroporins from NIP and APQ3 subfamilies [45]. Therefore, this tree, which is supported by the scientific literature, presents the 24 aquaporin isoforms identified by HT-SuperSAGE unitags expressed after 24 h of continuous dehydration stress. Moreover, considering only the nine isoforms identified from *S. officinarum* ESTs, this smaller set was also distributed across the four aquaporin subfamilies described for higher plants.

Additionally, the heat maps (Figure 2) revealed by the expression modulation of the unitags (FCs) in the tolerant or sensitive bulks (both with their respective unstressed controls) show that some PIP isoforms are divergently regulated in the bulks of genotypes. Thus, from the 12 PIP transcripts, eight were repressed in the tolerant genotypes under stress. At the same time, eight of those transcripts were induced in the sensitive bulk. Furthermore, the majority of the PIP transcripts showed divergent modulations (contrasting results) when the response of both bulks of stressed genotypes is compared. Taken together, different genotypes may have developed different survival strategies.

On the other hand, the TIP transcripts similarly responded to the stress (comparing the modulation between both bulks of genotypes, Figure 2). Of the seven TIP subfamily isoforms studied (Figure 2), five were induced in both bulks of genotypes responding to stress, suggesting the participation of these isoforms in

water transport. Finally, the only SIP subfamily representative studied here showed distinct regulation between the analyzed bulks, whereas the two NIP subfamily representatives distinctly responded: NIP3-1 was induced in the tolerant bulk and suppressed in its sensitive counterpart, while NIP3-2 was not modulated in the tolerant bulk, but was induced in the sensitive bulk (Figure 2). For these subfamilies, a larger amount of data is required for further analysis.

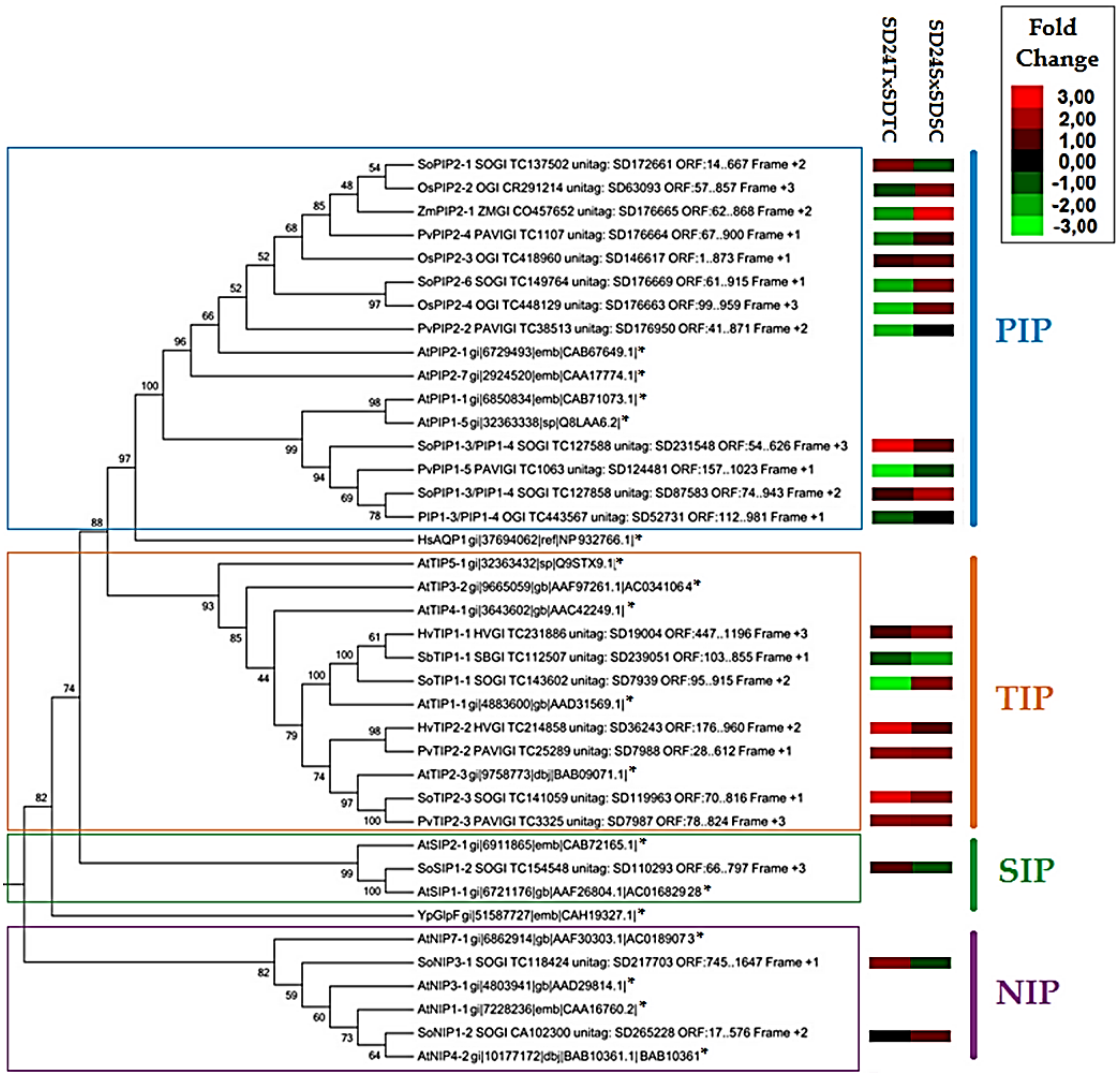

FIGURE 2: Neighbor Joining dendrogram (MEGA v.5.2.1 software [27]) and integrated heat map (bootstrap values of 1,000 replications), showing the phenetic grouping of 24 potential aquaporin amino acid sequences identified by HT-SuperSAGE unitags from sugarcane accessions after 24 h of continuous dehydration (with their respective EST and unitag identifiers), and aquaporins sequences of *Arabidopsis thaliana*, human and *Yersinia pseudotuberculosis* (all these labeled by an asterisk). Nomenclature: Isoforms are preceded by the abbreviated species name (*At-Arabidopsis thaliana; Hs-Homo sapiens; Hv-Hordeum vulgare; Os-Oryza sativa; Pv-Panicum virgatum; Sb-Sorghum bicolor; So-Saccharum officinarum; Yp-Yersinia pseudotuberculosis; Zm-Zea mays*).

Transcriptional Profile of Putative Aquaporins Based on Unitags. The 30 most expressed unitags, based on their normalized frequencies (tpm) in the HT-SuperSAGE libraries, associated to the aquaporin subfamilies PIP (15), TIP (10), SIP (3) and NIP (2) are displayed in Table 3. According to Kjellbom et al. [46] many aquaporin genes are constitutively expressed, with a large number of transcripts (as presented in Table 3), while others are temporally and spatially regulated during plant development or stress responses, as is, for example, the case with unitag SD173276 (Table 3).

TABLE 3: The 30 SuperSAGE unitags most expressed and annotated as aquaporins from sugarcane libraries with contrasting genotypes under stress (24 h of continuous dehydration) or normal daily irrigation.

			Tags *(per million)*			
	Tag id	**Aquaporin**	**SD24T**	**SDTC**	**SD24S**	**SDSC**
1	SD173282	*So*TIP2-2	1096	3784	1643	1816
2	SD231437	*So*PIP1-3/PIP1-4	819	1551	1140	990
3	SD87583	*So*PIP1-3/PIP1-4	964	956	1186	520
4	SD119746	*So*TIP2-3*	1162	1041	771	530
5	SD173276	*So*TIP2-2	564	1879	496	0
6	SD182865	*So*TIP2-2	535	876	501	674
7	SD87593	*So*PIP1-3 /PIP1-4	579	601	750	377
8	SD80613	*So*TIP1-1	318	234	775	571
9	SD80612	*So*TIP1-1	437	393	423	453
10	SD250744	*So*PIP2-1	321	577	265	508
11	SD19004	*Hv*TIP1-1**	422	395	411	210
12	SD176669	*So*PIP2-6	275	591	310	183
13	SD243880	*So*PIP2-1	176	326	334	496
14	SD28080	*So*PIP2-4	406	329	224	312
15	SD176663	*So*PIP2-6	135	340	227	136
16	SD241279	*So*PIP1-5	151	184	257	107
17	SD84960	*Ss*TIP1-1***	202	216	29	136
18	SD243849	*So*PIP2-1	108	123	113	40
19	SD54852	*So*PIP2-1	107	143	22	52
20	SD96918	*So*SIP1-2	106	66	96	49
21	SD96922	*So*SIP1-2	89	43	114	49
22	SD202395	*Pv*NIP1-1****	39	53	40	98
23	SD243867	*So*PIP2-1	37	42	79	43
24	SD87586	*So*PIP1-3/PIP1-4	31	54	28	55
25	SD250859	*So*PIP2-1	39	96	0	32
26	SD198883	*So*PIP2-5	39	27	30	37
27	SD21811	*So*SIP1-1	26	16	26	26
28	SD84958	*Ss*TIP1-1	29	15	9	17
29	SD217703	*So*NIP3-1	29	15	13	13
30	SD36243	*Hv*TIP2-2	31	9	12	9

*Isoforms preceded by the abbreviated species names (*Hv* - *Hordeum vulgare*; *Pv* - *Panicum virgatum*; *So* - *Saccharum officinarum*; *Ss* - *Saccharum* spp). SuperSAGE libraries: SD24T (bulk of

tolerant genotypes under stress); SD24S (bulk of sensitive genotypes under stress); SDTC (control bulk of tolerant genotypes; SDSC (control bulk of sensitive genotypes).

After necessary redundancy exclusion, we identified 42 potential aquaporin isoforms. The contribution of each aquaporin subfamily is presented in Figure 3. In each comparison (SD24T vs SDTC: 26; SD24S vs SDSC: 28; SD24T vs SD24S: 28; SDTC vs SDSC: 28), 26–28 isoforms were identified as being expressed in sugarcane roots after onset of stress (24 h of continuous dehydration) or under normal daily irrigation conditions. This number of isoforms is close to that of other higher plants (31 aquaporin isoforms in maize [43, 47], 35 in *A. thaliana* [48], 39 in rice [47]), and more than twice the amount predicted for vertebrates (11 to 13 isoforms) [49, 50]. The number of aquaporin isoforms in sugarcane may be even higher, since some isoforms respond only to specific tissues [51] or after salinity [52], freezing [53], mycorrhization [54], light [55, 56], cell growth stresses [10]. Therefore, the real number of aquaporin isoforms can only be estimated approximatively by whole genome sequencing. Since sugarcane has one of the most complex genomes of the plant kingdom, with a diploid number of chromosomes ranging from 100 to 130 as a result of aneuploidy and polyploidy events [41], this approach would require significant efforts and investments.

