IGOR CESARINO

"ESTUDOS INICIAIS DE CARACTERIZAÇÃO FUNCIONAL DE PEROXIDASES E LACCASES POTENCIALMENTE ENVOLVIDAS NO PROCESSO DE LIGNIFICAÇÃO EM CANA-DE-AÇÚCAR"

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Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Vegetal.

Orientador: Prof. Dr. Paulo Mazzafera

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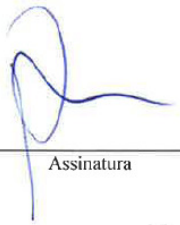

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ÍNDICE

Resumo

A lignina é um heteropolímero complexo depositado principalmente na parede celular secundária de tipos celulares especializados, conferindo força mecânica e rigidez para as plantas se manterem eretas e também proporcionando hidrofobicidade para que células condutoras transportem água e nutrientes por longas distâncias. Embora a lignina seja essencial para o desenvolvimento da planta, este polímero é o principal componente da parede celular responsável pela recalcitrância da biomassa vegetal, sendo que sua presença afeta negativamente o uso do material lignocelulósico para a produção de biocombustíveis e biomateriais. Diversas evidências suportam um papel para peroxidases e laccases no processo de polimerização da lignina. No entanto, identificar genes/isoformas relacionados(as) com o processo de polimerização de lignina e caracterizar seu mecanismo de ação estão entre as tarefas mais desafiadoras acerca do metabolismo deste polímero fenólico. Neste trabalho, uma abordagem técnica abrangente foi aplicada com o objetivo de se identificar potenciais candidatos envolvidos na oxidação dos monômeros de lignina. No capítulo I, atividade enzimática e o perfil proteômico de peroxidases de classe III foram analisados durante o desenvolvimento do colmo de cana-de-açúcar. No capítulo II, células em suspensão foram usadas como valiosa ferramenta para isolar e caracterizar peroxidases de classe III potencialmente envolvidas na polimerização de lignina. Finalmente, no capítulo III, a combinação de análises de co-expressão, expressão tecido/tipo celular específica e complementação de um mutante de *Arabidopsis thaliana* permitiu a caracterização de uma laccase fortemente relacionada com a polimerização de lignina em cana-de-açúcar. Acreditamos que estes foram os primeiros trabalhos a caracterizar peroxidases e laccases em cana-de-açúcar, além de terem contribuído para aumentar o conhecimento acerca do metabolismo de lignina nesta importante cultura dedicada à bioenergia.

Abstract

Lignin is a complex heteropolymer deposited in the secondarily thickened walls of specialized plant cells to provide strength and rigidity for plants to stand upright and hydrophobicity to conducting cells for long-distance water transport. Although lignin is essential for plant growth and development, this phenolic polymer is the major plant cell wall component responsible for biomass recalcitrance and its presence negatively affects the use of lignocellulose as a source for biofuels and bio-based materials. Several evidences support the role of peroxidases and laccases in lignin polymerization. However, the identification and characterization of peroxidases/laccases involved in lignin polymerization is still a major bottleneck**.** Here, we carried out a comprehensive approach to identify candidate genes related to the combinatorial coupling of lignin monomers. In chapter I, we analyzed the enzymatic activity and proteomic profile of class III peroxidases during sugarcane stem development. In chapter II, suspension cell culture was used as a tool for the characterization of class III peroxidases potentially involved in lignin polymerization. Finally, in chapter III, we provide evidence for the role of a laccase gene in lignin biosynthesis in sugarcane, by using a combination of co-expression analysis, tissue-specific expression analysis and genetic complementation of an *Arabidopsis thaliana* mutant. To our knowledge, these are the first reports on the characterization of peroxidases and laccases in sugarcane, which might ultimately improve our understanding of the lignin metabolism in this important bioenergy crop.

Introdução Geral

Lignina: aspectos gerais

Cerca de 450 milhões de anos atrás, durante o Ordoviciano Superior, as primeiras plantas pioneiras saíram do seu ambiente aquático para ocupar um nicho totalmente novo e desafiador: o ambiente terrestre (Ferrer *et al.*, 2008). A mudança fez com que as plantas pioneiras confrontassem uma série de novos desafios adaptativos, incluindo a exposição à danosa radiação UV-B, da qual estavam protegidos seus ancestrais aquáticos, falta de suporte físico outrora proporcionado pela flutuabilidade, desidratação e a contínua co-evolução de herbívoros e patógenos (Weng & Chapple, 2010). Para superar estas restrições e estresses, uma série de vias metabólicas especializadas, coletivamente chamadas de metabolismo secundário, evoluíram nas plantas terrestres, das quais a via dos fenilpropanóides é uma das mais críticas. Os fenilpropanóides contribuem em todos os aspectos das respostas vegetais a estímulos bióticos e abióticos, desempenhando papéis-chave desde respostas às variações de luz e nutrientes minerais até no processo de resistência contra herbívoros e patógenos (Vogt, 2010). A aquisição da capacidade de catalisar a desaminação de fenilalanina e hidroxilação do anel aromático dos metabólitos subsequentemente derivados resultou na habilidade de acumular fenilpropanóides simples com absorbância máxima na faixa da radiação UV-B (280-320 nm) e, consequentemente, permitiu que as plantas primitivas e seus vulneráveis esporos haploides resistissem à radiação UV e sobrevivessem no ambiente terrestre (Lowry *et al.*, 1980; Weng & Chapple, 2010). Embora a ocorrência e o acúmulo de fenilpropanóides protetores contra raios ultra-violeta tenha facilitado o passo inicial de conquista do nicho terrestre, por milhões de anos o corpo vegetal permaneceu pequeno devido à falta de suporte

mecânico (Bateman *et al.*, 1998). Somente com o surgimento das traqueófitas, que desenvolveram a capacidade de depositar o polímero lignina em suas paredes celulares, é que as plantas realmente prosperaram e deram início ao real domínio do ecossistema terrestre. A deposição de lignina foi essencial para a integridade estrutural da parede celular, conferindo rigidez e suporte físico para erguer o corpo vegetal, além de prover hidrofobicidade às células condutoras do sistema traqueal para transporte de água e nutrientes por longas distâncias. A natureza complexa e randômica da lignina também faz com que este biopolímero seja extremamente difícil de ser degradado e, portanto, consistiu em uma barreira defensiva ideal contra patógenos e herbívoros (Boerjan *et al.*, 2003). Como as plantas também não possuem um mecanismo de degradação de lignina, o processo de lignificação representa um significante e não-recuperável investimento de carbono e energia. Em vista disso, é provável que as plantas regulem o processo de lignificação de tal forma que o requerimento de esqueletos carbônicos e energia para a produção de lignina não ultrapasse a disponibilidade de recursos (Rogers *et al.*, 2005).

A lignina é um heteropolímero aromático complexo derivado do acoplamento oxidativo dos alcoóis *p*-hidroxicinamil (monolignóis) ou compostos relacionados. Originando-se da via do ácido chiquímico, pela qual as plantas produzem os aminoácidos aromáticos, a biossíntese dos monolignóis começa com a desaminação da fenilalanina pela enzima fenilalanina amônia-liase (PAL), seguida de modificações no anel aromático por meio de sucessivas hidroxilações e metilações e da transformação da cadeia lateral carboxílica por esterificação e redução (figura 1) (Liu, 2012). Este processo culmina na produção dos três alcoóis *p*-hidroxicinamil, considerados os monolignóis canônicos, álcool *p*-cumaril, álcool coniferil e álcool sinapil, que diferem entre si quanto ao grau de metoxilação (Boerjan *et al.*, 2003). Quando os monolignóis são incorporados ao polímero, eles passam a ser chamados de

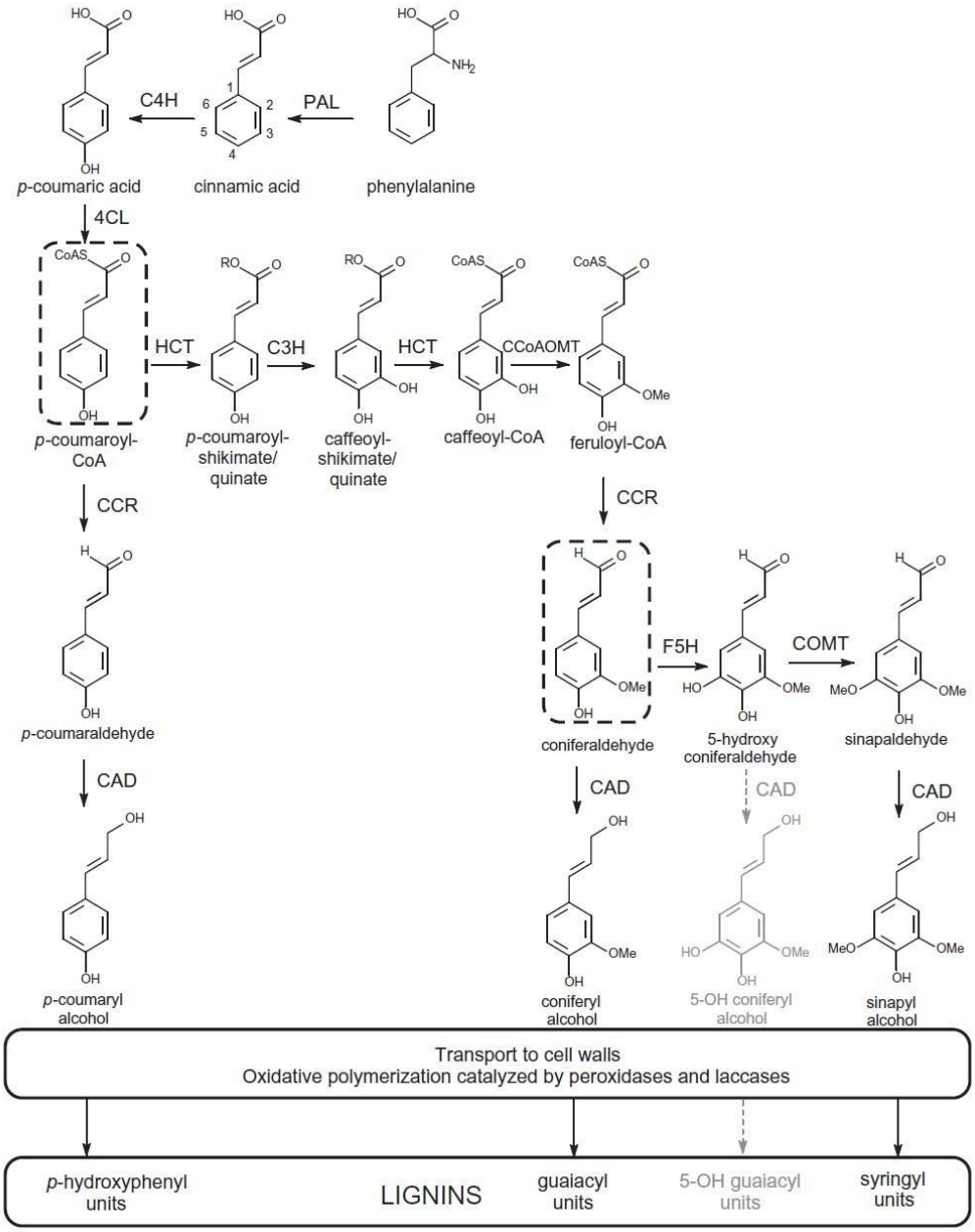

Figura 1. Via de biossíntese de lignina, modelo atual e simplificado. Esta via inclui diversos passos de redução, (tio)esterificação, hidroxilação e metilação para gerar os alcoóis *p*-cumaril, coniferil e sinapil, os respectivos precursores das unidades H, G e S de lignina. Legeda: PAL, fenilalanina amônia-liase; C4H, cinamato 4-hidroxilase; 4CL, 4-hidroxicinamoil CoA ligase; HCT, *p*-hydroxycinnamoyl-CoA:shikimate/quinate *p*-hydroxycinnamoyl transferase; C3'H, *p*-cumaroilchiquimato 3'-hidroxilase; CCoAOMT, cafeoil CoA *O*-metiltransferase; CCR, hidroxicinamoil CoA redutase; F5H, coniferaldeído/ferulato 5-hidroxilase; COMT, cafeato/5-hidroxiferulato 3/5-*O*-metiltransferase; e CAD, (hidroxi)cinamil álcool desidrogenase. Fonte: "Advances in Botanical Research – Lignins: Biosynthesis, Biodegradation and Bioengineering", Jouanin and Lapierre, 2012. Ed. Elsevier.

unidades *p*-hidroxifenil (H), guaiacil (G) e siringil (S), respectivamente. Esta via metabólica é catalisada por dez enzimas sequenciais, sendo que três delas, cinamato 4-hidroxilase (C4H), *p*-cumaroilchiquimato 3'-hidroxilase (C3'H) e coniferaldeído/ferulato 5-hidroxilase (F5H) são citocromos P450 que se associam com a superfície externa do retículo endoplasmático por meio de um motivo hidrofóbico na região N-terminal (Li *et al.*, 2008). Por outro lado, várias outras enzimas da via, como fenilalanina amônia-liase (PAL), 4-hidroxicinamoil CoA ligase (4CL), cafeoil CoA *O*-metiltransferase (CCoAOMT), hidroxicinamoil CoA redutase (CCR), cafeato/5-hidroxiferulato 3/5-*O*-metiltransferase (COMT) e (hidroxi)cinamil álcool desidrogenase (CAD), foram encontradas na forma funcional em diversas espécies vegetais como proteínas solúveis e muito provavelmente localizam-se no citoplasma (Liu, 2012). Aparentemente, a propensão à compartimentalização distinta das enzimas da via dos monolignóis, assim como a localização da via do ácido chiquímico exclusivamente nos plastídios, implica (i) na ocorrência de múltiplos sequestros subcelulares dos metabólitos intermediários ou (ii) em uma organização física ideal das vias de modo a eliminar a toxicidade e aumentar a eficiência de conversão destes intermediários aromáticos (Liu, 2012). Esta organização física de vias metabólicas é conhecida como canal metabólico (*metabolic channeling*). Este canal metabólico é formado por conjuntos de enzimas consecutivas e fisicamente associadas, agregadas com membranas ou estruturas físicas e interagindo diretamente umas com as outras. Tal associação física permite que o produto de uma enzima seja canalizado diretamente como substrato da enzima seguinte, sem liberar o intermediário para o ambiente intracelular (Chen *et al.*, 2011). Por exemplo, Dixon et al. (2001) propõe a existência de um complexo canal metabólico para a produção de lignina siringil, em que os três citocromos P450, C4H, C3'H e F5H ancoram outras enzimas solúveis funcionais como COMT e talvez possíveis isoformas específicas de PAL, 4CL e CCR na superfície do retículo

endoplasmático. No entanto, embora este mecanismo tenha sido demonstrado para algumas enzimas da via, a presença de um canal metabólico para a maioria das outras enzimas ainda necessita de comprovação experimental.

Além dos canônicos monolignóis, outros compostos fenilpropanóides também podem ser incorporados à lignina, como aldeídos hidroxicinamil, acetatos hidroxicinamil, ésteres hidroxicinamatos, entre outros (Raes *et al.*, 2003). De fato, a deposição e composição de lignina variam consideravelmente entre taxa, tipos celulares e fases de desenvolvimento, além de serem afetadas por condições ambientais como estresses bióticos e abióticos (Moura *et al.*, 2010). Embora existam exceções, ligninas provenientes de dicotiledôneas (angiospermas) são formadas por unidades G e S, com traços de unidades H, enquanto ligninas provenientes de gimnospermas são compostas basicamente por unidades G com baixos níveis de unidade H. Ligninas de gramíneas (monocotiledôneas) incorporam unidades G e S em níveis similares, e apresentam mais unidades H quando comparadas às dicotiledôneas (Boerjan *et al.*, 2003). Outra característica única da lignina de gramíneas é a presença de ácidos hidroxicinâmicos (como ácido ferúlico e ácido *p*-cumárico) em quantidades significantes, o que não ocorre em dicotiledôneas (Vogel, 2008). Organismos não-vasculares (incluindo algas e briófitas) não sintetizam lignina, embora briófitas acumulem fenilpropanóides solúveis, como flavonóides e lignanas (Weng & Chapple, 2010). Mesmo com este proposto cenário evolutivo, notáveis exceções foram encontradas nos últimos anos, como a descoberta de lignina em células periféricas da alga vermelha *Calliarthron cheilosporioides* (Martone *et al.*, 2009), a presença de lignina em tecidos não-vasculares da hepática *Marchantia polymorpha* (Espineira *et al.*, 2011) e a biossíntese de lignina do tipo S em células em suspensão da gimnosperma basal *Ginkgo biloba* (Uzal *et al.*, 2009).

A deposição de lignina, bem como a incorporação de cada tipo de monômero, é regulada espaço-temporalmente e varia entre parede celular primária e secundária (Grabber *et al.*, 2004). O conteúdo de lignina aumenta com a maturidade do tecido, enquanto a composição segue um padrão mais complexo. Nas fases iniciais de lignificação, álcool coniferil e pequenas quantidades de álcool *p*-cumaril são co-polimerizados na parede primária para formar um polímero de lignina constituído por mistura de unidades G e H. Posteriormente, durante a formação da parede celular secundária, álcool coniferil e quantidades crescentes de álcool sinapil são incorporados de modo a formarem um polímero de unidades G e S (Grabber, 2005). No caso das gramíneas, cuja parede celular apresenta características únicas, hidroxicinamatos também são significativamente incorporados (Vogel, 2008). Enquanto o ácido ferúlico é o principal derivativo hidroxicinâmico das paredes celulares jovens das gramíneas, o ácido p-cumárico pode ser considerado um indicador da maturidade da parede celular, já que é principalmente esterificado à cadeia lateral das unidades S e sua incorporação segue o mesmo padrão de deposição destas unidades (Riboulet *et al.*, 2009). Distribuição diferencial de monômeros de lignina também é observada no caso de tipos celulares específicos. Na maioria das plantas dicotiledôneas, o espessamento da parede secundária ocorre principalmente em células condutoras do xilema, que tendem a conter altos níveis de unidades G, e fibras interfasciculares, que são tipicamente ricas em unidades S (Bonawitz & Chapple, 2010). Em gramíneas (monocotiledôneas), parênquima e outros tecidos como epiderme e hipoderme apresentam limitado, porém significante nível de lignificação, enquanto os elementos condutores do xilema e as fibras de esclerênquima acumulam grandes quantidades de lignina, enriquecida em unidades S (Grabber *et al.*, 2004). A proporção S/G é considerada um parâmetro importante para predizer o nível e a natureza das ramificações no polímero de lignina. Tecidos ricos em unidades G apresentam lignina mais ramificada com

maior proporção de ligações bifenil e outras ligações carbono-carbono, enquanto ligninas ricas em unidades S são menos condensadas, conectadas por ligações mais passíveis de serem clivadas do tipo éter na posição 4-hidroxil (Ferrer *et al.*, 2008). Grupos metoxila adicionais no monômero de lignina resultam em menos combinações possíveis durante a polimerização devido ao reduzido número de sítios reativos disponíveis. Consequentemente, ligninas ricas em unidades S são mais facilmente degradadas do que ligninas ricas em unidades G (Ziebell *et al.*, 2010).

O último passo na via de síntese de lignina

Os monolignóis são sintetizados no citoplasma, mas precisam ser transportados para o apoplasto onde serão oxidados e então incorporados ao polímero em formação. O exato mecanismo pelo qual os monolignóis são transportados permanece pouco compreendido. Pelo menos três modelos foram propostos: i) exocitosis por meio de vesículas oriundas do sistema retículo endoplasmático-Golgi; ii) difusão passiva via interações hidrofóbicas com a membrana celular; e iii) transporte ativo por meio de transportadores localizados na membrana plasmática (Liu *et al.*, 2011). No entanto, estudos recentes parecem corroborar fortemente a terceira hipótese. Miao e Liu (2010) conduziram um teste de captação de monolignóis *in vitro* usando vesículas produzidas com membranas plasmáticas e vacuolares de *Arabidopsis thaliana*. Este estudo revelou diversas evidências do envolvimento de transportadores ABC no transporte e sequestro de monolignóis: (1) o transporte de monolignóis foi amplamente dependente de energia; (2) diversos inibidores dos transportadores ABC reduziram o sequestro de monolignóis pelas vesículas mesmo na presença de ATP; (3) o sequestro de monolignóis e seus derivados glicosilados apresentou a típica cinética proteína-ligante, indicando que este processo bioquímico é mediado por proteínas e não somente um processo passivo; e (4) o

transporte de precursores de lignina pelas vesículas apresentou óbvia seletividade. O transporte seletivo foi evidenciado ao observar que vesículas de membrana vacuolar sequestraram ativamente monolignol glicoconjugados, enquanto que as vesículas de membrana plasmática transportaram seletivamente as formas agliconas.

O último passo na biossíntese de lignina acontece na parede celular, onde ocorre a oxidação dos monolignóis e subsequente polimerização por meio de um mecanismo de acoplamento combinatorial dos radicais formados (Vanholme *et al.*, 2010a). O primeiro passo é a oxidação/desidrogenação do monolignol, que resulta na formação de radicais com densidade eletrônica desemparelhada nas posições 1-, 3-, *O*-4-, 5- e 8- (Morreel *et al.*, 2010a). Dois radicais se acoplam para formar um (dehidro)dímero, estabelecendo uma ligação covalente entre as subunidades. Como o acoplamento de radicais favorece a posição 8- (β), os dímeros resultantes são basicamente 8-8-, 8-*O*-4- e 8-5-. Este acoplamento radical-radical ocorre de maneira combinatorial, com natureza essencialmente química. Portanto, a proporção de cada possível dímero formado depende da natureza química de cada monômero e das condições da parede celular. A polimerização continua pela oxidação/desidrogenação do dímero formado para se acoplar com outro radical monomérico, sendo que o processo ocorre pela assimilação de uma unidade radicalar por vez (Morreel *et al.*, 2010b). Devido ao fato da lignificação ser um processo essencialmente químico, praticamente qualquer fenol que esteja presente na zona de lignificação da parede celular é capaz de entrar no processo de acoplamento combinatorial, de acordo com simples parâmetros químicos, como compatibilidade estrutural da molécula, e típicos parâmetros físicos como pH, temperatura, força iônica, fornecimento de monolignóis, concentração de enzimas oxidativas e seus cofatores (Vanholme *et al.*, 2008). Recentemente, uma nova hipótese de que existe um controle proteináceo da polimerização dos precursores de lignina por ação das chamadas

proteínas dirigentes foi proposta (Davin & Lewis, 2005). No entanto, até o momento não existem provas científicas do envolvimento das proteínas dirigentes no controle da polimerização de lignina.

As principais enzimas relacionadas com a formação de precursores radicalares de lignina são peroxidases de classe III e laccases. No entanto, este processo é pouco compreendido devido às dificuldades intrínsecas a estes dois tipos de enzimas: i) peroxidases e laccases apresentam baixa especificidade *in vitro*, não oferecendo informações precisas sobre os reais substratos *in planta*; e ii) produção de plantas transgênicas geralmente resulta em mutantes sem alterações fenotípicas observáveis devido à redundância genética (Ranocha *et al.*, 2002; Sato & Whetten, 2006; Cosio & Dunand, 2010; Fagerstedt *et al.*, 2010). Identificar genes/isoformas relacionados com o processo de polimerização de lignina e caracterizar seu mecanismo de ação estão entre as tarefas mais desafiadoras acerca do metabolismo deste polímero fenólico.

Peroxidases

A superfamília de peroxidase não-animal é formada por três classes de peroxidases, chamadas peroxidases de classe I, II e III. Estas três classes compartilham características como a presença de um grupo heme formado por protoporfirina IX de ferro III (forma férrica no estado fundamental) e estruturas tridimensionais similares. Todavia, estas proteínas apresentam baixa identidade em termos de sequência primária de aminoácidos, bem como diferentes localizações subcelulares e estão associadas a funções fisiológicas distintas (Mathe *et al.*, 2010). Peroxidases de classe I são glicoprotéinas intracelulares encontradas em plantas, fungos e procariotos e que não contém Ca^{2+} ligado ou pontes dissulfeto. A classe é composta pelas citocromo c peroxidases (CcP; EC 1.11.1.5), catalase peroxidases (CP; EC 1.11.1.6) e

ascorbato peroxidases (APx; EC 1.11.1.11) e sua principal função celular é eliminar o excesso de H_2O_2 (Passardi *et al.*, 2007). Peroxidases de classe II são glicoproteínas exclusivas de fungos que contém Ca^{2+} ligado, pontes dissulfeto e um peptídeo-sinal. A família consiste nas lignina peroxidases (LiP; EC 1.11.1.14), manganês peroxidases (MnP; EC 1.11.1.13) e nas peroxidases versáteis (VP; EC 1.11.1.16), sendo a degradação dos detritos do solo sua principal função biológica, visto que nenhuma outra heme peroxidase é capaz de degradar lignina (Cosio & Dunand, 2009). Peroxidases de classe III estão presentes em todas as plantas terrestres mas são ausente nas algas verdes unicelulares (Passardi *et al.*, 2004b). Assim como as peroxidases de classe II, as peroxidases de classe III também são glicoproteínas contendo Ca^{2+} ligado e quatro ou cinco pontes dissulfeto. Elas apresentam um peptídeo-sinal para serem secretadas via retículo endoplasmático, enquanto certas peroxidases de classe III podem apresentar uma extensão na região C-terminal para direcioná-las ao vacúolo (Passardi *et al.*, 2005; Mathe *et al.*, 2010).

Peroxidases de classe III estão presentes em grandes famílias multigênicas. Desde o aparecimento da primeira peroxidase de classe III, pouco antes do surgimento das plantas terrestres, até a distribuição e predomínio das Angiospermas, o número de genes aumentou exponencialmente (Passardi *et al.*, 2004a). A origem e diversificação de peroxidases de classe III estariam relacionadas à colonização do ambiente terrestre pelas plantas primitivas, que encontraram um ambiente com maiores concentrações de oxigênio e, portanto, necessitariam de um sistema de eliminação das espécies reativas mais frequentemente formadas (Passardi *et al.*, 2004b). Em Arabidopsis, 73 genes estão anotados como peroxidases de classe III, enquanto que o genoma do arroz contém 138 membros desta família (Passardi *et al.*, 2004a). De fato, monocotiledôneas possuem clusters adicionais de peroxidases não encontrados em dicotiledôneas, frutos de um maior número de eventos de duplicação gênica (Luthje *et al.*,

2011). Ademais, modificações pós-transcricionais e pós-traducionais podem gerar isoformas adicionais e exacerbar a complexidade desta família de enzimas (Mika *et al.*, 2010). Como as peroxidases de classe III são o foco desta tese, o termo peroxidase será usado como sinônimo de peroxidase de classe III no texto subsequente.

Peroxidases possuem dois ciclos catalíticos distintos. No ciclo peroxidativo, H_2O_2 oxida a forma férrica da enzima (FeIII) em uma oxidação dupla (de dois elétrons) para gerar o intermediário enzimático chamado composto I (FeIII + H_2O_2 → composto I + H_2O). O composto I aceita um elétron de um ânion fenolato (R^-) para gerar seu correspondente radical ($R^\bullet$) e o intermediário oxiferril conhecido como composto II (composto I + R^- → composto II + $R^\bullet$). A reação subsequente é a redução simples (um elétron) do composto II por uma segunda molécula de fenolato que gera novamente a forma férrica da enzima, FeIII (composto II + R^- → FeIII + $R^\bullet$ + H_2O), fechando o ciclo catalítico da enzima (Ros Barcelo *et al.*, 2007). Diversos tipos de moléculas doadoras podem ser utilizados como ânion fenolato, especialmente compostos fenólicos, precursores de lignina, auxina e outros metabólitos secundários (Cosio & Dunand, 2010). No caso do ciclo hidroxílico, as peroxidases nativas podem ser convertidas em oxiferroperoxidases (também conhecidas como composto III) e gerar espécies reativas de oxigênio (ROS) (Liszkay *et al.*, 2003). A possibilidade de dois ciclos catalíticos distintos e o grande número de genes e isoformas presentes na família fazem com que as peroxidases estejam implicadas em diversos processos fisiológicos como biossíntese de lignina e suberina (Christensen *et al.*, 1998; Quiroga *et al.*, 2000; Christensen *et al.*, 2001), alongamento celular (Passardi *et al.*, 2006), degradação de auxina (Cosio & Dunand, 2009), reações de defesa contra patógenos (Almagro *et al.*, 2009) e geração de espécies reativas de oxigênio (Liszkay *et al.*, 2003). Além disso, os diferentes padrões espaço-temporais de expressão de genes individuais e o fato de que todas as peroxidases apresentam

regiões conservadas e variáveis em suas sequências primárias de aminoácidos sugere a existência de especialização funcional dos membros desta família (Cosio & Dunand, 2009).

