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INDUÇÃO DO ALCALÓIDE BRAQUICERINA POR UV-B EM FOLHAS DE *Psychotria brachyceras*

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“Passada a nuvem, o garotinho voltou a cabeça para o nível da rua e, ao cruzarem-se nossos olhares ele sorriu cheio de encantamento. Sorri de volta. Éramos ambos cúmplices daquele momento mágico.”

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Índice

Resumo 6

Abstract 7

I. Introdução geral 8

1. Metabolismo secundário e seu papel na defesa das plantas 8
2. *Psychotria* spp. e seus alcalóides 10
3. Incidência de radiação ultravioleta (UV) na superfície da Terra 13

II. Objetivos 15

III. Early Changes in Gene Expression Induced by Acute UV-B Exposure in Leaves of *Psychotria brachyceras* 17

ABSTRACT 19
INTRODUCTION 20
MATERIALS AND METHODS 22
RESULTS AND DISCUSSION 25
CONCLUSION 30
REFERENCES .... **Error! Bookmark not defined.**

IV. Discussão geral 44

1. Resultados referentes à expressão gênica relacionada ao aumento de braquicerina em resposta à radiação UV-B em *Psychotria brachyceras* 44

V. Considerações finais 51

VI. Referências bibliográficas 52

Anexo 1: 67

Anexo 2: 73

Resumo

Psychotria brachyceras, uma planta arbustiva de sub-bosque nativa do Rio Grande do Sul, produz o alcalóide monoterpeno indólico glicosilado braquicerina. Esse alcalóide e o extrato metanólico de folhas de *P. brachyceras* apresentaram atividades antimutagênica e antioxidante em estudos previamente publicados por nosso grupo. Além disso, foi observado um aumento do acúmulo de braquicerina em plantas expostas à radiação UV-B. Sabe-se que com a diminuição da camada de ozônio, uma maior proporção do espectro UV-B atinge a superfície da Terra, trazendo sérias implicações para todos os organismos vivos. A radiação UV-B tem o potencial de danificar biomoléculas, como DNA, RNA, proteínas e lipídios, gerar espécies reativas de oxigênio, e prejudicar processos celulares. Em função disso, as plantas utilizam mecanismos de proteção, envolvendo desde proteção física (cutícula foliar) até a biossíntese de moléculas que absorvem UV, dentre as quais estão os alcalóides. A partir dessas observações, hipotetisamos um papel para braquicerina como molécula protetora contra a radiação UV-B através de sua atividade antioxidante e espectro de absorção UV. Para testar nossa hipótese, utilizamos a técnica de Hibridização Subtrativa para buscar genes relacionados ao metabolismo de braquicerina em plantas de *P. brachyceras* expostas à radiação UV-B. Através de qRT-PCR, confirmamos a indução de 5 genes potencialmente envolvidos com o metabolismo de braquicerina em resposta à UV-B, indicando que a indução de braquicerina em resposta à UV-B é regulada, pelo menos parcialmente, em nível de mRNA.

Abstract

Psychotria brachyceras is an understory shrub, native of Rio Grande do Sul state that produces the glucoside indole monoterpene alkaloid brachycerine. Both brachycerine and the foliar methanolic extract of *P. brachyceras* showed antimutagenic and antioxidant activities in previous studies published by our group. In addition, we also observed an increased accumulation of brachycerine in plants exposed to UV-B radiation. It is known that with the decrease of the ozone layer, a higher proportion of UV-B spectrum reaches the Earth's surface, with serious implications for all living organisms. The UV-B radiation can damage biomolecules such as DNA, RNA, proteins and lipids, generate reactive oxygen species, and impair cellular processes. Plants have developed several protection mechanisms, ranging from physical protection (leaf cuticle) to biosynthesis of molecules that absorb UV, including alkaloids. From these observations, a role for brachycerine as a protective molecule against UV-B through its antioxidant activity and UV absorption spectrum has been proposed. We hypothesized that brachycerine response to UV occurred at the level of transcript steady state. To test our hypothesis, Suppressive Subtractive Hybridization was applied to search for genes related to brachycerine metabolism in UV-B exposed *P. brachyceras* plants. We confirmed the UV-B upregulation of five genes potentially related to brachycerine metabolism by qRT-PCR, indicating that UV-B induction of brachycerine is at least partly regulated at the level of transcription.

I. Introdução geral

1. Metabolismo secundário e seu papel na defesa das plantas

Como organismos sésseis, uma das estratégias de defesa adotada pelas plantas ao longo da evolução foi a aquisição de uma variedade incrível de rotas metabólicas levando à produção de moléculas capazes de responder pronta e eficientemente a situações de estresse impostas por fatores bióticos e abióticos. Essas moléculas, frequentemente referidas como produtos naturais, são metabólitos secundários e compreendem uma ampla variedade de moléculas não-protéicas de peso molecular relativamente baixo que ocorrem em determinados táxons e são consideradas não essenciais para o crescimento e desenvolvimento da planta, embora sejam constantemente requisitados para a adaptação ambiental (Nascimento & Fett-Neto, 2010, no anexo 2). Metabólitos secundários são representados pelos alcalóides, terpenóides, compostos fenólicos (flavonóides, taninos, etc.), glicosinolatos, glicosídios cianogênicos, aminoácidos não protéicos, entre outros (Roberts & Wink, 1998). Em geral, alguns grupos destes compostos são dominantes dentro de determinados táxons, e, dentro de uma mesma planta, o padrão de distribuição dos metabólitos secundários é complexo, variando entre diferentes órgãos e tecidos, estádios de desenvolvimento, entre indivíduos e entre populações (Wink, 2003).

Muitas vezes recrutadas durante a evolução biológica a partir de rotas essenciais do metabolismo primário através de eventos de duplicação gênica, seguidos de acúmulo de mutações e diversificação funcional, as rotas do metabolismo secundário combinam carbono, hidrogênio, oxigênio, nitrogênio e enxofre em moléculas que são capazes de desempenhar diversas funções importantes. Algumas destas funções são: 1) deterrência à herbivoria; 2) sinalização tritrófica (i.e., atraindo predadores e parasitas de herbívoros); 3) inibição de patógenos; 4) respostas integradas de defesa; 5) absorção de radiação ultravioleta (UV); 6) mitigação de espécies reativas de oxigênio (ROS - do inglês, *reactive oxygen species*); 7) atividade alelopática; 8) atração de polinizadores e dispersores de frutos e sementes; 9) dissipação de calor, entre outras (Hartmann, 2007; Nascimento & Fett-Neto, 2010). A indução da expressão de genes envolvidos na biossíntese de metabólitos secundários em resposta a ferimento, moléculas derivadas de herbívoros, elicitores patogênicos, e estresse oxidativo causado por calor, seca, alagamento, radiação UV, ou extremos de temperatura, é frequentemente mediada pela integração de moléculas sinalizadoras, tais como ácido salicílico, ácido jasmônico (AJ), e seus derivados (Nascimento & Fett-Neto, 2010; Zhao et al., 2005).

Do ponto de visto humano, muitos destes metabólitos possuem considerável valor econômico e são extensivamente utilizados em medicamentos pela indústria farmacêutica (e.g., antitumoral taxol, e antileucêmico vincristina), em alimentos na forma de flavorizantes, como fragrâncias em cosméticos ou, ainda, como inseticidas, fungicidas, antivirais e herbicidas na agricultura (Croteau et al., 2000; Nascimento & Fett-Neto, 2010). A enorme biodiversidade vegetal do Brasil coloca o país em posição estratégica para desenvolver a exploração racional e sustentada de novos compostos de valor terapêutico. Estima-se que apenas cerca de 10 % das 400.000 espécies de plantas estimadas têm sido utilizadas com fins medicinais. Aproximadamente 25 % dos medicamentos de uso terapêutico são derivados de plantas e muitos outros são utilizados como compostos puros ou como chás e extratos. Além disso, constituintes de plantas têm também servido como modelos de drogas modernas (p.ex. atropina para tropicamida, quinino para cloroquina e cocaína para procaína e tetracaína) (Basso et al., 2005).

1.1 Alcalóides

Os alcalóides se destacam dentre os metabólitos secundários com propriedades terapêuticas e ocorrem em aproximadamente 20 % das espécies de plantas, e o número de estruturas já identificadas excede os 16000 (Memelink et al., 2001). O termo 'alcalóide' é derivado da palavra árabe *al-quali*, nome popular da planta da qual a soda foi obtida originalmente. Essas moléculas são representadas por compostos orgânicos com baixo peso molecular e caráter básico, formando uma estrutura heterocíclica com a presença de nitrogênio (Kutchan, 1995).

Alguns exemplos de alcalóides utilizados na indústria farmacêutica incluem: o analgésico morfina, o analgésico e antitussivo codeína (ambos de *Papaver somniferum*), os agentes quimioterápicos vincristina e vinblastina (de *Catharanthus roseus*), o antimalárico quinina (de *Cinchona officinalis*), o supressor de gota colchicina, o relaxante muscular (+)-tubocurarina (de *Chondodendrum tomentosum*), o antiarrítmico ajmalicina, o antibiótico sanguinarina (de *Eschscholtzia californica*) e o sedativo escopolamina (de *Hyoscyamus niger*). Outros alcalóides conhecidos oriundos de plantas incluem cafeína, nicotina e cocaína (Kutchan, 1995). Em média, cerca de dez mil substâncias são testadas para o desenvolvimento de um novo medicamento (Payne et al., 1991). Para alguns alcalóides, a síntese química foi desenvolvida; porém, para diversos desses compostos, como os da classe dos monoterpeno indólicos, o baixo rendimento e o alto custo do processo, devido à complexidade química estrutural, inviabilizam o uso comercial da síntese. Sendo assim, em muitos casos o cultivo e a extração de alcalóides a partir da planta

produtora (ou de precursores de alcalóides para semi-síntese) permanecem sendo a alternativa mais viável economicamente (Kutchan, 1995).

Apesar da grande importância dos alcalóides para uso medicinal, relativamente pouco é conhecido sobre a regulação da sua biossíntese e sua função nas plantas. Estudos sugerem que alcalóides desempenham um importante papel ecoquímico na defesa da planta contra patógenos e herbívoros (Rhodes, 1994). Além disso, foi verificado que a biossíntese dos alcalóides nas plantas é freqüentemente controlada durante o desenvolvimento (De Luca & Laflamme, 2001) e em resposta a estresses ambientais como infecção por microorganismos, herbivoria, íons de metais pesados, radiação UV e choque osmótico (Facchini, 2001; Memelink et al., 2001). Assim, possíveis funções desses compostos nas plantas incluem proteção contra patógenos, atividade anti-herbivoria, proteção contra radiação UV, reserva de nitrogênio e alelopatia em função de seu poder quelante e efeitos citotóxicos (Gregianini et al., 2003; Hartley & Jones, 1997; Rhodes, 1994; Wink, 1988; Wink, 1999).

Os alcalóides são divididos em diferentes classes como alcalóides tropânicos, esteroidais e indólicos. Cerca de 2000 alcalóides indólicos são conhecidos e dentre estes estão os alcalóides monoterpeno indólicos (MIAs - do inglês, *monoterpene indole alkaloids*) (Schripsema et al., 2001). Esse tipo de alcalóide tem sido isolado em três principais famílias de plantas tropicais, Loganiaceae, Apocynaceae e Rubiaceae, todas pertencentes à Ordem Gentianales, além da família Nyssaceae da Ordem Cornales (Memelink et al., 2001; Roberts & Strack, 1999). A biossíntese da maioria dos MIAs possui como etapa inicial a condensação da triptamina (precursor indólico) com o monoterpeno secologanina (precursor terpênico) formando o alcalóide glicosilado estrictosidina, através da atividade da enzima estrictosidina sintase. A estrictosidina é o precursor comum de uma grande variedade de alcalóides em diversas espécies de plantas. Passos adicionais de modificação por enzimas espécie-específicas e conversões espontâneas determinam os tipos de alcalóides que serão formados (Kutchan, 1993).

2. *Psychotria* spp. e seus alcalóides

O gênero *Psychotria*, pertencente à subfamília Rubioideae e à tribo Psychotrieae, é o maior da família Rubiaceae, abrangendo quase 2000 espécies amplamente distribuídas no estrato arbustivo das matas tropicais, e caracterizando-se pelo acúmulo de alcalóides indólicos (Davis et al., 2001; Nepokroeff et al., 1999; Pasquali et al., 2006). Foram identificadas 18 espécies de *Psychotria* no sul do Brasil (Santos, 1999), e a investigação química de algumas dessas plantas revelou diversas estruturas inéditas. Entre as espécies produtoras de alcalóides, aparecem *P.*

suterella (Santos et al., 2001), *P. brachyceras* (Kerber et al., 2001), *P. leiocarpa* (Henriques et al., 2004), *P. myriantha* (Simões-Pires et al., 2006), e *P. umbellata* (Kerber et al., 2008). Das 6 espécies investigadas, apenas uma não apresentou alcalóides, *P. carthagenensis* (Leal & Elisabetsky, 1996). Os alcalóides deste gênero são derivados, em sua maioria, do aminoácido triptofano, e são do tipo poliindólico ou derivados metiltriptamínicos principalmente (Hart et al., 1974; Libot et al., 1987; Adjibade et al., 1992; Santos, 1999). A análise de várias espécies do sul do Brasil mostrou que os alcalóides identificados são do tipo monoterpeno indólico glicosilado, uma característica química peculiar para este gênero (Kerber et al., 2001; Santos et al., 2001).

Foram demonstrados diferentes efeitos farmacológicos para alcalóides e/ou extratos alcaloídicos de espécies de *Psychotria*. Por exemplo, a psicolatina de *P. umbellata* apresentou atividades analgésica (Both et al., 2002a) e psicoativa (Both et al., 2005) específicas em testes com camundongos; além dessas, nosso grupo demonstrou propriedades antioxidantes e antimutagênicas desse alcalóide (Fragoso et al., 2008). Extratos de *P. brachyceras, P. leiocarpa, P. suterella*, *P. myriantha* e *P. umbellata* demonstraram atividade analgésica (Both et al*.,* 2002b; Elisabetsky et al., 1997; Leal, 1994). O extrato de *P. myriantha* apresentou ainda atividade antiinflamatória em testes com camundongos (Simões-Pires et al*.,* 2006).

