UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL (UFRGS)
INSTITUTO DE BIOCIÊNCIAS
DEPARTAMENTO DE GENÉTICA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

FERNANDA FLEIG ZENKNER

METABOLISMO DE NICOTINA NO GÊNERO *NICOTIANA*: Identificação e análise evolutiva da subfamília CYP82E e seu papel na conversão de nicotina em precursores de nitrosaminas com potencial cancerígeno

Porto Alegre
2017

FERNANDA FLEIG ZENKNER

METABOLISMO DE NICOTINA NO GÊNERO *NICOTIANA*:
Identificação e análise evolutiva da subfamília CYP82E e seu papel na conversão de nicotina em precursores de nitrosaminas com potencial cancerígeno

Dissertação apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para obtenção do título de mestre em Genética e Biologia Molecular.

Orientadora: Dra. Márcia Pinheiro Margis
Co-orientador: Dr. Alexandro Cagliari

Porto Alegre – RS
2017

DISSERTAÇÃO DE MESTRADO

DADOS DE IDENTIFICAÇÃO

Executora: Fernanda Fleig Zenkner, Bacharel em Ciências Biológicas, graduada pela Universidade de Santa Cruz do Sul (UNISC). E-mail: fzenkner@gmail.com

Linha de Pesquisa: Genética e Biologia Molecular Vegetal

Orientadora: Dra. Márcia Pinheiro Margis

Co-orientador: Dr. Alexandro Cagliari

Local de execução: Laboratório de Genética Molecular Vegetal da UFRGS

Palavras-chave: *Nicotiana tabacum*, CYP82E, nicotina, nornicotina.

AGRADECIMENTOS

Em primeiro lugar agradeço à JTI, empresa onde trabalho desde 2012 e que sempre apoiou a minha formação e desenvolvimento profissional. Mauro Feuerborn, Franque Specht e Paolo Donini: obrigada pela oportunidade e auxílio!

Ao profº Alexandre Rieger, por todas as conversas e ensinamentos desde o início da faculdade, em 2008. Obrigada pela amizade, pela preocupação comigo e pelo carinho. Não posso deixar de agradecer também pelas discussões produtivas, e por ter me apresentado ao profº Alexandro, que orientou este trabalho.

Ao profº Alexandro Cagliari, que topou o desafio de me orientar mesmo sabendo das minhas limitações de tempo em função do trabalho na JTI. Obrigada pelos ensinamentos e conselhos.

À profª Márcia Margis, pela abertura e acolhimento demonstrados desde a primeira conversa. Obrigada por ter aceitado me orientar durante o mestrado, pelos conhecimentos compartilhados e pelas críticas sempre construtivas.

Família e amigos: sem vocês eu não teria chegado até aqui! Agradeço pelo incentivo, pelos abraços, e por escutarem os meus desabafos.

Mãe (Tania) e mana (Luiza), obrigada por compreenderem a minha ausência, e por me acompanharem sempre. Simplesmente agradeço por vocês estarem sempre junto a mim!

Pai (Luiz Fernando), obrigada pelo incentivo e pela força. Conseguimos!

Hiuri, obrigada por estar ao meu lado, pelo apoio e pelas conversas sobre os mais variados assuntos. Obrigada por acompanhar de perto os desafios que o mestrado me trouxe, e por sempre acreditar em mim. Obrigada pelo nosso dia-a-dia!

À Carol Schmitt, agradeço pelos 16 anos de amizade, por ter o melhor abraço e a melhor energia do mundo! Obrigada por estar sempre perto, mesmo longe.

Mireila (Mika) e Simoni, obrigada pelas conversas diárias e por estarem sempre dispostas a ouvir e a ajudar.

Aos colegas do ADET (JTI), obrigada por tudo o que me ensinam no dia-a-dia. Muito mais do que ensinamentos teóricos, aprendemos na prática a respeitar as diferenças e a conviver em harmonia.

Obrigada, obrigada, obrigada!

RESUMO

O tabaco (*Nicotiana tabacum*) pertence à família Solanaceae, sendo uma cultura de grande importância econômica. O Brasil é o segundo maior produtor e o maior exportador de tabaco em folha do mundo, e os três estados do sul do país são responsáveis por mais de 90% da produção. As plantas do gênero *Nicotiana* são conhecidas pela sua composição de alcaloides. A nicotina é o principal alcaloide encontrado nas plantas desse gênero, seguida por nornicotina, anabasina e anatabina. Contudo, a composição de alcaloides pode variar muito dependendo da espécie de *Nicotiana*. Os alcaloides são compostos muito importantes para as plantas, pois atuam na defesa contra herbívoros. A nicotina é sintetizada nas raízes das plantas através de um processo complexo, envolvendo diversas enzimas e transportadores. Após sintetizada, a nicotina é translocada pelo xilema até as folhas, onde é estocada nos vacúolos e/ou metabolizada em outros alcaloides. O primeiro capítulo deste trabalho consistiu em um artigo de revisão sobre a biossíntese, o transporte e o metabolismo da nicotina em *Nicotiana*. Assim, pode-se identificar as questões ainda não esclarecidas sobre essas vias, e que merecem mais atenção em futuros trabalhos. A nornicotina é o segundo alcaloide mais encontrado em *Nicotiana*, sendo sintetizada a partir da desmetilação da nicotina. A reação de desmetilação é catalisada por enzimas nicotina *N*-desmetilases (NND), codificadas por genes da subfamília *CYP82E*. A nornicotina, por sua vez, pode reagir espontaneamente com óxidos de nitrogênio presentes nas folhas de tabaco, dando origem a *N*-nitrosonornicotina (NNN), uma substância cancerígena do grupo das Nitrosaminas Específica do Tabaco (TSNA – *Tobacco Specific Nitrosamines*). Considerando a importância dessa TSNA para a saúde humana, diversos estudos têm sido realizados a fim de reduzir a produção do seu precursor, a nornicotina, pelas plantas de tabaco. No segundo capítulo deste trabalho, apresentamos a identificação de novos genes putativos da subfamília *CYP82E* em diferentes espécies do gênero *Nicotiana*, e uma filogenia incluindo os novos genes identificados. Além disso, as sequências encontradas foram caraterizadas utilizando ferramentas *in silico*, e algumas predições em comum foram observadas para a maioria das sequências. Assim, conhecer todos os genes que codificam enzimas NND, bem como caracterizar essas enzimas e entender a organização da subfamília gênica *CYP82E*, podem ser fatores essenciais para desenvolver novas soluções com o objetivo de reduzir substâncias cancerígenas em produtos de tabaco, especialmente as TSNA.

Palavras-chave: *Nicotiana tabacum*. *CYP82E*. Nicotina. Nornicotina.

ABSTRACT

Tobacco (*Nicotiana tabacum*) belongs to the Solanaceae family, being a crop of great economic importance. Brazil is the second largest producer and the largest exporter of tobacco leaf in the world, and the three southern states account for more than 90% of production. *Nicotiana* plants are known by its alkaloid composition. Nicotine is the main alkaloid found in plants of this genus, followed by nornicotine, anabasine and anatabine. However, the alkaloid composition may vary widely depending on the *Nicotiana* species. The alkaloids are very important compounds to the plants, since they act in the defense against herbivores. Nicotine is synthesized in the plant roots though a complex process involving several enzymes and transporters. After synthesized, nicotine is translocated via xylem to the leaves, where it is stored in the vacuoles and/ or metabolized in other alkaloids. The first chapter of this work consisted of a review article on nicotine biosynthesis, transport and metabolism in *Nicotiana*. Thus, it was possible to identify the questions not yet clarified about these pathways, and that need further investigation. Nornicotine is the second most commonly found alkaloid in *Nicotiana*, being synthesized from nicotine demethylation. The demethylation reaction is catalyzed by nicotine *N*-demethylase enzymes (NND), encoded by genes of the *CYP82E* subfamily. Nornicotine, on its turn, can spontaneously react with nitrogen oxides present in the plant leaves, giving rise to *N*-nitrosonornicotine (NNN), a carcinogen from Tobacco Specific Nitrosamine (TSNA) group. Considering the importance of NNN to the human health, several studies have been carried on to reduce the production of its precursor, nornicotine, by tobacco plants. In the second chapter of this work, we present the identification of new putative *CYP82E* genes in different *Nicotiana* species, and a phylogenetic analysis including the new genes identified. In addition, the sequences found were characterized using *in silico* tools, and we observed some identical predictions among most of the sequences. Thus, the identification of all genes encoding NND enzymes, as well as the characterization of these enzymes and the understanding of *CYP82E* subfamily organization, can be essential factors in the development of new solutions aiming the reduction of carcinogenic substances in tobacco products, especially the TSNA.

Keywords: *Nicotiana tabacum*. *CYP82E*. Nicotine. Nornicotine.

SUMÁRIO

1 INTRODUÇÃO

O tabaco (*Nicotiana tabacum*) é uma planta da família Solanaceae, de relevante importância econômica para o Brasil, especialmente para os três estados do sul do país (Bonato, 2007; Sinditabaco, 2017). Os alcaloides são os principais compostos encontrados em plantas de tabaco e em outras espécies do gênero *Nicotiana*. A composição de alcaloides pode variar conforme a espécie de *Nicotiana*, mas sabe-se que a nicotina é o mais encontrado na maioria das espécies (Hung et al., 2013).

Além da nicotina, nornicotina, anatabina e anabasina são alcaloides que também podem ser encontrados nessas plantas. A nornicotina é o segundo alcaloide mais encontrado nas espécies de *Nicotiana*, sendo sintetizada a partir da nicotina através da reação de desmetilação (Lewis et al., 2010). A nornicotina, por sua vez, pode ser precursora da *N*-nitrosonornicotina (NNN), que é uma nitrosamina específica do tabaco (TSNA) com grande potencial carcinogênico (Gavilano; Siminszky, 2007).

Algumas das enzimas responsáveis por metabolizar a nicotina em nornicotina já foram identificadas em *Nicotiana*, sendo que todas fazem parte da subfamília gênica *CYP82E* (Lewis et al., 2010). Entretanto, poucos genes são conhecidos em espécies selvagens do gênero, e o controle da expressão gênica de *CYP82E* e a caracterização dessas enzimas não foram completamente elucidados.

Devido à importância econômica do tabaco para o país e considerando a questão de saúde da população que faz uso de produtos de tabaco, ressalta-se a relevância de estudos relacionados aos alcaloides de *Nicotiana*. A compreensão das bases genéticas da produção e metabolização dos alcaloides pelas plantas de tabaco pode contribuir para a produção de tabaco de alta qualidade e com baixos teores de TSNA.

Portanto, o presente trabalho teve como objetivo realizar uma revisão sobre a biossíntese e o metabolismo de nicotina no gênero *Nicotiana*, além de identificar e caracterizar os genes da subfamília *CYP82E*, responsáveis pela conversão de nicotina em nornicotina no gênero *Nicotiana*, buscando contribuir para o entendimento do processo de acúmulo de Nitrosaminas Específicas do Tabaco (TSNA).

2 REVISÃO BIBLIOGRÁFICA

2.1 Tabaco

A família Solanaceae compreende 90 gêneros contendo mais de 3000 espécies, incluindo plantas alimentícias, de uso medicinal, ornamental e econômico (Albuquerque et al., 2006). O tabaco pertence ao gênero *Nicotiana*, com cerca de 75 espécies conhecidas (Clarkson et al., 2004). Dentre essas, *Nicotiana tabacum* é uma das principais espécies utilizadas para fins comerciais, sendo cultivada há milhares de anos pelos aborígenes da Índia, México, América Central, Colômbia, Venezuela, Guianas e Brasil (Tso, 1990).

O tabaco comercial é uma espécie anfidiplóide (2n=4x=48), proveniente de duas espécies silvestres hibridizadas no passado, *Nicotiana sylvestris* (2n=2x=24) e *Nicotiana tomentosiformis* (2n=2x=24) (Shibata et al., 2013). Existem indícios de que esta hibridação ocorreu no norte da Argentina ou no sul da Bolívia, já que as espécies parentais são encontradas nessa região (Sierro et al., 2013).

2.2 Histórico do tabaco

Acredita-se que o tabaco seja consumido há mais de 4 mil anos, sendo cultivado inicialmente pelos Maias na região da América do Norte e América Central, e por diversos outros povos indígenas que utilizavam a planta em cerimônias religiosas (Bonato, 2007). Os nativos chamavam de “tobago” o utensílio que utilizavam para fumar as folhas, e por isso a planta ficou conhecida como “tabaco”. As sementes de tabaco foram levadas primeiramente para a Europa, e entre 1650 e 1750 o seu consumo espalhou-se rapidamente por todo o mundo (Collins e Hawks, 1983).

Já no Brasil, a hipótese mais provável é que o tabaco tenha se difundido a partir dos Andes Bolivianos pelas migrações indígenas, principalmente de Tupi-Guaranis. Na época da chegada dos europeus no Brasil o tabaco era cultivado em toda a costa brasileira, e logo os colonizadores utilizaram esta produção para abastecer o mercado europeu. Nas três primeiras décadas do século XX o tabaco passou a ser cultivado em outros estados: Minas Gerais (MG), Goiás (GO), São Paulo (SP), Paraná (PR), Santa Catarina (SC) e Rio Grande do Sul (RS), sendo nos últimos três estados o cultivo relacionado aos imigrantes alemães e italianos, particularmente nas colônias de São Leopoldo e Santa Cruz do Sul (Bonato, 2007).

2.3 Importância econômica do tabaco

O tabaco é consumido em diversos lugares do mundo, na forma de produtos para uso de diferentes maneiras: para fumar, mascar ou inalar (rapé) (Viegas, 2008). A produção do tabaco pode ocorrer em todos os locais onde o ambiente é favorável para o seu crescimento e desenvolvimento, o que está geograficamente representado entre as latitudes 60ºN a 40ºS. Entretanto, os maiores produtores de tabaco do mundo então localizados quase inteiramente entre as latitudes 45ºN e 30ºS (Tso, 1990).

O Brasil é o segundo maior produtor de tabaco do mundo, e o maior exportador de tabaco em folha, com 85% da produção destinada ao mercado externo (Sinditabaco, 2017). Dessa forma, a importância econômica da cultura do tabaco para o país traduziu-se em 2016 em uma receita de US$ 2,09 bilhões, com 481 mil toneladas de tabaco exportado (Sinditabaco, 2017).

No Brasil, os três estados do Sul concentram aproximadamente 95% da produção nacional de tabaco. De acordo com o Sindicato Interestadual da Indústria do Tabaco - SindiTabaco, são 574 municípios produtores de tabaco no RS, SC e PR, sendo que 52% da área produtiva está no RS, 28% em SC e 20% no PR (Sinditabaco, 2017).

2.4 Métodos culturais e a composição química da folha de tabaco

Após transplantadas para a lavoura, as plantas de tabaco continuam o seu crescimento, e desenvolvem-se até atingirem a maturidade, o que condiz com o início da senescência (Tso, 1990). Após colhidas, as folhas de tabaco devem passar por um processo chamado de cura. Esse processo tem dois objetivos principais: fornecer a temperatura e umidade ideias para que as modificações químicas e biológicas desejadas ocorram nas folhas de tabaco; e preservar as folhas através da sua secagem gradual para reter a sua qualidade (Collins e Hawks Jr., 1993).

A cura é diferente dependendo do tipo de tabaco em questão. Para o tabaco do tipo Burley, também chamado de tabaco de galpão, as plantas são curadas em um galpão de estrutura simples, sem nenhum equipamento para controle de temperatura e umidade (Tso, 1990). Já o tabaco Virgínia, ou tabaco de estufa, é curado em estufas que apresentam um sistema de aquecimento (Collins e Hawks Jr., 1993; Lewis et al., 2012).

A composição química da folha de tabaco é determinada por fatores genéticos, práticas culturais, condições climáticas e métodos de cura. As práticas de manejo da lavoura, como a adubação e o desponte, por exemplo, podem exercer efeitos consideráveis na composição química da folha curada (Cai et al., 2013).