The most transcribed 19 aquaporin unitags belonged to the PIP and TIP subfamilies (Table 3), which matches a report by Alexandersson et al. [51], who analyzed the transcriptional profile of 35 Arabidopsis aquaporins in three different tissues (roots, leaves, and flowers) during water deficit stress (watering suppression). These authors concluded that in all the studied tissues, the PIP, and TIP aquaporins showed higher expression levels, whereas NIPs aquaporins exhibited particularly low transcriptional levels under stress. Zhu et al. [52] also confirmed a lower amount of NIP and SIP in corn under controlled conditions (continuously aerated hydroponic medium, and parameters described by Gibeaut et al. [57]), as compared to the PIP and TIP, which could be related to the aquaporin transport specificity [58]. NIPs are related to the transport of small solutes [31], whereas the physiological functions of SIPs, in addition to water transport [59], still remain unclear. Otherwise, PIPs form primary channels mediating efficient water uptake

and thereby control plasma membrane potentials of permeability, while TIPs, in addition to their high water transport capacity in tonoplasts [60], also transport CO2 [13] and urea [12].

In the present work, SIP and NIP subfamilies were less responsive to the applied stress. We noticed, that NIPs were not up-regulated in the sensitive bulks SD24S versus SDSC (Figure 3b), as well as among the down-regulated unitags in the tolerant bulks SD24T versus SD24S (Figure 3c). On the other hand, the SIP subfamily also harbored no isoform among the down-regulated unitags in the contrast SD24T versus SDTC (Figure 3a). Alexandersson et al. [51] also confirmed that some SIP isoforms presented little expression variation in Arabidopsis plants under drought stress (watering suppression extended until 12 days). Therefore, AtSIP1-1 was considered as constitutively expressed. This low responsiveness to water deficit can be explained by the unique location of these aquaporins in the endoplasmic reticulum [59], an organelle with tortuous structure and high surface-to-volume ratio with high demand for osmotic balance volume and, therefore, may not require the water transport mediated by aquaporins [59]. Thus, further studies are necessary to define SIP functions more clearly.

The level of aquaporin transcripts varied less than 10 times based on the unitags in the contrasts, except for the SD173276 unitag (a potential SoTIP2-2), which was almost two thousand times higher in the tolerant SDTC versus SDSC contrast, and almost 500 times in the sensitive contrast SD24S versus SDSC (data not shown).

Alexandersson et al. [51] also observed that most aquaporins do not alter their expression under water deficit stress, and no Arabidopsis aquaporin isoforms varied their expression more than twice until the seventh day of stress treatment.

The contrast analysis of the tolerant bulks defined four possible targets: one exclusively up-regulated PIP1-1 isoform (SD264077 unitag, FC 3.58), and three exclusively down-regulated PIP2-2 (SD176950 unitag, FC -2.34), PIP2-1 (SD176664 unitag, FC -1.73) and NIP1-1 (SD202395 unitag, FC -1.36).

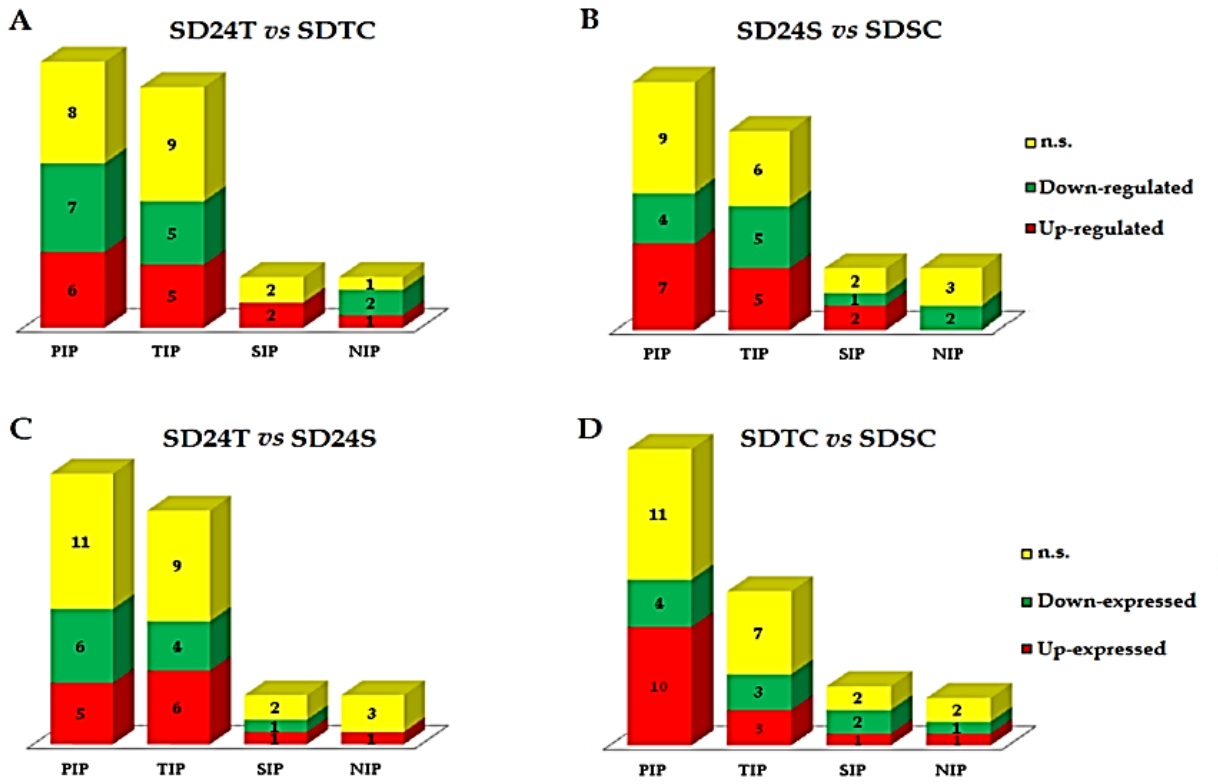

FIGURE 3: Representativeness of the aquaporin subfamilies expressed in sugarcane after 24h water suppression (A, B and C) and in normal irrigation (D), involving bulks of genotypes tolerant and sensitive to stress. HT-SuperSAGE libraries: SD24T (bulk of tolerant genotypes under stress); SD24S (bulk of sensitive genotypes under stress); SDTC (bulk of tolerant genotypes under control conditions); SDSC (bulk of sensitive genotypes under control conditions).

In rice, the PIP1-1 isoform promoted salt stress tolerance [23], and it was involved in the rehydration after cooling stress in tolerant genotypes [61]. PIP1-1 overexpression conferred tolerance to water deficit in rice and to salt stress in transgenic Arabidopsis [62]. This isoform also responded to drought and daytime in grapevine [56]. The up-regulated SD264077 unitag, as a possible PIP1-1 isoform, was validated in the present work by RT-qPCR analysis, as detailed in the next chapter, and represents a potential target for further studies, including the development of molecular markers for marker-assisted selection in breeding (real-time PCR-assisted selection) [63] or cisgenesis (insertion of genes in different accessions of the same species [64]), already successfully applied by Joshi et al. [65]. These authors inserted resistance genes to apple scab under the control of the RubisCO promoter in varieties known to be susceptible to the pathogen.

Isoform PIP2-2 is down-regulated over four times in Arabidopsis under 12 days of drought [51] and in barley under salt stress [66]. As expected, it was also observed in the tolerant bulk analysis of the present study, showing FC -2.34. The subsequent PIP2-2 monitoring revealed that aquaporin expression increased sensitivity to salt stress in transgenic rice [67]. This point is relevant, since crosstalks involving shared pathways in response to drought and salinity stress are regular [68, 69]. Thus, this isoform, after appropriate RT-qPCR validation, could be useful as stress-indicator in breeding programs.

On the other hand, the potential usefulness of unitags related to PIP2-1, PIP2-5, PIP2-6, TIP1-1, TIP2-2, SIP1-1, and SIP1-2 in breeding programs still need to be confirmed. In relation to the PIP2-5 isoform (up- and down-regulated simultaneously in the present study, depending on unitags), Jang et al. [70] observed that overexpressing this aquaporin reduced drought tolerance of transgenic Arabidopsis and tobacco. The same group proposed that PIP2-5 expression influenced the transcription levels of other PIPs and H^+-ATPases (enzymes that regulate the cytoplasmic pH in which levels of H+ interfere with the control of the opening and closing of the aquaporins channels known as the aquaporin gating [71]). Lembke et al. [25] also observed this isoform to be down-regulated under water deficit (72 hours of watering suppression), despite the detected induction via oligonucleotide array hybridization.

Therefore, for the tolerant bulk of genotypes, this isoform is expected to restrain its expression under root dehydration (24 h). Basically, up- or down-regulation and constitutive expression were all observed in the contrast analysis of tolerant bulks, (except that down-regulation was not observed in the SIP subfamily; Figure 3(a). The sensitive bulk of genotypes also presented all three expression levels for each aquaporin subfamily (with the exception of the NIP subfamily, in which up-regulation was not observed; Figure 3(b)). The analysis of the sensitive bulks allowed the identification of only up-regulated [PIP2-2 (SD176665 unitag, FC 4.83), PIP2-4 (SD176663 unitag, FC 1.66), PIP2-6 (SD176669 unitag, FC 1.70)] or only down-regulated [PIP1-1 (SD264077 unitag, FC -4.56), NIP1- 1 (SD202395 unitag, FC -2.00)] aquaporin isoforms. These exclusively up- or down-regulated isoforms, respectively,

may represent a panel of markers based on real-time PCR, and suggesting high stress sensitivity. In this way, at least two candidates are particularly appealing: (a) PIP1-1, that was up-regulated (SD264077 unitag, FC 3.58) in the tolerant bulks and entirely differently regulated (SD264077 unitag, FC - 4.56) in the sensitive bulks; (b) PIP2-2 isoform, which was exclusively down-regulated in the tolerant bulks (SD176950 unitag, FC -2.34) and up-regulated in the sensitive bulks (SD176665 unitag, FC 4.83). Thus, both isoforms are strong candidates for further research aiming at molecular marker development and cisgenesis. Finally, further studies are needed to determine the true meaning of each stress responsive isoform.

When comparing both genotype bulks under stress (SD24T versus SD24S), all three expression levels (up- or down-regulation and constitutive expression) were observed for each aquaporin isoform subfamily. Notably, a specific isoform in the SIP subfamily was down-regulated in the tolerant bulk, but not in the sensitive bulk (Figure 3(c)). Two more isoforms are worth mentioning: PIP2-4 (SD176664 unitag, FC 3.64), which was more transcribed in the stressed tolerant bulk than in the stressed sensitive one, and PIP2-1 (SD176669 unitag, FC -1.13), which is being less transcribed in the stressed tolerant bulk as compared to the stressed sensitive bulk.

The unitag related to PIP2-4 (SD176664) was down- regulated in the tolerant bulks after onset of the stress, and it had no relevant expression changes in the sensitive bulks. Nevertheless, it was more expressed in the tolerant bulk when compared with the sensitive bulk, upon stress or even under control conditions. Thus, the tolerant genotypes seemed to produce more PIP2-4 transcripts than the sensitive genotypes. In maize, this aquaporin isoform was up-regulated after only two hours of salt stress, in which time the recovery phase of the osmotic potential falls [52].

