UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

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"ACC SINTASE EM CANA-DE-AÇÚCAR: ESTUDOS DE REGULAÇÃO GÊNICA E APLICAÇÃO BIOTECNOLÓGICA"

Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Genética e Biologia Molecular, na área de Genética Vegetal e Melhoramento.

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Campinas – SP

2011

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

T197a

Tavares, Rafael Garcia
ACC sintase em cana-de-açúcar: estudos de regulação gênica e aplicação biotecnológica / Rafael Garcia Tavares. – Campinas, SP: [s.n.], 2011.

Orientador: Marcelo Menossi Teixeira.
Dissertação (mestrado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. ACC sintase. 2. Etileno. 3. Maturação. 4. Cana-de-açúcar. I. Teixeira, Marcelo Menossi. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

(rcdt/ib)

Título em inglês: Sugarcane ACC synthase: studies of gene regulation and biotechnology application.
Palavras-chave em inglês: ACC synthase; Ethylene; Maturation; Sugarcane.
Área de concentração: Genética Vegetal e Melhoramento.
Titulação: Mestre em Genética e Biologia Molecular.
Banca examinadora: Marcelo Menossi Teixeira, Adriana Silva Hemerly, Marlene Aparecida Schiavinato.
Data da defesa: 23/02/2011.
Programa de Pós-Graduação: Genética e Biologia Molecular.

Campinas, 23 de fevereiro de 2011

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Dedicação exclusiva deste trabalho à minha avó Tereza Vetere Garcia (*in memorium*) pelo seu imenso amor, carinho, atenção e dedicação maternal. Através da sua vida exemplar, hoje colhemos mais um fruto.

Agradecimentos

À DEUS, pela minha vida, tranquilidade, coragem e a graça desse imenso privilégio de estudar na UNICAMP.

Ao Prof. Dr. Marcelo Menossi, uma pessoa fantástica e um exemplo de formação profissional e pessoal. Obrigado pela sua confiança, pela sua orientação e por me proporcionar a oportunidade de fazer parte de sua equipe.

Aos membros da pré-banca examinadora, o Prof. Dr. Luís Eduardo Aranha Camargo e o Prof. Dr. Michel Vicentz pelas críticas e sugestões.

Aos membros da banca examinadora, a Profa. Dra. Marlene A. Schiavinato e a Profa. Dra. Adriana Silva Hemerly pelas críticas e sugestões.

À toda equipe do Laboratório Genoma Funcional, meus companheiros e amigos de bancada, Daniela, Agustina, Diego, Kevin, Wilson, Tiago, Éder, Fábio, Ilse, José Sérgio, José Pedro, Thaís, César, Andrea, Edna, Giane, Lúcia, Grazi e Raphael.

Aos pesquisadores e amigos que me ajudaram muito e contribuíram na minha formação profissional, Agustina Gentile, Juliana Felix, Renato Vicentini e Sandra Camargo.

Aos meus grandes amigos da graduação, que sempre me proporcionaram momentos de apoio, torcida, tranquilidade, alegria e risadas, Maicon Grella e Daniela Feltrim.

À minha esposa Camila, pelo seu companheirismo, apoio, amor, carinho, atenção e compreensão. Muito obrigado!!!

À minha amada família por estar sempre ao meu lado. Obrigado pela confiança, pelo incentivo, pelo apoio e principalmente por todo o cuidado, carinho e amor. Pai, Mãe, Avó, Joel, Vera, Cris, tia Toninha e tia Edna, obrigado por tudo!!

Ao Programa de Pós-Graduação em Genética e Biologia Molecular.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPESP) pela bolsa de estudos concedida durante esse mestrado.

“Escolhe um trabalho de que gostes, e não terás

que trabalhar nem um dia na tua vida.”

Confúcio

Índice

Índice de Ilustrações

Introdução

Capítulo I

Índice de Ilustrações

Capítulo II

Índice de tabelas

Capítulo I

Capítulo II

Lista de Abreviaturas

SUCEST	Sugarcane EST project
SUCAST	Sugarcane signal transduction
ACS	Ácido 1-carboxílico-1-aminociclopropano sintase
ACO	Ácido 1-carboxílico-1-aminociclopropano oxidase
SAM	S-adenosil-metionina
RING	Really interesting new gene
E3	Ubiquitin-protein ligase
qRT-PCR	Quantitative real-time polymerase chain reaction
SD	Synthetic dropout medium
DDO	Double dropout medium
QDO	Quadruple dropout medium
GFP	Green fluorescent protein
PLP	Pyridoxal-5'-Phosphate
ETO1	Ethylene overproducer 1
TOE	Target of ETO1
SUMO	Small ubiquitin-like modifier
X-α-Gal	X-α-D-Galactoside
AbA	Aureobasidin A
CDPK	Calcium-dependent protein kinases
ERF	Ethylene-responsive element-binding factor
ATL	Arabidopsis toxicos para levedura

Resumo

A cultura da cana-de-açúcar (*Saccharum spp*) está entre as mais importantes do Brasil, tendo o setor sucroalcooleiro grande importância econômica no país devido ao crescente consumo de álcool e açúcar. A fase de maturação no desenvolvimento da cana-de-açúcar é especialmente interessante, pois está diretamente relacionada com o teor de sacarose. O hormônio etileno está envolvido em muitos aspectos do ciclo de vida da planta, incluindo a maturação. Como primeira parte deste projeto, o objetivo foi avaliar a relação entre etileno e maturação de cana-de-açúcar pela caracterização da expressão dos genes que codificam as isoformas da enzima ACC sintase, responsável por uma das vias da biossíntese desse hormônio. Assim, dois genes (*Scacs1* e *Scacs2*) que codificam para a enzima ACC sintase foram identificados no projeto SUCEST e caracterizados. Nossos resultados de localização subcelular confirmaram estudos anteriores sobre a localização citoplasmática das proteínas ACS. Todavia, nós identificamos por análise *in silico* a presença de um peptídeo sinal na região N-terminal da proteína ScACS2. Ensaios de duplo-híbrido com bibliotecas de cDNAs a partir de tecidos de folha +1 e entrenó 5, nos revelaram novas interações putativas com a proteína ScACS2, sendo uma delas, a proteína denominada sinaptotagmina envolvida no mecanismo de reparo da membrana celular. Ao contrário do que foi observado em Arabidopsis, nossos resultados de heterodimerização não identificaram uma interação entre as isoformas de cana-de-açúcar. Análise de expressão por PCR quantitativo em duas variedades contrastantes ao teor de sacarose, identificaram uma maior expressão nos entrenós madurados quando comparados aos entrenós jovens para os dois genes ACS. Em uma estudo *in situ* da expressão, o gene *Scacs1* apresentou uma expressão nas células do parenquima e nas células da bainha do feixe

vascular, entretanto, não mostrou expressão nos feixes vasculares. Por outro lado, o gene *Scacs2* apresentou expressão constitutiva nos diferentes tipos celulares e, inclusive, nos feixes vasculares. Os resultados apresentados nesse trabalho, abrem novas perspectivas e perguntas para os diversos mecanismos fisiológicos e celulares realizados pelo hormônio etileno nas plantas.

Todavia, uma prática comum antes da colheita da cana é a aplicação de precursores do hormônio etileno, como o etefon (ácido 2-cloroetil fosfônico) para acelerar o amadurecimento final das plantas, inibir o florescimento e consequentemente aumentar o teor de sacarose. Assim, a manipulação da biossíntese desse hormônio tem o potencial para aumentar a produtividade em cana. Desta forma, um segundo objetivo deste projeto foi desenvolver uma estratégia para promover o amadurecimento controlado da cana-de-açúcar no final da safra, usando o etanol em substituição aos análogos químicos do etileno. Para tal, foi avaliado o uso de um promotor ativado por etanol (sistema de expressão *alc* de *Aspergillus nidulans*) controlando a expressão do gene que codifica a ACC sintase, enzima chave na biossíntese de etileno. Nossos resultados foram condizentes com o esperado, mostrando claramente um aumento na produção do hormônio etileno quando plantas transgênicas de tabaco foram induzidas por spray foliar com 5% (v/v) de etanol. Esses resultados demonstram a possível utilização de ferramentas biotecnológicas no controle da expressão de genes envolvidos em características agrícolas interessantes, tais como na cultura de cana-de-açúcar.

Abstract

Sugarcane crop (*Saccharum spp*) is among the most important of Brazil, due to its economic importance in the country with the increasingly consume of its by-products such as the ethanol and sugar. Throughout development of the sugarcane, the maturation phase is especially interesting, whereas it is highly related to sucrose accumulate. The ethylene hormone is involved in several physiological processes of plants, including the maturation. As first part of this project, the goal was evaluated the correlation between ethylene hormone and sugarcane maturation through characterization of the genes expression encoded ACS enzymes, involved in biosynthesis pathway of this hormone. Thus, two genes (*Scacs1* and *Scacs2*), which encoding the ACS enzyme, were identified in SUCEST project and characterized. Our results of subcellular localization confirmed previous studies regarding to cytoplasmic localization of ACS proteins. Nonetheless, we identify through *in silico* analyses the presence of a signal peptide in the N-terminal region of the ScACS2 protein. Yeast two-hybrid assay using cDNAs libraries from leaf +1 and internode 5 tissues, revealed putative interactions with the ScACS2 protein, being one of them, a protein called plant synaptotagmin involved in the mechanism of cell membrane repair. Different from Arabidopsis, our results about heterodimerization did not identify an interaction between sugarcane ACS isoenzymes. Expression analyses of quantitative PCR in two sugarcane varieties contrasting to Brix content presented a higher expression of ACS genes in mature internodes than young internodes. *In situ* analyses for *Scacs1* gene showed an expression in parenchyma cells and bundles sheath, however, none expression was observed in vascular bundles. On the other hand, the *Scacs2* gene presented an ubiquitous expression in the different

cell types and, including in vascular bundles. Our results presented in this work, open further perspectives and issues about the underlying mechanisms by ethylene hormone in plants.

Nevertheless, a common practice before harvest of sugarcane is the application of precursors of ethylene hormone, such as ethephon (2-chloroethyl- dioxido-oxophosphorane) to accelerate plants ripening, delay opening of the inflorescence and to increase sucrose accumulate. As a result, the manipulation of biosynthetic pathway of this hormone is a potential tool to increase the productivity in sugarcane. Thus, other goal of this work was develop a strategy to promote a controlled ripening of sugarcane at the end of harvest, using the inductor ethanol instead of ethylene precursor. To achieve this goal, an ethanol-inducible system based on *alc* switch of the ascomycete fungus *Aspergillus nidulans* were evaluated controlling gene expression of ACS and consequently the production of the ethylene hormone. Our results were consistent with the expected, showing clearly an increase in the production of ethylene hormone when transgenic tobacco plants were induced for the leaf spray with 5% (v/v) ethanol. This result shows the possible utilization of biotechnology tools in the control of gene expression involved in important agricultural traits, such as in the sugarcane culture.

Introdução

1. Acumulação de açúcar em cana-de-açúcar

A maturação é influenciada por uma variedade de fatores incluindo umidade, nitrogênio, incidência da luz solar, altitude, latitude, temperatura, idade do cultivo e genótipo (Mamet and Galwey, 1999). É também uma das últimas fases dos processos fisiológicos da planta e está diretamente relacionada ao acúmulo de açúcares. Na cana-de-açúcar, a maturação ocorre da base para o ápice do colmo. A cana imatura apresenta teores de sacarose bastante distintos entre os segmentos da base e do ápice (Rodrigues, 1995).

A biologia do acúmulo da sacarose em cana-de-açúcar não é importante somente na agronomia, mas também proporciona um sistema fisiológico interessante para o estudo da quebra do carboidrato (Rae et al., 2005). O acúmulo de sacarose tem sido estudado mais em cana-de-açúcar do que em qualquer outra planta, devido à grande concentração de sacarose que é obtida em seu sistema considerado simples. A característica de simplicidade de estocagem da sacarose na cana-de-açúcar é o fato de que este açúcar é produzido na folha, translocado no floema e estocado no colmo. Assim, não é necessário levar em consideração a síntese e o metabolismo de carboidratos de estocagem alternativos, nem considerar a estocagem em estruturas reprodutivas especializadas como frutas e sementes (Moore, 1995).

A cana-de-açúcar é uma gramínea C4 que pode acumular sacarose no caule (colmo) em níveis excedendo 50% da massa seca (Rae et al., 2005). Em uma análise de folha de cana-de-açúcar exposta a $^{14}CO2$, foi sugerido que a sacarose fotoassimilada era translocada das células do mesófilo da folha até os vários tecidos drenos sem a sua quebra e ressíntese (Moore, 1995).

Nesses tecidos drenos, a sacarose pode ser importada para as células através do plasmodesmata (transporte simplástico) ou da parede celular e espaços intercelulares dos tecidos (transporte apoplástico) (Rolland et al., 2006). Em cana-de-açúcar cada feixe vascular está contido numa bainha de fibra celular que se torna altamente lignificada com o desenvolvimento. Pela coloração de tecidos de cana-de-açúcar com berberina, pode-se mostrar que a parede celular em volta do feixe provavelmente é suberizada. Alinhando essas observações com o perfil da concentração de sacarose no tecido, sugere-se que o movimento apoplástico de solutos através dessas camadas de células seria severamente restrita durante o período de máxima acumulação de sacarose (Rae et al., 2005). Assim, a via de acúmulo de sacarose começa com a sua translocação através do elemento crivado do floema para os entrenós do caule (Moore, 1995) por uma via predominantemente simplástica (Rae et al., 2005).

2. Controle da maturação pelo hormônio etileno

A maturação é um complexo processo, mais conhecido em frutos, sendo caracterizado por um número de processos bioquímicos e fisiológicos que alteram a coloração, textura, sabor e aroma do fruto (Nishiyama et al., 2007). Frutos com diferentes mecanismos de maturação têm sido classificados como climatéricos, nos quais a maturação é acompanhada por um pico na respiração e uma concomitante produção de etileno; e não-climatéricos, nas quais a respiração não mostra nenhuma mudança drástica e a produção de etileno continua num nível muito baixo durante a maturação (Liu et al., 1999; Alexander and Grierson, 2002). Em frutos climatéricos, tais como tomate, abóbora, abacate, banana, pêssego, ameixa e maça (Giovannoni, 2001), o etileno realiza uma importante função na maturação em que uma produção maciça desse hormônio é iniciada no começo do período climatérico respiratório (Liu et al., 1999), embora

frutos não-climatéricos tais como os citrus, possam responder também ao hormônio etileno (Alonso et al., 1995). A produção do etileno endógeno é uma parte essencial da maturação do fruto climatérico e provavelmente atua como um reostato para processos dependentes de etileno (Watkins, 2006). Na maturação de frutos climatéricos, ambos ACC sintase e ACC oxidase são induzidos e contribuem para a regulação da biossíntese do etileno (Liu et al., 1999).

O etileno é produzido pela maioria dos tecidos da planta (Stearns and Glick, 2003), estando no estado gasoso sob condições fisiológicas normais (Theologis, 1992). Sua produção varia de quase zero a mais de 500 nl/g de tecido de planta por hora, mas sua atividade biológica é observada até mesmo quando sua concentração é muito baixa, da ordem de 10-100 nl/l de ar (Theologis, 1992).

Esse hormônio apresenta diversas funções regulatórias no crescimento e desenvolvimento das plantas (Kende, 1993). Como um hormônio de senescência, promove o amarelamento das folhas, a maturação das folhas climatéricas e a abscissão das folhas e flores. Como um hormônio de estresse, está envolvido na resposta de estresse biótico e abiótico de plantas (Wang et al., 2005), sendo sua produção nos tecidos induzida por uma variedade de fatores, incluindo ferimento, tratamento com auxina, frio, anaerobiose, patógenos e íons Li^+ (Yamagami et al., 2003).

Em cana-de-açúcar existem poucos estudos detalhados na literatura sobre a relação do hormônio etileno com o controle da maturação e o acúmulo da sacarose nos colmos. Entretanto, é uma prática comercial comum em canaviais a aplicação de etefon, (ácido 2-cloroetilfosfônico), que é degradado a fosfato, cloreto e etileno quando em contato com células vegetais. Seu uso abrange várias espécies de plantas como maçã, cevada, trigo, café, algodão, uva, abacaxi, etc (Thomson, 1992).

Em um dos estudos em cana foram analisados os efeitos do tratamento com etefon no florescimento e na produção de açúcar em cana-de-açúcar, sendo observado uma redução de 20% do florescimento e um aumento de 10% na produção de açúcar, ambos em relação ao campo controle (Moore and Osgood, 1989). Dados obtidos no Estado de São Paulo por Salata (1992), citada por Caputo (2006) mostraram que a aplicação de etefon causou um aumento entre 6 e 8% (pol % cana). Pontin (1995) também descreveu aumentos significativos de pol % cana decorrentes da aplicação de etefon. Mais recentemente, Castro et al. (2001) e Caputo (2006) observaram aumento do teor de sacarose, redução da isoporização dos entrenós e antecipação de 21-30 dias da colheita. A aplicação do etefon causa paralisia do crescimento vegetativo do meristema intercalar (nos entrenós), com consequente aumento do açúcar armazenado nos entrenós (Martins and Castro, 1999). No entanto, estudos mais aprofundados em termos de fisiologia e genética não foram encontrados na literatura científica.

O índice de maturação (IM) da cana-de-açúcar é determinado em função da porcentagem de sólidos solúveis do caldo (Brix) correlacionado ao teor de sacarose e é usado para determinar o ponto de maturação e a época da colheita. A aplicação de etefon ou aumento da síntese de etileno endógeno, motivada por aplicações de reguladores vegetais, herbicidas ou estresses de qualquer natureza, levam à inibição do crescimento do colmo e ao seu engrossamento, contribuindo para o aumento do teor de sacarose no colmo e a antecipação da colheita (Rodrigues, 1995).

3. A biossíntese do etileno

A biossíntese desse fitormônio está indicada na Figura 1 e começa com a conversão do aminoácido L-metionina para o composto S-adenosil-metionina (SAM) pela ação da enzima S-adenosil-metionina sintase (Stearns and Glick, 2003). Após esse processo, a enzima ácido 1-carboxílico-1-aminociclopropano sintase (ACS) converte SAM em ácido 1-carboxílico-1-aminociclopropano (ACC) e 5-Metil-tio-adenosina (MTA), sendo que este é reciclado para L-Metionina pelo ciclo de Yang (Stearns and Glick, 2003). Esse ciclo permite altas taxas da produção de etileno sem grandes concentrações intracelulares de metionina (Jakubowicz, 2002). Esse ciclo é um passo importante na via biossintética, já que a enzima ACS é extremamente lábil. Devido a essa característica, a enzima ACS é o passo limitante para os níveis de etileno nos tecidos das plantas (Stearns and Glick, 2003), diferentemente das enzimas S-adenosil-metionina sintase, que é constitutivamente sintetizada e também participa em outras reações, tais como a metilação de lípidios e proteínas e a síntese de poliaminas, e também a ácido 1-carboxílico-1-aminociclopropano oxidase (ACO) que geralmente é constitutivamente expressa (Jakubowicz, 2002). Finalmente, o ACC é oxidado pela enzima ACC oxidase (ACO), para formar etileno, CO_2 e cianeto, do qual é detoxificado para β-cianoalanina pela β-cianoalanina sintase (β-CAS) (Wang et al., 2002).

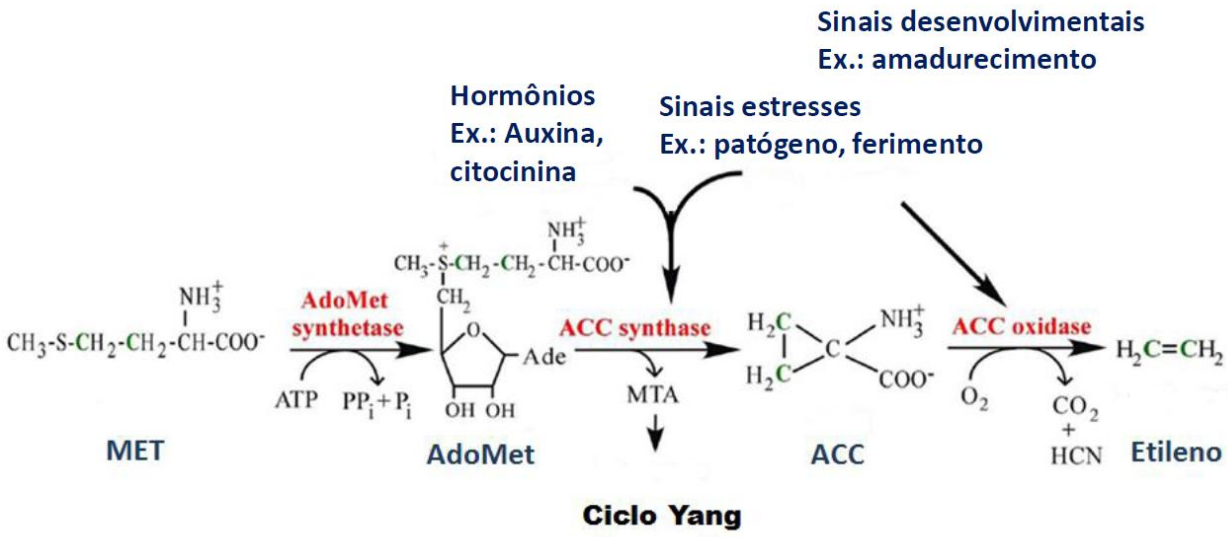

Figura 1- A via biossintética do etileno. MET - metionina, AdoMet - S-adenosilmetionina, ACC - ácido 1-carboxílico-1-aminociclopropano, ACC sintase - ácido 1-carboxílico-1-aminociclopropano sintase, ACC oxidase - ácido 1-carboxílico-1-aminociclopropano oxidase, MTA - 5-Metil-tio-adenosina. Alguns sinais de regulação transcricional e pós-transcricional são mostrados atuando nas enzimas ACC sintase e ACC oxidase. Modificado de Argueso et al., (2007)

4. A ACC sintase

A ACC sintase, localizada no citoplasma (Jakubowicz, 2002), é codificada por uma família multigênica, cujos membros assemelham-se estruturalmente à família subgrupo-I das aminotransferases dependente-5`-fosfato piridoxal (PLP) (Wang et al., 2002).

Apesar das características polimórficas da família multigênica da ACC sintase, seus membros têm estruturas primárias bastante similares. Apesar do tamanho proteíco entre 441 a 496 aminoácidos, todas as enzimas apresentam sete regiões fortemente conservadas (Wong et al., 1999) e 11 resíduos de aminoácidos invariantes em seu subgrupo, incluindo 4 resíduos conservados (Gly-197, Asp-222, Lys-258 e Arg-386) presentes em todas as aminotransferases, às quais as enzimas ACS apresentam semelhança estrutural (Tsuchisaka and Theologis, 2004).