Embora a expressão gênica e atividade de peroxidases possam ser facilmente detectadas em diversos tecidos ao longo de todo o ciclo de vida das plantas, determinar precisamente uma função biológica para uma isoforma específica continua sendo uma tarefa complexa, provavelmente devido ao enorme número de genes pertencentes à família e à possibilidade de dois ciclos catalíticos distintos (Passardi *et al.*, 2005). Na tentativa de se identificar funções específicas para peroxidases de classe III, diversos estudos reportaram o uso de engenharia genética para superexpressar ou silenciar um gene específico. Não obstante, apenas 9 das 73 peroxidases de classe III em *Arabidopsis thaliana* foram identificadas/caracterizadas por esta estratégia (Cosio & Dunand, 2010), visto que a maioria dos mutantes forneceu informações inconclusivas. Além disso, testes *in vitro* utilizando peroxidases purificadas ou recombinantes e diferentes substratos geraram poucas informações sobre sua possível função *in planta* devido à alta promiscuidade destas enzimas (Passardi *et al.*, 2004b; Cosio & Dunand, 2009; Cosio & Dunand, 2010). Desse modo, acredita-se que informações sobre a expressão espaço-temporal e localização subcelular da proteína são essenciais para indicar a função desempenhada por um gene específico. De fato, a maioria dos estudos que associaram peroxidases de classe III com algum mecanismo fisiológico utilizou métodos de análise de expressão gênica como microarrays e PCR em tempo real (Cosio & Dunand, 2009). No entanto, somente a combinação de diversas técnicas de transcriptômica, engenharia genética e bioquímica poderá trazer informações conclusivas acerca da função funcional dos membros da família de peroxidases de classe III.

O envolvimento de peroxidases na formação de monolignóis radicalares é uma das funções mais estudadas para este grupo de enzimas. Durante o seu ciclo peroxidativo, as

peroxidases oxidam monolignóis, formando seu correspondente intermediário radical que, por sua natureza instável participa de uma variedade de reações não-enzimáticas e, em última instância, é responsável pelo acoplamento combinatorial e consequente formação do polímero de lignina (Ros Barcelo *et al.*, 2007). Estudos envolvendo tecidos e sistemas de células em suspensão de diversas espécies vegetais como *Arabidopsis thaliana* (Tokunaga *et al.*, 2009), *Nicotiana tabbacum* (Blee *et al.*, 2003), *Zinnia elegans* (Gabaldon *et al.*, 2005), *Populus trichocarpa* (Christensen *et al.*, 1998) e espécies lenhosas de Gimnospermas (Marjamaa *et al.*, 2006) levantaram evidências importantes para correlacionar peroxidases e polimerização de lignina. No entanto, somente os estudos funcionais envolvendo estratégias transgênicas trouxeram provas convincentes e conclusivas sobre o papel de peroxidases neste processo. Em tabaco, o silenciamento de um gene codificando para a peroxidase catiônica *NtPrx60* (*TP60*) resultou em uma redução de 50% nos níveis de lignina (Blee *et al.*, 2003). De modo semelhante, o silenciamento da peroxidase aniônica *PkPrx03* (*PrxA3a*) em álamo transgênico resultou em 20% menos lignina, enquanto que a composição deste polímero também foi alterada, gerando uma maior proporção de unidades S (Li *et al.*, 2003). Ademais, a superexpressão de um gene codificante para uma peroxidase catiônica resultou em lignificação ectópica em plantas de tomates transgênicos (El Mansouri *et al.*, 1999). Embora suportado por um pequeno número de estudos, provavelmente devido ao problema de redundância genética, o sucesso na obtenção de plantas transgênicas com alterado conteúdo e/ou composição de lignina comprova a participação de peroxidases de classe III no passo final da biossíntese de lignina em plantas.

Laccases

Oxidases multicobre (MCOs) constituem uma família enzimática que inclui laccases, ferroxidases, metal oxidases bacterianas e ceruloplasminas e cujos membros apresentam de um a seis átomos de cobre por molécula e no mínimo 100 resíduos de aminoácidos em uma única cadeia polipeptídica (Hoegger *et al.*, 2006). Laccases (*p*-difenol:O_2 oxidorredutase; EC 1.10.3.2) são oxidases encontradas em plantas superiores, fungos, bactérias e artrópodes e que estão envolvidas em numerosos processos biológicos. Laccases de origem micótica são as laccases mais extensivamente estudadas e caracterizadas, já que sua principal função biológica é a degradação de lignina (Gavnholt & Larsen, 2002). No entanto, a maioria dos fungos ligninolíticos expressa tanto peroxidases (como visto anteriormente, as peroxidases de classe II) quanto laccases, o que torna difícil a elucidação do papel real de cada classe de enzimas na biodegradação de lignina. De qualquer forma, laccases e peroxidases de classe II são essenciais para a manutenção dos ecossistemas, pois degradam o material vegetal morto e fazem a reciclagem do solo (Gavnholt & Larsen, 2002; Passardi *et al.*, 2007).

Laccases em plantas também constituem famílias multigênicas grandes e complexas, cujos membros apresentam potencial para catalisar reações oxidativas múltiplas e divergentes (McCaig *et al.*, 2005). Essas glicoproteínas são amplamente distribuídas ao longo de todas as ordens de plantas superiores, tendo evoluído em seis grupos filogenéticos divergentes e sendo expressas em diversos tecidos/órgãos vegetais ao longo de todo o desenvolvimento da planta (McCaig *et al.*, 2005). As laccases estão associadas com diversas funções biológicas nas plantas, mas elas são particularmente relacionadas com a biossíntese de lignina. Na verdade, as laccases foram as primeiras enzimas caracterizadas *in vitro* com participação na polimerização de monômeros de lignina (Freudenberg, 1959). Além disto, estas enzimas foram intimamente relacionadas com o processo de lignificação por neio de detecção de transcritos (Sato & Whetten, 2006) e imunolocalização de proteínas (Driouich *et al.*, 1992) em

sítios de deposição de lignina. Entretanto, enquanto o envolvimento de peroxidases de classe III foi indubitavelmente comprovado por um significante número de estudos reportando a produção de plantas transgênicas cujo conteúdo/composição de lignina foi afetada pela superexpressão ou silenciamento de um gene específico (Marjamaa *et al.*, 2009; Fagerstedt *et al.*, 2010), evidências genéticas ainda são raras no caso de laccases. Ranocha et al. (1999) purificaram três laccases de tecidos em lignificação de *Populus trichocarpa* e observaram sua capacidade de oxidar álcool coniferil *in vitro*. Tais enzimas foram consideradas potenciais candidatas a desempenhar o mesmo papel *in planta*. No entanto, o silenciamento individual das três laccases não afetou nem composição nem o conteúdo de lignina nas plantas transgênica, embora tenha havido um acúmulo no conteúdo de compostos fenólicos totais (Ranocha *et al.*, 2002). A expressão de uma laccase de algodão (*GaLAC1*) sob controle do promotor constitutivo do vírus do mosaico da couve-flor 35S em *Populus deltoides* resultou em umaumento discreto no teor de lignina no caule das plantas transgênicas (Wang *et al.*, 2008). No caso da planta modelo *Arabidopsis thaliana*, a análise de mutantes com inserção de T-DNA (linhagens SALK) de todos os genes de laccase revelou fenótipo observável em apenas três casos (Cai *et al.*, 2006). Mutantes de *AtLAC2* apresentaram comprometimento no alongamento radicular sob estresse hídrico; mutantes de *AtLAC8* floresceram prematuramente; e mutantes de *AtLAC15* continham sementes com diferente coloração da testa (Cai *et al.*, 2006). Na verdade, a primeira função comprovada para uma laccase nesta planta foi a polimerização oxidativa de flavonoides na testa das sementes observada para AtLAC15 (TRANSPARENT TESTA10) (Pourcel *et al.*, 2005). Um estudo subsequente sugeriu que o conteúdo de lignina na testa dos mutantes *AtLAC15* também era menor que aquele do tipo selvagem (Liang *et al.*, 2006). No entanto, somente com o trabalho de Berthet *et al.* (2011) é que se comprovou por engenharia genética o papel de laccases na lignificação de caules em

Arabidopsis thaliana. Esses autores mostraram que os mutantes individuais de *lac4* e *lac17* apresentaram menores níveis de lignina do que o controle, enquanto o mutante duplo *lac4lac17* apresentou níveis ainda mais baixos. Os autores ainda demonstraram que LAC17 está envolvida na deposição de unidades G especificamente em fibras interfasciculares (Berthet *et al.*, 2011).

Finalmente, como ambos os tipos de enzimas, peroxidases e laccases, encontram-se em forma ativa nos tecidos em lignificação, alguns autores propuseram diferentes hipóteses para explicar como seria a contribuição de cada grupo neste processo. Sterjiades *et at.* (Sterjiades *et al.*, 1993) sugeriram que as laccases atuariam em fases iniciais de lignificação nas células visto que essas enzimas funcionam apenas na ausência de H_2O_2. Este poderia ser o caso, por exemplo, dos estágios iniciais de lignificação da testa da semente em formação, próxima dos sensíveis embriões em desenvolvimento (Gavnholt & Larsen, 2002). Outra teoria sugere que as laccases são as principais enzimas atuando durante condições em que a concentração de lignina atingiu um determinado nível em que a lamela média se tornou tão hidrofóbica a ponto de impedir o fornecimento de água e H_2O_2 enquanto O_2 ainda está disponível (Gavnholt & Larsen, 2002).

Lignina: influência no aproveitamento da biomassa vegetal

Os principais biocombustíveis disponíveis no mercado atualmente são o bioetanol e o biodiesel. O bioetanol é produzido pela fermentação de carboidratos ou amido, derivados principalmente de cana-de-açúcar, milho, trigo, beterraba, mandioca, dentre outros, sendo comumente chamado de biocombustível de primeira geração (Waclawovsky *et al.*, 2010). No entanto, um esforço emergente está relacionado aos chamados biocombustíveis de segunda geração, cuja produção se baseia no uso da biomassa lignocelulósica vegetal (Dias *et al.*,

2009). Lignocelulose refere-se à biomassa vegetal composta pelos polímeros biológicos celulose, hemicelulose e lignina (Yuan *et al.*, 2008). Lignocelulose apresenta natureza extremamente complexa devido à sua estrutura molecular e heterogeneidade, onde a rede de microfibras de celulose está embebida em uma matriz de hemicelulose covalentemente interligada com o heteropolímero aromático lignina (Vega-Sanchez & Ronald, 2010). A natureza recalcitrante desta mistura é um dos maiores obstáculos para a conversão dos polissacarídeos presentes na parede celular vegetal em carboidratos fermentáveis para a produção de biocombustíveis (Vanholme *et al.*, 2010c). A capacidade da lignina em resistir à degradação faz deste polímero o principal componente da parede celular responsável pela recalcitrância da biomassa vegetal. O fato de a lignina ser um polímero não-linear, construído com diferentes subunidades conectadas por ligações covalentes impossibilita a ação de enzimas, que não conseguem reconhecer e degradar este composto (Weng *et al.*, 2008). Além disto, alguns derivados de lignina formados durante a remoção industrial do polímero também podem inibir as enzimas durante o processo de fermentação, diminuindo o rendimento na produção de biocombustíveis (Simmons *et al.*, 2010). Consequentemente, a conversão de biomassa a biocombustível requer pré-tratamentos caros (e também tóxicos) para remoção/degradação da lignina, afrouxando a rígida estrutura da parede celular e disponibilizando os carboidratos monoméricos para a sacarificação (Vanholme *et al.*, 2010c). Desse modo, as indústrias de biocombustíveis, bem como a produção de papel que também é negativamente afetada pela presença de lignina, seriam beneficiadas pela utilização de plantas com menor conteúdo de lignina ou que apresentem lignina mais susceptível à degradação química (Vanholme *et al.*, 2010a).

O estudo acerca da biossíntese e deposição de lignina na parede celular de plantas é um dos principais focos de pesquisa na área de biocombustíveis. O objetivo principal seria

modificar a lignina (em conteúdo e/ou composição) de modo a diminuir a recalcitrância da biomassa vegetal (Simmons *et al.*, 2010). A estratégia mais comumente adotada é a modificação da expressão de enzimas responsáveis pela biossíntese de lignina por meio de engenharia genética. Chen *et al.* (2006) reportaram o silenciamento independente de sete genes da via de síntese de lignina em alfafa. Em todos os casos, o fluxo total de carbonos direcionado para a biossíntese de lignina foi menor, com efeitos mais drásticos no caso do silenciamento das enzimas iniciais da via. A composição de lignina também foi afetada. No entanto, o efeito variou dependendo da posição da enzima na via, resultando, por exemplo, em maiores teores de unidades H, no caso do silenciamento de *HCT* e *C3'H*, e menores quantidades de unidades S no caso da supressão da expressão de *COMT* e *F5H*. Um estudo posterior do mesmo grupo provou que estas linhagens transgênicas de alfafa apresentaram melhores performances na sacarificação enzimática, gerando maiores quantidades de carboidratos monoméricos produzidos quando comparados ao controle (Chen & Dixon, 2007). Este estudo também sugere que o conteúdo de lignina, ao contrário de sua composição, é o fator principal responsável pela recalcitrância da biomassa. No entanto, outros estudos demonstram que não necessariamente o conteúdo de lignina precisa ser modificado para afetar a hidrólise enzimática: a modificação da composição monomérica da lignina pode ser uma alternativa. A redução da expressão do gene *CAD* em alfafa não alterou o conteúdo de lignina mas modificou a sua composição, o que por fim resultou em melhor digestibilidade (Baucher *et al.*, 1999). De forma semelhante, a superexpressão de *F5H* em *Populus* produziu um aumento considerável nos níveis de unidades S, aumentando significativamente a eficiência da polpação química (Huntley *et al.*, 2003).

De fato, como a lignina é um componente natural da parede celular e essencial para o correto desenvolvimento da planta, a modificação da composição da lignina via engenharia

genética é uma tendência. Um dos focos atuais é a produção de lignina com monômeros não tradicionais, aqueles que não seriam normalmente incorporados ao polímero. Neste sentido, um estudo de biologia de sistemas foi executado combinando a superexpressão do gene *F5H* com o silenciamento do gene seguinte da via, *COMT* em *Arabidopsis thaliana* (Vanholme *et al.*, 2010b). Como a etapa de hidroxilação catalisada por F5H é irreversível, o silenciamento de COMT provoca o acúmulo de 5-hidroxiconiferaldeído nas células, que não pode ser convertido em sinapaldeído, mas pode ser reduzido por CAD formando o álcool 5-hidroxiconiferílico (Figura 1). Este novo monômero é então exportado para a parede celular onde é normalmente incorporado ao polímero. O resultado foi a formação de lignina composta por aproximadamente 92% de unidades benzodioxanas, com massiva substituição dos monolignóis tradicionais por álcool 5-hidroxiconiferílico. Tais resultados mostram a plasticidade do transporte e da polimerização de monômeros de lignina e mais uma vez reforçam a ideia de que a formação deste polímero é um processo basicamente químico, sem o envolvimento de um controle proteináceo.

Cana-de-açúcar: aspectos gerais e aplicações

A cana-de-açúcar é uma monocotiledônea pertencente à família Poaceae e originária do Sudeste Asiático, cujo cultivo se estendeu por toda região tropical e subtropical de ambos os lados do equador (Lisboa *et al.*, 2011). Por ser uma planta C4, as variedades de cana-de-açúcar (híbridos *Saccharum* spp.) apresentam alta capacidade de captação de radiação solar para a conversão em biomassa, além de possuírem a característica única entre os membros da família Poaceae de acumular até 60% da massa seca dos entrenós maduros do colmo em sacarose (Casu *et al.*, 2004). O colmo da cana-de-açúcar consiste em uma série de entrenós em diferentes estádios de desenvolvimento, com a maturidade aumentando à medida que se

aproxima da base do colmo. As células em entrenós jovens estão em processo de alongamento com mínimo de espessamento celular. Quando o alongamento celular cessa, ocorre grande aumento no espessamento celular e na deposição de lignina. Entrenós maduros já completaram o desenvolvimento e acumularam sacarose, enquanto entrenós jovens continuam crescendo (Jacobsen *et al.*, 1992; Casu *et al.*, 2007). Por ser membro da subclasse Commelinoíde, a cana-de-açúcar apresenta parede celular do Tipo II, composta por fibras de celulose envolvidas por glicuronoarabinoxilanos, altos níveis de hidroxicinamatos e baixos níveis de pectina e proteínas estruturais (Vogel, 2008). A parede celular secundária é depositada somente após o fim do processo de expansão celular, para garantir estabilidade mecânica para tipos celulares particulares, como células do xilema e esclerênquima (Casu *et al.*, 2007).

Atualmente, o Brasil é o maior produtor mundial de cana-de-açúcar, com uma produção total de 612 milhões de toneladas durante a safra 2009/2010, das quais metade foi destinada para a produção de açúcar e o restante usado para a produção de 25 bilhões de litros de etanol (Cheavegatti-Gianotto *et al.*, 2011). O Brasil é também o líder mundial na produção de etanol, produzido a partir de cana-de-açúcar, seguido dos Estados Unidos que utilizam amido proveniente de grãos de milho. No entanto, a produção de etanol a partir de cana-de-açúcar tem a vantagem de não competir diretamente com a produção de alimentos, além de apresentar melhor balanço de energia e maior potencial de consumir gases-estufa (Lisboa *et al.*, 2011). Além do grande aproveitamento da cana-de-açúcar para a produção dos biocombustíveis de primeira geração, esta cultura também pode se beneficiar das tecnologias para produção dos chamados biocombustíveis de segunda geração, visto que a cana está entre as espécies vegetais mais eficientes na produção de biomassa (Vermerris, 2011). O resíduo formado após a extração do açúcar, chamado de bagaço, é uma fonte abundante de material lignocelulósico. Uma tonelada de cana-de-açúcar origina 280 kg de bagaço, que é composto

de 39% de celulose, 25% de hemicelulose e 23% de lignina, além de outros componentes menos abundantes (Carroll & Somerville, 2009; Rabelo *et al.*, 2011). Atualmente, o bagaço é usado na geração de calor e energia para a própria indústria durante a produção de açúcar e etanol (Carroll & Somerville, 2009). Portanto, a produção de etanol de segunda geração poderia ser vantajosa para a indústria de cana-de-açúcar, uma vez que o bagaço está prontamente disponível após a extração da sacarose e o etanol celulósico poderia ser co-produzido e compartilhar parte da estrutura já utilizada para a produção do etanol tradicional (Dias *et al.*, 2009; Rabelo *et al.*, 2011; Dias *et al.*, 2012). Para tal propósito, um maior conhecimento acerca da recalcitrância da biomassa vegetal e consequentemente do metabolismo de lignina é fundamental para o total aproveitamento do potencial dos biocombustíveis celulósicos.

O nosso conhecimento atual sobre o metabolismo de lignina é derivado basicamente de estudos com dicotiledôneas herbáceas como Arabidopsis e alfafa. Todavia, as culturas potencialmente destinadas à produção de bioetanol celulósico incluem também dicotiledôneas lenhosas (como *Populus* e eucalipto) e gramíneas, como cana-de-açúcar (Li *et al.*, 2008). Embora a lignina esteja presente em todas as plantas vasculares, isso não obrigatoriamente significa que todos os mecanismos referentes à regulação, síntese e polimerização de lignina são conservados (Weng *et al.*, 2008). No caso específico de cana-de-açúcar, pouco se sabe a respeito do metabolismo de lignina em geral, e informações genéticas são limitadas a alguns estudos reportando análises transcriptômicas em larga escala em que genes da via dos fenilpropanóides foram diferencialmente expressos durante o desenvolvimento do colmo ou em relação ao acúmulo de sacarose (Casu *et al.*, 2004; Casu *et al.*, 2007; Papini-Terzi *et al.*, 2009). Estudos genéticos funcionais, no entanto, são dificultados pelo alto grau de complexidade do genoma de cana-de-açúcar. Os cultivares modernos de cana-de-açúcar

originaram-se do cruzamento entre a espécie domesticada *Saccharum officinarum*, que é um auto-octaplóide (2n = 8x = 80), com a espécie selvagem *Saccharum spontaneum*, que também é poliplóide, mas o número de cromossomos varia de 2n = 5x = 40 a 2n = 16x = 128. Portanto, estes cultivares modernos apresentam um genoma de aproximadamente 10 Gb com cerca de 120 cromossomos, sendo 70-80% oriundos de *S. officinarum*, 10-20% oriundos de *S. spontaneum* e alguns se originando de recombinações interespecíficas (Garsmeur *et al.*, 2011). Além da complexidade genômica, que resulta em alta redundância genética, outra questão nos estudos funcionais em cana-de-açúcar está relacionada com a transformação genética problemática. A transformação de cana é principalmente realizada por biobalística em calos embriogênicos, já que o uso de *Agrobacterium* é limitado pela baixa eficiência de transformação, alta variabilidade entre experimentos e especificidade de genótipos (Anderson & Birch, 2012). No entanto, as plantas transgênicas apresentam alta atividade em estágios de plântulas jovens, mas a expressão do transgene é "desligada" nas plantas adultas. Esta instabilidade de expressão do transgene é atribuída ao fenômeno de silenciamento gênico induzido pelo alto número de cópias introduzidas no genoma pela técnica de biobalística. Ademais, também foi sugerido que este fenômeno ocorre mais frequentemente nesta planta pela alta ploidia e complexidade do seu genoma (Manners & Casu, 2011). Independentemente do método de transformação utilizado, cultura de tecidos e regeneração de plantas ainda são pré-requisitos para a transformação genética de cana-de-açúcar, passos que são longos e laboriosos, além de sujeitos à contaminação (Arruda, 2012). Devido a todas essas dificuldades, poucos estudos obtiveram sucesso na elucidação da função de genes específicos em cana-de-açúcar. Somente com uma abordagem mais abrangente, com a utilização de diversas técnicas em biologia molecular e bioquímica, é que se conseguirá atingir este objetivo.

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Objetivo Geral

O objetivo deste trabalho foi selecionar e caracterizar genes candidatos de peroxidases e laccases potencialmente envolvidos na etapa final da biossíntese de lignina em cana-de-açúcar, a formação oxidativa de radicais e sua subsequente polimerização.

Objetivos específicos

- Analisar a atividade total e o perfil proteômico de peroxidases ao longo do desenvolvimento do colmo de cana-de-açúcar
- Utilizar células em suspensão como modelo para isolamento e caracterização de peroxidases potencialmente envolvidas em lignificação nesta espécie
- Caracterizar um gene de laccase que é coordenadamente expresso com outros genes da via dos fenilpropanóides em cana-de-açúcar

Capítulo I

“Enzymatic activity and proteomic profile of class III peroxidases during sugarcane stem development”

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ABSTRACT

Class III peroxidases are present as large multigene families in all land plants. This large number of genes together with the diversity of processes catalysed by peroxidases suggests possible functional specialization of each isoform. However, assigning a precise role for each individual peroxidase gene has continued to be a major bottleneck. Here we investigated the enzyme activity and translational profile of class III peroxidases during stem development of sugarcane as a first step in the estimation of physiological functions of individual isoenzymes. Internodes at three different developmental stages (young, developing and mature) were divided into pith (inner tissue) and rind (outer tissue) fractions. The rind of mature internodes presented the highest enzymatic activity and thus could be considered the ideal tissue for the discovery of peroxidase gene function. In addition, activity staining of 2DE gels revealed different isoperoxidase profiles and protein expression regulation among different tissue fractions. In-gel tryptic digestion of excised spots followed by peptide sequencing by LC-MS/MS positively matched uncharacterized peroxidases in the sugarcane database SUCEST. Multiple spots matching the same peroxidase gene were found, which reflects the generation of more than one isoform from a particular gene by post-translational modifications. The identified sugarcane peroxidases appear to be monocot-specific sequences with no clear orthologue in dicot model plant *Arabidopsis thaliana*.

Keywords: Saccharum officinarum, peroxidase, lignification, enzymatic activity, proteome

1. Introduction

Class III peroxidases are heme-containing proteins present as large multigene families in all land plants but absent in unicellular green algae [1]. The origin and diversification of class III peroxidases may be related to land colonization by plants, by allowing the formation of cell wall structures or by adapting the organism to elevated oxygen concentrations [2,3]. Indeed, it appears that the number of genes encoding class III peroxidases increased dramatically during the evolution of land plants, with the most recent plants often containing more than 100 members of class III family [4]. For example, 73 genes were annotated as peroxidase-encoding genes in *Arabidopsis thaliana* [5], whereas rice contains 138 members [3] and maize approximately 200 [6]. Moreover, additional isoenzymes can be produced by post-transcriptional and post-translational modifications [7].

Most of class III peroxidases are secreted into the apoplast. Structurally, they are glycosylated, calcium-containing proteins that present four or five disulfide bridges in conserved positions and a signal peptide for their secretion across the endoplasmic reticulum [8]. Additionally, some peroxidases may present a C-terminal extension that seems to direct the protein to the vacuole [4].

Peroxidases play a role in two distinct catalytic cycles. In the standard peroxidative cycle, peroxidases catalyze the reduction of H_2O_2 by taking electrons from a variety of donor molecules, especially phenols, lignin precursors, auxin and secondary metabolites [1]. A separated hydroxylic cycle has also been described, which leads to the formation of various reactive oxygen species [9]. Probably as a consequence of these two catalytic cycles and the large number of genes and protein isoforms, class III peroxidases are implicated in a broad range of physiological processes such as lignin and suberin formation [10-12], auxin metabolism [2], cell elongation [13], plant defense [14], as well as the generation of highly

reactive oxygen species [9,15]. Moreover, the heterogeneous regulation of their expression and the fact that all peroxidase sequences present both conserved and variable parts suggest the existence of functional specialization of the members of this enzyme family [2].

Despite the fact that peroxidase activity can be easily detected in different phases and different tissues during the whole lifespan of various plants, it is difficult to assign a specific function to any particular peroxidase. Two main problems inherent in peroxidases can be highlighted: i) overexpression/silencing of a single gene results in no visible phenotypes, due to overlapping functions of redundant genes; and ii) broad substrate specificity *in vitro*, which cannot offer precise information about their real substrates [16]. Therefore, information about the timing and tissue specificity of gene expression, as well as the final localization of isoforms *in planta*, may be extremely important for determination of the putative function of a specific peroxidase [2]. The combination of several approaches may be the only way to unambiguously gain insight into the precise biological role of an individual class III peroxidase isoenzyme.

One of the most extensively studied functions of peroxidases is their involvement in the polymerization of lignin [17]. Lignin polymerization occurs via oxidative radicalization of monolignols (i.e. *p*-coumaryl, coniferyl and sinapyl alcohols), a reaction catalyzed by class III peroxidases and/or laccases, followed by combinatorial radical coupling [18,19]. The oxidation of monolignols results in an electro-delocalized radical with unpaired electron density at positions 1-, 3-, *O*-4-, 5- and 8- [20]. The coupling of monolignol radicals results in a mixture of dehydrodimers with 8-8′, 8-5′, and 8-O-4′ linkages after re-aromatization, since radical coupling at the 8-position is favored [21]. Despite the fact that sinapyl alcohol is more prone to oxidation than either coniferyl or *p*-coumaryl alcohols, most class III peroxidases are uncapable of oxidizing syringyl moieties, while they present noticeable specificity for guaiacyl

moieties [22]. Therefore, the ability of oxidizing syringaldazine (SYR), an analogue of sinapyl alcohol, has been used to correlate class III peroxidase isoenzymes with lignification in a wide range of woody species [10,23].