2.1 *Psychotria brachyceras* e seu alcalóide braquicerina

Psychotria brachyceras Müll. Arg. (Figura 1) é uma planta lenhosa de sub-bosque e de hábito arbustivo, podendo atingir até 3 metros de altura, que apresenta ampla distribuição, ocorrendo desde o Estado do Rio de Janeiro até o Rio Grande do Sul, na área da Mata Pluvial Costeira e Mata da Planície Litorânea (Dillenburg & Porto, 1985). Suas flores são brancas e os frutos drupáceos são de cor azul-violácea. Essa espécie caracteriza-se pela produção do MIA majoritário braquicerina (Figura 1, Kerber et al., 2001), o qual possui uma estrutura peculiar, pois apresenta um resíduo de glicose (incomum na forma final da maioria dos alcalóides conhecidos) ligado à sua porção terpênica, a qual é possivelmente derivada de uma forma de *epi*-loganina, diferindo dos demais alcalóides indólicos, cuja porção terpênica é geralmente derivada de secologanina e da subseqüente formação de estrictosidina, o precursor geral de pelo menos 3000 alcalóides indol-terpênicos conhecidos. A sua porção indólica é provavelmente derivada do aminoácido triptofano, o qual é convertido em triptamina pela enzima citossólica TDC (triptofano descarboxilase). A porção terpênica em alcalóides monoterpeno indólicos de *C. roseus* é fornecida por isopentenil difosfato (IPP) ou dimetilalil difosfato (DMAPP) oriundo da rota biossintética triose fosfato/piruvato em plastídios, a rota independente de mevalonato (ou rota

MEP - metil-eritrol fosfato) (Contin et al., 1998). Estudos com o fornecimento exógeno de inibidores específicos das rotas citosólica (rota MVA - mevalonato) e plastídica (rota MEP) de biossíntese de IPP em *P. brachyceras* (sendo a mevinolina um inibidor da enzima HMGR da rota MVA, e a fosmidomicina um inibidor da enzima DXR da rota MEP) revelaram que a porção terpênica da braquicerina também é predominantemente originada do IPP fornecido pela rota plastídica MEP (Nascimento et al., dados não publicados). A estrutura final da braquicerina parece resultar da condensação direta da triptamina com *epi*-loganina, proveniente da rota MEP. Essa hipótese biogenética indica uma rota alternativa para biossíntese dessa molécula, representando uma nova classe de alcalóides indólicos (Kerber et al., 2001).

A braquicerina é sintetizada nas partes aéreas da planta e não é acumulada nas raízes e em tecidos indiferenciados. Observou-se acúmulo preferencial durante a fase reprodutiva, com variação sazonal e entre os diferentes indivíduos de uma mesma população (Gregianini et al., 2004). Dentre os fatores capazes de induzir o acúmulo de braquicerina estão: dano mecânico, estresse osmótico, ocasionado pela presença de agentes osmóticos como cloreto de sódio, sorbitol e polietilenoglicol, e aplicação exógena de AJ, principal molécula sinalizadora de herbivoria, e do fitormônio ácido abscísico (ABA), envolvido em respostas a estresse de seca (Gregianini et. al., 2004; Nascimento et al., dados não publicados). Esses resultados sugerem um papel desse alcalóide na proteção da planta contra herbivoria e contra possíveis danos ocasionados por estresse osmótico ou hídrico. Além disso, o acúmulo do alcalóide nas folhas é significativamente aumentado em estacas de *P. brachyceras* expostas à radiação UV-C e UV-B (Gregianini et al., 2003; Porto, 2009). A proteção contra radiação UV é provavelmente relacionada à atividade antioxidante da braquicerina observada *in vitro* sobre oxigênio singleto, radical hidroxila e ânion superóxido (Gregianini et al., 2003; Nascimento et al., 2007; Porto, 2009). Esses últimos resultados que sugerem um papel do alcalóide como um antioxidante protetor contra os efeitos da radiação UV foram os principais motivadores para o desenvolvimento da presente tese.

As atividades biológicas já identificadas da braquicerina incluem: efeito antiinflamatório específico em testes de migração de leucócitos em ratos por meio de ensaio de quimiotaxia (F. Farias e A.T. Henriques, comunicação pessoal), efeitos antioxidante e antimutagênico em ensaios com leveduras, sendo estes também observados para o extrato foliar metanólico de *P. brachyceras* (Nascimento et al., 2007), além de atividade alelopática observada para o extrato foliar aquoso de *P. brachyceras* (Correa et al, 2008).

Até o momento não existem dados disponíveis sobre o genoma de *P. brachyceras*, os quais auxiliariam na melhor compreensão da regulação da biossíntese do alcalóide braquicerina e

seu papel *in planta*, além de possibilitarem estudos envolvendo a engenharia genética dessa espécie a fim de manipular a biossíntese de moléculas de interesse.

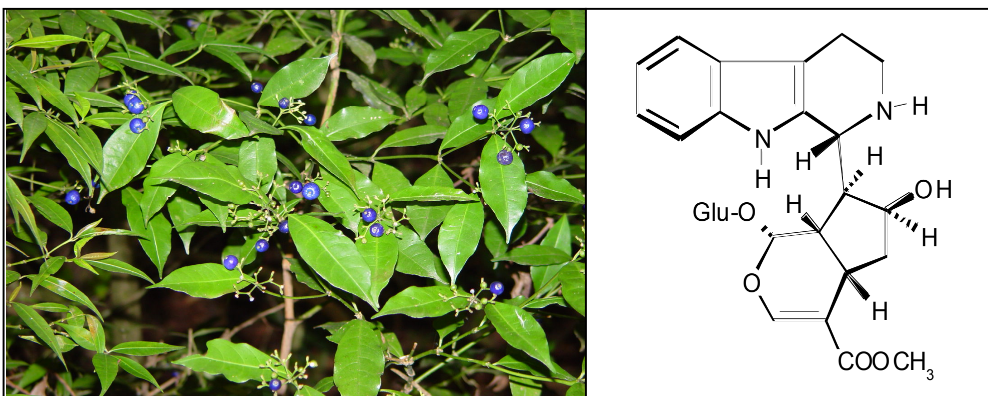

Figura 1. Fotografia de *Psychotria brachyceras* em seu ambiente natural (à esquerda); e estrutura do alcalóide monoterpeno indólico braquicerina presumivelmente resultante da condensação direta de triptamina (oriunda da descarboxilação do aminoácido triptofano) com *epi*-loganina (proveniente majoritariamente da rota plastídica mevalonato-independente - MEP) (à direita).

3. Incidência de radiação ultravioleta (UV) na superfície da Terra

Sete por cento da radiação eletromagnética emitida pelo sol está na faixa da radiação UV (200-400 nm). À medida que passa através da atmosfera, o fluxo total transmitido é muito reduzido, e a composição da radiação UV é modificada. A radiação UV-C (200-290 nm) é completamente absorvida pelos gases atmosféricos. A radiação UV-B (290-315 nm) é adicionalmente absorvida pelo ozônio estratosférico e, portanto, apenas uma percentagem muito pequena é transmitida para a superfície da Terra, enquanto a radiação UV-A (315-400 nm) é dificilmente absorvida pela camada de ozônio (Frohnmeyer & Staiger, 2003). Um estudo de 1996 (Pyle) revela uma diminuição de 5 % na concentração de ozônio estratosférico, principalmente devido ao lançamento de poluentes antropogênicos como os clorofluorocarbonetos. Por conseguinte, uma maior proporção do espectro UV-B atinge a superfície da Terra trazendo sérias implicações para todos os organismos vivos (Caldwell et al., 2003; Xiong & Day, 2001). A radiação UV-B tem o potencial de danificar biomoléculas, como DNA, RNA, proteínas e lipídios, de gerar ROS, e de prejudicar processos celulares (Frohnmeyer & Staiger, 2003; Jansen et al., 1998; Jenkins, 2009; Ban et al., 2007).

3.1 As plantas e suas defesas contra a radiação UV-B

Nas plantas, elevada radiação UV-B tem efeitos pleiotrópicos sobre o seu desenvolvimento, e, por causa de sua condição séssil, as plantas são obrigadas a se adaptarem localmente a mudanças ambientais (Frohnmeyer & Staiger, 2003). Ao longo da evolução, as plantas desenvolveram várias adaptações para tolerar o estresse imposto pela UV-B (Jansen et al., 1998; Hollósy, 2002; Frohnmeyer & Staiger, 2003), incluindo defesas estruturais, enzimáticas e químicas. A cutícula foliar é a primeira barreira à penetração de UV, e suas características morfológicas (forma) e químicas (composição e quantidade de ceras epicuticulares) modulam a reflexão e a absorção de UV pelas folhas das plantas (Krauss et al., 1997; Grant et al., 2003). A biossíntese de compostos que absorvem radiação UV é o mecanismo mais comum de proteção nas plantas contra essa radiação potencialmente prejudicial (Hahlbrock & Scheel, 1989). Esses compostos são metabólitos secundários, principalmente compostos fenólicos, flavonóides e ésteres hidroxicinamatos, os quais se acumulam nos vacúolos das células epidérmicas em resposta à radiação UV-B e atenuam a penetração da radiação UV-B do espectro solar em camadas celulares mais profundas, causando pouco efeito sobre a absorção da região da luz visível (Frohnmeyer & Staiger, 2003; Schmitz-Hoerner & Weissenbock, 2003).

Os alcalóides terpeno-indólicos (TIAs - do inglês, *terpenoid indole alkaloids*) também fazem parte desses compostos que absorvem radiação UV-B, supostamente atuando na proteção da planta contra essa radiação nociva (Binder et al., 2009; Gregianini et al., 2003; Hartmann, 1991). A produção e o acúmulo dessa classe de moléculas pelas plantas são bem documentados como respostas de defesa a ataques de herbívoros e patógenos (Wittstock & Gershenzon, 2002; Gatehouse, 2002; Siciliano et al., 2005); no entanto, relativamente pouco se sabe sobre a participação dos alcalóides em respostas à radiação UV.

Estudos prévios mostraram que a radiação UV entre 290 e 380 nm, incluindo a faixa de UV-B (290-315 nm), estimulou a produção de alcalóides em *C. roseus*: alcalóides diméricos em partes aéreas (Hirata et al., 1992; Hirata et al., 1993), alcalóides monoméricos em culturas de células em suspensão (Ramani & Chelliah, 2007), e TIAs em cultura de raízes em cabeleira (Binder et al., 2009). Também foi observada a indução da expressão de vários genes envolvidos nos estágios iniciais da biossíntese de TIAs em *C. roseus* em resposta à radiação UV-B (Ouwerkerk et al., 1999). Outros eventos induzidos pela radiação UV-B sugerem a participação de ROS como componentes de sinalização; por exemplo, sabe-se que a irradiação do tecido vegetal com UV-B *per se* causa a geração de ROS, tais como oxigênio singleto (Frohnmeyer & Staiger, 2003), sendo que a expressão de proteínas relacionadas à patogênese (PR-1), induzida

por UV-B, é mediada por ROS em folhas de tabaco (*Nicotiana tabacum*) (Green & Fluhr, 1995). Além disso, mais genes induzidos por UV-B cuja expressão pode ser modulada pela eliminação de ROS têm sido encontrados em *Arabidopsis* (Mackerness et al., 2001).

O possível envolvimento relatado de alcalóides participando da defesa das plantas contra danos causados pela radiação UV-B, juntamente com as nossas observações sobre a indução de braquicerina em resposta à essa radiação e sua atividade antioxidante sobre diferentes ROS, sugerem um papel protetor desse alcalóide em *P. brachyceras* contra a radiação UV-B.

3.2 Braquicerina e seu possível papel protetor contra UV-B *in planta*:

Hipótese de trabalho:

Com base nos resultados publicados por nosso grupo de pesquisa, relativos à indução de braquicerina por UV-B, apresentados anteriormente na seção 2.1, foi formulada a seguinte hipótese de trabalho:

"A indução de braquicerina in planta pela radiação UV-B ocorre em nível transcricional."

Como atualmente não existem dados disponíveis sobre o genoma de *P. brachyceras* que facilitem uma melhor compreensão das vias de biossíntese do alcalóide e do estabelecimento do seu papel na planta, a identificação de genes cuja expressão é induzida pela radiação UV-B é uma estratégia útil para a elucidação dos mecanismos ligados ao processo fisiológico que conduz ao aumento do acúmulo de braquicerina.

II. Objetivos

- Identificar genes relacionados ao metabolismo de braquicerina em *P. brachyceras* na presença de radiação UV-B através da técnica de Hibridização Subtrativa (SSH - do inglês, *Suppressive Subtractive Hybridization*), uma estratégia que tem sido amplamente utilizada para identificar genes diferencialmente expressos (Diatchenko et al., 1996; Liang & Pardee, 1992).

- Definir genes normalizadores em folhas completamente expandidas de *P. brachyceras* na condição experimental (exposição à UV-B) para serem usados na confirmação das seqüências

diferencialmente expressas encontradas na biblioteca de SSH.

- Confirmar a expressão diferencial dos genes identificados e selecionados no SSH em resposta à radiação UV-B através de RT-PCR quantitativo (qRT-PCR).

III. Early Changes in Gene Expression Induced by Acute UV-B Exposure in Leaves of *Psychotria brachyceras*

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Early Changes in Gene Expression Induced by Acute UV-B Exposure in Leaves of *Psychotria brachyceras*

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ABSTRACT

UV-B radiation can damage biomolecules, such as DNA, RNA, proteins, and lipids, halting essential cellular processes; part of this damage is due to reactive oxygen species (ROS) generation. Plants developed several adaptations to tolerate UV-B imposed stress, including the accumulation of secondary metabolites, which may act as UV-B shields and antioxidants. *Psychotria brachyceras* Müll. Arg. (Rubiaceae), an Atlantic Forest shrub, produces brachycerine, a monoterpene indole alkaloid mainly accumulated in leaf tissues, which displays antioxidant and antimutagenic activities in yeast. Exposure of *P. brachyceras* cuttings to UV-B radiation significantly increases leaf brachycerine concentration. It has been suggested that this alkaloid might protect the plant against possible UV-B damages through its activity on ROS. To identify differentially expressed genes of *P. brachyceras* in response to UV-B and investigate a possible role in the metabolism of brachycerine, suppressive subtractive hybridization was applied. Complementary DNA from UV-B-treated leaves for 24h was used as tester, and cDNA from untreated leaves, as driver. After BLASTX alignments, 134 sequences matched with plant genes. Using quantitative RT-PCR, 5 out of the 9 selected genes possibly related to brachycerine metabolism showed significant increases in transcription after UV-B exposure ($P<0.05$): tryptophan decarboxylase, ACC oxidase, UDP-glucose glucosyltransferase, lipase, and serine/threonine kinase. Taken together, our results suggest a possible involvement of brachycerine in UV-B defense and show that the alkaloid accumulation response might be at least partly regulated at transcriptional level.