A adubação nitrogenada das lavouras é diretamente proporcional aos níveis de alcaloides nas plantas de tabaco cultivadas (Roton et al., 2005). Isso ocorre, pois os alcaloides são compostos nitrogenados, e a sua síntese pode variar de acordo com a quantidade de nitrogênio disponível no meio (Baldwin et al., 1994). O desponte, por sua vez, consiste na remoção do botão floral junto com algumas folhas jovens da planta (Hibi et al., 1994). Essa prática estimula o crescimento de raízes, e a concentração de nicotina aumenta nas folhas de tabaco após o desponte (Xi et al., 2005; Shi et al., 2006). Já durante a cura, fatores como a alta temperatura e a baixa ventilação podem influenciar na formação de nitrosaminas específicas do tabaco (TSNA) a partir dos alcaloides, alterando a composição da folha (Roton et al., 2005).

Além disso, a maturidade das folhas e as técnicas de colheita são de grande importância na qualidade e usabilidade do tabaco. Assim, as práticas culturais devem preservar o sabor, o aroma, e a composição química da folha que é desejada pela indústria e pelo consumidor (Tso, 1990).

A nicotina é um alcaloide produzido como o principal metabólito secundário nas espécies do gênero *Nicotiana*, representando em *N. tabacum* até 95% do total de alcaloides e cerca de 2 a 5% do peso seco da folha (Hung et al., 2013). Quando as plantas de tabaco são feridas, a síntese de nicotina é estimulada nas raízes, onde são expressos os principais genes envolvidos em sua biossíntese (Shitan et al., 2014).

Após a síntese, a nicotina é translocada através do xilema para as folhas, onde é acumulada nos vacúolos. No xilema, a concentração de nicotina é de cerca de 1mM, e nos vacúolos das células epidérmicas e nas pontas das folhas essa concentração pode ser de até 60mM (Lochmann et al., 2001). Nas folhas, a nicotina desempenha um papel muito importante na defesa anti-herbivoria em função de ser extremamente tóxica ao sistema nervoso de insetos (Shitan et al., 2014).

2.5 Nitrosaminas específicas do tabaco

Os alcaloides podem sofrer transformações dando origem a outros compostos, como as Nitrosaminas Específicas do Tabaco (TSNA – *Tobacco Specific*

Nitrosamines). Já foram identificadas 7 TSNA em produtos de tabaco: *N*-nitrosonornicotina (NNN), 4-(metil-nitrosamino)-1-(3-piridil)-1-butanona (NNK), 4-(metil-nitrosamino)-1-(3-piridil)-1-butanol (NNAL), *N*'-nitrosoanatabina (NAT), *N*'-nitrosoanabasina (NAB), 4-(metil-nitrosamino)-4-(3-piridil)-1-butanol (*iso*-NNAL) e ácido 4-(metil-nitrosamino)-4-(3-piridil) butírico (*iso*-NNAC). Dessas, NNN, NNK e NAT geralmente ocorrem em maiores quantidades que as outras (Hecht, 1998).

A Figura 1 ilustra as estruturas químicas das TSNA, bem como os alcaloides precursores de cada uma. Os precursores das TSNA são os alcaloides e os nitritos. Alcaloides como a nornicotina, a anatabina e a anabasina podem reagir diretamente com óxidos de nitrogênio para formar NNN, NAT e NAB, respectivamente. Já a nicotina primeiro precisa sofrer oxidação nas pontes 1', 2' do anel pirrólico para formar a pseudo-oxi-nicotina (PON), seguida de nitrosação (adição de grupamento N=O) para produzir NNK (Shi et al., 2013). Além disso, apesar de a maior parte da NNN ser sintetizada diretamente a partir da nornicotina, sabe-se que uma pequena parte dessa TSNA pode ser formada diretamente através da nicotina (** na Figura 1), principalmente ao final da cura do tabaco (IARC, 2007).

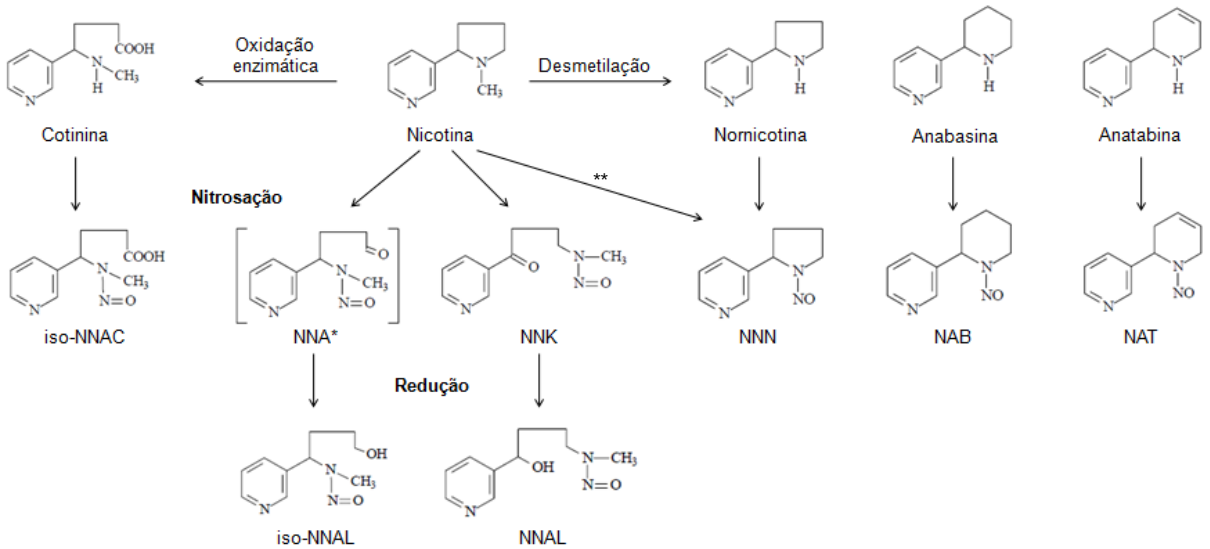


Figura 1: Estruturas químicas das Nitrosaminas Específicas do Tabaco (TSNA) e dos seus alcaloides precursores. *iso*-NNAC: ácido 4-(metil-nitrosamino)-4-(3-piridil) butírico; *iso*-NNAL: 4-(metil-nitrosamino)-4-(3-piridil)-1-butanol; NAB: *N*′-nitrosoanabasina; NAT: *N*′-nitrosoanatabina; NNA: 4-(metil-nitrosamino)-4-(3-piridil) butanal; NNAL: 4-(metil-nitrosamino)-1-(3-piridil)-1-butanol; NNK: 4-(metil-nitrosamino)-1-(3-piridil)-1-butanona; NNN: *N*′-nitrosonornicotina.
* NNA é um aldeído altamente reativo e nunca foi quantificado no tabaco ou na fumaça de cigarros.
** É possível que uma pequena parte da NNN seja sintetizada diretamente a partir da nicotina.
Fonte: Iarc, 2007.

Esses compostos podem ser formados por 2 vias diferentes. Em tabaco do tipo Burley, o rompimento das membranas celulares durante a cura libera exsudatos que servem como substratos para a proliferação de microrganismos naturalmente presentes nas folhas, e esses reduzem nitratos a nitritos. Os nitritos reagem com os alcaloides do tabaco formando TSNA (Shi et al., 2013). Já em tabaco do tipo Virgínia, os microrganismos têm sua atividade reduzida em função da temperatura mais alta dentro da estufa de cura. Dessa forma, a via de formação de TSNA nesse tipo de tabaco envolve a reação de óxidos de nitrogênio (NOx) produzidos como um subproduto da combustão utilizada para aquecimento da estufa, com os alcaloides presentes nas folhas de tabaco (Reed, 2008).

O grupo das nitrosaminas tem grande relevância do ponto de vista da saúde humana, pois muitas delas foram relacionadas à carcinogênese (Hecht, 1998; El-Bayoumy et al., 1999; Brown et al., 2003; Iarc, 2007). As nitrosaminas NNN e NNK estão presentes em maiores quantidades no tabaco, e são consideradas carcinógenos grupo I, ou seja, carcinogênicas para humanos (Hecht, 1998; Iarc, 2007). Além disso, NNN é considerada ainda mais nociva que NNK em função da sua taxa de formação ser muito mais alta (Gavilano; Siminszky, 2007).

Acredita-se que a maior parte da NNN seja formada a partir da nornicotina, que é o segundo alcaloide mais encontrado na maioria das variedades de tabaco. Por sua vez, a nornicotina é sintetizada diretamente a partir da nicotina através da reação de desmetilação, que é catalisada por enzimas nicotina *N*-desmetilases (NND) (Figura 2) (Lewis et al., 2010).

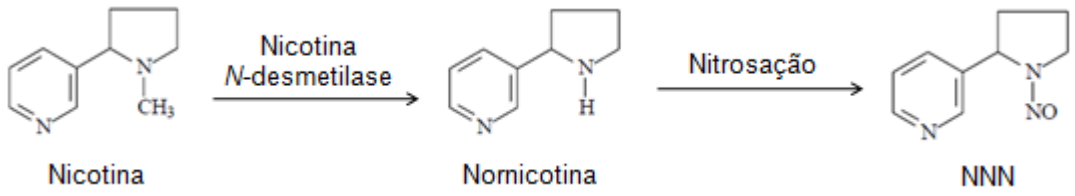


Figura 2: Formação de *N'*-nitrosonornicotina (NNN) a partir da nicotina.
Fonte: Siminszky et al., 2005.

Adicionalmente aos efeitos carcinogênicos e mutagênicos caracterizados para as TSNA, a redução dos níveis de nornicotina também é muito importante em função de esse ser um alcaloide precursor da TSNA NNN. Foi previamente demonstrado que a nornicotina por si só é capaz de induzir a glicosilação anormal de proteínas, o que pode estar relacionado a doenças metabólicas em humanos (Dickerson; Janda,

2002). Além disso, a nornicotina também tem sido relacionada à degeneração macular relacionada à idade, uma das maiores causas de cegueira no mundo (Brogan et al., 2005).

Algumas práticas culturais podem auxiliar na redução de TSNA nas folhas de tabaco. A adubação nitrogenada, por exemplo, está diretamente relacionada com a formação de alcaloides, visto que o nitrogênio é o seu principal componente (Chandrasekhararao et al., 2014). O tabaco Burley possui níveis mais altos de nicotina do que o tabaco Virgínia, além de apresentar um conteúdo de nitratos cerca de 10 vezes maior (Shi et al., 2013). Assim, naturalmente espera-se que o tabaco Burley possua taxas maiores de nornicotina e, consequentemente, de NNN (Lewis et al., 2012). Além disso, o desponte, a colheita, a cura e a estocagem também podem influenciar na síntese de maior ou menor quantidade de TSNA em tabaco (Shi et al., 2013).

Entretanto, fatores genéticos também influenciam as taxas de conversão de nicotina em nornicotina. Em algumas plantas de tabaco, as chamadas plantas conversoras, alterações na expressão gênica em um lócus instável do genoma leva plantas que inicialmente produziam quantidades pequenas de nornicotina a originarem uma progênie que converte até 98% da nicotina das folhas em nornicotina (Lewis et al., 2010). Por razões ainda desconhecidas, a frequência de reativação deste lócus é muito maior em plantas de tabaco Burley do que de outros tipos de tabaco (Lewis et al., 2008).

2.6 Genes envolvidos na conversão de nicotina em nornicotina

Com relação à formação de nornicotina em tabaco, seis genes da superfamília citocromo P450 que codificam enzimas nicotina *N*-desmetilases (NND) já foram caracterizados, todos pertencentes à subfamília gênica *CYP82E*: *CYP82E2*, *CYP82E3*, *CYP82E4*, *CYP82E5*, *CYP82E10* e *CYP82E21* (Lewis et al., 2010; Liedschulte et al., 2016). Entretanto, dois desses genes, *CYP82E2* e *CYP82E3*, sofreram mutações ao longo da evolução de *N. tabacum* e não apresentam atividade de *N*-desmetilase (Siminszky et al., 2005).

Quanto às enzimas funcionais, CYP82E5 e CYP82E10 possuem mínima atividade de *N*-desmetilase em folhas verdes e em raízes, respectivamente (Gavilano e Siminszky, 2007; Lewis et al., 2010). CYP82E21 apresenta atividade apenas nas flores de tabaco (Liedschulte et al., 2016). Já CYP82E4 é a principal

enzima responsável pela *N*-desmetilação da nicotina nas folhas de tabaco (Siminszky et al., 2005; Xu et al., 2007). Alguns estudos comprovaram que *CYP82E4* é expresso em níveis muito baixos em folhas verdes saudáveis, e que a sua expressão é fortemente induzida durante a senescência (Figura 3) (Siminszky et al., 2005; Gavilano et al., 2006; Chakrabarti et al., 2008).

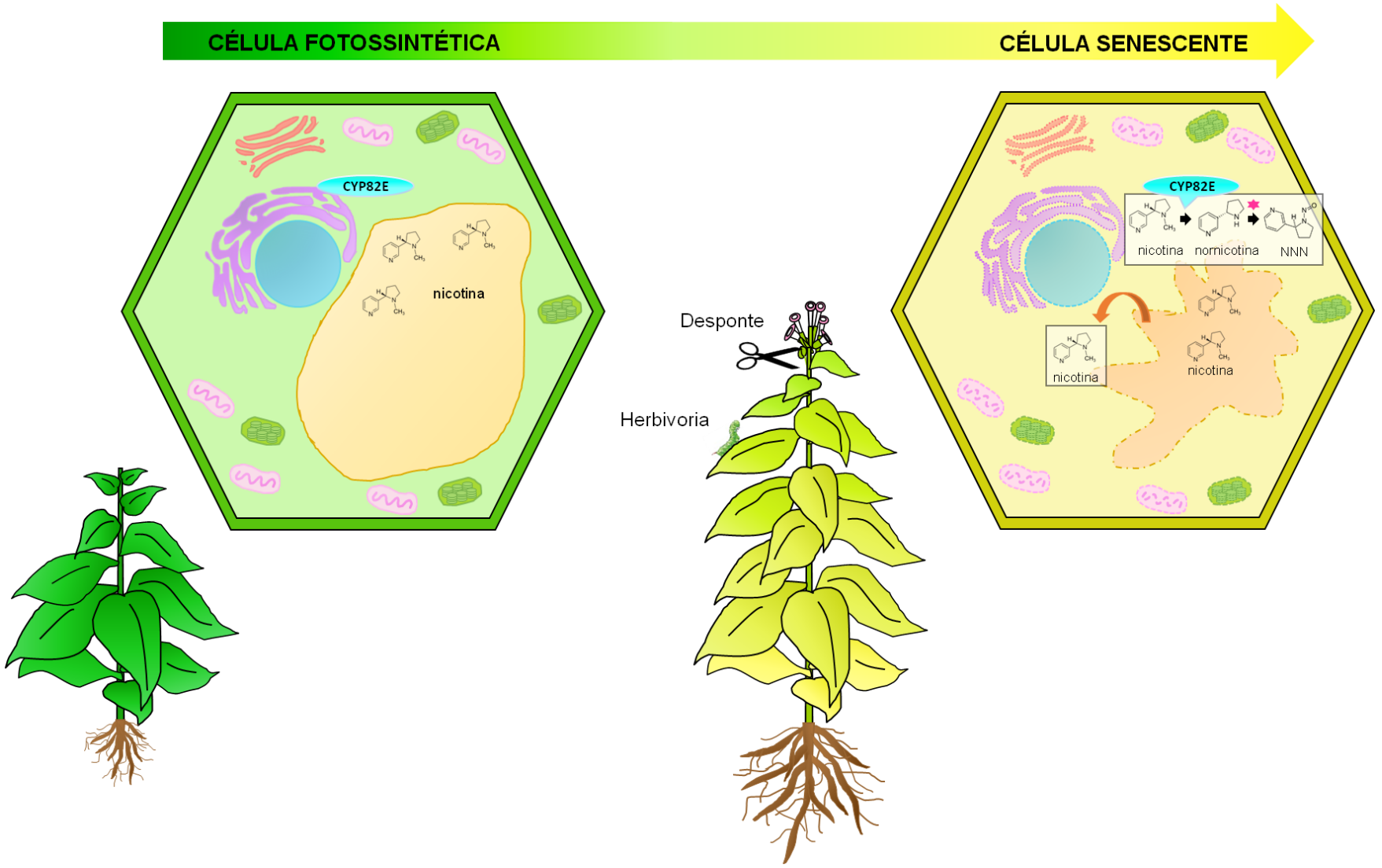


Figura 3: A nicotina estocada nos vacúolos das células das folhas é liberada para o citoplasma durante a senescência, e convertida em nornicotina em uma reação catalisada por enzimas da subfamília gênica *CYP82E*. A nornicotina produzida pode reagir espontaneamente com óxidos de nitrogênio presentes na folha, dando origem à *N*-nitrosonornicotina (NNN) (representado pela estrela de cor rosa na imagem).