In turn, the PIP2-1 related unitag (SD176669) behaved differently under stress and its reaction depends on the genotype (it was down-regulated as compared to the tolerant bulks, and up-regulated as compared to the sensitive bulks). When considering the contrast between both control and stressed bulks of genotypes, this unitag was better expressed in the sensitive bulk than in the tolerant one. By taking into account that this aquaporin isoform increases insensitivity to salinity [67] in

transgenic rice and the fact that salinity and drought share many response pathways [68, 69], this isoform deserves further investigation.

Analysis of controls of the two different genotype bulks showed that the aquaporin isoforms are present in all subfamilies and expressed in the three studied levels during normal daily irrigation (Figure 3(d)). Four isoforms [PIP1-2 (SD92576 unitag); PIP2-1 (SD176664 unitag); PIP2-4 (SD176663 and SD176950 unitags); PIP2-6 (SD176669 unitag)] presented significantly higher abundance in the tolerant bulk, while only one (NIP1-1) was less expressed in relation to the sensitive bulk. In turn, five isoforms (PIP2-2, PIP2-3, PIP2-5, PIP2-6, and TIP4-2) were similarly transcribed in both bulks, while another five isoforms (PIP2-1, PIP2-4, TIP2-2, TIP2-3, and SIP1-2) presented all the three expression levels. Differences in the transcriptional profiles of both controls bulks reinforce the expression modulation of genes presenting in the genotypes composing the bulks.

The two main comparisons SD24T versus SDTC and SD24S versus SDSC revealed that from a total of 18 up-regulated unitags in the tolerant bulks, eight were down-regulated in sensitive bulks, while from 22 other unitags, down-regulated in the tolerant bulks, eight were up-regulated in the sensitive bulks (Table 4). The same isoforms showing different expression levels (Table 4) can be explained by the similarities between aquaporins sequences, in part a consequence of the high level of duplicated plant MIP genes, which is higher than that observed in vertebrates, possibly reflecting the environmental pressures plants are exposed to [45, 49], and also the aneuploidy and polyploidy events observed in the *Saccharum* complex [41].

Unitag Expression Validation by RT-qPCR. The use of RT-qPCR for the confirmation of aquaporin gene expression changes in grass (maize [72] and sugarcane [25]) has already been reported. In the present work we attempted to determine which genotype was responsible for the bulk of expression in tolerant or sensitive genotypes. To that end, each genotype composing that bulk was independently tested by RT-qPCR analysis. Thus, two unitags [SD264077 (PIP1-1) and SD231548 (PIP1-3/PIP1-4)] considered UR in the tolerant bulk as well as DR and n.s. in the sensitive

were selected for expression validation using two reference genes (25S rRNA and GAPDH), both reported to be suitable for sugarcane (Table 5). The relative expression results of the tolerant and sensitive genotypes for the two target genes are shown in Table 6, together with their respective unitag results.

TABLE 4: Unitags annotated as aquaporins with distinct responses regarding the level of expression between two contrast analysis involving HT-SuperSAGE libraries originated from sugarcane drought tolerant and sensitive genotypes (SD24T *vs* SDTC and SD24S *vs* SDSC) under watering suppression (24 h).

Unitag	**Anotation**	**FC_T**	**Unitag $Regulation_T$**	**FC_S**	**Unitag $Regulation_S$**
SD264077	*Ss*PIP1-1	3.58	UR	-4.56	DR
SD2444	*So*PIP1-3/PIP1-4	5.93	UR	-2.66	DR
SD231548	*So*PIP1-3/PIP1-4	3.18	UR	1.28	n.s.
SD243866	*So*PIP2-1	13.51	UR	-15.97	DR
SD243874	*So*PIP2-1	3.18	UR	-2.14	DR
SD28082	*So*PIP2-4	15.10	UR	-6.39	DR
SD28080	*So*PIP2-4	1.23	UR	-1.39	n.s.
SD198883	*So*PIP2-5	1.46	UR	-1.25	n.s.
SD36536	*So*TIP1-1	2.97	UR	-1.90	n.s.
SD80612	*So*TIP1-1	1.11	UR	-1.07	n.s.
SD84958	*Ss*TIP1-1	2.02	UR	-1.82	DR
SD36243	*Hv*TIP2-2	3.53	UR	1.34	n.s.
SD182891	*So*TIP2-2	2.78	UR	-5.85	DR
SD119963	*So*TIP2-3	5.56	UR	1.93	n.s.
SD119859	*So*TIP2-3	3.18	UR	-2.66	DR
SD217703	*So*NIP3-1	1.93	UR	-1.01	n.s.
SD21811	*So*SIP1-1	1.65	UR	1.01	n.s.
SD96919	*So*SIP1-2	2.38	UR	-1.06	n.s.
SD233575	*So*PIP1-3/PIP1-4	-6.56	DR	4.21	UR
SD231438	*So*PIP1-3/PIP1-4	-5.16	DR	1.38	n.s.
SD231437	*So*PIP1-3/PIP1-4	-1.89	DR	1.15	UR
SD205705	*So*PIP1-3/PIP1-4	-1.87	DR	-1.14	n.s.
SD231440	*So*PIP1-3/PIP1-4	-2.79	DR	-2.14	n.s.
SD241279	SoPIP1-5	-1.22	DR	2.40	UR
SD91837	SoPIP2-4	-17.81	DR	1.32	n.s.
SD243847	*So*PIP2-1	-7.50	DR	-1.60	n.s.
SD243911	*So*PIP2-1	-5.62	DR	-1.33	n.s.
SD54851	*So*PIP2-1	-2.53	DR	1.87	n.s.
SD176663	*Os*PIP2-4*	-2.51	DR	1.66	UR
SD176664	*So*PIP2-4	-1.73	DR	1.02	n.s.
SD84616	*So*PIP2-5	-4.42	DR	1.32	n.s.
SD176669	*So*PIP2-6	-2.15	DR	1.70	UR

Table 4 Cont.

Unitag	Anotation	FC_T	Unitag $Regulation_T$	FC_S	Unitag $Regulation_S$
SD19005	*Hv*TIP1-1*	-1.87	DR	1.84	n.s.
SD19006	*Hv*TIP1-1	-5.16	DR	4.67	UR
SD7939	*Ta*TIP1-1*	-2.95	DR	1.76	n.s.
SD80616	*Ss*TIP1-1*	-2.81	DR	2.34	UR
SD182871	*So*TIP2-2	-6.09	DR	-1.63	n.s.
SD173276	*So*TIP2-2	-3.33	DR	496.29	UR
SD119919	*So*TIP2-3*	-6.56	DR	1.61	n.s.
SD194892	*So*NIP3-1	-2.34	DR	-1.60	n.s.

*Isoforms preceded by the species name abbreviation (*Hv* - *Hordeum vulgare*; *Os* - *Oryza sativa*; *So* - *Saccharum officinarum; S*s - *Saccharum* sp. and *Ta* - *Triticum aestivum*). HT-SuperSAGE libraries: SD24T (bulk of tolerant genotypes under stress); SD24S (bulk of sensitive genotypes under stress); SDTC (bulk of tolerant genotypes under control conditions); SDSC (bulk of sensitive genotypes under control conditions). FC ratio between the frequencies of a unitag present in the library under stress relative to the control library of same genotypes. n.s.: not significant at the level studied. Underline T: contrast SD24T *vs* SDTC; Underline S: contrast SD24S *vs* SDSC.

PIP1-1 (SD264077 unitag) was induced by stress in two of the tolerant genotypes (CTC6 and SP83-2847), in comparison to the respective controls (Table 6, Figure 4). Nevertheless, in the remaining tolerant genotypes (CTC15 and SP83-5073) both PIP1-1 genes were down-regulated under the same conditions (Table 6, Figure 4). Thus it can be concluded that CTC6 and SP83-2847 were responsible for the unitag upregulation. The overexpression of rice PIP1-1 in root and leaf (within 24h) enhanced the tolerance to drought (200mM mannitol) and salt stress (100mM NaCl) in transgenic Arabidopsis [62]. Also, PIP1-1 aquaporin isoforms in grapevine were highly expressed in roots (RT-qPCR) in response to water deficit (8 days of constant dehydration [56]).

PIP1-3/PIP1-4 (SD231548 unitag), were stress-induced in genotype SP83-2847 (Table 6, Figure 4), in agreement with the HT-SuperSAGE data. It should be noted that in phylogenetic analyses PIP1-3 and PIP1-4 are highly similar with barley PIP amino acid sequences, being grouped together as one isoform, while they are phylogenetically more distant from PIP1-1 (from barley and rice, [73]). *A. thaliana* PIP1-3 and PIP1-4 isoforms had their transcription level increased more than five times, covering the first 48 h of drought stress (250mM mannitol), in leaves and roots, as well as in response to salt (150mM NaCl) and cold stresses [48]. In turn, PIP1-3

overexpression in transgenic rice, combining aquaporin coding sequence with a constitutive corn promoter, showed enhanced stress tolerance to cold [74].

However, in relation to water transport by PIP1-3, which appears to be less permeable to water [74], this isoform could work best in conjunction with PIP2 subgroup members, in which *in silico* analysis showed them to be mostly DR. Considering the remaining components of the tolerant bulk, the genotype CTC6 did not show significant differences in transcript levels, while CTC15 and SP83-5073 presented down-regulated transcription at the onset of root dehydration (Table 6, Figure 4).

The strategy of opening bulks in the RT-qPCR validation reinforced the transcription modulation of sugarcane aquaporins and gave hints to genotype-specific expression. Thus, plants considered physiologically tolerant or sensitive to root dehydration (24 h) varied in the expression of aquaporin isoforms. The same was observed with up (*O. sativa* L. cv. Zhonghan 3) and lowland (cv. Xiushui) rice under water deficit [75]. The RT-qPCR results revealed genotype-specific differences for PIP1-2, PIP1-3, PIP2-1, and PIP2-5 isoforms in roots, and PIP1-2 and PIP1-3 in leaves. The above mentioned isoforms were up-regulated in upland rice, whereas they remained unchanged or DR in lowland rice [75].

Finally, the RT-qPCR protocol, in the present work applied for unitags validation, as well as the identified unitags for PIP1-1 and PIP 1-3/PIP1-4, define a set of functional molecular markers based on the expression profiles validated with appropriate genotypes. This expression marker set will assist breeders in marker-assisted selection of elite genotypes more tolerant to abiotic stresses.

TABLE 5: Primers sequences for RT-qPCR of *Ss*PIP1-1, *So*PIP1-3/PIP1-4 (designed from sugarcane ESTs), 25S rRNA, and GAPDH (as reference genes).