INTRODUCTION

Solar ultraviolet-B radiation (UV-B; 290–315nm) is a small fraction of the solar spectrum that reaches ground level. In spite of its modest contribution to the total quantum flux, UV-B can be an important modulator of biological processes in terrestrial ecosystems (1-3). UV-B has the potential of damaging biomolecules, such as DNA, RNA, proteins, and lipids, generating reactive oxygen species (ROS), and impairing cellular processes (3-6). The effects of UV-B are multiple, affecting both morphological and physiological aspects of plants. The sessile life of plants requires effective adaptation to environmental changes, such as increased levels of UV radiation (4). In the course of evolution, plants developed several adaptations to tolerate UV-B imposed stress (5,7), including structural, enzymatic and chemical defenses. The accumulation of UV-absorbing compounds is often observed as a protection strategy against the damages of UV exposure (8). Secondary products, mostly phenolics and their derivatives, accumulate in vacuoles of epidermal cells, reducing penetration of UV in deeper tissues without major effects on the visible region of the spectrum (4,9). Among these putative protective compounds terpenoid indole alkaloids (TIAs) may also be included, since they absorb light in the UV range (10-11). It has been shown that an increased production of truxillines alkaloids in *Erythroxylum novogranatense* var. *novogranatense* is induced by UV exposure (12).

Previous studies with *Catharanthus roseus* have shown that UV-B can stimulate the acumulation of alkaloids, both in cell and organ cultures (13-15). UV-B light can induce the expression of genes encoding enzymes of the initial stages of TIA biosynthesis in vinca (16). Exposure of plants to UV-B stimulates the production of ROS, such as singlet oxygen, which may also act as signaling components in UV-B induced responses (4). ROS can trigger the expression of various genes, including pathogenesis-related proteins (PR-1) in tobacco (17) and several UV-B inducible genes in *Arabidopsis* (18).

Psychotria brachyceras Müll. Arg. (Rubiaceae) is an Atlantic Forest understorey shrub that

produces brachycerine, a glucoside monoterpene indole alkaloid (MIA) mainly accumulated in leaf tissues, flowers and fruits, independent of the presence of roots (19). Brachycerine has an unusual structure compared to other alkaloids within the same class because its terpene moiety is apparently derived from epiloganin instead of the conventional precursor secologanin; its structure also has a glucose residue, usually absent in related molecules (19-20). Exposure of *P. brachyceras* cuttings to UV-B radiation increases brachycerine concentration, and the purified alkaloid displays antioxidant activities, both for *in vitro* and *in vivo* assays, besides *in vivo* antimutagenic effects (21-22).

Based on these results, we hypothesized that this alkaloid might protect the plant against possible UV-B damages through its activity on free radicals. Currently, there is no data available on the genome of *P. brachyceras* that would contribute for a better understanding of which molecular mechanisms this plant might use to protect itself against UV radiation. The identification of genes expressed in response to UV-B irradiation is a useful strategy for investigating the mechanisms underlying the physiological response leading to increased brachycerine accumulation. Suppressive Subtractive Hybridization (SSH), a widely used strategy to identify differentially expressed genes (23), was applied to identify genes of *P. brachyceras* that are related to UV-B response and possibly involved in the brachycerine metabolism. To confirm the UV-B differentially expressed sequences quantitative RT-PCR (qRT-PCR) was carried out.

MATERIALS AND METHODS

Plant materials and UV-B exposure

Psychotria brachyceras Müll Arg. (Rubiaceae) tip cuttings (20 cm long, with four to six leaves per cutting) collected from field-grown shrubs growing at Morro Santana – UFRGS, Porto Alegre, Brazil – were used in the experiments. Voucher specimens are deposited at UFRGS ICN

herbarium (7899). Before the UV-B experiment, cuttings were incubated for 10 days in solution of 0.1X MS salts for adaptation to lab conditions. During the adaptation time, cuttings were incubated in a growth room and illuminated by white fluorescent tubes (Sylvania Luz do Dia Plus, F40W/T10/5000K, Made in China - SX023, CE mixed with Osram Universal 40W, KOB8A 6, Osasco, SP, Brazil) with a 16:8h day: night regimen (photosynthetic active radiation-PAR of approximately 73 μmol m^{-2} s^{-1}), at 25±3°C. For UV-B treatment, cuttings were exposed for 24 h to an UV-B lamp (UVB-313EL, Q-Lab, Ohio, USA, max. wavelength 313 nm) with 68.89 KJ m^{-2} day^{-1} of UV-B radiation (39.37 KJ m^{-2} day^{-1} biologically effective radiation [24], corresponding to the UV-B radiation that reached the cuttings) plus a white light lamp (PAR of approximately 73 μmol m^{-2} s^{-1}). Control plants were maintained in white light only with a 16 h photoperiod. After UV-B radiation treatment, leaves (20 replicates for both control and UV-B treatment, with 4 leaves in each one) were collected, immediately frozen in liquid nitrogen, and stored at -80 ºC until RNA isolation.

Total RNA extraction and first strand cDNA synthesis

Total RNA was prepared using NucleoSpin RNA Plant Kit (Macherey-Nagel) including DNAse I treatment, according to the manufacturer recommendations. Fractions of 100 mg, corresponding to each replicate of 4 leaves, had their RNA extracted separately. After extraction, the corresponding fractions (total of 20 fractions for both control and UV-B treatment) were pooled for RNA quantification. Total RNA concentration was measured using Quant-iT™ RNA Assay Kit and the Qubit fluorometer (Invitrogen), whereas nucleic acid quality was checked by 1% agarose gel electrophoresis (data not show). Complementary DNA (cDNA) was prepared using the SMART PCR cDNA Synthesis Kit by Clontech Laboratories (Mountain View, CA, USA), in presence of RNase OUT, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was performed with reverse transcriptase (M-

MLV, Invitrogen, Carlsbad, CA, USA) using 1.0 μg of total RNA from each condition (control or UV-B treatment).

Construction of subtractive cDNA libraries, DNA sequencing and sequence data analysis

Suppression subtractive hybridization (SSH) was carried out between UV-B-treated leaves ('tester') and control leaves ('driver') using the PCR-Select cDNA Subtraction Kit, according to the manufacturer's instructions (Clontech, Mountain View, CA, USA). Second strand cDNA was prepared using first-strand cDNA as template. After digestion with Rsa I, tester and driver cDNAs were ligated to adaptors. Hybridization and PCR amplification were carried out in two rounds to enrich for differentially expressed sequences. After the last PCR of SSH, 60 ng of the subtracted cDNAs was purified and cloned into the pCR2.1-TOPO Vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA). Electrocompetent cells of *Escherichia coli* XL1 Blue were transformed with the ligated products and individual colonies were picked and grown in 96-well plates. A total of 150 clones were sequenced using the ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems®). All of the inserted sequences were checked for similarity using BLASTX program against the GenBank non-redundant protein sequence database.

PCR primer design

Prior to the SSH results confirmation, primers were designed for conventional PCR amplification of possible reference genes for *Psychotria brachyceras*. A set of reference genes for *P. brachyceras* under the studied condition (UV-B exposure) was determined with GeNorm and NormFinder software based on suitable reference genes described for *Coffea arabica*, *Arabidopsis thaliana* and *Oryza sativa* (25-27). Eight genes were chosen and tested for use in *P. brachyceras*: actin, elongation factor-1 alpha, glucose-6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, polyubiquitin 10, protein phosphatase 2A, tubulin, and yellow-leaf-specific protein 8 gene. Alignments with sequences from other plants for each of

these genes, that are available at the NCBI database, were used to design new primer pairs based on the most conserved areas (Table 1). PCR was composed of an initial denaturation step of 1 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C, and a final step of 5 min at 72°C. Platinum® Taq DNA Polymerase (Invitrogen) was used as DNA polymerase, and cDNA first strand from SSH RNA as template. The resulting amplicons were visualized in 1% agarose gel under UV light. Fragments with expected size were purified from the agarose gel with GFX PCR DNA and Gel Band Purification kit (GE Healthcare, formerly Amersham Biosciences), quantified, cloned, transformed into *E. coli*, and sequenced as previously described in subsection 4.3. The resulting sequences (all of them specific to *P. brachyceras*) from the conventional PCR (reference genes) as well as from SSH (differential expressed sequences) were used to design primers (Tables 2 and 3, respectively) to be used in the confirmation of SSH results by quantitative RT-PCR (qRT-PCR). Primer pairs for qRT-PCR were designed using Oligo Perfect™ Designer software (Invitrogen) for each gene. The primer specification ranges included: melting temperatures (Tm) of 59-61°C, primer lengths of 20-22 nucleotides, guanine-cytosine contents of 45-55%, and PCR product lengths of 100-150 base pairs.

>Table1<

>Table2<

>Table3<

Quantitative RT-PCR and data analysis

Independent cDNA synthesis was performed for all of the samples starting from 100 ng of total RNA. All qRT-PCR procedures were carried out as described by Almeida et al. (2010) (28). Each plate was repeated three times in independent runs for all reference and SSH selected genes. Determination of reference genes expression stability was performed using the GeNorm and NormFinder software as previously described (28). Gene expression was evaluated by the $2^{-\Delta CT}$

method (29). For each sample, a ΔC_T value was calculated by subtracting the reference gene C_T value from the C_T of the gene under evaluation. Three true biological replicate samples were used to generate each data point.

Statistical Analyses

The relative expression profile of SSH selected genes between UV-B and control condition was compared by *t* test ($P \leq 0.05$) using the SPSS Base 12.0 for Windows (SPSS Inc., USA).

RESULTS AND DISCUSSION

Considerations on brachycerine responses to UV-B radiation

Most vascular plants rely on inducible defenses to cope with increased UV-B radiation reaching the Earth's surface, which has become an important environmental issue due to the thinning of the stratospheric ozone layer by human generated pollutants. When exposed to higher UV-B levels, plants deploy several defensive metabolic pathways (30). These defense mechanisms include production of reactive oxygen scavenging compounds and enzymes (31-32), UV-absorbing molecules such flavonoid derivatives and their biosynthetic machineries (33-34), pathogenesis-related defense proteins (35), and initiation of DNA repair pathways (36). There are evidences suggesting that brachycerine may play a role as a protective molecule against UV-B: it was increased in cuttings of *P. brachyceras* upon UV-B radiation exposure (21); it absorbs within UV-B range of the spectrum (21); and it displays antioxidant activities against singlet oxygen, hydroxyl radicals and superoxide anion (21-22, Porto and Fett-Neto, unpublished results). Therefore, this alkaloid may act as a scavenger of ROS and/or UV shield in *P. brachyceras*.

A significant increase of brachycerine concentration in *P. brachyceras* leaves occurred at the third day of UV-B exposure, but because the molecular events involved in the increase of the

alkaloid must occur earlier, we chose the time of 24 hours to analyze the expression of genes possibly involved in this increase.

SSH library

The SSH technique was chosen to identify genes differentially expressed under specific condition (UV-B exposure) because it allows the identification of genes without prior knowledge of the genome of the studied species. SSH is a useful strategy both to identify abundant differentially expressed genes and to enrich for low expression genes. The technique relies on the generation of libraries of differentially expressed clones obtained by subtracting tester cDNA (*e.g.* UV-B treated) with an excess of driver cDNA (*e.g.* white light as control condition) (23, 37). A total of 150 clones were sequenced. Alignments were performed using BLASTX against the GenBank non-redundant protein sequence database. A maximum restrictive cut-off of E-value (equal to e-05) was applied and 134 sequences (size ranging from 200 to 900 base pairs) were selected (Supplementary Table 1). Among these, 119 different sequences were identified (corresponding to 119 putative genes), and only 10 out of these 119 sequences appeared more than once in the SSH library (maximum redundancy= 4.5%, corresponding to ACC oxidase gene). The 119 sequences obtained were grouped into 7 functional categories: molecular (20.2%), enzymatic (25.2%), structural (9.2%), transport (3.4%), alkaloid metabolism (7.6%), hypothetical (2.5%), and unknown (31.9%) functions. Considering the number of sequences and the low redundancy obtained, we assume that SSH was a suitable and efficient method to find differentially expressed sequences in the studied condition, even without previous information about the genome of *P. brachyceras*, as also reported by recent studies with other plant species (6, 38-40).

Determination of reference genes

Amplification by conventional PCR for 5 out of the 8 genes selected as possible reference

genes for *P. brachyceras* in the studied condition (UV-B) generated products with expected size (see subsection 4.4 in Materials and Methods section). Sequencing and BLASTX results of these amplicons confirmed their identity with the corresponding genes in other plant species. The 5 sequences corresponding to fragments of the amplified genes specific of *P. brachyceras* were submitted to the GenBank database as follows: actin (*Act*) (JG017739), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (JG017740), protein phosphatase 2A (*PP2A*) (JG017741), tubulin (*Tub*) (JG017743), and yellow-leaf-specific protein 8 (*YLS8*) (JG017742) genes. After qRT-PCR, suitable parameters to choose the best reference genes to be used for the normalization of the transcription levels of the selected genes from SSH library were calculated using the GeNorm and NormFinder softwares and analyzed as previously described (28).

The average expression stability (M value) of all genes and the pairwise variation, which evaluates the optimum number of reference genes for reliable normalization, were calculated by geNorm (version 3.5). Although geNorm indicates an M value threshold of 1.5 for identifying stably expressed genes, a maximum value of 0.5 has been proposed for achieving more accurate results (41-42). Our results indicated that *Act* and *GAPDH* were the most stably expressed genes (i.e., with the lowest M value = 0.109) and *Tub* was the least (M value = 0.703) (Table 4). Based on geNorm results *Act* and *GAPDH* are adequate for accurate normalization in *P. brachyceras* in the studied condition (43).

NormFinder, another software for gene expression data analyses, considers most stable the gene with least intergroup variation (44). Both *Act* and *GAPDH* were shown to be suitable reference genes in several plant species (25-27), and this was also the case in this study (Table 4). Therefore *Act* and *GAPDH* were used as reference genes to evaluate the transcription levels of the genes of interest (SSH) comparing untreated and UV-B exposed *P. brachyceras* cuttings. The observed transcription profiles of the genes of interest (discussed in the next subsection) were similar using both reference genes (results are shown relative to *Act* expression, Fig. 1).

>Table4<

Confirmation of differentially expressed sequences in response to UV-B

Nine sequences, corresponding to 9 putative genes, possibly related to brachycerine metabolism were selected for further confirmation of their up-regulation by UV-B exposure using qRT-PCR (Table 5). Five out of these 9 selected genes corresponding to the isolated sequences from *P. brachyceras* had significantly increased transcription ($P<0.05$) in response to UV-B (Fig. 1), and were submitted to the GenBank database under the following accession numbers: tryptophan decarboxylase (JG017744) (Fig. 1A), UDP-glucose glucosyltransferase (JG017745) (Fig. 1B), lipase (JG017747) (Fig. 1C), ACC oxidase (JG017746) (Fig. 1D), and serine/threonine protein kinase (JG017748) (Fig. 1E). Amplification was not consistent with primers used for the other 4 sequences.