A senescência é um processo fisiológico complexo que ocorre em resposta ao envelhecimento, podendo ser acelerado por estresses bióticos ou abióticos, como a seca e o ataque de patógenos, mas também pela ação de hormônios, como o ácido abscísico (ABA) e o etileno (Chakrabarti et al., 2008). Em tabaco, o amarelamento das folhas é um indicador visível da senescência. Nesse estágio, as folhas são consideradas maduras e apropriadas para a colheita (Lim et al., 2007).

Ao mesmo tempo que a colheita ocorre nesse estágio do desenvolvimento, é durante a senescência que a maior parte da nornicotina é formada através do processo de conversão da nicotina, catalisado pela enzima CYP82E4 (Chakrabarti et al., 2008). Entretanto, não é possível alterar a época de colheita com o objetivo de evitar a conversão da nicotina, pois as transformações químicas que ocorrem nas folhas durante a senescência, e os compostos que agregam valor comercial ao produto, seriam perdidos (Peek et al., 2008).

Diversos estudos tentando reduzir a formação de nornicotina e TSNA durante a senescência foram realizados, sendo a utilização da tecnologia de RNA de interferência (RNAi) (Gavilano et al., 2006; Lewis et al., 2008) e de mutações induzidas por etilmetanosulfonato (EMS) (Lewis et al., 2010) empregadas com a finalidade de reduzir a formação de nornicotina pelas plantas de tabaco. Entretanto, apesar do tabaco ser uma planta de fácil manipulação via técnicas de DNA recombinante, há certa relutância em implantar variedades comerciais de tabaco transgênico (Lewis et al., 2008).

As empresas que produzem sementes de tabaco normalmente realizam uma triagem em todas as plantas a fim de eliminar indivíduos que apresentem a taxa de conversão de nicotina maior que 3% (Carvalho et al., 2014). Embora o processo de triagem reduza significativamente o teor de nornicotina (e de NNN) em comparação as populações de tabaco não triadas, este processo nunca será perfeito, uma vez que plantas conversoras com alta produção de nornicotina podem espontaneamente surgir a cada geração (Lewis et al., 2010).

Portanto, o controle genético desta característica não é tão simples, e acredita-se que as plantas conversoras possam inesperadamente surgir a partir de alterações epigenéticas (Carvalho et al., 2014). Assim, apesar das informações disponíveis na literatura, ainda não está totalmente esclarecido como ocorre e quais são os fatores que regulam a transcrição de *CYP82E4*, o principal gene relacionado à formação de nornicotina em tabaco. Considerando o risco à saúde humana,

representado pela nornicotina e TSNAs, bem como pelo sabor desagradável que a nornicotina causa no cigarro, não há justificativa para impedir a redução da nornicotina em tabaco (Sun et al., 2015).

3 OBJETIVOS

3.1 Objetivo Geral

Identificar e caracterizar os genes da subfamília *CYP82E*, responsáveis pela conversão de nicotina em nornicotina no gênero *Nicotiana*, buscando contribuir para o entendimento do processo de acúmulo de Nitrosaminas Específicas do Tabaco (TSNA).

3.2 Objetivos Específicos

a) Elaborar uma revisão sobre a biossíntese e metabolização da nicotina no gênero *Nicotiana*;
b) Identificar os genes pertencentes à subfamília *CYP82E* em espécies do gênero *Nicotiana*;
c) Realizar a análise filogenética dos genes pertencentes à subfamília *CYP82E* no gênero *Nicotiana*;
d) Realizar a caracterização, *in silico*, das proteínas CYP82E identificadas*;*

4 RESULTADOS

Os resultados serão apresentados em dois capítulos. O primeiro capítulo apresenta uma revisão sobre a biossíntese e o metabolismo da nicotina no gênero *Nicotiana*. Neste capítulo são descritos os genes envolvidos no processo de síntese da nicotina e os transportadores responsáveis pelo seu transporte das raízes até a parte aérea das plantas, onde os alcaloides exercem um importante papel na defesa contra herbívoros. Além disso, a metabolização da nicotina em nornicotina e *N*-acil-nornicotina é discutida.

O segundo capítulo apresenta os resultados quanto à identificação e caracterização dos genes da subfamília *CYP82E*, responsáveis pela conversão de nicotina em nornicotina no gênero *Nicotiana*. Neste capítulo os genes identificados são caracterizados através de análises *in silico*, e uma filogenia para a subfamília *CYP82E* é proposta.

CAPÍTULO I: NICOTINE BIOSYNTHESIS IN *NICOTIANA*: A METABOLIC OVERVIEW

NICOTINE BIOSYNTHESIS IN *NICOTIANA*: A METABOLIC OVERVIEW

Fernanda Fleig Zenkner[1,2]; Márcia Margis-Pinheiro[1]; Alexandro Cagliari[3]

[1] Departamento de Genética, Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul (UFRGS), PO Box 15053, Porto Alegre, RS CEP 91501‑970, Brazil

[2] JTI Kannenberg Comércio de Tabacos do Brasil LTDA, Santa Cruz do Sul, RS, Brazil

[3] Universidade Estadual do Rio Grande do Sul (UERGS), Santa Cruz do Sul, RS, Brazil

Abstract

The alkaloids are important compounds found in *Nicotiana* plants, essential in the plant defense against herbivores. The main alkaloid of *N. tabacum*, nicotine, is produced in the roots and translocated to the leaves. Nicotine is formed by a pyrrolidine and a pyridine ring, in a process involving several enzymes. The pyridine ring of nicotine is derived from the nicotinic acid, whereas the pyrrolidine ring is originated from the polyamine putrescine metabolism. After nicotine synthesis in the root cortical cells, a set of transporters is known to transport it upwards the aerial part and to store it in the leaf vacuoles. Moreover, nicotine can be metabolized in the leaves, giving rise to nornicotine through the *N*-demethylation process. Some *Nicotiana* wild species present acyltransferase enzymes, which allow the plant to produce *N*-acyl-nornicotine, an alkaloid with higher insecticide capacity than nicotine. However, although we can find a wealth of information about the alkaloid production in *Nicotiana*, our understanding about the nicotine biosynthesis, transport and metabolism is still incomplete. Thus, this review will summarize these pathways based on recent literature, as well as highlighting the questions that need further investigation.

1 Introduction

Plants produce a diverse array of secondary metabolites, among which are some important compounds mainly used in the defense against pathogens and herbivores, the alkaloids (Memelink *et al.*, 2001; Steppuhn *et al.*, 2004). Historically, the use of alkaloid-containing plant extracts as medicines, drugs and poisons is widely known since the beginning of the human civilization (Kutchan, 1995). The Solanaceae (nightshade family) is a botanical family with several species producing alkaloids (Rushton *et al.*, 2008). Tobacco (*Nicotiana tabacum*) is one of those

species, and it has become an economically important crop plant due to alkaloid production, although the entire *Nicotiana* genus is recognized for producing these type of metabolites (Kutchan, 1995; Sun *et al.*, 2013).

The alkaloids found in *Nicotiana* plants are toxic compounds, which play a major role in the defense against generalist herbivores (Shi *et al.*, 2006). The root represents the particular site of alkaloid synthesis in the *Nicotiana* species (Sisson e Severson, 1990; Sun *et al.*, 2013). After their production, the alkaloids are transported through the xylem and accumulated in the leaves which are the areas more susceptible to a herbivore attack (Shi *et al.*, 2006).

The alkaloid synthesis is elicited upon herbivore attacks via the canonical jasmonate-signaling pathway (Shoji *et al.*, 2008; Zayed and Wink, 2009). In undamaged tobacco plants, nicotine represents 0.1-1% of its dry mass, while in wounded plants or plants attacked by herbivores, the nicotine concentration increases to 1-4% (Baldwin, 1998).Thus, a basal level of alkaloids is constantly maintained in the plant tissues, and increases after wounding stimuli (Zayed e Wink, 2009).

Regarding its ecological importance, the alkaloid biosynthesis, specifically of nicotine and its derivatives, is a complex process that involves several enzymatic steps. Nicotine has two ring moieties, a pyrrolidine ring and a pyridine ring, derived from two branch pathways (Wang *et al.*, 2015). The pyridine ring of nicotine is derived from the nicotinic acid, whereas the pyrrolidine ring originates from the polyamine putrescine metabolism, which is gradually modified to *N*-methylpyrrolinium (Naconsie *et al.*, 2014). The condensation of a nicotinic acid derivative and *N*-methylpyrrolinium forms the nicotine (Sun *et al.*, 2013). Other *Nicotiana* alkaloids, such as anatabine and anabasine, are synthesized solely through the pyridine ring pathway, while nornicotine is mainly produced via a nicotine *N*-demethylation process (Siminszky *et al.*, 2005; Shoji *et al.*, 2010).

Although a wealth of information is available on the alkaloid production in *Nicotiana* spp., some parts of the process are not fully understood. Thus, this review will present a reconstruction of nicotine biosynthesis, transport and metabolism pathways based on the current literature, and highlight the questions that would merit further investigation. Moreover, we will discuss the ecological importance of nicotine and the nicotine-derived alkaloids, nornicotine and *N*-acyl-nornicotine, to different *Nicotiana* species.

2 The structural genes of the nicotine pathway

The pathway leading to the formation of the pyrrolidine ring initiates with putrescine (Sun *et al.*, 2013) (Figure 1). Putrescine can be produced via two alternate routes: directly from ornithine or indirectly from arginine (Shi *et al.*, 2006). The direct route is catalyzed by ornithine decarboxylase (ODC), whereas the indirect route is catalyzed by arginine decarboxylase (ADC) (Cohen *et al.*, 1982; Bortolotti *et al.*, 2004). In the indirect route, the arginine is decarboxylated to agmatine, which is subsequently hydrolyzed to *N*-carbamoylputrescine by agmatine iminohydrolase (AIH) and further to putrescine by *N*-carbamoylputrescine amidohydrolase (CPA) (Janowitz *et al.*, 2003) (Figure 1). Then, putrescine is converted to *N*-methylputrescine by the putrescine *N*-methyltransferase (PMT) (Hibi *et al.*, 1994). Finally, *N*-methylputrescine is deaminated oxidatively by the *N*-methylputrescine oxidase (MPO) to 4-methylaminobutanal, which spontaneously cyclized to form the *N*-methylpyrrolinium cation that contains the pyrrolidine ring (Naconsie *et al.*, 2014) (Figure 1).

The pyridine ring of nicotine is derived from the nicotinic acid, which is formed by the same enzymes involved in the early steps of NAD biosynthesis, such as aspartate oxidase (AO), quinolinic acid synthase (QS) and quinolinic acid phosphoribosyl transferase (QPT) (Shoji *et al.*, 2010) (Figure 1). The exact metabolite derived from nicotinic acid that is used in the nicotine biosynthesis is not known (Naconsie *et al.*, 2014). However, it was suggested that nicotinic acid needs to be reduced to 3,6-dihydronicotinic acid before its condensation with *N*-methylpyrrolinium cation to form nicotine (Deboer *et al.*, 2009). Moreover, a PIP family oxidoreductase member, A622, also participates in the last step of pyridine alkaloid formation in *Nicotiana*, although it is not clear if it is related to nicotinic acid reduction or not (Deboer *et al.*, 2009; Kajikawa *et al.*, 2009). All the tobacco genes encoding the proteins mentioned above are known as the structural genes of the nicotine pathway (Shoji *et al.*, 2010) (Figure 1).

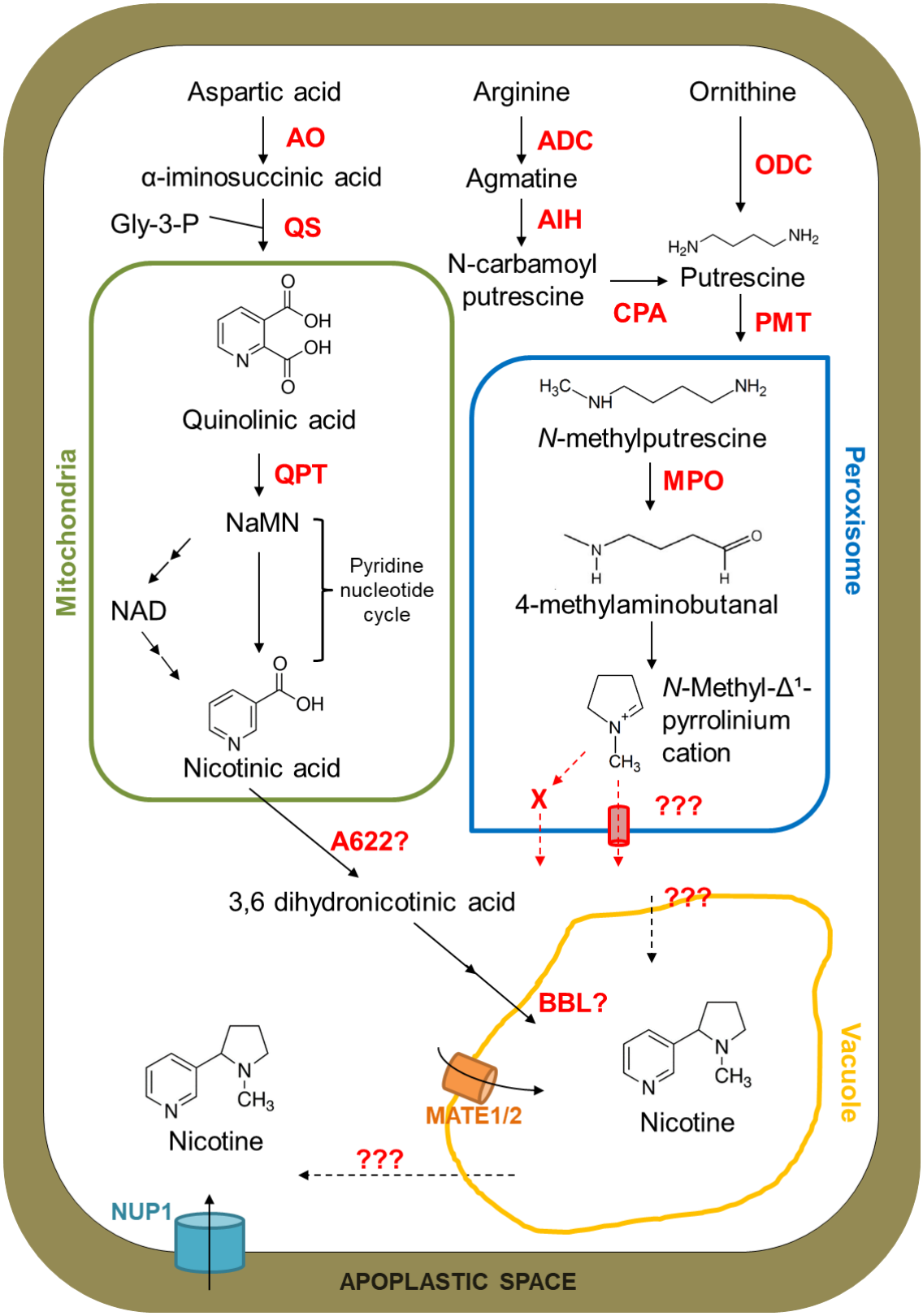


Figure 1: Nicotine biosynthesis in a root cortical cell of *Nicotiana*. The enzymes are indicated in red letters. AO: Aspartate oxidase; QS: Quinolinic acid synthase; Gly-3-P: Glyceraldehyde-3-P; QPT: Quinolinate phosphoribosyltransferase; ADC: Arginine decarboxylase; ODC: Ornithine decarboxylase; AIH: Agmatine iminohydrolase; CPA: *N*-carbamoylputrescine amidohydrolase; PMT: Putrescine *N*-methyltransferase; MPO: *N*-methylputrescine oxidase; BBL: Berberine bridge enzyme-like; MATE1/2: Multidrug and toxic compound extrusion 1 and 2; NUP1: Nicotine uptake permease 1; ???: undefined step.