Unitag	EST / Cluster	Database *	Gene	Primers sequences (Foward/Reverse)	Tm (°C)	Amplicon (bp)	Regression Coefficient (R^2)	Amplification Efficiency (%)
SD264077	gi|35203438|	dbEST	*Ss*PIP1-1	(F)GTTCCTATCCTTGCCCCACT (R)AGGCGTGATCCCTGTTGTAG	84.6	134	0.995	95.55
SD231548	TC127588	SoGI	*So*PIP1-3/PIP1-4	(F)GACTCCCATGTTCCTATCCTTG (R)CGTGATCCCTGTTGTAGATGAT	84.3	142	0.992	93.47
-	gi|33464288	dbEST	25S rRNA	(F)-GCAGCCAAGCGTTCATAG (R)CGGCACGGTCATCAGTAG	82.9	172	0.999	99.82
-	TC531505	SoGI	GAPDH	(F)GGTTCACTTGAAGGGTGGTG (R)TGAGGTGTACCTGTCCTCGTT	81.8	100	0.984	100.89

*Database [NCBI (dbEST), Gene Index (SOGI)];

TABLE 6: Relative expression rates of aquaporins PIP1-1 (SD264077 unitag) and PIP1-3/PIP1-4 (SD231548 unitag) in bulks of tolerant or sensitive genotypes, respectively, and RT-qPCR data (both in bulks and each genotype)

Technique and genetic material		**Target gene**	
		PIP1-1	**PIP1-3/PIP1-4**
		Modulation of gene expression&	
HT-SuperSAGE*	Tolerant bulk	3.580* / UR	3.180* / UR
	Sensitive bulk	- 4.560* / DR	1.280* / ns
RTqPCR# and Tolerant Genotypes	CTC6	1.652# / UR	1.271# / ns
	CTC15	0.740# / DR	0.670# / DR
	SP83-2847	1.836# / UR	1.468# / UR
	SP83-5073	0.324# / DR	0.383# / DR
RTqPCR# and Sensitive Genotypes	CTC9	1.030# / ns	1.205# / ns
	CTC13	0.635# / DR	0.644# / DR
	SP90-1638	1.536# / ns	1.236# / ns
	SP90-3414	0.324# / DR	0.383# / DR

Bulk of the four tolerant or sensitive genotypes; &$P < 0.05$; **fold change* [FC: ratio of the frequencies (normalized to 1,000,000) observed in the stressed library in relation to the control library]; #relative expression level using the REST software (v. 2.0.13) [26]; DR: down-regulated; UR: up-regulated; ns: not significant at $P < 0.05$.

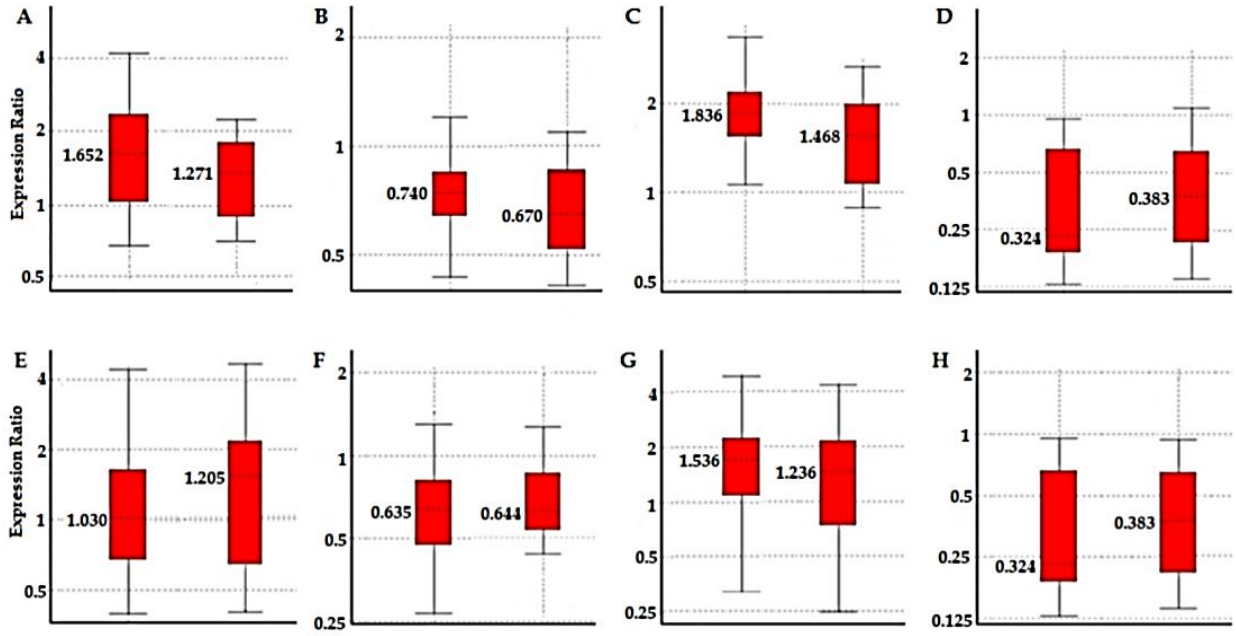

FIGURE 4. Relative quantifications to SsPIP1 aquaporin-1 (Unitag SD264077) and SoPIP1-3/PIP1-4 (Unitag SD231548) obtained from cDNAs of sugarcane drought tolerant genotypes [(A) CTC6, (B) CTC15, (C) SP83-2847 and (D) SP83-5073] and drought sensitive genotypes [(E) CTC9, (F) CTC13, (G) SP90-1638 and (H) SP90-3414] the stress condition (suppression of watering 24 h) with respect to the same genotypes in the control situation normalized by reference genes GAPDH and 25S rRNA. #Relative expression (REST software version 2.0.13)

with the median value (dotted horizontal at the colored box) and range comprising 100% of the observations (among horizontal bars), being 50% of them in colored box (confidence interval at 95%).

Conclusions

The present work is a pioneer study specifically addressing the aquaporin transcripts in sugarcane transcriptomes established from HT-SuperSAGE transcription profiles from roots of tolerant and sensitive genotypes after 24h of continuous dehydration. Almost all 26 bp unitags were annotated using a public sugarcane EST databases, allowing the identification of potential aquaporins. Categorizing the EST-anchored unitags by Gene Ontology (GO) enhanced the annotation efficiency by almost 15%. These procedures identified potential isoforms of the four aquaporin subfamilies (PIP, TIP, NIP, and SIP) already described for higher plants, together with their respective expression profiles in sugarcane under abiotic stress. Moreover, an efficient protocol for RTqPCR was developed, enabling gene expression validation of SuperSAGE unitags related to PIP aquaporins (PIP1-1 and PIP1-3/PIP1-4) and involving reference genes encoding GAPDH and 25S rRNA, testing each genotype individually the employed, validation strategy revealed genotype-specificity of the response to the applied stress.

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Conflict of Interests

The authors declare that there is no conflict of interests.

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Artigo 3 - Validation of Novel Reference Genes for Reverse Transcription Quantitative Real-time PCR in Drought-stressed Sugarcane

Artigo científico submetido ao periódico The Scientific World Journal

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Abstract

One of the most challenging aspects of reverse transcription quantitative real time PCR (RT-qPCR) data analysis is the identification of stable reference genes. Ideally, reference genes should be neither induced nor repressed under different experimental conditions. For sugarcane (*Saccharum* spp.) only four reference genes (GAPDH, 25S rRNA, ß-actin and ß-tubulin) have been described to date, despite increasing transcriptomic studies in sugarcane aiming RT-qPCR validation. In this work, in addition to GAPDH, 25S rRNA and ß-actin, other potential reference genes (28S rRNA, α-tubulin, histone H1, elongation factor-1α, S-adenosylmethionine decarboxylase - SAMDC, 14-3-3, ubiquitin and 14-3-3 gene family) were tested for gene expression normalization of sugarcane root tissues from drought-tolerant and sensitive accessions. After evaluation with different statistical methods (GeNorm, NormFinder and BestKeeper), H1, αTUB and GAPDH were considered the most reliable genes for normalization purposes. Their suitability as reference genes was validated using two target genes (Glutamine-dependent asparagine synthase and Pyrophosphate-fructose 6-phosphate 1-phosphotransferase) showing differential regulation, as previously revealed by HT-SuperSAGE assay. In conclusion, H1, αTUB and GAPDH genes are considered most useful reference genes for dissecting gene expression in sugarcane roots, particularly in transcriptomic studies using SuperSAGE or RNA-Seq approaches, being H1 and αTUB genes reported for the first time for sugarcane studies.

Introduction

Sugarcane (*Saccharum* spp.) is a major crop with easy vegetative propagation and capacity to accumulate high levels of sucrose in the culms [1-3]. World production of this crop in 2011 resulted in about two billion tons of raw material, which corresponded to a gross income of more than US$ 52 billion [4]. Despite the economic importance of sugarcane, the understanding of important genetic mechanisms remains challenging, due to the fact that this crop presents one of the largest and most intricate genomes of the plant kingdom, with diploid numbers ranging from 100 to 130 chromosomes, indicating a high ploidy level as well as recurrent aneuploidy events [5-7]. Because of this complexity, the application of molecular tools represents an attractive approach to the improvement of sugarcane breeding programs. Moreover, transcriptomic studies have been prioritized, allowing identification of candidate genes involved in developmental processes and plant responses to environmental cues, which have ultimately lead to the discovery of functional molecular markers [8].

Quantitative real time PCR (RT-qPCR) is based on a high specific polymerase chain reaction associated to sensitive fluorescence, allowing the detection of variations in gene expression, including discreetly transcribed genes [9, 10]. This technology has been widely used as a diagnostic tool for detection of plant pathogens, transgene expression [9], human diseases [11, 12] and to validate transcriptional profiles generated by several methodologies, such as EST libraries [13], Microarray [14], HT-SuperSAGE [15] and RNAseq [16]. The reliability of RT-qPCR data based on relative quantification is depending upon comparative transcription of target genes to stable reference genes [17, 18]. The use of reference genes that undergo changes in transcription across experimental groups, tissues, or developmental stage can dramatically alter the conclusions on targeted gene expression [18]. In order to test for normalization of reference gene expression, several statistical algorithms, such as geNorm [17], NormFinder [19] and BestKeeper [20] have recently been developed.

Despite the importance of proper reference gene choice for reliable and accurate RT-qPCR assays, most reports involving sugarcane have not described or

compared methods in order to identify the efficiency of reference genes [21, 22, 23], suggesting arbitrary criteria for this selection. To our knowledge, only one systematic study aimed to test, standardize and validate reference genes (GAPDH, ß-tubulin, ß-actin and 25S rRNA) for tissue and genotype-specific gene expression analysis in sugarcane [24]. Additionally, no such evaluation was carried out before under stress conditions, a situation that often alters the behavior of some genes. Thus, rigorous selection of reference genes for expression profiling validation in sugarcane under biotic or abiotic stress remained to be described.

In the present work screening and validation of novel reliable reference genes for expression analysis in sugarcane roots was carried out. Besides, additional resources for target validation were evaluated, especially considering comprehensive transcription profiling, like those provided by HT-SuperSAGE [25], carried out by our group in sugarcane, revealing hundreds of candidate genes responsive against drought stress, requiring subsequent validation.