>Table5<

>Figure1<

There are only a few reports about UV-induction of alkaloid biosynthesis. The model plant for monoterpene indole alkaloids (MIAs) studies, *C. roseus*, synthesizes over 100 different MIAs, several of them up-regulated by UV-B treatment (16,45). This accumulation seems to be a consequence of higher expression of the tryptophan decarboxylase (*TDC*) gene, which encodes the enzyme that produces tryptamine, the indole precursor to alkaloid synthesis (16). In the present study, the up-regulation of *TDC* in leaves of *P. brachyceras* upon UV-B exposure was also observed (Fig. 1A), indicating that TDC might participate in brachycerine biosynthesis induced by this abiotic stress. The UV-B up-regulation of UDP-glucose glucosyltransferase gene (Fig. 1B) might also be related to brachycerine biosynthesis, since this alkaloid retains a glucose residue, a peculiar feature of its structure (19-20).

The involvement of jasmonic acid (JA) in UV-B responses was shown in previous reports (46-47), including its participation in a proposed model for signal transduction during UV-B irradiation of *A. thaliana* plants (30). The induction of lipase gene in UV-B exposed *P. brachyceras* (Fig. 1C) might indirectly contribute for brachycerine biosynthesis, acting in the

production of JA or related molecules previously shown to be involved in brachycerine accumulation (19).

The transcription of ACC oxidase gene was increased in UV-B exposed *P. brachyceras* (Fig. 1D). ACC oxidase catalyzes the last step in the biosynthesis of the plant hormone ethylene (48), which has also been shown to be involved in UV-B responses and as a signaling molecule in signal cascades during UV-B irradiation in *A. thaliana*, leading to altered expression of defense genes against UV-B (30,46). Therefore, up-regulation of ACC oxidase gene in *P. brachyceras* may be involved in a defense response against UV-B that could include brachycerine induction at some point.

Finally, the induction of serine/threonine kinase gene, probably only one representative of the members of this class of enzymes, in *P. brachyceras* in response to UV-B exposure (Fig. 1E) might be related to phosphorylation cascades that occur in response to UV-B, perhaps involving the phosphorylation of transcription factors, the activity of which may result in activation of TIA biosynthetic genes, such as *TDC* (30,45).

Based on the up-regulated genes and the pathways that they may participate, it is suggested that these molecular events might contribute for the brachycerine accumulation in *P. brachyceras* in response to UV-B. Upon UV-B irradiation, ROS levels as well as JA and ethylene signaling components increase in plant cells initiating a defense response that includes induction of genes possibly related to brachycerine metabolism: tryptophan decarboxylase, UDP-glucose glucosyltransferase, lipase, ACC oxidase, and serine/threonine protein kinase gene). Brachycerine can protect the plant against UV-B scavenging ROS and acting as a shield (21-22).

CONCLUSION

This study is the first significant step to understanding the response of *P. brachyceras* to UV-B irradiation at the transcriptional level. Our results indicate that the induction of the alkaloid glucoside brachycerine by UV-B exposure likely occur at the level of mRNA. A potential

involvement of brachycerine in UV-B defense is suggested, however functional characterization studies of the genes up-regulated by UV-B in *P. brachyceras* leaves are needed to confirm their role in the metabolism of brachycerine.

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Figure legend

Figure 1. Relative expression profile (Actin as reference gene) of SSH selected genes after 24 hours of UV-B irradiation on leaves of *P. brachyceras*. **A)** tryptophan decarboxylase gene. **B)** UDP-glucose glucosyltransferase gene. **C)** lipase gene. **D)** ACC oxidase gene. **E)** serine/threonine kinase gene.

Untreated columns refer to relative gene expression in *P. brachyceras* leaves 24 hours exposed to white light only (control condition).

*: indicates significant difference between UV-B and control condition by *t-test* ($P \leq 0.05$). **: indicates significant difference between treatments by *t-test* ($P \leq 0.01$).

Table 1. Primer sequences for conventional PCR and product sizes for each of the 8 possible reference genes of *Psychotria brachyceras*.

Gene Symbol	Gene Name	Primer sequence (5'-3') Forward/Reverse	Amplicon Length (bp)
Act	Actin	TGAGCATGGTATTGTCAGCA/ ACGAAGGATGGCATGTGGA	300
EF1a	Elongation factor-1 alpha	ACTGGTACAAGGGCCCAAC/ TGACCTGGGAGGTGAAGTTG	372
G6PD	Glucose-6-phosphate dehydrogenase	GGACAAGGGTCATTGTTGAGA/ CTGACAGGCGTCTCCATTGC	343
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	ATCGGTATCAACGGATTTGG/ ACCACCCTTCAAGTGAGCAG	336
UBQ10	Polyubiquitin 10	GGACCAGCAGAGGTTGATCT/ CACCAAGTGAAGGGTGGACT	325
PP2A	Protein phosphatase 2A	GGCGTGTTCGCTATATGGTT/ GCATGCAAAGAGCACCAAG	564
Tub	Tubulin	GTGGCTGTGCTTCTTGACAAT/ GGCACCAGTCAACAAACTGA	445
YLS8	Yellow-leaf-specific protein 8	GAAACTTGCATGCAGATGGA/ TGGAGTAGTCTTTGGGAGCAA	307

Table 2. Primer sequences for qRT-PCR amplification and product sizes for each of the 5 reference genes of *Psychotria brachyceras*.

Gene Symbol	Gene Name	Primer sequence (5'-3') Forward/Reverse	Amplicon Length (bp)
Act	Actin	CTTAATCCCAAGGCCAACAG/ AAAGGACAGAACGGCCTGA	120
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	TCAAGGTCTTCGGAGTCAGG/ GCAGCCTTGTCCTTGTCAGT	106
PP2A	Protein phosphatase 2A	CTGCCTTGGCATCAGTCATA/ CCGAACATCAGGGAACTCAT	116
Tub	Tubulin	ATTCCAGACCAACCTTGTGC/ TGGTGATCTCCGCAACAGTA	116
YLS8	Yellow-leaf-specific protein 8	ATCTTGGCACTGGGAACAAC/ TTGGGAGCAATAACCAGACC	124

Table 3. Primer sequences for qRT-PCR amplification and product sizes for each of the 5 genes of interest of *Psychotria brachyceras*.

Gene Symbol	Gene Name	Primer sequence (5'-3') Forward/Reverse	Amplicon Length (bp)
TDC	Tryptophan decarboxylase	AGATGTTCGAAGGGTTCGTG/ TCCACCAACAATTCACTGGA	118
ACCox	ACC oxidase	TTTCCAGAAGTCCTGGTGCT/ TACAAAAGCGTGCTCCACAG	118
UDP-Gluc	UDP-glucose glucosyltransferase	GCTCAAGGAAGCAAAGGATG/ TTGTGAGTGCCGATGTGATT	140
Ser/Thre	Serine/threonine kinase	CGCAAGGCTCTTTTGGTTTA/ TGTATCGAGGGGGAACAAAG	103
Lip	Lipase	TGTGAGAATCCAGGGGACTATT/ AAAATGCGAAGCTGGAAGAG	146

Table 4. Ranking of candidate reference genes for *Psychotria brachyceras* in decreasing order of expression stability calculated by *geNorm* and *NormFinder*.

Ranking Order	***geNorm*** **(M value)**	***NormFinder*** **(Stability value ± EB*)**
1	*Act/GAPDH* (0.109)	*GAPDH* (0.261 ± 0.008)
2	*PP2A* (0.154)	*PP2A* (0.307 ± 0.092)
3	*YLS8* (0.258)	*Act* (0.332 ± 0.059)
4	*Tub* (0.703)	*YLS8* (0.346 ± 0.003)
5		*Tub* (0.954 ± 0.779)

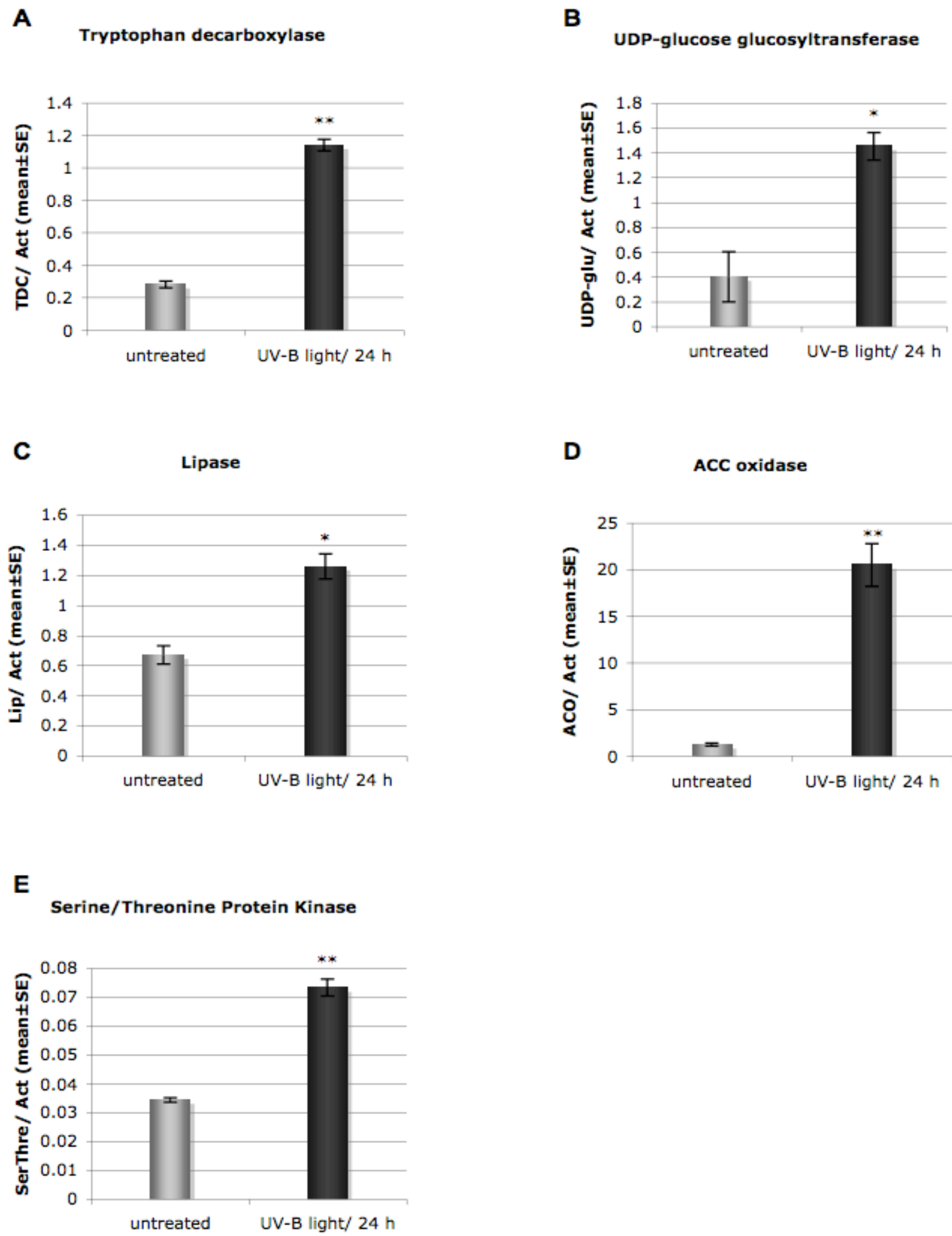
*EB= error bars

Table 5. BLASTX results of selected up-regulated genes in leaves of *Psychotria brachyceras* after 24 hours of UV-B exposure.

Functions	**Description**	**Species**	**GenBank accession Number**	**Clone redundancy (%)**	**BLASTX (E value)**
Ethylene production	ACC oxidase 1*	*Ziziphus jujuba*	ABW91146.1	6 (4.48)	2e-62
Oxidoreductase activity, acting on sulfur group of donors, disulfide as acceptor; response to hydrogen peroxide	Thioredoxin m	*Populus trichocarpa*	XP_002313121 .1	1 (0)	3e-18
Indole alkaloid biosynthesis	Tryptophan decarboxylase*	*Camptotheca acuminata*	AAB39708.1	1 (0)	1e-50
Chorismate biosynthetic process; Phenylalanine, tyrosine and tryptophan biosynthesis	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (2-dehydro-3-deoxyphosphohepto-nate aldolase)	*Morinda citrifolia*	CAA75092.1	1 (0)	2e-14
Metabolic process, protein amino acid phosphorylation	Serine/threonine-protein kinase cx32, putative*	*Ricinus communis*	EEF35235.1	1 (0)	8e-36
Oxidoreductase activity; response to abscisic acid stimulus, desiccation and salt stress	Aldehyde dehydrogenase family 7 member A1	*Euphorbia characias*	AAX09646.1	1 (0)	2e-26
Alkaloid biosynthesis (vindoline in *C. roseus*)	Desacetoxyvindoline 4-hydroxylase, putative	*Ricinus communis*	EEF33073.1	1 (0)	6e-33
Transferase activity, transferring glycosyl groups	UDP-glucose glucosyltransferase*	*Catharanthus roseus*	BAD29721.1	1 (0)	2e-28
Lipid metabolic process	Lipase SIL1*	*Brassica rapa* subsp. *pekinensis*	AAM47031.1	1 (0)	3e-10

Sequences were obtained from SSH experiments and differential expression of genes marked with an asterisk(*) was confirmed by quantitative RT-PCR. A complete list of all genes obtained from the SSH experiments is shown in Supplementary Table 1.

Figure 1.



IV. Discussão geral

1. Resultados referentes à expressão gênica relacionada ao aumento de braquicerina em resposta à radiação UV-B em *Psychotria brachyceras*

1.1 Resultados anteriores

A diminuição da camada de ozônio está aumentando a incidência de UV-B na superfície da Terra, particularmente em altas latitudes (Hollósy, 2002). A maioria das plantas vasculares não é adaptada para lidar com esse aumento do fluxo de radiação UV-B, empregando mecanismos de defesa à UV-B constantemente ativos. Quando expostas a níveis de radiação UV-B superiores aos mais frequentes em seu ambiente, as plantas valem-se de uma série de rotas metabólicas de defesa diferentes (Brosché & Strid, 2003). Dentre esses mecanismos de defesa estão compostos e enzimas antioxidantes que detoxificam ROS (Strid, 1993; Willekens et al., 1994); moléculas que absorvem radiação UV, tais como derivados de flavonóides (Chapell & Hahlbrock, 1984; Demkura et al., 2010; Izaguirre et al., 2007); proteínas de defesa relacionadas à patogênese (Surplus et al., 1998); e mecanismos de reparo de DNA (Ries et al., 2000).