2.1 Arginine decarboxylase (ADC) and Ornithine decarboxylase (ODC) –The origin of putrescine

Putrescine may be synthesized from arginine, via a three enzymatic step process initiated by ADC, or directly from ornithine via the action of ODC (Deboer *et al.*, 2011) (Figure 1). ADC activity is predominant in the putrescine biosynthesis of plants, while ODC is more frequently found in animals and fungi (Bortolotti *et al.*, 2004). However, in the case of nicotine biosynthesis in tobacco, ODC seems to have a more prominent role (Deboer *et al.*, 2011). The impairment of ODC activity is related to plants with increased capacity of anatabine production (Shoji *et al.*, 2010; Deboer *et al.*, 2011). Thus, the imbalance in the putrescine supply (pyrrolidine pathway) causes an oversupply of derivatives from the pyridine pathway, from which anatabine is entirely derived (Deboer *et al.*, 2011).

2.2 Putrescine Methyltransferase (PMT)

Putrescine methyltransferase (PMT) is the enzyme responsible for the *N*-methylation of putrescine, generating *N*-methylputrescine (Heim *et al.*, 2007) (Figure 1). *PMT* is expressed in the pericycle tissue, and its activity takes place in the apical portion of the roots (Suzuki *et al.*, 1999; Shoji *et al.*, 2002; Xu and Timko, 2004). *N. tabacum* presents five genes encoding functional PMT, three of them are similar to those of *Nicotiana sylvestris*, whereas the other two are similar to *PMT* genes present in *Nicotiana tomentosiformis* (Riechers and Timko, 1999).

PMT genes are not exclusive of *Nicotiana* spp., and have been identified in many species that produce pyridine and tropane alkaloids (Hashimoto *et al.*, 1989; Walton *et al.*, 1990; Hibi *et al.*, 1992; Naconsie *et al.*, 2014). The sequence similarities indicate that tobacco *PMT* has evolved from *Spermidine synthase* (*SPDS*) during the diversification of the Solanaceae (Hibi *et al.*, 1994; Hashimoto *et al.*, 1998). SPDS can be found in various organisms, e.g., *Escherichia coli* (Bowman *et al.*, 1973)*, Bos taurus* (Raina *et al.*, 1984), and soybean, *Glycine max* (Yoon *et al.*, 2000). In contrast, PMT activity was purified only from plants (Biastoff *et al.*, 2009). Thus, the concept of gene duplication and mutation of one gene copy until the neofunctionalization may explain *PMT* evolution, as current PMT and SPDS catalyze different reactions with high specificities (Teuber *et al.*, 2007).

2.3 N-Methylputrescine Oxidase (MPO)

N-methylputrescine oxidase (MPO) is an amine oxidase that requires copper as a cofactor (Heim *et al.*, 2007; Katoh *et al.*, 2007). MPO catalyzes the oxidative deamination of *N*-methylputrescine in the second step of the nicotine biosynthesis (Naconsie *et al.*, 2014) (Figure 1). *N*-methylputrescine is the preferable substrate of MPO, although this enzyme can also use other polyamines, as putrescine and cadaverine (Moschou *et al.*, 2012). When putrescine is used as a substrate, MPO produces an unmethylated-pyrrolinium salt, resulting in the direct biosynthesis of nornicotine, while when cadaverine is used, anabasine is synthesized (Heim *et al.*, 2007). It was suggested that the *N. tabacum* MPO preference for *N*-methylputrescine instead of other substrates, may have contributed to the alkaloid pool characteristic of the tobacco plants, with nicotine being the main alkaloid, followed by nornicotine and anabasine, respectively (Saitoh *et al.*, 1985; Heim *et al.*, 2007).

N. tabacum MPO1 (*NtabMPO1*) possibly evolved from a diamine oxidase (*NtabDAO1*) after gene duplication and subsequent neofunctionalization (Naconsie *et al.*, 2014). Throughout this process, the *cis*-elements that placed *MPO* under the control of the NIC2 locus emerged in its promoter (Naconsie *et al.*, 2014). Moreover, during the neofunctionalization process, the substrate specificity of MPO1 was altered to prefer *N*-methylputrescine (Naconsie *et al.*, 2014). *MPO1* homologs were found in *Solanum lycopersicum* and *Solanum tuberosum* genomes, indicating that molecular evolution of MPO1 occurred early in the diversification of the Solanaceae family (Naconsie *et al.*, 2014). In fact, in other Solanaceae species MPO is involved in the biosynthesis of several alkaloids, such as calystegines, hyoscyamine and scopolamine (Bedewitz *et al.*, 2014).

Recently, it was described that MPO1 acts in the peroxisome, the same subcellular localization of DAO1 (Naconsie *et al.*, 2014). This revelation added a new cellular compartment to the nicotine biosynthesis pathway, and this is important for the full understanding of the route (Rajabi *et al.*, 2017). The product derived from the MPO1 reaction, 4-methyl-aminobutanal, spontaneously undergoes intramolecular cyclization to form the *N*-methylpyrrolinium cation (Katoh *et al.*, 2007). As a cation, it is not permeable to biological membranes, and a transporter should be required to move it to the cytoplasm, where the final steps of nicotine formation occur (Naconsie *et al.*, 2014) (Figure 1). Another possibility is that an unknown peroxisomal enzyme

may condensate the cation with another compound, generating a membrane-permeable product (Naconsie *et al.*, 2014) (Figure 1).

2.4 Quinolinate Phosphoribosyltransferase (QPT)

Quinolinate phosphoribosyltransferase (QPT) is an enzyme that converts quinolate to nicotinate ribonucleotide (Moghe and Last, 2015) (Figure 1). The activity of QPT is required for both primary and secondary metabolism in *Nicotiana* spp. (Sinclair *et al*., 2000). As a primary metabolic enzyme, QPT is essential for NAD synthesis and in the secondary metabolism it is involved in the formation of several alkaloids (Ryan *et al.*, 2012). QPT is encoded by duplicate genes (*QPT1* and *QPT2*) in *N. tabacum*, *N.* sylvestris, *N. tomentosiformis* and *Nicotiana glauca* (Ryan *et al.*, 2012). *QPT2* activity increased several fold after wounding of aerial tissues, and it was correlated with wounding-induced nicotine production (Sinclair *et al.*, 2000).

2.5 A622 and BBL – The late steps in the nicotine biosynthesis

A622 is a member of the PIP family of NADPH-dependent reductases, with homology to phenylcoumaran benzylic ether reductase (PCBER), pterocarpan reductase (PTR) and isoflavone reductase (IFR) (Kajikawa *et al.*, 2009). However, considering that pterocarpans and isoflavonoids are phytoalexins not produced by wild type tobacco, and A622 did not presented PCBER activity, the specific substrate of A622 remains unclear (Yu *et al.*, 2000; Shoji *et al.*, 2002; Kajikawa *et al.*, 2009). It was suggested that because of the high similarity, these genes may have diverged relatively recently, and possibly the substrate for A622 may be structurally similar to phytoalexins (Hibi *et al.*, 1994).

The suppression of *A622* in tobacco roots caused the inhibition of the condensation reactions to form several pyridine alkaloids and resulted in the accumulation of nicotinic acid *N*-glucoside (NaNG). Since nicotinic acid itself is not a substrate for A622, and NaNG is a nicotinic acid metabolite, there are speculations whether a derivative of nicotinic acid may be used as A622 substrate (Kajikawa *et al.*, 2009) (Figure 1).

Like other structural genes of the nicotine pathway, *A622* expression occurs in the root, more strongly in the cortex of the root tip and in the outer cortex layer and endodermis in the differentiated region (Kajikawa *et al.*, 2009). In *N. sylvestris*, the expression of *A622* is strongly up regulated by methyl-jasmonate (MeJA), and

ethylene may work as an antagonist, suppressing the MeJA-induced expression of *A622* (Shoji *et al.*, 2000; Shoji *et al.*, 2002). At a subcellular level, A622 protein was detected mainly in the cytoplasm of *N. sylvestris* (Shoji *et al.*, 2002).

Recently, a *Berberine Bridge Enzyme-Like* (*BBL*) gene family was identified for *N. tabacum* and was found to act downstream in the nicotine biosynthetic pathway (Lewis *et al.*, 2015). *BBL*s are expressed in the vacuoles of tobacco root cells, and its suppression inhibited anatabine biosynthesis, demonstrating that this gene participates in the pyridine pathway (Kajikawa *et al.*, 2011).

3 Nicotine: from the bottom to the top of tobacco plants

Nicotine is synthesized in root cortical cells and needs to be transported to the leaves, where it is accumulated and used as anti-herbivory defense (Kato *et al.*, 2014). The nicotine path from the root to the leaves implies a long-distance transport through the xylem, and it should include several transporters (Shitan *et al.*, 2015).

Although most nicotine produced in roots is transported to and accumulates in above-ground tissues, a portion of nicotine may stay in root cells (Hildreth *et al.*, 2011). High levels of nicotine in the roots might trigger feedback inhibition of its synthetic genes (Wang *et al.*, 2015). Thus, maintaining low cytoplasmic concentrations of this alkaloid in the root cells may be important to ensure its active synthesis to continue (Shoji *et al.*, 2009).

The multidrug and toxic compound extrusion (MATE)-type transporters, NtMATE1 and NtMATE2 (collectively called NtMATE1/2), were identified in alkaloid-synthesizing root cells. These transporters are located in the tonoplast, and use an antiport of protons from the vacuole to move cytoplasmic nicotine through the tonoplast of the root cells (Figure 1). Additionally, the vacuolar sequestration of nicotine may be necessary to protect the nicotine-synthesizing cells from a potential cytotoxicity caused by this alkaloid (Shoji *et al.*, 2009).

Another transporter from the MATE family, *N. tabacum* jasmonate-inducible alkaloid transporter 1 (Nt-JAT1), is involved in the vacuolar accumulation of nicotine in the aerial parts of tobacco plants. The expression of *Nt-JAT1* was detected in roots, stems and leaves, but its subcellular localization in the tonoplast was showed just in the green leaves (Morita *et al.*, 2009). Further detailed investigation is needed to clarify its role and location in the root cells.

A similar transporter, called Nt-JAT2, was found in the tonoplast of tobacco leaves. Nt-JAT2 acts in the nicotine transport to the vacuolar lumen specifically in the leaves. It was suggested that Nt-JAT1 is responsible for steady-state alkaloid transport in various tissues, whereas Nt-JAT2 works on the nicotine accumulation in leaves upon herbivore attack (Shitan *et al.*, 2014).

After synthesis, the nicotine from the roots may move to the apoplast, and it is energetically important for the plant to avoid this nicotine to be secreted into the rhizosphere (Hildreth *et al.*, 2011). Nicotine uptake permease 1 (NUP1) (Figure 1) is a plasma membrane-localized transporter of the purine uptake permease (PUP) family, presenting a high degree of substrate specificity for nicotine (Hildreth *et al.*, 2011; Kato *et al.*, 2014). This transporter uses proton symport for the uptake of nicotine (Hildreth *et al.*, 2011). *NUP1* expression occurs primarily in the epidermal cells of the root tip, and low expression was also detected in the leaf and stem of tobacco plants. NUP1 may prevent the loss of apoplastic nicotine, or it even may retrieve secreted nicotine back into the root tissues (Kato *et al.*, 2014).

Transporters responsible for loading nicotine into the xylem in the root, as well as unloading of nicotine from xylem into leaf cells remain unknown (Wang *et al.*, 2015) (Figure 2). Alkaloids are protonated in the apoplastic space due to the acidic condition, becoming more hydrophilic and consequently harder to transpose the plasma membrane (Morita *et al.*, 2009). Thus, it was speculated that a plasma membrane-localized transporter, like NUP1, may import the nicotine from xylem to leaf cells (Jelesko, 2012). Further studies are needed to unravel the whole translocation mechanism of nicotine.

4 Genetic and hormonal regulation of nicotine structural-genes expression

There are two regulatory *loci* specifically controlling the expression of nicotine-related structural genes in tobacco, *NIC1* and *NIC2*. Studies using mutants showed that *nic1* and *nic2* mutations are semidominant or show dose-dependent effects on nicotine levels, but the effect of *nic1* was 2.4 times stronger than that of *nic2* (Hibi *et al.*, 1994; Shoji *et al.*, 2010). The downregulation of nicotine biosynthesis genes using *nic* mutants has been confirmed in several studies (Legg and Collins, 1971; Hibi *et al.*, 1994; Reed and Jelesko, 2004; Cane *et al.*, 2005).

When compared to the wild type, *nic1* and *nic2* double mutants (*nic1nic2*) present the lowest *N*-methylputrescine content, whereas the total content of

putrescine, spermidine and spermine were the highest (Hibi *et al.*, 1994). It suggests that the metabolism of putrescine is blocked through the impairment of *PMT*, and this polyamine accumulates in the roots (Hibi *et al.*, 1994). Moreover, *A622*, *QPT*, *QS* and *BBL* were also showed to be regulated by *NIC* loci (Shoji *et al.*, 2002; Kidd *et al.*, 2006; Kajikawa *et al.*, 2009; Shoji and Hashimoto, 2011a).

Although the *NIC1* locus remains uncharacterized, the impact of *NIC2* locus on leaf nicotine content becomes considerably stronger in the presence of *nic1* mutation (Legg and Collins, 1971). The *NIC2* locus was shown to encode for a group of transcription factor genes from the IXa Ethylene Response Factor (ERF) subfamily (Lewis *et al.*, 2015). At least seven *NIC2*-locus ERF genes were found as downregulated in *nic2* mutants: ERF189, ERF115, ERF221, ERF104, ERF179, ERF17 and ERF168 (Shoji *et al.*, 2010).

Regarding hormonal regulation, it was already showed that most of the genes participating in nicotine biosynthesis and transport, such as *PMT*, *QPT*, *A622*, *MATE1/2*, *Nt-JAT1* and *NtJAT-2*, are upregulated by jasmonates (Hibi *et al.*, 1994). Tobacco uses canonical jasmonate signaling components to activate structural genes involved in nicotine biosynthesis (Paschold *et al.*, 2007; Shoji *et al.*, 2008). The Coronative Insensitive 1 (COI1) acts in the perception of jasmonate, degrading Jasmonate Zim Domain (JAZ) proteins, which allows the release of transcription factors to activate the transcription of jasmonate-responsive genes (Wasternack and Hause, 2013).

One of the transcription factors released after JAZ degradation is the highly conserved basic Helix-Loop-Helix (bHLH) MYC2. MYC2 was shown to controls the expression of nicotine biosynthetic genes by directly binding its *cis*-elements in the target promoters and also by up-regulating the *NIC2*-locus *ERF* genes (Shoji and Hashimoto, 2011b). Additionally, MYC2 also induces the expression of *NUP1*, which in turn positively controls the expression of *ERF189,* one of the major regulators of the nicotine biosynthetic pathway (Kato *et al.*, 2014).

Another phytohormone involved in the nicotine pathway is the auxin, which downregulates the nicotine biosynthesis (Hibi *et al.*, 1994). A very common practice among tobacco growers is the removal of the flower head and several young leaves of the tobacco plants, also called “topping” (Hibi *et al.*, 1994). This practice switches the plant from its reproductive to its vegetative phase, eliminates the apical dominance and favors the root growth (Li *et al.*, 2016). After topping, the nicotine

concentration increases in tobacco leaves, and it is highly dependent on the removal of apical meristems, the primary source of auxin in the plant (Xi *et al.*, 2005; Shi *et al.*, 2006). Indeed, it was suggested that the decrease of auxin supplied to the root might result in activation or derepression of *PMT* (Hibi *et al.*, 1994).