Alguns artigos recentes indicam que a estabilidade da atividade da ACC sintase pode ser regulada, com uma meia-vida oscilando de 20 minutos a 2 horas (Chae and Kieber, 2005). A expressão dos polipeptídeos da ACC sintase de maçã, abobrinha e tomate em *E. coli* produziu uma enzima funcional, de 52, 53 e 54 kDa respectivamente (Li et al., 1996). Além disso, quando comparada a massa da enzima purificada com a tradução *in vitro*, em vários casos, o produto *in vitro* é maior em 8-9 kDa, aumentando a possibilidade que a proteína seja regulada pós-traducionalmente (Nakajima et al., 1988). A estrutura quartenária da ACS é controversa (Huxtable et al., 1998). Diferenças existem na literatura em se a enzima natural existe como um monômero ou dímero (Li and Mattoo, 1994).

A região N-terminal da ACS é caracterizada por dois resíduos, leucina e serina, altamente conservados entre todas ACC sintase funcionais descritas até o momento (Jakubowicz, 2002). No entanto, as regiões carboxi-terminal de todas ACC sintase conhecidas são bastante divergentes e consistem em uma região hipervariável de 18-85 resíduos (Wong et al., 1999). Entretanto, o domínio não conservado em quanto à sequência de aminoácidos é altamente conservado num aspecto: sua rede de carga positiva (Li and Mattoo, 1994). Essa região variável não é importante para a atividade da enzima, mas serve como um domínio regulatório responsável pela regulação pós-traducional da enzima (Tsuchisaka and Theologis, 2004).

Estudos fisiológicos e farmacológicos têm sugerido o envolvimento da fosforilação no controle pós-traducional da atividade da ACC sintase (Spanu et al., 1994). Tatsuki and Mori (2001) observaram que a ACS de tomate, LeACS2, é fosforilada na região carboxi-terminal conservada entre as várias isoformas da ACC sintase por uma atividade quinase induzida por ferimento, sugerindo o envolvimento da regulação pós-traducional. Bostick et al., (2004)

demonstraram uma inibição da atividade ACS pela família protéica ETO1 dependente de função proteossômica.

Adicionalmente, usando a técnica de duplo-híbrido, foi revelado que a interação com a proteína ETO1 é restrita a um grupo de ACC sintase (tipo 2), as quais possuem uma sequência de aminoácidos C-terminal específica. Essa sequência consenso das ACS tipo 2 compreende os motivos WVF (triptofano-valina-fenilalanina) e RLSF (arginina-leucina-serina-fenilalanina) e uma região rica em R/D/E [rica em aminoácidos ácidos (ácido aspartico ou ácido glutâmico) e básico (arginina ou histidina)]. Yoshida et al., (2005) identificaram uma seqüência alvo em Le-ACS3 chamada TOE (alvo de ETO1) que incluem os motivos WVF, RLSF e ricos em R/D/E. Estudos de proteínas quiméricas revelaram que a TOE atua como um sinal para a degradação protéica em células de planta dependente de ETO1. Em células de levedura, a adição dessa sequência consenso C-terminal em ACS tipo 1, LE-ACS2, conferiu uma forte habilidade em ligar a proteína ETO1 (Yoshida et al., 2005).

Muitos estudos têm detalhado as diferentes respostas transcricionais da família do gene da ACS, mas existem poucos dados a respeito da função do mecanismo pós-transcricional da ACS no controle da biossíntese do etileno (Chae and Kieber, 2005). Em todas as espécies de plantas examinadas, a ACC sintase é codificada por uma família multigênica, sendo que cada gene é diferencialmente expresso durante o desenvolvimento e em resposta ao estímulo ambiental (Liang et al., 1992).

Vários genes da ACC sintase têm sido identificados e clonados de diferentes espécies de plantas, incluindo tomate, cravo, abobrinha, arroz, batata, tabaco, banana, feijão, alface e *Arabidopsis thaliana* (Wang et al., 2002). O genoma de Arabidopsis contém nove genes da ACS, que codificam oito proteínas funcionais e uma não funcional (Chae and Kieber, 2005), oito genes

em tomate (Matarasso et al., 2005), seis genes em feijão, cinco em batata e cinco em arroz (Wang et al., 2005). Os genes da ACC sintase são induzidos em tecidos específicos tais como hipocótilo, folha, raiz, tubérculo, pecíolo, pétalas da flor, pistilo, estame, e fruto ou em resposta a fatores ambientais bióticos e/ou abióticos como radiação, Cu^{2+}, Li^{+}, ozônio, ferimento, ácido amino-oxiacético, ciclohexamida, EIX, inibidor proteína Kinase, anaerobiose por inundação, etileno, IAA, benziladenina, frio e patógenos (Wang et al., 2005). A expressão de cada membro da família multigênica da ACS é também diferentemente regulada em resposta para esses fatores (Wang et al., 2005). Por um lado, um membro da família do gene *ACS* é capaz de responder a numerosos sinais ambientais e desenvolvimentais (Ge et al., 2000). Por outro lado, um simples sinal relacionado ao desenvolvimento ou fator ambiental é capaz de induzir a expressão coordenada de vários genes ACS (Schlagnhaufer et al., 1997).

Não há qualquer relato na literatura caracterizando os genes da ACC sintase de cana-de-açúcar.

5. Uso de indutores químicos

O controle da expressão de transgenes em plantas é um ponto importante para caracterização da função dos genes em estudo bem como para fins biotecnológicos, permitindo sua manipulação durante o crescimento e desenvolvimento da planta (Roslan et al., 2001). A expressão controlada de genes de interesse utilizando promotores induzíveis apresenta importantes vantagens: permite que a atividade gênica seja induzida em determinadas fases do desenvolvimento da planta; possibilita modular a expressão e então determinar a resposta da planta para diferentes níveis do produto gênico; e permite que a expressão possa ser usada para determinar se o efeito fenotípico é reversível ou permanece estável em estágios específicos do

desenvolvimento (Garoosi et al., 2005). Deste modo, sistemas de expressão gênica que dependem de indução química têm sido desenvolvidos para o uso em plantas (Gatz and Lenk, 1998; Jepson et al., 1998; Zuo and Chua, 2000).

6. O Sistema *alc*

Um sistema de expressão controlado por etanol foi desenvolvido por Salter el al., (1998). Esse sistema está baseado no gene *alc*, do fungo *Aspergillus nidulans*, que usa o etanol como indutor (Pateman et al., 1983). O etanol é um indutor químico e não tóxico nos níveis requeridos para a indução. Além disso, é de fácil utilização, podendo ser aplicado nas plantas por pulverização, submersão das raízes ou adicionado ao meio de cultura (Salter et al., 1998).

A indução por etanol ocorre pela ação do gene *alcR* , cuja proteína ativa a expressão de dois genes estruturais *alcA* e *aldA,* que codificam a álcool desidrogenase I e aldeído desidrogenase, respectivamente (Pateman et al., 1983). O gene alcR é positivamente auto-regulado, necessitando da proteína AlcR funcional mesmo para a expressão em níveis basais (Fillinger et al., 1995). Da mesma forma, para a ativação dos genes *alcA* e *aldA* é preciso a presença do etanol para que a proteína AlCR seja capaz de se ligar às regiões promotoras desses genes e permitir a transcrição.

A figura 2 mostra o funcionamento do sistema. O gene *alcR* é controlado por um promotor geralmente constitutivo, como o CaMV35S, garantindo altos níveis da proteína ALCR (fig. 2A). Essa proteína só é ativa após a ligação com etanol. O gene de interesse é posto sob controle do promotor alcA, cuja atividade é dependente da proteína ALCR. Desta forma, o gene de interesse só será ativo na presença de etanol (fig. 2B) (para uma revisão, veja Li et al., (2005).

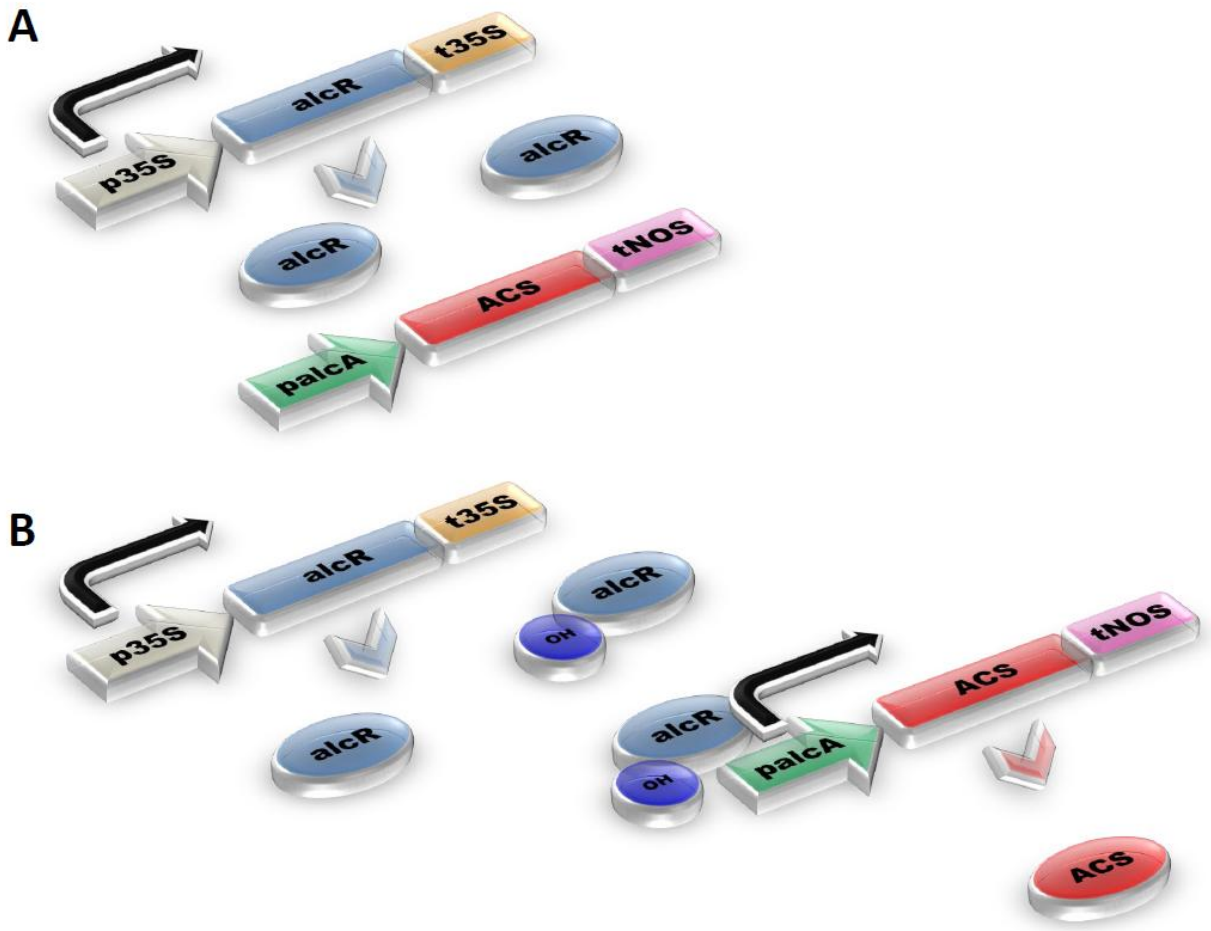

Figura 2: Funcionamento do sistema *alc*. **(A)** Sem etanol, a proteína ALCR é produzida, mas fica na sua forma inativa, e não há produção da proteína de interesse. **(B)** A aplicação de etanol ativa a proteína ALCR, que aciona o promotor *alcA*, sendo produzida a proteína de interesse.

O sistema *alc* é muito sensível ao etanol, com rápida indução em baixa concentração; 0,01% (1,7 mM) de etanol em meio de cultura inicia a expressão do gene *CAT* (*chloranphenicol acetyl transferase*) nas primeiras 4 horas de exposição (Salter et al., 1998). A indução da expressão gênica usando o sistema *alc* tem sido estudada através da absorção do etanol pelas raízes ou pulverização das folhas. Esses métodos têm sido utilizados com sucesso em tabaco, no qual a expressão de um gene repórter foi observada depois de duas horas de aplicação atingindo um pico após 96 horas por absorção radicular e 24 horas depois da pulverização das folhas. De

acordo com Sweetman et al., (2002), baixas concentrações de vapor de etanol induzem eficientemente o sistema de expressão do gene *alc* em plantas transgênicas de tabaco, batata e canola. Tratamentos de tubérculos de batata com vapor de etanol resultam em uma expressão uniforme do gene *uidA*, que codifica a enzima beta-glucoronidase (GUS). O uso de vapor de etanol como um indutor químico também tem sido relatado em estudos com *Arabidopsis* (Roslan et al., 2001). A eficácia desse sistema de expressão de transgene foi demonstrada recentemente também em tomate (Garoosi et al., 2005). Adicionalmente, o sistema *alc* tem sido usado para promover a expressão em tecidos específicos, através da fusão do gene alcR a promotores apropriados, cuja atividade só exista no tecido alvo (Deveaux et al., 2003; Maizel and Weigel, 2004).

Por esses motivos, o promotor *alc* destaca-se como um excelente candidato para a estratégia de amadurecimento controlado da cana-de-açúcar e será usado como modelo para validar a estratégia. Convém destacar que a proposta para testar o sistema *alc* de controle de expressão genética visando à manipulação da maturação de variedades de cana-de-açúcar encontra-se baseada em dois fatores que envolvem principalmente os custos desta operação e como ela é realizada nas usinas. Pode-se controlar a maturação através de baixas dosagens de alguns herbicidas como glifosato e fluazifop-P-butil obtendo-se a paralisação do desenvolvimento, mas com acúmulo pouco significativo de açúcar e com evidentes danos para a rebrota e a produtividade da cultura no próximo ciclo. Essa estratégia tem custo relativamente baixo, mas pelos danos mencionados e por ser feita de avião, pode ainda causar outros problemas, como a deriva do produto para outras áreas plantadas com a própria cana ou outras culturas, resultando em efeitos não desejados.

Outra estratégia é o uso do etefon, de baixíssima toxicidade, muito mais seguro que os herbicidas e que, por deriva, também pode causar efeitos indesejados em outras culturas. No entanto, o

grande inconveniente do uso do etefon como maturador em cana é o alto custo, sendo que, em muitos casos, o aumento na produção de açúcar resultante da aplicação não consegue cobri-lo. É fácil entender essa situação considerando a necessidade de aplicação de 2 litros/ha do produto (de acordo com o fabricante) a um custo de R$ 78,63/litro em outubro de 2010 (fonte IEA – Instituto de Economia Agrícola).

A concentração de álcool a ser aplicada em uma situação de campo para promover a indução eficiente do sistema precisa ser determinada, mas podemos afirmar que existe um grande potencial de redução dos custos da usina usando a estratégia de regular o gene da ACS com o sistema *alc* baseada nos seguintes dados:

- o custo do álcool anidro na usina é bem baixo: R$ 1,21/litro, ;

- a literatura indica que uma solução de álcool a 2,5% é capaz de ativar o sistema *alc*;

- nesses níveis o álcool não apresenta toxicidade;

- a aplicação aérea utiliza cerca de 60 litros de solução por hectare;

- 60 litros a 2,5% de etanol correspondem a 1,5 litros de álcool anidro por hectare;

- portanto, o custo da aplicação com etanol seria de apenas R$ 1,81, contra R$ 157,26 de custo do etefon.

É importante destacar que devido à volatilidade do etanol talvez seja necessária a aplicação em uma concentração mais elevada para compensar o possível efeito da evaporação. Ainda que isso ocorra, dado que o custo com solução de 2,5% é quase 100 vezes menor, há ampla margem para se garantir que a pulverização com solução de etanol seria economicamente viável. Deve-se lembrar que o

custo da aplicação aérea (cerca de 60 litros por hectare), de aproximadamente US$ 10,00/ha seria igual nos dois casos (etefon e álcool).

Outra possibilidade a ser explorada utilizando esse sistema é a de uma possível redução no tempo da safra, que hoje está restrita, no centro-sul do Brasil ao período maio/novembro. A recomendação para o uso de maturadores ocorre entre meados de fevereiro e março, permitindo às variedades que estão recebendo a aplicação um maior acúmulo de sacarose no início da colheita, no início de maio, conseguindo-se em alguns casos, dependendo da variedade, uma pequena antecipação desse período para as últimas semanas de abril. A indução do gene da ACS poderia se tornar uma oportunidade para conseguir uma maturação mais precoce, permitindo a colheita nos meses de entre safra, atendendo à necessidade premente do mercado, que pode ser constatada pelos aumentos de preço do álcool combustível nesta época, uma situação que vivenciamos neste exato momento, com grande divulgação na mídia.

Objetivos

Objetivo Geral

Esse projeto tem por objetivo principal contribuir para o entendimento da relação entre etileno e maturação de cana-de-açúcar. Para tal propomos estudar a expressão de genes que codificam a ACS de cana-de-açúcar e validar o uso do sistema *alc* em plantas de tabaco como uma primeira etapa de uma estratégia de amadurecimento controlado por etanol.

Objetivos Específicos

a) Clonar e caracterizar genes que codificam isoformas da ACC sintase de cana-de-açúcar (*Saccharum* spp) em diferentes tecidos, como entrenós e folhas;

b) Comparar os perfis de expressão das duas variedades contrastantes para teor de Brix, para verificar se há correlação com a acumulação de sacarose e assim poder aferir a relação entre etileno e maturação na planta;

c) Identificar interações protéicas das isoformas da ACS em bibliotecas de cDNA de entrenó e folha de cana-de-açúcar em leveduras.

d) Validar a estratégia de controle temporal e espacial utilizando etanol como indutor químico do sistema alc em uma planta modelo, *Nicotiana tabacum*, utilizando o gene repórter que codifica a enzima GUS;

e) Avaliar se a expressão de um gene *ACS* de cana sob controle do promotor *alcA* promove a acumulação de etileno em *Nicotiana tabacum*.

Apresentação do trabalho

O presente trabalho da família multigênica ACC sintase de cana-de-açúcar fundamentou-se em duas frentes de pesquisa. O primeiro capítulo apresenta a caracterização dos genes das isoformas ACS através da análise das sequências protéicas, análise de expressão gênica em diferentes tecidos, localização subcelular e interação protéica utilizando bibliotecas de folha e entrenó 5 de cana-de-açúcar. O segundo capítulo apresenta os dados da validação do sistema *alc* para o controle temporal da expressão gênica da ACS de cana-de-açúcar em plantas de tabaco transgênicos.

Capítulo I

Molecular Cloning and Characterization of Two Members of the Sugarcane 1-Aminocyclopropane-1-Carboxylate Synthase Gene Family

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Abstract

A common commercial practice in sugarcane culture is the application of ethylene precursors, such as ethrel, to anticipate the maturation and increase sucrose levels in culms. The ethylene hormone is involved in several physiological processes of plants, including the maturation. The 1-aminocyclopropane-1-carboxylate synthase (ACS) enzyme, the rate-limiting step in the ethylene biosynthesis, has been identified and characterized in several higher plants. Our major goals were to characterize sugarcane ACS genes and establish their relation with sugarcane maturation. For that, two ACS genes named *Scacs1* and *Scacs2* were cloned and characterized. The full-length cDNA of *Scacs1* and *Scacs2* encoded 487 and 466 amino acid proteins, respectively. Alignment analysis with various ACS from different species showed that both isoforms have all seven conserved regions and 11 invariant amino acid residues, characteristic of ACS multigene families. Moreover, phylogenetic analysis indicated that ScACS1 and ScACS2 proteins belong to group I and III, respectively, because of the differences in their C-terminal domain of post-translational regulation. Through two-hybrid assay we demonstrated that ACS enzymes were not able to form heterodimeric interactions. Besides, using ScACS2 protein as bait, novel protein interactions in leaf and internode cDNA libraries were identified. One of them encoded an E3 ubiquitin ligase protein, homolog to RING-H_2 finger proteins responsible by targeting proteins through the ubiquitin-proteasome pathway. We suggest a possible novel mechanism of post-translational regulation of group III ACS. Subcellular localization studies of ScACS1-GFP and ScACS2-GFP proteins in onion epidermal cells showed a cytoplasmic localization pattern. The results of expression analysis by quantitative RT-PCR showed a higher expression in leaf and mature internodes of low Brix cultivars for both isoforms. *In situ*

hybridization assay demonstrated that the *Scacs1* localization was found in parenchyma cells and fibrous sheath in internodes 1, 5 and 9, but except in vascular bundles. On the other hand, the *Scacs2* transcript expression showed an ubiquitous expression pattern, including in phloem cells. Taken together, these results provide novel insights in the correlation between the ethylene biosynthesis and the maturation process in sugarcane, and a better understanding of molecular mechanisms involved in the regulation and action of ACS proteins in plants.

Keywords: Sugarcane, maturation, ethylene, ACC synthase

Introduction

The observation that gaseous materials in the air could cause abnormal effects in the plant growth has been known for a long time. Nonetheless, the identification and discovered of ethylene as a bioactive gas produced from plant tissues only was established by Neljubow (1901), Cousins (1910) and Gane's studies (1934). In this way, despite its chemical simplicity, the ethene or ethylene (C_2H_4) is used as a hormone by plants, in which act as a potent regulator of plant growth and development (Yang and Hoffman, 1984; Kende, 1993; Bleecker and Kende, 2000). Although its synthesis has been identified in most plant tissues and cell types, the ethylene hormone, a volatile organic compound, is able to permeate through cell membrane by diffusion and to play its role far away from its local synthesis. This allows the plant to respond rapidly to different biotic (hypoxia, and exposure to drought and cold) and abiotic stresses (pathogen attack, wounding).

In addition, ethylene is involved in the regulation of many physiological and developmental processes such as seed germination, root initiation, root hair development, flower development, sex determination, fruit ripening, abscission and senescence (Tsuchisaka et al., 2009). To accomplish these diverse functions in plants, several factors have been recently identified acting in the regulation of its biosynthetic pathway. Factors such as light, circadian clock (Thain et al., 2004; Robertson et al., 2009) and application of others plant hormones like auxin, abscisic acid and cytokinins have been recognized as regulators in ethylene biosynthesis (Tsuchisaka and Theologis, 2004; Chae and Kieber, 2005).

The biosynthetic pathway of ethylene can be described basically in three steps: from amino acid methionine to S-adenosyl-L-methionine (SAM) by S-adoMet synthetase enzyme, the

convertion of SAM into 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase enzyme, which generally is considered the rate-limiting step, and finally the oxidation of ACC by ACC oxidase enzyme that yield ethylene and CO2 (Adams and Yang, 1979) (Figure 1). In many cases, the fine-tuning regulation of ethylene biosynthesis occurs upon transcription and translational levels of ACC synthase (ACS) and ACC oxidase (ACO) enzymes (Wang et al., 2002).