The participation of peroxidase in lignin biosynthesis has been studied in many plant species and cell culture systems, such as *A. thaliana* [16,24], tobacco [25], *Zinnia elegans* [26], poplar [10,11], and gymnosperm tree species [27,28]. However, very few studies were concerned with peroxidases from monocotyledonous species [29], despite the fact that such species appears to have many more peroxidases genes than eudicotyledonous [3].

Many studies on the evaluation of peroxidase involvement in a specific mechanism are based on gene expression data, especially microarrays [30,31], northern blot and *in situ* hybridization [32]. However, since gene expression does not always reflect protein abundance and activity, proteomic approaches working directly on target proteins could be an interesting alternative. Recent reports on the analysis of plant cell wall and secretory proteomes in many species had identified peroxidase isoforms putatively involved in different biological processes, such as secondary cell wall synthesis in a tobacco cell line [33], wall stiffening after fungal elicitation in *Medicago truncatula* cell culture [34], primary root elongation in maize [35], hypocotyls elongation in *A. thaliana* seedlings [36] and oxidative stress in maize roots [6]. Moreover, additional features such as thermal stability [14] and the fact that these enzymes retain activity after separation on SDS-PAGE [37] and two dimensional electrophoresis (2DE) [38], make proteomic approaches extremely suitable to study class III peroxidases. Activity staining using artificial phenolic substrates such as guaiacol and tetramethylbenzidine (TMB) can be used for the identification of all class III peroxidases independently of their *in vivo* substrate [7], and can dramatically reduce the complexity of protein profiles in 2DE gels.

Sugarcane is an important monocotyledonous crop grown in all tropical and subtropical regions of the world [39]. The utilization of sugarcane for ethanol production in Brazil, since the 1970 decades, is the best example of use of renewable energy [40]. Currently, Brazil is the world's largest ethanol producer, with approximately 25 billion liters of ethanol produced in the 2009/2010 crop season [39]. Sugarcane not only accumulates large amounts of sucrose in the stem, but also produces large lignocellulosic biomass [41], which can be hydrolyzed and used for second generation bioethanol production [42]. This lignocellulosic biomass, known as bagasse, is produced after sucrose extraction and is partially burnt to produce thermoelectricity to sustain the alcohol industrial plants. One ton of sugarcane originates 280 Kg of bagasse [43]. However, recalcitrance of plant cell walls due to the presence of lignin is still a major bottleneck for the utilization of plant biomass as a source for biofuels and bio-based materials [19].

Since peroxidase specificity may be partially responsible for the structure of the final lignin polymers, the modification of expression of specific isoenzymes could be an alternative strategy for reducing cell wall recalcitrance [44]. For such purpose, the identification of isoperoxidases related to lignification in bioenergy crops such as sugarcane requires immediate attention. Therefore, here we investigated the enzyme activity and translational profile of class III peroxidases during stem development of sugarcane as a first step in the estimation of physiological functions of individual isoenzymes.

2. Results and Discussion

In this study, internodes from three different positions in the sugarcane stem, i.e. at three different developmental stages, were used for analysis: young (top), developing (intermediary) and mature (basal) internodes (Fig. 1A). In young internodes, the cells elongate

rapidly with minimal wall thickening, and lignification is restricted to tracheary elements of protoxylem, which stained yellow-brown with Mäule reagent, indicating the presence of mainly G units (Fig. 2A, 2C, 2F). As maturation proceeds, elongation and sucrose accumulation are completed and cell wall becomes thickened and lignified in different cell types [41]. Lignified cell types from developing and mature internodes included tracheary elements of metaxylem, sclerenchyma surrounding the vascular bundles, epidermis, hypodermis and most of the parenchyma cells (Fig. 2B, 2D, 2E). Most of the lignified cells stained in red-brownish, indicating the presence of S units, except the already mentioned protoxylem vessels. The outermost cell layers of the stem, usually named the rind, is of particular importance since both epidermis and hypodermis become highly lignified, as well as many vascular bundles that are distributed throughout the rind. In fact, as 75% of the vascular bundles are densely packed within the outer 3 mm of the stem [45], the rind is probably a high metabolically active region for lignin deposition and peroxidase activity. Conversely, vascular bundles are more sparsely distributed in the center of the stem, also known as pith, and some isolated storage parenchyma cells remain not completely lignified. In addition, vascular bundles located in the rind seem to present a broader sclerenchyma sheath compared to the ones located deeper in the pith. Due to these developmental differences, internodes were divided here in rind and pith tissues (Fig. 1B) for all enzymatic and electrophoretic analyses.

2.1. In situ and in vitro peroxidase activity

Transverse sections of sugarcane internodes were incubated in the presence of TMB or SYR, and H_2O_2 for *in situ* visualization of peroxidase activity. This assay is based on the H_2O_2–dependent oxidation of TMB or SYR by cell wall peroxidases [23,46]. Treatment of stem sections with TMB resulted in little blue staining only in vascular bundles, regardless of

the developmental stage of the internode (Fig. 3A, 3B, 3C). On the other hand, internodes were differentially stained when SYR was used (Fig. 3D, 3E, 3F). In young internodes, activity was only detected in vascular bundles, while no activity was observed in the outer cell layers (Fig. 3D). Curiously, an intense pink deposit was also observed when the stem tissue was damaged (Fig. 3D, arrow), probably due the release of peroxidases or other types of intracellular oxidases, like polyphenoloxidase [47]. In developing and mature internodes, a gradient of SYR-peroxidase activity was observed from the rind to the pith (Fig. 3E, 3F). An intense staining could be detected in the rind, which comprises epidermis, hypodermis and developing vascular bundles, but inwards the activity was limited to vascular bundles. These results are in total agreement to the pattern of developmental lignification of sugarcane stems [45].

To refine the analysis, high salt extraction proteins from the six tissue samples were used in spectrophotometric analyses of peroxidase activity (Fig. 4). Three different substrates were used: guaiacol, syringaldazine and coniferyl alcohol. Comparison of different tissues from the same internode shows that in young internodes peroxidase activity is equivalent between rind (YR) and pith (YP), while in developing and mature internodes the activity in the rind is much greater. By comparing distinct internodes, peroxidase activity was highest in MR and DR respectively, regardless the substrate. Moreover, SYR oxidation activity was negligible in pith from all internode ages and rind of young internodes. Altogether, these results reinforce the idea that the rind region of the sugarcane stem is a high metabolically active tissue for peroxidase activity.

Despite the fact that sinapyl alcohol is more prone to oxidation than either coniferyl or *p*-coumaryl alcohols, most of class III peroxidases present steric restrictions at the substrate binding site that do not allow the oxidation of this doubly methoxylated monolignol, while

they can readily oxidize 4-hydroxyphenyl and guaiacyl moieties, i.e. *p*-coumaryl and coniferyl alcohol, respectively [48]. Indeed, oxidation of coniferyl alcohol is no proof for peroxidase involvement in monolignol dehydrogenation since most peroxidases isolated from a variety of species could catalyze the reaction with this singly methoxylated hydroxycinnamoyl alcohol [17]. On the other hand, oxidation of syringaldazine, which possesses a syringyl moiety, has been observed exclusively in lignifying tissues, suggesting that lignin polymerization is correlated to SYR-oxidizing capacity of peroxidases [11,23]. However, the relevance of syringyl peroxidases to radicalization of sinapyl alcohol has been questioned by recent studies on the potential role of hydroxycinnamates as a radical transfer mechanism, where it was demonstrated that peroxidases isolated from corn cell wall can readily oxidize *p*-coumarate (*p*CA) in the presence of H_2O_2, whereas sinapyl alcohol radical coupling proceeds more slowly [49]. The addition of *p*CA in the reaction mixture enhanced the rate of sinapyl alcohol oxidation by 10-20 fold. Therefore, they suggested a possible role for *p*-coumarate as an oxidation shuttle, in which the readily oxidized *p*CA can transfer its oxidation state to an acceptor molecule such as sinapyl alcohol. If such an oxidation shuttle operates within the lignifying cell wall matrix, sinapyl alcohol can be incorporated in lignin polymer without the presence of syringyl peroxidases. Thus, SYR-oxidizing peroxidases observed in lignifying tissues could be involved in other processes during cell wall formation apart from lignification, such as hydrogen peroxide production, cross-linking of cell wall proteins and carbohydrates, and biosynthesis or degradation of low-molecular weight compounds in the cell wall [11].

2.2. Class III peroxidase isoforms pattern during sugarcane stem development

The protein profile of the class III peroxidase isoforms at distinct stages of stem development was analyzed using in-gel detection of peroxidase activity [7]. Previous works reported the use of 1DE to identify genetic variation among sugarcane cultivars [50] or to analyze peroxidase isoforms in sugarcane cultivars in response to *Colletotrichum falcatum* inoculation, the causal agent of red rod disease [51]. However, to our knowledge, this is the first report on the characterization of class III peroxidases protein profiles of sugarcane stem using a developmental perspective. A modified mono-dimensional electrophoresis in 0.8 % agarose gels was performed to roughly generate cationic/anionic isoperoxidase profiles. Although agarose is not a suitable matrix to separate proteins, our analysis showed that anionic peroxidases (at pH 8.0) were found as predominant isoforms in all analyzed tissue samples (Fig. 5A). Moreover, an intense cationic band could be observed almost exclusively in samples DR and MR (arrow, Fig. 5A), which are supposed to be highly active regions for lignification. This cationic band was extracted for proteins and used for peroxidase detection with guaiacol as substrate in 10 % polyacrilamide activity gel. At least two peroxidase bands could be resolved by semi-denaturing SDS-PAGE (data not shown), both of which were excised for further MS analysis. Unfortunately, the sequenced peptides (RSPATASRRA and RQRVFLRR) did not match any peroxidase hit when SUCEST or even PeroxiBase databases were searched. Since the PeroxiBase database presents only a few sequences from sugarcane peroxidases and the SUCEST database is based on ESTs rather than the whole genome, is possible that the peptides generated from the tryptic digestion of such proteins belong to peroxidases not recorded in these databases and/or to peroxidases whose sequences are only partially recorded in SUCEST.

Activity staining after semi-denaturing SDS-PAGE was also performed to identify the expression of isoenzymes in such tissues (Fig. 5B). For all internodes ages, high molecular

mass isoenzymes (>76 kDa) were predominantly observed in rind samples, whose expression seems to be up-regulated during stem maturation (Fig. 5B). On the other hand, low molecular mass isoforms (≤ 52 kDa) were predominant in pith preparations and their levels of expression were reduced during maturation (Fig. 5B). These results suggest that rind and pith not only accumulate different isoperoxidase forms but also showed different kinects of isoenzyme expression during stem development. However, since semi-denaturing PAGE does not allow protein separation exclusively according to molecular weight, the indicated isoform molecular weight might not be precise.

In order to elucidate the class III peroxidase profiles from the six sugarcane stem tissues, 2DE was carried out and activity staining with guaiacol was used for the detection of peroxidases in the second dimension run (Fig. 6). Analysis of 2-DE profiles can be separated in two parts: (i) comparison of rind and pith from the same internode; and (ii) comparison of same tissue type (rind or pith) from internodes of different developmental stages. Representative gel images for each tissue protein fraction are shown in Fig. 6. In young internodes (Fig. 6A, D), most of spots were common to both rind and pith. It should be noted that only in young internodes, the overall protein activity of pith profile was similar to rind profile, and such observation is in agreement with *in vitro* peroxidase activity results (Fig. 4). In addition and as already indicated by agarose 1-DE, anionic peroxidases were predominantly expressed in both tissue samples. By contrast, significant differences were observed among the 2-DE activity profiles of pith and rind from developing and mature internodes (Fig. 6B, C, E, F). As shown by 1-DE analysis, there was an evident higher activity of high molecular weight peroxidases (>76 kDa) in rind protein fractions than pith proteins, and the overall protein activity in rind fractions was greater than pith fractions. Isoperoxidase profiles can also be analyzed in a developmental perspective, comparing the same tissue type from different

internode stages. In the case of pith preparations (Fig. 6A-C), slight differences in activity could be noticed throughout the stem development. On the other hand, the already mentioned increase of high molecular weight isoperoxidases activity in the rind is characteristic from the transition of young to maturing internode and it is maintained during the mature stage (Fig. 6D-F). Noteworthy, anionic isoperoxidases were predominantly expressed in all tissues analyzed.

Casu et al. [41] reported on the identification of differentially expressed transcripts in the sugarcane stem during development by expression profiling using the GeneChip Sugarcane Genome Array. Transcriptional activity in young parts of the stem was primarily involved in growth and development, while maturing and mature internodes presented transcripts that are involved in sugar transport, fiber metabolism and protein metabolism. Moreover, cell wall metabolism-associated genes were up-regulated with maturity. Nevertheless, peroxidases expression was higher in the immature stem and down-regulated in maturing and mature internodes. The authors justified such unexpected expression profile as a consequence of the lack of a representative probe set for a peroxidase involved in lignification, since those enzymes are also implicated in other physiological processes. However, since class III peroxidase can be highly stable due to different levels of protein glycosylation [8], those genes may present a different expression profile than the expected for lignification or cell wall-related peroxidase genes. Gene expression and protein production could take place already in young stem and highly stable peroxidases would still be available during the whole developmental process, even with their gene down-regulation. In addition, secondary cell walls are already present in cells from young sugarcane stem [41], which in part depends on peroxidase activity. Our 2-DE profiles revealed high diversity of isoperoxidases in both maturing and mature internodes. The presence of active peroxidases in mature stem, a tissue

that is mostly already lignified, would be advantageous for lignification during radial growth or cell wall stiffening to respond to stresses like wounding and pathogen attack [52].

2.3. Peptide sequencing by LC-MS/MS

Aiming to identify peroxidase genes expressed during sugarcane stem development, individual spots were excised from 2DE gels (Fig. 6), digested with trypsin and analyzed by LC-MS/MS. SUCEST database was uploaded and searched for peroxidase peptides. From 22 spots used for MS analysis, 7 matched contigs from SUCEST database which code for peroxidase genes (Table 1). Most of protein identifications were based on at least 2 peptide matches and had scores equal or greater than two times the accepted significance threshold, although all proteins whose score was equal or exceeded the identity score was positively reported. Occasionally, the same peroxidase was identified from multiple spots. For example, all protein spots from region II (spots 2 to 6) matched the same peroxidase contig SCCCAD1001C08.g in the SUCEST database. Interestingly, these proteins present the same isoelectric point but different molecular weights, suggesting post-translational modifications such as glycosylation or protein truncation. Laugesen et al. [53] reported on a proteomic analysis of peroxidase isoenzymes from barley seed, using 2DE followed by mass spectrometry, the identification of the same BSSP1 peroxidase in nine different spots. In addition, BSSP1 was always present as doublets of identical pI and different molecular weight in 2DE gels, which also suggest different levels of protein glycosylation. It has been postulated that glycosylation may be important not only for substrate specificity and protein folding and stabilization [8] but also for protein/cell wall interactions [54]. Finally, the majority of identified peroxidases were of lower molecular weight (≤ 52 kDa) because those

of high molecular weight were located in a region of low resolution in the gel, where activity was spread, making difficult the excision of a single band.

The phylogenetic relationships among all 73 Arabidopsis class III peroxidases and the identified sugarcane peroxidases are shown in Fig. 7. In addition, the putative orthologues of sugarcane peroxidases in *Sorghum bicolor* were also included, since sorghum is the closest relative species to sugarcane in the Andropogonea tribe [55]. Despite the fact that many studies report no clear relation between amino acid sequence similarity and physiological function of peroxidases, a phylogenetic tree using sequences from different plant species can be useful for the identification of putative orthologues and, as a consequence, to gain insights into the physiological function of unknown class III peroxidases. As already expected, sugarcane peroxidases cluster preferentially with their sorghum orthologues in clades always supported by high bootstrap values. Interestingly, Arabidopsis seems to contain no clear co-orthologue corresponding to any of the identified sugarcane peroxidases, since each sugarcane/sorghum clade is sister to a clade containing several Arabidopsis sequences. Thus, those sugarcane/sorghum peroxidases are products of monocot-specific gene duplication events. Since no information about the putative role of the identified sugarcane peroxidases was obtained from the phylogenetic analysis, tissue-specific expression and inducers data were extracted from PeroxiBase database. For such an approach, both sorghum co-orthologues and sugarcane hits from PeroxiBase (when available) were analyzed. Local Blastp analysis for SCBFRZ2017C11.g retrieved no sugarcane hit in PeroxiBase and the corresponding co-orthologue in sorghum (SbPrx75 – ID 878) presented no information about inducers, while is reported to express in seedlings. The contig SCCCCL3002E11.b seems to be SofPrx06 (ID 1856), which is reported to express in buds, callus and seeds of sugarcane and induced by temperature stress. No information is available for the corresponding co-orthologue in

sorghum (SbPrx170 – ID 6388). Finally, the contig SCCCAD1001C08.g hits SsPrx04 in PeroxiBase, which is expressed in stems of sugarcane. The co-orthologue in sorghum is SbPrx18 (ID – 816), which is expressed in seedlings, roots and leaves and is induced by biotic and abiotic stresses and light growth. Interestingly, this peroxidase was the most abundant in our 2-DE profiles, possibly playing an important role during the developing of sugarcane stem.

3. Conclusions

Sugarcane stem development involves distinct processes such as cell elongation, lignification, suberization and sugar concentration, most of which are known as physiological processes related with activity or expression of plant class III peroxidases. Despite years of scientific interest in peroxidases, the precise function of most of the individual isoenzymes remains elusive, especially for monocotyledonous plants, which evolved additional gene clusters not found in dicotyledonous plants. This report represents a significant advancement in the number of peroxidases characterized from sugarcane, since almost nothing related to this protein family had been previously reported in this crop. Here we observed that the rind of developing and mature internodes presented the highest enzymatic activity and thus could be considered the ideal tissue for studies on the determination of peroxidase gene function. In addition, a proteomic approach using activity staining of 2DE revealed that different morphological regions of internodes also present specific isoperoxidase profiles and that expression of these isoenzymes are differently regulated over time. Excised spots from 2DE gels were sequenced and some of them positively matched peroxidases in SUCEST database. The identified sugarcane peroxidases appear to be monocot-specific sequences with no clear orthologue in Arabidopsis. Currently, more detailed expression analysis is being performed

using such sugarcane peroxidase contigs, since spatio-temporal expression may gain insights into the precise role of specific peroxidase isoforms. The combination of several approaches seems to be essential for the understanding of this multifunctional enzyme family.

4. Methods

4.1. Plant materials and protein extraction

Stem tissue samples were harvest from three biological replicates of adult cultivar IACSP04-529 of sugarcane plants cultivated in the field, at the Centro de Cana of the Instituto Agronômico de Campinas (IAC), at Ribeirão Preto, State of São Paulo. The canes were kindly supplied by Dr Ivan Antônio dos Anjos. Plants were 2 years old and tissues were separated as follows and immediately frozen in liquid nitrogen and stored at -80°C until protein extraction. The stem tissue samples comprised: i) rind and pith of young internodes (internodes 1 to 3; named YR and YP), ii) rind and pith of developing (intermediary) internodes (internodes 5 to 7; named DR and DP), and iii) rind and pith of mature internodes (internodes 15 to 17; named MR and MP). Internodes were numbered according to Jacobsen et al. [45] and cut as follows: the most external 0.3 cm corresponded to the rind sample, while a fragment of 1-2 cm in diameter from the inner part corresponded to the pith sample.

Frozen tissues were ground to a powder using a mill (IKA® A11) with liquid nitrogen and extracted with 50 mM Tris-HCl buffer (pH 7.5) supplemented with 1 M NaCl, in a proportion of 0.25 g FW per mL buffer, for extraction of ionically-bound proteins. After 2 h incubation on ice with continuous shaking (150 rpm), the samples were centrifuged (20,000 xg, 30 min, 4°C) and the recovered supernatants were then filtered once through paper filter and once through a 60 µm NY60 Millipore nylon membrane. Subsequently, the extracts were concentrated using an Amicon ultra-filtration apparatus (YM 10 membrane) and buffer

exchanged by passage through Sephadex G25 columns (PD10 columns, GE Healthcare). Proteins were eluted from the PD10 columns with 20 mM Tris-HCl buffer (pH 7.5), and then lyophilized and stored at -80°C until use.

4.2. Lignin histochemical staining

Stem material from internodes 2, 9 and 15 was fixed in formalin/acetic acid/ethanol/water (5:5:60:30, v/v/v/v) and embedded in Paraplast® X-tra (Fisher). Sections of 20 μm thickness were obtained with a microtome and the Paraplast resin was removed by immersion in xylene. The sections were stained for lignin with Mäule [56] and Safranin-O/Astra-Blue [57]. Mäule reagent estimates lignin composition, while Safranin-O/Astra-Blue was used to follow developmental deposition of lignin, since Astra-Blue stains cellulose blue only in the absence of lignin and Safranin-O stains lignin red regardless of the presence of cellulose. Photographs were taken using Olympus BX 51 microscope with an Olympus DP 71 camera.

4.3. In situ peroxidase activity

In situ activity was carried out using syringaldazine (SYR) [23] and 3,3′,5,5′-tetramethylbenzidine (TMB) [46]. Briefly, whole pieces of sugarcane internodes were hand cut with a razor blade, incubated in 1 mM ascorbic acid for 1 min and rinsed with distilled water prior to staining with 0.1 % SYR (Sigma) solution in N,N-dimethylformamide or 0.1 $mg.mL^{-1}$ TMB, both cases supplemented with 0.03% H_2O_2. Stained internodes were photographed with a Nikon D40 digital camera.

4.4. In vitro activity assays

Lyophilized protein samples were solubilized in 5 M urea and 2 % CHAPS for enzyme assays and electrophoresis, and the protein concentration was determined using Qubit® Protein Assay (Invitrogen). Guaiacol peroxidase (GPrx) activity was obtained by measuring the increase of absorbance at 470 nm for 2 min relative to guaiacol oxidation to tetraguaiacol (ε = 26.6.mM^{-1}.cm^{-1}). The assay (1 mL) contained 10 µg protein, 8.26 mM guaiacol and 0.03% H_2O_2 (v/v) in 50 mM Na-acetate buffer (pH 5.0), as previously described [7]. GPrx activity was expressed as µmol tetraguaiacol.min^{-1}.μg^{-1} total protein. Syringaldazine peroxidase (SyrPrx) activity was measured by following the increase of absorbance at 530 nm (ε = 27.1.mM^{-1}.cm^{-1}) for 2 min in 1 mL reaction mixture containing 50 µM SYR, 0.03% H_2O_2 and 10 µg protein in 20 mM Tris-HCl buffer (pH 7.5) [10]. SyrPrx activity was expressed as µmol SYR oxidized.min^{-1}.μg^{-1} total protein. Coniferyl alcohol peroxidase (CaPrx) activity was determined by following the decrease of absorbance at 262 nm (ε = 9.6.mM^{-1}.cm^{-1}) for 2 min in a 1 mL reaction mixture containing 50 µM coniferyl alcohol, 0.03% H_2O_2 and 10 µg protein in 50 mM Na-acetate buffer (pH 5.0) [58]. CaPrx activity was expressed as µmol coniferyl alcohol oxidized.min^{-1}.μg^{-1} total protein.

4.5. Mono- and bidimensional electrophoresis

Mono-dimensional electrophoresis (1DE) was carried out in two distinct systems. In the first system, separation of proteins was carried out on 0.8% (w/v) agarose gels (Mini-Sub Cell GT system, BioRad) using TAE (Tris-acetate EDTA) buffer (pH 8.0). The comb was placed in the middle of the gel to allow separation of cationic and anionic proteins and

approximately 100 µg of protein was loaded into each well. Class III peroxidases were actively stained with 1 % guaiacol and 0.03% H_2O_2 in 50 mM Na-acetate buffer (pH 5.0) [7]. In the second system, 1DE was carried out as semi-denaturing PAGE [59] using 10% gels, at 4°C. Sample buffer was prepared without SDS or any reducing agent and proteins were not boiled, although SDS was used in the gel and buffer preparation. Approximately 80 µg of protein was loaded into each well. Proteins were stained with guaiacol, as described above. Pre-stained molecular marker (Rainbow Full Range, GE Healthcare) was used to determine the apparent molecular mass of protein bands.

For the two-dimensional electrophoresis (2DE), the first dimension (isoelectric focusing, IEF) was made with Immobiline Dry Strip pH 3-10 NL 7 cm (GE Healthcare). Protein samples (200 µg) were diluted to 125 µL with rehydration buffer (5 M urea, 2 % CHAPS, 0.8 % ampholytes, 0.006 % bromophenol blue), incubated at room temperature for 30 min and centrifuged at 10,000 xg for 5 min. Gel strips were rehydrated overnight with protein solutions at room temperature by placing the strip gel-side down in IEF tray. IEF was carried out in an IPGphor system (GE Healthcare) with the current limited to 50 µA per strip until focusing reached total 8 kVh. After focusing, gel strips were equilibrated in 50 mM Tris-HCl buffer (pH 8.8), 30 % glycerol, 2 % SDS, 0.006 % bromophenol blue for 10 min and subjected to semi-denaturing PAGE on 10 % gels. Electrophoresis was performed at 4°C and the peroxidases were stained for activity [7]. Pre-stained molecular marker (Rainbow Full Range, GE Healthcare) was used in the second dimension. Although SDS was used in the gel and buffer preparation, protein samples were not boiled or reduced with SDS and reducing agent, thus, the second dimension was a semi-denaturing electrophoresis. The method was performed in triplicates for each tissue. Images were taken by a Nikon D40 digital camera and evaluated using ImageJ (Version 1.45; http://imagej.nih.gov/ij).

4.6. Mass spectrometry analysis

The identification of 2DE spots was made by peptide sequencing using LC-ESI-Q-TOF after in-gel digestion. The spots were excised from gels manually with a blade and washed twice with 500 µL 50 % methanol, 2.5 % acetic acid for 2 h for destaining and SDS removal. Then they were dehydrated with 200 µL 100% acetonitrile (ACN) and dried under vacuum after removal of ACN. Protein in the spots were reduced by adding 30 µL of 10 mM dithiothreitol in 100 mM NH_4HCO_3 solution and incubation for 30 min, followed by alkylation with 30 µL of 50 mM iodoacetamide in 100 mM NH_4HCO_3 solution for 30 min. Then, the gel spots were submitted to consecutive washing and dehydration steps with 200 µL of 100 mM NH_4HCO_3 and 200 uL of 100% ACN respectively, and dried under vacuum. The dried gel pieces were rehydrated with 30 µL of porcine trypsin (20 $ng.\mu L^{-1}$ in 50 mM NH_4HCO_3, Promega) and incubated on ice for 30 min. The excess of trypsin solution was removed and the gel spots were covered with 50 mM NH_4HCO_3. Digestion was carried out overnight at 37°C. The peptides were extracted (i) by adding 10 µL of 5% formic acid, incubating for 10 min at room temperature and transferring the solution to a new tube; and (ii) by adding 12 µL of 5% formic acid, 50% ACN and incubating for 10 min at room temperature. Both fractions were combined and concentrated under vacuum to a final volume of 1 µL. Each sample was injected into an analytic C18 RP-UPLC column (1.7 µm BEH 130, 100 µm x 100 mm, nanoAcquity UPLC, Waters) coupled to a nano-electrospray tandem mass spectrometry on a Q-TOF Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 $nL.min^{-1}$. The gradient was 0-50% ACN in 0.1% formic acid over 45 min. Sample desalting was performed using a trapping column Symmetry C18 (180 µm x 20 mm) at a flow rate of 20 $\mu L.min^{-1}$ over 1 min. The equipment was operated in MS positive mode, data

continuum acquisition from m/z 100-2,000 Da at a scan rate of 1 sec and an interscan delay of 0.1 sec.