Materials and methods

Plant Material and Treatments. Sugarcane accessions were previously evaluated in a glasshouse trial conducted by the Center for Sugarcane Technology (CTC), in Piracicaba, Brazil (22º41'S; 47º33'W, altitude 554 m), aiming to identify drought-tolerant and drought–sensitive sugarcane accessions. Four accessions were selected as drought-tolerant (CTC6, CTC15, SP83-2847 e SP83-5073) and other four as drought-sensitive (CTC9, CTC13, SP90-3414, e SP90-1638) for the experiment. Briefly, plants of each selected accession were grown under glasshouse conditions [30.2 ± 5.7ºC (maximum), 16.8 ± 1.9ºC (average) and 9.3 ± 3.0ºC (minimum) temperatures and in 71.5 ± 5.1% (average) relative humidity under natural photoperiod] in 40 L pods, in a randomized experimental design (comprising six repetitions) under daily irrigation (4L.h^{-1}) until reaching three months of age. Plants were submitted to drought conditions by continuous dehydration caused by interruption of irrigation during 24 hours. Roots of both, stressed and unstressed plants, were collected, immediately frozen in liquid nitrogen and stored at -80ºC until further use. These accessions were previously reported as tolerant and sensitive to drought by Thiebaut

et al. [26] based on chlorophyll and water and content measurements and field observations.

Total RNA isolation, purification, cDNA synthesis and HT-SuperSAGE Libraries. Total RNA was extracted using RNeasy Mini kit (Qiagen®), according to the manufacturer's instructions. RNAs samples were measured using Qubit (Invitrogen®), after DNAse (Qiagen®) digestion aiming to remove any residual genomic DNA contamination and purified with the aid of RNeasy Mini kit (Qiagen®). RNA integrity was verified in 1.5% agarose gel electrophoresis with Blue-Green Loading Dye (LGC Biotechnology®) staining. The first-strand cDNA was synthesized from equal amounts of purified total RNA (1µg) of drought-tolerant and drought- sensitive sugarcane accessions using QuantiTect Reverse Transcription Kit (Qiagen®) according to the manufacturer's instructions. All cDNA samples were tested by standard PCR before further analysis. The bulks were composed with equimolar amounts of poli-A^+ messenger RNA from all accessions comprising the HT-SuperSAGE libraries. Four libraries were generated as described by Kido et al. [25]: **TD** (bulk of tolerant accessions under stress); **TC** (bulk of tolerant accessions without stress, as tolerant negative control); **SD** (bulk of sensitive accessions after stress); **SC** (bulk of unstressed sensitive accessions, as sensitive negative control). The procedures for HT-SuperSAGE library generation followed Matsumura et al. [27], including the attachment of library-specific adaptors carried out by GenXPro GmbH (Frankfurt, Germany) allowing the identification of library-specific reads after SOLEXA sequencing.

Primer design, amplification efficiency and RT-qPCR analysis. Sugarcane ESTs from dbEST database (http://www.ncbi.nlm.nih.gov/nucest) were used for primers design. All primer pairs were synthesized by Bioneer Corporation® (South Korea) and details of primers sequences are given in Table 1. Specific primers were designed using Primer 3 software [28], with minor modifications. Amplicon length range was 70-200 bp, melting temperatures between 40ºC (minimum), 50ºC (optimum) and 60ºC (maximum), and CG content ranging from 45 - 55% (optimum of 50%). A dissociation

curve analysis was performed to investigate the amplification to the reference genes, and PCR products were analyzed on 1.5% agarose gel electrophoresis followed by Blue-Green Loading Dye staining (LGC Biotechnology®) aiming to verify primer specificity. A No Template Control (NTC) was also performed for each primer pair. Standard curves using a dilution series (1, 1/10, 1/100, 1/1000 e 1/10.000) of the cDNA pool was made to calculate the gene-specific PCR efficiency and regression coefficient (R^2) for each gene. The RT-qPCR amplifications were performed on LineGene 9660 model FQD-96A (Bioer®), using SYBR Green detection. Each reaction mixture comprised 1µL of template cDNA (diluted 5-fold), 5µL of HotStart-IT SYBR Green qPCR Master Mix 2x (USB®), 0.05µL of ROX, 1.95µL of ultrapure water, and 1µL of each primer (0.05µM) to a final volume of 10µL. The reactions were subjected to an initial denaturation step of 95°C for 2min, followed by 40 cycles of 95°C for 15 s, 58°C for 30s, and 72°C for 30s in 96-well reaction plates. The dissociation curves were analyzed at 65 – 95°C for 20min after 40 cycles. Three biological and three technical replicates were used in each run for RT-qPCR analysis.

Data analysis. Expression levels of candidate genes were evaluated based on the number of amplification cycles needed to achieve a fixed threshold (Cycle threshold – Ct) in the exponential phase of PCR. Statistical analysis was performed by GeNorm (version 3.5) [17], NormFinder (version 0.953) [19] and BestKeeper (version 1) [20] softwares. Software packages were used according to the manufacturer's instructions. Reference genes were ranked according to their stability in tested sample, and the number of housekeeping genes (HKGs) necessary for an optimal normalization was also indicated. Relative quantification data were analyzed with the aid of the REST (Relative Expression Software Toll) software version 2.0.13 [29].

Results

RNA integrity, specificity and efficiency amplifications. Standard PCR analyzes were initially performed with candidate reference genes in order to confirm the amplification of designed primers described above using sugarcane cDNA samples as templates. From the set of candidate genes (αTUB, 14-3-3, GAPDH, ß-actin, H1,

EF-1α, SAMDC, UBQ, 25S rRNA and 28S rRNA), six (αTUB, GAPDH, H1, SAMDC, UBQ, 25S rRNA) were amplified and showed a single product based on the gel electrophoresis (Figure 1A) and the dissociation curve analysis (Figure 1B). Despite the pooled cDNA (bulk of tolerant and sensitive accessions) used in the differential expression approach for HT-SuperSAGE as highlighted by Kido et al. [25], those six genes were selected for further RT-qPCR analysis. Thus, standard curves were generated using a serial dilution of the cDNA pool (Figure 2) and PCR amplification efficiency for the six reference genes ranged from 98.34% to 100.89%, whereas regression coefficients (R^2) varied from 0.984 to 0.999 (Table 1). The results showed favorable conditions for amplification, efficiency in successive dilution and little variation in gene expression across samples. Collectively, these conditions represent significant criteria for choosing a suitable reference gene. Thus, in expression studies, fluctuations due to pipetting errors, variations in the quantification of samples or the concentration of reagents can be normalized with the aid of an appropriate reference gene [30].

Expression stability of sugarcane reference genes. Six potential reference genes (αTUB, GAPDH, H1, SAMDC, UBQ and 25S rRNA) were selected for normalization tests of sugarcane gene expression. The expression level of the candidate genes showed Ct (Cycle threshold) values ranging from 13.06 to 28.00 (Table 2). All potential reference genes showed constant expression and variations below one cycle, except for UBQ and SAMDC. The 25S rRNA gene was the most abundantly transcribed, reaching the threshold fluorescence peak after 13 amplification cycles, while αTUB was the least abundant gene having an average Ct value of 27.48.

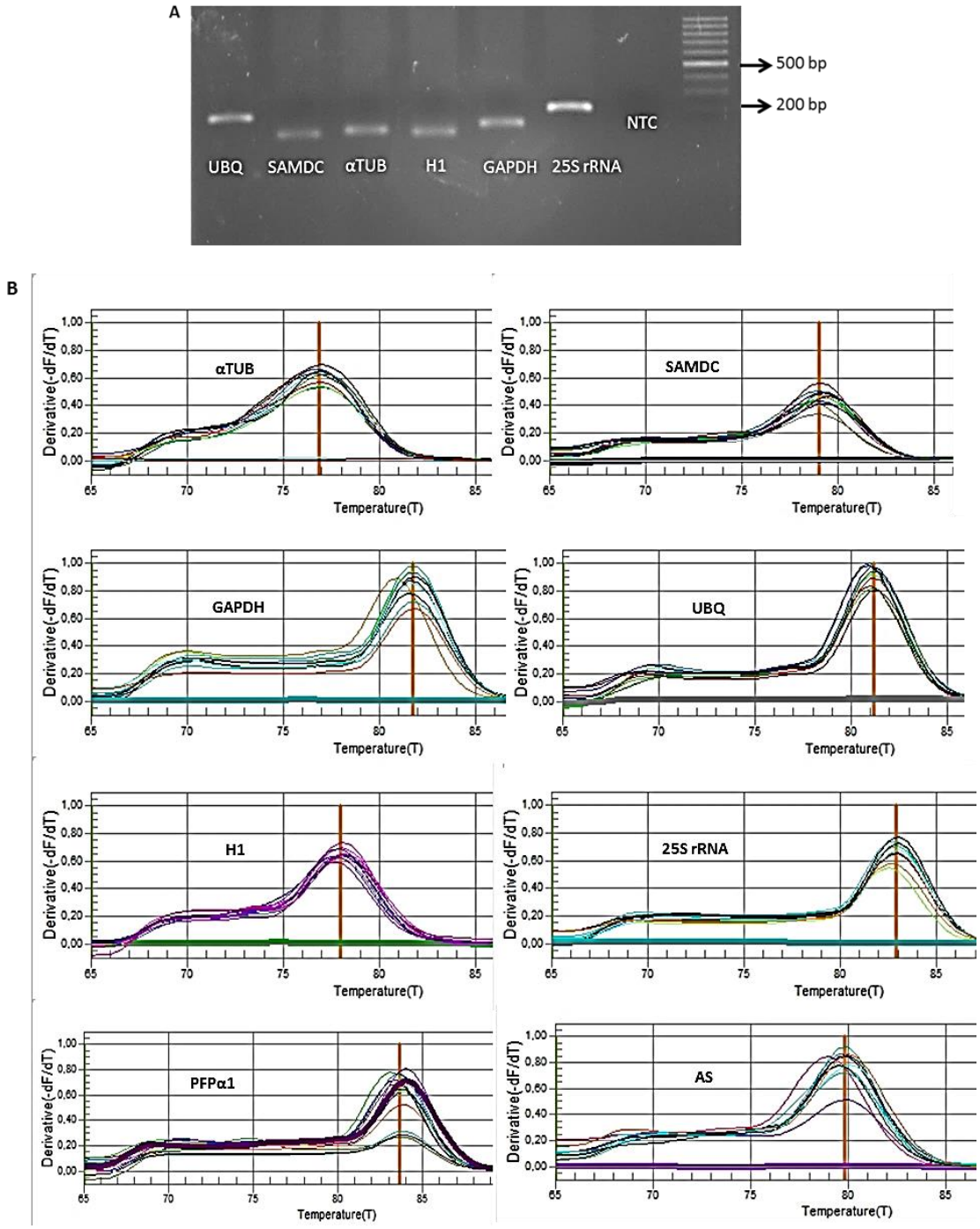

FIGURE 1: (A) Agarose gel (1.5 %) showing the specific products of expected size for each gene used in the study, displaying primer specificity as required for RT-qPCR amplification. (B) Dissociation curves showing a single peak for six potential reference genes **(**αTUB: Alpha-tubulin; GAPDH: Glyceraldehyde 3 phosphate dehydrogenase; H1: Histone H1; SAMDC: S-adenosylmethionine decarboxylase; UBQ: Ubiquitin; 25S rRNA: 25S ribosomal RNA) and two targets (PFPα1: Pyrophosphate fructose-6-phosphate 1-phosphotransferase alpha subunit; AS: Glutamine-dependent asparagine synthetase).