In all plant species that have been studied, ACS and ACO protein are encoded by multigene families. Among numerous article published, we can cite nine ACS functional genes from arabidopsis (*Arabidopsis thaliana*) (Yamagami et al., 2003), eight ACS genes from tomato (*Lycopersicon esculentum*) (Zarembinski and Theologis, 1994; Yokotani et al., 2009), three ACS genes from maize (*Zea mays*) (Gallie et al., 2009), five ACS genes from rice (*Oryza sativa*) (Zhou et al., 2002), and two ACS genes from wheat (*Triticum spp*) (Subramaniam et al., 1996). The biological significance of the gene redundancy found in ACS family and among others multigene families is still unknown. Nevertheless, different biochemical properties of each ACS isoform may be associated to the biochemical environment of the cells or tissue in which each isoform is expressed (Rottmann et al., 1991). This tissue-specific expression pattern may be related to the distinct biological function of ACS isoforms, which has been reported in all plant species studied (Rottmann et al., 1991; Yamagami et al., 2003; Tsuchisaka and Theologis, 2004).

Although ACS subunits show seven highly conserved domains along the protein sequence, their carboxylic ends are widely variable. This C-terminus region serves as a regulatory domain responsible for the stabilization of the enzyme through the post-translational regulation. Thus, ACS isoforms can be classified in three phylogenetic clades: Type 1 isoforms, which own the longest C-terminal region with a putative calcium-dependent protein kinase (CDPK) phosphorylation domain and three mitogen-activated protein Kinase (MAPK)

phosphorylation sites; Type 2 isoforms, which own an intermediate length C-terminus containing a single putative CDPK phosphorylation site; and Type 3 isoforms, owing a truncated C-terminal region and no predicted kinase phosphorylation sites (Chae and Kieber, 2005; Yoshida et al., 2005; Hansen et al., 2009).

One of the most studied roles of ethylene phytohormone is the ripening of climacteric fruit (Pech et al., 2008). Although diverse plants has been extensively characterized in studies of fruit ripening, species such as sugarcane show distinct features in this economically important process and provide new knowledge of the specific mechanisms of ethylene hormone. Moreover, a commercial practice common in agriculture has been the application of an artificial ripener, ethrel (2-chloroethyl phosphonic acid), which is rapidly metabolized in plants, producing ethylene gas, phosphoric acid and hydrochloric acid. As result, innumerous papers have demonstrated different roles performed by ethylene hormone in sugarcane plants such as delay opening of the inflorescence (Eastwood and Davis, 1997), earlier maturation process (Page, 1983) and higher tillering and sucrose content (Li and Solomon, 2003; Caputo et al., 2007).

In this study, we have identified the members of the ACS multigene family in sugarcane. Sugarcane ACSs were characterized and their gene expression patterns were evaluated in different tissues using two sugarcane cultivars contrasting for sucrose accumulation. Our data bring further insights into molecular mechanisms of ACS enzyme in plants and the correlation of ethylene hormone in the sugarcane maturation.

Materials and Methods

Plant material

Field-grown plants (8 months old) were supplied by Centro de Ciências Agrárias – UFSCAR (Araras - SP, Brazil). Samples of mature leaf, first, fifth and ninth internodes were collected from sugarcane cultivars with different agronomical trait concerning Brix content. The RB855156 sugarcane cultivar (high Brix) shows an early physiological maturation and higher sucrose content in mature internodes than SP832847 and RB935744 sugarcane cultivars (low Brix). All tissues were sectioned, frozen in liquid nitrogen, and stored at -80°C.

Identification and cloning of ACC synthase genes

Through local Blast alignment on the SUCEST database (http://sucest-fun.org) using ACS sequences from others plant species, two SASs (sugarcane assembled sequences) encoding ACC synthase protein homologs were found in sugarcane. The SCJLRT1006C03 and SCQGAM2028D11 SASs were called *Scacs1* and *Scacs2*, respectively. The complete coding region of each ACC synthase gene was amplified by PCR using gene-specific primers as follows: ScACS1Fw (5'- CTGCAGGGAAAAAACCGACGTCAA – 3') and ScACS1Rv (5' – GAGCTCTGTCTTACCGAATGCATGC – 3'), ScACS2Fw (5'- CTGCAGATGGGTGGCAAGTTGTTG-3') and ScACS2Rv (5'- GATATCCTAATGGCGGTTGCTG -3'). The PCR reaction for *Scacs1* was performed using the full-length cDNA of SCJLRT1006C03 SAS as template. On the other hand, for *Scacs2,* root cDNA from the same sugarcane variety utilized in the SUCEST project (SP80-3280) was used as template, because the SCQGAM2028D11 SAS is truncated at the 5' end. The amplified product was subcloned into

pGEMT-easy (Promega, USA) and transformed into *Escherichia coli* DH5α cells. Oligonucleotides T7 and SP6 were used for sequencing and the nucleotide sequence determined was used for further analysis.

Sequence alignment and phylogenetic analysis

The ACS sequences obtained were aligned with other homolog sequences from various plant species using the ClustalX program (Larkin et al., 2007). ACS sequences were aligned to construct a distance tree with neighbor-joining algorithm (Saitou and Nei, 1987). The phylogenetic tree was visualized with the TreeViewX program (Page, 1996). Bootstrap values from 1000 replicates were used to access the reliability of each node.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from mature leaf +1, internode 1, internode 5 and internode 9 of two sugarcane cultivars (RB855156 – high Brix and RB935744 – Low brix) using the RNeasy Plant Mini kit (QIAGEN, USA) according to the manufacter's instructions. First-strand cDNAs were synthesized using 2.5 μg DNAse I-treated RNA, (500 ng/uL) oligo-dT primer (Invitrogen), 1 uM RT primer and 10 mM dNTP mix. All RNA samples were denaturated at 65°C for 10 min., cooled on ice for 2 min. and added 5X First Strand Buffer, 0.1 M DTT, RNAse out (40 U/μL) and Superscript III RT enzyme (200 U/μL) (Life Technologies, USA). The reaction was carried out at 16°C for 30 min. followed of 60 cycles at 30°C for 30 s., 42°C for 30 s. and 50°C for one s. in a Verity thermocycler (Applied Biosystems, USA) as described by Varkonyi-Gasic et.al., (2007). First-strand cDNAs synthesis was finished by heating to 85°C for 5 min. to inactivate the RT enzyme.

Real time PCR was performed in the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, USA). To each cDNA sample, SybrGreen PCR Master mix (Applied Biosystems, USA), 10uM primer forward, 10uM primer reverse and water were added. The 3'UTR region of *Scacs1* and *Scacs2* cDNAs were used as template to design primers. The reactions were incubated at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute as described by Varkonyi-Gasic et al. (2007). A polyubiquitin gene from sugarcane (SCCCST2001G02.g) was used as internal control (Papini-Terzi et al., 2009). All reactions were done with two biological replicates and in experimental triplicates. qPCR result values displayed are relative to samples of the high Brix cultivar (RB855156), which were set to a relative value of 1.

In situ hybridization analysis

Digoxigenin labeling of RNA probes, tissue preparation, and hybridization conditions were performed as described by Malcomber, (2003) with minor modifications, using the DIG RNA labeling (SP6/T7) kit (Roche Applied Sciences, USA). Transversal sections of internodes 1, 5 and 9 of two sugarcane cultivars were used for *in situ* hybridization analysis. The 3'UTR region of *Scacs1* and *Scacs2* cDNAs were amplified with specific primers and cloned into pGEMT-easy vector (Promega, USA). Both sense and antisense digoxigenin-labeled riboprobes were transcribed with RNA polymerases SP6 and T7 using the above constructs as template. The hybridized sections were viewed as a purple precipitated after an overnight stain and were photographed under an Olympus BX51 microscope (Olympus, USA).

Construction of leaf and internode 5 cDNA libraries

Sugarcane leaf and internode 5 cDNA libraries were constructed using the Make Your Own "Mate & Plate™" Library System (Clontech, USA). Two micrograms of total RNA from leaf and internode 5 tissues of low Brix cultivar (RB935744) were used for cDNA synthesis according to the manufacturer's instruction. First strand cDNA was amplified by Long Distance PCR using the Advantage 2 PCR Kit (Clontech, USA) and purified with a ChromaSpin + TE-400 column to reduce small fragments. The purified cDNA was then fusion in frame with the GAL4 activation domain (GAL4AD) of pGADT7-Rec (Clontech, USA) expression vector by homologous recombination in Y187 yeast strain (MATα, trp1-901, leu2-3, 112, ura3-52, his3-200, ade2-101, gal4Δ, gal80Δ, met-, URA3::$GAL1_{UAS}$-$Gal1_{TATA}$ -LacZ MEL1) (Harper *et al*., 1993) (Figure 1A).

Yeast two-hybrid screen

Two-hybrid screens were carried out with *Saccharomyces cerevisiae* strain Y2HGold (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$GAL1_{UAS}$-$Gal1_{TATA}$ -HIS3, $GAL2_{UAS}$-$Gal2_{TATA}$ -Ade2, URA3::$MEL1_{UAS}$-$Mel1_{TATA}$,AURI-C MEL1) using the Matchmaker Gold Yeast Two-Hybrid System (Clontech, USA). The pGBKT7::Scacs2 bait construct expresses the ScACS2 protein as a fusion to the GAL4 DNA binding domain (GAL4DNA-BD), already present in the pGBKT7 vector (Louvet et al., 1997) (Figure 1B). The screens were carried out by mating according to the manufacturer's instructions (Clontech, USA). Briefly, an overnight culture of strain Y2HGold expressing ScACS2 was mixed with strain Y187, expressing the cDNA library, for 24 hours, in rich medium at 30°C in a shaker at low rotation (40 rpm). The yeast mixture was plated onto synthetic dropout (SD) medium

lacking tryptophan, leucine, histidine and adenine (QDO = Quadruple DropOut medium). Positive colonies appearing within 5 days of plating and after activation of the HIS3 and ADE2 reporter genes were subsequently tested for expression of MEL1 (β- galactosidase). The interactions were initially confirmed by restreaking the positive clones on QDO medium and after 5 days, three-replica plating were done onto the same medium with X- α-Gal and the antibiotic aureobasidin A to verify the stability of the correct phenotype and to test the strength of the interaction.

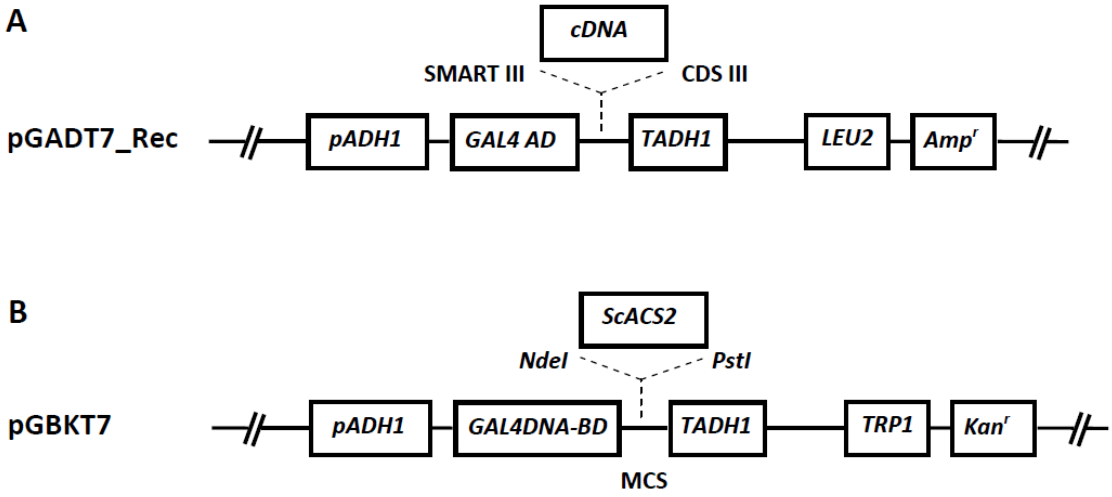


Figure 1. Maps and cloning strategies used in the two-hybrid assay. (A) cDNAs originated from sugarcane libraries were inserted by homologous recombination between SMARTIII and CDSIII regions in pGADT7 vector. (B) The complete coding region of *Scacs2* was inserted in frame to *GAL4DNA-BD* in the pGBKT7 vector; pADH1: constitutive *ADH1* promoter*; GAL4DNA-BD*: GAL4 DNA binding domain; *TADH1*: *ADH1* transcriptional terminator; *TRP1*: Nutritional marker (tryptophan) for selection in yeast; Kan^r: Kanamycin gene for selection in *E.coli*; GAL4 AD: GAL4 activation domain; SMART III: Oligonucleotide sequence; CDS III; primer sequence; LEU2: Nutritional marker (Leucine) for selection in yeast; Amp^r: Ampicilin gene for selection in *E.coli*.

Yeast two-hybrid direct mating

To analyze the heterodimeric interaction between ScACS1 and ScACS2 proteins, a small-scale yeast mating was performed. One colony of each yeast carrying each construction Y2HGold (pGBKT7::Scacs2) and Y187 (pGADT7::Scacs1) was picked and placed together in a 1.5 ml microcentrifuge tube containing 0.5 ml of 2xYPDA medium for 24 hours at 30°C in a shaker at low rotation (40 rpm). The yeast mixture was plated on QDO medium and positive colonies were evaluated within 5 days of plating.

Protein interaction analysis

After the confirmation of the phenotype, plasmid isolation of positive clones was done using a modified lyticase protocol from Philippsen et al., (1991). Because the plasmid DNA isolated was a mixture of both plasmids (bait and library), a strain of *E. coli* was transformed and plated on LB medium containing ampicillin to select for the AD/library plasmid only. Three colonies were chosen for each clone to analyze the number of AD library plasmids they contained. The cDNA inserts were amplified using the Advantage 2 PCR kit (Clontech, USA). Each positive clone generated only one PCR product (one type of AD library plasmid), so the PCR product was sequenced and compared in GenBank, EMBL, or other databases.

Subcellular localization

The *Scacs1* and *Scacs2* complete coding sequences amplified by PCR were cloned downstream of the CaMV35S promoter and in frame with the GFP coding region in the modified vector pRT104 (Töpfer et al., 1987). The resulting plasmid DNA was precipitated onto tungsten

microparticles using 2.5 M $CaCl_2$ and 100 mM spermidine (Sigma-Aldrich, USA). The onion layers were bombarded with DNA-coated particles using a helium biolistic device (Biomics, Brazil) at a pressure of 1,100 psi. Bombarded onion layers were maintained on the plates and incubated at 25°C in darkness for 24 h. Samples were examined under a LEICA DMI 4000B fluorescence microscope (Leica, Germany), using a GFP filter. Two control constructs were used in this experiment: the pRT104::GFP vector (cytoplasmatic) and the pGFPKDEL vector containing KDEL-GFP sequence (endoplasmic reticulum).

Results

Phylogenetics analysis of ScACS1 and ScACS2 proteins

Sequence analysis revealed that the entire coding region of *Scacs1* (GenBank acession n° JF274985) had 1464 bp and encoded a protein of 487 amino acids with a predictive molecular mass of 54kDa and pI 6.35 (Figure 2). BLAST searches against the GenBank non-redundant protein database showed that ScACS1 is homolog to the ZmACS2 from maize, OsACS2 from rice, AtACS6 from arabidopsis and LeACS2 from tomato proteins, showing around 94%, 82%, 66%, 61% of identity, respectively.

The *Scacs2* isoform (GenBank acession n° JF274986) showed a coding region of 1401 bp and encoded a protein of 466 amino acids with a predictive molecular mass of 51kDa and Pi 6.47 (Figure 3). Different from ScACS1, BLAST searches using ScACS2 as a query showed high (around 84%) identity to OsACS5 from rice, and 71% to AtACS7 from Arabidopsis.

Although ScACS1 and ScACS2 isoenzymes are substantially divergent each other (55% amino acids identity), both contain the seven conserved boxes found in ACS from various plant

species and eleven out of twelve amino acids residues conserved in aminotransferase and ACS proteins (Figure 4). Moreover, both sequences from sugarcane have the highly conserved tripeptide TNP (Thr-Asn-Pro) in box IV which is crucial for ACS enzymatic activity (Liang et al., 1995) and the Y^{93} (ScACS1), Y^{105} (ScACS2) residues in box II, which is involved in substrate recognition (Capitani et al., 1999).

Despite local Blast alignment on SUCEST database with ACS sequences described from others plant species to have identified the SCSGCL6070B03 SAS as another putative sugarcane ACS, a phylogenetic analysis clustered this protein with AtACS10 and AtACS12 Arabidopsis aminotransferases proteins (data not shown). Thus, our analyses on SUCEST database did not identify any other protein that could be classified as sugarcane ACS.

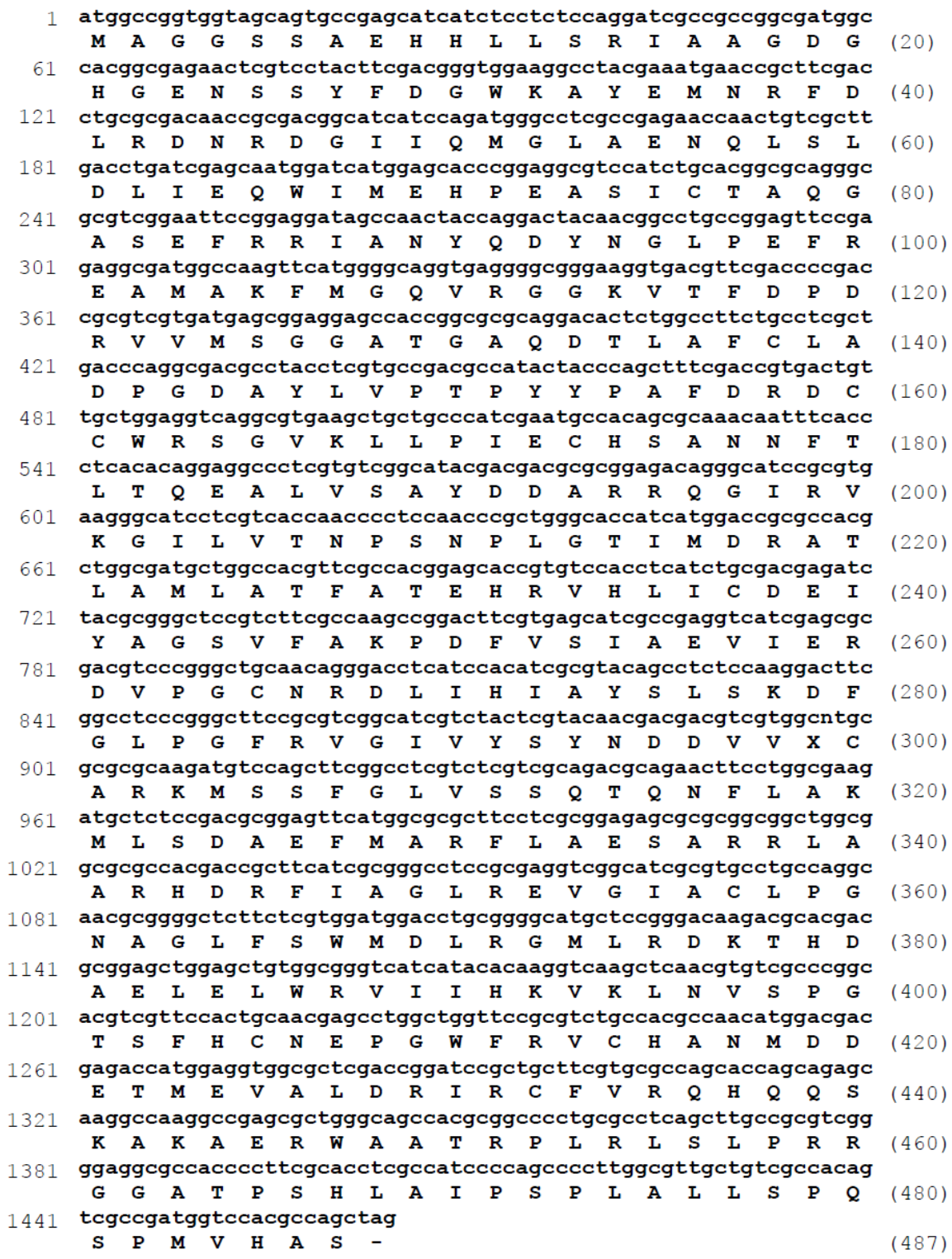


Figure 2. Nucleotide and deduced amino acid sequences of *Scacs1*. The numbers on the left refer to the nucleotide sequence, while those on the right, between parenthesis, refer to the amino acid sequence.