For peptide identification, the MS/MS ion search was performed with Mascot Distiller (version 2.3.2.0, 2009, Matrix Science, Boston, MA) using the following restrictions: (i) trypsin as digesting enzyme; (ii) oxidation of methionine as variable modifications; (iii) carbamidomethylation of cysteine as fixed modifications; (iv) mass values were monoisotopic; (v) protein mass was unrestricted; (vi) peptide mass tolerance was ± 0.1 Da; (vii) fragment mass tolerance was ± 0.1 Da; (viii) max missed cleavages was 1; (ix) significant threshold was $p < 0.05$. SUCEST database was uploaded and searched for sugarcane-specific peroxidase peptides.

4.7. Phylogenetic tree

Multiple amino acid sequence alignment was performed with ClustalW [60] using sequence data extracted from PeroxiBase (http://peroxibase.toulouse.inra.fr/) and SUCEST (http://www.sucest-fun.org/) databases. A phylogenetic tree was constructed with MEGA software version 4.02 [61] and evolutionary relationships were inferred using Neighbor-joining method [62]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Information concerning tissue-specific expression and inducers of class III peroxidases was retrieved from PeroxiBase database.

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Figure Captions and Tables

Fig. 1. Sugarcane internodes at three different developmental stages were used: young (bottom), developing (intermediary) and mature (top) (A). Each internode was divided into pith (inner tissue) and rind (outer tissue) for protein preparation (B).

Fig. 2. Transversal sections of sugarcane internodes. Young internodes (A, C, F), developing internodes (B, D, G), and mature internodes (E and H). Sections were stained with Safranin (A) and Astra Blue (B), or histochemically stained for lignin using Mäule reagent (C to H) - sections of young (C), developing (D) and mature (E) rinds; and sections of young (F), developing (G) and mature (H) piths. Pvb, peripheric vascular bundle; cvb, central vascular bundle; e, epidermis; pc, parenchymatic cells; vb, vascular bundle; ph, phloem; f, fibers; px, protoxylem; mx, metaxylem; pl, protoxylem lacuna. Bars: A-B, 200 μm; C-H, 50 μm.

Fig. 3. *In situ* activity staining of peroxidases in sugarcane stem sections. TMB activity staining of young (A), developing (B) and mature (C) internodes, respectively. The blue staining is limited to vascular bundles in all three internodes. SYR activity staining of young (D), developing (E) and mature (F) internodes, respectively. SYR activity was higher in the rind of developing and mature internodes, as well as in damaged tissues (D, arrow).

Fig. 4. *In vitro* activity of guaiacol peroxidase – GPrx (A), syringaldazine peroxidase – SyrPrx (B), and coniferyl alcohol peroxidase – CAPrx (C), in pith and rind protein preparations of young, developing and mature internodes. Total activity was higher in rind of developing and mature internodes, regardless the substrate. Vertical bars indicate standard

deviation. YP, young pith; YR, young rind; DP, developing pith; DR, developing rind; MP, mature pith; MR, mature rind.

Fig. 5. Monodimensional electrophoresis was carried out in two different systems. (A) Agarose electrophoresis showed the distribution of positively and negatively charged isoforms. Anionic peroxidases were found as predominant isoforms in all analyzed tissue samples, while a cationic major band (arrow) was found mainly in developing and mature rind preparations. (B) Activity staining after semi-denaturing SDS-PAGE showed a predominance of high molecular mass isoenzymes (>76 kDa) in all rind samples and low molecular mass isoforms (≤ 52 kDa) in pith preparations. YP, young pith; YR, young rind; DP, developing pith; DR, developing rind; MP, mature pith; MR, mature rind.

Fig. 6. 2D-PAGE analysis of peroxidases during sugarcane stem development, in the pith (A, B, C) and rind (D, E, F) of young (A, D), developing (B, E) and mature (C, F). Isoforms were separated by isoelectric focusing in the first dimension in a pH range 3-10, followed by a non-reducing and non-denaturing SDS-PAGE in the second dimension. The detection of haem groups of class III peroxidases for the visualization of protein activity was performed by staining with guaiacol and H_2O_2. Numbers in A indicate molecular mass markers. Proteins discussed in the text are indicated by numbers in B.

Fig. 7. Phylogenetic relationships between *Arabidopsis thaliana* and identified sugarcane peroxidases. The co-orthologues of sugarcane peroxidases in sorghum were also included. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is indicated next to the branches, when over 50%. Sugarcane

peroxidases cluster preferentially with their sorghum co-orthologues and showed no clear corresponding orthologue in *A. thaliana*.

Table 1

Identification of sugarcane peroxidases after in-gel digestion with trypsin using LC-ESI-Q-TOF and homology-based search against SUCEST database.

Spot Number	Tryptic Peptide	SUCEST contig	Mascot Score (Significance Level)	Sorghum Orthologue (PeroxiBase ID)
1	R.DAAPNNPSLR.G R.SFCTSFLAR.I	SCCCCL3002E11.b	29 (>14)	SbPrx170 (6388)
2	R.TGASLLR.M K.SAVDAAVMQEAR.T + Oxidation (M)	SCCCAD1001C08.g	68 (>14)	SbPrx18 (872)
3	R.TGASLLR.M K.SAVDAAVMQEAR.T + Oxidation (M)	SCCCAD1001C08.g	95 (>14)	SbPrx18 (872)
4	R.TGASLLR.M R.RFDVVDNIK.A K.SAVDAAVMQEAR.T + Oxidation (M)	SCCCAD1001C08.g	116 (>14)	SbPrx18 (872)
5	R.TGASLLR.M K.SAVDAAVMQEAR.T + Oxidation (M)	SCCCAD1001C08.g	114 (>14)	SbPrx18 (872)
6	R.TGASLLR.M R.RFDVVDNIK.A K.SAVDAAVMQEAR.T + Oxidation (M)	SCCCAD1001C08.g	128 (>14)	SbPrx18 (872)
7	R.DAVPNNPSLR.G	SCBFRZ2017C11.g	58 (>14)	SbPrx75 (878)

Figure 1

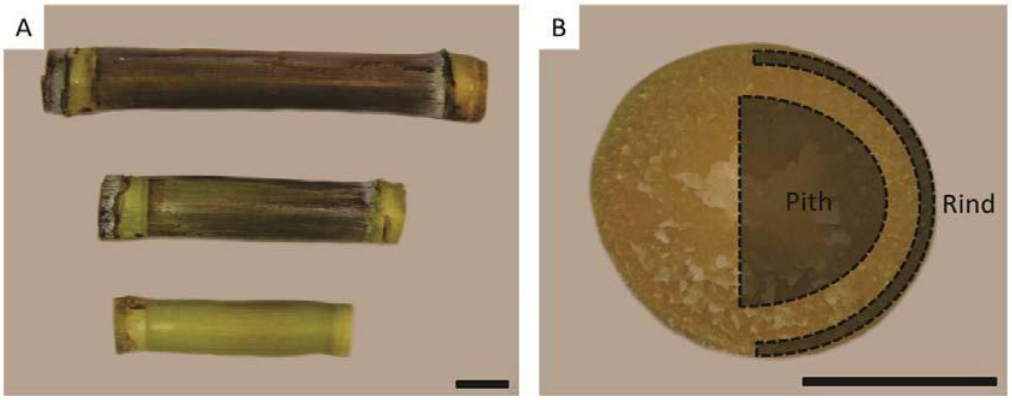


Figure 2

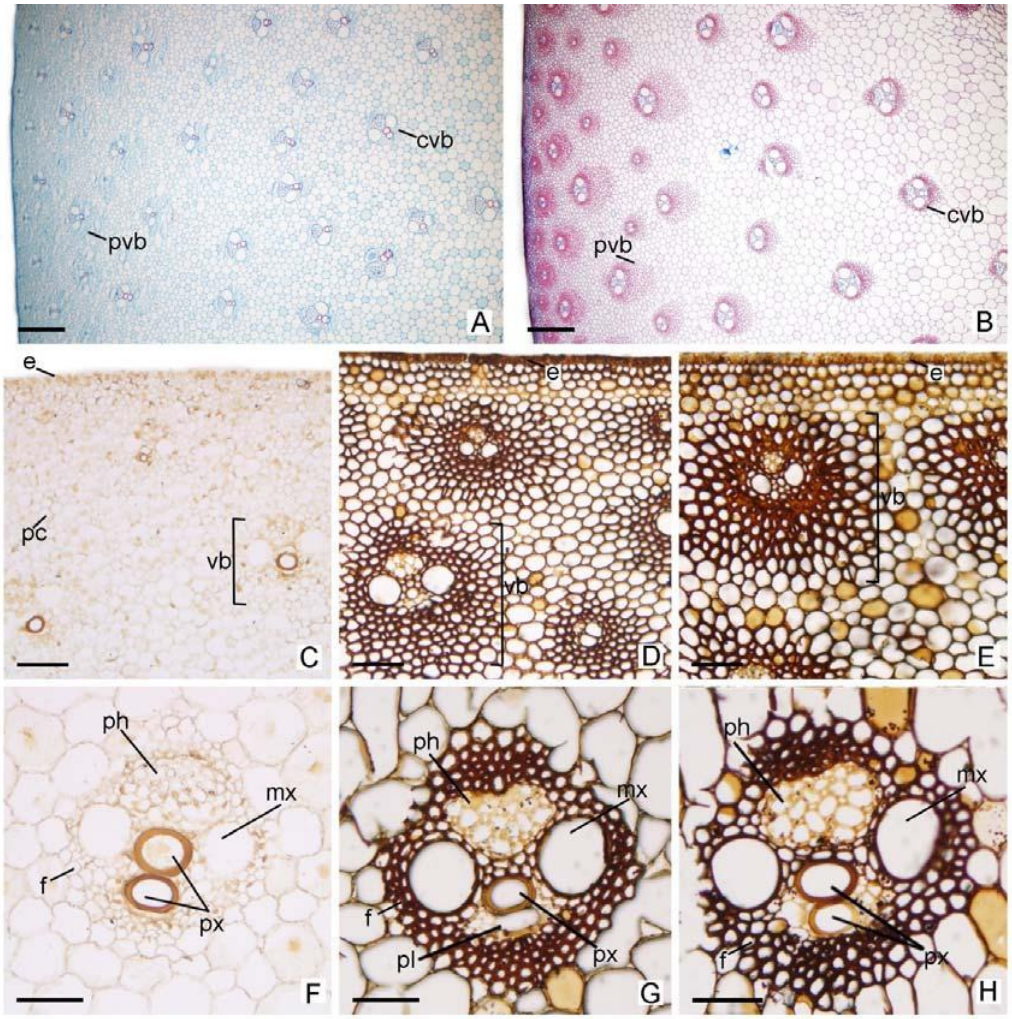


Figure 3

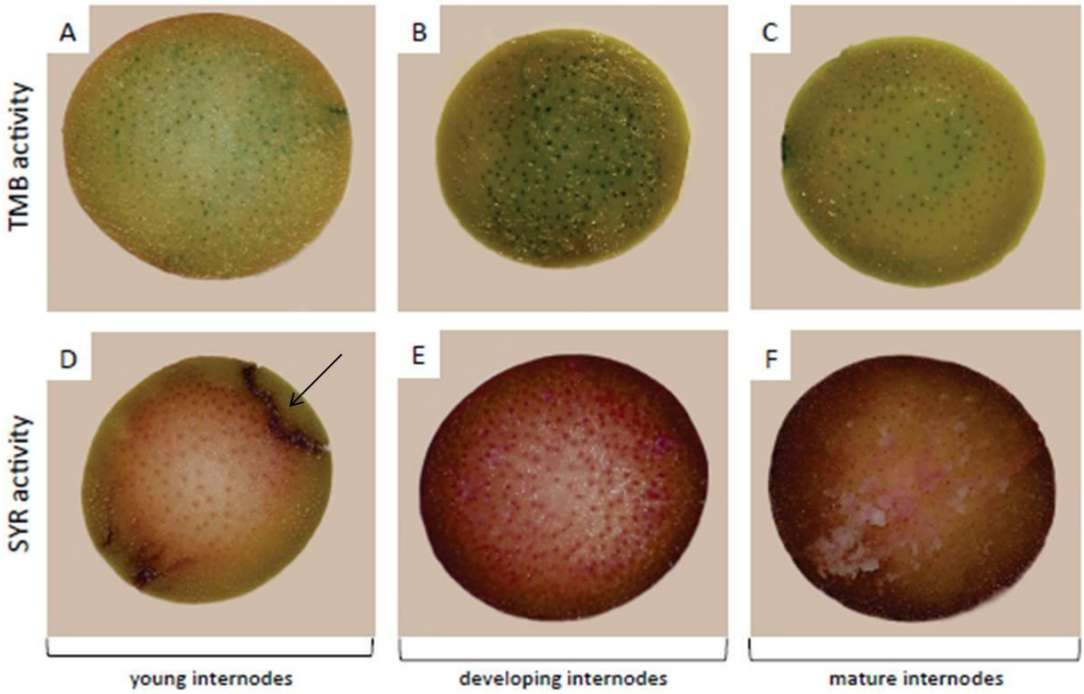

A
B
C
TMB activity
D
E
F
SYR activity
young internodes
developing internodes
mature internodes

Figure 4

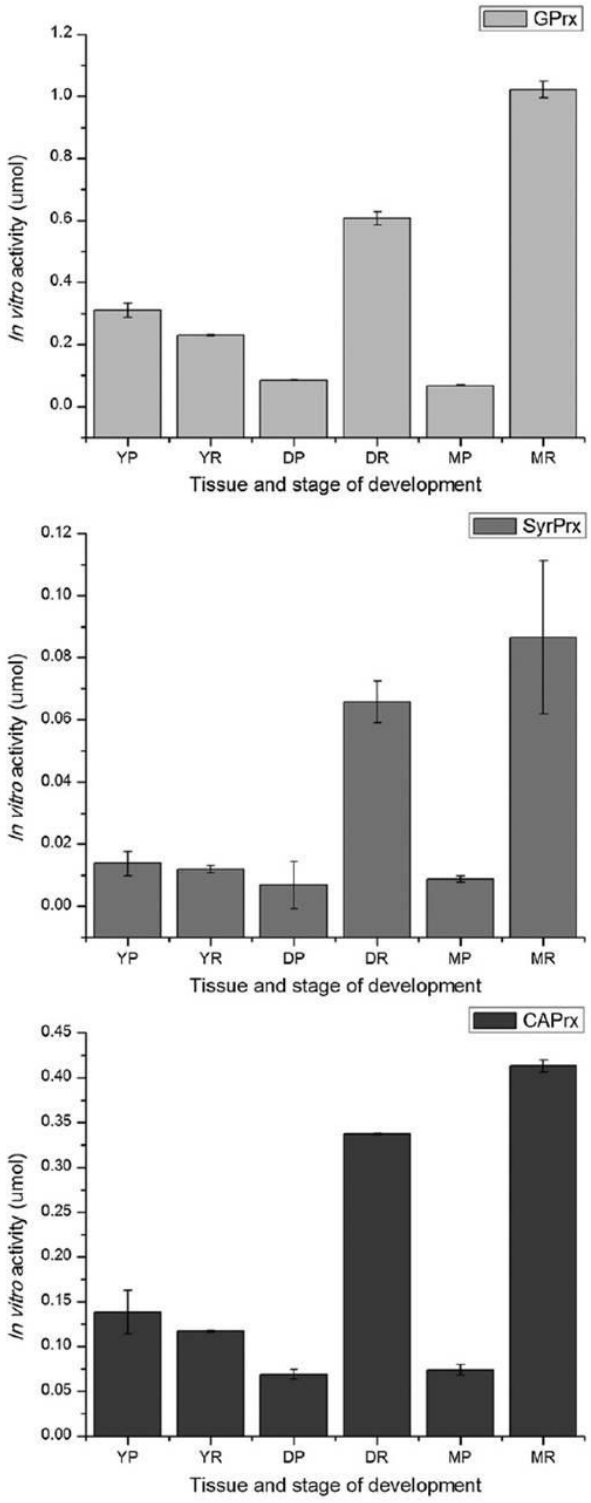

GPrx
1.2
1.0
0.8
0.6
0.4
0.2
0.0
In vitro activity (umol)
YP
YR
DP
DR
MP
MR
Tissue and stage of development
SyrPrx
0.12
0.10
0.08
0.06
0.04
0.02
0.00
In vitro activity (umol)
YP
YR
DP
DR
MP
MR
Tissue and stage of development
CAPrx
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
In vitro activity (umol)
YP
YR
DP
DR
MP
MR
Tissue and stage of development

Figure 5

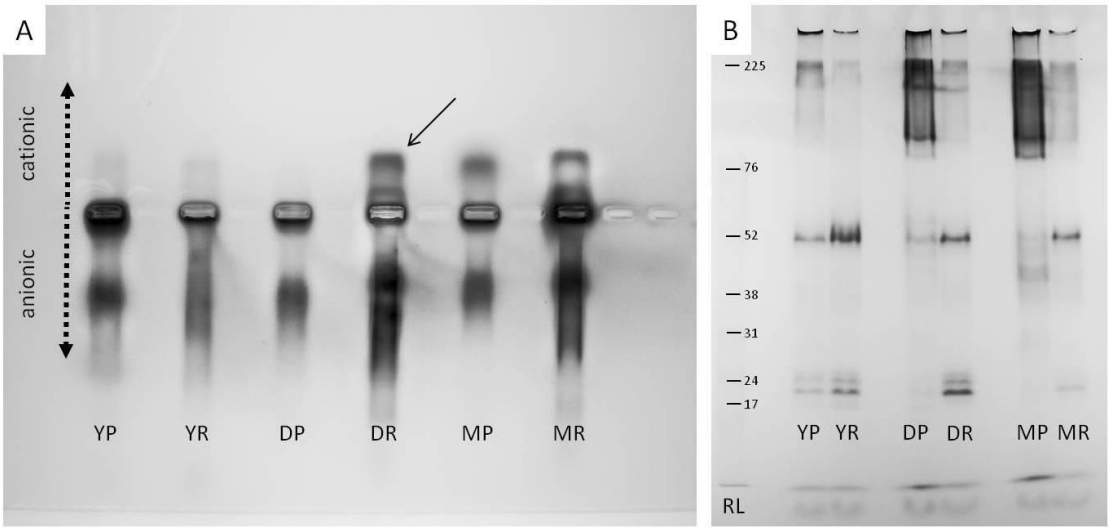


Figure 6

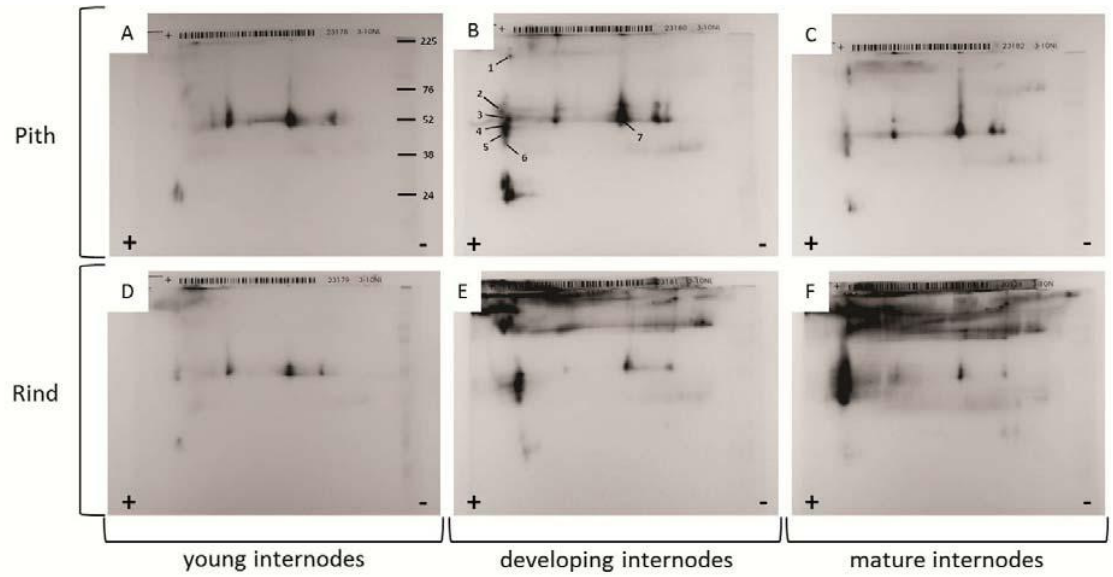
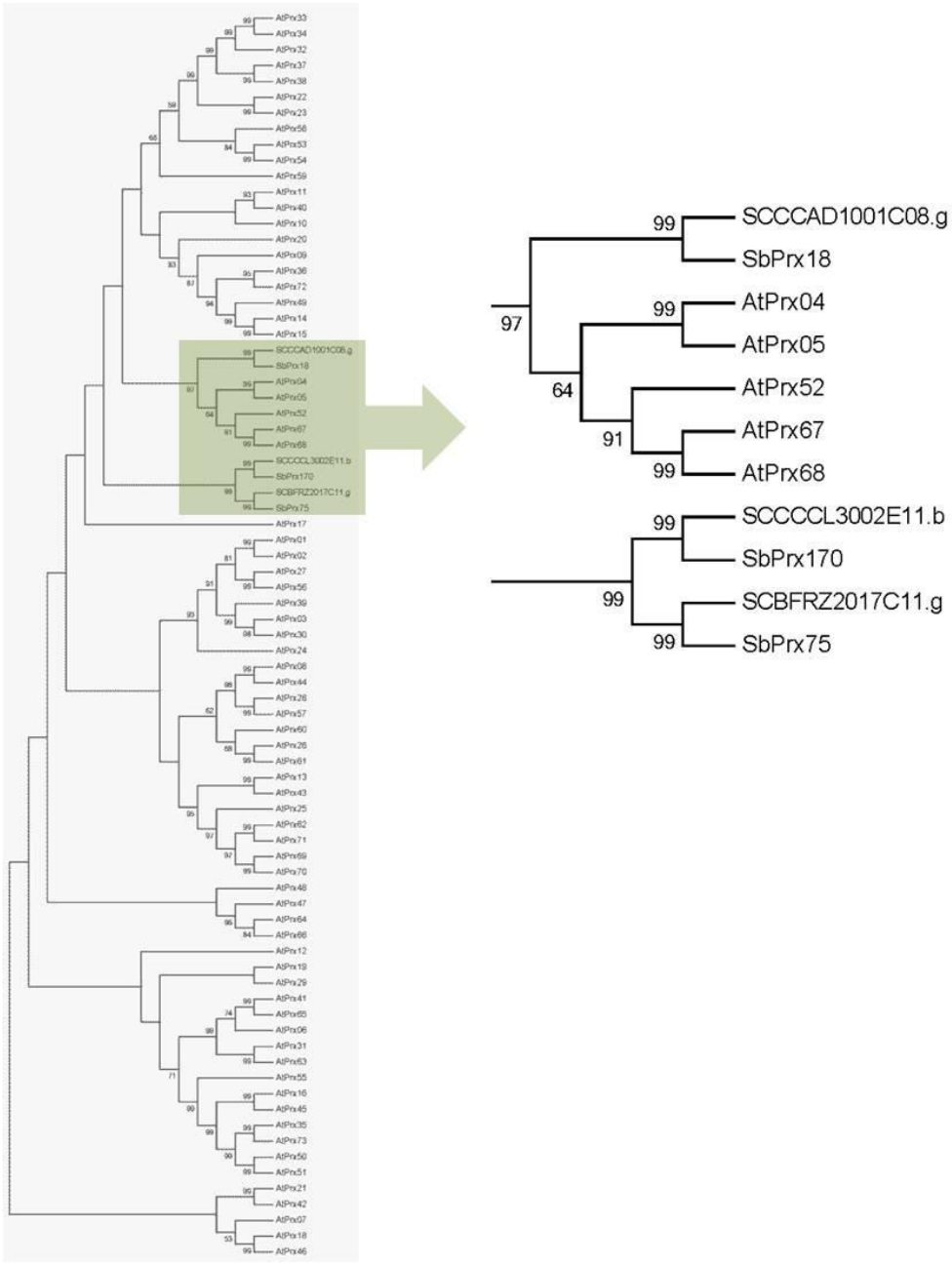


Figure 7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SCBFRZ2017C11.g
SbPrx75

Capítulo II

Suspension cell culture as a tool for the characterization of class III peroxidases in sugarcane

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Abstract

Secreted class III peroxidases are implicated in a broad range of physiological processes throughout the plant life cycle. However, the unambiguously determination of the precise biological role of an individual class III peroxidase isoenzyme is still a hard task due to genetic redundancy and broad substrate specificity in vitro. In addition, many difficulties are encountered during extraction and analysis of cell wall proteins. Since class III peroxidases are also secreted into the apoplast, the use of suspension cell cultures can facilitate isolation and functional characterization of individual isoforms. Here, we report on the characterization of class III peroxidases secreted in the spent medium of sugarcane suspension cell cultures. After treatment with specific inducers of cell wall lignification, peroxidases were isolated and activities assayed with guaiacol, syringaldazine and coniferyl alcohol. Enzymatic activity was not significantly different after treatments, regardless the substrate, with the exception of methyl-jasmonate, which led to a decreased guaiacol peroxidase activity. Remarkably, peroxidases isolated from the medium were capable to oxidize syringaldazine, an analog to sinapyl alcohol, suggesting that sugarcane cultures can produce peroxidases putatively correlated to lignification process. A proteomic approach using activity staining of 2-DE gels revealed a complex isoperoxidase profile, composed predominantly of cationic isoforms. Individual spots were excised and analyzed by LC-ESI-Q-TOF and homology-based search against the Sugarcane EST Database resulted in the identification of several proteins. Spatio-temporal expression pattern of selected genes was determined for validation of identified class III peroxidases that were preferentially expressed during sugarcane stem development.

Keywords: suspension cell cultures, class III peroxidases, lignin, proteomics, gene expression analysis

Introduction

The non-animal peroxidase superfamily is divided into three classes of peroxidases: class I, II and III. Class I peroxidases are intracellular non-glycosylated proteins found in plants, fungi and prokaryotes, that do not contain disulfide bridges or calcium ions (1). The family is divided into cytochrome *c* peroxidases (EC 1.11.1.5), catalase peroxidases (EC 1.11.1.6) and ascorbate peroxidases (EC 1.11.1.11), and their main cellular function is the scavenging of the excess H_2O_2 (2). Class II peroxidases are fungi-exclusive glycosylated proteins containing calcium ions, disulfide bridges and a signal peptide for protein secretion. The family consists of the lignin peroxidases (EC 1.11.1.14) and the manganese peroxidases (EC 1.11.1.13) and their major role is the degradation of soil debris, since these enzymes are the only peroxidases able to degrade plant-derived lignin (3). Class III peroxidases (EC 1.11.1.7) are present as large multigenic families in all land plants but absent from unicellular green algae (4). Like class II peroxidases, they are glycosylated proteins that also contain calcium ions and four or five disulfide bridges. They also possess a signal peptide for the secretion across the endoplasmic reticulum, while a C-terminal extension may be present in some peroxidases for vacuole targeting (1).

Class III peroxidases play a role in two distinct catalytic cycles. In the peroxidative cycle, peroxidases take electrons from a variety of donor molecules, such as phenolic compounds, to catalyze the reduction of H_2O_2 (5). A separated hydroxylic cycle has also been described, which leads to the formation of various reactive oxygen species (6). As a consequence of the large number of genes and protein isoforms and the two catalytic cycles, class III peroxidases are implicated in a broad range of physiological processes such as plant defense reactions, cell elongation, reactive oxygen species production and lignin and suberin formation (3). Their implication in such a number of physiological processes makes

peroxidase activity very easy to be detected throughout the plant life cycle, from germination to senescence, and under different conditions such biotic and abiotic stresses (3, 5, 7). However, to assign a precise role for an individual peroxidase gene is still a major bottleneck. Two difficulties are inherent in peroxidases: (i) generation of transgenic plants results in no visible mutant phenotype due to gene redundancy, and (ii) broad substrate specificity *in vitro*, which cannot offer precise information about their real substrates (8). Therefore, the determination of spatio-temporal gene expression pattern as well as protein isoform localization is recognized as an important step for functional studies concerning class III peroxidases. The combination of several approaches may be the only way to unambiguously gain insight into the precise biological role of an individual class III peroxidase isoenzyme.