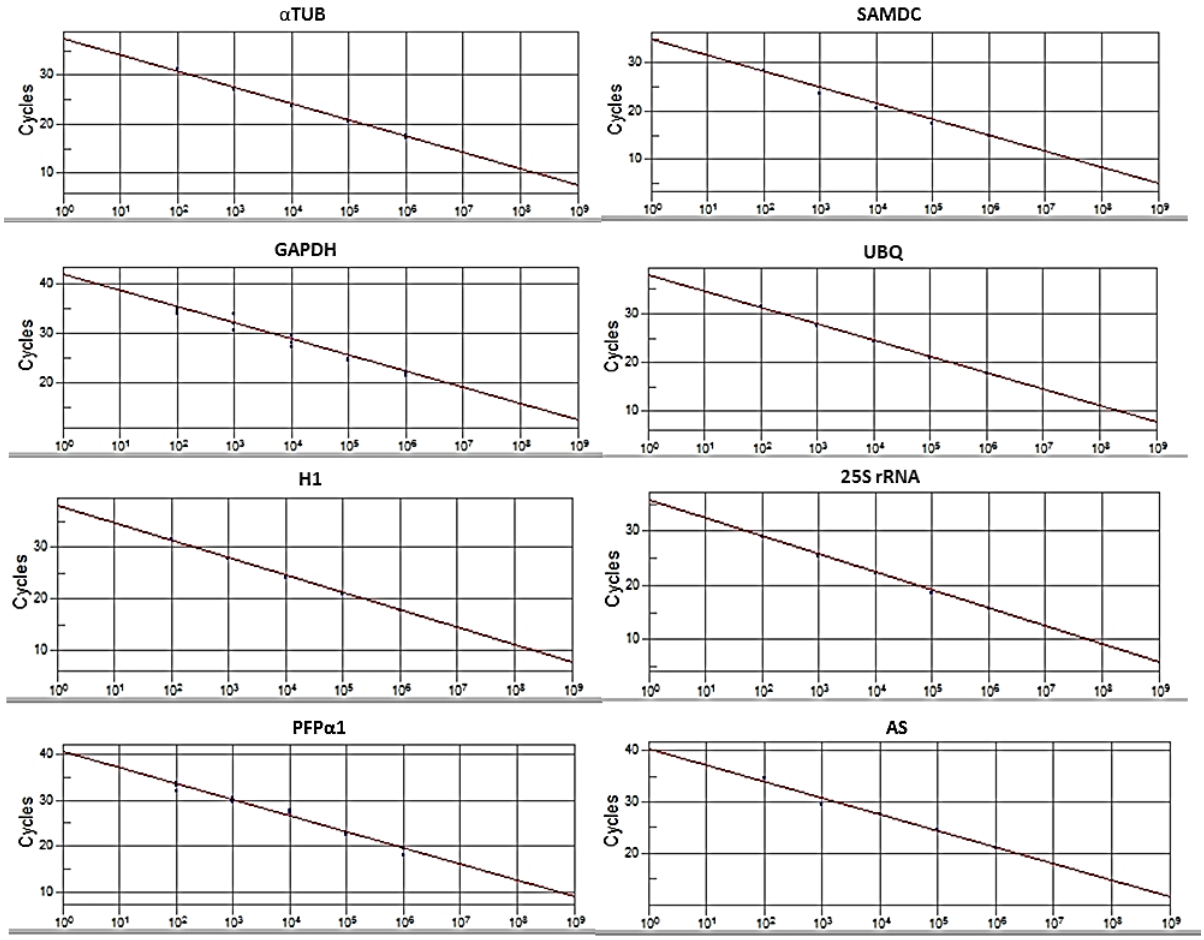

FIGURE 2: Standard curves using a dilution series (1, 1/10, 1/100, 1/1000 e 1/10.000) of potential genes for sugarcane roots under drought stress (αTUB: Alpha-tubulin; GAPDH: Glyceraldehyde 3 phosphate dehydrogenase; H1: Histone H1; SAMDC: S-adenosylmethionine decarboxylase; UBQ: Ubiquitin; 25S rRNA: 25S ribosomal RNA) and the respective curves for two targets (PFPα1: Pyrophosphate fructose-6-phosphate 1-phosphotransferase alpha subunit; AS: Glutamine-dependent asparagine synthetase).

TABLE 1: Potential reference genes and two targets, the respective accession number (NCBI), primer sequences and efficiencies in the RT-qPCR reactions

Gene	Accession	Description	Primer sequence (Forward/Reverse)	Tm (°C)	Product size (bp)	Regression Coefficient (R^2)	Amplification efficiency (%)
αTUB	CN607271	Alpha-tubulin	(F)CCATTGGCAAGGAGATTGTT (R)TCCACCAACTGCATTGAAGA	75.9	104	0.998	99.53
GAPDH	CA254672	Glyceraldehyde 3 phosphate dehydrogenase	(F)GGTTCACTTGAAGGGTGGTG (R)TGAGGTGTACCTGTCCTCGTT	81.8	100	0.984	100.89
H1	CA116806	Histone H1	(F)CGCACACGCACACTGAAAG (R)CGGTGGCCATGATCAAAAA	78.0	57	0.999	97.41
SAMDC	CA127376	S-adenosylmethionine decarboxylase	(F)TGCTGCTGAAGACGCTGTTG (R)TCGCCTTCAAAGCAGTGTAGAAC	79.0	60	0.992	99.66
UBQ	CA077378	Ubiquitin	(F)ACCGAAGGTTGCATTCAAGAC (R)GGGTTTGGGTCCGTTAGAAG	81.2	153	0.999	98.34
25S rRNA	BQ536525	25S ribosomal RNA	(F)GCAGCCAAGCGTTCATAG (R)CGGCACGGTCATCAGTAG	82.9	108	0.999	99.82
PFPα1	XM_004973200.1	Pyrophosphate fructose-6-phosphate 1-phosphotransferase alpha subunit	(F)CCAGAGAACACACCCACAAC (R)AGGCCACACTAGGACCTCCA	83.6	151	0.988	92.99
AS	FM212633.1	Glutamine-dependent asparagine synthetase	(F)TTATGAGTTGCTGCGAGAGAAG (R)TATCTCGATATCGCCCATGTAG	79.8	112	0.990	105.05

Tm: melting temperature (ºC)

TABLE 2: CT values of reference genes used for gene expression normalization of sugarcane roots under drought stress

Sample*	αTUB	GAPDH	H1	SAMDC	UBQ	25S rRNA
1	27.18	23.89	25.56	22.43	23.70	14.85
2	28.00	23.51	24.42	22.00	24.55	14.46
3	27.96	23.97	24.67	23.22	25.66	13.88
4	27.47	23.48	24.94	22.03	24.21	14.29
5	27.15	23.78	24.48	22.05	25.11	14.61
6	27.38	23.10	25.14	22.17	25.19	14.53
7	27.41	23.91	24.38	23.47	24.07	13.43
8	27.19	24.23	24.55	24.24	24.52	13.21
9	27.71	24.23	24.97	23.49	23.74	13.68
10	27.08	23.29	24.54	22.47	24.88	13.06
11	27.23	23.47	24.71	22.62	24.88	13.48
12	28.00	23.39	24.77	22.73	25.11	14.54
Average	27.48	23.69	24.76	22.74	24.64	14.00

*1-3: bulk of tolerant genotypes without stress (control); 4-6: bulk of tolerant genotypes under stress; 7-9: bulk of sensitive genotypes without stress (control); 10-12: bulk of sensitive genotypes under stress. αTUB: Alpha-tubulin; GAPDH: Glyceraldehyde 3 phosphate dehydrogenase; H1: Histone H1; SAMDC: S-adenosylmethionine decarboxylase; UBQ: Ubiquitin; 25S rRNA: 25S ribosomal RNA.

In order to test the stability of candidate reference genes, raw data was submitted to GeNorm [17], NormFinder [19] and BestKeeper [20] software analysis.

GeNorm Analysis. The measurement of gene expression stability for a recommended gene by the software GeNorm is based in the average pairwise variation (V) for that gene, when compared with all other tested. Moreover, the expression stability mean-value (M-value) is a parameter for quantification of steady endogenous gene candidates, in which a low M-value indicates more stable expression, making a certain candidate more appropriate for an endogenic gene control under evaluation [7].

Accordingly, GeNorm analyses of αTUB (M = 0.61), GAPDH (M = 0.62) and Histone H1 (M = 0.63) were the most stable while SAMDC represented the more variable (M = 0.87) gene. However, all genes showed high stability based on M-values below 1 (ranging 0.61 - 0.87; Figure 3), suggesting that all six candidates may be considered appropriate for normalizing expression data under the conditions described above. Therefore, based on the pairwise variation (V) data (Figure 3), it was possible to determine the optimal number of reference genes required for the relative quantitation assay and to systematically check the expression stability with the addition of other genes. Data suggested that the addition of the two most stable genes αTUB and GAPDH (Figure 4) and a random third gene (V2/3 = 0.15;

Figure 4), a fourth gene (V3/4 = 0.14) or even more (V4/5 and V5/6; Figure 4) still exhibited desirable values (below to the 0.15 proposed by Vandesompele et al. [17]). However, the combination of αTUB, GAPDH and H1 should be sufficient to normalize the mentioned sugarcane samples, considering individual performance of each gene (M < 1) (Figure 3).

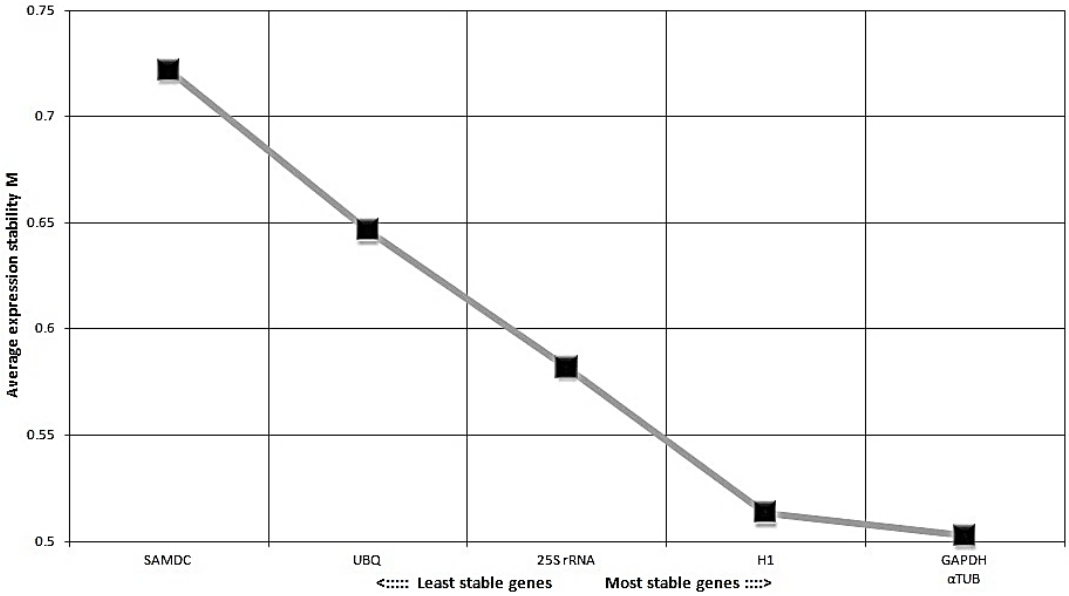

FIGURE 3: Average expression stability values (M) of six potential reference genes of sugarcane (αTUB: Alpha-tubulin; GAPDH: Glyceraldehyde 3 phosphate dehydrogenase; H1: Histone H1; SAMDC: S-adenosylmethionine decarboxylase; UBQ: Ubiquitin; 25S rRNA: 25S ribosomal RNA) based on the GeNorm analysis [17].