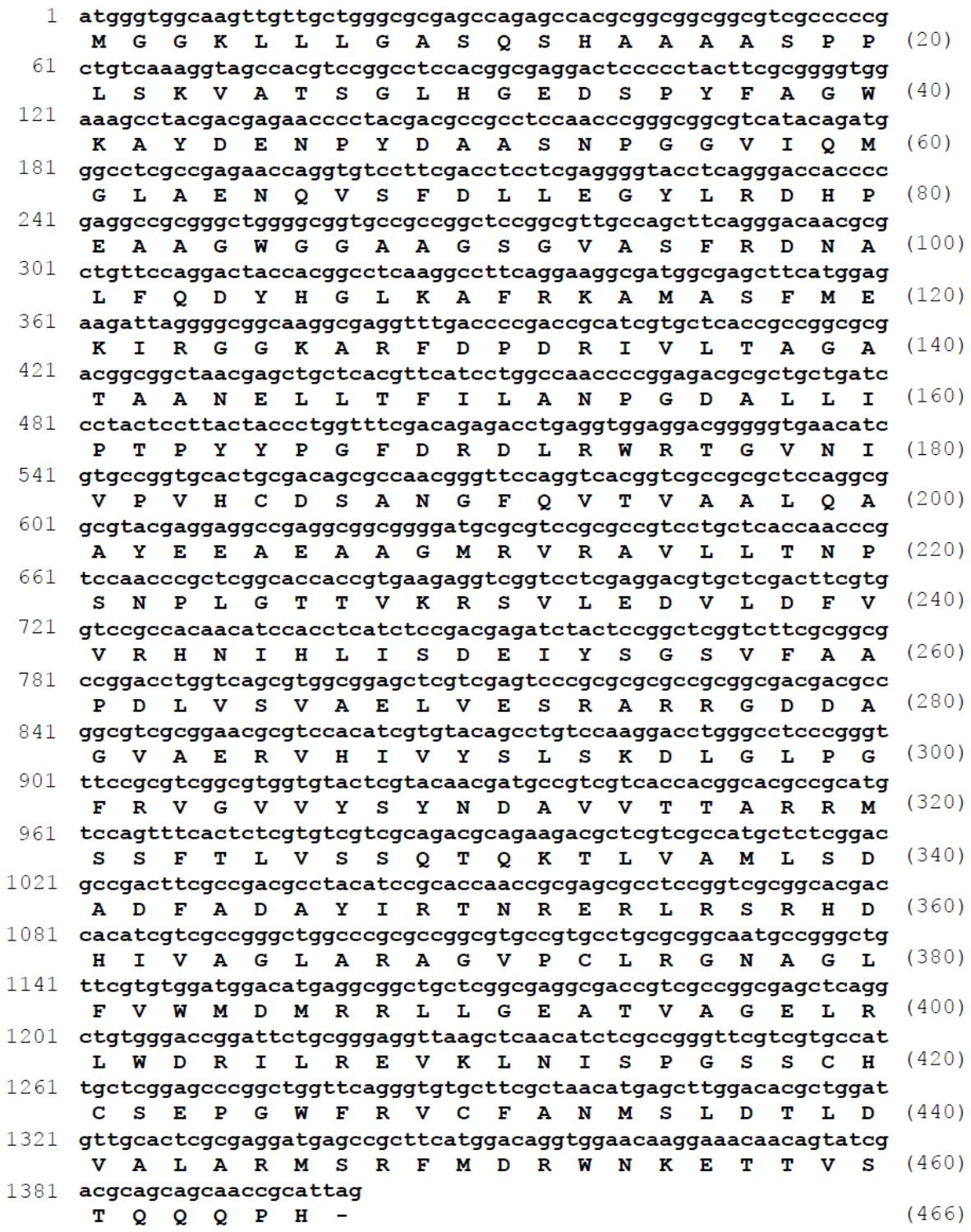
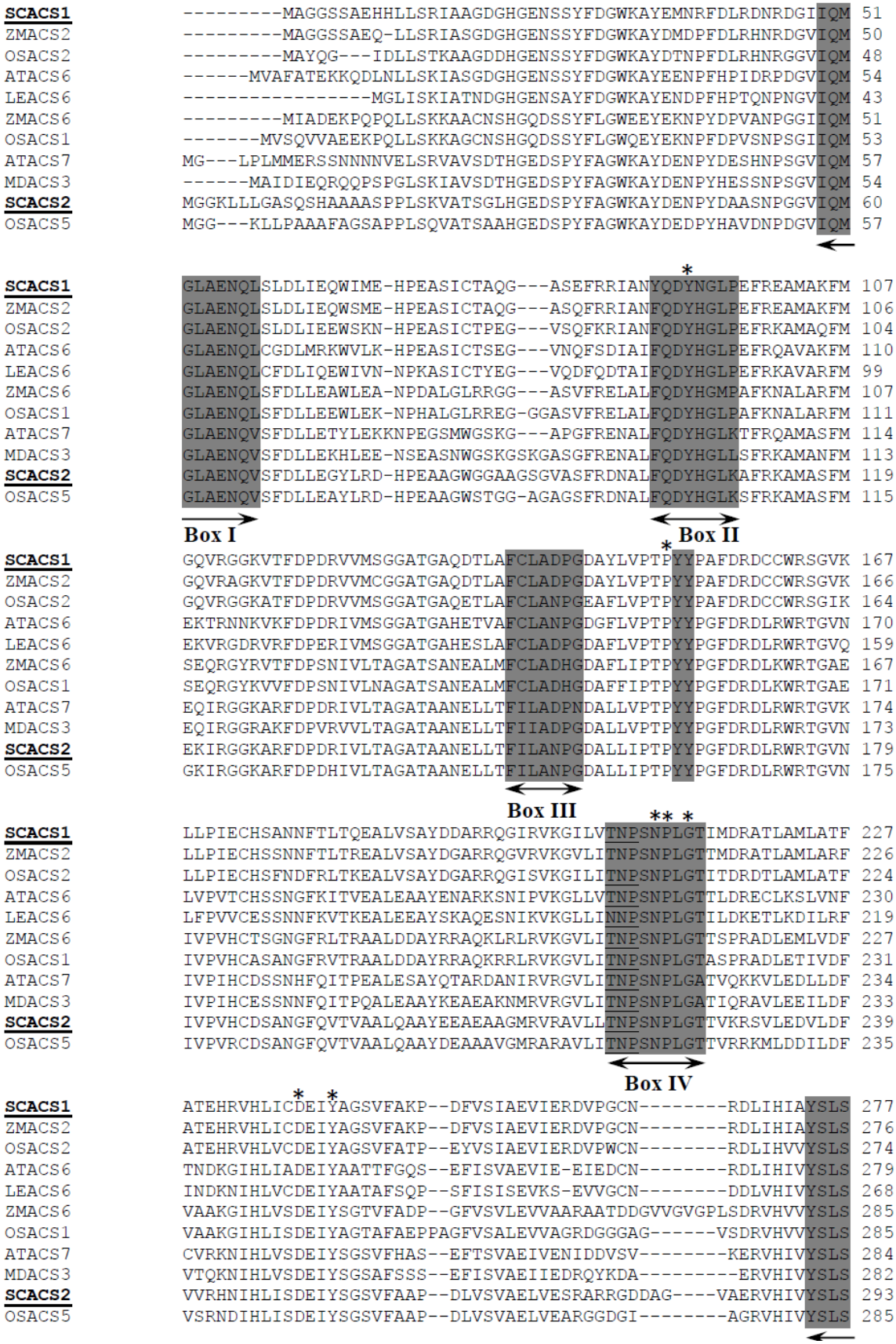


Figure 3. Nucleotide and deduced amino acid sequences of *Scacs2*. The numbers on the left refer to the nucleotide sequence, while those on the right, between parenthesis, refer to the amino acid sequence.

Analysis of the C-terminal region of ACC synthase proteins revealed that ScACS1 belongs to the Type 1 class because it contains three target Ser residues for MPK6 phosphorylation and the 'RLSF' motif for CDPK phosphorylation. In addition, Joo et al. (2008) have reported two invariable acidic residues (Asp/Asp), upstream of the phosphorylation site, important for AtACS6 stabilization, so that these residues contribute additional negative charges. Interestingly, ScACS1, ZmACS2 and OsACS2 amino acid sequences did not show these two conserved residues. In spite of that, the C-terminal region of these ACS proteins from monocotyledons showed a large number of neutral and non-polar amino acids. On the other hand, the ScACS2 isoform showed a truncated C-terminal region lacking all the important residues for phosphorylation, belonging to Type III class of ACS proteins (Figure 4).


SCACS1 ---------MAGGSSAEHHLLSRIAAGDGHGENSSYFDGWKAYEMNRFDLRDNRDGIIQM 51
ZMACS2 ---------MAGGSSAEQ-LLSRIASGDGHGENSSYFDGWKAYDMDPFDLRHNRDGVIQM 50
OSACS2 ---------MAYQG---IDLLSTKAAGDDHGENSSYFDGWKAYDTNPFDLRHNRGGVIQM 48
ATACS6 ------MVAFATEKKQDLNLLSKIASGDGHGENSSYFDGWKAYEENPFHPIDRPDGVIQM 54
LEACS6 ----------------MGLISKIATNDGHGENSAYFDGWKAYENDPFHPTQNPNGVIQM 43
ZMACS6 ---------MIADEKPQPQLLSKKAACNSHGQDSSYFLGWEEYEKNPYDPVANPGGIIQM 51
OSACS1 -------MVSQVVAEEKPQLLSKKAGCNSHGQDSSYFLGWQEYEKNPFDPVSNPSGIIQM 53
ATACS7 MG---LPLMMERSSNNNNVELSRVAVSDTHGEDSPYFAGWKAYDENPYDESHNPSGVIQM 57
MDACS3 ------MAIDIEQRQQPSPGLSKIAVSDTHGEDSPYFAGWKAYDENPYHESSNPSGVIQM 54
SCACS2 MGGKLLLGASQSHAAAASPPLSKVATSGLHGEDSPYFAGWKAYDENPYDAASNPGGVIQM 60
OSACS5 MGG---KLLPAAAFAGSAPPLSQVATSAAHGEDSPYFAGWKAYDEDPYHAVDNPDGVIQM 57
SCACS1 GLAENQLSLDLIEQWIME-HPEASICTAQG---ASEFRRIANYQDYNGLPEFREAMAKFM 107
ZMACS2 GLAENQLSLDLIEQWSME-HPEASICTAQG---ASQFRRIANFQDYHGLPEFREAMAKFM 106
OSACS2 GLAENQLSLDLIEEWSKN-HPEASICTPEG---VSQFKRIANFQDYHGLPEFRKAMAQFM 104
ATACS6 GLAENQLCGDLMRKWVLK-HPEASICTSEG---VNQFSDIAIFQDYHGLPEFRQAVAKFM 110
LEACS6 GLAENQLCFDLIQEWIVN-NPKASICTYEG---VQDFQDTAIFQDYHGLPEFRKAVARFM 99
ZMACS6 GLAENQLSFDLLEAWLEA-NPDALGLRRGG---ASVFRELALFQDYHGMPAFKNALARFM 107
OSACS1 GLAENQLSFDLLEEWLEK-NPHALGLRREG-GGASVFRELALFQDYHGLPAFKNALARFM 111
ATACS7 GLAENQVSFDLLETYLEKKNPEGSMWGSKG---APGFRENALFQDYHGLKTFRQAMASFM 114
MDACS3 GLAENQVSFDLLEKHLEE-NSEASNWGSKGSKGASGFRENALFQDYHGLLSFRKAMANFM 113
SCACS2 GLAENQVSFDLLEGYLRD-HPEAAGWGGAAGSGVASFRDNALFQDYHGLKAFRKAMASFM 119
OSACS5 GLAENQVSFDLLEAYLRD-HPEAAGWSTGG-AGAGSFRDNALFQDYHGLKSFRKAMASFM 115
Box I
Box II
SCACS1 GQVRGGKVTFDPDRVVMSGGATGAQDTLAFCLADPGDAYLVPTPYYPAFDRDCCWRSGVK 167
ZMACS2 GQVRAGKVTFDPDRVVMCGGATGAQDTLAFCLADPGDAYLVPTPYYPAFDRDCCWRSGVK 166
OSACS2 GQVRGGKATFDPDRVVMSGGATGAQETLAFCLANPGEAFLVPTPYYPAFDRDCCWRSGIK 164
ATACS6 EKTRNNKVKFDPDRIVMSGGATGAHETVAFCLANPGDGFLVPTPYYPGFDRDLRWRTGVN 170
LEACS6 EKVRGDRVRFDPERIVMSGGATGAHESLAFCLADPGDAFLVPTPYYPGFDRDLRWRTGVQ 159
ZMACS6 SEQRGYRVTFDPSNIVLTAGATSANEALMFCLADHGDAFLIPTPYYPGFDRDLKWRTGAE 167
OSACS1 SEQRGYKVVFDPSNIVLNAGATSANEALMFCLADHGDAFFIPTPYYPGFDRDLKWRTGAE 171
ATACS7 EQIRGGKARFDPDRIVLTAGATAANELLTFILADPNDALLVPTPYYPGFDRDLRWRTGVK 174
MDACS3 EQIRGGRAKFDPVRVVLTAGATAANELLTFIIADPGDALLVPTPYYPGFDRDLRWRTGVN 173
SCACS2 EKIRGGKARFDPDRIVLTAGATAANELLTFILANPGDALLIPTPYYPGFDRDLRWRTGVN 179
OSACS5 GKIRGGKARFDPDHIVLTAGATAANELLTFILANPGDALLIPTPYYPGFDRDLRWRTGVN 175
Box III
SCACS1 LLPIECHSANNFTLTQEALVSAYDDARRQGIRVKGILVTNPSNPLGTIMDRATLAMLATF 227
ZMACS2 LLPIECHSSNNFTLTREALVSAYDGARRQGVRVKGVLITNPSNPLGTTMDRATLAMLARF 226
OSACS2 LLPIECHSFNDFRLTKEALVSAYDGARRQGISVKGILITNPSNPLGTITDRDTLAMLATF 224
ATACS6 LVPVTCHSSNGFKITVEALEAAYENARKSNIPVKGLLVTNPSNPLGTTLDRECLKSLVNF 230
LEACS6 LFPVVCESSNNFKVTKEALEEAYSKAQESNIKVKGLLINNPSNPLGTILDKETLKDILRF 219
ZMACS6 IVPVHCTSGNGFRLTRAALDDAYRRAQKLRLRVKGVLITNPSNPLGTTSPRADLEMLVDF 227
OSACS1 IVPVHCASANGFRVTRAALDDAYRRAQKRRLRVKGVLITNPSNPLGTASPRADLETIVDF 231
ATACS7 IVPIHCDSSNHFQITPEALESAYQTARDANIRVRGVLITNPSNPLGATVQKKVLEDLLDF 234
MDACS3 IVPIHCESSNNFQITPQALEAAYKEAEAKNMRVRGVLITNPSNPLGATIQRAVLEEILDF 233
SCACS2 IVPVHCDSANGFQVTVAALQAAYEEAEAAGMRVRAVLLTNPSNPLGTTVKRSVLEDVLDF 239
OSACS5 IVPVRCDSANGFQVTVAALQAAYDEAAAVGMRARAVLITNPSNPLGTTVRRKMLDDILDF 235
Box IV
SCACS1 ATEHRVHLICDEIYAGSVFAKP--DFVSIAEVIERDVPGCN--------RDLIHIAYSLS 277
ZMACS2 ATEHRVHLICDEIYAGSVFAKP--DFVSIAEVIERDVPGCN--------RDLIHIAYSLS 276
OSACS2 ATEHRVHLVCDEIYAGSVFATP--EYVSIAEVIERDVPWCN--------RDLIHVVYSLS 274
ATACS6 TNDKGIHLIADEIYAATTFGQS--EFISVAEVIE-EIEDCN--------RDLIHIVYSLS 279
LEACS6 INDKNIHLVCDEIYAATAFSQP--SFISISEVKS-EVVGCN--------DDLVHIVYSLS 268
ZMACS6 VAAKGIHLVSDEIYSGTVFADP--GFVSVLEVVAARAATDDGVVGVGPLSDRVHVVYSLS 285
OSACS1 VAAKGIHLISDEIYAGTAFAEPPAGFVSALEVVAGRDGGGAG------VSDRVHVVYSLS 285
ATACS7 CVRKNIHLVSDEIYSGSVFHAS--EFTSVAEIVENIDDVSV--------KERVHIVYSLS 284
MDACS3 VTQKNIHLVSDEIYSGSAFSSS--EFISVAEIIEDRQYKDA---------ERVHIVYSLS 282
SCACS2 VVRHNIHLISDEIYSGSVFAAP--DLVSVAELVESRARRGDDAG----VAERVHIVYSLS 293
OSACS5 VSRNDIHLISDEIYSGSVFAAP--DLVSVAELVEARGGDGI--------AGRVHIVYSLS 285

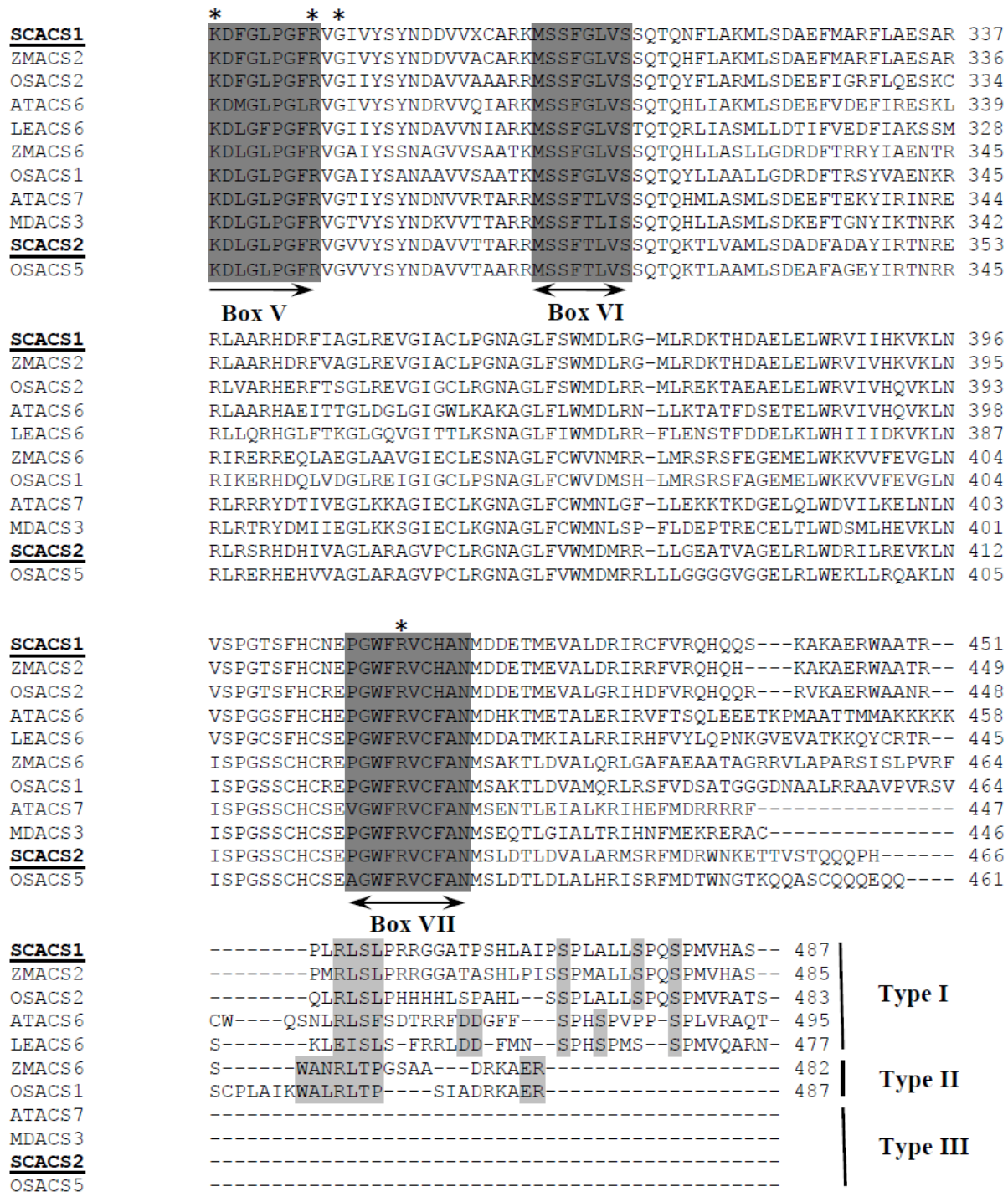


Figure 4. Multiple alignment analysis of ACS proteins. ScACS1 (JF274985), ZmACS2 (AAR25558), OsACS2 (BA689048), LeACS6 (AAK72433), AtACS6 (NP_192867), OsACS1 (AAT77071), ZmACS6 (ACN25592), ScACS2 (JF274986), OsACS5 (BAB12704), AtACS7 (NP_194350), MdACS3 (AAB67989) were aligned using the ClustalX program. Dark grey shading indicates the 7 conserved boxes in all ACS amino acid sequences. The 11 asterisks

designate conserved amino acids in aminotransferase and ACS proteins. Light grey shading shows conserved domain and residues responsible by post-translational regulation in C-terminal region. The conserved tripeptide TNP in Box IV is underlined (see the text).

To analyze the evolutionary scenario of these isoforms, the two identified sugarcane protein sequences were compared with homologous sequences from maize, rice, tomato, Arabidopsis and apple by a phylogenetic analysis (Figure 5). The neighbor-joining tree generated using the alignment above also divided the ACS family into three distinct subgroups. These three evolutionary clades grouped ACS sequences that showed identical domains to post-translational regulation in C-terminal region. Thus, ScACS1 and ScACS2 isoenzymes were clustered in different clades. In Type I branch, the ScACS1 isoenzyme was clustered together with ZmACS2 from maize and with OsACS2 from rice, sharing a common ancestor and reflecting what was seen in the protein alignment (Figure 4). The ScACS2 isoenzyme was clustered together with OsACS5, both belonging to Type III branch. Interestingly, the branch that grouped ACS isoenzymes classified as Type I showed a higher evolutionary rate than ACS isoenzymes classified as Type III.