Trabalhos anteriores publicados por nosso grupo de pesquisa mostraram que a concentração de braquicerina aumentou em estacas de *P. brachyceras* expostas à radiação UV-B, e que esse alcalóide apresentou atividade antioxidante contra oxigênio singleto, radical hidroxila, e ânion superóxido (Gregianini et al., 2003; Nascimento et al., 2007; Porto, 2009). Assim, a braquicerina pode ser considerada uma molécula do repertório de defesas ativadas em resposta ao aumento de irradiação UV-B em *P. brachyceras*, atuando na detoxificação de ROS. Como o aumento de braquicerina ocorreu no terceiro dia de exposição das estacas à UV-B, escolhemos o período de 24 horas para avaliar a expressão de genes possivelmente envolvidos no aumento do alcalóide em resposta à UV-B, uma vez que os possíveis eventos moleculares que levam a esse processo devem ser anteriores.

1.2 Resultados do SSH

A técnica de SSH foi escolhida em detrimento de outras abordagens moleculares para identificar genes diferencialmente expressos em condições específicas, porque permite a

identificação de genes sem o conhecimento prévio do genoma das espécies estudadas, como era o caso do genoma de *P. brachyceras* até o presente estudo. Além disso, o SSH é uma ferramenta poderosa para identificar em abundância os genes diferencialmente expressos e também para enriquecer a biblioteca de genes expressos em baixas quantidades (Diatchenko et al., 1996; Sperotto et al., 2009). A técnica é baseada na geração de bibliotecas de clones diferencialmente expressos pela subtração resultante da hibridização da população de cDNAs (DNAs complementares aos RNA mensageiros) da condição estudada (referida como *tester*; e.g., exposição à radiação UV-B) com um excesso da população de cDNAs controle (referida como *driver*; e.g., exposição à luz branca). Nossa biblioteca de SSH foi construída a partir de cDNAs provenientes de folhas de estacas de *P. brachyceras* expostas por 24 horas à radiação UV-B (população *tester*) subtraídos de cDNAs provenientes de folhas expostas por 24 horas à luz branca apenas (população *driver*) (para detalhes de metodologia, consultar *Material and Methods* da seção III).

Um total de 150 clones foi sequenciado. Após o alinhamento das sequências através de BLASTX contra a base de dados de sequências de proteínas do GenBank, identificamos 134 sequências que alinharam com sequências de plantas (Tabela 1, no anexo 1). Dentre essas 134, identificamos 119 sequências (correspondentes a 119 genes putativos), e apenas 10 delas apareceram mais de uma vez na biblioteca, sendo a sequência relativa ao gene da ACC oxidase a de maior redundância (igual a 4,5 %).

As 119 sequências obtidas foram agrupadas em diferentes categorias funcionais: molecular (20,2 %), enzimática (25,2 %), estrutural (9,2 %), transporte (3,4 %), metabolismo de alcalóides (7,6 %), sequências com funções hipotéticas (2,5 %), e sequências com funções desconhecidas (31,9 %) (Figura 1). Considerando o número de sequências obtidas na biblioteca de SSH e a baixa redundância encontrada, podemos confirmar que a técnica de SSH é adequada e eficiente para encontrar genes diferencialmente expressos, até mesmo sem o conhecimento prévio do genoma estudado, assim como foi constatado por estudos recentes com outras espécies (Ban et al., 2007; Li et al., 2009; Park et al., 2007; Sperotto et al., 2009; Zinser et al., 2007).

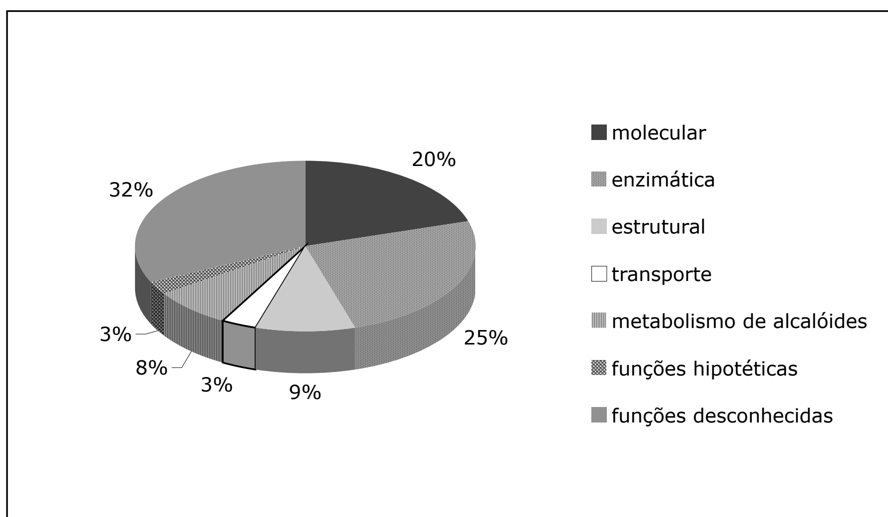


Figura 1. Categorias funcionais das 119 sequências identificadas na biblioteca de SSH de *Psychotria brachyceras* exposta à radiação UV-B.

1.3 Escolha de genes normalizadores para *P. brachyceras*

Um gene normalizador ou de referência, também conhecido como gene de controle interno ou *housekeeping gene*, é considerado ideal se apresenta expressão constitutiva, i.e., se sua expressão é constante na maioria dos tipos celulares, ao longo dos estágios de desenvolvimento, e nos diferentes órgãos do organismo estudado (Almeida et al., 2010). Além disso, sua expressão deve ser considerada inalterada frente a diferentes parâmetros experimentais (Thellin et al., 1999). Genes envolvidos em processos celulares básicos, tais como manutenção da estrutura celular ou metabolismo primário, são frequentemente escolhidos como normalizadores (Czechowski et al., 2005). No entanto, os níveis de transcritos desses supostos genes normalizadores não são sempre estáveis e seu uso sistemático sem prévia validação pode levar à interpretação errada de resultados.

Em função da ausência de informações sobre o genoma de *P. brachyceras*, foi necessária uma busca de genes normalizadores a partir de trabalhos realizados com outras espécies de plantas (para detalhes de metodologia, consultar *Material and Methods* da seção III). Dos 8 genes selecionados como possíveis normalizadores para *P. brachyceras*, 5 apresentaram produtos de tamanho esperado amplificados por PCR convencional. O sequenciamento desses produtos confirmou sua identidade com as sequências originalmente utilizadas para a projeção dos primers (Tabela 1, seção III). Essas sequências foram depositadas no GenBank como ESTs de *P.*

Brachyceras sob os seguintes números de acesso: actina (*Act*) (JG017739), gliceraldeído 3-fosfato desidrogenase (*GAPDH*) (JG017740), proteína fosfatase 2A (*PP2A*) (JG017741), tubulina (*Tub*) (JG017743), e proteína 8 específica da folha amarela (*YLS8*) (JG017742). A partir dessas sequências, foram projetados os primers para a amplificação desses possíveis genes normalizadores por qRT-PCR (Tabela 2, seção III). Após a amplificação por qRT-PCR, os parâmetros adequados para a escolha dos melhores genes normalizadores para a condição estudada foram calculados usando os software GeNorm e NormFinder e analisados segundo descrito por Almeida et al. (2010).

A estabilidade de expressão média (valor de M) de todos os genes e a variação par-a-par, que avalia o número ideal de genes de referência para uma normalização confiável, foram calculadas pelo geNorm (versão 3.5) (Tabela 4, seção III). Segundo o geNorm, o valor de M recomendado deve ser abaixo de 1,5 para identificar genes normalizadores com expressão estável; no entanto, alguns autores propõem que o valor máximo de M seja 0,5 para obter resultados mais precisos e conclusões mais confiáveis (Gutierrez et al., 2008; Hellemans et al., 2007). Nossos resultados indicaram que *Act* e *GAPDH* foram os genes de expressão mais estável (ou seja, com o menor valor de M = 0,109), enquanto *Tub* foi o menos estável (valor M = 0,703) (Tabela 4, seção III). Considerando o valor de corte de 0,15 para a variação par-a-par, proposto por Vandesompele et al. (2002), abaixo do qual a inclusão de um gene normalizador adicional não é necessária, o uso dos dois genes que apresentaram expressão mais estável (*Act* e *GAPDH*) é suficiente para uma normalização correta em *P. brachyceras*, pois o valor calculado foi de V2/3= 0,058.

Segundo a análise pelo NormFinder, o melhor gene normalizador é aquele que apresentar o valor de variação inter-grupo mais próximo de zero, e, ao mesmo tempo, a menor barra de erro padrão possível, ambos calculados pelo próprio programa (Andersen et al., 2004). Após análise, verificamos que *GAPDH* foi o gene mais estável baseado nos parâmetros do NormFinder (Tabela 4, seção III). Estudos mostraram o uso tanto de *Act* como de *GAPDH* como genes normalizadores adequados em diferentes espécies de plantas em diferentes condições estudadas (Barsalobres-Cavallari et al., 2009; Czechowski et al., 2005; Kim et al., 2003). Assim, decidimos utilizar *Act* e *GAPDH* como normalizadores para a análise de expressão de genes de *P. brachyceras* no presente estudo. Os perfis de expressão dos genes de interesse foram semelhantes com ambos os genes normalizadores. Portanto, os resultados nos gráficos são mostrados em relação à expressão de *Act* somente (Figura 1, seção III).

1.4 Confirmação da indução dos genes de interesse pela radiação UV-B

Selecionamos da biblioteca de SSH as 9 sequências de genes supostamente envolvidos com a biossíntese de alcalóides para testar nossa hipótese de que a braquicerina seria induzida pela radiação UV-B conferindo proteção à *P. brachyceras*. O resultado do BLASTX referente às 9 sequências escolhidas é mostrado na tabela 5 da seção III. Projetamos primers para a amplificação dessas sequências por qRT-PCR (Tabela 3, seção III). Analisando os resultados do qRT-PCR, confirmamos a indução da expressão em resposta à UV-B de 5 dos 9 genes selecionados ($P<0,05$). As sequências desses 5 genes, obtidas a partir da biblioteca de SSH, foram depositadas no GenBank como ESTs de *P. brachyceras* sob os seguintes números de acesso: triptofano descarboxilase (JG017744) (Figura 1A, seção III), UDP-glicose glicosiltransferase (JG017745) (Figura 1B, seção III), lipase (JG017747) (Figura 1C, seção III), ACC oxidase (JG017746) (Figura 1D, seção III), e proteína serina/treonina quinase (JG017748) (Figura 1E, seção III).

O conhecimento sobre a biossíntese de alcalóides induzida em resposta à radiação UV é escasso. Um número maior de estudos revela a atuação de compostos antioxidantes na proteção contra a radiação UV-B. Um exemplo de alcalóide indólico com atividade antioxidante que parece proteger contra UV-B é a pitiriacitrina, produzida pela levedura *Malassezia furfur*, a qual é capaz de diminuir o efeito inibitório da UV-B sobre o crescimento da levedura (Mayser et al., 2002). A maior parte das nossas conclusões é baseada na espécie *C. roseus*, planta modelo para o estudo de alcalóides monoterpeno indólicos (MIAs). Essa espécie sintetiza mais de 100 MIAs diferentes, e muitos deles são induzidos pelo tratamento com UV-B (Ouwerkerk et al., 1999; Ramani & Jayabaskaran, 2008). O acúmulo desses alcalóides parece ser consequência da maior expressão do gene *TDC* em resposta à UV-B, o qual codifica a enzima triptofano descarboxilase, responsável pela conversão do triptofano em triptamina, o precursor indólico para a biossíntese de MIAs (Ouwerkerk et al., 1999).

Nós também observamos uma indução do gene *TDC* em folhas de *P. brachyceras* expostas à UV-B (Figura 1A, seção III), indicando que a enzima TDC participa da biossíntese de braquicerina induzida por esse estresse abiótico. A indução do gene que codifica UDP-glicose glicosiltransferase também foi verificada em *P. brachyceras* em resposta à UV-B (Figura 1B, seção III), a qual pode estar relacionada com o fornecimento do resíduo de glicose para a montagem de braquicerina, característica peculiar da estrutura desse alcalóide (Kerber et al., 2001; Gregianini et al., 2004).

Estudos prévios mostraram o envolvimento de ácido jasmônico (AJ) e do fitormônio

etileno em resposta à UV-B (A-H-Mackerness et al., 1999; Conconi et al., 1996), incluindo a participação de ambos em um modelo de transdução de sinal proposto para plantas de *A. thaliana* durante sua irradiação por UV-B (Figura 2, Brosché & Strid, 2003). A indução do gene da lipase em *P. brachyceras* exposta à UV-B (Figura 1C, seção III) pode contribuir indiretamente para a biossíntese de braquicerina, atuando na produção de AJ ou moléculas relacionadas que foram previamente mostradas estando envolvidas no acúmulo do alcalóide (Gregianini et al., 2004). Já a transcrição aumentada do gene de ACC oxidase em resposta à UV-B em *P. brachyceras* (Figura 1D, seção III) pode estar relacionada com a biossíntese do etileno, catalisada pela enzima ACC oxidase, fitormônio envolvido em respostas à UV-B que culminam na expressão alterada de genes de defesa, os quais possivelmente incluem genes de biossíntese de alcalóides (Ramani & Jayabaskaran, 2008).

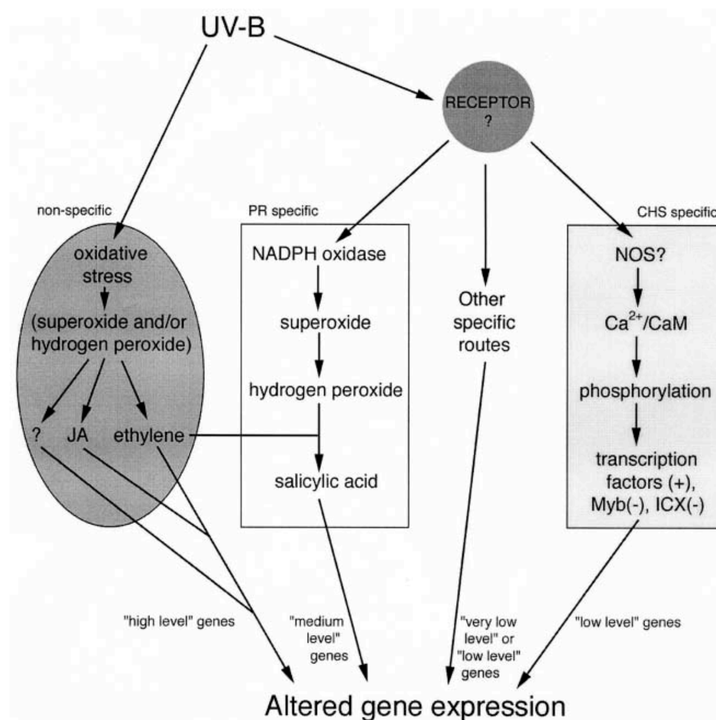


Figura 2. Extraída de Brosché & Strid, 2003. Modelo proposto para a transdução de sinal durante a irradiação de plantas de *Arabidopsis thaliana* por UV-B. O modelo foi construído com base em modelos propostos anteriormente (A-H-Mackerness, 2000; A-H-Mackerness et al., 2001), a partir de resultados de Brosché & Strid (2003), e de resultados de outros grupos (Jenkins, 1997; Schäfer et al., 1997). O modelo indica o possível envolvimento de um fotorreceptor de UV-B na sinalização, bem como de uma via de transdução de sinal em resposta a níveis elevados de UV-B, que causam dano às células vegetais. JA, ácido jasmônico; PR, proteínas relacionadas à patogênese; CHS, chalcona sintase; NOS, óxido nítrico sintase; CaM, calmodulina; MYB e ICX, repressores putativos da expressão gênica.