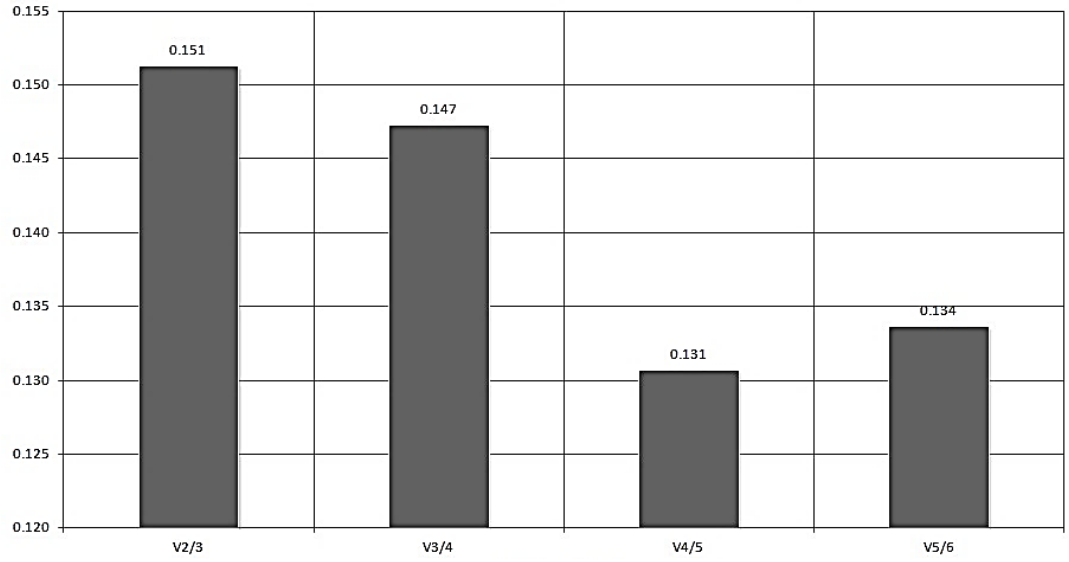

FIGURE 4: Pairwise variation (V) analysis for six potential reference genes of sugarcane (α-tubulin; Glyceraldehyde 3 phosphate dehydrogenase; Histone H1; S-adenosylmethionine decarboxylase; Ubiquitin; 25S rRNA) based on the GeNorm analysis [17]. The addition of the two most stable genes αTUB and GAPDH (Figure 4) of a random third gene (V2/3 = 0.15), a

fourth gene (V3/4 = 0.14) or even more (V4/5 and V5/64) still exhibited desirable (basically below than 0.15).

Normfinder and Bestkeeper Analysis. NormFinder is a tool based on an algorithm for identifying and ranking the most suitable normalization amounts from a set of candidate genes and estimating intra-group and inter-group variation. However, BestKeeper is a Microsoft Excel based software, using Ct values as inputs, which determine the most stable expressed genes by a Pearson correlation coefficient (geometric mean of Ct values of candidate genes), calculating the standard deviation (S.D) of Ct values among the whole data set.

The results using Normfinder and Bestkeeper softwares are presented in Table 3. The stability ranking provided by both softwares exhibited the same order: Histone < αTUB < GAPDH < 25S rRNA < UBQ < SAMDC (Table 3). The two most stable genes were Histone H1 (M = 0.28) and αTUB (M = 0.32), followed by GAPDH, 25S rRNA and UBQ with M-values of 0.37, 0.56 and 0.57, respectively. In contrast, SAMDC showed the highest instability (M = 0.61, Table 3), in agreement with GeNorm analysis results. Both softwares identified Histone H1 and αTUB as the most stable and thus reliable genes for normalization.

TABLE 3: Expression stability values for sugarcane candidate calculated by NormFinder and BestKeeper.

NormFinder analysis			**BestKeeper analysis**		
Ranking	**Gene name**	**Total**	**Ranking**	**Gene name**	**Total**
1	H1	0.28[1]	1	H1	(1.06±0.26)[2]
2	αTUB	0.32	2	αTUB	(1.06±0.29)
3	GAPDH	0.37	3	GAPDH	(1.33±0.31)
4	25S rRNA	0.56	4	25S rRNA	(3.89±0.55)
5	UBQ	0.57	5	UBQ	(2.04±0.50)
6	SAMDC	0.61	6	SAMDC	(2.53±0.57)

[1]Expression stability and ranking of six candidate HKG calculated by NormFinder. [2]Ranking of candidate HKG identified by the lowest values of the coefficient of variance (CV) and standard deviation (SD) using BestKeeper. αTUB: Alpha-tubulin; GAPDH: Glyceraldehyde 3 phosphate dehydrogenase; H1: Histone H1; SAMDC: S-adenosylmethionine decarboxylase; UBQ: Ubiquitin; 25S rRNA: 25S ribosomal RNA.

Despite the fact that all three softwares display distinct methodologies, results were quite consistent identifying H1, αTUB and GAPDH as the most stable genes for normalization of RT-qPCR data. More importantly, two of these genes (H1 and αTUB) are here reported as normalizing genes suitable for sugarcane under drought stress for the first time.

Normalization of two target genes. In order to confirm the applicability of previously recommended reference genes (Histone H1, αTUB and GAPDH), the relative expression of Glutamine-dependent asparagine synthetase (AS, EC 6.3.5.4) and Pyrophosphate fructose-6-phosphate 1-phosphotransferase alpha subunit (PFPα1, EC 2.7.1.90) genes were evaluated by RT-qPCR. AS is an important component of the asparagine synthesis, acting as a key member in the assimilation, nitrogen recycling and storage in higher plants [31]. PFPα1 is responsible for the addition of phosphate to the second D-fructose 6-phosphate in the glycolysis pathway [32] and is essential for maintenance of carbohydrate metabolism and other processes in plant cells [33].

HT-SuperSAGE analysis indicated that the SD282748 unitag (annotated as AS, TC127333) was up-regulated 1.92 times in the drought-tolerant bulk after 24h irrigation suppression as compared to the unstressed control. Simultaneously no relevant frequency change (at the $p < 0.05$) was observed with regard to the transcription levels of the sensitive accessions bulk after the drought stress stimulus. Relative quantification, using specific primers to this enzyme, was confirmed as being overexpressed. This indicated a 1.473 fold change considering the tolerant bulk as compared to its stressed control group (Figure 5A). However, no significant change (at the $p < 0.05$) was observed considering the relative quantification (0.86 times) of the susceptible bulk compared to its negative control (Figure 5B).

The SD179780 unitag, possibly a PFPα1 gene, did not respond ($p < 0.05$) to water deficit in any of the contrasts using the data of the four HT-SuperSAGE libraries analyzed. The results obtained in RT-qPCR also show constitutive expression of this gene and thus validate the HT-SuperSAGE data (Figure 5A and 5B).

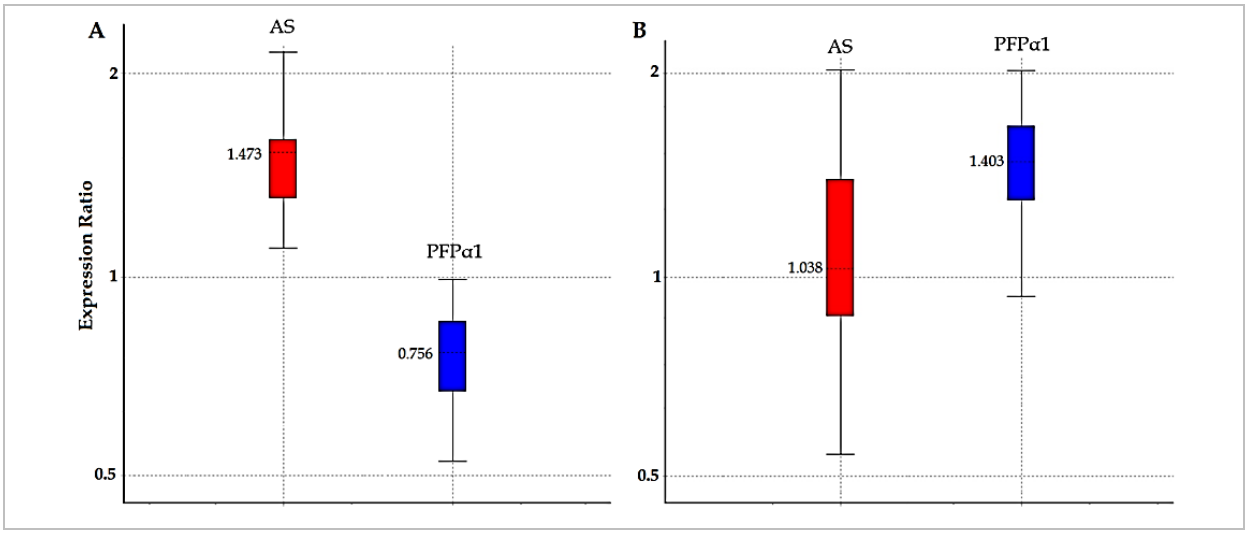

FIGURE 5: Relative quantification of Glutamine-dependent asparagine synthetase (AS) and Pyrophosphate-fructose 6-phosphate 1-phosphotransferase (PFPα1) in cDNA pools of sugarcane drought tolerant genotypes[CTC6, CTC15, SP83-2847 and SP83-5073] and drought sensitive genotypes [CTC9, CTC13, SP90-1638 and SP90-3414] under drought, after normalized by reference genes H1, αTUB and GAPDH using the REST software version 2.0.13. (A) Tolerant bulk compared to control. (B) Susceptible bulk compared to control. Relative expression with the median value (dotted horizontal at the colored box) and range (horizontal bars) comprising 100% of the observations, being 50% of them in colored box (confidence interval at 95%).