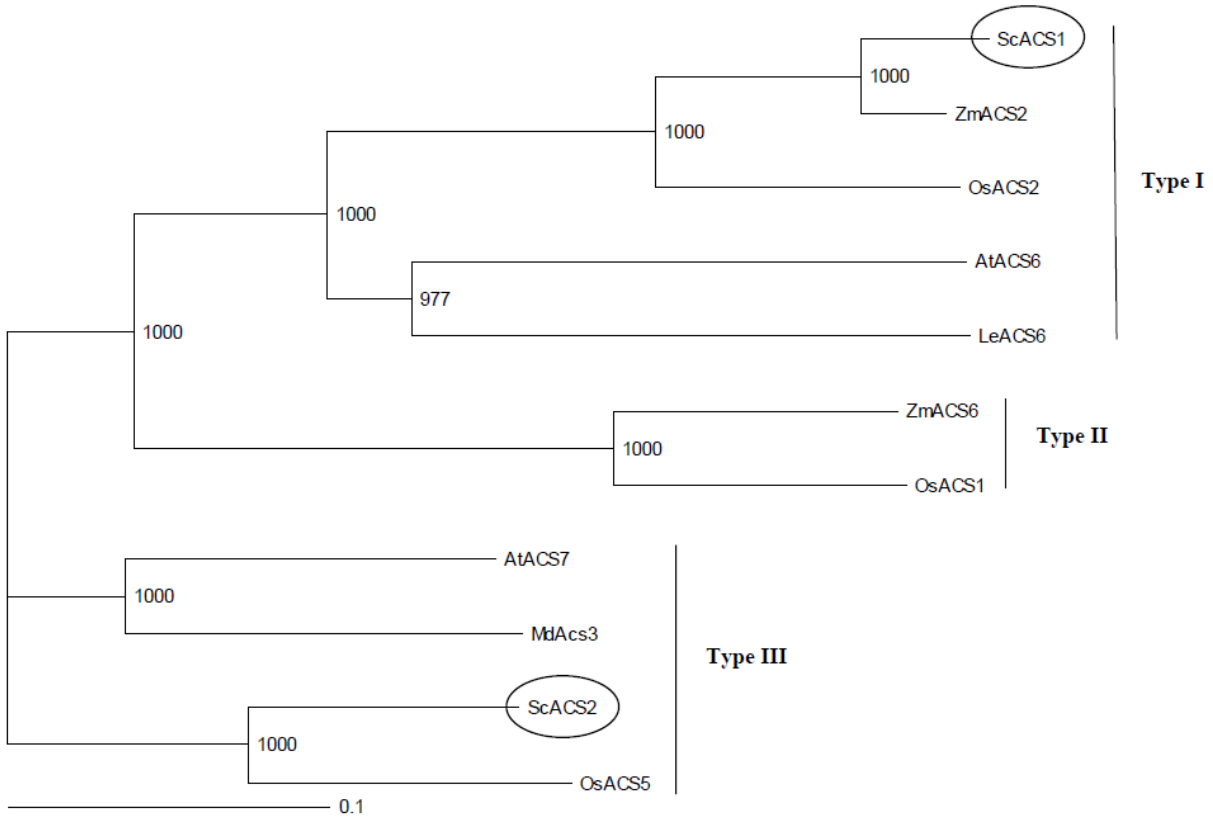


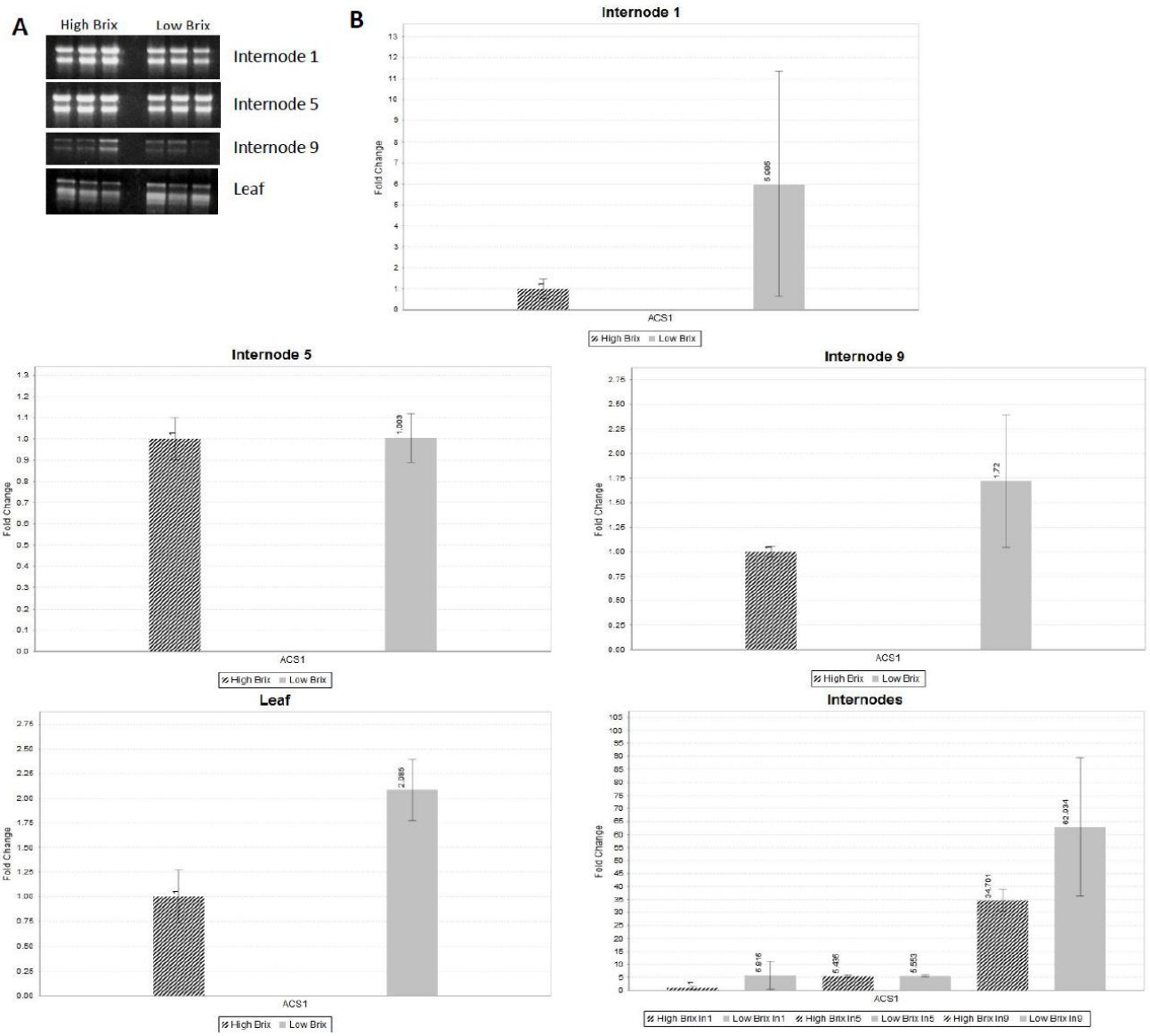
Figure 5. Neighbor-joining tree of the ACS protein sequences from sugarcane and other species. Branches were classified in different ACS protein subfamilies (Yamagami et al., 2003). Circles indicate the sequences from sugarcane. Sequences accession numbers: SCACS1 (JF274985), ZMACS2 (AAR25558), OSACS2 (BA689048), LEACS6 (AAK72433), ATACS6 (NP_192867), OSACS1 (AAT77071), ZMACS6 (ACN25592), SCACS2 (JF274986), OSACS5 (BAB12704), ATACS7 (NP_194350) and MDACS3 (AAB67989). The bootstrap value is above each node. These values indicate the bootstrap replicates that support this node out of 1000 samples.

Expression of ACS genes in sugarcane tissues

Quantitative RT-PCR was performed to analyze the expression of the genes encoding the two ACS isoforms in sugarcane cultivars differing in their Brix content. To determine the integrity of total RNAs of biological triplicates, an electrophoresis gel was performed before quantitative RT-PCR (Figure 6A). All total RNA samples showed a satisfactory integrity for

expression analysis. Figure 6B shows the expression of *Scacs1* in mature leaf and throughout the development of the stalk (internodes 1, 5 and 9). *Scacs1* transcripts were detected in all tissues and the higher levels were observed in tissues of the cultivar with lower Brix content. Note that *Scacs1* were detected at a higher level in internode 9 when compared with internodes 1 and 5 in both sugarcane cultivars.

A similar pattern of expression was observed for *Scacs2* in all internodes (Figure 6C). Nevertheless, in the mature leaf of the high Brix cultivar, it was identified a higher level of expression, in opposition to the observed for *Scacs1*.


A
High Brix
Low Brix
Internode 1
Internode 5
Internode 9
Leaf
B
Internode 1
Fold Change
ACS1
High Brix
Low Brix
Internode 5
Internode 9
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High Brix In9
Low Brix In9

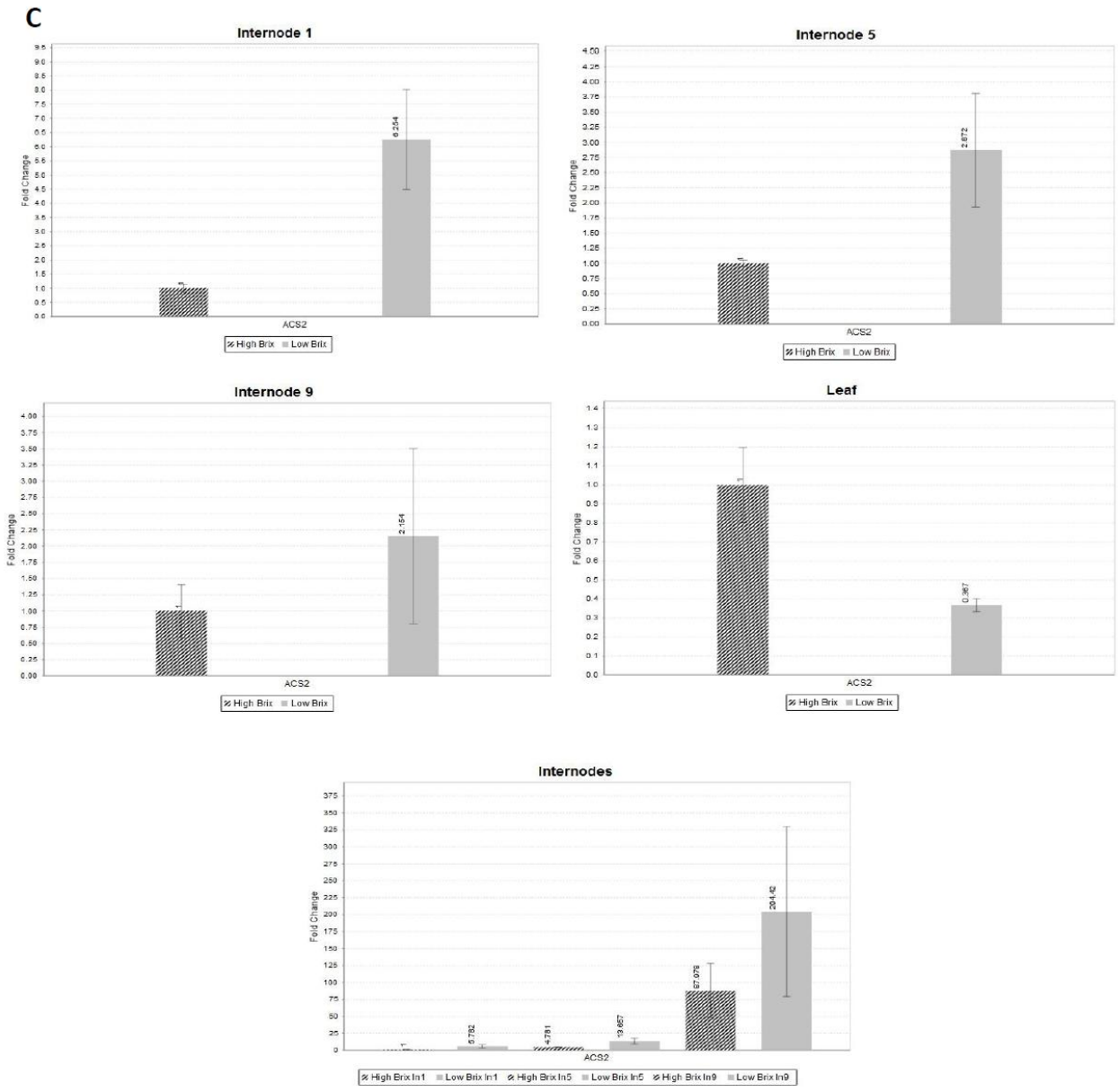
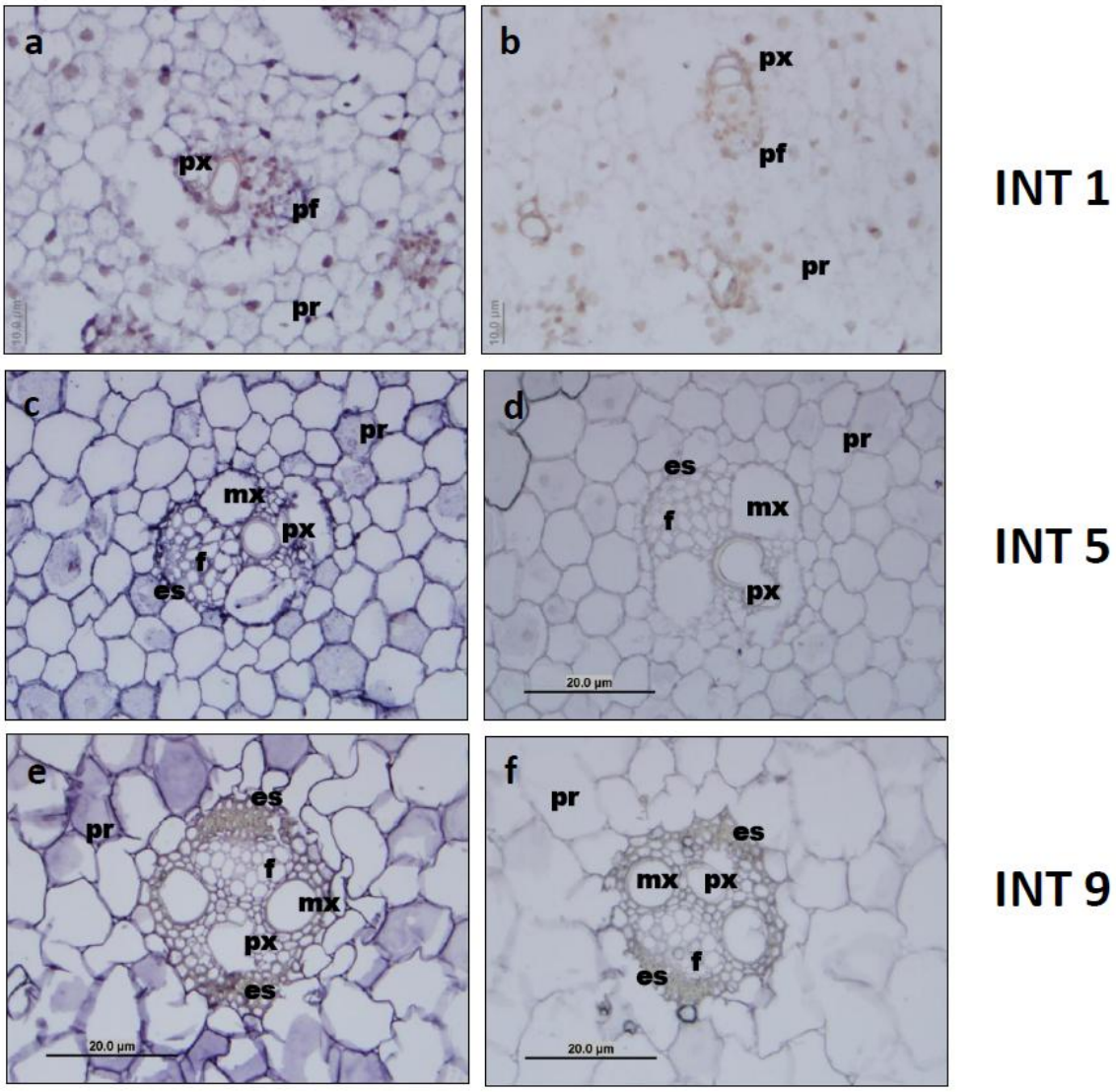


Figure 6. Quantitative real time PCR of *Scacs1* and *Scacs2* transcripts in mature leaf +1, internodes 1, 5 and 9 from two sugarcane cultivars contrasting to Brix content. (A) Electrophoresis gel of total RNAs of biological triplicates of RB855156 (High Brix) and RB935744 (Low Brix) sugarcane cultivars. (B) Expression analysis of *Scacs1* transcripts. (C) Expression analysis of *Scacs2* transcript. The samples were tested in experimental triplicate and normalized to polyubiquitin gene from sugarcane. The bars represent the mean from two biological replicates ± standard deviation.

In situ hybridization

To further characterize the expression of the ACS genes in sugarcane tissues, *Scacs1* and *Scacs2* probes were hybridized to transversal sections of mature leaf, internodes 1, 5 and 9 from two sugarcane cultivars to contrasting Brix content (Figure 7). *In situ* hybridization revealed a similar expression pattern of both *Scacs1* and *Scacs2* transcripts in parenchyma cells and sclerenchyma cells of the bundle sheath. However, the expression of *Scacs1* mRNA was not observed in the vascular bundles from internodes.

A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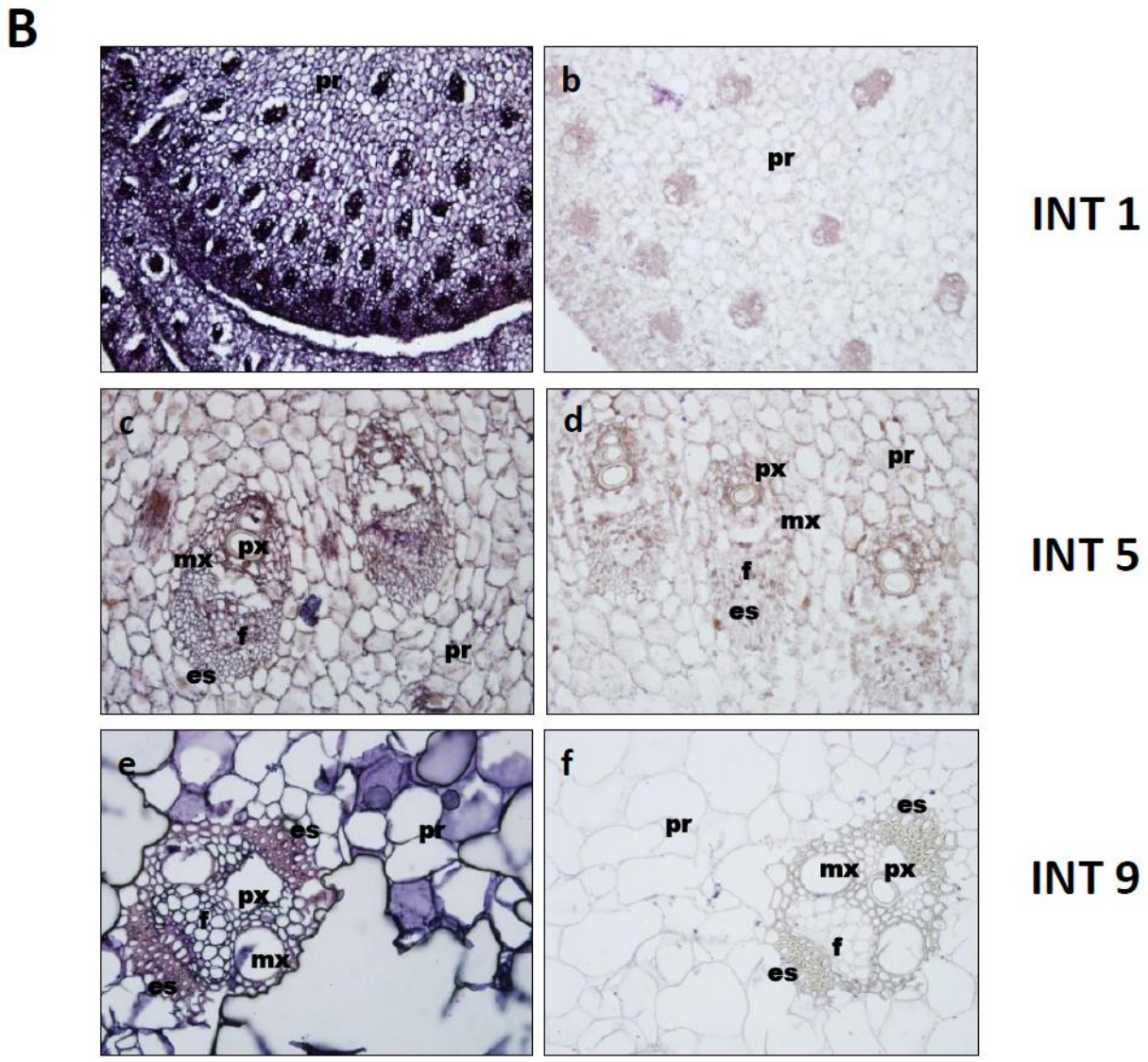

Figure 7. *In situ* hybridization of *Scacs1* and *Scacs2* mRNAs. The paraffin-embedded sections were hybridized with digoxigenin-labeled antisense and sense riboprobes generated from *Scacs1* and *Scacs2* 3'UTR region. (A) *Scacs1* transcript. (B) *Scacs2* transcript. All sections are transversal. The hybridization signal was observed as a purple precipitate. ´The figures b,d and f were hybridized with sense probe. pr, parenchyma; pf, protophloem; px, protoxylem; es, sclerenchyma; f, phloem; mx, metaxylem; INT 1, 5 and 9, first, fifth and nineth internodes, respectively.

Subcellular localization

To investigate the subcellular localization of sugarcane ACS proteins, two chimeric genes containing the ScACS1::GFP and ScACS2::GFP fusion, under the control of CaMV35S promoter, were constructed (Figure 8A). These expression cassettes were introduced in onion epidermal cells by particle bombardment and analyzed by fluorescence microscopy. Transient expression analysis for both ACS proteins showed a well-distributed localization in the cytoplasm of the onion epidermal cells (Figures 8C). Interestingly, the Signal P program (http://www.cbs.dtu.dk/services/SignalP/) indicated that a high probability (91.4%) of a signal peptide containing 19 amino acids at the N-terminal region from ScACS2, as depicted figures 8A and 8B. When other ScACS2 orthologs were analyzed the probability of a signal peptide was observed in some proteins, and increased from dicotyledonous to monocotyledonous sequences. For instance, AtACS7 protein from Arabidopsis and MdACS3 from apple did not show signal peptide in their sequences. In contrast, OsACS5 protein from rice (Figure 5) and the Sb03g003070 Sorghum EST sequence showed a high probability of 94.3% and 64.3%, respectively. Although, GFP fluorescence for ScACS2 showed a cytoplasmic staining, further analysis is needed to elucidate this intriguing highlight in sugarcane ACS.

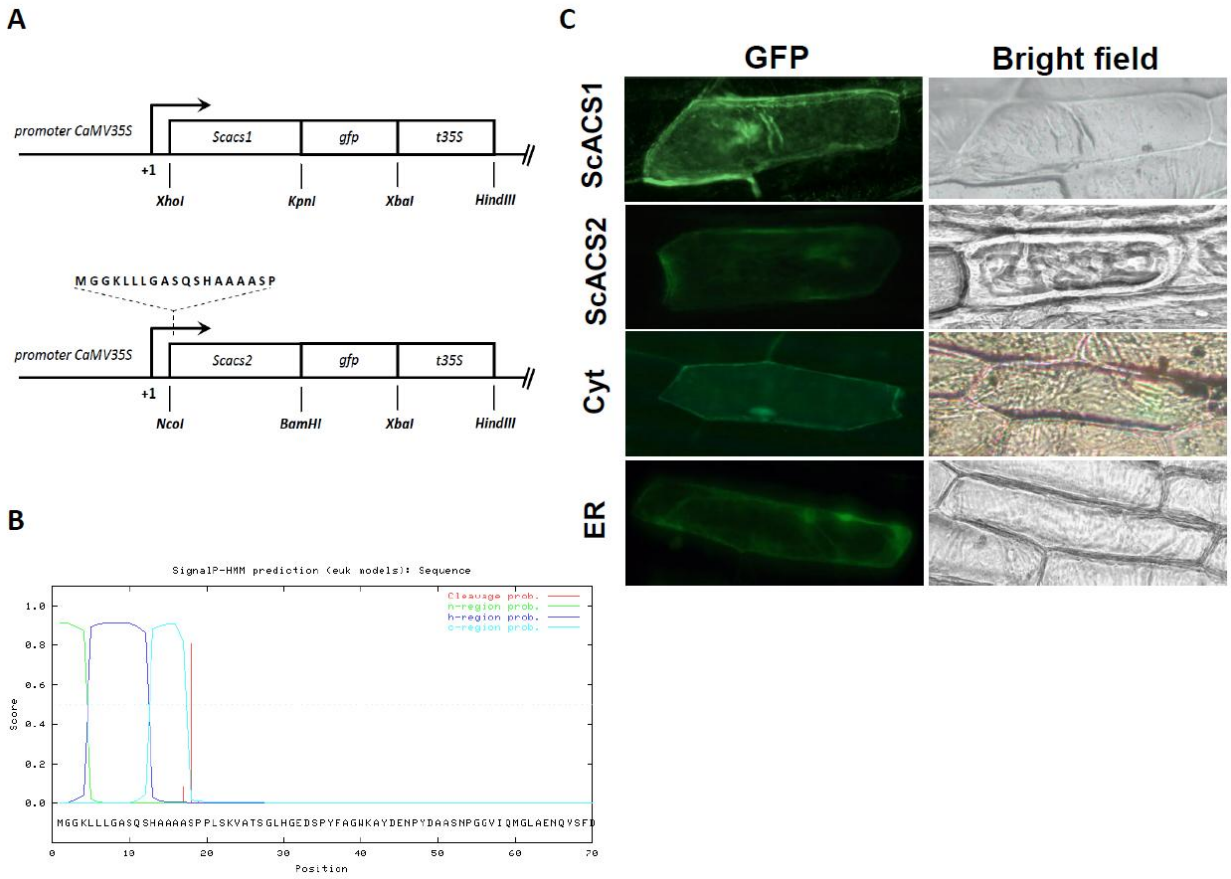

Figure 8. Subcellular localization of sugarcane ACS proteins. (A) Expression cassettes that were transiently expressed in onion epidermal cells. In detail, the putative signal peptide of 19 amino acids at the N-terminal region of ScACS2. (B) The graphical output from SignalP 3.0 server predicted a potential signal peptide of ScACS2 protein. The green, dark blue and light blue colors indicate the probability score for N-terminal region, hydrophobic core and C-terminal region of the signal peptide, respectively. The red color indicates the cleavage site of the signal peptide. (C) Transient expression of sugarcane ACS proteins, ER (pKDEL) and Cyt (pRT104_GFP) controls in onion epidermal cells.