The nicotine biosynthesis must be extremely regulated, as it is highly costly to the plant, reducing even the seed yield (Baldwin, 1998). An example of this fine regulation was observed when nicotine-tolerant herbivores fed on tobacco leaves, triggering an ethylene burst that suppressed the jasmonate-mediated activation of nicotine biosynthesis genes (Kahl *et al.*, 2000). In this case, the ethylene signaling prevents the plant to waste its resources on an ineffective nicotine defense, and to instead allocate them to other defense mechanisms (Winz and Baldwin, 2001).

The cross-talk between different signaling pathways, mainly jasmonates and ethylene in the case of nicotine biosynthesis in tobacco, may be critical to managing the destination of the plant energy sources (Shoji *et al.*, 2000). In a herbivore attack scenario, most of the nitrogen available is redirected to alkaloid biosynthesis, targeting plant defense (Baldwin *et al.*, 1994). However, as it is not possible to recover alkaloid nitrogen and reinvest it in other metabolic processes, its biosynthesis requires optimum regulation (Kutchan, 1995).

5 The alkaloid content in different Nicotiana *species*

The alkaloid composition within the *Nicotiana* genus is species-specific and extremely variable (Saitoh *et al.*, 1985; Sisson and Severson, 1990). The alkaloids most frequently found in *Nicotiana* are nicotine, nornicotine, anatabine and anabasine, but a single alkaloid usually predominates in each species (Saitoh *et al.*, 1985; Sun *et al.*, 2013). Most of the *N. tabacum* cultivars currently used present nicotine as the primary alkaloid (Wang *et al.*, 2015).

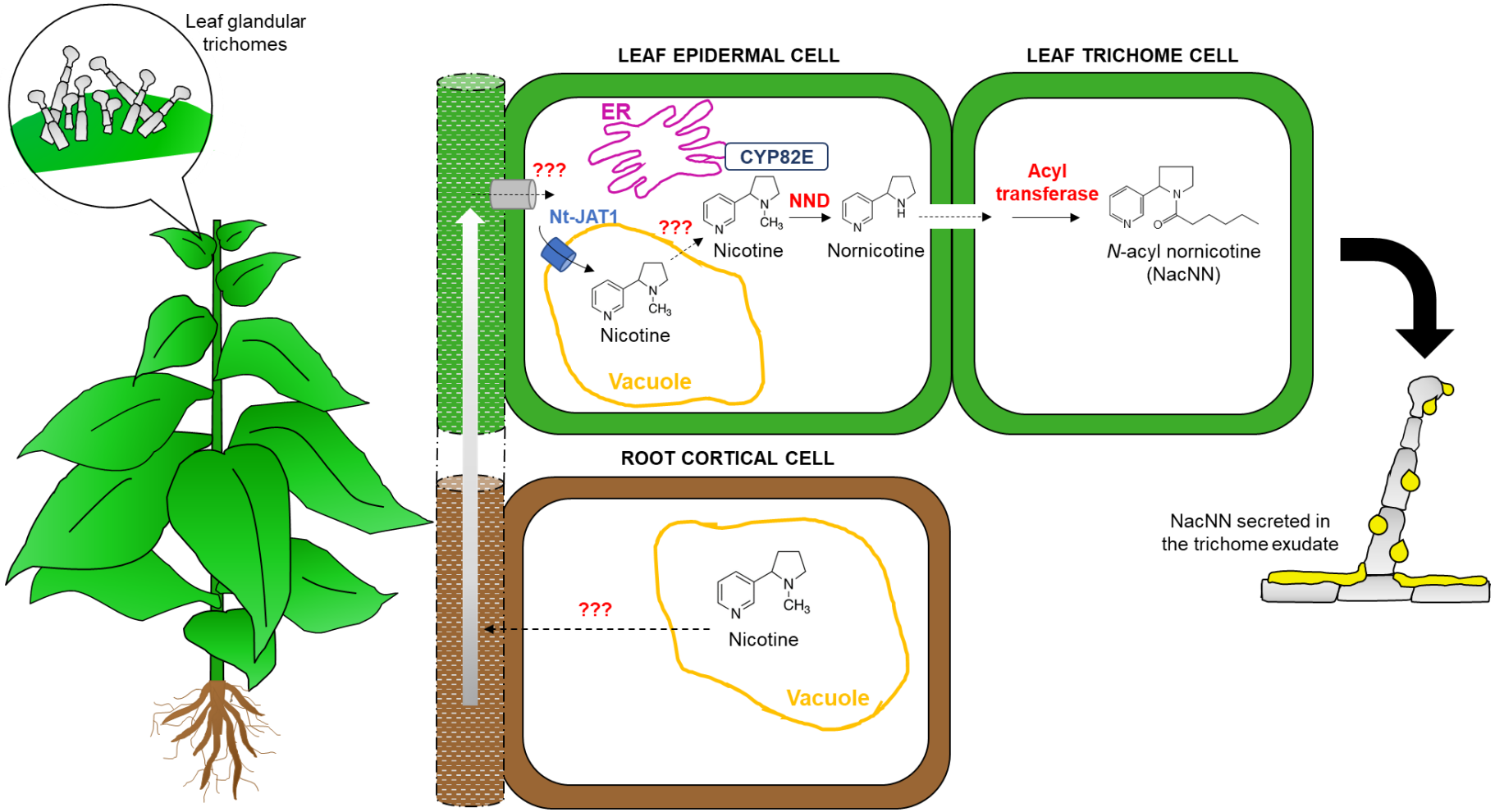


Figure 2: The nicotine produced in the *Nicotiana* root cells is translocated through the xylem to the leaves, where it can be demethylated to nornicotine. Nornicotine, by the action of an acyltransferase, is acylated to *N*-acylnornicotine (NacNN) in the trichomes. NacNN is secreted by the glandular trichome, and acts in the anti-herbivory defense. ER: Endoplasmic Reticulum.

However, despite being a natural allotetraploid derived from the interspecific hybridization between ancestral *N. sylvestris* and *N. tomentosiformis, N. tabacum* remarkably presents an alkaloid profile different from both of its progenitor species (Gavilano *et al.*, 2007). *N. tomentosiformis* primarily accumulates nornicotine, whereas nicotine and nornicotine can be found as the predominant alkaloids in *N. sylvestris* green and senescing leaves, respectively (Sisson and Severson, 1990).

Nicotine and nornicotine are the alkaloids more frequently found in *Nicotiana* spp., and the insecticide activity of these alkaloids varies widely depending on the insect species (Feinstein, 1952). Additionally, in some of *Nicotiana* wild species, nornicotine has an important role as a precursor of *N*-acyl-nornicotine (NacNN), an alkaloid that exhibits 1000-fold higher activity against nicotine-resistant *Manduca sexta* (tobacco hornworm) than nicotine (Gavilano *et al.*, 2007).

NacNN was found in the trichome exudate produced at the epidermis of *Nicotiana repanda, Nicotiana stocktonii* and *Nicotiana nesophila* aerial parts (Zador and Jones, 1986; Huesing *et al.*, 1989; Laue *et al.*, 2000). It was suggested a route where nicotine is *N*-demethylated to nornicotine in the leaves, followed by its mobilization to the trichomes upon herbivory (Zador e Jones, 1986) (Figure 2). Then, nornicotine is acylated with straight-chain fatty acids, rising to the hydrophobic NacNN, which is secreted from the gland to coat the leaf surface (Figure 2) (Zador e Jones, 1986; Schilmiller *et al.*, 2008). Other species of the *Nicotiana* genus lack the acyltransferase enzyme necessary for NacNN biosynthesis (Huesing *et al.*, 1989).

6 Concluding remarks

Alkaloids, specially nicotine, have an essential role in the anti-herbivory defense of *Nicotiana* plants (Kutchan, 1995).The site of nicotine production is the root cortical tissue. After been synthesized, the alkaloid is translocated via xylem to the leaves, where it is stored in the vacuoles (Sun *et al.*, 2013). Depending on the *Nicotiana* species, nicotine can be metabolized in the leaves, resulting in different alkaloids, such as nornicotine and NacNN (Zador and Jones, 1986; Gavilano *et al.*, 2007) (Figure 2). Nicotine biosynthesis, transport to the leaves and metabolism are multi-enzymatic and complex processes, which have not been completely elucidated yet. This review presented the nicotine biosynthesis, transport and metabolism, and highlighted the points that need further investigation: a) the late steps of nicotine biosynthesis, and b) the involvement of the long-distance transport to the leaves.

The suppression of both *A622* and *BBL* in tobacco roots inhibited the pyridine pathway, demonstrating the importance of these enzymes to the pyridine alkaloid formation (Kajikawa *et al.*, 2009; Kajikawa *et al.*, 2011). Although there is a consensus that they act downstream in this route, it is not clear what is the substrate of each enzyme (Lewis *et al.*, 2015). Moreover, the translocation of nicotine upwards the aerial part of *Nicotiana* involves several transporters. There are currently five transporters known to conduct nicotine translocation in *Nicotiana* spp.: NtMATE1, NtMATE2, NUP1, NtJAT1 and NtJAT2 (Morita *et al.*, 2009; Shoji *et al.*, 2009; Hildreth *et al.*, 2011; Kato *et al.*, 2014; Shitan *et al.*, 2014). However, the transporters responsible for loading nicotine into the xylem in the root, and for unloading of nicotine from xylem into leaf cells remain unknown (Wang *et al.*, 2015). Thus, further studies are needed to unravel the whole biosynthesis and translocation pathways of nicotine in *Nicotiana species*.

ACKNOWLEDGMENTS

We thank Tomoyuki Komatsu for his contribution on Figure 2 design, and to Paolo Donini and Christelle Bonnet for their review of this manuscript and helpful comments.

7 References

BALDWIN, I. T. Jasmonate-induced responses are costly but benefit plants under attack in native populations. **Proc Natl Acad Sci USA,** v. 95, n. 14, p. 8113-8, Jul 1998. ISSN 0027-8424. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/9653149>.

BALDWIN, I. T.; KARB, M. J.; OHNMEISS, T. E. Allocation of 15N from nitrate to nicotine: production and turnover of a damage-induced mobile defense. **Ecology,** v. 75, n. 6, p. 1703-1713, 1994.

BEDEWITZ, M. A. et al. A root-expressed L-phenylalanine:4-hydroxyphenylpyruvate aminotransferase is required for tropane alkaloid biosynthesis in Atropa belladonna. **Plant Cell,** v. 26, n. 9, p. 3745-62, Sep 2014. ISSN 1532-298X. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/25228340>.

BIASTOFF, S.; BRANDT, W.; DRÄGER, B. Putrescine N-methyltransferase--the start for alkaloids. **Phytochemistry,** v. 70, n. 15-16, p. 1708-18, 2009 Oct-Nov 2009. ISSN 1873-3700. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/19651420>.

BORTOLOTTI, C. et al. Localization of arginine decarboxylase in tobacco plants. **Physiol Plant,** v. 120, n. 1, p. 84-92, Jan 2004. ISSN 1399-3054. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/15032880>.

BOWMAN, W. H.; TABOR, C. W.; TABOR, H. Spermidine biosynthesis. Purification and properties of propylamine transferase from Escherichia coli. **J Biol Chem,** v. 248, n. 7, p. 2480-6, Apr 1973. ISSN 0021-9258. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/4572733>.

CANE, K. A. et al. Molecular analysis of alkaloid metabolism in *AABBv.aabb* genotype *Nicotiana tabacum* in response to wounding of aerial tissues and methyl jasmonate treatment of cultured roots. **Functional Plant Biology,** v. 32, n. 4, p. 305-320, 2005.

COHEN, E. et al. Participation of ornithine decarboxylase in early stages of tomato fruit development. **Plant Physiol,** v. 70, n. 2, p. 540-3, Aug 1982. ISSN 0032-0889. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/16662530>.

DEBOER, K. D. et al. RNAi-mediated down-regulation of ornithine decarboxylase (ODC) leads to reduced nicotine and increased anatabine levels in transgenic Nicotiana tabacum L. **Phytochemistry,** v. 72, n. 4-5, p. 344-55, Apr 2011. ISSN 1873-3700. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/21232776>.

_____. The A622 gene in Nicotiana glauca (tree tobacco): evidence for a functional role in pyridine alkaloid synthesis. **Plant Mol Biol,** v. 69, n. 3, p. 299-312, Feb 2009. ISSN 0167-4412. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/19011764>.

FEINSTEIN, L. Insecticides from plants. **Yearbook of Agriculture**, p. 222-229, 1952.

GAVILANO, L. B. et al. Functional analysis of nicotine demethylase genes reveals insights into the evolution of modern tobacco. **J Biol Chem,** v. 282, n. 1, p. 249-56, Jan 2007. ISSN 0021-9258. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17102129>.

GOODIN, M. M. et al. Nicotiana benthamiana: its history and future as a model for plant-pathogen interactions. **Mol Plant Microbe Interact,** v. 21, n. 8, p. 1015-26, Aug 2008. ISSN 0894-0282. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/18616398>.

HASHIMOTO, T. et al. Molecular cloning of plant spermidine synthases. **Plant Cell Physiol,** v. 39, n. 1, p. 73-9, Jan 1998. ISSN 0032-0781. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/9517003>.

HASHIMOTO, T.; YUKIMUNE, Y.; YAMADA, Y. Putrescine and putrescine N-methyltransferase in the biosynthesis of tropane alkaloids in cultured roots of Hyoscyamus albus : I. Biochemical studies. **Planta,** v. 178, n. 1, p. 123-30, May 1989. ISSN 0032-0935. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/24212557>.

HEIM, W. G. et al. Cloning and characterization of a Nicotiana tabacum methylputrescine oxidase transcript. **Phytochemistry,** v. 68, n. 4, p. 454-63, Feb 2007. ISSN 0031-9422. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/17174363>.

HIBI, N. et al. Putrescine N-Methyltransferase in Cultured Roots of Hyoscyamus albus: n-Butylamine as a Potent Inhibitor of the Transferase both in Vitro and in Vivo. **Plant Physiol,** v. 100, n. 2, p. 826-35, Oct 1992. ISSN 0032-0889. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/16653064>.

_____. Gene expression in tobacco low-nicotine mutants. **Plant Cell,** v. 6, n. 5, p. 723-35, May 1994. ISSN 1040-4651. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/8038607>.

HILDRETH, S. B. et al. Tobacco nicotine uptake permease (NUP1) affects alkaloid metabolism. **Proc Natl Acad Sci U S A,** v. 108, n. 44, p. 18179-84, Nov 2011. ISSN 1091-6490. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/22006310>.

HUESING, J. et al. Biochemical investigations of antibiosis material in leaf exudate of wild *Nicotiana* species and interspecific hybrids. **Journal of Chemical Ecology,** v. 15, n. 4, p. 1204-1217, 1989.

JANOWITZ, T.; KNEIFEL, H.; PIOTROWSKI, M. Identification and characterization of plant agmatine iminohydrolase, the last missing link in polyamine biosynthesis of plants. **FEBS Lett,** v. 544, n. 1-3, p. 258-61, Jun 2003. ISSN 0014-5793. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/12782327>.

JELESKO, J. G. An expanding role for purine uptake permease-like transporters in plant secondary metabolism. **Front Plant Sci,** v. 3, p. 78, 2012. ISSN 1664-462X. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/22639664>.

KAHL, J. et al. Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. **Planta,** v. 210, n. 2, p. 336-42, Jan 2000. ISSN 0032-0935. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/10664141>.

KAJIKAWA, M.; HIRAI, N.; HASHIMOTO, T. A PIP-family protein is required for biosynthesis of tobacco alkaloids. **Plant Mol Biol,** v. 69, n. 3, p. 287-98, Feb 2009. ISSN 0167-4412. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/19002761>.

KAJIKAWA, M. et al. Vacuole-localized berberine bridge enzyme-like proteins are required for a late step of nicotine biosynthesis in tobacco. **Plant Physiol,** v. 155, n. 4, p. 2010-22, Apr 2011. ISSN 1532-2548. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/21343426>.

KATO, K.; SHOJI, T.; HASHIMOTO, T. Tobacco nicotine uptake permease regulates the expression of a key transcription factor gene in the nicotine biosynthesis pathway. **Plant Physiol,** v. 166, n. 4, p. 2195-204, Dec 2014. ISSN 1532-2548. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/25344505>.