Plant cell wall proteins (CWPs), such as secreted class III peroxidases, are responsible for approximately 10% of the cell wall weight (9). Depending on their interactions with cell wall components, CWPs can be divided into three groups: loosely bound proteins, with few or no interactions with cell wall components; weakly bound proteins, presenting hydrophobic or ionic interactions with the matrix; and strongly bound proteins, which are cross-linked with cell wall components by covalent bonds (10). Due to these possible interactions, many technical difficulties are encountered during extraction and analysis of cell wall proteins. Moreover, accessibility and availability of CWPs from specific tissues in a living plant are not always straightforward, making the analysis experimentally limited (11). Hence, tissue culture systems provide a convenient and attractive source of cell wall proteins. Culture medium can be considered as a large apoplastic space that contains proteins involved in all the particular aspects of cell wall development and it is easily isolated for further analysis (12). Indeed, several studies have reported the proteomic analysis of the medium of suspension cell cultures of a number of plant species like *Arabidopsis thaliana* (13), *Cycas revoluta* (14) and *Zinnia*

elegans (15). An additional advantage of plant cell cultures is that they allow studies to be performed under controlled conditions, and can also be used for studies other than proteomics, such as gene expression and natural product biosynthesis analyses.

Since class III peroxidases are secreted into the apoplast, where they can be soluble and freely move in the intercellular space or ionically or covalently bound to the cell wall, the use of suspension cell cultures can facilitate isolation and functional characterization of individual isoforms. For such a purpose, proteomic approaches can be an attractive option due to specific features of class III peroxidases such as thermal stability and retention of enzymatic activity after separation on SDS-PAGE (16) and two dimensional electrophoresis (2-DE) (17). Activity staining of peroxidases using artificial phenolic substrates such as guaiacol and *o*-dianisidine can be performed to dramatically reduce the complexity of 2-DE protein profiles. Moreover, since gene expression does not always reflect protein abundance and activity, proteomic approaches working directly on target proteins could be an interesting alternative for functional studies of class III peroxidases.

Here, we report on the characterization of class III peroxidases secreted in the spent medium of sugarcane suspension cell cultures. After treatment with specific inducers of cell wall lignification, peroxidases were isolated and their enzymatic activities were determined with the substrates guaiacol, syringaldazine and coniferyl alcohol. A proteomic approach using activity staining of 2-DE gels unveiled the isoperoxidase profile secreted by the cells and individual spots were excised and analyzed by LC-ESI-Q-TOF. Homology-based search against the Sugarcane EST Database SUCEST led to the identification of several spots and bioinformatic approaches in addition to qPCR were used for validation of identified class III peroxidases which were preferentially expressed in sugarcane stem.

Material and Methods

Suspension cell cultures, treatments and protein fractions

Transversal sections of meristematic stem tissue from 6-month old of sugarcane cultivar IACSP2008 were surface-sterilized in a 70 % (v/v) ethanol for 1 min followed by 7 % (w/v) sodium hypochlorite and 0.1 % (v/v) Triton-100 for 10 min. After sequential washes with sterilized distilled water, the sections were placed on Murashige and Skoog (MS) Basal Medium with Vitamins (M519, PhytoTechnology, USA) supplemented with 3 % (w/v) sucrose, 3 mg l^{-1} 2,4-dichlorophenoxiacetic acid (2,4-D) and 7 g l^{-1} agar and grown in the dark at 25 ºC. Cells derived from friable calluses were sub-cultured every 2 weeks in 30 ml (1:1, v/v) of fresh culture medium and grown in the dark on a rotatory shaker (130 rpm) at 25 ºC. Suspension cells treatments were performed according to a list of inducers of tracheary element differentiation or extracellular lignin formation (12). For treatment with 6-benzylaminopurine (BAP), cultures were diluted to 60 ml in fresh MS medium to a final concentration of 3 % (w/v) sucrose, 1.5 mg l^{-1} 2,4-D and 3 mg l^{-1} BAP. For methyl-jasmonate (MeJA) treatment, cultures were diluted to 60 ml in fresh MS medium containing 3 % (w/v) sucrose and 3 mg l^{-1} 2,4-D and MeJA (95 %, Aldrich, USA) was added to a final concentration of 73 μM. For treatment with brassinolide/boric acid, cultures were diluted to 60 ml in fresh MS medium to a final concentration of 3 % (w/v) sucrose, 3 mg l^{-1} 2,4-D and 10 mM boric acid and epibrassinolide was added to a final concentration of 1 μM. In addition, cells were also exposed to light from fluorescent lamps (photosynthetic active radiation was approximately 80 μmol photons m^{-2} s^{-1}). All treatments were performed in triplicates and the flasks were maintained for 3 days in the dark (except for the light treatment) at 25 ºC on a rotatory shaker (130 rpm).

The cells were separated from the culture medium by filtration through paper filter and the medium was saved as the soluble protein fraction. Fresh cells were transferred into 50 mM Tris-HCl buffer, pH 7.5 supplemented with 1 M NaCl and incubated at 4 ºC with shaking (130 rpm) for 1 h to extract ionically bound peroxidases. After another filtration through paper filter, both protein fractions were pooled and centrifuged for 30 min at 20000 x *g* and 4 ºC. Ammonium sulfate was added to supernatant to a final concentration of 80 % (w/v) to precipitate proteins and the solution was incubated on ice for 30 min, followed by centrifugation (20000 x *g*, 4º C, 30 min). Protein pellet was solubilized in 20 mM Tris-HCl buffer, pH 7.5 and passed through PD10 desalting columns (Sephadex G-25, GE Healthcare). Aliquots of 1 ml were lyophilized overnight and stored at -70 ºC until use.

***In vitro* activity assays**

Lyophilized protein samples were solubilized in 5 M urea and 2 % CHAPS for enzyme assays and electrophoresis. The protein concentration was determined using Qubit® Protein Assay (Invitrogen). Guaiacol peroxidase (GPrx), syringaldazine peroxidase (SyrPrx) and coniferyl alcohol peroxidase activities were determined according to Cesarino et al. (18). Peroxidase activity was expressed as µmol substrate oxidized min^{-1} μg^{-1} total protein. Student's t test was used to compare the means of replicates.

Lignin determination

Lignin content was determined by thioglycolic acid (TGA) method, as previously described (19). Triplicates of cell samples were separated from the culture medium by filtration through paper filter and freeze-dried. Lignin content was expressed as µg lignin mg-1 dry weight.

Mono- and bidimensional electrophoresis

Mono-dimensional electrophoresis (1DE) was performed in two distinct systems. First, 100 µg of protein was loaded on 0.8 % (w/v) agarose gels (Mini-Sub Cell GT system, BioRad) using TAE (Tris-acetate EDTA) buffer pH 8.0. The comb was placed in the middle of the gel to allow separation of cationic and anionic proteins. Activity staining of class III peroxidases was carried out with 1% guaiacol and 0.0 3% H_2O_2 in 50 mM Na-acetate pH 5.0. Second, 80 µg of protein was separated in semi-denaturing PAGE using 10 % gels, at 4 °C. Despite the fact that SDS was used in gel and buffer preparation, no reducing agents or SDS were used in sample buffer and proteins were not boiled. Peroxidase activity was revealed by staining with guaiacol, as described above. Pre-stained molecular marker (Rainbow Full Range, GE Healthcare) was used to determine the apparent molecular mass of protein bands.

For the two-dimensional electrophoresis (2-DE), 200 µg of proteins were diluted to 125 µl with rehydration buffer (5 M urea, 2 % CHAPS, 0.8 % ampholytes, 0.006 % bromophenol blue), incubated at room temperature for 30 min and centrifuged at 10,000 x g for 5 min. Gel strips (Immobiline Dry Strip pH 3-10 NL 7 cm – GE Healthcare) were rehydrated overnight with protein solutions at room temperature. Isoelectric focusing (IEF) was carried out in an IPGphor system (GE Healthcare) with the current limited to 50 µA per strip until focusing reached total of 8 kVh, followed by equilibration in 50 mM Tris-HCl pH 8.8, 30 % glycerol, 2 % SDS, 0.006 % bromophenol blue for 10 min. Semi-denaturing PAGE on 10 % gels was performed at 4 °C and peroxidases were stained for activity. Pre-stained molecular marker (Rainbow Full Range, GE Healthcare) was used in the second dimension. The 2-DE separations were performed in triplicates.

Mass spectrometry analysis

Peptide sequencing with LC-ESI-Q-TOF after trypsin in-gel digestion was used for the identification of 2-DE. The spots were manually excised from gels and washed twice with 500 µl 50 % methanol, 2.5 % acetic acid for 2 h for destaining and removal of SDS. After dehydration with 200 µl 100 % acetonitrile (ACN), they were dried under vacuum. Proteins were reduced by adding 30 µl of 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 solution and incubation for 30 min, followed by alkylation with 30 µl of 50 mM iodoacetamide in 100 mM NH_4HCO_3 solution for 30 min. Consecutive washing and dehydration steps using 200 µl of 100 mM NH_4HCO_3 and 200 uL of 100 % ACN respectively were performed before drying under vaccum. The dried gel pieces were rehydrated with 30 µl of porcine trypsin (20 ng.μl^{-1} in 50 mM NH_4HCO_3, Promega) on ice for 30 min. After removal of excess of trypsin solution, gel spots were covered with 50 mM NH_4HCO_3 and digestion was carried out overnight at 37 °C. The peptides were extracted (i) by adding 10 µl of 5 % formic acid, incubating for 10 min at room temperature and transferring the solution to a new tube; and (ii) by adding 12 µl of 5 % formic acid, 50 % ACN and incubating for 10 min at room temperature. Fractions were pooled and concentrated to a final volume of 1 µl. Samples were injected into an analytic C18 RP-UPLC column (1.7 µm BEH 130, 100 µm x 100 mm, nanoAcquity UPLC, Waters) coupled to a nano-electrospray tandem mass spectrometry on a Q-TOF Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl min-1. The gradient was 0-50 % ACN in 0.1 % formic acid over 45 min. Sample desalting was performed using a trapping column Symmetry C18 (180 µm x 20 mm) at a flow rate of 20 µl min^{-1} over 1 min. The equipment was operated in MS positive mode, data continuum acquisition from m/z 100-2,000 Da at a scan rate of 1 sec and an interscan delay of 0.1 s.

MS/MS ion search was carried out with Mascot Distiller (version 2.3.2.0, 2009, Matrix Science, Boston, MA) and the following restrictions were used: (i) trypsin as digesting enzyme; (ii) oxidation of methionine as variable modifications; (iii) carbamidomethylation of cysteine as fixed modifications; (iv) mass values were monoisotopic; (v) protein mass was unrestricted; (vi) peptide mass tolerance was ± 0.1 Da; (vii) fragment mass tolerance was ± 0.1 Da; (viii) max missed cleavages was 1; (ix) significant threshold was $p < 0.05$. Homology-based searches were performed against Sugarcane EST Database (SUCEST, http://www.sucest-fun.org/) for the identification of sugarcane-specific peroxidase peptides.

Phylogenetic tree

Multiple amino acid sequence alignment and phylogenetic tree construction were carried out with MEGA5 software (20). Evolutionary relationships were inferred using Neighbor-joining method (21) with bootstrap test for 1000 replicates. Amino acid sequences were downloaded from PeroxiBase (http://peroxibase.toulouse.inra.fr/) and SUCEST databases.

RNA isolation, cDNA synthesis and quantitative RT-PCR

Stem material was harvest from three biological replicates of adult cultivar Sabura of sugarcane plants cultivated in the field, at the Centro de Cana of the Instituto Agronômico de Campinas (IAC), Ribeirão Preto, Brazil. Internodes at three developmental stages, young, developing and mature, were separated and cut on the longitudinal axis into circular pieces. These pieces were used to separate rind and pith samples, as following: an internal fragment of 1-2 cm in diameter corresponded to pith fraction, while the most external 0.3 cm corresponded to rind fraction. The material was immediately frozen in liquid N_2 and stored at – 80ºC until

use. Frozen stem fractions were ground into powder using a pre-cooled IKA® A11 mill and the resulting pulverized tissue was used for total RNA extraction (22). cDNA synthesis was performed with SuperScript III (Invitrogen) after treatment with TURBO DNAse-free (Ambion). Quantitative RT-PCR was carried out in an optical 96-well plate with an iCycler iQTM5 Multicolor Real-Time PCR Detection System (Bio-Rad) using QuantiFastTM SYBR® Green PCR Kit (Quiagen) to monitor dsDNA synthesis. Confirmation of amplicon specificity was based on the dissociation curve at the end of each run. Gene-specific primers were designed in a manner that at least one of the primers was present in the 5'-UTR or 3'-UTR sequence, to discriminate between members of the class III peroxidase gene family (for primer list, see Table 2). The expression stability of three house-keeping genes (ubiquitin, UBQ; tubulin, TUB; and glyceraldehyde 3-phosphate dehydrogenase, GAPDH) was compared in order to obtain an accurate internal normalization. Since GAPDH presented the highest stability (data not shown), transcript levels were standardized to transcripts of GAPDH as a reference gene. Each reaction was repeated three times and negative control was performed in absence of cDNA template. Relative quantification (RQ) was determined by a method derived from the 2-ΔΔCt method (23), using the formula: 2-ΔCt, where ΔCt = (Cttag - Ctref), Ct = threshold cycle, tag = tag gene, and ref = reference gene, where the expression of tagged genes was compared to the reference gene GAPDH.

Results

In vitro peroxidase activity after treatments

Suspension cells were treated with different compounds reported to induce phenylpropanoid biosynthesis, extracellular lignin formation or tracheary element differentiation (Fig. 1). Peroxidase activity was measured *in vitro* using the substrates guaiacol

(GPrx), syringaldazine (SyrPrx) and coniferyl alcohol (CAPrx). Enzymatic activity was not significantly different after treatments, regardless the substrate, with the exception of MeJA, which led to a decreased GPrx activity when compared with the control treatment (Fig. 1). Interestingly, peroxidase activity with syringaldazine and coniferyl alcohol was not affected by MeJA. Moreover, despite the treatments with brassinolide/H_3BO_3 and BAP led to a trend for increased peroxidase activity with all substrates tested, this higher activity was not statistically significant. Finally, this enzymatic assay showed that sugarcane suspension cells are able to produce and secrete class III peroxidases capable to oxidizing not only coniferyl alcohol but also syringaldazine, an analogue of sinapyl alcohol which also presents a syringyl moiety.

Treatments with secondary cell wall inducers led to no significant induction of peroxidase activity, especially peroxidase activity specifically related to the lignin monomer analog syringaldazine. In addition, lignin production in control cells was validated by thioglycolic acid (TGA) lignin quantification method (lignin content in cells was 3.17 $\mu g.mg^{-1}$ dry weight). Therefore, further analyses were carried out exclusively with control cells.

Mono- and bidimensional isoperoxidase profiles and protein identification

To gain insights into the cationic/anionic distribution of isoperoxidases in spent medium of sugarcane suspension cell culture, a modified 1-DE in 0.8 % agarose gels was performed. Although agarose is not a suitable matrix to separate proteins, our analysis clearly showed that cationic peroxidases (at pH 8.0) were found as predominant isoforms in the enzyme preparation (Fig. 2A). In addition, at least 9 different peroxidase isoforms could be resolved by semi-denaturing PAGE, ranging from 30 to 250 kDa in molecular mass (Fig. 2B). Strongest in-gel activity could be observed in a molecular mass range between 24 and 38 kDa

and above 76 kDa. However, since charge is still important for protein separation in semi-denaturing PAGE, high molecular mass peroxidase bands may reflect cationic isoforms that cannot migrate properly in the gel or even protein complexes. The 2-DE analysis showed that the number of peroxidase isoforms secreted by sugarcane suspension cells is much higher than suggested by 1-DE gels (Fig. 2C). The analysis also revealed spots with a wide range of molecular weight but with a clear predominance of basic isoforms (cationic, pI > 7.0), although several anionic peroxidases could also be observed, which is in agreement with the results obtained with agarose 1-DE.

Individual spots were excised, digested with trypsin and analyzed by mass spectrometry for further identification of sugarcane peroxidases. From 28 spots analyzed, 12 generated peptides that matched peroxidases contigs recorded in SUCEST database (Table 1). The majority of protein identifications had scores greater than or equal to two times the accepted significance threshold, although all other proteins whose score exceed the minimum accepted were positively reported. While some protein identifications were based on multiple peptide sequences (spots 1, 2, 3, 7, 8, 11 and 12), some others were based on single peptide match (spots 4, 5, 6, 9 and 10), but their scores were still higher than the accepted threshold. Two or more proteins were identified in the same spot in the case of spots 1, 2, 3, 7 and 11, while the other spots were related to a single identification. In addition, the same peroxidase contig was often identified in multiple spots, which reflects the generation of more than one isoform from a particular gene possibly by post-translational modifications.

Phylogenetic relationships and expression analysis

SUCEST virtual northern results were analyzed to select only the identified suspension culture contigs whose reads were present in stem cDNA libraries. This analysis showed that,

from 13 peroxidase contigs retrieved from homology-based search with Mascot, 4 contigs presented reads from stem cDNA libraries: SCMCSB1113A08.g, SCCCCL4012A01.g, SCCCST3005E08.g and SCCCAD1001C08.g. Therefore, phylogenetic and expression analyses were carried out exclusively with this selected contigs.

Phylogenetic relationships between Arabidopsis peroxidases and the selected identified sugarcane amino acid sequences were investigated using Neighbor-joining method (Fig. 3). The closest protein homologue hit of each sugarcane contig in local BLASTP from PeroxiBase database was also included in the analysis. With the exception of SCCCCL4012A01.g, all other sugarcane peroxidases clustered in the same sub-clade, together with Arabidopsis AtPrx04, AtPrx05, AtPrx52, AtPrx67 and AtPrx68, none of which has its function unambiguously identified. While SCCCST3005E08.g and SCCCAD1001C08.g seem to be product of a monocot-specific gene duplication event, SCMCSB1113A08.g clustered with Arabidopsis AtPrx52, in a clade supported by a significant bootstrap value. In addition, Arabidopsis seems to contain no clear co-orthologue to SCCCCL4012A01.g, since the clade containing this sugarcane protein is sister to a clade containing several Arabidopsis peroxidases.

Spatio-temporal expression pattern of selected peroxidase genes was evaluated during sugarcane stem development by RT-qPCR. Internodes at three developmental stages, young, developing and mature, divided in pith (inner tissue) and rind (outer tissue) were used for RNA isolation and cDNA synthesis. Despite the fact that all four sugarcane contigs presented reads retrieved from stem cDNA libraries, expression of SCCCCL4012A01.g was not observed in any stem preparation. The other three genes presented similar gene expression profiles over time but remarkably different regulation between pith and rind (Fig. 4). In all cases, relative gene expression was higher in young internodes, while transcript levels strongly

decrease in developing and mature internodes. SCCCST3005E08.g was the only gene whose expression in young tissues was higher in pith than rind samples, while in developing and mature tissues the opposite was observed (Fig. 4A). In the case of SCCCAD1001C08.g, the transcript levels were not statistically different between pith and rind samples, regardless of the developmental stage (Fig. 4B). Finally, SCMCSB1113A08.g presented the highest relative expression level among the investigated genes, and changes in expression level over time were different between pith and rind (Fig. 4C). In both cases the expression strongly decreases from young to developing tissues, but no significant changes in gene expression was observed between developing and mature rind, while a slight increase occurred between developing and mature pith. Additionally, SCMCSB1113A08.g transcript levels were always higher in rind than pith in all developmental stages.

Discussion

Here, we investigated enzymatic activity and proteomic profile of class III peroxidases secreted in the medium of sugarcane suspension cell culture. Cell cultures were treated with different inducers of extracellular lignin formation, tracheary element differentiation or phenylpropanoid biosynthesis, since peroxidase involvement in lignin polymerization during secondary wall formation is one of the most extensively studied functions of peroxidases (24). In addition, the identification of isoperoxidases involved in lignin polymerization in bioenergy crops such as sugarcane is important since the presence of lignin increases the recalcitrance of plant cell walls and negatively affects the utilization of plant biomass as a source for biofuels and bio-based materials (25). From all treatments selected in literature known to induce lignification of tracheary elements (12), only MeJA affected peroxidase activity by exclusively decreasing guaiacol peroxidase activity. Exposure of maize seedlings to methyl-jasmonate

resulted in a reduction of specific guaiacol activity of plasma membrane-bound class III peroxidases (26). On the other hand, MeJA application in cultured Arabidopsis cells increased the monolignol biosynthesis and led to an up-regulation of peroxidase transcripts (27). Despite the fact that application of cytokinin (BAP) or brassinolide/H_3BO_3 to sugarcane suspension cells led to a trend for increased peroxidase activity with all substrates tested, this higher activity was not statistically significant. Auxins, cytokinins and brassinosteroids are the main hormones involved in tracheary element differentiation (28). Gutierrez et al. (29) showed differential responsiveness of lignin peroxidase ZePrx from *Z. elegans* to hormone treatments. Auxin and cytokinin induce ZePrx activity and secondary growth in seedlings, while brassinosteroids had an inhibitory effect on both ZePrx activity and secondary growth. Finally, although light exposure is known to induce the expression of genes involved in the first steps in the phenylpropanoid pathway, such as phenylalanine ammonia-lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*) and 4-coumarate:CoA ligase (*4CL*) (30), cultured cells exposed to light did not present any significant changes in peroxidase activity. Reports in the literature showed that the effect of light irradiance in peroxidase expression and activity varies significantly. Mungbean seedlings exposed to light showed higher levels of lignin content and increased anionic peroxidase activity (31), while additional peroxidase isoforms were expressed in seedlings of *Ebenus cretica* grown in dark conditions (32).

Since none of the treatments used previously led to an increased syringaldazine peroxidase activity, lignin content was determined for validation of an active lignin biosynthesis pathway in control suspension cell walls. Although low lignin content was found in these cell walls, it was already reported that lignin levels in suspension cell walls are about 20-30% of those found in plant tissues (33). Therefore, the lignin levels measured in sugarcane suspension cells (average 3.17 $\mu g.mg^{-1}$ dry weight) are in total agreement with expected, since

lignin content of young sugarcane internodes is around 15 μg.mg^{-1} dry weight (Bottcher, A., Cesarino, I., Mazzafera, P. et al., unpublished data). In addition, significant variation can be found when different lignin determination methods are employed (19).

Although no induction of peroxidase activity was observed after hormone or elicitor treatments, the enzymatic assay showed that peroxidases secreted by sugarcane suspension cells have the ability to oxidize syringaldazine, an analog of sinapyl alcohol. Such information is important since most of class III peroxidases cannot oxidize the doubly methoxylated sinapyl alcohol (see discussion below), while they can readily oxidize 4-hydroxyphenyl and guaiacyl moieties (i.e. *p*-coumaryl and coniferyl alcohol, respectively) (34). While oxidation of coniferyl alcohol is no proof for peroxidase involvement in monolignol dehydrogenation (35), oxidation of syringaldazine, which possesses a syringyl moiety, has been observed exclusively in lignifying tissues, suggesting that lignin polymerization is correlated to SYR-oxidizing capacity of peroxidases (36). Therefore, our data suggest that peroxidases isolated from the medium of sugarcane suspension cells can be used in studies involving *in vitro* polymerization of lignin (37) and kinetic and specificity of peroxidases (38).

Cationic peroxidases were found as dominant isoforms in the enzyme preparation from the medium of sugarcane suspension culture. Interestingly, the opposite was found in sugarcane stems, in which anionic isoforms were clearly dominant throughout the referred organ (18). Although no simple correlation has been established between pI and enzymatic function of class III peroxidases (2), in the specific case of peroxidases involved in lignin polymerization, typical anionic peroxidases are generally regarded as poor catalysts with sinapyl alcohol, while cationic peroxidases are universally accepted as enzymes with the ability to oxidize syringyl moieties (39). The oxidation of sinapyl alcohol by most anionic peroxidases is sterically hindered due to unfavourable hydrophobic interactions between the

atoms of the extra methoxy group from sinapyl alcohol and conserved amino acids residues at the substrate binding site of the enzyme (34). However, the relevance of syringyl peroxidases to radicalization of sinapyl alcohol has been questioned by recent studies suggesting a possible role for *p*-coumarate (pCA) as an oxidation shuttle, in which the readily oxidized pCA can transfer its oxidation state to an acceptor molecule such as sinapyl alcohol (38). If such a system operates within the lignifying cell wall matrix, sinapyl alcohol could be incorporated in lignin polymer without the presence of syringyl peroxidases.

The proteomic analysis of the medium of sugarcane suspension cell culture allowed the peptide sequencing and peroxidase identification of 12 out of 28 spots digested with trypsin. Database queries often yielded different peroxidase identifications for the same spot, which was already expected since peroxidases present conserved amino acid regions. Additionally, peptides that would permit isoforms differentiation were probably not sequenced. In addition, the same peroxidase could be identified in more than one spot and such identifications were often based on multiple peptide sequences, which reflect the presence of different isoforms from the same gene possibly generated by post-translational modifications. Gabaldón et al. (40) reported the characterization of co-translational and post-translational modifications of the major basic peroxidase (ZePrx) from *Z. elegans* suspension cell cultures. ZePrx is post-translationally modified by the formation of disulphide bridges, *N*-terminal pyroglutamate residues and N-glycosilation, which give rise to at least ten different isoforms, hampering the establishment of a reliable structure-function relationship. Glycosylation could be potentially involved not only with substrate specificity and protein folding and stabilization (2) but may well participate in protein/cell wall interactions (40).

Although the cell walls of plant cells cultured in suspension are comparable to the primary cell walls found in meristematic cells of living plants, gene expression and regulation

of metabolic pathways, such as the phenylpropanoid biosynthesis, may be different. Therefore, while a convenient and attractive source of cell wall proteins, cell cultures may not always be an appropriate model. Hence, to validate peroxidase identifications in the spent medium of sugarcane suspension cultures as possible candidates for peroxidases playing an important role in the development of sugarcane stem, phylogenetic and expression analyses of selected genes were performed. Only the contigs whose reads could be identified in stem cDNA libraries were used in further analysis, since the stem of sugarcane is the target tissue for genetic engineering to improve biomass processing. Neighbor-joining method was selected to unveil the phylogenetic relationships among all 73 Arabidopsis class III peroxidases and the peroxidases identified from sugarcane suspension cells. Although there is no clear correlation between amino acid sequence similarity, including intron/exon pattern, and physiological function of peroxidases, a phylogenetic analysis can be useful for the identification of putative orthologues and, as a consequence, to gain insights into the physiological function of unknown class III peroxidases. Phylogenetic analysis showed that *Z. elegans* peroxidase ZPO-C, suggested to be involved in lignification of tracheary element *in vivo* and *in vitro* (41), was most closely related to AtPrx66 and formed an independent group together with AtPrx47 and AtPrx64 (42). Further investigation suggested that these Arabidopsis peroxidases are also associated with lignification, with AtPrx66 and AtPrx47 being related to lignification of vessels, while AtPrx64 would be responsible for lignification of sclerenchyma (42). Our phylogenetic analysis revealed that, with the exception of SCMCSB1113A08.g, all other identified sugarcane peroxidases are products of gene duplication events within monocots and seem to have no clear co-orthologue in eudicots. Indeed, is believed that monocotyledonous plants evolved additional peroxidase clusters not found in dicotyledonous plants (43). SCMCSB1113A08.g and its closest homolog in *Sorghum bicolor* SbPrx152 are co-

orthologous to AtPrx52. Despite no study reported the functional characterization of this specific Arabidopsis peroxidase, microarray expression analysis of several groups revealed the putative involvement of this gene in cellular/physiological mechanisms such as ozone stress (44), insect defense (45), infection by *Pseudomonas syringae* (46) and, more interesting, cell wall modification during stamen abscission (47).