ScACS1 and ScACS2 interactions

Tsuchisaka and Theologis (2004) uncovered that ACS enzymes from Arabidopsis can form heterodimers. To address the question whether sugarcane ACS non-homologous subunits can form heterodimers through protein-protein interaction, a small-scale yeast mating assay was

performed (Figure 9A). Diploid yeast cells were able to grow on DDO medium (Figure 9B), indicating that these cells harbored both plasmids with nutritional marker for the leucine and tryptophan amino acids. However, they were not able to grow on QDO medium, showing that the Gal4-responsive *ADE2* and *HIS3* genes were not activated due to the lack of interaction between ScACS1 and ScACS2 proteins (Figure 9C). Therefore, sugarcane ACS proteins were not able to interact and form heterodimers, contrasting to the findings in Arabidopsis.

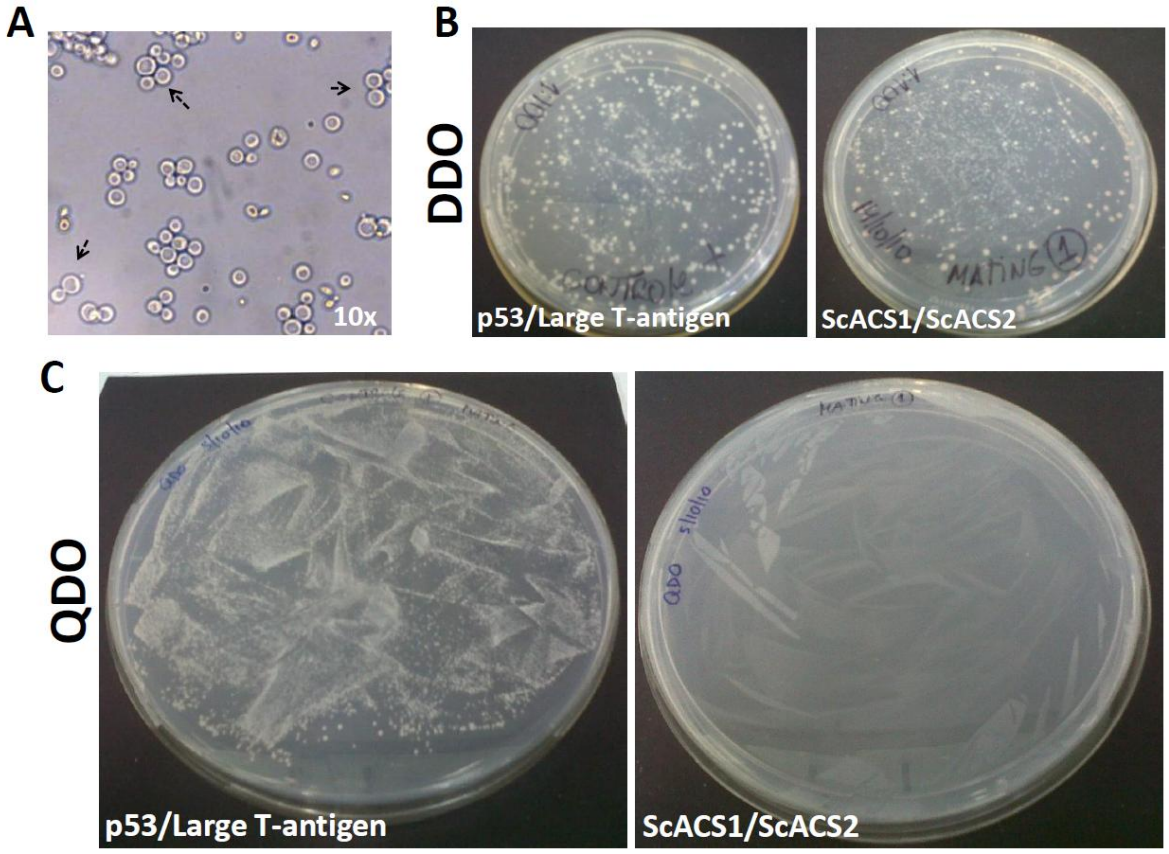

Figure 9. Small-scale mating of yeast expressing ScACS1 and ScACS2. (A) Fluorescence microscope imaging of the yeast cells culture after the mating. The presence of zygotes (showed by arrows) in culture indicates the success of the mating. (B) To verify the mating efficiency, a dilution of 1:100 of control and ScACS1/ScACS2 cultures were plated on DDO medium (-Leu/-Trp). (C) Mated culture plated on QDO medium (-Leu/-Trp/-His/-Ade). On the left, positive control mating of p53 and large T-antigen proteins, which are known to interact in a yeast two-hybrid assay (Li & Fields, 1993; Iwabuchi et al., 1993). On the right, diploid yeast cells containing both pGBKT7::ScACS2 and pGADT7::ScACS1 plasmids were not able to grow on QDO medium.

Identification of ScACS2-interacted proteins by yeast two-hybrid assay

The ethylene hormone has extensively been reported to play several roles in plants. Thus, in order to identify novel protein interactions of ACS proteins belong to type III and, consequently, to understand the molecular mechanisms involved by this key enzyme in the ethylene biosynthesis pathway, a yeast two hybrid assay using the ScACS2 isoform as bait was carried out using leaf and internode cDNA libraries from sugarcane (Figure 10).

Assays to verify toxicity and autoactivation were conducted and negative results supported the use of ScACS2 as bait (data not shown). The yeast strain expressing ScACS2 protein fused to *GAL4* DNA-BD was combined with the library strain and, after 24 hours of mating, the mixture was plated on low stringency media (DDO) for analysis of number of colonies screened (Figure 10D; Table 1). Concomitantly, this same mixture was plated on QDO medium for detection of positive interactions. As a result, two screens were done with each leaf and internode 5 cDNA libraries, yielding 27 and 16 independent clones, respectively, that grew on QDO medium (Table 1).

Table 1 Yeast two-hybrid screening using ScACS2 as bait

Library	Library aliquot[a] (LA)	Number of screened Clones[b]	QDO medium[c]	Genuine Positive[d]
Leaf +1	LA1	$1.15x10^6$	27	25
	LA2	$> 6.0x10^6$		
Internode 5	LA1	$0.92x10^6$	16	06
	LA2	$1.15x10^6$		

[a]. Library aliquots were chosen randomly;

[b]. Number of screened clones = cfu/ml of diploids x resuspension volume (ml);

[c]. Number of positives clones that grew on -His/-Ade/-Leu/-Trp medium;

[d]. Both bait and prey are required to activate all the Gal4-responsive reporters;

Subsequently, these clones were streaked on high stringency medium to identify potential binding proteins for the sugarcane ACS. Thus, at the first screen, 25 out of 27 and 6 out of 16 clones grew on QDO plus X-α-Gal medium and stained blue (Figure 10B) These clones were further streaked on QDO plus AbA (Aureobasidin A) antibiotic medium and the same 25 and 6 positives clones have grown, showing a strong interaction with ScACS2 and the activation of all the Gal4–responsive *MEL1*, *ADE2*, *HIS3* and *AUR1-C* reporter genes (Figure 10C; Table 1). The library plasmids from yeast cells that grown on QDO plus AbA were successfully rescued and sequenced (Table 2).

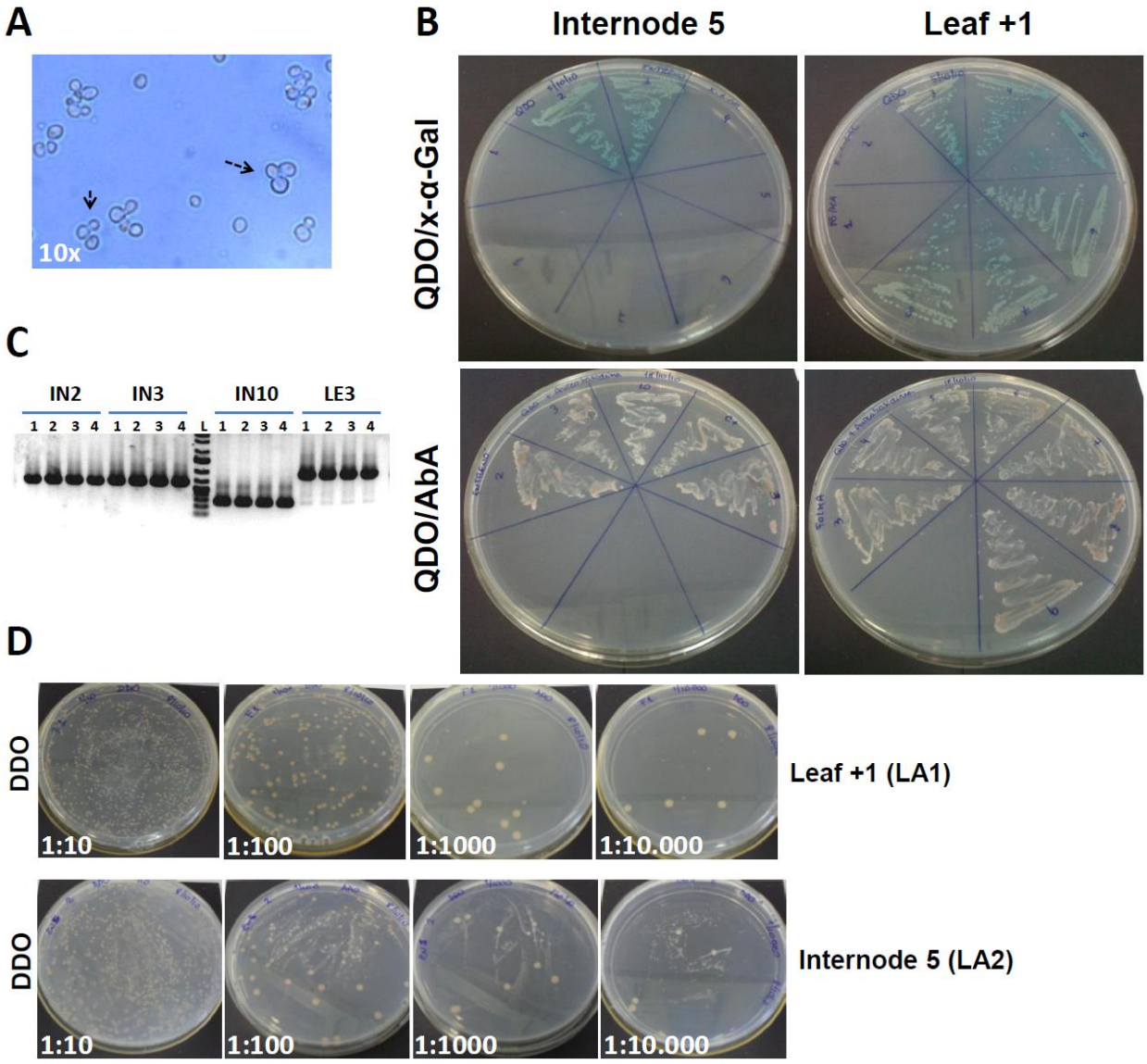

Figure 10. Yeast two-hybrid assay using ScACS2 as bait. (A) Fluorescence microscope imaging of the yeast cells culture after the mating. Arrows indicate the presence of zygotes in culture showing the success of the mating. (B) Positives clones screened by the two-hybrid assay. ScACS2-interacting clones grew on QDO medium with AbA antibiotic and showed β-galactosidase activity (blue color). (C) PCR product of positive clones. In detail, only the IN2, IN3, IN10 internode positive clones and LE3 leaf positive clone. For each positive clone, four plasmid-transformed *E.coli* colonies were used to analyze the inserted cDNA in pGADT7 vector. (D) Screened clones of leaf and internode cDNA libraries on DDO medium (-Leu/-Trp), as described in Table 1.

Among the six independent clones screened from internode 5 cDNA library, two presented similarity with a plant synaptotagmin from sorghum (Table 2). This membrane-trafficking protein has been characterized in Arabidopsis as a component of the machinery of plasma membrane repair allowing cellular integrity and viability under abiotic stress conditions such as salt (Yamazaki et al., 2010), osmotic stress (Schapire et al., 2008), freezing and wounding (Yamazaki et al., 2008).

Interestingly, regarding to ScACS2-interacted positive clones screened from the leaf +1 cDNA library, four showed similarity with an ATLM2 protein from maize (Table 2). These proteins presented a RING-H2 finger motif, which has been shown to interact with components in the ubiquitin-mediated protein degradation pathway, such as E2 ubiquitin-conjungating enzymes. Stone et al., (2005) have identified more than 450 predicted E3 ligases proteins belonging to the RING finger class in Arabidopsis. Likewise, Prasad et al., (2010) has identified and characterized a RING E3 ligase protein called XBAT32 in Arabidopsis which showed that negatively regulates the ethylene biosynthesis by interaction and ubiquitination to both AtACS4 and AtACS7 proteins.

Table 2 Yeast two-hybrid interacting clones selected with ScACS2 bait construct

Yeast two-hybrid interactors	Protein ID	N°. of times interactors identified with bait	
		Leaf +1	Internode 5
Plant synaptotagmin	EER91867		2
RING-H2 finger protein ATL2M	ACG30216	4	
Transcription factor MYC7E	AAD15818	1	
Hypothetical Protein	AAM08574	1	
Hypothetical Protein	EER88180	1	

Discussion

The ACS proteins present an extraordinary performance in plant physiology, whereas they are key regulatory enzyme for production of ethylene hormone in response to various environment and developmental cues in plants. Thus, innumerous studies have been conducted to identify and characterize ACS enzymes in several plants species from dicotyledonous to monocotyledonous. In our present study, two sugarcane ACS genes were identified and characterized. Similar to the size of others ACS isoenzymes from different plant species, the *Scacs1* and *Scacs2* genes presented a total coding region of 1464 bp and 1401 bp, respectively, encoding deduced polypeptides that comprised 487 and 466 amino acids with 55% of identity each other (Figure 2 and 3). In spite of the low similarity along all protein sequence between ACS isoenzymes, all the seven conserved boxes and the eleven invariants amino acids were identified in sugarcane ACS, the hallmark of the ACS multigene family (Figure 4). Different from AtACS1 non-functional protein from Arabidopsis, which it does not presented the

conserved tripeptide TNP (Liang et al., 1995), the ScACS1 and ScACS2 proteins presented all the conserved region and residues responsible for the substrate recognition and enzymatic activity (Figure 4).

The ACS proteins have been classified in three types according to the post-translational regulation domains found at the C-terminal region (Chae et al., 2003; Wang et al., 2004). ScACS1 predicted protein was closely related to ZmACS2 protein (Type 1), presenting the Ser target of CDPK phosphorylation in 'RL**S**F' motif and three conserved Ser residues that are the target for MPK6 phosphorylation (Figure 5). Interestingly, ACS proteins from monocotyledonous (ScACS1, ZmACS2 and OsACS2) showed a replacement of the conserved Phe residue in 'RLS**F**' motif with a Leu residue 'RLS**L**'. Hernández Sebastià et al., (2004) identified and analyzed the new motif of recognition of CDPK proteins in the ACS family from Arabidopsis and the LeACS2 protein from tomato. These authors found that the probably 'ACS motif' would be ϕ_{-3}-R_{-2}-ϕ_{-1}-$\mathbf{S_0}$-ϕ_{+1}-x-K_{+3}-R_{+4}, where ϕ is a hydrophobic residue and x is any amino acid. As a result, this point mutation, possibly, does not interfere in recognition of the enzyme to the RLSL motif, since both amino acids (Phe/Leu) are hydrophobic (Figure 5). In contrast, the ScACS2 protein fell into group type 3 showing high similarity to the OsACS5 protein from rice (Figure 4 and 5). Different from ACS proteins belonging to type 1 and -2, these proteins presented a short C-terminal region lacking all residues important for post-translational regulation identified until now. Our study also identified a putative interaction between the ScACS2 protein and an ATL protein, a RING H2 finger protein. This protein acts in the ubiquitination of target proteins for degradation by ubiquitin-proteasome pathway. Takai et al., (2002) has reported that the EL5 protein from rice, which share common structural features to the plant-specific ATL family, is an ubiquitin ligase and its RING H2 finger domain is necessary

for this activity. Similar to our results, Prasad et al., (2010) has demonstrated that the XBAT32 protein, a RING E3 ligase, was able to ubiquitinate in vitro the AtACS4 and AtACS7 proteins belonging to type 2 and 3, respectively, and that the *xbat32* mutant overproduces ethylene hormone. Together these results suggest for the first time a possible post-translational mechanism of ACS proteins belonging to type 3.

Remarkably, ACS proteins from monocotyledonous and dicotyledonous were found in the three groups according to well-defined branches of the phylogenetic tree (figure 5), indicating that the polymorphisms and the post-translational regulation domains have occurred prior of the time of divergence of plant species. Moreover, (LIANG et al., 1992; Liang et al., 1992; Jakubowicz, 2002; Huang et al., 2006) have proposed a possible correlation between ACS genes clustered in a branch and their expression patterns. Based on this hypothesis, quantitative PCR analysis has revealed that *Zmacs2,* which presented high similarity to *Scacs1* gene, was expressed in maize roots and during leaf development (Young et al., 2004; Gallie et al., 2009). Likewise, the SUCAST project showed that the SCJLRT1006C03 cluster, called *Scacs1*, presented a higher expression in sugarcane roots and leaf roll (data not shown). With respect to *Osacs5* gene from rice, similar to *Scacs2,* a restrict expression to vascular tissues of stems, shoot apex and young leaves have been reported (Zhou et al., 2002). Our *in situ* analysis of *Scacs2* gene revealed an ubiquitinous expression in all the cell types of the internodes 1, 5 and 9 in sugarcane, including the vascular bundles (Figure 7B). This expression pattern was also identified in the *in situ* analysis for the *Scacs1* gene, except in vascular bundles, where no expression signals were detected (Figure 7A). It is worthwhile to further discuss the expression of the *Scacs2* gene in vascular bundles. Jackson, (1997) and Zhou et al., (2002) have reported the

importance of ACC transport in root-to-shoot signaling in flooded tomato and rice. Nonetheless, future research will be necessary to gain further insight into this process in sugarcane.

Additionally, our results of quantitative PCR revealed a slight expression of the *Scacs1* and *Scacs2* genes in internodes 1, 5 and 9 (Figure 6B). Interestingly, when a relative expression analysis was performed, the expression of sugarcane ACS genes was correlated with the development of the internodes (Figure 6). The higher expression of the sugarcane ACS genes in the most mature internodes (5 and 9) might be related to the osmotic stress imposed to the high levels of sucrose abiotic stress tolerance, particularly osmotic and oxidative stress, due to the high accumulation of sucrose in the storage parenchyma. In a recent report, Iskandar et al., (2011) showed that transcription factors of the ERF family were more expressed in older internodes when compared to young internodes. Other evidence in this line, coming from our data, is the putative interaction of the ScACS2 protein with the plant synaptotagmin protein in the internode, since this synaptotagmin is involved in the cellular integrity and viability (Schapire et al., 2008). In view of this new scenario, the ethylene hormone would be required for mediating the plasma membrane repair mechanism, facilitating the accumulation of high levels of sucrose in most mature internodes. In literature, ethylene has been well documented in mediating mechanical wounding responses (O'Donnell et al., 1996). To our knowledge, the present study is the first to identify a possibly participation of the ethylene hormone in the plasma membrane repair through of ACS enzyme.

As above-mentioned, the ethylene hormone plays an important role in defense mechanism in plants (Ecker and Davis, 1987). Nevertheless, recent studies on jasmonate (JA) and ethylene signaling pathways have identified a cross-talk regulation in the activation of the defense genes in response to abiotic and biotic stresses (Penninckx et al., 1998; Zhao et al.,

2004). Recently, Lorenzo et al., (2003) found a synergy between these hormones in the transcriptional activation of ERF1 gene, indicating it as a convergence point in synergistic signaling of ethylene/JA. Another point of convergence between JA and ethylene signaling is CEV1 (Constitutive Expressor of VSP1), which is also known as cellulose synthase (Ellis et al., 2002). Nevertheless, our data presented here have identified a putative interaction between ScACS2 protein and the MYC transcription factor through yeast two hybrid assay (Table 2). The MYC protein is a helix-loop-helix-leucine zipper (bHLHzip)-type transcription factor that has been reported regulating the JA signaling pathway (Lorenzo et al., 2004). Thus, the present study suggests a new upstream regulation point of ERF1 transcription factor between ethylene and JA hormones. However, further analyses are necessary to decipher the molecular mechanisms underlying this interaction.

On the other hand, our findings confirmed previously studies about the cytosolic localization of the ACC synthase proteins (Figure 8C). Nevertheless, none conclusion was achieved to ScACS2 protein, whereas a computational prediction presented a probable signal peptide of 19 amino acids at the N-terminal region (Figure 8A and 8B). To assess whether the ScACS2 protein present really this signal peptide and is directed to some organelle, further analyses will be necessary.

Finally, with respect to dimerization of sugarcane ACS proteins, our preliminary results demonstrated that there is no interaction between the ScACS1 and ScACS2 monomers for the formation of heterodimers (Figure 9). However, Tsuchisaka and Theologis, (2004) have reported the capacity of all Arabidopsis ACS proteins to heterodimerize. According to Tsuchisaka et al., (2009) the formation of homo- and heterodimers (active or inactive) increase the combinatorial

interplay among ACS isoforms to performe ethylene-mediated processes. Thus, our data indicate that regulation mechanisms through ACS dimerization can be different among distinct species.

In conclusion, our work has identified and characterized two ACC synthase genes in sugarcane. In this report, novel highlights presented here will allow a better understanding of the underlying mechanisms by ethylene hormone in sugarcane.

Acknowledgements

We thank the Prof. Sandra Maria Carmello-Guerreiro, Plant Anatomy of the Department of Plant Biology – UNICAMP, Campinas – Brazil. The authors RGT and AG were supported by CAPES and FINEP/CNPq. MM received a research fellowship from CNPq.

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Capítulo II

Chemically-controlled synthesis of sugarcane ACC synthase increases ethylene production in transgenic tobacco plants.