KATOH, A.; SHOJI, T.; HASHIMOTO, T. Molecular cloning of N-methylputrescine oxidase from tobacco. **Plant Cell Physiol,** v. 48, n. 3, p. 550-4, Mar 2007. ISSN 0032-0781. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/17283012>.

KIDD, S. K. et al. The A and B loci in tobacco regulate a network of stress response genes, few of which are associated with nicotine biosynthesis. **Plant Mol Biol,** v. 60, n. 5, p. 699-716, Mar 2006. ISSN 0167-4412. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/16649107>.

KUTCHAN, T. M. Alkaloid Biosynthesis[mdash]The Basis for Metabolic Engineering of Medicinal Plants. **Plant Cell,** v. 7, n. 7, p. 1059-1070, Jul 1995. ISSN 1532-298X. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/12242397>.

LAUE, G.; PRESTON, C. A.; BALDWIN, I. T. Fast track to the trichome: induction of N-acyl nornicotines precedes nicotine induction in Nicotiana repanda. **Planta,** v. 210, n. 3, p. 510-4, Feb 2000. ISSN 0032-0935. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/10750910>.

LEGG, P. D.; COLLINS, G. B. Inheritance of percent total alkaloids in *Nicotiana tabacum* L. II. genetic effects of two loci in Burley21 x L.A. Burley21 populations **Canadian Journal of Genetics and Cytology,** v. 13, p. 287-291, 1971.

LEWIS, R. S. et al. Transgenic and mutation-based suppression of a berberine bridge enzyme-like (BBL) gene family reduces alkaloid content in field-grown tobacco. **PLoS One,** v. 10, n. 2, p. e0117273, 2015. ISSN 1932-6203. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/25688975>.

LI, F. et al. Identification of Topping Responsive Proteins in Tobacco Roots. **Front Plant Sci,** v. 7, p. 582, 2016. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/27200055>.

MEMELINK, J.; VERPOORTE, R.; KIJNE, J. W. ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. **Trends Plant Sci,** v. 6, n. 5, p. 212-9, May 2001. ISSN 1360-1385. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/11335174>.

MOGHE, G. D.; LAST, R. L. Something Old, Something New: Conserved Enzymes and the Evolution of Novelty in Plant Specialized Metabolism. **Plant Physiol,** v. 169, n. 3, p. 1512-23, Nov 2015. ISSN 1532-2548. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/26276843>.

MORITA, M. et al. Vacuolar transport of nicotine is mediated by a multidrug and toxic compound extrusion (MATE) transporter in Nicotiana tabacum. **Proc Natl Acad Sci U S A,** v. 106, n. 7, p. 2447-52, Feb 2009. ISSN 1091-6490. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/19168636>.

MOSCHOU, P. N. et al. The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. **J Exp Bot,** v. 63, n. 14, p. 5003-15, Sep 2012. ISSN 1460-2431. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/22936828>.

NACONSIE, M. et al. Molecular evolution of N-methylputrescine oxidase in tobacco. **Plant Cell Physiol,** v. 55, n. 2, p. 436-44, Feb 2014. ISSN 1471-9053. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/24287136>.

PASCHOLD, A.; HALITSCHKE, R.; BALDWIN, I. T. Co(i)-ordinating defenses: NaCOI1 mediates herbivore- induced resistance in Nicotiana attenuata and reveals the role of herbivore movement in avoiding defenses. **Plant J,** v. 51, n. 1, p. 79-91, Jul 2007. ISSN 0960-7412. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/17561925>.

RAINA, A. et al. Polyamine synthesis in mammalian tissues. Isolation and characterization of spermidine synthase from bovine brain. **Biochem J,** v. 219, n. 3, p. 991-1000, May 1984. ISSN 0264-6021. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/6743257>.

RAJABI, F. et al. Combination of Plant Metabolic Modules Yields Synthetic Synergies. **PLoS One,** v. 12, n. 1, p. e0169778, 2017. ISSN 1932-6203. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/28081182>.

REED, D. G.; JELESKO, J. G. The *A* and *B* loci of *Nicotiana tabacum* have non-equivalent effects on the mRNA levels of four alkaloid biosynthetic genes. **Plant Science,** v. 167, n. 5, p. 1123-1130, 2004.

RIECHERS, D. E.; TIMKO, M. P. Structure and expression of the gene family encoding putrescine N-methyltransferase in Nicotiana tabacum: new clues to the evolutionary origin of cultivated tobacco. **Plant Mol Biol,** v. 41, n. 3, p. 387-401, Oct 1999. ISSN 0167-4412. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/10598105>.

RUSHTON, P. J. et al. Tobacco transcription factors: novel insights into transcriptional regulation in the Solanaceae. **Plant Physiol,** v. 147, n. 1, p. 280-95, May 2008. ISSN 0032-0889. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/18337489>.

RYAN, S. M. et al. Structure and expression of the quinolinate phosphoribosyltransferase (QPT) gene family in Nicotiana. **Plant Sci,** v. 188-189, p. 102-10, Jun 2012. ISSN 1873-2259. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/22525250>.

SAITOH, F.; NOMA, M.; KAWASHIMA, N. The alkaloid contents of sixty Nicotiana species. **Phytochemistry,** v. 24, n. 3, p. 477-480, 1985. ISSN **0031~9422/8**.

SCHILMILLER, A. L.; LAST, R. L.; PICHERSKY, E. Harnessing plant trichome biochemistry for the production of useful compounds. **Plant J,** v. 54, n. 4, p. 702-11, May 2008. ISSN 1365-313X. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/18476873>.

SHI, Q.; LI, C.; ZHANG, F. Nicotine synthesis in Nicotiana tabacum L. induced by mechanical wounding is regulated by auxin. **J Exp Bot,** v. 57, n. 11, p. 2899-907, 2006. ISSN 0022-0957. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/16868042>.

SHITAN, N.; HAYASHIDA, M.; YAZAKI, K. Translocation and accumulation of nicotine via distinct spatio-temporal regulation of nicotine transporters in Nicotiana tabacum. **Plant Signal Behav,** v. 10, n. 7, p. e1035852, 2015. ISSN 1559-2324. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/26251879>.

SHITAN, N. et al. Involvement of the leaf-specific multidrug and toxic compound extrusion (MATE) transporter Nt-JAT2 in vacuolar sequestration of nicotine in Nicotiana tabacum. **PLoS One,** v. 9, n. 9, p. e108789, 2014. ISSN 1932-6203. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25268729>.

SHOJI, T.; HASHIMOTO, T. Recruitment of a duplicated primary metabolism gene into the nicotine biosynthesis regulon in tobacco. **Plant J,** v. 67, n. 6, p. 949-59, Sep 2011a. ISSN 1365-313X. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/21605206>.

_____. Tobacco MYC2 regulates jasmonate-inducible nicotine biosynthesis genes directly and by way of the NIC2-locus ERF genes. **Plant Cell Physiol,** v. 52, n. 6, p. 1117-30, Jun 2011b. ISSN 1471-9053. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/21576194>.

SHOJI, T. et al. Multidrug and toxic compound extrusion-type transporters implicated in vacuolar sequestration of nicotine in tobacco roots. **Plant Physiol,** v. 149, n. 2, p. 708-18, Feb 2009. ISSN 0032-0889. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/19098091>.

SHOJI, T.; KAJIKAWA, M.; HASHIMOTO, T. Clustered transcription factor genes regulate nicotine biosynthesis in tobacco. **Plant Cell,** v. 22, n. 10, p. 3390-409, Oct

2010. ISSN 1532-298X. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/20959558>.

SHOJI, T.; NAKAJIMA, K.; HASHIMOTO, T. Ethylene suppresses jasmonate-induced gene expression in nicotine biosynthesis. **Plant Cell Physiol,** v. 41, n. 9, p. 1072-6, Sep 2000. ISSN 0032-0781. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/11100780>.

SHOJI, T.; OGAWA, T.; HASHIMOTO, T. Jasmonate-induced nicotine formation in tobacco is mediated by tobacco COI1 and JAZ genes. **Plant Cell Physiol,** v. 49, n. 7, p. 1003-12, Jul 2008. ISSN 1471-9053. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/18492687>.

SHOJI, T. et al. Expression patterns of two tobacco isoflavone reductase-like genes and their possible roles in secondary metabolism in tobacco. **Plant Mol Biol,** v. 50, n. 3, p. 427-40, Oct 2002. ISSN 0167-4412. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/12369619>.

SIMINSZKY, B. et al. Conversion of nicotine to nornicotine in Nicotiana tabacum is mediated by CYP82E4, a cytochrome P450 monooxygenase. **Proc Natl Acad Sci U S A,** v. 102, n. 41, p. 14919-24, Oct 2005. ISSN 0027-8424. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16192354>.

SINCLAIR, S. J. et al. Molecular characterization of quinolinate phosphoribosyltransferase (QPRtase) in Nicotiana. **Plant Mol Biol,** v. 44, n. 5, p. 603-17, Nov 2000. ISSN 0167-4412. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/11198422>.

SISSON, V.; SEVERSON, R. **Alkaloid composition of the Nicotiana species**: Beiträge zur Tabakforschung International. 14 1990.

STEPPUHN, A. et al. Nicotine's defensive function in nature. **PLoS Biol,** v. 2, n. 8, p. E217, Aug 2004. ISSN 1545-7885. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/15314646>.

SUN, B. et al. Genetic variation in alkaloid accumulation in leaves of Nicotiana. **J Zhejiang Univ Sci B,** v. 14, n. 12, p. 1100-9, Dec 2013. ISSN 1862-1783. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24302710>.

SUZUKI, K.; YAMADA, Y.; HASHIMOTO, T. Expression of Atropa belladonna putrescine N-methyltransferase gene in root pericycle. **Plant Cell Physiol,** v. 40, n. 3, p. 289-97, Mar 1999. ISSN 0032-0781. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/10353217>.

TEUBER, M. et al. Putrescine N-methyltransferases--a structure-function analysis. **Plant Mol Biol,** v. 63, n. 6, p. 787-801, Apr 2007. ISSN 0167-4412. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/17221359>.

WALTON, N. J.; ROBINS, R. J.; PEERLESS, A. C. Enzymes of N-methylputrescine biosynthesis in relation to hyoscyamine formation in transformed root cultures of

Datura stramonium and Atropa belladonna. **Planta,** v. 182, n. 1, p. 136-41, Aug 1990. ISSN 0032-0935. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/24197009>.

WANG, B. et al. Genetic Factors for Enhancement of Nicotine Levels in Cultivated Tobacco. **Sci Rep,** v. 5, p. 17360, Dec 2015. ISSN 2045-2322. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/26626731>.

WASTERNACK, C.; HAUSE, B. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. **Ann Bot,** v. 111, n. 6, p. 1021-58, Jun 2013. ISSN 1095-8290. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/23558912>.

WINZ, R. A.; BALDWIN, I. T. Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. IV. Insect-Induced ethylene reduces jasmonate-induced nicotine accumulation by regulating putrescine N-methyltransferase transcripts. **Plant Physiol,** v. 125, n. 4, p. 2189-202, Apr 2001. ISSN 0032-0889. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/11299398>.

XI, X. Y.; LI, C. J.; ZHANG, F. S. Nitrogen supply after removing the shoot apex increases the nicotine concentration and nitrogen content of tobacco plants. **Ann Bot,** v. 96, n. 5, p. 793-7, Oct 2005. ISSN 0305-7364. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/16100227>.

XU, B.; TIMKO, M. Methyl jasmonate induced expression of the tobacco putrescine N -methyltransferase genes requires both G-box and GCC-motif elements. **Plant Mol Biol,** v. 55, n. 5, p. 743-61, Jul 2004. ISSN 0167-4412. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/15604714>.

YOON, S. O. et al. Polyamine synthesis in plants: isolation and characterization of spermidine synthase from soybean (Glycine max) axes. **Biochim Biophys Acta,** v. 1475, n. 1, p. 17-26, Jun 2000. ISSN 0006-3002. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/10806333>.

YU, O. et al. Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues. **Plant Physiol,** v. 124, n. 2, p. 781-94, Oct 2000. ISSN 0032-0889. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/11027726>.

ZADOR, E.; JONES, D. The Biosynthesis of a Novel Nicotine Alkaloid in the Trichomes of Nicotiana stocktonii. **Plant Physiol,** v. 82, n. 2, p. 479-84, Oct 1986. ISSN 0032-0889. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/16665055>.

ZAYED, R.; WINK, M. Induction of pyridine alkaloid formation in transformed root cultures of Nicotiana tabacum. **Z Naturforsch C,** v. 64, n. 11-12, p. 869-74, 2009 Nov-Dec 2009. ISSN 0939-5075. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/20158160>.

CAPÍTULO II: THE CYP82E SUBFAMILY IN THE *NICOTIANA* GENUS: IDENTIFICATION OF PUTATIVE NEW GENES INVOLVED WITH NICOTINE TO NORNICOTINE CONVERSION

THE CYP82E SUBFAMILY IN THE *NICOTIANA* GENUS: IDENTIFICATION OF PUTATIVE NEW GENES INVOLVED WITH NICOTINE TO NORNICOTINE CONVERSION

Fernanda Fleig Zenkner[1,2], Alexandre Rieger[3], Márcia Margis-Pinheiro[1], Alexandro Cagliari[4]

[1] Departamento de Genética, Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul (UFRGS), PO Box 15053, Porto Alegre, RS CEP 91501-970, Brazil

[2] JTI Kannenberg Comércio de Tabacos do Brasil LTDA, Santa Cruz do Sul, RS, Brazil

[3]Universidade de Santa Cruz do Sul (UNISC), Santa Cruz do Sul, RS, Brazil

[4]Universidade Estadual do Rio Grande do Sul (UERGS), Santa Cruz do Sul, RS, Brazil

ABSTRACT

Introduction: *Nicotiana tabacum* plants are known by their alkaloid content, especially nicotine, which can be converted to nornicotine through the *N*-demethylation reaction catalyzed by nicotine *N*-demethylase (NND) enzymes. Nornicotine may give rise to a harmful Tobacco Specific Nitrosamine (TSNA), undesirable in tobacco products due to its carcinogenic risk to humans. Genes encoding NND enzymes belong to *CYP82E* subfamily.

Methods: Using a high-throughput comparative genomic approach, we conducted a broad survey analysis in the available sequenced *Nicotiana* genomes, in order to identify the presence of homologous genes from the subfamily *CYP82E*, coding for NND. Phylogenetic reconstruction of CYP82E subfamily in the genus *Nicotiana* was performed using Bayesian inference. A set of bioinformatic tools were used to carry out *in silico* characterization of putative *Nicotiana CYP82E* genes.

Results: We have identified 23 new putative *CYP82E* genes being 2 from *N. benthamiana*, 7 from *N. sylvestris*, 10 from *N. tabacum* and 4 from *N.*

tomentosiformis. After *in silico* analysis, we selected 15 candidate genes with the highest potential of being NND. *In silico* analysis of *N. tabacum* NND indicated endoplasmic reticulum (ER) localization for CYP82E1, E2, E3, E4, E5, E8 and E21, and cytoplasmic localization for CYP82E10.The interaction with small ubiquitin-like modifiers (SUMOs) was predicted for all CYP82E, except CYP82E5.

Conclusions: Here we present the first phylogenetic and *in silico* analysis performed with CYP82E subfamily in tobacco and wild *Nicotiana* species. The phylogeny demonstrated that putative *CYP82E* genes form an independent cluster from other *CYP* families and *CYP82* subfamilies. We have identified uncharacterized putative *CYP82E* genes in *Nicotiana*. The identification of all potential NND could contribute to clarify the conversion process in *Nicotiana* species.

Key words: *Nicotiana*; nornicotine; nicotine; CYP82E; tobacco-specific nitrosamine.

INTRODUCTION

The Solanaceae are a large eudicot family including many economically important crop plants such as tomato, potato and tobacco. The genus *Nicotiana* L. is the fifth largest genus in this family, with 75 naturally occurring species (Clarkson *et al.*, 2004). Amphidiploid plants are common in the genus *Nicotiana*, and the cultivated tobacco (*Nicotiana tabacum*) is one of those species, its progenitors being *Nicotiana sylvestris* and *Nicotiana tomentosiformis* (Knapp, S. *et al.*, 2004). For thousands of years *N. tabacum* has been cultivated by Native Americans in the Americas (Tso, 1990). After its discovery, tobacco was introduced to Europe and rapidly spread throughout the world (Dewey and Xie, 2013).