Determination of peroxidase expression profiles was used as a final step for selection of candidate genes with a role in cell wall modification during sugarcane stem development. The determination of timing and tissue specificity of peroxidase gene expression is considered an important step during functional characterization of individual peroxidase isoforms (3). Internodes at three developmental stages were separated in pith and rind fractions since previous work described the rind as a high metabolically active region for lignin deposition and peroxidase activity, while the pith is an important region for sucrose storage (18). This analysis revealed similar kinetics of expression during maturation of sugarcane stem for the selected peroxidase genes, but a remarkable difference in gene regulation between pith and rind preparations. With the exception of SCCCCL4012A01.g, whose expression was not detected in any stem preparation, all other studied peroxidase genes were preferentially expressed in young tissues. These findings are in agreement with the results found in maize, in which the majority of expressed phenylpropanoid genes had a maximum expression in young stems, with a decrease during following stages (48). Moreover, it was suggested that the young stems are an excellent indicator of lignification-associated gene expression in maize (49). Casu et al. (50) also found low expression of peroxidase genes in developing and mature internodes of sugarcane, with increased expression in immature internodes. Since secondary cell walls are already present in cells from young stems in sugarcane (50), it is possible that the expression of peroxidases takes place in early stages of stem development and, even with

subsequent down-regulation, peroxidase activity is maintained during the whole developmental process due to high stability of peroxidase proteins (18). Since the pith region of sugarcane stem is characterized by less lignified parenchyma cells, while the rind region contains predominantly fibers and vessels which are highly lignified (18), it is expected that a peroxidase gene putatively involved in stem lignification in sugarcane would also present preferential expression in rind samples. SCMCSB1113A08.g, the putative co-orthologue of Arabidopsis AtPrx52, not only showed higher relative expression throughout the whole stem but also presented preferential expression in rind fractions and thus can be considered a good candidate.

In conclusion, we showed that suspension cell culture is a convenient model system to study secreted class III peroxidases in sugarcane. Proteins isolated from spent medium were capable to oxidize not only coniferyl alcohol but also syringaldazine, an analog to sinapyl alcohol that also contains a syringyl moiety, suggesting that sugarcane culture can produce peroxidases correlated to lignification. Electrophoretic analysis revealed a complex isoperoxidase profile, composed predominantly of cationic isoforms. Several sugarcane peroxidases were identified from these profiles by mass spectrometry and their corresponding contig in SUCEST database were evaluated to contain reads belonging to stem cDNA libraries. Spatio-temporal expression pattern during stem development was determined for selected genes and showed that SCMCSB1113A08.g is a good candidate with a putative role in secondary cell wall formation in sugarcane stems.

Acknowledgments

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Figure Captions and Tables

Fig. 1. *In vitro* activity of guaiacol peroxidase - GPrx (A), syringaldazine peroxidase - SyrPrx (B) and coniferyl alcohol peroxidase - CaPrx (C) in protein fractions isolated from the medium of sugarcane cell cultures treated with different inducers of extracellular lignin biosynthesis or tracheary element differentiation. Only treatment with MeJA resulted in significant changes in peroxidase activity (*$p<0.05$, $n = 3$), exclusively when guaiacol was used as substrate. Vertical bars indicate standard error.

Fig. 2. Electrophoretic analysis of class III peroxidase present in the spent medium of sugarcane cell culture. Visualization of protein activity was performed by staining with guaiacol and H_2O_2. (A) Monodimensional electrophoresis in agarose gel showed that cationic isoforms were predominant in the protein fraction. (B) Several peroxidase isoforms could be resolved by semi-denaturing PAGE, ranging from 30 to 250 kDa in molecular mass. Strongest in-gel activity was observed in a molecular mass range between 24 and 38 kDa and above 76 kDa. Closed triangles correspond to estimated masses of protein bands. (C) Two-dimensional electrophoretic analysis revealed a much higher number of peroxidase isoforms secreted by sugarcane suspension cells than suggested by 1-DE gels and also showed a clear predominance of cationic isoforms.

Fig. 3. Neighbor-joining tree showing the phylogenetic relationships between Arabidopsis peroxidases and selected peroxidases identified from sugarcane suspension cell cultures. The closest protein homologue hit of each sugarcane contig in local BLASTP from PeroxiBase database was also included in the analysis. SCMCSB1113A08.g clustered with Arabidopsis AtPrx52, while the other sugarcane peroxidases seem to be product of a monocot-specific

gene duplication event. The values indicated next to the branches represent the percentage of replicate trees, in which the associated taxa clustered together in a bootstrap test (1,000 replicates), and are showed when over 50%.

Fig. 4. Expression pattern of selected peroxidase genes was evaluated during sugarcane stem development by RT-qPCR. The analysis was carried out in internodes at three developmental stages, divided in pith and rind fractions. The selected genes (A) SCCCST3005E08.g, (B) SCCCAD1001C08.g and (C) SCMCSB1113A08.g presented similar kinetics of gene expression but different regulation between pith and rind fractions. Vertical bars indicate standard errors. No detectable expression of SCCCCL4012A01.g was found in such stem preparations.

Table 1

Identification of sugarcane peroxidases isolated from the spent medium of suspension cell cultures. Peptides were identified using LC-ESI-Q-Tof and homology-based search against SUCEST database.

Spot	Tryptic Fragment	Mascot Score (Significant Level)	SUCEST	Closest Homolog	Organism
1		33 (>14)	SCMCSB1113A08.g	SbPrx103	*Sorghum bicolor*
	R.GFEVIDAAK.S	33 (>14)	SCCCCL4012A01.g	ZmPrx30	*Zea mays*
		33 (>14)	SCUTCL6037A04.g	ZmPrx110	*Zea mays*
	K.GLDVSDMVTLSGAHSIGR.S + Oxidation (M)	51 (>13)	SCCCCL4012A01.g	ZmPrx30	*Zea mays*
2	R.AFIEGMVK.M K.DALSNIGSLR.S R.VFDNQYFK.D R.SFEVVDEIK.E K.GGDEEVTGGLDATPR.V R.DGSDAFQK.D	109 (>13)	SCCCCL7037A10.g	SbPrx38	*Sorghum bicolor*
	K.SGQNPTVPNNAR.D K.NNPAEWNSDFAAAMAK.L R.DAADILSGGK.I	92 (>13)	SCRLAD1099B04.g	SofPrx15	*Saccharum officinarum*
	K.TPTDGTNTEK.K K.AMANNPSIGPALVR.L	74 (>13)	SCCCCL7C05F08.g	SofPrx15	*Saccharum officinarum*
	R.VGASIVR.L R.GFEVIDAAK.S	63 (>13)	SCMCSB1113A08.g	SbPrx103	*Sorghum bicolor*
3	R.GFEVIDAAK.A R.SHCSSFTR.D K.AELEAACPGK.V K.AELEAACPGK.V K.FGPPNFPSLR.G K.VSCADIVAFAAR.D K.GLDVSDMVTLSGAHSIGR.S + Oxidation (M)	272 (>13)	SCCCCL4012A01.g	ZmPrx30	*Zea mays*
		57 (>13)	SCCCLB1002D05.g	SbPrx90	*Sorghum bicolor*
	K.TAADGEIR.R	57 (>13)	SCJLRT1014B03.g	SbPrx90	*Sorghum bicolor*
		57 (>13)	SCRUFL1112E01.b	SbPrx90	*Sorghum bicolor*
	K.TAADGEIR.R R.TIVLYYTMG.-	57 (>13)	SCJLRT1014B03.g	SbPrx90	*Sorghum bicolor*
4	K.MGNISPLTGTAGQIR.A	17 (>12)	SCSGHR1069A02.g	SbPrx132	*Sorghum bicolor*
5	K.SGQNPTVPNNAR.D	21 (>13)	SCRLAD1099B04.g	SofPrx15	*Saccharum officinarum*
6	K.FGPPNFPSLR.G	21 (>13)	SCCCCL4012A01.g	ZmPrx30	*Zea mays*
7	K.FGPPNFPSLR.G	56 (>13)	SCCCCL4012A01.g	ZmPrx30	*Zea mays*
	R.TPMPPILTMAAAK.S + Oxidation (M)	17 (>13)	SCCCST3005E08.g	SbPrx23	*Sorghum bicolor*
8	R.GFEVIDAAK.A R.SHCSSFTR.D K.AELEAACPGK.V K.FGPPNFPSLR.G K.GLDVSDMVTLSGAHSIGR.S + Oxidation (M)	138 (>14)	SCCCCL4012A01.g	ZmPrx30	*Zea mays*
9	K.GLDVSDMVTLSGAHSIGR.S + Oxidation (M)	26 (>13)	SCCCCL4012A01.g	ZmPrx30	*Zea mays*
10	K.AAAAGEIR.R	32 (>13)	SCSGRT2063H01.g	SbPrx156	*Sorghum bicolor*
11		48 (>13)	SCMCSB1113A08.g	SbPrx103	*Sorghum bicolor*
	R.GFEVIDAAK.S	48 (>13)	SCCCCL4012A01.g	ZmPrx30	*Zea mays*
		48 (>13)	SCUTCL6037A04.g	ZmPrx110	*Zea mays*
12	R.TGASLLR.M R.FDVVDNIK.A R.RFDVVDNIK.A K.AQVEAVCPGVVSCADILAVAAR.D	138 (>14)	SCCCAD1001C08.g	SbPrx18	*Sorghum bicolor*

Table 2

List of primers used for RT-qPCR.

SUCEST Contig/Gene	Forward (5' to 3')	Reverse (5' to 3')	Amplicon size (bp)
SCCCCL4012A01.g	CAGGAGGAATTAAGCGACGA	GTAAACGGGTGGTGGTGGT	146
SCCCST3005E08.g	CGAGCGTTCAGGGATTTATT	CTTGGACTGGCGAAAGTGAT	101
SCCCAD1001C08.g	CACACAGCCAAAGAGCAGAA	CCAACAGGCTAAGGCAAGAA	118
SCMCSB1113A08.g	ACAATAACTTGGCGCCTTTG	GCTCCACCGTTGAAGAGC	121
GAPDH (SCCCCL3001G02.g)	TTGGTTTCCACTGACTTCGTT	CTGTAGCCCCACTCGTTGT	122

Figure 1

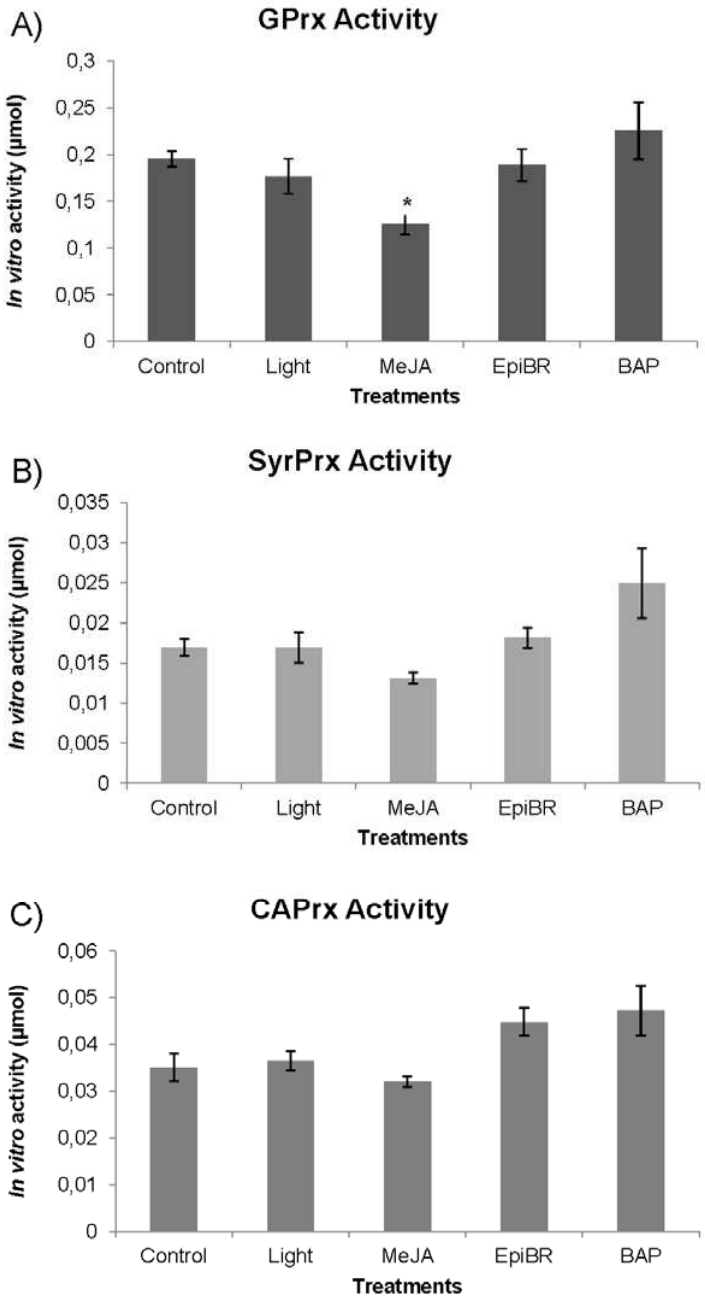

A)
GPrx Activity
In vitro activity (µmol)
0,3
0,25
0,2
0,15
0,1
0,05
0
*
Control
Light
MeJA
EpiBR
BAP
Treatments
B)
SyrPrx Activity
In vitro activity (µmol)
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
Control
Light
MeJA
EpiBR
BAP
Treatments
C)
CAPrx Activity
In vitro activity (µmol)
0,06
0,05
0,04
0,03
0,02
0,01
0
Control
Light
MeJA
EpiBR
BAP
Treatments

Figure 2

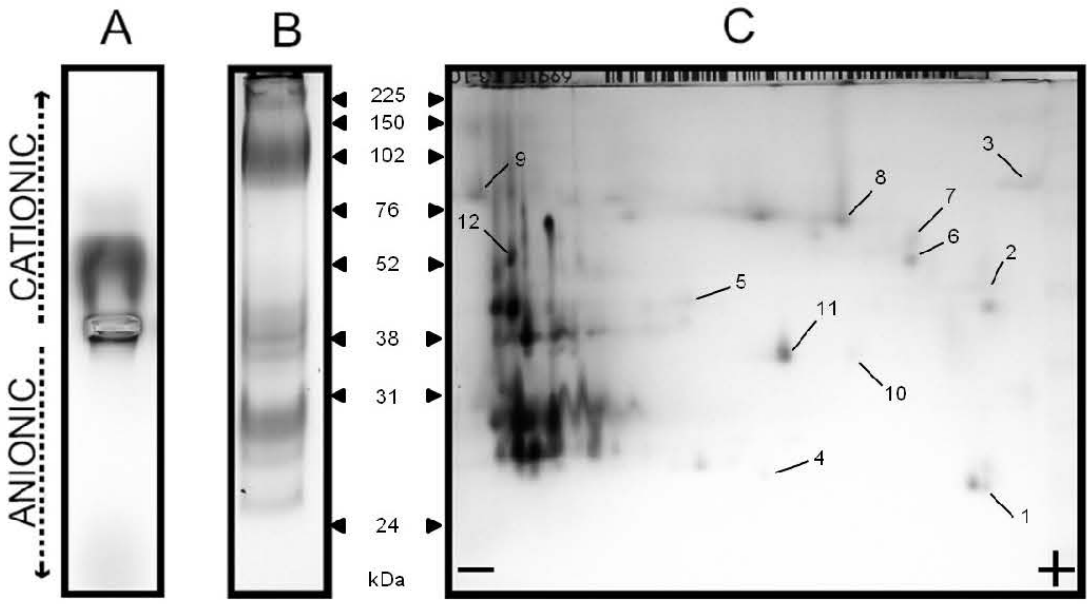

A
B
C
ANIONIC
CATIONIC
225
150
102
76
52
38
31
24
kDa
1
2
3
4
5
6
7
8
9
10
11
12

Figure 3

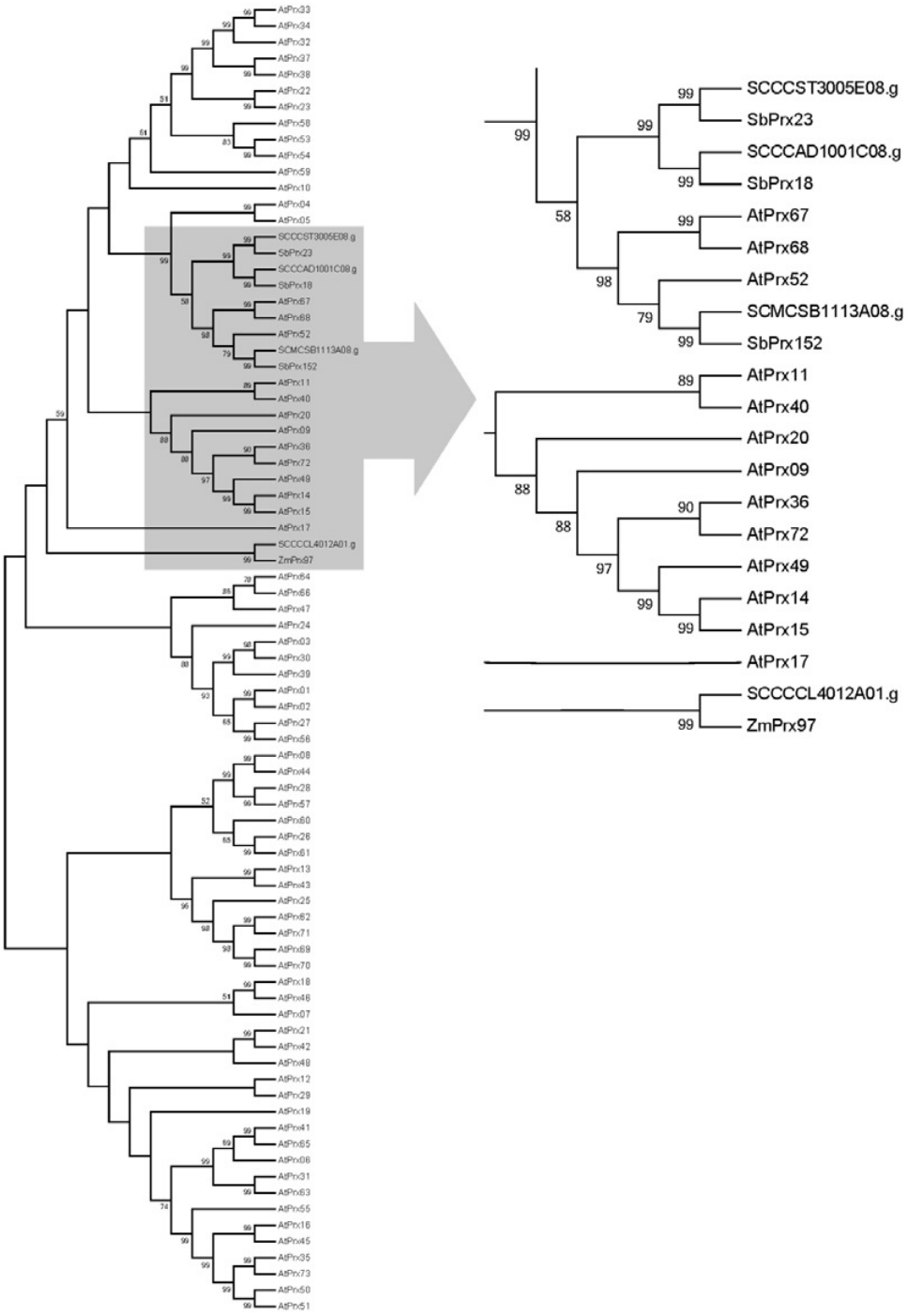
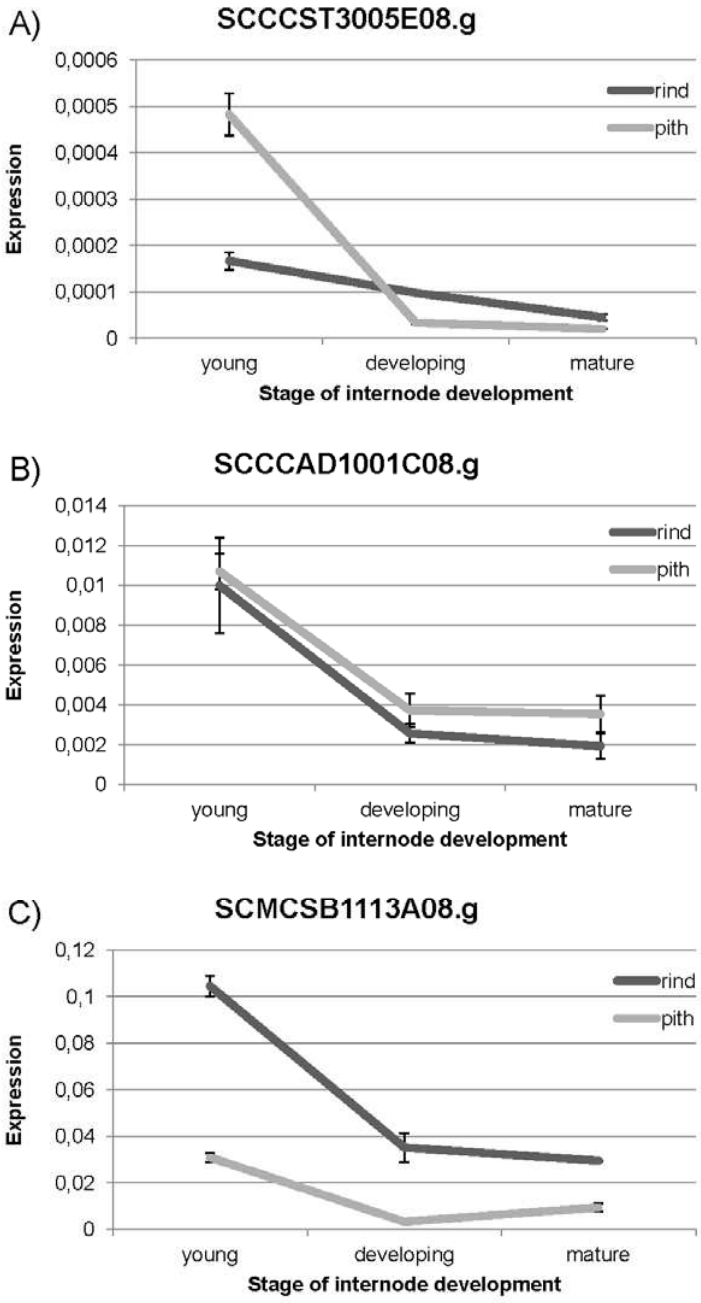

SCCCST3005E08.g
SbPrx23
SCCCAD1001C08.g
SbPrx18
AtPrx67
AtPrx68
AtPrx52
SCMCSB1113A08.g
SbPrx152
AtPrx11
AtPrx40
AtPrx20
AtPrx09
AtPrx36
AtPrx72
AtPrx49
AtPrx14
AtPrx15
AtPrx17
SCCCCL4012A01.g
ZmPrx97

Figure 4


A)
SCCCST3005E08.g
0,0006
0,0005
0,0004
0,0003
0,0002
0,0001
0
Expression
rind
pith
young
developing
mature
Stage of internode development
B)
SCCCAD1001C08.g
0,014
0,012
0,01
0,008
0,006
0,004
0,002
0
Expression
rind
pith
young
developing
mature
Stage of internode development
C)
SCMCSB1113A08.g
0,12
0,1
0,08
0,06
0,04
0,02
0
Expression
rind
pith
young
developing
mature
Stage of internode development

Capítulo III

Expression of *SofLAC*, a new laccase in sugarcane, restores the altered lignin profile of Arabidopsis *lac17* mutant

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Abstract

Lignin is a complex heteropolymer deposited in the secondarily thickened walls of specialized plant cells to provide strength and rigidity for plants to stand upright and hydrophobicity to conducting cells for long-distance water transport. Although lignin is essential for plant growth and development, this phenolic polymer is the major plant cell wall component responsible for biomass recalcitrance and its presence negatively affects the use of lignocellulose as a source for biofuels and bio-based materials. While the involvement of peroxidases in monolignol dehydrogenation has been clearly demonstrated, the contribution of plant laccases in this specific mechanism still needs further investigation. Here, we used a combination of co-expression analysis, tissue/cell type-specific expression analysis and a genetic complementation approach to correlate a *laccase* gene to the lignification process in sugarcane. A co-expression network constructed from 37 cDNA libraries showed that one sugarcane laccase gene, *SofLAC*, was coordinately expressed with some phenylpropanoid genes. Tissue-specific expression analysis by quantitative RT-PCR showed that *SofLAC* is preferentially expressed in young internodes and that expression levels decrease with stem maturity. Cell-type expression analysis by *in situ* hybridization demonstrated the localization of *SofLAC* mRNA in lignified cell types, mainly in inner and outer portions of sclerenchymatic bundle sheaths. For a conclusive demonstration of the involvement of SofLAC in the lignification process, the genetic complementation of Arabidopsis *lac17* mutant with *SofLAC* coding sequence was performed. *SofLAC* fully complements the altered lignin profile of *lac17* mutant. Therefore, we suggest that SofLAC plays a role in lignification of mainly sclerenchymatic sheats in early stages of sugarcane stem development.

Keywords: laccase, lignin, sugarcane, gene expression, genetic complementation

Introduction

Lignins are complex aromatic heteropolymers derived from the oxidative combinatorial coupling of mainly three *p*-hydroxycinnamyl alcohol monomers, *p*-coumaryl, coniferyl and sinapyl alcohols, differing in their degree of methoxylation (Boerjan *et al.*, 2003). After incorporation into lignin, these monolignols are called *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively (Raes *et al.*, 2003). These biopolymers are mainly deposited in the secondarily thickened cell walls of tracheary elements and fibers to provide strength and rigidity for plants to stand upright and hydrophobicity to conducting cells for long-distance water transport (Vanholme *et al.*, 2008; Weng and Chapple, 2010). Cell wall lignification, as well as the incorporation rate of each monomer, is regulated in a spatio-temporal fashion and varies between primary and secondary cell walls and among tissues (Grabber *et al.*, 2004). In addition, the deposition of lignin not only follows a developmental program but can also be triggered by a variety of stresses and environmental conditions (Moura *et al.*, 2010).