O último gene selecionado para o qual confirmamos sua indução por UV-B em *P. brachyceras* foi o gene que codifica uma proteína Serina/treonina quinase (Figura 1E, seção III). Sugerimos que a indução desse gene pode estar relacionada com cascatas de fosforilação que ocorrem em resposta à radiação UV-B, especialmente envolvendo a fosforilação de fatores de transcrição cuja atividade pode resultar na ativação de genes biossintéticos de alcalóides indólicos, tais como o gene de *TDC* (Brosché & Strid, 2003; Ramani & Jayabaskaran, 2008).

Reunindo os resultados obtidos do SSH e sua posterior confirmação por qRT-PCR com os resultados prévios do nosso grupo relativos à regulação de braquicerina por UV-B, propomos um modelo para a indução de braquicerina em *P. brachyceras* em resposta à radiação UV-B (Figura 3). Sugerimos que quando *P. brachyceras* é irradiada por altos níveis de UV-B, as concentrações celulares de ROS, bem como as de AJ e etileno, aumentam. Com isso, é iniciada uma resposta de defesa que envolve a indução de genes relacionados ao metabolismo de braquicerina, tais como os genes de UDP-glicose glicosiltransferase, lipase, ACC oxidase, e serina/treonina quinase, incluindo o gene que codifica a enzima-chave da biossíntese de alcalóides indólicos, triptofano descarboxilase (TDC), levando ao aumento do acúmulo de braquicerina. A braquicerina protege a planta contra UV-B absorvendo esta radiação e detoxificando ROS, evitando possíveis danos causados por esses radicais livres (Gregianini et al., 2003; Nascimento et al., 2007; Porto, 2009).

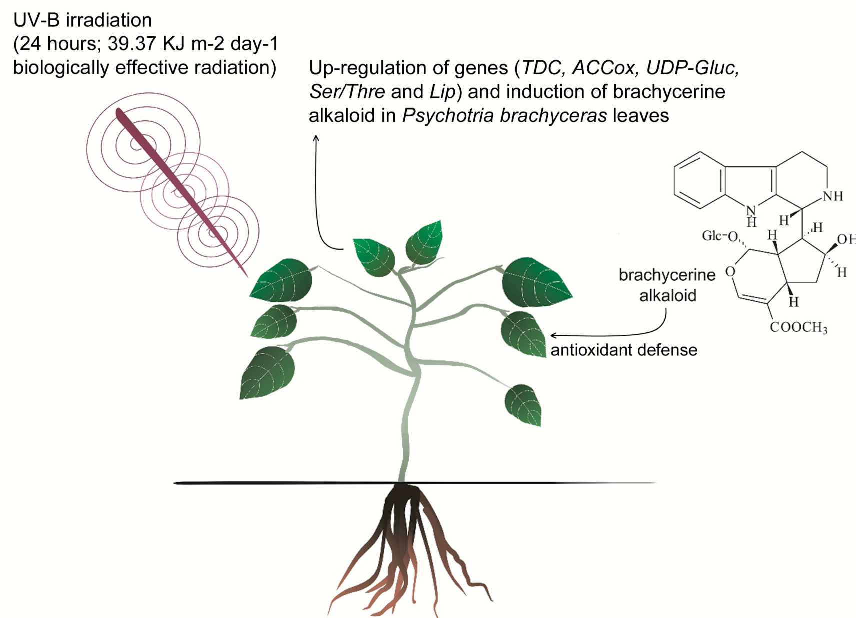


Figura 3. Esquema representativo da indução de braquicerina em folhas de *Psychotria brachyceras* expostas à UV-B: na presença de UV-B, os níveis de espécies reativas de oxigênio (ROS) (*e.g.* peróxido de hidrogênio, ânion superóxido) e dos sinalizadores celulares ácido jasmônico e etileno aumentam na célula vegetal. Esses eventos são parte das respostas de defesa de *P. brachyceras* que incluem a expressão de genes possivelmente relacionados à transdução de sinal e biossíntese de alcalóide [triptofano

descarboxilase (*TDC*), ACC oxidase (*ACCox*), UDP-glicose glicosiltransferase (*UDP-Gluc*), serina/treonina quinase (*Ser/Thre*) e lipase (*Lip*)], os quais levam ao aumento de braquicerina. Braquicerina protege a planta contra os danos da radiação UV-B detoxificando ROS (radical hidroxila e ânion superóxido) e possivelmente atuando como um "escudo" já que essa molécula absorve no espectro da radiação UV-B.

V. Considerações finais

Foi construída uma biblioteca de SSH feita a partir de folhas de *P. brachyceras* expostas à radiação UV-B. Pela primeira vez, acessamos informações sobre o genoma dessa espécie, disponibilizando 10 sequências inéditas no banco de dados do GenBank. Também pela primeira vez, mostramos que a indução de braquicerina em resposta à UV-B é regulada, pelo menos parcialmente, em nível de mRNA. Os nossos resultados reunidos sugerem fortemente um potencial envolvimento da braquicerina na defesa contra a radiação UV-B em *P. brachyceras*.

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Anexo 1:

Tabela 1. Resultado do alinhamento por BLASTX das 134 sequências de genes vegetais identificadas na biblioteca de SSH de folhas de *Psychotria brachyceras* expostas por 24 horas à radiação UV-B.

Functions	Description	Species	Acession number	Clone redundancy (number of copies)	BlastX (E value)
DNA binding; nucleosome assembly	Hystone H3	*Zea mays*	ACG27450.1	1	3e-34
Binding protein	Ubiquitin fusion protein	*Vitis vinifera*	XP_002282071	1	3e-31
Ethylene production	ACC oxidase ACC oxidase 1	*Ziziphus jujuba*	ABW91146.1	6	2e-62
Purine nucleotide biosynthetic process	SAICAR synthetase (succinoaminoimidazole-carboximide ribonucleotide synthetase)	*Nicotiana tabacum*	AAR06292.1	1	9e-50
Rho GTPase activator activity; signal transduction	Rho GTPase activator, putative	*Ricinus communis*	EEF51594.1	2	8e-18
Senescence	Putative senescence-associated protein	*Trichosanthes dioica*	ABN50029.1	1	6e-14
Hypothetical function	Hypothetical protein	*Vitis vinifera*	CAN65763.1	1	3e-25
Response to heat	Hsp20.1 protein	*Solanum peruvianum*	CAA12387.1	2	1e-28
Methyltransferase	S-adenosylmethionine-dependent methyltransferase, putative	*Ricinus communis*	EEF29862.1	2	3e-06
Oxidoreductase activity, acting on sulfur group of donors, disulfide as acceptor; response to hydrogen peroxide	Thioredoxin m	*Populus trichocarpa*	XP_002313121	1	3e-18
Molecular	Pre-mRNA cleavage factor im, 25kD subunit, putative	*Ricinus communis*	EEF51404.1	1	9e-16
Binding protein	Nucleic acid binding protein, putative	*Ricinus communis*	EEF33857.1	2	7e-40
Response to heat	Chaperone	*Agave*	ABF61872.1	1	1e-19

		tequilana			
Lipid metabolic process	Lipase SIL1	*Brassica rapa subsp. pekinensis*	AAM47031.1	1	3e-10
Oxidative phosphorylation	NADH dehydrogenase subunit 2	*Arabidopsis thaliana*	ABS50624.1	1	6e-05
Translation release factor activity	PEL1 (PELOTA); translation release factor	*Arabidopsis thaliana*	NP_194495.3	1	6e-58
Elongation cycle during protein biosynthesis	Elongation factor 1-gamma	*Oryza sativa Japonica Group*	ACA50518.1	2	1e-23
Glycine hydroxymethyl-transferase activity	Serine hydroxymethyltransferase	*Populus tremuloides*	ABO61384.1	2	7e-19
Ferric iron binding	Ferritin	*Conyza canadensis*	CAH05075.1	1	2e-16
AMP deaminase activity	FAC1 (embryonic factor 1); AMP deaminase	*Arabidopsis thaliana*	NP_565886.1	1	7e-15
Hydrolase activity; response to salt stress	Putative dienelactone hydrolase family protein	*Salvia miltiorrhiza*	ABR92334.1	1	4e-19
Structural constituent of ribosome; translation	40S ribosomal protein S3a-like protein	*Solanum tuberosum*	ABB72801.1	1	1e-22
Molecular function; leaf morphogenesis, ubiquitin-dependent protein catabolic process	RPN8	*Nicotiana benthamiana*	ABB18116.1	1	1e-42
Hydrolase activity; metabolic process	Hydrolase, putative	*Ricinus communis*	EEF27950.1	2	6e-14
Hypothetical function	Conserved hypothetical protein	*Ricinus communis*	EEF31901.1	1	7e-58
ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	Phospholipid-transporting ATPase, putative	*Ricinus communis*	EEF42234.1	1	2e-49
Protein binding; signal transduction	Leucine-rich repeat family protein	*Arabidopsis thaliana*	NP_187250.1	1	4e-14
Indole alkaloid biosynthesis	Tryptophan decarboxylase	*Camptotheca acuminata*	AAB39708.1	1	1e-50

L-aspartate:2-oxoglutarate aminotransferase activity; nitrogen compound metabolic process	Aspartate aminotransferase	*Lotus japonicus*	CAA63894.1	1	8e-09
Biological process; DNA binding	tbp-associated factor taf, putative	*Ricinus communis*	EEF34770.1	1	3e-08
Hypothetical function	Conserved hypothetical protein	*Ricinus communis*	EEF45017.1	1	4e-40
Oxidative phosphorylation	Cytochrome P450 like_TBP	*Nicotiana tabacum*	BAA10929.1	1	1e-46
Inorganic ion transport and metabolism	Porin/voltage-dependent anion-selective channel protein	*Populus trichocarpa*	XP_002329628	1	8e-53
Protein binding; biological process	Protein binding	*Arabidopsis thaliana*	NP_194285.2	1	6e-17
Structural constituent of ribosome; translation	Putative 23S ribosomal RNA	*Vigna unguiculata*	CAO02532.1	3	7e-23
Structural constituent of ribosome; translation	60S ribosomal protein L8, putative	*Ricinus communis*	EEF46635.1	1	6e-56
First enzyme of general phenylpropanoid pathway; response to wounding, defense response	Phenylalanine ammonia lyase	*Robinia pseudoacacia*	ACF94716.1	1	2e-45
Cysteine biosynthesis	Serine O-acetyltransferase 1	*Glycine max*	AAR31185.1	1	8e-28
Structural constituent of cytoskeleton	Actin, macronuclear, putative	*Ricinus communis*	EEF30627.1	1	6e-54
Structural constituent of ribosome; translation	60S ribosomal protein L23	*Zea mays*	ACG48540.1	1	6e-18
Hydrolase activity; metabolic process	Hydrolase	*Arabidopsis thaliana*	NP_566047.1	1	2e-17
Protein binding; protein transporter activity	Putative adapitin protein	*Ipomoea trifida*	AAS79593.1	1	7e-16
Glucose metabolic process	Glucose-6-phosphate dehydrogenase	*Arabidopsis thaliana*	NP_563844.1	1	9e-29
Chorismate biosynthetic process; Phenylalanine, tyrosine and tryptophan biosynthesis	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (2-dehydro-3-deoxyphosphoheptonate aldolase)	*Morinda citrifolia*	CAA75092.1	1	2e-14

Lysine decarboxylase	Carboxy-lyase, putative	*Ricinus communis*	EEF52702.1	1	7e-56
Sructural constituent of ribosome; translation	40S ribosomal protein S25-1, putative	*Ricinus communis*	EEF49429.1	1	3e-17
Protein binding	Ring finger protein, putative	*Ricinus communis*	EEF35887.1	1	6e-09
Water channel activity; response to water deprivation, water transport	Aquaporin, MIP family, PIP subfamily	*Populus trichocarpa*	XP_002336348	1	1e-19
Cell wall macromolecule catabolic process	Endochitinase MCHT-2	*Cucumis sativus*	ABK55751.1	1	4e-39
Molecular function	Ubiquitin-protein ligase, putative	*Ricinus communis*	EEF44763.1	1	8e-31
Transporter activity; phosphate transport	Phosphate abc transporter, putative	*Ricinus communis*	EEF40243.1	1	2e-10
Splicing, ribosome biogenesis and RNA degradation	Dead box ATP-dependent RNA helicase, putative	*Ricinus communis*	EEF41044.1	1	2e-08
Structural constituent of ribosome; translation	60S ribosomal protein L13A (RPL13aA)	*Arabidopsis thaliana*	NP_001030654	1	2e-13
Acridone alkaloid biosynthesis	Anthranilate N-methyltransferase	*Ruta graveolens*	ABI93949.1	1	2e-20
Metabolic process, protein amino acid phosphorylation	Protein kinase, putative	*Ricinus communis*	EEF41091.1	1	6e-18
Metabolic process, protein amino acid phosphorylation	Serine/threonine-protein kinase cx32, putative	*Ricinus communis*	EEF35235.1	1	8e-36
Hydrolase activity, protein serine/threonine phosphatase activity	Protein serine/threonine phosphatase	*Arabidopsis thaliana*	ATFYPP3	1	1e50
Structural constituent of ribosome; translation	60S ribosomal protein L34	*Zea mays*	ACG30531.1	2	1e-10
Glutamate-ammonia ligase activity; nitrate assimilation	Glutamine synthetase isoform GS1b	*Triticum aestivum*	AAZ30058.1	1	8e-11
Transcription factor activity; defense response	WRKY6	*Nicotiana attenuata*	AAS13440.1	1	6e-10
RNA binding	Heterogeneous nuclear ribonucleoprotein 27C, putative	*Ricinus communis*	EEF34049.1	1	1e-08
Metabolic process, protein amino acid phosphorylation	Adenosine kinase, putative	*Ricinus communis*	EEF30697.1	1	3e-48