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Abstract

The use of constitutive promoters to control transgene expression has been a key tool in plant molecular biology. However, in some cases, a precise temporal regulation is desired to characterize gene function in a specific time during plant growth and development. Additionally, for many genes, constant induction leads to undesirable or deleterious phenotypes. In this scenario, an ethanol-inducible expression system, the *alc* gene-expression system, has successfully been used in tomato, potato, Arabidopsis and tobacco. In this way, the use of this system in sugarcane plants would be a valuable tool in the control of the maturation by application of ethanol instead of plant regulators such as ethrel. Here, the utilization of the *alc* system in the regulation of ACS gene and, consequently, in the control of the ethylene biosynthesis was evaluated. In this study, transgenic tobacco plants expressing the *Scacs1* gene encoding a sugarcane ACS under the control of the *alc* system were generated. Plants presented a normal growth and no abnormalities were observed. A significant increase of ethylene levels were observed upon ethanol treatment of the transgenic plants. Our data highlights the usefulness of the strategy of using a chemically controlled system to control ethylene levels in plants. The implications for sugarcane cultivation are discussed

Key words: Ethylene, ACC synthase, *alc* system

Introduction

Plants produce the ethylene hormone to regulate a wide range of developmental processes and signal different responses due to biotic and abiotic stresses (Bleecker and Kende, 2000). As a result, the production of this phytohormone is a highly regulated process. The main point of regulation is the rate-limiting step in biosynthesis, catalyzed by the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase enzyme (Jakubowicz, 2002). Various external and developmental cues have been reported acting in the regulation of the expression of this enzyme (Lin et al., 2009). Additionally, studies on transgenic plants have focused on the ACC synthase gene for manipulation of ethylene synthesis in plants. Knoester et al., (1997) have demonstrated in tobacco that overexpression of an ACC synthase (ACS) gene lead to an increase of 320% more ethylene. However, this alteration in ethylene levels affected stem length and leaf chlorophyll levels. On the other hand, Oeller et al., (1991) have shown that antisense suppression of an ACS gene in tomato reduced ethylene synthesis by 99%. Nevertheless, the fact that ethylene plays diverse biological roles in plants and the fine-tuning regulation of its synthesis, an ubiquitious expression or silenced of ACC synthase during all plant cycle could result in agriculturally undesirable phenotypes.

A very common practice in agriculture has been the use of Ethrel. This compound (2-chloroethyl- dioxido-oxophosphorane) is a synthetic molecule and upon contact with plant it is rapidly metabolized and converted to ethylene. In sugarcane, various field studies have demonstrated large benefits of the application of etephon such as the delay opening of the inflorescence (Eastwood and Davis, 1997), earlier maturation process (Page, 1983) and higher tillering and sucrose content (Li and Solomon, 2003; Caputo, 2006; Caputo et al., 2007).

With the advances in plant biotechnology, one essential tool for the temporal, spatial and quantitative control of genes has been developed, based in the utilization of chemical gene induction systems (Gatz, 1997). Several articles and excellent reviews about these systems have been published and are available (Zuo and Chua, 2000; Padidam et al., 2003; Zuo et al., 2006). Of them, the ethanol-inducible gene expression (*alc* switch) have been widely utilized in diverse plants species such as Arabidopsis (*Arabidopsis thaliana*) (Roslan et al., 2001), tobacco (*Nicotiana tabacum*) (Schaarschmidt et al., 2004), potato (*Solanum tuberosum*), oilseed rape (*Brassica napus*) (Sweetman et al., 2002), and tomato (*Lycopersicon esculentum*) (Garoosi et al., 2005).

The goal of this study was to demonstrate that the *alc* system could be used to control ACC synthase gene expression in plants and therefore to increase ethylene biosynthesis. We used an ACS gene from sugarcane, *Scacs1*, recently cloned (Tavares, unpublished results). A rapid transient assay was performed to test the dose-response of ethanol (inductor) on the expression of β-glucuronidase (GUS) reporter gene in lettuce leaves. Ours results confirm the ability of the *alc* system in transient expression assay and the temporal control of ethylene hormone synthesis in plants, indicating a possible application of this system in maturation control in sugarcane.

Materials and Methods

Plasmid Construction

Standard DNA cloning procedures were used for plasmid constructions (Sambrook et al., 1989). Four constructs were used in this study (Figure 1). As a positive control, a construct based in the alc system using the GUS gene was produced as follows. The coding region of β-

glucuronidase (GUS) reporter gene was PCR-amplified from pBI101.2 (Jefferson et al., 1987), subcloned into pGEMTEasy vector (Promega, USA) and then cloned downstream of the alcA:CaMV35S fusion promoter from pACN vector (Syngenta, USA). Concomitantly, the coding region of alcR transcription factor was PCR-amplified from pbinSRNACatN vector (Syngenta, USA), subcloned into pGEMTEasy vector (Promega, USA) and subsequently cloned into the pRT104 vector (Töpfer et al., 1987). Both cassettes were inserted into the binary vector pCAMBIA2300 (CAMBIA, Australia) (Figure 1A.I). The test construction was done similarly, except that the coding region of *Scacs1* gene from sugarcane replaced the GUS gene. (Figure 1A.II). As negative controls (Figure 1B.I and 1B.II), similar constructs were obtained, except that the cassette carrying the alc regulon was not inserted in the MCS. Constructs were cloned and transformed into *Escherichia coli* DH5α cell and then introduced into A*grobacterium tumefaciens* GV3101 by the freeze–thaw method (Chen et al., 1994).

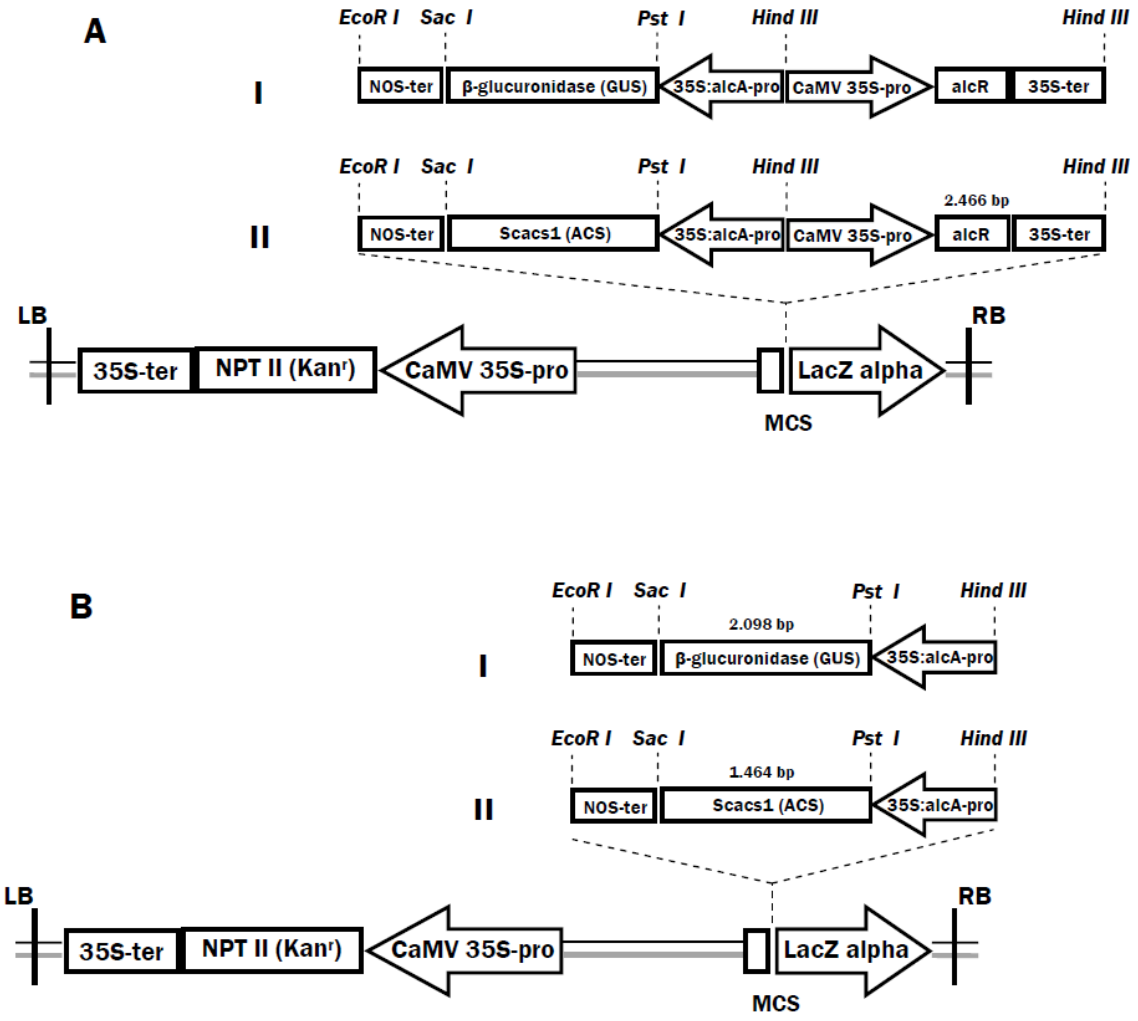


Figure 1. Constructs using the alc gene expression system. **(A)** Constructions containing complete alc systems. The constructs included the CaMV35S promoter (CaMV35S-pro) driving the alcR cDNA, positioned upstream of the CaMV35S terminator (35S-ter). The chimeric promoter (35S:alcA-pro), comprised by the CaMV35S minimal promoter (-23 to +1) fused with the alcA promoter (Caddick et, al., 1998) drives the β-glucuronidase (GUS) reporter gene in construction **(AI)** or the Scacs1 cDNA in construction **(AII)**, in both cased using the NOS terminator (NOS-ter). **(B)** Negative controls of the alc system. The expression cassette with the alcR transcription factor was not included in the constructs **(BI)** and **(BII)**. Plant transformation vector pCAMBIA2300 has the resistance gene *nptII* for selection in plants neomycin phosphotransferase II, and the lacZ alpha gene for screen blue/white colonies. Left border, LB; Right border, RB; Multiple cloning site, MCS;

Transient expression assay

Lettuce (*Lactuca sativa*) transformation using vacuum infiltration method with *Agrobacterium* were performed as described by Joh et al., (2005). The pCAMBIA2301 vector containing the β-glucuronidase (GUS) gene under control of the CaMV35S promoter was utilized as positive control.

Determination of the ethanol dose-response in lettuce.

Small foliar pieces from each transformed lettuce carrying the constructs of *alc* system and control vectors were exposed to 0,5%, 1%, 5% and 10% ethanol (% v/v) to assess their ability to induce the *alc* system. All samples were incubated at 37 °C for 24 hours. The solution of ethanol was applied directly to the surface of each sample. Ultrapure water was used as a negative control of the *alc* system.

GUS histochemical staining

Control and ethanol-treated samples were assessed by GUS histochemical assay according to Jefferson et al., (1987). Each sample was incubated at 37 °C for 16 hours. Images were taken using a Leica MZ10F stereomicroscope (Leica, Germany).

Plant material and transformation

Tobacco plants (*Nicotiana tabacum* var SR1) were grown in a growth room under a 16 h light/8 h dark cycle and 25 °C. Transformation was achieved by co-cultivating leaf discs from sterile grown plantlets with *Agrobacterium* strain GV3101 harboring the expression plasmids (Gallois and Marinho, 1995). Shoots were regenerated from transformed calli selected on MS

medium containing 100 mg/L kanamycin and subsequently rooted on MS medium (Murashige and Skoog, 1962) supplemented with 100 mg/L kanamycin and transplanted to soil.

Analysis of transgenic plants

Initial screen of transformants (T_1 generation) was performed on kanamycin selective medium, followed by PCR tests for the presence of *alcR* and *Scacs1* genes. The seeds from individual plants of T_0 generation were collected separately and grown on agar plates containing kanamycin (150mg/L) and modified MS medium (0,2x MS salt, 0,5% sucrose, absent vitamins) to screen for the transformants.

For PCR analysis, the genomic DNA of positive transgenic plants on the selective medium described above was extracted as described by Edwards et al., (1991). A segment of coding region of *Scacs1* not conserved when compared to ACC synthases from tobacco was chosen to design of the specific primers of the *Scacs1* gene. Wild-type plants were used as negative control.

RNA isolation

In order to verify the constitutive expression of alcR gene in transgenic lines, total RNA was isolated using Trizol (Invitrogen) according to the manufacter's instructions. First-strand cDNAs were synthesized using 2.0 μg DNAse I-treated RNA, (500 ng/μL) oligo-dT primer and 10 mM dNTP mix. All RNA samples were denaturated at 65°C for 10 minutes, cooled on ice for 2 minutes and added 5X First Strand Buffer, 0.1 M DTT, RNAse out (40 U/μL) and Superscript III RT enzyme (200U/μL) (Life Technologies, USA). The reaction was carried out at 42°C for

50 minutes. First-strand cDNAs syntheses were finished by heating to 70°C for 15 minutes for the inactivation of the RT enzyme.

Induction of ACC synthase in mature plants and quantification of ethylene hormone in the airspace

Two alcR alcA::Scacs1 transgenic lines (16 and 32) carrying the construction with both *alcR* and *Scacs1* expression cassettes (Figure 1AII) and one alcA::*Scacs1* transgenic line (10) carrying the construction only with *Scacs1* expression cassette (Figure 1BII) were propagated *in vitro* under growth chamber conditions (25°C; 16 h light/8 h dark) in tightly closed vials. These 4-months old tobacco plants were induced with 5% (v/v) ethanol for 24 h by foliar spray and subsequently the ethylene hormone was quantified. Gas samples of internal space were withdrawn from the vials by a gas-tight syringe and analyzed by gas chromatography (Shimadzu, Japan). An ethylene gas standard was used in the analysis. Triplicate experimental was performed for each plant. Previously, these same plants were mock-induced with ultra-pure water in the same conditions above as a negative experimental control. A non-transgenic control plant and biological triplicates of each transgenic line were used in the experiment.

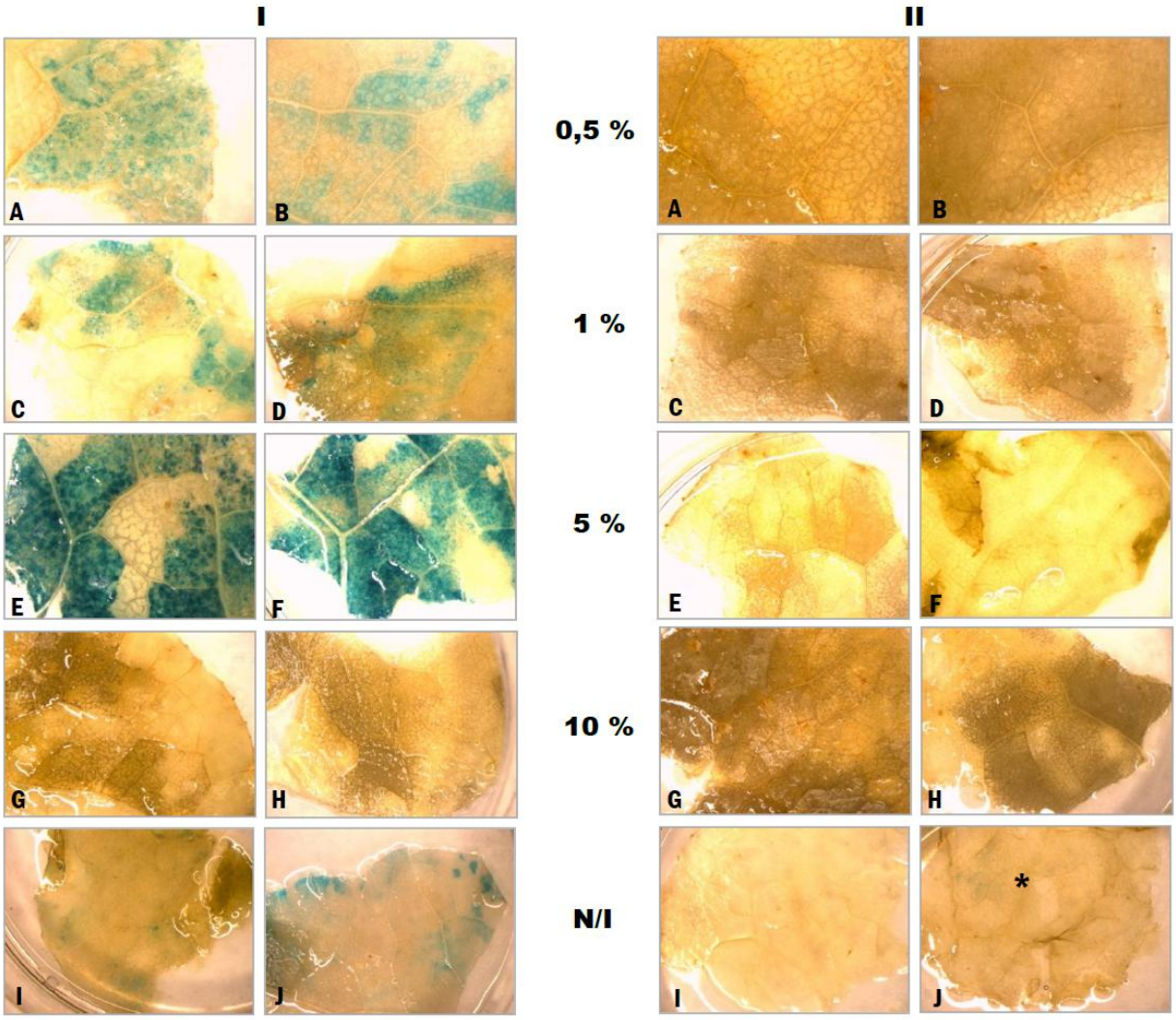
Results

The alc system is active in transient expression

In order to determine the ethanol dose-response for induction of the GUS reporter gene, our constructions were assessed transiently in lettuce. According to Garoosi et al., (2005) the application of ethanol inductor shows a faster activation and efficient of alc system than other

alternative inductors. Ethanol concentrations in the range 0.5%-10% (v/v) applied to the foliar surface resulted in a remarkable difference in β-glucuronidase (GUS) activity (Figure 2.I). Different from concentrations of 0.5% and 1% (v/v) that showed a relatively slight increase in activity (Figure 2.I. A-D), the concentration of 5% showed strong induction of the alc system (Figure 2.I. E and F). In contrast, application of ethanol at higher concentration 10% (v/v) seemed to be toxic to the plant, whereas the reporter gene has no expression in all assays (Figure 2.I and III. G and H). A weak background expression due to alcA promoter leakage was found in non-inducible samples as depicted in figure 2.I. I-J.

Regarding the construction alcA-GUS, negative control of the alc system due to lack of the alcR transcription factor, there was an insignificant GUS activity in the non-inducible assay (Figure 2.II.J). Nevertheless, the activation of alc system was not observed in the presence of ethanol inductor in different concentrations (Figure 2.II.A-H). Agro-infiltration and GUS histochemical assay controls provided reliability in the results, whereas positive control showed expression of the reporter gene in all samples and the negative control, non-transformed lettuce, did not show any expression signal of GUS gene (Figure 2.III and IV). The table 1 summarizes the results of induction of the alc system by ethanol. Therefore, these results showed the activation of the constructed alc system and the optimal concentration of 5% (v/v) of ethanol for induction.


I
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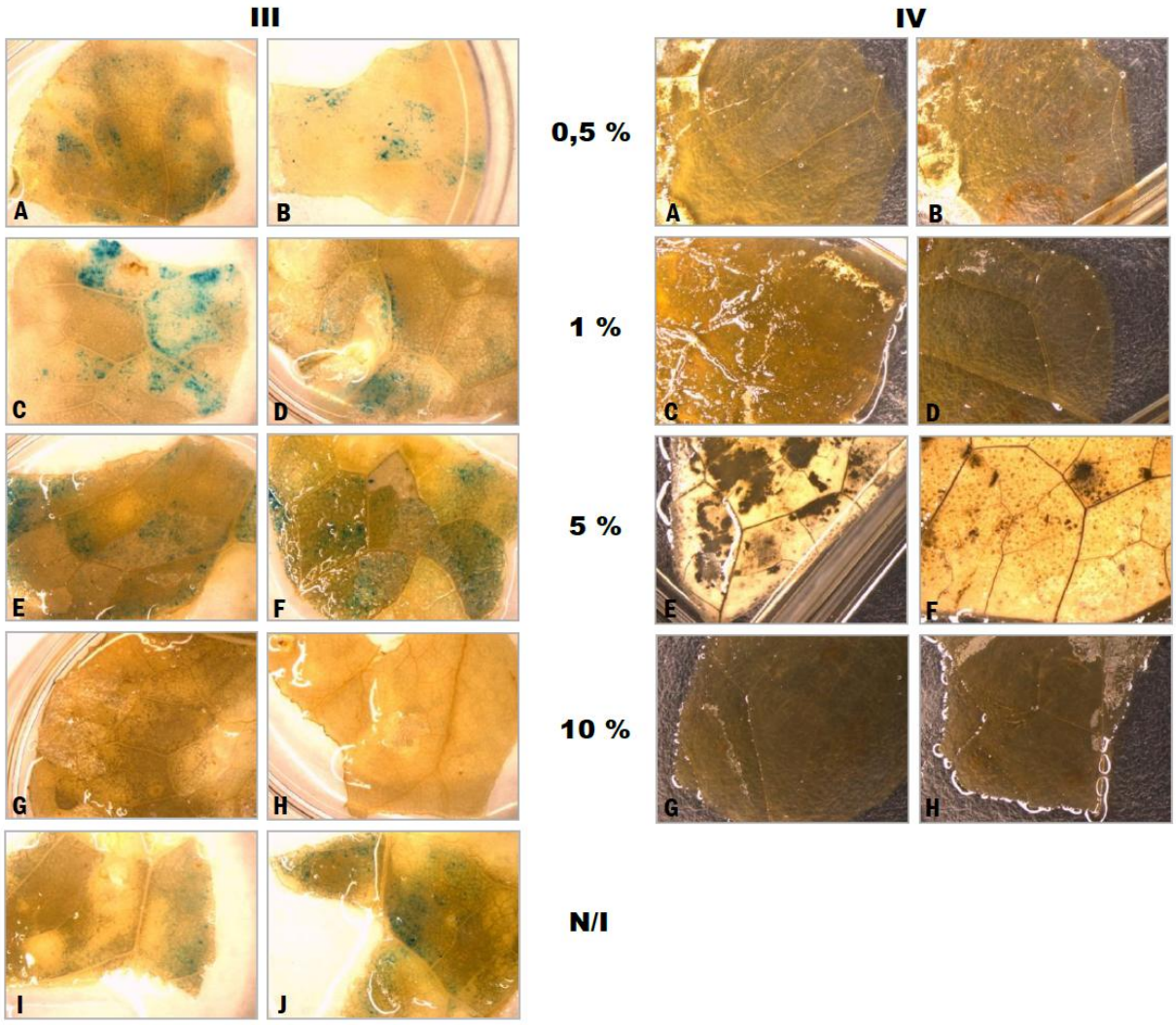


Figure 2. Transient GUS gene expression in lettuce using alc gene inducible system. Lettuce leaves were stained for GUS activity 2 days after infiltration with *A. tumefaciens* strain GV3101 harboring either the alcR-alcA-GUS construction (I), the alcA-GUS construction (II), the control plasmid pCAMBIA2301 (III) or non-transformed lettuce, used as negative control of the assay (IV). Ethanol concentrations of 0,5%, 1%, 5%, 10% (v/v) and ultra pure water correspond the figures AB, CD, EF, GH and IJ, respectively. N/I – non-inducible samples treated with ultrapure water. Asterisks (*) indicate the presence of the GUS staining as depicted in figure II.J. Cuts were made in the leaves to enable penetration of the GUS substrate.