Tobacco's relevance as an economically important crop is primarily due to its use as a source of the psychoactive alkaloid nicotine (Sun *et al.*, 2013). Nicotine is also the chemical compound associated with the addiction to tobacco products (Benowitz, 2009). Nicotine and alkaloids in general are also important to the plants that produce them because these substances are used as a defence against herbivores and insects (Steppuhn *et al.*, 2004; Gavilano *et al.*, 2006; Dicke and Baldwin, 2010). Stresses such as tissue wounding or insect attacks, further stimulate nicotine biosynthesis from the root apparatus, which is the site where nicotine biosynthetic genes are specifically expressed (Shitan *et al.*, 2014). After been

produced, nicotine is transported via the xylem to the leaves, where it is stored in the cell vacuoles (Sun *et al.*, 2013).

The Cytochrome P450 (CYP) is one of the largest known enzyme families in plants (Bak *et al.*, 2011). CYP enzymes participate in a diverse range of toxicological and physiological processes such as the bioconversion of xenobiotics and the biosynthesis of important hormones and structural components of cell (Bernhardt, 2006). Remarkably, the functional redundancy of these genes is limited, reflecting the complexity of plant metabolism (Bak *et al.*, 2011).

Currently, 5100 CYP sequences are known in plants, which is a relatively large number when compared to those found in other taxa, such as vertebrates (1461 CYPs), insects (2137 CYPs), fungi (2960 CYPs), bacteria (1042 CYPs) or archaea (around 30 CYPs) (Nelson and Werck-Reichhart, 2011). In the model plant *Arabidopsis thaliana*, a panel of more than 200 CYPs was found (Bak *et al.*, 2011). *Nicotiana* species are known by their alkaloid content, such as nicotine, nornicotine, anabasine and anatabine (Sun *et al.*, 2013), and CYP enzymes largely participate in alkaloid biosynthesis (Siminszky *et al.*, 2005; Xu *et al.*, 2007).

Nornicotine is the source of a harmful Tobacco Specific Nitrosamine (TSNA): *N*-nitrosonornicotine (NNN), which derives from the reaction of nornicotine with Nitrogen Oxide compounds (NO_X), and nitrite in particular (Shi *et al.*, 2013). NNN is also one of the toxicants recommended for mandated lowering by the WHO (WHO, 2007).

Nornicotine is generated through nicotine *N*-demethylation, a process catalyzed by *N*-demethylases enzymes (NND). Genes known to encode NND enzymes belong to *CYP82E* subfamily. Seven *CYP82E* genes have already been described in *N. tabacum*, and six of them are involved in this conversion process (Pakdeechanuan, Teoh*, et al.*, 2012; Liedschulte *et al.*, 2016). Up to now, two and four *CYP82E* genes were described in tobacco progenitors, *N. sylvestris* and *N. tomentosiformis*, respectively (Chakrabarti *et al.*, 2007; Gavilano *et al.*, 2007; Gavilano and Siminszky, 2007; Lewis *et al.*, 2010; Liedschulte *et al.*, 2016).

Different approaches have been employed to reduce nornicotine levels in commercial tobacco, aiming to correspondent decrease in NNN formation in tobacco products. Transgenic tobacco lines carrying an RNA interference (RNAi) construct to inhibit the expression of a *CYP82E* gene resulted in plants with decreased nornicotine content (Gavilano *et al.*, 2006; Lewis *et al.*, 2008). However, considering that some tobacco producing countries do not allow GMO crops, and consumers in

many countries reject the use of GMO products, it would be important to develop alternative solutions that would allow to lower nornicotine in tobacco (Lewis *et al.*, 2008). Alternative methods, such as mutation induction through ethyl methane sulfonate (EMS), have been used with the aim of obtaining mutants with an altered nornicotine profile (Lewis *et al.*, 2010).

Despite the importance of NNN to human health, there are relatively few studies that have focused on the genetic regulation involved with the conversion of nicotine to nornicotine. The study of the *CYP82E* subfamily organization and its evolution in different *Nicotiana* species represent an important step in the understanding of the nicotine-nornicotine conversion process. The use of genomic and phylogenomic approaches could contribute to the development of new solutions that can be used in breeding programs to the aim of lowering NNN emissions from tobacco products. Here we report on the identification and phylogenetic reconstruction of the CYP82E subfamily in the genus *Nicotiana*. We also performed an *in silico* characterization of the tobacco CYP82E proteins. The evolution of the CYP82 family and the emergence of the CYP82E subfamily in tobacco are also discussed.

METHODS

CYP82E gene annotation

Putative *CYP82E* subfamily genes were identified in NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) database using as a query the protein sequence of *N. tabacum* CYP82E4, the main CYP82E in *N. tabacum* leaves, deposited under accession number ABA07805.1. When using other sequences as queries (e.g. CYP82E5 or CYP82E10), the search did not return any different sequences in the results.

Sequences were identified through BLAST (Basic Logical Alignment Search Tool) searches against genomes of *Nicotiana* species available at the database. We considered the species included in the classification by Knapp (Knapp, Sandra *et al.*, 2004) (Supplementary Table 1). The sequences with at least 25% similarity were selected for further analysis.

Deduced amino acid sequences from the putative CYP82E subfamily were aligned using the CLUSTAL W Algorithm in the Molecular Evolutionary Analysis (MEGA v.6) software (Tamura *et al.*, 2011). Contiguous insertion-deletion events

(indels) with more than one base pair were treated as single mutations (Simmons and Ochoterena, 2000).

A Bayesian inference were generated using BEAST v.1.8.2 software (Drummond and Rambaut, 2007); a run of 40,000,000 chains was performed, and the trees were sampled every 1000 generations. The Yule tree prior, and the uncorrelated log-normal relaxed clock were used in the BEAST analysis. The TRACER v.1.6 (http://beast.bio.ed.ac.uk/Tracer) was used to check the convergence of the Monte Carlo Markov chains (MCMCs) and for adequate effective sample sizes (EES >200) after the first 10% of the generations had been deleted as burn-in. The final joint sample was used to estimate the maximum clade credibility tree with the TreeAnnotator program, which is part of the BEAST package. The statistical support for the clades was determined by accessing the Bayesian posterior probability (PP). The tree was visualized using FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

The following sequences were used as outgroup for phylogenetic analysis: well-described CYP85A3 from *Solanum lycopersicum* (Nomura *et al.*, 2005), CYP82C2 and CYP82G1 from *Arabidopsis thaliana* (Lee *et al.*, 2010; Liu *et al.*, 2010), CYP82D1, CYP82D2 and CYP82D3 from *Gossypium hirsutum* (Sun *et al.*, 2014), CYP82A1 from *Pisum sativum* (Whitbred and Schuler, 2000), CYP82N2v2 from *Eschscholzia californica* (Takemura *et al.*, 2013)*;* and other genes from CYP82 family, as CYP82H1 from *Ammi majus*, CYP82C3, CYP82C4 and CYP82F1 from *A. thaliana*, CYP82B1 from *E. californica*, CYP82A1, CYP82A3, CYP82A4 from *Glycine max,* CYP82D62 from *Mentha* x *piperita*, CYP82D33 from *Ocimum basilicum*, CYP82X1 and CYP82Y1 from *Papaver somniferum*, CYP82K1 and CYP82L1 from *Populus trichocarpa*, CYP82Q1 from *Stevia rebaudiana*, CYP82E11 and CYP82E13 from *Solanum tuberosum*, CYP82E11 from *Solanum lycopersicum*. We also included in the phylogenetic analysis five *N. tabacum* genes from different CYP families and subfamilies: CYP81B2, CYP81C6, CYP82M1, CYP92A2 and CYP92B2, as outgroup. The sequences used as outgroup were already named and found in the literature. All sequences used in the phylogenetic analysis are presented in the Supplementary Table 2.

The alignment of CYP82E sequences from *N. tabacum* was performed using CLUSTAL W Algorithm in GeneDoc (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html).

In silico protein analysis

In silico analysis was performed with the following software: MEME (http://meme-suite.org/) for motif analysis, Protter (http://wlab.ethz.ch/protter/) for protein topology, Phobius (http://phobius.sbc.su.se/) (Käll *et al.*, 2004) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (Krogh *et al.*, 2001) for transmembrane domain prediction. Regarding subcellular location and signal peptide prediction, we followed the protocol described by Emanuelsson et al. (Emanuelsson *et al.*, 2007), which indicates to start the analysis with TargetP and in case of low reliable prediction, or secretory pathway prediction, further confirm with SignalP. We also assessed subcellular location using CELLO (http://cello.life.nctu.edu.tw/) and Predotar (https://urgi.versailles.inra.fr/predotar/predotar.html).

The proteins were also tested for post-translational modifications (PTMs) using GPS-SUMO (http://sumosp.biocuckoo.org/) to predict both covalent SUMOylation sites and non-covalent SUMO-interaction motifs (Zhao *et al.*, 2014) and GPS-Lipid (http://lipid.biocuckoo.org/), which is a predictor for multiple protein lipid modification sites, as S-palmitoylation, prenylation and N-myristoylation (Xie *et al.*, 2016). The analyses were run using the default software parameters.

RESULTS

Using a high-throughput comparative genomic approach we conducted a broad survey analysis in the available sequenced *Nicotiana* genomes, in order to identify the presence of homologous genes from the subfamily *CYP82E*, coding for NND.

BLAST searches and domain analysis (See material and methods) allowed the identification of 42 putative *CYP82E* subfamily genes in *Nicotiana* (Supplementary Table 3). We have identified 4 putative *CYP82E* genes belonging to *Nicotiana alata*, 2 to *Nicotiana benthamiana*, 1 to *Nicotiana langsdorffii*, 9 to *N. sylvestris*, 18 to *N. tabacum* and 8 to *N. tomentosiformis*.

From the total of *CYP82E* subfamily identified in *Nicotiana*, 23 were never described previously (Supplementary Table 3): 2 for *N. benthamiana*, 7 for *N. sylvestris*, 10 for *N. tabacum* and 4 for *N. tomentosiformis*. These new putative *CYP82E* genes were not yet characterized, and there is no information about their function or possible roles in the nicotine conversion process.

***In silico* analysis of CYP82E proteins**

Motif analysis showed a high degree of conservation for the three well known motifs described for the CYP superfamily: a) the K-helix consensus sequence (EXXR), b) PERF consensus sequence and c) F-xx-G-xb-xx-C-x-G motif (Supplementary Figure 1). The first two sequences (**E**xx**R** and PE**R**F) form the triade E-R-R (represented by bold letters above).

In order to analyze the protein conservation among CYP82E subfamily, amino acid alignment focusing on sequences from *N. tabacum* was performed. In spite of the high amino acid similarity among CYP82E sequences (up to 95%) (Siminszky *et al.*, 2005; Gavilano *et al.*, 2007), different important residues changes were also observed (red letters in Figure 1).

New putative CYP82E identified for all *Nicotiana* species (Supplementary Table 3) present no substitution in the conserved amino acid positions shown in Figure 1, suggesting that these genes may potentially encode functional NND enzymes.

Regarding *in silico* post-translational modification analysis, the software GPS-SUMO predicted SUMO interaction at the residues 504-508 for most of the NND enzymes tested ($P<0.05$), except for CYP82E3 and CYP82E5 from *N. tabacum* and *N. tomentosiformis*, and for all *N. alata, N. benthamiana* and *N. langsdorffii* NND (Table 1). The same PTM was also predicted for some new putative CYP82E: Ntab1, Ntab5, Nsyl1, Nsyl5, Nsyl6 and Ntom1. The lipid modification analysis indicated the S-Palmitoylation at the position C330 for NtabCYP82E3, C23 for Ntab3 and Nsyl2, C161 for Ntab4 and C160 for Nsyl4. *N*-Myristoylation was predicted at the position G10 for NsylCYP82E10 and Nsyl1.

Transmembrane domains were predicted for most of the CYP82E proteins, except for NtabCYP82E10, Ntab1 and Nsyl3 using TMHMM software, and CYP82E5 from *N. tabacum* and *N. tomentosiformis*, CYP82E10 from *N. tabacum* and *N. sylvestris*, Ntab1, Ntab2, Nsyl1 and Nsyl3 using Phobius software (Table 1).

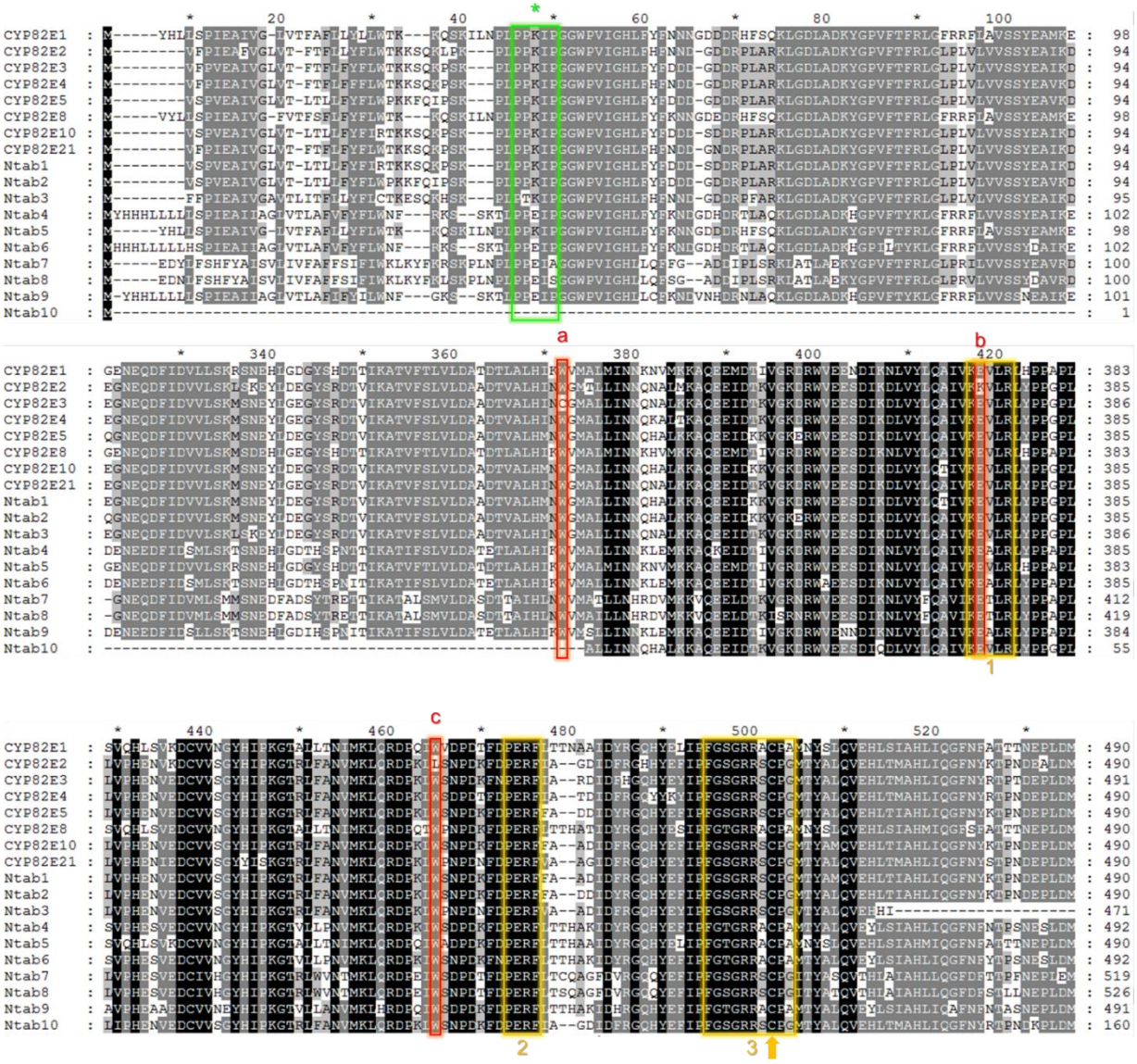


Figure 1: Comparison of already described and putative new *Nicotiana tabacum* CYP82E sequences regarding amino acid substitutions and consensus sequences. The proline-rich motif is highlighted in green (*). The amino acid substitutions are highlighted in red: **a)** W330C substitution in CYP82E3; **b)** E375K substitution in CYP82E2; **c)** W422L substitution in CYP82E2. The consensus sequences are highlighted in yellow: **1)** ExxR consensus sequence; **2)** PERF consensus sequence; **3)** F-xx-G-xb-xx-C-x-G consensus sequence. The arrow indicates heme-ligand cysteine. Amino acids are colored according conservation: black represents 100%, dark gray 80% and light gray 60% similarity.