Oxidoreductase activity	Formate dehydrogenase, putative	*Ricinus communis*	EEF44880.1	1	6e-18
Transporter	Oligopeptide transporter, putative	*Ricinus communis*	EEF37056.1	1	2e-45
Metabolic process, protein amino acid phosphorylation	Putative adenosine kinase	*Populus alba x Populus tremula*	AAS00533.1	1	2e-36
Structural constituent of ribosome; translation	Ribosomal protein L15	*Elaeis guineensis*	ACF06564.1	1	4e-13
Tansferase activity, transferring glycosyl groups; carbohydrate biosynthetic process	Galactinol synthase	*Capsicum annuum*	ABQ44212.1	1	1e-19
Transcription factor	GRAS transcription factor	*Capsicum annuum*	ABB54445.1	1	2e-39
Protein binding, ubiquitin-protein ligase activity; ubiquitin-dependent protein catabolic process	Ubiquitin ligase SINAT5, putative	*Ricinus communis*	EEF49577.1	1	2e-50
Molecular function; splicing factor-related protein	YT521-B-like family protein	*Arabidopsis thaliana*	NP_974537.1	1	8e-15
Binds calcium; phosphatase	Annexin p35	*Lycopersicon esculentum*	AAC97493.1	1	5e-06
Calmodulin-dependent protein kinase activity; protein amino acid phosphorylation	regulator of gene silencing	*Nicotiana tabacum*	AAK11255.1	1	7e-08
ATP synthase	ATP synthase beta subunit	*Durandea pentagyna*	AAX62969.1	1	8e-28
Transferase activity, transferring glycosyl groups	UDP-glucose glucosyltransferase	*Catharanthus roseus*	BAD29721.1	1	2e-28
Structural constituent of ribosome; translation	60S ribosomal protein L7, putative	*Ricinus communis*	EEF45058.1	1	8e-08
Posttranscriptio-nal gene silencing	RNA-directed RNA polymerase	*Nicotiana tabacum*	CAA09697.1	1	1e-31
Oxidoreductase activity; response to abscisic acid stimulus, desiccation and salt stress	Aldehyde dehydrogenase family 7 member A1	*Euphorbia characias*	AAX09646.1	1	2e-26
Cell wall macromolecule catabolic process	Chitinase	*Helianthus annuus*	ABO26878.1	1	2e-12

Alkaloid biosynthesis (vindoline in *C. roseus*)	Desacetoxyvindoline 4-hydroxylase, putative	*Ricinus communis*	EEF33073.1	1	6e-33
Cochaperone	Suppressor of G2 allele of SKP1	*Zea mays*	NP_001149123	1	4e-33
ATPase activity	ATP binding protein, putative	*Ricinus communis*	EEF44983.1	1	1e-47
Others	Predicted, unknown, unnamed proteins	Several plant species	N/A	38	< e-05
Total				**134**	

Anexo 2:

Capítulo publicado durante o doutoramento "Plant secondary metabolism and challenges in modifying its operation: an overview" In: Plant secondary metabolism engineering, Methods and Applications

Chapter 1

Plant Secondary Metabolism and Challenges in Modifying Its Operation: An Overview

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Abstract

Plants have metabolic pathways leading to tens of thousands of secondary products capable of effectively responding to stress situations imposed by biotic and abiotic factors. These pathways, often recruited from essential primary metabolism pathways upon initial gene duplication, are frequently restricted to specific taxonomic groups and play a major role in the plant × environment interaction. A strict spatial and temporal control of gene expression ensures the correct accumulation pattern of various secondary products. The required transport of metabolic intermediates constitutes an additional level of regulation. The induction of secondary metabolism gene expression by wounding, herbivore-derived molecules, pathogen elicitors, and oxidative stress caused by heat, drought, flooding, UV light, or temperature extremes is often mediated by integrating signaling molecules such as jasmonate, salicylic acid, and their derivatives. Ontogeny and circadian clock-controlled gene expression are also important features of plant secondary metabolism, as are master regulatory transcription factors. These regulators are attractive targets for engineering secondary metabolic pathways. In spite of the complexity of secondary metabolism, important advances have been achieved, leading to success stories in engineering this diverse reservoir of useful molecules.

Key words: Plant secondary metabolism, environmental stress, development, elicitors, metabolic engineering, transcription factors, microbial expression, plant transformation.

1. Introduction

Plant secondary metabolites, often referred to as natural products, comprise a wide array of non-protein molecules, often of relatively low molecular weight, that occur in certain taxa and are not essential for basic growth and development, but are frequently involved in environmental adaptation to both biotic and abiotic stresses. Secondary metabolites also have important roles in pollination and seed dispersion, as well as in the interaction of plants with mutualist species of bacteria and fungi. In addition, chemical

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interactions with other plants (e.g., allelopathy), host recognition by herbivores, and indirect defense based on the attraction of parasites and predators of herbivores (tri-trophic interactions) often involve secondary metabolites (1). Therefore, the adaptive and evolutionary significance of plant secondary metabolites is undisputed.

From the human point of view, many of these thousands of molecules have interesting applications in pharmacology, chemical industry, novel materials, agriculture, and forestry. Even with a sparse and fragmentary knowledge of plant secondary metabolites in plants, mostly centered in a few medicinal, crop, and forestry species, this type of natural chemicals has made a huge impact in human life. Take the example of quinine as an antimalarial. Without this remedy, it is quite probable that the colonization of the Americas would have had a different outcome. The discovery and development of secondary metabolism-derived drugs, such as the antitumoral taxol from yew and the antileukemic vincristine from vinca, have been making possible the continuity of many human lives (2). Because of their structural complexity, often including several chiral centers, plant secondary metabolites are very difficult to synthesize chemically in adequate yields, making plants or cells and tissues derived thereof the only commercially viable sources of these molecules (3).

Detailed knowledge of plant secondary pathways is restricted to a few plant species. The chemical profile of the vast majority of the 300,000 estimated plant species is unknown, particularly considering the wealth of tropical and subtropical species, which are either understudied or unknown to science. The picture that has emerged from the few cases in which plant secondary metabolism has been well described indicates that there is a large variation in the biochemical pathways involved and in their regulation. This observation points to the need of intensive investigations to unveil the "big picture" of this metabolic diversity and paves the way to uncover and explore its myriad of applications.

The pathways leading to the biosynthesis of natural products in plants have been the object of significant research efforts to better understand their operation and to optimize production of useful metabolites (3). Nonetheless, effective metabolic engineering of numerous plant natural product pathways remains a challenge. Among the hurdles on the way are (a) lack of sustainable source of biomass, (b) poor effectiveness of transformation and regeneration systems, (c) need to evaluate accumulation profile throughout developmental stages and upon environmental changes, (d) difficult synthesis and labeling of biosynthetic intermediates, (e) problems in the establishment of axenic cultures to separate genuine plant metabolism from endophyte or plant–endophyte interaction metabolism, and (f) limited knowledge of transport systems for metabolites in organelles and between cells.

In this chapter, an outline of the main features of plant secondary metabolic pathways will be presented, with emphasis on the bottlenecks associated with the improvement of biosynthetic flow to metabolites of interest. This will be a preamble to the ingenious techniques described in this volume of *Methods in Molecular Biology* which aims at assisting researchers in manipulating this dazzling complex, dynamic, and relevant part of plant metabolism. Some of the regulatory points in plant secondary metabolism and potential strategies to engineer them are shown in **Fig. 1.1**.

2. Diversity from Common Backbones: Primary Origins, Core Compounds, Modifying Enzymes, and Pathway Merging

One of the most impressive aspects of plant secondary metabolites is their diversity. Whereas a few hundred metabolites can be assigned to the core of primary metabolism (photosynthesis, respiration, and metabolism of carbohydrates, fatty acids, and nitrogen), estimates indicate that 200,000 metabolites take part in secondary metabolism (terpenes, polyketides, phenolics, alkaloids, cyanogenic glycosides, glucosinolates, and non-protein amino acids) (3). The biochemical and evolutionary origin of secondary metabolites can be traced to primary compounds and genes of primary metabolism, respectively. Secondary metabolites often arise from amino acids, nitrogenous bases, and intermediates of photosynthetic, respiratory, carbohydrate, or fatty acid metabolism. From an evolutionary point of view, most secondary metabolism genes appear to have arisen from the recruitment of primary metabolism genes by events of duplication, followed by accumulation of mutations and functional diversification (1).

The impressive numbers of secondary metabolites are the product of relatively small modifications in core compounds from which a large number of derivatives arise. This provides plants with a “natural combinatorial chemistry” approach to select ecologically relevant compounds for various adaptive functions in the course of evolution. The catalysts of secondary metabolism can be roughly divided into parent compound enzymes and modifying enzymes. The former catalyze reactions that provide the basic skeletons of compound series, whereas the latter change parent compound structure (monoxygenases, methyltransferases, glucosyltransferases, hydroxilases, etc.). In terpene metabolism, some terpene cyclases have been found to convert one substrate into many different products, significantly adding to chemical diversity (4). The predominant products formed by these enzymes are likely controlled by cofactor availability and other aspects of enzyme subcellular environment.

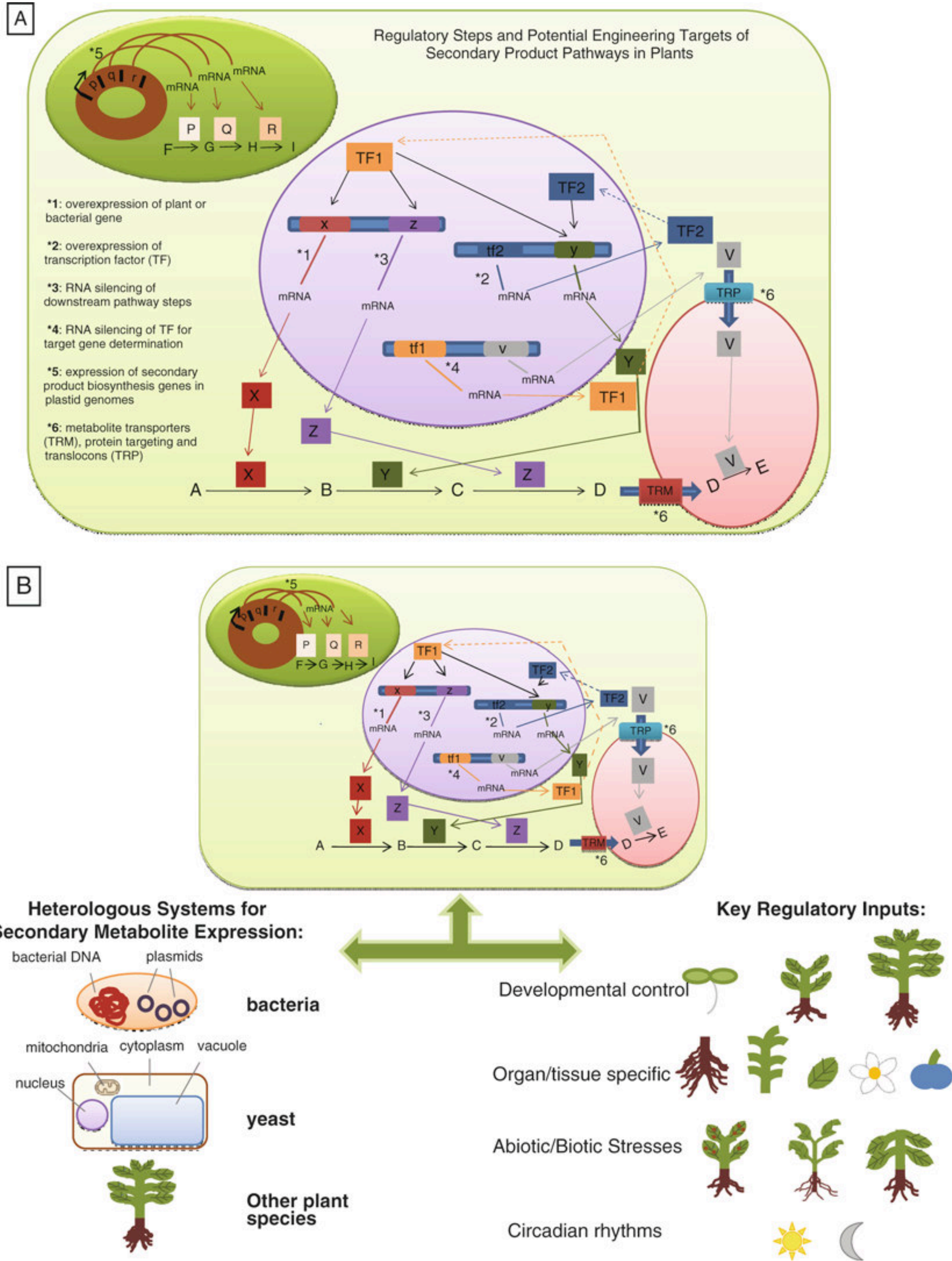

Fig. 1.1. (**a**) Outline of main regulatory points involved in secondary metabolite production. Numbers with asterisks indicate potential strategies for modifying carbon flux through secondary pathways or for insertion of new pathways. Organelles from left to right: chloroplast, nucleus, and vacuole. *Lower case letters* represent genes, *upper case letters* represent metabolites, and *boxed upper case letters* represent proteins. TRP, protein transport; TRM, metabolite transport; and TF, transcription factor. *Dashed lines* represent nuclear entry of transcription factors. *Solid dark lines* are catalytic steps. (**b**) Alternatives of heterologous systems for partial or total expression of plant secondary pathways (*left*) and main regulatory inputs that must be considered and can be manipulated in pathway engineering (*right*).

Diversity of secondary metabolites also results from mixed origin compounds. It is quite common to find compounds that arise from the combination of intermediates derived from different metabolic pathways. Typical examples are the flavonoids, which are assembled from intermediates of the acetate–malonate and shikimate pathways, and taxol, a diterpene amide that derives from isopentenyl biphosphate (terpene pathway), and phenylalanine (phenolic pathway) (5). The involvement of two or more metabolic pathways in the biosynthesis of some secondary metabolites contributes to a high degree of complexity in the regulation of metabolic flux leading to these compounds.

3. Signal Transduction and Environmental Responsiveness

One of the hallmarks of plant secondary metabolism is its capacity to respond to environmental signals. As sessile organisms, plants acquired a complex metabolic machinery to produce chemicals that allow them to cope with various types of stresses, both biotic and abiotic. In that regard, phytohormones and other signal molecules often play a key role in the transduction of the environmental information to trigger a metabolic response that is adaptive to the particular circumstances. The activation of secondary pathways is often triggered by molecules, such as jasmonate, ethylene, abscisic acid, salicylic acid, and nitric oxide, which are produced upon herbivore or pathogen attack. Ethylene and abscisic acid are also induced by a variety of abiotic stresses, including flooding, drought, salinity, UV-B, and extreme temperatures (6).