Table 1. Comparison of the GUS activity in the transient expression in lettuce

Leaves of lettuce (*Lactuca sativa*) were incubated with or without the ethanol inductor at 25 °C for 24 h in a growth chamber.

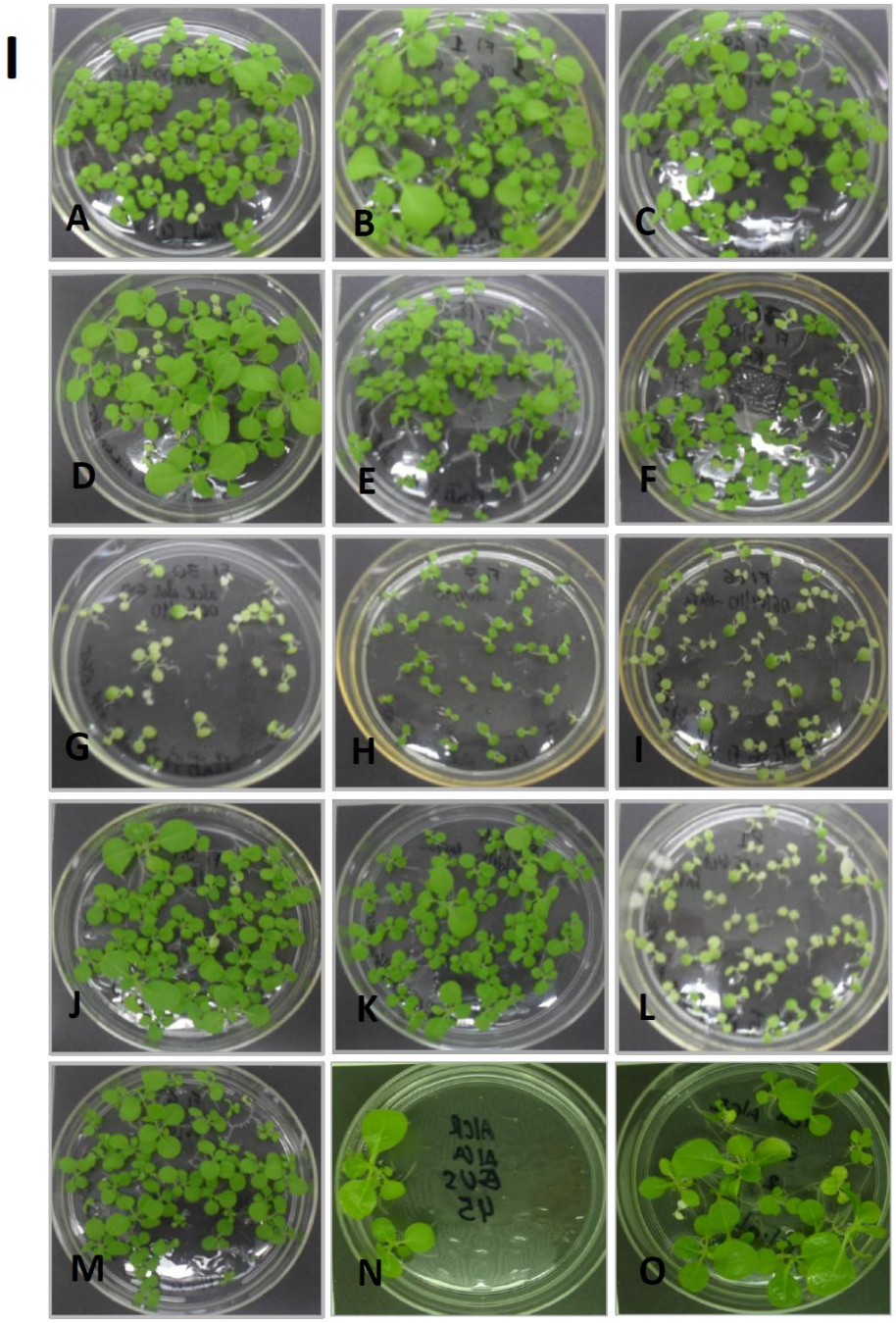
The signs (+ / –) represent the presence or absence of GUS staining, respectively.

Line	Induced (v/v)				Non-induced
	0.5%	1%	5%	10%	
pCAMBIA2301	+	+	+	-	+
alcA-GUS	-	-	-	-	-
alcR-alcA-GUS	+	+	+	-	+
Wild Type	-	-	-	-	-

Preparation and selection of transgenic lines

Tobacco plants (*Nicotiana tabacum var. SR1*) grown *in vitro* were transformed separately with Agrobacterium strain GV3101 carrying the constructions alcR alcA::GUS, alcR alcA::*Scacs1* and alcA::*Scacs1* (Figure 1) and a total of 15, 6 and 6 independently kanamycin-resistant lines (transformation events) were generated, respectively. Kanamycin-sensitive plants showed chlorotic spots in leaves, early senescence, developmental arrest and absent of root (data not shown). The T_0 transgenic lines were transferred to the greenhouse to obtain T_1 progeny seeds by self-pollination.

Segregation analysis on modified MS medium of T_1 progeny seeds showed a mendelian segregation ratio 3:1 for almost all events. However, some transformed tobacco plants were false positives (Figure 3).



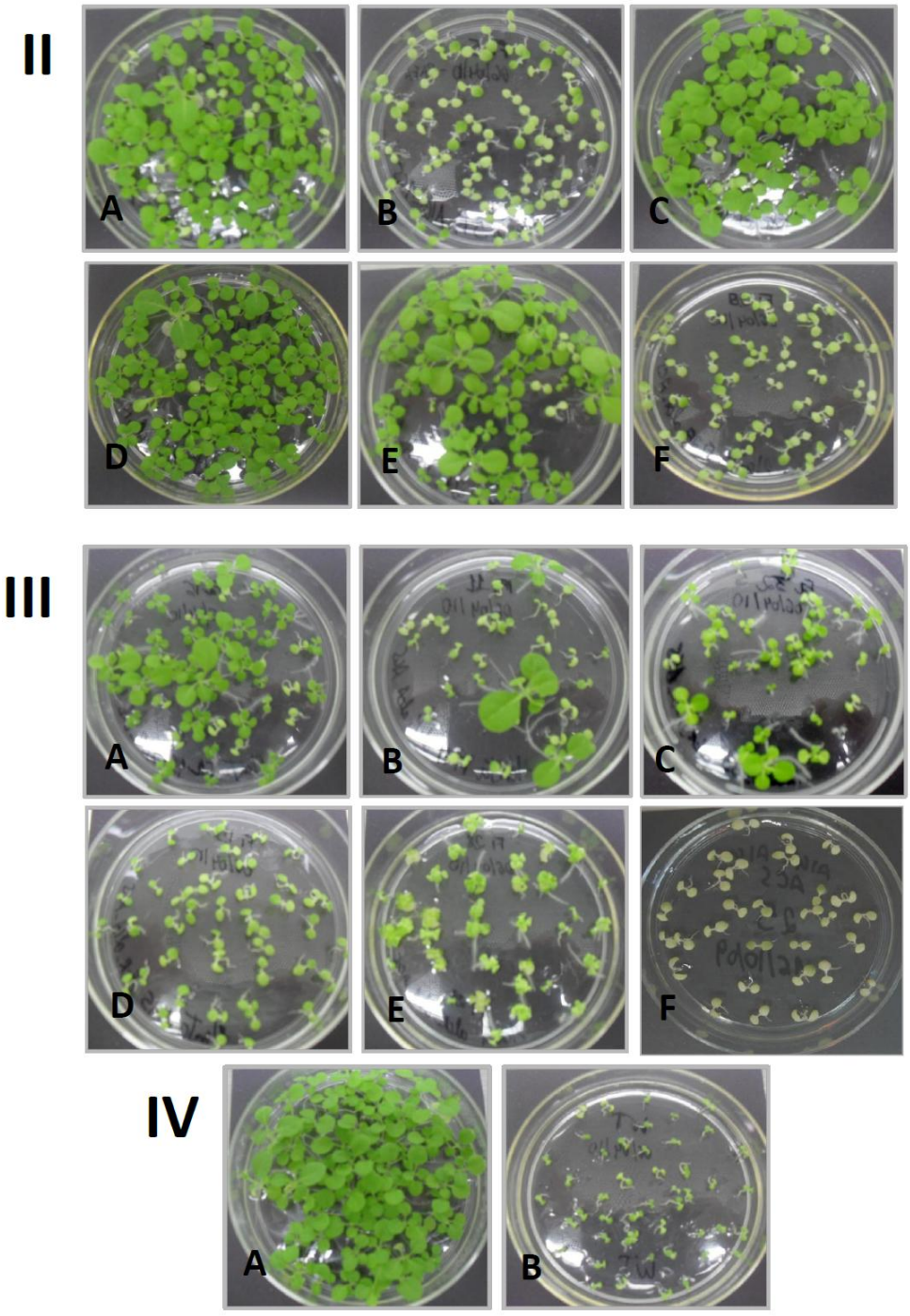


Figure 3. Phenotype of T_1 progeny tobacco plants 32 days after the germination on Kanamycin-selective MS medium. Independent events of alcR alcA::GUS (I) alcA::*Scacs1* (II) alcR alcA::*Scacs1* (III) constructions and untransformed control (IV). As controls, untransformed plants were grown on MS medium without (IV-A) and with (IV-B) kanamycin.

Further analysis of these transgenic plants was performed to confirm the integration of the transgenes. Genomic DNA was extracted from kanamycin-resistant T_1 tobacco plants and PCR assay were subsequently analyzed for integration of alcR and Scacs1 coding regions (Figure 4).

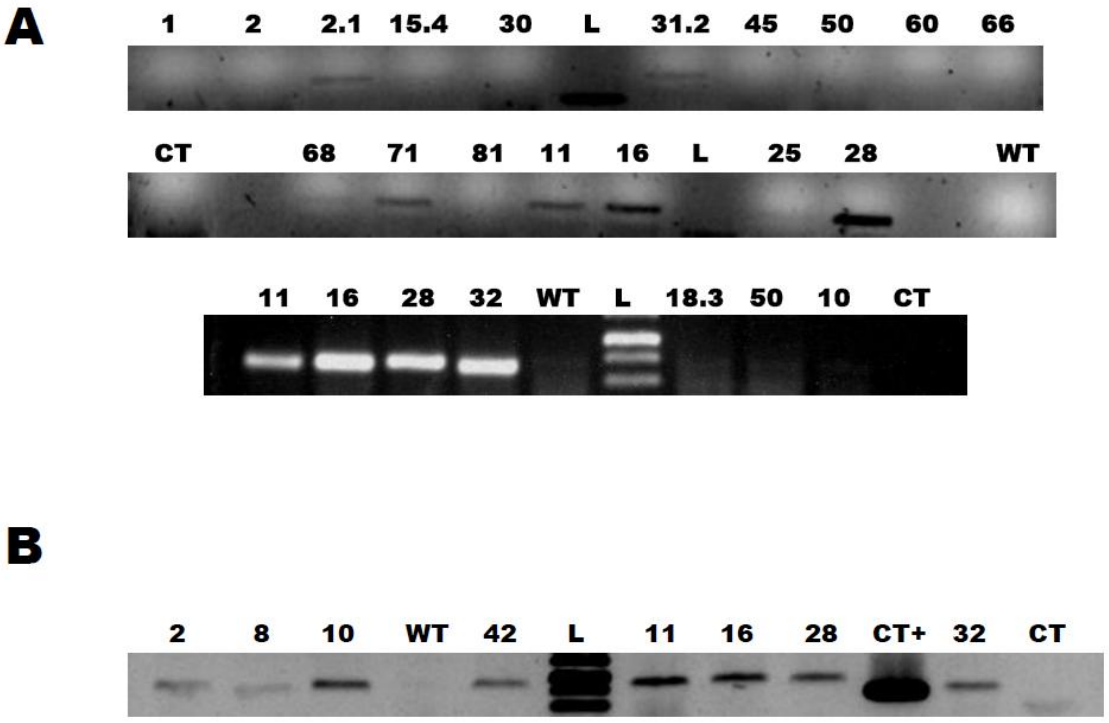


Figure 4. PCR analysis of transgenic plants. Primers for the *alcR* (A) and the *Scacs1* (B) were used. WT, wild-type; 1, 2, 2.1, 15.4, 18.3, 30, 31.2, 45, 50, 60, 66, 68, 71 and 81, independently lines for alcR alcA::GUS construct; 11, 16, 25, 28 and 32 independently lines for alcR alcA::*Scacs1* construct; 2, 8, 10 and 42 independently lines for alcA::*Scacs1* construct; CT+, alcR alcA::*Scacs1* vector; CT, no template.

Stability of the system

According to Roslan et al., (2001) the silencing has been observed in the alcR alcA::GUS lines in Arabidopsis. Nevertheless, the frequency and extent varies between and within lines.

With the exception of the alcR alcA::Scacs1 lines, all the lines alcR alcA::GUS showed silencing of the alcR transcription factor in tobacco plants (Figure 5). Metzlaff et al., (1997) has attributed the problem of silencing to the design of the constructs, which include a repeat of CAMV35S promoter sequence around 91bp in the *alc* promoter. Furthermore, a uniformity of expression was not observed among alcR alcA::Scacs1 lines. The 28 line showed a highest level of expression and the 16 line a lower expression compared to 32 line expression level.

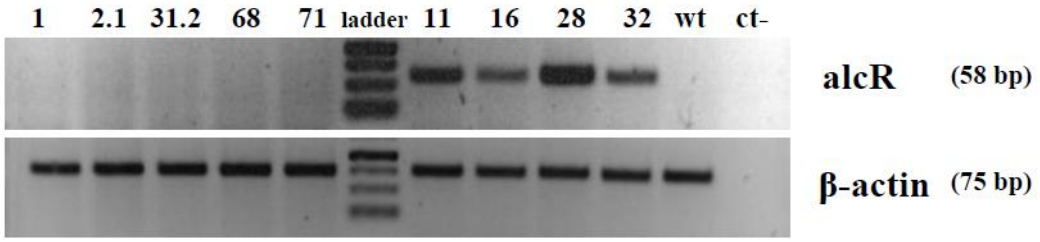


Figure 5. RT-PCR analysis of alcR expression in transgenic plants. On the left, 1, 2.1, 31.2, 68 and 71 are independently lines of alcR alcA::GUS construct; on the right, 11, 16, 28 and 32 are independently lines of alcR alcA::Scacs1 construct; wt, wild type; ct-, no template; The β-actin control is also shown in the lower panel.

The ACC synthase is the rate-limiting step in the ethylene hormone biosynthesis

To determine whether transgenic plants carrying *alc* system increase the ethylene hormone biosynthesis through of ACS expression modulation, two alcR alcA::Scacs1 lines (16 and 32) and one alcA::Scacs1 control line (10) were assessed. As depicted in figure 6, all the plants presented a similar production of ethylene, using water as inductor. Thus, the uninduced background level of ethylene of plants carrying *alc* system was negligible, being equivalent to wild-type control. After 24 h of the induction of the plants with ethanol 5% (v/v), the production

of ethylene was significantly higher compared to the non-induced condition in both 32 and 16 lines.

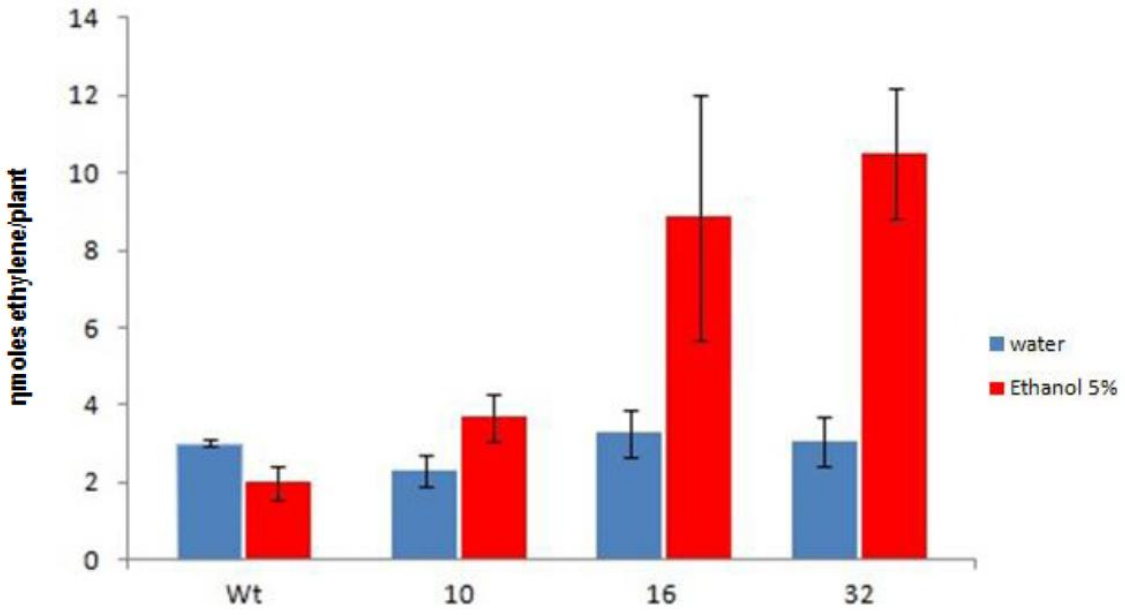


Figure 6. Ethanol-induced ethylene production in transgenic tobacco plants. Ethylene quantification was done in plants treated with water or 5% ethanol (v/v). Wt, wild-type plants; 10, line of the alcA::*Scacs1* construct; 16 and 32, independently lines of the alcR alcA::*Scacs1* construct.

Discussion

With the progress in the whole-genome sequencing technology, a vast resource of sequences data from plants and animals have been available. As a result, novel tools and approaches have been developed to evaluate gene function at morphological and physiological levels, such as chemically regulated expression systems which enable an accurate control of temporal and spatial expression of target gene (Li et al., 2005). In this study, the ethanol-

inducible system (*alc* switch) was used to control the ethylene hormone biosynthesis using the sugarcane ACS gene in transgenic tobacco plants.

A preliminary characterization in lettuce, the *alc*::GUS system was functional under ethanol induction ranging from 0.5% to 5% (v/v) (Figure 2). Nevertheless, the most effective expression was identified at concentration of 5% (v/v). Although background levels of GUS expression were detectable without induction of alc system, our results were consistent with previous work in which also demonstrated a background activity of the system (Sweetman et al., 2002; Garoosi et al., 2005). The transient assay in lettuce allowed faster and easier analyses of the *alc* system, providing an excellent tool in the study. Unfortunately, a deeper study of *alc*::GUS system in tobacco plants was not possible due to the silencing of the alcR transcription factor (Figure 5). Recent studies on transgenes regulated expression in plants have considered this issue and novel chemically-inducible systems have been developed, using less active promoters to drive alcR, such as tissue-specific promoters (Roslan et al., 2001).

However, our data presented here showed clearly an increase in the ethylene released in the airspace by transgenic plants carrying *alc*::Scacs1 system under the induction of 5% (v/v) of ethanol (Figure 6). This result not only confirmed that the *Scacs1* gene encodes an ACC synthase that is able to increase ethylene biosynthesis in transgenic plants. Additionally, our data indicates that the ethylene response pathway is conserved in different plants so that elements of this pathway can be genetically manipulated. Although the underlying mechanisms of action of the ethylene hormone in sugarcane maturation are still unclear, approaches and studies in field with the application of Ethrel ethylene precursor have gained new insights (Caputo et al., 2007). Nevertheless, the utilization of ethanol inductor in the control of the sugarcane maturation, not

only would present advantages as less toxicity in its manipulation, but also a lower cost compared Ethrel plant regulator.

Our results demonstrated a new tool to control ethylene biosynthesis through the ethanol-inducible alc system, with potential uses in the near future in sugarcane.

Acknowledgements

We thank Dr. Eugenio Ulian, who enthusiastically supported this project during his work at the Cane Technology Center (Piracicaba, Brazil) and Prof. Marlene Schiavinato (University of Campinas, Brazil) for her help with ethylene quantification. The authors RGT and AG were supported by CAPES and FINEP/CNPq. MM is a research fellow from CNPq.

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Conclusões

O objetivo principal deste projeto foi a identificação e caracterização dos genes que codificam ACC sintase de cana-de-açúcar. O estudo do padrão de expressão desses genes e sua caracterização gênica e protéica são de fundamental importância para que se possa inferir sua relação com a maturação e o acúmulo de sacarose. Este presente trabalho teve dois capítulos, sendo que os resultados obtidos permitiram concluir que:

I. A análise dos dados do projeto genoma SUCEST permitiram inferir que a cana de açúcar apresenta dois genes que codificam ACC sintases: *ScACS1* e *ScACS2*.

II. O gene da isoforma ScACS1 (JF274985) apresenta 1.464 bp e codifica uma proteína de 487 aminoácidos, sendo classificada como Tipo I. Sua localização subcelular é citoplasmática e apresenta uma maior expressão gênica nos entrenós mais maduros. Na localizaçao *in situ*, apresenta uma expressão gênica em todos os tipos celulares dos entrenós 1, 5 e 9, exceto no feixe vascular.

III. O gene da isoforma ScACS2 (JF274986) é um pouco menor, com 1.401 bp e 466 aminoácidos, sendo classificada como Tipo III. Sua localização subcelular é citoplasmática e apresenta uma maior expressão gênica nos entrenós mais maduros. Na localização *in situ,* apresenta uma expressão gênica em todos os tipos celulares dos entrenós 1, 5 e 9, inclusive nos feixes vasculares.

IV. A isoforma ScACS2 interage com uma E3 ubiquitina ligase, identificando um provável mecanismo de regulação pós-traducional. A interação com duas proteínas, sinaptotagmina e o fator myc7, indicam uma relação da síntese de

etileno com mecanismos de reparo de membrana celular e mecanismos de defesa, respectivamente.

V. Diferentemente dos ortólogos AtACS6 e AtACS7, as isoformas ScACS1 e ScACS2 não formaram heterodímeros.

VI. O sistema *alc* permite a produção controlada do hormônio etileno, através da ativação da síntese da enzima ACC sintase de cana. Este dado abre perspectivas para aplicações biotecnológicas na cultura da cana.

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Declaro para os devidos fins que o conteúdo de minha dissertação/tese de Mestrado/Doutorado intitulada ACC sintase em cana-de-açúcar: Estudos de regulação gênica e aplicação biotecnológica.

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