Monolignols are synthesized in the cytoplasm and transported to the cell wall, where they are oxidized by laccases and/or peroxidases prior to their incorporation into the polymer (Vanholme et al., 2008; Bonawitz and Chapple, 2010; Vanholme et al., 2010). However, studying the lignin polymerization process is still a major bottleneck, since (i) both types of enzymes show low substrate specificity *in vitro*, which cannot offer precise information about their real substrates *in planta*, and (ii) generation of transgenic plants often results in no visible phenotype due to gene redundancy (Ranocha et al., 1999; McCaig et al., 2005; Cai et al., 2006; Cosio and Dunand, 2009, 2010). Nevertheless, the involvement of peroxidases in lignin polymerization has been clearly demonstrated by a small number of studies reporting the generation of transgenic plants in which lignification was affected by the up- or down-

regulation of a particular peroxidase gene (Marjamaa *et al.*, 2009; Fagerstedt *et al.*, 2010). The down-regulation of a cationic peroxidase *NtPrx60* in tobacco resulted in up to 50% reduction in lignin content, with no visible differences in overall growth and development but with vascular tissue modification (Blee *et al.*, 2003). It has also been shown that over-expression of a cationic peroxidase led to ectopic lignification in transgenic tomato plants (El Mansouri *et al.*, 1999), while transgenic aspen lines down-regulated in an anionic peroxidase *PkPrx03* presented 20% less lignin compared to the wild-type (Li *et al.*, 2003). By contrast, laccases have been correlated to lignin biosynthesis mainly due to their ability to oxidize lignin precursors *in vitro* (Karkonen et al., 2002; Liang et al., 2006; Sato and Whetten, 2006) and their localization in lignin forming tissues in various plant species (Ranocha et al., 2002; Caparrós-Ruiz et al., 2006; Turlapati et al., 2011), but there is almost no genetic evidence on the role of laccases in lignin polymerization. Repression of laccase expression in transgenic poplar plants had no effect on overall growth and development and, although those lines exhibited an increase in total soluble phenolic content, neither lignin content nor composition was affected (Ranocha *et al.*, 2002). Arabidopsis *tt10* (*TRANSPARENT TESTA10*) mutants provided evidence for the role of AtLAC15 in the oxidative polymerization of flavonoids in the seed coat (Pourcel *et al.*, 2005). Mutant seeds displayed a 59% increase of soluble proanthocyanidin or condensed tannin and nearly 30% less extractable lignin compared to wild-type seeds (Liang et al., 2006). The overexpression of a cotton laccase in transgenic poplars (*Populus deltoides*) resulted in a slight increase of lignin content in stems (Wang *et al.*, 2008). Only recently, the role of laccases in lignification of stems was unambiguously demonstrated by a genetic engineering approach. By studying laccase T-DNA insertion mutants in *Arabidopsis thaliana*, Berthet *et al.* (2011) demonstrated that single mutants of *AtLAC4* and *AtLAC17* had moderately low lignin levels, whereas stems of double mutants

displayed up to 40% less lignin when compared to the control. Moreover, tissue-specific alterations were found, since *AtLAC17* is specifically involved in the deposition of G-units in fibers. Still, genetic evidence for the functions of laccases in plants is mostly lacking, especially for monocotyledonous plants.

Although lignin is essential for plant growth and development, the presence of this biopolymer negatively affects the use of lignocellulosic biomass as a source for biofuels and bio-based materials (Vanholme et al., 2010). The ability of lignin to resist degradation, mainly due to the chemically diverse and low reactive linkages and different monomer units, makes this phenolic polymer the major plant cell wall component responsible for biomass recalcitrance (Weng *et al.*, 2008). Therefore, the identification and characterization of genes responsible for lignin composition, polymerization and regulation is a fundamental goal to allow tailoring energy crops for industrial purposes. Here, we provide evidence for the role of a laccase gene (SofLAC) in lignin biosynthesis in sugarcane, the most important and productive bioenergy crop, by using a combination of co-expression analysis, tissue-specific expression analysis and genetic complementation of lower lignin Arabidopsis *lac17* mutant.

Material and Methods

Plant materials and growth conditions

All experiments were performed using plant material harvest from adult sugarcane plants of cultivar IACSP04-627 cultivated in the greenhouse of the Department of Plant Biology, Institute of Biology, State University of Campinas, Campinas, Brazil. In the case of genetic complementation of *lac17* mutant in *Arabidopsis thaliana*, all plants were from the Columbia background (Col-0) and cultures were performed in long-day conditions in a growth

chamber (21 °C, 60% relative humidity). The *lac17* mutant (S_016748) was obtained from the Salk Institute T-DNA insertion collection.

Analysis of SUCEST Database and bioinformatic analysis

Laccase genes were identified from the Brazilian Sugarcane EST Database (http://www.sucest-fun.org) by performing a local tblastn using laccase protein sequences of *Arabidopsis thaliana* (The Arabidopsis Information Resource; http://www.arabidopsis.org), *Populus trichocarpa* (Joint Genome Institute; http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.info.html), *Oryza sativa* (Phytozome; http://www.phytozome.net/rice.php) and *Sorghum bicolor* (Phytozome; http://www.phytozome.net/sorghum) as queries. The resulting sugarcane hits were then re-blasted against nr-NCBI database to confirm protein identity. Multiple amino acid sequence alignment and phylogenetic analysis were carried out with MEGA5 software (Tamura *et al.*, 2011). Evolutionary relationships were inferred using Neighbor-joining method (Saitou and Nei, 1987) with bootstrap test for 1000 replicates. The evolutionary distances were computed using the Dayhoff matrix-based method. Target sugarcane laccase gene (*SofLAC*) was selected based on phylogeny, co-expression analysis and data from literature (Casu *et al.*, 2007). Multiple amino acid sequence alignment figure was generated with ESPript 2.2 (Gouet *et al.*, 1999).

In silico co-expression network between SofLAC and lignin biosynthesis-related genes

For the co-expression network construction, a set of 37 cDNA libraries developed by the SUCEST project (Vettore et al., 2003) and originated from different sugarcane tissue types and developmental stages was used. For all pairs of SASs (Sugarcane Assembled Sequences)

the number of libraries in which each SAS is present was determined and the number of common libraries for the both SASs was computed, as described by Faccioli et al. (2005). To join two SAS by an edge in our network, the one-sided Fisher's exact test (p-value 0.05) was applied. The final co-expression network, with 50 nodes, was analyzed in the Medusa software (Hooper and Bork, 2005) using the affinity propagation clustering method.

RNA isolation, cDNA synthesis and quantitative RT-PCR

Roots, internodes at three developmental stages (young, internodes 1, 2 and 3; developing, internodes 5, 6 and 7; mature, internodes 15, 16 and 17) and leaves from three biological replicates were harvested and immediately frozen in liquid N_2, ground into fine powder using a pre-cooled IKA® A11 mill and stored at – 80 °C until use. Total RNA isolation was performed as described previously (Chang *et al.*, 1993), and first-strand cDNA was synthesized using SuperScript III (Invitrogen) after treatment with TURBO DNAse-free (Ambion). Quantitative RT-PCR was carried out using QuantiFastTM SYBR® Green PCR Kit (Quiagen) on an iCycler iQTM5 Multicolor Real-Time PCR Detection System (Bio-Rad). Sugarcane *SofLAC*-specific primers (list of primers used in this work is found in supplementary table S1) were designed in the 3'-untranslated (UTR) region to discriminate the target laccase gene from other members of the gene family. Transcript levels were standardized to transcripts of *GAPDH* as a reference gene. Relative quantification (RQ) was determined by a method derived from the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), using the formula: $2^{-\Delta Ct}$, where $\Delta Ct = (Ct_{tag} - Ct_{ref})$, Ct = threshold cycle, tag = tag gene, and ref = reference gene, where the expression of tagged genes was compared to the reference gene *GAPDH*.

Lignin histochemical staining

Stem material from young internodes (internode 3) was fixed in formalin/acetic acid/ethanol/water (5:5:60:30, v/v/v/v), embedded in Paraplast® X-tra (Fisher) and sections of 20 μm were obtained with a rotary microtome. Lignin deposition and composition were histochemically investigated by staining with Mäule reagent as described previously (Cesarino *et al.*, 2012).

In situ hybridization

The same pair of primers designed for RT-qPCR was used for the amplification of a 136 bp fragment from the 3'-UTR region of a *SofLAC* clone ordered from the Brazilian Clone Collection Center (BCCCenter, FCAV-UNESP, Campus Jaboticabal). The fragment was cloned into pGEM®-T Easy Vector (Promega, USA) using T4 DNA Ligase (Promega, USA) and sequenced to confirm identity and check insert orientation. Plasmid linearization was done with *Sal*I (Fermentas) and *Nco*I (Fermentas) and probes were synthesized by *in vitro* transcription with SP6 and T7 RNA polymerases using DIG RNA labeling kit (SP6/T7) (Roche Applied Science), according to manufacturer's protocol. Sense probe was used as negative control.

Stem material from internode 3 was fixed in 4% paraformaldehyde (w/v) in phosphate buffered saline (PBS) (130 mM NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4) for 16h at 4 °C, under vacuum, dehydrated in a graded ethanol series and stored at 4 °C until use. Ethanol was replaced by tertiary butyl alcohol (TBA) in a graded series (70, 85, 95 and 100% TBA), with samples maintained in each solution for 48h. Incubation with 100% TBA was repeated 3-times. Solid Paraplast® X-tra (Fisher) was added to samples in 100% TBA (3:1, w/v) and incubated at 58 °C to melt Paraplast® and evaporate TBA. After changing Paraplast® 3-times

(every 12h), samples were placed on moulds for solidification. Serial sections of 12 μm thick were cut on a rotary microtome (Leica®), placed on pre-cleaned and electrically charged slides (Probe-On Plus®, Fisher) and distended in a heated plate at 48 °C.

In situ hybridization protocol was performed according to Kidner and Timmermans (2006), with few modifications. Slides were immersed in xylene for removal of Paraplast®, subsequently washed in 100% ethanol and dried at room temperature. Slides were then incubated at 37 °C in 1 μg/mL Proteinase K solution (50 mM Tris-HCl pH 7.5) for 10 minutes, washed in DEPC-treated water for 10 minutes and further incubated for 10 minutes in 100 mM triethanolamine-HCl and 0,5% acetic anhydride (v/v). A hybridization solution containing 50% deionized formamide, 100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM EDTA, 1x Denhardt's solution and 10% dextran sulfate was used to dilute 400 ng/mL of DIG-labeled sense and antisense *SofLAC* probes. Hybridization was carried out overnight at 42 °C. Subsequently, slides were washed twice in 4x SSC (600 mM NaCl, 60 mM tri-sodium citrate dehydrate pH 7.0) followed by two washes in 2x SSC (300 mM NaCl, 30 mM tri-sodium citrate dehydrate pH 7.0), for 20 minutes at 42 °C each. The slides were rinsed with 100 mM Tris-HCl pH 7.5 at room temperature and then incubated with blocking solution (2% w/v blocking reagent, Roche, in 100 mM Tris-HCl pH 7.5) for 10 minutes at 37 °C. For staining, an anti-DIG-alkaline phosphatase (Roche) diluted in 100 mM Tris-HCl pH 7.5, 150 mM NaCl (1:1000) was added and samples incubated at 37 °C for an additional hour. Slides were washed twice with 100 mM Tris-HCl pH 7.5 and then incubated for 10 min in 10 mM Tris-HCl pH 9.0, 10 mM $MgCl_2.6H_2O$ at room temperature. For immunological detection, slides were maintained in a phosphatase substrate buffer (NBT/BCIP in 100 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$) at room temperature in the dark, overnight. The color reaction was stopped by washing with 10 mM Tris-HCl pH 8.0, 50 mM EDTA. Photomicrographs

were captured with an Olympus BX 51 photomicroscope equipped with an Olympus DP71 camera.

Complementation of Arabidopsis thaliana lac17 mutant

The full length open reading frame (ORF) of *SofLAC* was obtained from the Brazilian Clone Collection Center (BCCCenter, FCAV-UNESP, Campus Jaboticabal). This clone was used as template for introduction of *att*B1 and *att*B2 recombination sites by PCR, for further cloning into Gateway pDONR221 vector via BP clonase. *SofLAC*-specific primers were designed to also include the 3'-UTR. For *AtLAC17* promoter cloning, the region directly upstream of the *AtLAC17* (1997 bb; At5g60020) ORF was PCR amplified from genomic DNA using gene-specific primers containing the *att*B4 and *att*B1R recombination sites and BP cloned into pDONR-P4P1R. Sequence identity of all pENTR was confirmed by sequencing. The recombination of pENTR-pro*AtLAC17* and pENTR-SofLAC in the destination vector pK7m24GW-FAST was performed via MultiSite LR Clonase Plus, resulting in the expression clones proAtLAC17::SofLAC, which was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Positive colonies were confirmed by PCR and used for genetic transformation of Arabidopsis *lac17* mutant plants using floral dip method (Clough and Bent, 1998). The identification of transformed seeds (T1 line) and further identification of homozygous seeds were based on seed fluorescence (Shimada *et al.*, 2010).

Lignin analysis

For preparation of cell wall material, 5 mg of senescent inflorescence dry stems were sequentially washed with 500 µL water, ethanol, chloroform and acetone for 30 min each at 98, 76, 59 and 45 °C, respectively. Acetyl bromide lignin extraction was then performed as

described previously (Vanholme et al., 2010). Lignin analyses were performed with four independent transformed lines and four biological replicates each. Statistical analysis was performed using Student's *t* test ($p < 0.01$; $n = 4$).

Results

SofLAC is coordinately expressed with phenylpropanoid genes and is phylogenetically related to AtLAC17

tBlastn analysis of Sugarcane EST Database (SUCEST) based on the annotated Arabidopsis, rice, poplar and sorghum laccase genes resulted in the identification of 12 putative sugarcane *laccase* genes. A co-expression analysis using sugarcane cDNA libraries was used to further infer function and select candidate *laccase* genes putatively related to cell wall lignification. A total of 37 diverse cDNA libraries originated from different tissue types and developmental stages (Vettore *et al.*, 2003) were used for the construction of the network (Fig. 1). This analysis showed that one specific *laccase* gene, named *SofLAC*, (corresponding to two different tentative consensus number SCVPRZ3027A08.g and SCUTST3084C11.g) was transcriptionally linked with the putative phenylpropanoid genes *cinnamoyl-CoA reductase* (*CCR*; SCBFRT1064A01.g), *p-hydroxycinnamoyl-CoA:quinate shikimate p-hydroxycinnamoyltransferase* (*HCT*; SCUTRZ3072B08.g) and *caffeoyl-CoA O-methyltransferase* (*CCoAOMT*; SCJLRT2050C09.g). Moreover, several other putative phenylpropanoid-related genes may be found in close vicinity of *SofLAC*, including two *hydroxycinnamoyl/benzoyl transferase* (*HCT*; SCVPRT2082B07.g and SCCCCL4009E02.g), *caffeic acid O-methyltransferase* (*COMT*; SCJLRT1023B09.g), *phenylalanine ammonia-lyase* (*PAL*; SCJFLR1017B11.g) and *cinnamyl alcohol dehydrogenase* (*CAD*; SCEPRZ1011A02.g). In addition, *SofLAC* presented 99% identity to a sugarcane *laccase* gene previously shown to

be co-expressed with a *cellulose synthase* gene putatively involved in secondary cell wall synthesis (Casu *et al.*, 2007). Thus, *SofLAC* was the best candidate among all 12 identified laccase genes to play a role in secondary cell wall synthesis in sugarcane.

A phylogenetic analysis was carried out to gain insights into the evolutionary relationships between SofLAC and other plant laccases (Fig. 2). Previously characterized laccases from *Zea mays* (Caparrós-Ruiz et al., 2006; Liang et al., 2006), *Populus trichocarpa* (Ranocha *et al.*, 1999; Ranocha *et al.*, 2002) and *Gossypium arboretum* (Wang *et al.*, 2008), as well as the whole protein family from *Arabidopsis thaliana* and *Sorghum bicolor* were included in this analysis. Consistently with previous studies, laccases clustered into six subgroups (McCaig *et al.*, 2005; Caparrós-Ruiz *et al.*, 2006; Turlapati *et al.*, 2011). Sorghum and Arabidopsis laccases were identified in each phylogenetic group, except for subgroup 6, which is formed exclusively by the divergent AtLAC1. Although not true for all subgroups, monocot laccases usually clustered separately from dicot laccases. In agreement with previous results, there was no clear relationship between sequence and function, since laccases with similar putative functions clustered in different subgroups. SofLAC is clustered within the subgroup 1, together with ZmLAC2, ZmLAC4 and ZmLAC5 from maize, PtLAC110 from poplar and Arabidopsis AtLAC2 and AtLAC17, as well as several sorghum laccases. The closest protein to SofLAC was its co-orthologue in sorghum SORBIDRAFT_01g039690, which share an overall sequence identity of 95%, as already expected since sorghum is the closest relative species to sugarcane in the Andropogoneae tribe (Garsmeur *et al.*, 2011). Interestingly, the closest Arabidopsis homolog to SofLAC is AtLAC17 (overall sequence identity of 68%), which was shown to be specifically involved in the deposition of G-units in fibers in Arabidopsis stems (Berthet *et al.*, 2011).

The presumably full-length *SofLAC* coding sequence is 1722 bp long and the predicted protein has 574 amino acids, with a predicted molecular weight of 62.6 kDa and theoretical *p*I of 8.28. SofLAC has a putative N-terminal signal peptide predicted by SignalP program (Petersen *et al.*, 2011), suggesting that this protein is targeted to the cell wall via the typical secretory pathway. After cleavage, which is predicted to occur between amino acids 25 and 26, the mature peptide contained 548 amino acids, with a predicted molecular weight of 60 kDa and theoretical *p*I of 8.31, confirming the basic nature of this laccase. According to typical features of plant laccases (Fig. 3), SofLAC presents four highly conserved copper ligands, two N-terminal and two C-terminal, and previously reported strictly conserved motifs with unknown function such as proline-glycine-proline (PGP) motif, upstream the first copper-binding region, a TQCP motif downstream the first copper-binding region and the NPGxW motif in the C-terminal region (McCaig *et al.*, 2005).

SofLAC is preferentially expressed in sclerenchymatic and parenchymatic cells of young internodes

In order to investigate whether SofLAC could be involved in lignification of sugarcane stems, the tissue/development expression pattern of *SofLAC* was analyzed by quantitative RT-PCR (Fig. 4). Relative expression was determined in roots, internodes at three developmental stages (i.e young, developing and mature internodes) and leaves. Comparison among tissues showed that *SofLAC* is preferentially expressed in sugarcane stems, with lower expression levels in roots and especially in leaves. Analysis during sugarcane stem development showed higher expression of *SofLAC* in young internodes, where secondary cell walls are already present and deposition of lignin already started, while expression level decreases with maturity.

In order to localize the expression of *SofLAC* more precisely at the cell-type level, mRNA localization was performed by *in situ* hybridization. Thus, young internodes were the chosen tissue for this experiment since they correspond to the tissue with highest accumulation of *SofLAC* mRNA. In addition, histochemical staining with Mäule reagent was carried out to compare developmental lignin deposition to cell-specific *SofLAC* mRNA accumulation. Lignified cell types from internode 3 included the protoxylem and metaxylem elements, sclerenchyma sheath and, to a lesser extent, parenchymatic cells close to the periphery of the stem (Fig. 5A, C and E). Antisense *SofLAC* probe was strongly detected in inner and outer portions of the sclerenchymatic bundle (Fig. 5B, D and F). Signal was also detected in parenchymatic cells surrounding the vascular bundles (Fig 5G). No signal was detected when sense probe (control) was used (Fig. 5H). Therefore, the expression pattern and mRNA localization of *SofLAC* are in agreement with that expected for genes involved in the lignification process in sugarcane stems.

Sugarcane SofLAC can complement Arabidopsis lac17 mutant

For confirmation of the function of SofLAC in lignin biosynthesis, lower lignin content Arabidopsis *lac17* mutant was complemented with two different constructs (Fig. 6A). Complementation was assayed with *SofLAC* coding region including the 3' untranslated region under the control of the endogenous *AtLAC17* specific promoter. The 3'-UTR region was included during cloning strategy because its importance was proved for the effectiveness of complementation of Arabidopsis *lac17* mutant by *AtLAC17* itself as reported by Berthet et al. (2011). Several independent transformants were obtained, and the confirmation of *SofLAC* expression was performed by RT-PCR (Fig. 6B). Four independent transformants with high *SofLAC* expression were further analyzed.

Arabidopsis *lac17* mutant has 14% less Klason lignin than the corresponding control and altered S/G ratio, since *LAC17* deficiency affects G units deposition in interfascicular fibers (Berthet *et al.*, 2011). Therefore, the effectiveness of complementation was evaluated by subjecting dry stems to lignin quantification using acetylbromide method. In our conditions, *lac17* mutant presented 19% less lignin than the corresponding WT control (Fig. 6C). Expression of *SofLAC* in the *lac17* background resulted in the complete restoration of WT lignin levels in all four analyzed transformed lines (Fig. 6C). These results strongly indicate a role for SofLAC in lignin biosynthesis in sugarcane stems.

Discussion

Sugarcane is a C4 plant with high capacity to convert solar radiation into biomass and also with the ability to accumulate up to 50–60% of dry weight of stem as sucrose in the mature tissues (Casu *et al.*, 2004). The worldwide demand for sustainable energy production resulted in a significant interest in sugarcane in recent years, which was responsible for approximately 25 billion liters of ethanol in the 2009/2010 crop season only in Brazil (Cheavegatti-Gianotto *et al.*, 2011). Although the production of sugar-based ethanol, often referred to as first-generation ethanol, represents the most convenient and efficient option for sustainable energy in the case of sugarcane, sugarcane biofuel industry would also benefit from recent advances in processing lignocellulosic biomass, especially because sugarcane is among the most efficient biomass producers known (Vermerris, 2011). The so-called second generation ethanol is produced from the plant biomass composed of the polymers cellulose, hemicellulose and lignin (Yuan *et al.*, 2008). However, the recalcitrant nature of this mixture negatively affects the conversion of plant cell wall polysaccharides into fermentable sugars for biofuels (Vanholme et al., 2010). In this regard, the ability of lignin to resist degradation

makes this complex phenolic polymer the major plant cell wall component responsible for biomass recalcitrance (Weng *et al.*, 2008). Therefore, an enhanced understanding of gene expression, function and regulation during lignin biosynthesis is required in order to fully benefit from cellulosic biofuels potential.

Even though cellulosic biofuel crops may ultimately include dicotyledonous woody plants (e.g. eucalyptus and hybrid poplar) and monocotyledonous grasses, our current knowledge regarding lignin biosynthesis is mainly derived from studies in dicotyledonous herbaceous plants, such as *Arabidopsis thaliana* and alfalfa (Li *et al.*, 2008). Genetic information on lignin metabolism in sugarcane is scarce and limited to few studies reporting general expression profiling in which some phenylpropanoid genes were differentially expressed during sugarcane stem development or in relation to sucrose accumulation (Casu et al., 2004; Casu et al., 2007; Papini-Terzi et al., 2009). To our knowledge, no functional characterization of lignin biosynthetic genes was performed in sugarcane so far. Here, a combination of co-expression analysis, tissue- and cell-specific expression analyses and genetic complementation experiment were employed for functional characterization of a laccase protein potentially involved in lignin biosynthesis in sugarcane.

Co-expressed gene pairs, i.e. genes that are transcriptionally coordinated, have the potential to be involved in similar processes and to have similar function (Ruprecht and Persson, 2012). The expression of *SofLAC* was directly linked with the expression of phenylpropanoid genes *HCT*, *CCR* and *COMT*, while several other lignin-related genes, although not directly coordinated with *SofLAC*, were found in close vicinity and connected to the same nodes as *SofLAC*. In Arabidopsis, non-annotated genes whose expression was highly co-regulated with expression of *cellulose synthase* (*CesA*) genes implicated in secondary cell wall synthesis were analyzed genetically and their function in the same physiological process

was confirmed (Brown *et al.*, 2005; Persson *et al.*, 2005). In the same study (Persson *et al.*, 2005), *AtLAC4* and *AtLAC17* were among the most highly co-regulated genes for *CesA* genes involved in secondary wall synthesis, and their involvement in lignin polymerization in Arabidopsis stems was further demonstrated (Berthet *et al.*, 2011). Moreover, the comparison of co-expression networks of primary and secondary *CesA* genes from seven different plant species suggests that many components in these networks are conserved across species (Ruprecht *et al.*, 2011). Co-expression approaches have been considered a valuable tool for identifying new genes especially involved in secondary cell wall biosynthesis (Brown et al., 2005; Persson et al., 2005; Ruprecht et al., 2011; Ruprecht and Persson, 2012). In this regard, the transcriptional coordination of *SofLAC* with genes from the lignin biosynthesis pathway suggests a potential role of this particular laccase in lignin polymerization in sugarcane.

Similar to class III peroxidases, functional characterization of plant laccases has been a major challenge due to genetic redundancy among members of the multigene family, the diversity of physiological processes catalyzed by them and the broad substrate specificity *in vitro* (Ranocha et al., 2002; Cai et al., 2006; Liang et al., 2006). Transcriptome/expression approaches seem to offer a valuable option to overcome part of these difficulties (Cosio and Dunand, 2010). Tissue/cell-type specificity and timing of *laccase* gene expression may provide important information about putative function of a specific *laccase* gene. Accordingly, a *laccase* gene whose expression is activated during lignin deposition and is preferentially expressed in plant tissues that undergo lignification is likely to be involved in lignin polymerization in this specific tissue type. *SofLAC* mRNA preferentially accumulated in sclerenchymatic bundle sheaths of sugarcane young internodes. Despite the fact that lignification process is highly active in developing and mature internodes, secondary walls are already present in cells from young internodes, where lignin is deposited in protoxylem and

metaxylem elements and inner and outer portions of sclerenchymatic bundle sheats (Jacobsen et al., 1992; Cesarino et al., 2012). Casu et al. (2007) reported similar results for this specific *laccase* gene in sugarcane (i.e. higher expression in young stems), which was similar to the expression pattern of *ShCesA12*, a *cellulose synthase* gene putatively involved in secondary cell wall synthesis in sugarcane. Therefore, the expression pattern of *SofLAC* is in agreement with the hypothesis that laccases play a role in the early stages of lignification in living cells since they operate in the absence of toxic H_2O_2 (Sterjiades *et al.*, 1993). Interestingly, most expressed phenylpropanoid genes in maize had a maximum expression in young ear internodes, with lower levels during the following stages of development, while lignin content remarkably increased with maturity (Riboulet *et al.*, 2009). In another study, Guillaumie et al. (2008) reported that all genes known to be involved in constitutive monolignol biosynthesis in maize were also preferentially expressed in younger ear internodes. Finally, transcripts and proteins of AtLAC17, SofLAC closest homolog in Arabidopsis, could only be detected in interfascicular fibers but not in vessels (Berthet *et al.*, 2011), which resembles the cell type-specific localization of SofLAC expression. Altogether, the results from expression analyses suggest that SofLAC is involved in lignification of mainly sclerenchymatic sheaths during early stages of sugarcane stem development.

Although co-expression networks and expression analyses can be used to link a laccase gene to a specific mechanism, transgenic approaches are in principle more reliable for elucidation of gene function (Cai et al., 2006; Cosio and Dunand, 2009). However, genetic transformation of sugarcane has continued to be a major bottleneck. Sugarcane transformation has been mostly performed by particle bombardment of embryogenic calli, but transgene expression is generally switched off in mature plants due to both transcriptional and post-transcriptional gene silencing (Arruda, 2012). Expression instability in mature sugarcane

plants is attributed to gene silencing induced by integration of high copy number of transgenes, as well as to a high degree of ploidy of sugarcane genome (Manners and Casu, 2011; Arruda, 2012). Alternatively, *Agrobacterium*-mediated transformation has also been employed, but this approach is still limited by low transformation efficiency, high variability between experiments and genotype specificity (Anderson and Birch, 2012). Furthermore, regardless of the transformation protocol, tissue culture and plant regeneration are prerequisites for sugarcane genetic transformation, which are laborious and time-consuming. To overcome these limitations and to provide a conclusive genetic proof of the involvement of SofLAC in lignin biosynthesis, the genetic complementation of Arabidopsis *lac17* mutant was performed with the coding sequence of *SofLAC* under the control of *AtLAC17* promoter. Arabidopsis *lac17* mutant was selected because i) knockdown T-DNA line is available and presents visible phenotype (i.e. lower lignin content and altered S/G ratio); ii) AtLAC17 is the closest homolog to SofLAC in Arabidopsis; and iii) cell-type specificity of *SofLAC* expression seems to be more related to the one observed for *AtLAC17* in Arabidopsis stems. In addition, the 3' untranslated region of *SofLAC* cDNA was included in the cloning strategy since previous studies highlighted the importance of this region for effective complementation of *laccase* mutants. Complementation of Arabidopsis *lac17* mutant by expressing *AtLAC17* genomic sequence was only partial in the absence of its 3'-UTR (Berthet *et al.*, 2011), while the reversion to the wild-type phenotype in complemented *tt10* (*AtLAC15*) mutants was only observed when the 3'-UTR was included in the expression cassette (Pourcel *et al.*, 2005). It seems likely that the 3'-UTR is essential for the stabilization of gene transcript, avoiding the mRNA turnover by the mRNA decay machinery present in plant cells (Gutierrez *et al.*, 1999).