Most stresses result in the transient accumulation of reactive oxygen species (ROS), which are important in signaling, but become toxic if accumulated (7). Several secondary metabolites are induced by ROS. These chemical species may not only act as signaling molecules to regulate secondary metabolism genes but also be the target of the metabolites derived from the catalytic activity of the respective enzymes. Various natural products can quench and mitigate reactive species, contributing to regulate the antioxidant defenses and protect nucleic acids, proteins, and membranes. The shielding and antioxidant capacity of flavonoids to protect against UV and its ROS-driven effects on plant cells is well documented (8), and increasing evidence points to a similar role for some monoterpene indole alkaloids (9).

The signaling pathways connecting environmental status to changes in gene expression converge on four main regulation levels: transcriptional control, mRNA stability, post-translational protein modification, and selective proteolysis (10). The activation, release from membranes, inhibition, or proteolysis of

transcription factors (TF) or TF-sequestering proteins, affecting their concentration, nuclear mobility, or DNA-binding activity, often determine the production of enzymes that are responsible for the phenotypic metabolic profiles (11, 12). Another very important level of regulation that is just starting to be disclosed for plant secondary metabolism is RNA stability, orchestrated by a wide array of small RNA molecules. Effective RNA interference techniques have been applied to modify the profile of secondary metabolites by changing the expression of regulatory proteins or to knock down biosynthetic steps (13).

4. Secondary Metabolism and Defense Strategies: Phytoanticipins, Phytoalexins, and Everything in Between

Defense strategies based on secondary metabolism can vary between plant species and with interactions involving specific types of aggressors, both herbivores and pathogens. In general, plant defense metabolites can be pre-existent or constitutive (phytoanticipins) or their biosynthesis may be induced upon challenge (phytoalexins) (14). A special case of constitutive defense corresponds to the accumulation of precursor compounds often stored in vacuoles; upon damage, these metabolites are released and modified by enzymes which are either co-activated in these circumstances or made available by de-compartmentalization (e.g., glucosinolates) (1).

Numerous species, however, have intermediate profiles in which defense compounds can be accumulated in basal levels and further induced upon specific challenges. Examples of this can be found in the production of monoterpene indole alkaloids of *Psychotria* sp. (9). Although the strategies outlined above have been classified based on responses to biotic stresses, they could easily be extended to describe accumulation profiles of secondary metabolites in response to abiotic stresses.

5. Organ, Tissue, Cell, and Subcellular-Specific Metabolism: Developmental Controls Organize Structure and Function

In many cases, the expression of plant secondary pathways is strongly controlled in both time and space. Typical examples include the production of volatile terpenes and flavonoid pigments in floral tissues, the accumulation of flavonoids in the epidermis of UV-treated leaves, and the production of monoterpenes in leaf glandular trichomes of peppermint (2). The circadian pattern of expression of terpene biosynthetic genes, enzyme

activities, and specific metabolite profiles also illustrates the coupling of secondary metabolism with time cues (15).

Studies on alkaloids have shown that secondary metabolic pathways can be distributed in different tissues and cell types, each one of them expressing a portion of the pathway leading to a final product. A major site for the biosynthesis of monoterpenoid alkaloids of *Catharanthus roseus* is the leaf epidermis, although further steps of their biosynthesis have been shown to take place in other tissues, such as palisade idioblasts, laticifers, and possibly phloem parenchyma cells. The biosynthesis of the tropane alkaloid scopolamine in *Atropa belladonna* and *Hyoscyamus muticus* takes place in roots and involves different tissues. An early and a late enzyme of the alkaloid biosynthesis (putrescine *N*-methyl transferase and hyoscyamine 6β-hydroxylase) localize to the pericycle, whereas an intermediate enzyme in the pathway (tropinone reductase I) resides in the endodermis and inner cortical cells (16).

In spite of a relatively limited number of case studies, the distribution of portions of some pathways of secondary metabolism in various organs and tissues of plants implies the transport of intermediates between cell types. However, little is known on the mechanisms of transport, specificity characteristics, or nature of the transporters. The involvement of ABC-type transporter proteins has been shown for alkaloids and isoprenoids (17). This is certainly a field that demands much investigation to allow better metabolic engineering, since it represents a different checkpoint for regulation of carbon flux through secondary metabolic pathways.

Several subcellular compartments can be involved in the biosynthesis of secondary metabolites. In the production of monoterpene indole alkaloids, for example, the cytosol, the chloroplast, the endoplasmic reticulum, and the vacuole host enzymes that take part in assembling these metabolites (16). In the production of glucosinolates and cyanogenic glucosides, the vacuole can act as a storage compartment for final products, which are released upon wounding to generate isothiocyanites or cyanide, both having herbivore deterrence properties (1). The enzymes chalcone synthase and chalcone isomerase, from the flavonoid biosynthesis pathway, have been localized to the nucleus of *Arabidopsis thaliana* cells, where accumulation of some flavonoids was also observed, in agreement with reports on other plant species (8).

The adaptive value of subcellular compartmentalization of secondary metabolic pathways must be a driving force for its establishment in various unrelated species and molecular backbones. Reduced competition for common substrates, optimized control of pathway inhibition by end products, self-protection (safe storage of potentially harmful metabolites), better enzyme activity in subcellular niches, and better flux control are among

the possible advantages of this biochemical architecture. Once again, transporter systems for certain metabolites and enzyme sorting systems between compartments become a requirement and a potential regulatory level. The cellular and subcellular complexities associated with the expression of secondary pathways help to explain why in many cases undifferentiated cell cultures fail to form bioactive or industrially relevant metabolites (9).

6. Channeling, Regulatory Proteins, and Metabolic Flux

Metabolic channeling is one of the reasons that explain why some secondary pathways are capable of quickly producing relatively large amounts of required metabolites upon cell perception of specific signals. Co-localization of related enzymes to specific compartments or membranes and the establishment of multienzyme complexes provide prompt transfer of metabolic intermediates between catalysts as well as prevent the accumulation of potentially toxic compounds. The latter can be found in the metabolism of phenylpropanoids (18). The availability and production of co-factors may also be favored by these spatial arrangements.

The control of metabolic flux in biochemical pathways has been recognized as the result of the combined inputs of all enzymes in the pathway. Enzymes and metabolites behave as a system in such a way that changes in one of its components will often affect others (19). This is the general concept behind metabolic flux analysis. Every enzyme in a pathway contributes to flux control of metabolites, although some may exert a larger effect. Unlike constants of enzyme kinetics, the flux control coefficient (contribution of an enzyme to the flux control in a metabolic pathway) is not a fixed value, varying with factors such as developmental stage or physiological status.

Taking this systemic feature of metabolic pathways into account, it is not hard to understand why increasing the activity of a single enzyme often falls short of improving final metabolite yields (9). Whenever possible, strategies to engineer metabolism should take into account steady-state concentrations of intermediates, maintenance of minimum pools to avoid major unbalance in pathway operation, enzyme cofactor availability and regeneration capacity, product sequestration in vacuoles or other compartments to avoid pathway inhibition, transport of metabolites across membranes and cell types, and the role of transcription factors (19) and/or small RNAs in regulating the expression of functionally related biosynthetic genes (13). Some of these aspects are

poorly known for most plant species, especially when considering the modification of secondary metabolism.

Transcription factors (TFs), both positive and negative, are interesting targets for metabolic engineering, since they can regulate the expression of several biosynthetic enzyme genes in an integrated and coordinated fashion (19). However, as is the case of flavonoid metabolism, more than one class of transcription factors has to be modulated to affect the expression of target genes that receive mixed inputs from different transcriptional regulators (20). The existence of transcriptional networks characterized by complex interactions, such as TFs sequentially regulating the transcription of other counterparts, auto-regulation of TF gene transcription, and transactivating activity depending on TFs interactions at the protein level, is observed in plants (21). This reflects the systemic feature of transcriptional networks that control enzyme genes, just as is observed with the flux of metabolites at the biochemical level. In theory, the modular nature of these transcriptional regulation proteins, with DNA sequence-specific binding and protein transactivating domains, allows the design of tailor-made proteins to regulate metabolic pathways.

7. Plant Secondary Metabolic Steps in a Less Complex Setup: Plant Cell and Organ Cultures and Heterologous Systems

Metabolic engineering has a long history in microorganisms, but is much younger in plants. However, large-scale culture systems available for microorganisms are very attractive as platforms for producing metabolites of interest. In spite of the compartmentalization and tissue differentiation requirements for the correct function of plant secondary metabolism, some pathways can be entirely or at least partially introduced and expressed in bacteria and yeast (2) (**Fig. 1.1b**). Plant cell cultures can also be effective ways to produce secondary products, even without need for genetic modification, using environmental control and elicitors to improve production, reaching commercial scale viable yields (5).

Bacteria are versatile organisms to express plant secondary metabolism steps or even short pathways. In particular, *Escherichia coli* is widely used with this purpose. Fast growth rates, ease of transformation, simplicity of culture media, large variety of strains and mutants (also allowing codon bias modifications), high expression rates for foreign proteins, and plasmid-based expression systems are just some of the advantages of using this species to produce plant secondary metabolites and enzymes (22). Similar features can be listed for yeast, namely *Saccharomyces cerevisiae* and *Pichia pastoris*, with the additional advantage of "built-in" eukaryotic post-translation modification

capacity. Genetic manipulations using *P. pastoris* are similar to those of *S. cerevisiae*. However, because of the strong preference of *P. pastoris* for respiratory metabolism, extremely high cell densities can be achieved without the accumulation of high ethanol concentrations as is often the case in high-density cultures of *S. cerevisiae* (23). In baker's yeast, short pathways have been effectively expressed, and the organism has been used to produce much needed and hard to obtain labeled intermediate and parental compounds for biosynthetic studies of plant secondary metabolism, as exemplified by taxadiene (22).

Plant cell cultures resemble some of the features of microorganism cultures, although growth is much slower (increasing contamination chance), cells are less resistant to shearing stress in bioreactors, culture media are more complex, and subpopulations are often out of synchrony during culture (24). However, some plant cell cultures are capable of producing secondary metabolites of interest in large scale, using slightly modified bioreactors to accommodate for their specific features (e.g., impeller design, airlift technology, and aeration levels) (24). One of the advantages of cell cultures is the easy application of elicitors, such as jasmonate, and feeding of limiting precursors to boost metabolic flow toward products of interest. Two-step culture media systems, with a nutrient-rich growth media and nutrient-limited elicitor-containing production media, can be applied to cell cultures. A continuous two-phase organic solvent extraction layer (physically separated from cells) can assist in metabolite extraction and in reducing feedback inhibition of pathways. Cell sorting methods based on metabolite expression via fluorescent detection systems can afford more homogeneous cell populations for enhanced production of target compounds (25).

Cell immobilization techniques can also be used to promote the accumulation of secondary metabolites; the establishment of cell layers or "pseudotissues" creates an environment for different cell–cell signaling and metabolite gradients that may favor secondary metabolism pathway expression (24). Plant cell cultures can also be transformed via *Agrobacterium* or biobalistic strategies to improve yields. When metabolite release to culture medium is limited and preservation of cell biomass is desired, small concentrations of cell permeabilization agents (e.g., dimethyl sulfoxide) may be employed.

Organ cultures can be an alternative to produce secondary metabolites in bioreactor setups. Roots are especially useful for this purpose, since they have a tendency to exude various metabolites, facilitating recovery. Gas-phase trickle-bed reactors, in which medium is re-circulated from a reservoir, sprayed over the root mass, flowing down through the root surface, have been designed for root cultures. Transformation of plant explants with wild or engineered strains of *Agrobacterium rhizogenes* generally results

in profuse adventitious root development, which can display significantly higher secondary metabolite yields (26).

8. Using Bacterial Genes and Plastid Engineering to Improve Plant Secondary Metabolism

Bacteria are also a rich source of secondary metabolites, particularly carotenoids, and bacterial genes can be used to improve the profile of human health-promoting terpenes in plants. One of the most important examples of this strategy is the development of golden rice, expressing the pathway to the vitamin A precursor β-carotene, using both bacterial and plant genes under the control of an endosperm-specific promoter (27).

A similar successful strategy was used to insert a phytoene synthase gene (*crtB*) derived from a soil bacterium *Pantoea ananatis* into flax plants, yielding transgenic seeds with about 20-fold increase in total carotenoid content (28). Although these are examples of nuclear genome engineering, the expression of bacterial genes in plastid genomes can also be used, taking advantage of the prokaryotic nature of plastids stemming from their endosymbiotic origin. The introduction of nuclear genes in plastid genomes is also feasible with some further modifications. Plastids have many useful features for secondary metabolism engineering, including operon arrangement, no need for transit peptides, high genome copy number, and level of protein expression (29).

9. Transfer of Specific Secondary Pathways to Host Plant Species

The transfer of secondary metabolic pathways between plant species is of high interest in agriculture, pharmaceutical sciences, and forestry. Target metabolite accumulation and valuable traits conferred by secondary metabolites for plant defense against herbivores and pathogens, nutraceutical content, and abiotic stress resistance are in high demand in both non-cultivated species of interest and crops. Some limitations for the effective metabolic profile modification are related to difficulties in multigenic pathway transfer, transformation and regeneration of modified plants, and development of specific cell types involved in production and storage (e.g., glandular trichomes).

In spite of these potential difficulties, entire plant-derived metabolic pathways have been effectively transferred from one species to another (30). The availability of simpler and more

efficient cloning techniques, exemplified by the ligation-free cloning technique USER, allowing the one-step assembly of complex DNA constructs, has been the key element in some of these achievements (31).

The conversion of the model plant *A. thaliana* into a cyanogenic plant through the transfer of the complete pathway leading to the cyanogenic glycoside dhurrin, using *Sorghum bicolor* genes, was carried out (32). The transgenic plants showed improved resistance to herbivores. The final portion of the glucosinolate biosynthetic pathway (*C–S* lyase, glycosyltransferase, and sulfotransferase) from *Arabidopsis* has been introduced in tobacco. To achieve this important step toward the goal of engineering glucosinolates into non-cruciferous plant species, an expression construct made up of a single polycistronic open reading frame was used, allowing the control of the three coding sequences by a single promoter (31). Although still significantly based on model plant species, these examples show that wider engineering of crop species with secondary metabolic pathways is on its way.

10. Conclusion

The handful of plant species which have had their secondary metabolism investigated to significant detail, at the level of metabolites, proteins, and genes, provide us with a sketchy picture of the architecture and functioning of secondary metabolism and its diversity. There are various tools to engineer plant secondary metabolism, and several of them have been shown to be effective. However, to fully realize the benefits of manipulating the inner chemical factories of plants and their refined responses to the environment, there is a long way to go. This is a very fortunate time to work with plant secondary metabolism.

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