Discussion

RT-qPCR is currently one of the most used techniques for gene expression analysis, due to its rapid, specific and highly sensitive parameters. It allows amplicon quantification in each cycle of the reaction by issuing fluorescence [9]. However, problems with RNA samples variations, standardization and protocols efficiency (RNA extraction, RT and qPCR) have routinely been observed [34]. Furthermore, the selection of normalizing genes remains one of the most time consuming and difficult steps of RT-qPCR. It requires reference genes to be constitutively expressed also after external stimuli. Additionally, it needs to display little or no variation in different cell types or tissues, as well as in distinct developmental stages and experimental conditions [35-37]. Although some studies include reference genes for normalization without any validation of their expression stability, statistical algorithms present in softwares like GeNorm, Normfinder, and Bestkeeper have assisted in the selection of housekeeping genes (HKGs) suitable for normalization of expression profiling studies [38].

In the present study, six potential reference genes (αTUB, GAPDH, H1, SAMDC, UBQ, 25S rRNA) were evaluated by three methodologies (GeNorm, Normfinder and Bestkeeper) in order to assess their stability for normalization of expression profiles in sugarcane roots under drought stress (24h of continuous dehydration). With small changes in ranking order, all three statistical approaches pointed Histone H1, αTUB and GAPDH as the most stable candidate genes. Although some studies reported the participation of Histone H3 as a good normalizing gene in dicots such as pea [38], cotton [39] and chicory [40], there were no references for sugarcane. On the other hand, genes like GAPDH, αTUB, UBQ and 25S rRNA have been routinely used to normalize RT-qPCR data in several plant species, including grasses like rice [41, 42], wheat [43], *Brachypodium distachyon* [44] and bamboo [37].

The understanding of sugarcane physiology under different conditions remains under intensive research, due to socioeconomic importance of this crop and increasing unpredictability of environmental conditions worldwide. In this regard, gene expression analysis is an attractive approach to dissecting plant physiological response to stress conditions. Nevertheless, reference gene selection has received limited attention for RT-qPCR in sugarcane. Papini-Terzi et al. [21] described the transcriptional profile of signal transduction events indifferent sugarcane tissues, where HKGs were selected based upon the literature (Tubulin and Actin), microarray data, and EST sequencing from the SUCEST project (Polyubiquitin and 14-3-3 proteins). Rocha et al. [22] relied upon four reference genes (14-3-3, Polyubiquitin, GAPDH and 25S rRNA) to individually normalize gene expression in sugarcane under various conditions. Moreover, Rodrigues et al. [23] used ß-tubulin as the reference gene solely based upon previous data [24]. Although these methods are useful for prospecting candidate reference genes [21] or addressing gene expression using validated HKG for target tissues [22, 23], respectively, prospection followed by rigorous selection should be the method of choice for identification of novel reliable reference genes.

To our knowledge, Iskandar et al. [24] reported the only effort to prospect stable reference genes in sugarcane. The stability of four genes was evaluated (ß-

actin, ß-tubulin, GAPDH and 25S rRNA) in leaf, root and internode tissues of several sugarcane cultivars and representatives of *Saccharum* genus, but none of them under abiotic stress or using more suitable statistical approaches (not available in 2004). According to the authors, GAPDH was the more stable gene (CV = 51%) when comparing different tissues, followed by ß-actin and ß-tubulin (CV = 68% and 81%, respectively). When involving various species, ß-actin showed the lowest coefficient of variation (31%) followed by GAPDH (33%). In the present work, GAPDH was also one of the most stable genes, confirming it to be a suitable HKG for experiments involving sugarcane roots under water deficit conditions.

On the other hand, αTUB was indicated by GeNorm as the most stable gene in bulked genotypes, both under normal irrigation and after 24h of continuous dehydration. By Normfinder and Bestkeeper analysis, this gene was the second more appropriate HKG. However, Fan et al. [37] assessed the stability of reference genes in 14 different tissues and developmental stages of *Phyllostachys edulis* and observed that αTUB showed a larger variation (M = 1.94) among all candidates. Similar results were reported by Zhong et al. [45] with litchi (*Litchi chinensis* Sonn.) under several experimental conditions (tissues, organs, developmental stages and varieties), whereas αTUB was the most unstable gene among 10 candidates. Under biotic and abiotic stresses αTUB also displayed instability, as demonstrated by Die et al. [38], Hong et al. [44] and Zhu et al. [46]. Thus, this analysis is extremely useful to select suitable reference genes to normalize gene expression in sugarcane and other species, because reference genes may be differently regulated in diverse plant species, displaying distinctive gene expression patterns [41].

The 25S rRNA gene was the most abundant transcript (Ct 13.06), in agreement with results previously obtained from rice and sugarcane (Ct values of 15 and 16.6, respectively [24, 42]). Despite this, the gene was not one that proved to be stable HKGs. This can be explained by the fact that rRNA comprises the majority of total RNA present in a cell and therefore, requires further dilution for its use in RT-qPCR approaches [41]. Furthermore, the abundance of transcripts can affect the stability and consequently, the normalizing results for the reference gene candidates [47].

Accordingly, the 25S rRNA showed the highest variation in the present assay (Table 3).

Three software analyses indicated SAMDC as the most variable reference gene (M = 0.87) tested. However, Hong et al. [44] reported SAMDC as the most stable in grass *Brachypodium distachyon*, when evaluated under four abiotic stress conditions [high salt (300 mM), cold (4°C for 5h) heat (42°C for 2h) and drought (400 mM mannitol). In addition, Li and Chen [48] when describing SAMDC as a gene of interest (target) verified that this gene was induced in roots of rice seedlings at three leaf stages (after application of 171 mM salt for 24h, 20 mM exogenous abscisic acid (ABA), and dehydration using 15% PEG6000). These results highlight the need to select suitable reference genes for each experimental condition, especially under stress conditions.

Selected reference genes (H1, αTUB and GAPDH) were here used to validate the expression of two differentially expressed transcript (AS and PFPα1) by HT-SuperSAGE analysis. These results support those found in a previous microarray study showing AS overexpression in a wheat accession considered tolerant to drought after 36 h of irrigation suppression [49]. Transcription levels detected by RT-PCR using mRNA samples from roots and shoots (two week old plantlets) were significantly induced after salinity (250 mM) and osmotic stress [using mannitol 5.0% (w/v)] and exogenous abscisic acid (ABA) application (20 mM) [50]. Altogether, these results indicate the gene AS as a valuable target for future use in sugarcane breeding programs. Considering PFPα1, the constitutive expression observed in the present work is in agreement with Lim et al. [33] that demonstrated by semiquantitative RT-PCR that the expression of PFPα1 in wild-type Arabidopsis was also constitutive in different tissues (roots, leaves and flowers) and also distinct developmental stages (15, 25, and 45 days after planting). In sugarcane, PFPs are known to play an important role in sucrose accumulation, especially in immature and metabolically active tissues, taking part in glycolysis and in carbon compartmentalization [51]. Thus, PFP could be a valuable target or a reference gene for sugarcane roots, since its constitutive expression has been reported previously, being also confirmed in the present assay.

Conclusions

Among the HKG candidates, six reference genes (αTUB, GAPDH, H1, SAMDC, UBQ and 25S rRNA) tested with cDNAs from sugarcane roots under drought stress, revealing αTUB, H1 and GAPDH as the best HKGs for normalization of sugarcane expression profiling after three software analyses (GeNorm, Normfinder and Bestkeeper). With the successful identification of the selected HGKs, it was possible to validate two unitags (26 bp) annotated as Glutamine-dependent asparagine synthetase and Pyrophosphate-fructose 6-phosphate 1-phosphotransferase using a bulk of cDNAs relative to the drought-tolerant sugarcane accessions, confirming the HT-SuperSAGE data, with the aid of the REST software (Relative Expression Software Toll, v.2.0.13). These HKGs will be useful for dissecting gene expression in sugarcane roots, particularly in ongoing transcriptomic studies using SuperSAGE or RNAseq approaches, being H1 and αTUB reported for the first time for sugarcane.

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Conflict of Interests

The authors have declared that they have no conflict of interests.

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5 Considerações Finais

De acordo com os resultados obtidos pode-se ressaltar que:

1. A técnica de SuperSAGE utilizada no presente estudo, apresentou-se como uma ferramenta eficiente na identificação de candidatos potenciais na resposta de defesa à seca da cana-de-açúcar, e poderá auxiliar programas de melhoramento genético da cultura;

2. O uso de ferramentas de bioinformática e diferentes bancos de dados permitiu detectar nos transcriptomas avaliados, representantes das quatro subfamílias de aquaporinas descritas em vegetais superiores; sugerindo que a metodologia utilizada para a anotação e categorização obteve sucesso, podendo ser aplicada em outras espécies e particularmente, em estudos transcriptômicos que utilizem as abordagens de SuperSAGE ou RNA-Seq;

3. A maioria dos genes validados via RT-qPCR apresentaram respostas exclusivas e diferencialmente significativas entre os genótipos tolerantes e sensíveis à seca, apresentando potencial para o desenvolvimento de marcadores moleculares funcionais para aplicação em programas de melhoramento genético da cana-de-açúcar;

4. Os genes normalizadores selecionados neste estudo foram utilizados com sucesso nas análises de validação dos dados SuperSAGE via RT-qPCR, propriciando resultados consistentes e reprodutíveis, e poderão contribuir para estudos posteriores que abordem expressão gênica em raízes de cana-de-açúcar;

5. H1, αTUB e de GAPDH foram considerados como os HKGs mais adequados para estudos de RT-qPCR em genótipos de cana-de-açúcar tolerantes e sensíveis ao déficit hídrico, sob condições de supressão de rega por 24h, sendo H1 e αTUB relatados pela primeira vez para a espécie;

6. Os *primers* PIP1-1 e PIP1-3/PIP1-4 podem vir a se tornar marcadores associados à tolerância a estresse hídrico em acessos de cana-de-açúcar. É necessário, contudo, validar a expressão desses genes em outros clones tolerantes;

7. O genótipo SP83-2847 pode ser utilizado como referência para seleção ao estresse hídrico, podendo também, dependendo de seu valor como recurso genético industrial, ser utilizado como progenitor em programas de melhoramento da cana-de-açúcar.

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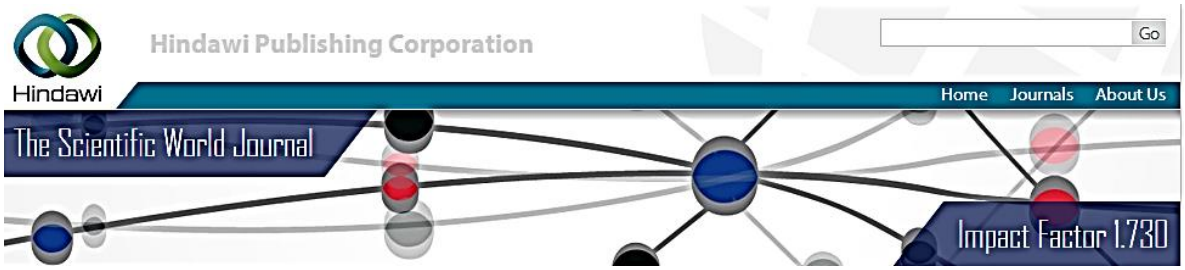
ANEXO B - Normas para submissão (The Scientific World Journal)



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