The expression of *SofLAC* under the control of the endogenous *AtLAC17* promoter in *lac17* background led to the complete recovery of the lignin levels from the WT. These results

were considered the definitive proof of the involvement of SofLAC in the constitutive lignification of sugarcane stems. Nevertheless, it is important to highlight that, due to the promiscuous nature of laccases, it seems likely that any laccase gene expressed under the control of *AtLAC17* promoter might be able to restore lignin levels of the WT. However, several other evidences support the hypothesis that SofLAC is involved in early lignification of sugarcane stems, as shown by co-expression and expression analyzes. In conclusion, here we report, for the first time to our knowledge, on the functional characterization of a lignin biosynthetic gene in sugarcane, and also provided additional confirmation of the involvement of laccases in the oxidative coupling of monolignols.

Supplementary Data

Supplementary Table S1. List of primers used in this work.

Primer name	Primer sequence (5'->3')	Use
SofLAC_REV	CAAGCCTCTTTAACCGTTGTGT	RT-qPCR
GAPDH_FWD	TTGGTTTCCACTGACTTCGTT	RT-qPCR
GAPDH_REV	CTGTAGCCCCACTCGTTGT	RT-qPCR
SofLAC_attB1	AAAAAGCAGGCTTCACCATGGGGGCGCCACCATGT	Cloning of SofLAC into Gateway system
SofLAC_attB2	AGAAAGCTGGGTCGAAGAATCTATGTGAAAGTAA	Cloning of SofLAC into Gateway system
proLAC17_FWD	ATAGAAAAGTTGAATTTTACTACGTAGTAATTGATATATAGTATC	Cloning of proAtLAC17 into Gateway system
proLAC17_REV	TGTACAAACTTGCGGTGAAGTGAGCTTGGACACGGA	Cloning of proAtLAC17 into Gateway system
SofLAC_RT_F	CGTCAAGGTGGTGAACAATG	RT-PCR
SofLAC_RT_R	TGAACGTGTCTTTGCTGGAG	RT-PCR
CBP20_FWD	GAGCATCTACAACGGTTTACATTGG	RT-PCR
CBP20_REV	TAGGCCGGTCATCGAGAATAGTC	RT-PCR

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Figure Captions

Figure 1. Co-expression gene network for *SofLAC*. Individual genes/contigs are represented by nodes, while edges indicate whether two genes are coordinately expressed above a certain threshold. Genes belonging to the same vicinity are indicated by the same color. *SofLAC* (yellow lozenges, contigs SCVPRZ3027A08.g and SCUTST3084C11.g) was directly linked with the putative phenylpropanoid genes *cinnamoyl-CoA reductase* (*CCR*; SCBFRT1064A01.g), *p-hydroxycinnamoyl-CoA:quinate shikimate p-hydroxycinnamoyltransferase* (*HCT*; SCUTRZ3072B08.g) and *caffeic acid O-methyltransferase* (*COMT*; SCJLRT2050C09.g).

Figure 2. Phylogenetic tree of SofLAC and other laccase proteins from *Arabidopsis thaliana*, *Sorghum bicolor*, *Zea mays*, *Populus trichocarpa* and *Gossypium arboretum*. Different colors indicate different laccase subgroups based on McCaig et al. (2005). SofLAC (black square) clustered in subgroup 1 together with AtLAC17 and other dicot and monocot laccases. The evolutionary relationships were inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 13.71005872 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Figure 3. Multiple amino acids sequence alignment of SofLAC and closely related laccases. Amino acid identity is highlighted in black shading box. Overall sequence identity between SofLAC and other proteins is 95% with Sb_01g039690, 68% with AtLAC17 and 66% with PtLAC110. Black triangle indicates the putative cleavage site, black bars correspond to strictly

conserved motifs with unknown function and black stars indicate typical conserved copper ligands.

Figure 4. Tissue-specific expression pattern of *SofLAC* determined by quantitative RT-PCR. Expression is higher in young internodes, while expression levels decrease with stem maturity. Values are the means of three measurements. Error bars indicate SE of three replicates.

Figure 5. Histochemical staining of lignin and localization of *SofLAC* mRNA by *in situ* hybridization in sugarcane young internodes. Developmental deposition of lignin occurred in the protoxylem and metaxylem elements, sclerenchyma sheath and, to a lesser extent, parenchymatic cells close to the periphery of the stem (A, C, E). Antisense probe showed SofLAC mRNA accumulation in lignified cell types, especially in schlerenchymatic budles (B, D, F). Signal was also observed in parenchymatical cells (G). Arrows indicate in situ hybridization signal. Sense probe was used as negative control (H).

Figure 6. Genetic complementation of Arabidopsis *lac17* mutant with sugarcane *SofLAC*. A) Schematic representation of the Gateway construct used for complementation of *lac17* mutant. B) RT-PCR analysis of *SofLAC* and *CBP20* transcript levels in WT, *lac17* and complemented lines (LAC1 to 4). C) Acetyl bromide lignin contents in dry stems of WT, *lac17* and complemented lines (LAC1 to 4). Error bars indicate SD of four replicates.

Figure 1

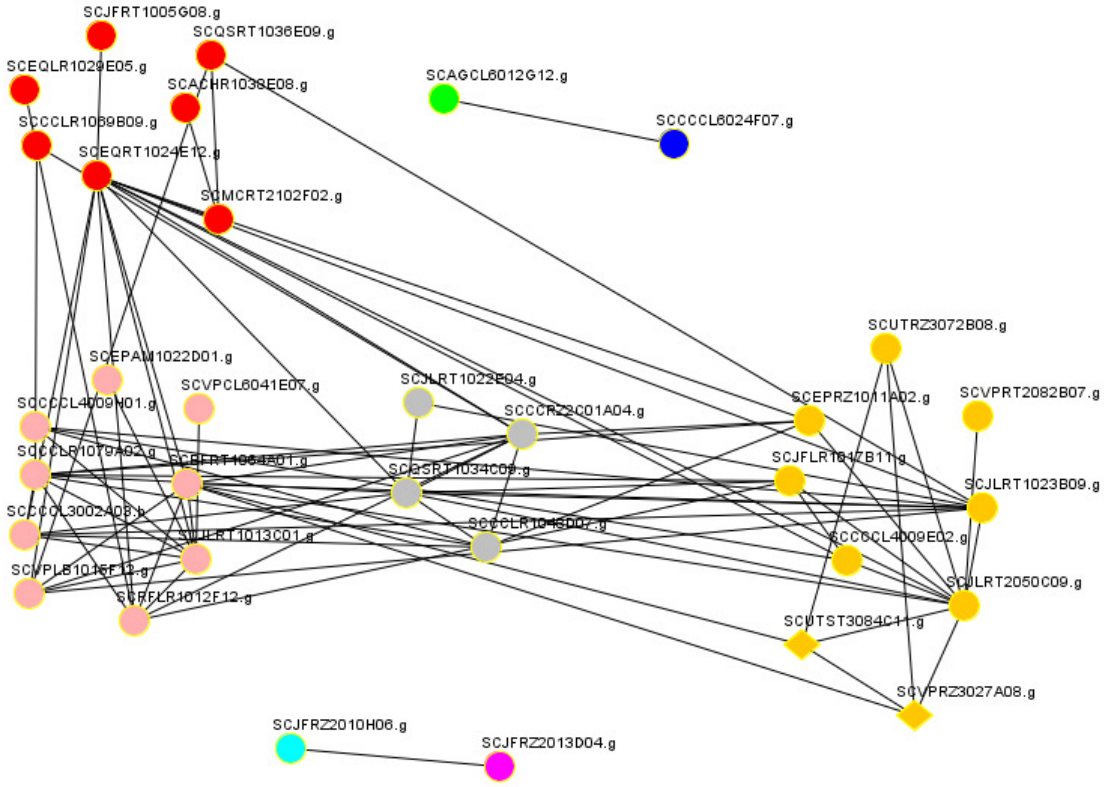
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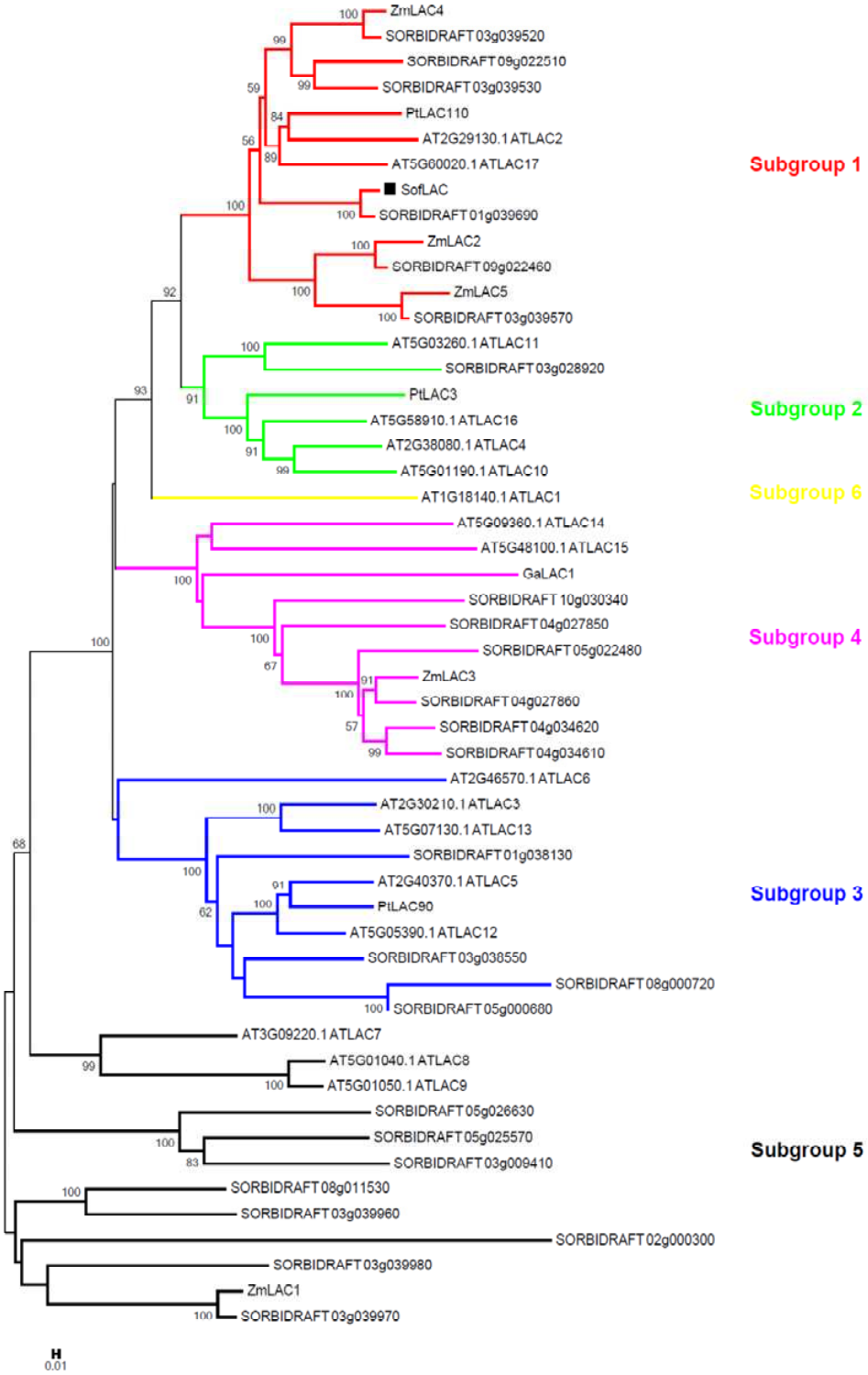
Figure 2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SofLAC
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ZmLAC2
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ZmLAC5
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0.01
Subgroup 1
Subgroup 2
Subgroup 6
Subgroup 4
Subgroup 3
Subgroup 5

Figure 3

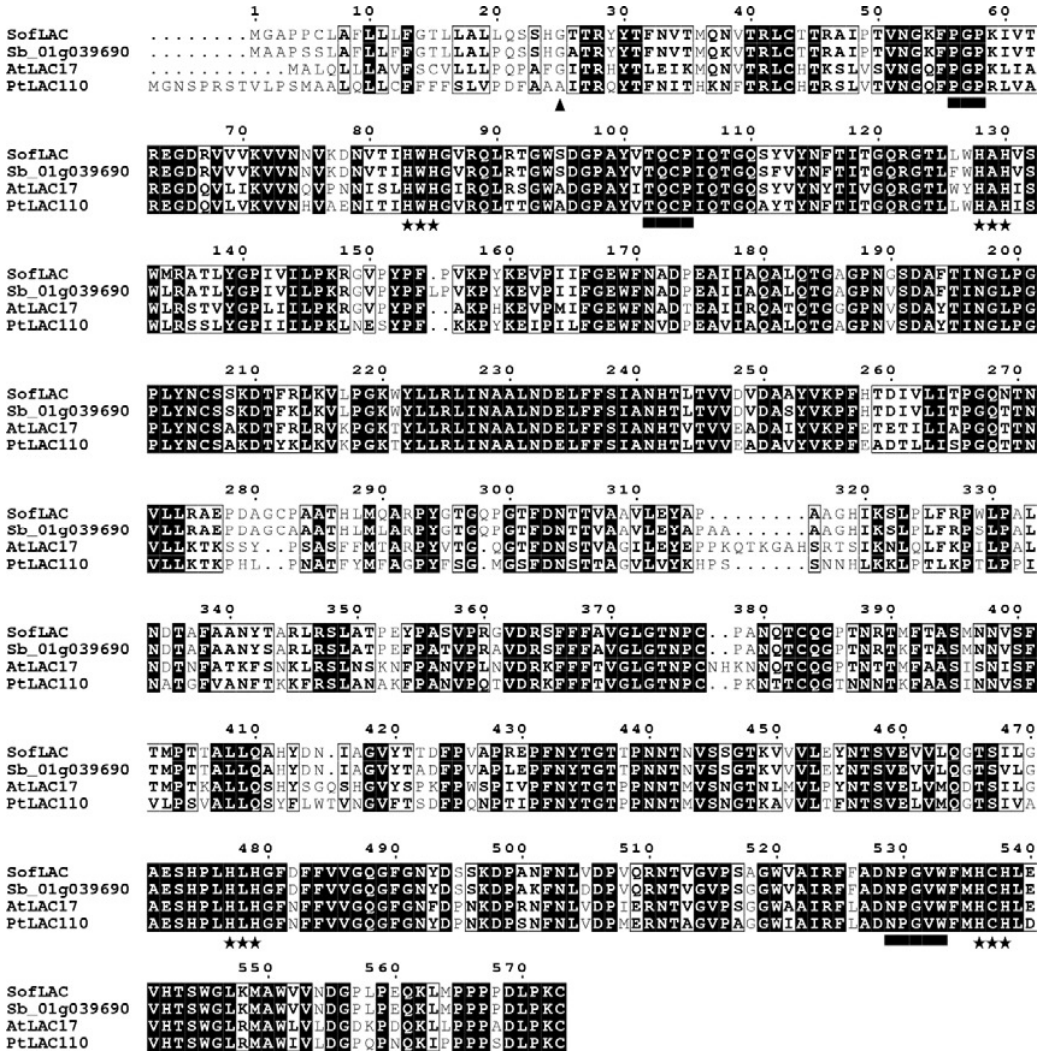


Figure 4

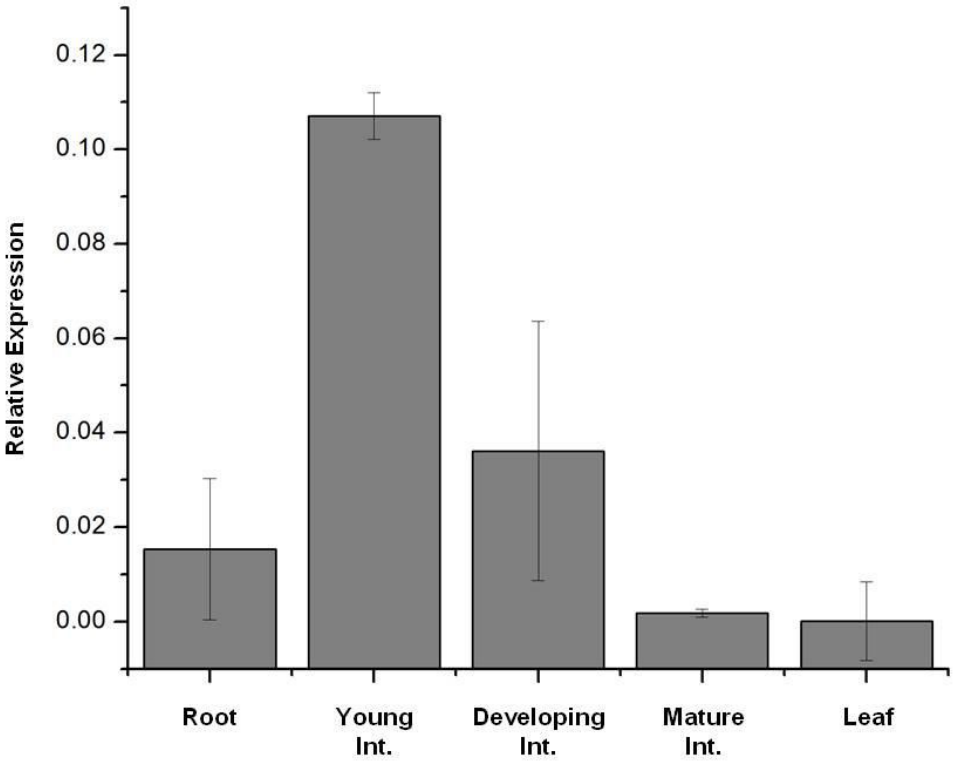

Relative Expression
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Root
Young Int.
Developing Int.
Mature Int.
Leaf

Figure 5

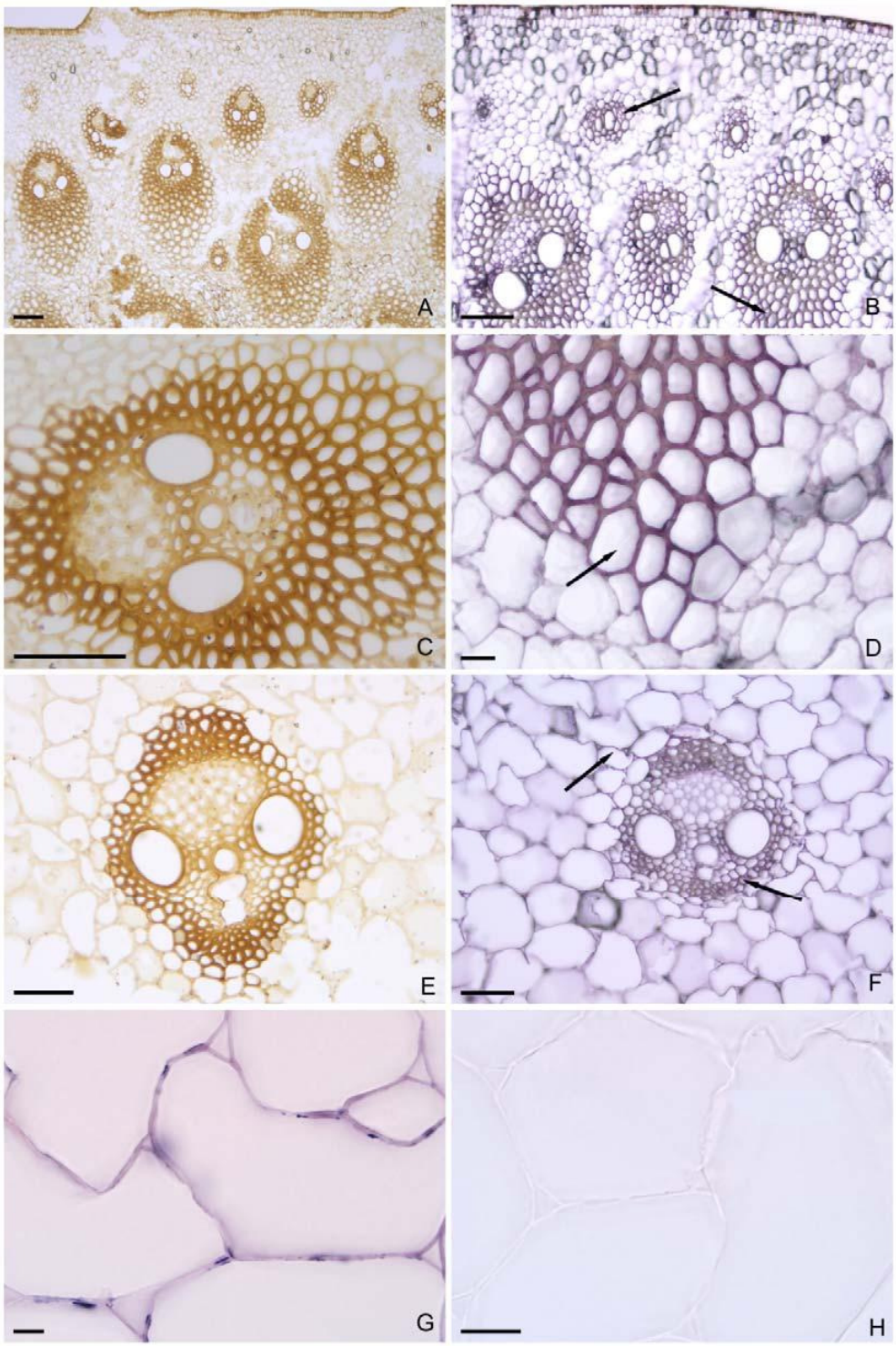
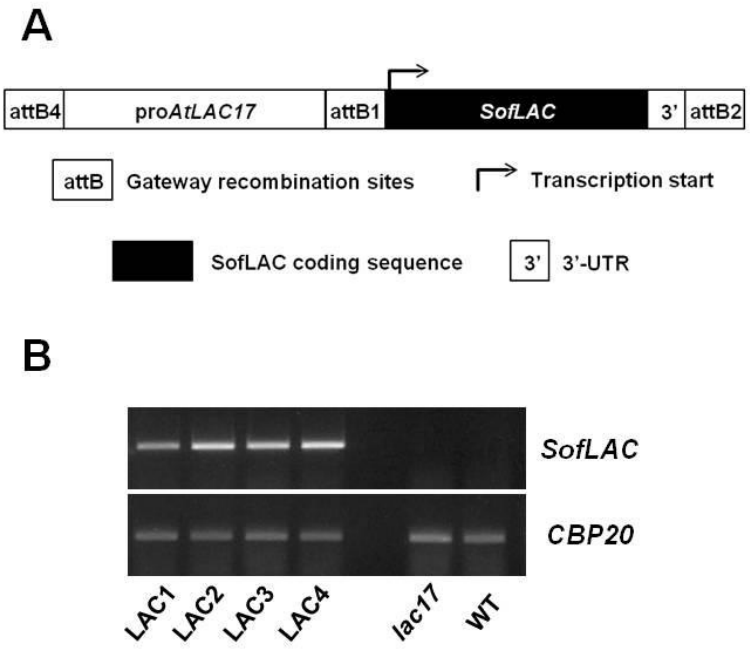


Figure 6


A
attB4
proAtLAC17
attB1
SofLAC
3'
attB2
attB
Gateway recombination sites
Transcription start
SofLAC coding sequence
3'
3'-UTR
B
SofLAC
CBP20
LAC1
LAC2
LAC3
LAC4
lac17
WT
C
% lignin/weight residue
20
18
16
14
12
10
8
6
4
2
0
WT
lac17 back
LAC1
LAC2
LAC3
LAC4

Conclusões Gerais

Embora diversos estudos tenham relacionado peroxidases e laccases com um processo biológico específico, a determinação precisa da função biológica de um gene individual de peroxidase e/ou laccase ainda continua sendo um desafio substancial. Uma das funções mais largamente correlacionadas com peroxidases e laccases é a polimerização oxidativa dos monômeros de lignina. A maioria dos estudos baseia-se na capacidade destas enzimas de metabolizarem monolignols *in vitro* e na determinação da expressão gênica no local (órgão e tipo celular) e no momento em que o processo de lignificação está ativo. Ainda assim, poucos foram os estudos que conseguiram provar, inequivocamente, o papel de uma peroxidase/laccase específica na biossíntese de lignina. Acredita-se que somente a combinação de diversas técnicas de transcriptômica, transgenia, fisiologia e bioquímica poderá revelar os verdadeiros papéis biológicos de cada membro destas famílias multigênicas. O presente trabalho tinha por objetivo selecionar genes de peroxidase/laccase candidatos a desempenharem um papel na biossíntese de lignina em cana-de-açúcar. Demonstrou-se que o colmo de cana-de-açúcar é um órgão complexo, formado por diversos tipos celulares com distintos programas genéticos para deposição de lignina. Este órgão também apresenta clara distinção anatômica entre a região periférica (*rind*) e a região interna (*pith*), sendo a primeira uma região altamente ativa para deposição de lignina e atividade de peroxidases. Ademais, tais regiões apresentaram diferentes perfis proteômicos de peroxidases de classe III, que são diferencialmente reguladas ao longo do desenvolvimento do colmo. Algumas peroxidases de classe III foram identificadas por espectrometria de massas, mas análises filogenéticas sugerem que estas proteínas são frutos de duplicações gênicas específicas de monocotiledôneas. Este estudo demonstrou que o número de peroxidases expressas no colmo

de cana-de-açúcar é extremamente alto, resultando em complexos perfis proteômicos e dificultando a realização de estudos funcionais.

Para facilitar o isolamento de peroxidases oriundas da parede celular e para evitar a contaminação das frações proteicas de origem intracelular, células em suspensão de cana-de-açúcar foram utilizadas como modelo. Este estudo demonstrou que as células em suspensão são capazes de produzir e excretar no meio de cultura, peroxidases com capacidade de oxidar siringaldazina, um análogo do monolignol álcool sinapil. Tal observação é importante porque nem todas as peroxidases são capazes de oxidar este composto, devido à presença de um grupo metoxila extra no anel aromático. Além disso, a oxidação de siringaldazina por peroxidases tem sido relacionada exclusivamente com tecidos em lignificação. Portanto, observou-se que o sistema de células em suspensão pode ser uma alternativa interessante para o isolamento e caracterização de peroxidases. Após identificação de isoformas por espectrometria de massas, genes candidatos a desempenharem um papel funcional na oxidação de monolignols foram selecionados a partir de seus perfis de expressão tecido-específica. Espera-se que genes relacionados com tal processo apresentem expressão preferencial em tecidos e órgãos em lignificação ativa. Dos genes analisados, somente um apresentou maior expressão nas regiões periféricas altamente lignificadas ao longo de todo o desenvolvimento do colmo e foi, consequentemente, considerado o melhor gene candidato.

Embora resultados interessantes tenham sido conseguidos nos estudos com peroxidases, somente no caso de laccases é que uma caracterização funcional mais profunda foi realizada. Isto se deve ao fato de que evidências genéticas são as mais confiáveis para se atribuir a função de um gene e, no caso da laccase de cana-de-açúcar, utilizou-se uma estratégia de complementação de um mutante com menores teores de lignina. Este experimento, juntamente com os dados adquiridos com análises de co-expressão e expressão

gênica tecido- e tipo celular-específica, proporcionaram evidências definitivas do envolvimento desta laccase na síntese de lignina durante fases iniciais de lignificação no colmo de cana-de-açúcar.

Portanto, os resultados obtidos neste trabalho contribuem não somente para a elucidação de funções biológicas de isoformas/genes específicos de peroxidases e laccases, mas também para aumentar o nosso conhecimento acerca do desenvolvimento, metabolismo e oportunidades de aplicação biotecnológica para cana-de-açúcar, uma cultura de grande importância na área de bioenergia.