Table 1: *In silico* characterization of *Nicotiana* CYP82E already named as CYP82E1, E2, E3, E4, E5, E8, E10 and E21, and new putative CYP82E. ER: endoplasmic reticulum.

	Subcellular location		Transmembrane domain		Post-translational modification	
	CELLO	**Predotar**	**TMHMM**	**Phobius**	**GPS-SUMO**	**GPS-lipid**
NtabCYP82E1	Cytoplasma	ER	Yes (7-26)	Yes (6-25)	SUMO interaction (504-508)	No
Ntab5	Cytoplasma/ Mitochondria	ER	Yes (7-26)	Yes (6-25)	SUMO interaction (504-508)	No
NtabCYP82E2	Cytoplasma	ER	Yes (5-23)	Yes (6-22)	SUMO interaction (504-508)	No
NtabCYP82E3	Cytoplasma	ER	Yes (2-23)	Yes (6-22)	SUMO interaction (505-509)	S-Palmitoylation (C330)
NtabCYP82E4	Cytoplasma	ER	Yes (2-23)	Yes (6-23)	SUMO interaction (504-508)	No
NtabCYP82E5	Cytoplasma/ ER	ER	Yes (5-22)	No	No	No
Ntab2	Cytoplasma/ ER	ER	Yes (5-22)	No	No	No
NtabCYP82E8	Cytoplasma	ER	Yes (2-24)	Yes (6-25)	SUMO interaction (504-508)	No
NtabCYP82E10	Cytoplasma	ER	No	No	SUMO interaction (504-508)	No
Ntab1	Cytoplasma	ER	No	No	SUMO interaction (504-508)	No
NtabCYP82E21	Cytoplasma	ER	Yes (2-23)	Yes (6-22)	SUMO interaction (504-508)	No
Ntab3	Cytoplasma	ER	Yes (5-24)	Yes (6-23)	No	S-Palmitoylation (C23)
Ntab4	Plasma membrane	ER	Yes (7-29)	Yes (6-31)	Sumoylation Nonconcensus (58); SUMO interaction (506-510)	S-Palmitoylation (C161)
Ntab6	Cytoplasma/ Plasma membrane	Possibly ER	Yes (7-29)	Yes (12-31)	Sumoylation Nonconcensus (58); SUMO interaction (506-510)	No
Ntab7	Plasma membrane	ER	Yes (7-26)	Yes (6-26)	Sumoylation Nonconcensus (298); SUMO interaction (89-93; 533-537)	No
Ntab8	Plasma membrane	ER	Yes (7-26)	Yes (6-26)	SUMO interaction (540-544)	No
Ntab9	Cytoplasma/ Plasma membrane	ER	Yes (7-29)	Yes (6-30)	Sumoylation concensus (57); SUMO interaction (505-509)	No

Ntab10	Cytoplasma	-	No	No	SUMO interaction (174-178)	No
NsylCYP82E2	Cytoplasma	ER	Yes (5-23)	Yes (6-22)	SUMO interaction (504-508)	No
Nsyl5	Cytoplasma/ Mitochondria	ER	Yes (7-26)	Yes (6-25)	SUMO interaction (504-508)	No
Nsyl6	Cytoplasma/ Mitochondria	ER	Yes (7-26)	Yes (6-25)	SUMO interaction (504-508)	No
NsylCYP82E10	Cytoplasma	ER	Yes (4-21 and 70-92)	No	SUMO interaction (504-508)	N-Myristoylation (G10)
Nsyl1	Cytoplasma	ER	Yes (4-21 and 70-92)	No	SUMO interaction (504-508)	N-Myristoylation (G10)
Nsyl2	Cytoplasma	ER	Yes (2-24)	Yes (6-23)	SUMO interaction (502-506)	S-Palmitoylation (C23)
Nsyl3	Cytoplasma	None	No	No	SUMO interaction (472-476)	No
Nsyl4	Plasma membrane	ER	Yes (5-27)	Yes (6-30)	Sumoylation concensus (57); SUMO interaction (505-509)	S-Palmitoylation (C160)
Nsyl7	Plasma membrane	ER	Yes (7-26)	Yes (6-26)	SUMO interaction (540-544)	No
NtomCYP82E3	Cytoplasma	ER	Yes (5-22 and 109-131)	Yes (6-22)	No	No
Ntom2	Cytoplasma	ER	Yes (5-22 and 109-131)	Yes (6-22)	No	No
NtomCYP82E4	Cytoplasma	ER	Yes (5-22)	Yes (6-22)	SUMO interaction (504-508)	No
Ntom1	Cytoplasma	ER	Yes (5-22)	Yes (6-22)	SUMO interaction (504-508)	No
NtomCYP82E5	Cytoplasma/ ER	ER	Yes (5-22)	No	No	No
NtomCYP82E21	Cytoplasma/ Plasma membrane	ER	Yes (5-22 and 70-92)	Yes (6-22 and 70-89)	SUMO interaction (504-508)	No
Ntom3	Plasma membrane	ER	Yes (7-29)	Yes (7-32)	Sumoylation concensus (59); SUMO interaction (508-512)	No
Ntom4	Plasma membrane	ER	Yes (7-26)	Yes (6-26)	SUMO interaction (89-93; 533-537); Sumoylation Nonconcensus (298)	No

NalaCYP82E15	Cytoplasma	ER	Yes (5-22 and 109-131)	Yes (6-22)	SUMO interaction (501-505)	No
NalaCYP82E16	Cytoplasma	ER	Yes (5-22 and 109-131)	Yes (6-22)	SUMO interaction (501-505)	No
NalaCYP82E17	Cytoplasma	ER	Yes (5-22 and 109-131)	Yes (6-22)	SUMO interaction (501-505)	No
NalaCYP82E18	Cytoplasma	ER	Yes (7-24)	Yes (6-23)	SUMO interaction (502-506)	No
NlanCYP82E19	Cytoplasma	ER	Yes (2-24)	Yes (6-23)	SUMO interaction (502-506)	No
Nben1	Plasma membrane	ER	Yes (7-29)	Yes (6-30)	No	No
Nben2	Plasma membrane	ER	Yes (7-26)	Yes (6-26)	SUMO interaction (89-93; 541-545)	No

Regarding subcellular location and signal peptide, our predictions in TargetP resulted in a reliability coefficient (RC) of 5 (not reliable prediction) for most of the proteins, and few proteins were predicted for the secretory pathway. Thus, the analysis was run in SignalP, resulting in no signal peptide predicted for all NND and new putative CYP82E tested.

The analysis in Predotar for subcellular location prediction indicated that all CYP82E proteins are directed to the endoplasmic reticulum (ER), except Ntab10 and Nsyl3, while CELLO indicated cytoplasmic location for most of the proteins. Besides cytosol, it was predicted additional location on ER for Ntab2 and for CYP82E5 from *N. tabacum* and *N. tomentosiformis*, on plasma membrane for CYP82E21 from *N. tomentosiformis,* Ntab6 and Ntab9, on mitochondria for Ntab5, Nsy5 and Nsyl6. The proteins Ntab4, Ntab7, Ntab8, Nsyl4, Nsyl7, Ntom3, Ntom4, Nben1 and Nben2 were not predicted to be in the cytosol.

Phylogenetic analysis of CYP82E family

We performed Bayesian analysis in order to evaluate the phylogenetic relationship within *CYP82E* subfamily of *Nicotiana* (Figure 2). We observed that the *CYP82E* subfamily, containing NND genes, formed a well-supported group separated from other CYP families, as *CYP81* and *CYP92*, and other *CYP82* subfamilies (Figure 2).

The phylogenetic analysis demonstrated that *N. tabacum* NND clustered together with its ancestral parent homologs: NsylCYP82E2 and NtabCYP82E2; NtomCYP82E3 and NtabCYP82E3; NtomCYP82E4 and NtabCYP82E4; NtomCYP82E5 and NtabCYP82E5; NsylCYP82E10 and NtabCYP82E10; NtomCYP82E21 and NtabCYP82E21 (Figure 2).

In addition, 23 uncharacterized genes also clustered in the NND group. Then, through the *in silico* analysis of the new putative *CYP82E* genes, we have identified 08 candidate genes with the highest potential of being NND: 03 from *N. sylvestris*, 03 from *N. tabacum* and 02 from *N. tomentosiformis* (Table 1).

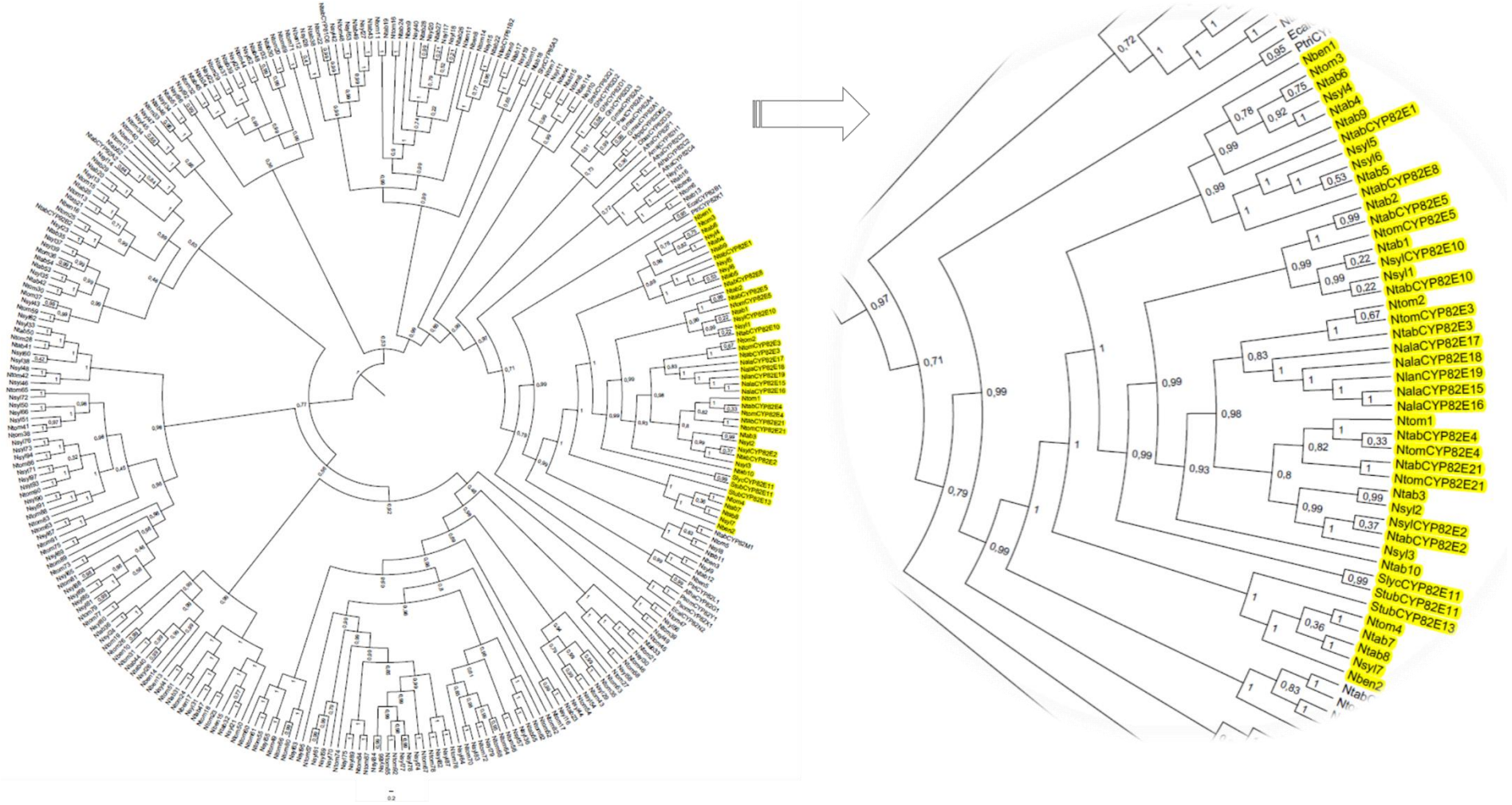


Figure 2: Phylogenetic analysis of Cytochrome P450 (CYP) genes, with emphasis to CYP82E subfamily (yellow). The Bayesian phylogenetic analysis was reconstructed following protein sequence alignments using a Bayesian approach by employing Beast software. The numbers in the branches of the tree indicate the posterior probability values. The acronym of CYP proteins were named using the first letter of the genus followed by the first three letters of the species: Amaj (*Ammi majus*); Atha (*Arabidopsis thaliana*)*;* Ecal (*Eschscholzia californica*); Ghir (*Gossypium hirsutum*); Gmax (*Glycine max*); Mpip (*Mentha x piperita*); Nala (*Nicotiana alata*); Nben (*N. benthamiana*); Nlan (*N. langsdorffii*); Nsyl (*N. sylvestris*); Ntab (*N. tabacum*); Ntom (*N. tomentosiformis*); Obas (*Ocimum basilicum*); Psat (*Pisum sativum*i); Ptri (*Populus trichocarpa*); Psom (*Papaver somniferum*); Slyc (*Solanum lycopersicum*), Stub (*S. tuberosum*).

DISCUSSION

In silico analysis of CYP82E proteins

Sequence alignment of P450s isolated from different organisms, from bacteria to plant and animals, showed strong conservation in some amino acid residues positions, although the most important one is the catalytic center formed by a heme group with iron coordinated to the thiolate of a conserved cysteine (Gavilano and Siminszky, 2007; Bak *et al.*, 2011).

The main motifs of the CYP superfamily were found in the sequences we have identified. The triad E-R-R was described as involved in locking the heme pocket into position and to assure stabilization of the conserved core structure (Bak *et al.*, 2011). F-xx-G-xb-xx-C-x-G motif facilitates the binding of heme iron to CYP enzyme. It is formed by a hydrophobic aromatic residue phenylalanine (F), an invariant hydrophobic cysteine (C) and small amino acid glycine (G). ‘x’ represents any residue and ‘xb’ refers a basic amino acid. This consensus sequence tend to differ among CYP subfamilies, although cysteine is always present (Paquette *et al.*, 2000).

It was previously demonstrated that *N. tabacum* CYP82E3 does not present NND activity because of an amino acid substitution at the position 330 (W330C) (Figure 1a). The comparison of CYP sequences from several organisms showed there is a strong conservation of aromatic amino acids (phenylalanine, tryptophan, tyrosine) in this position (Gavilano *et al.*, 2007). Other residue essential to CYP enzymatic function is the glutamate residue in the position 375 (the first amino acid of the conserved motif ExxR) (Figure 1.1). This motif, localized in K helix of most CYPs, was described as important to stabilize the association with heme group. In *N. tabacum* CYP82E2, the substitution E375K (Figure 1b) impairs the NND function (Chakrabarti *et al.*, 2007). Additionally, the substitution W422L of *N. tabacum* CYP82E2 (Figure 1c) was related to its inactivation (Chakrabarti *et al.*, 2007).

Post-translational modifications (PTMs) are extremely important as regulators of cellular processes, and influence activity, stability and localization of proteins (Ytterberg and Jensen, 2010).

Small ubiquitin-like modifiers (SUMOs) may regulate many biological processes, as transcriptional regulation and cellular signaling, by non-covalently interacting with proteins or covalently modifying specific lysine residues in protein substrates, which is called SUMOylation (Ytterberg and Jensen, 2010; Zhao *et al.*,

2014). The SUMO interaction was predicted for several NND enzymes tested in the residues 504-508, including five new putative CYP82E. Thus, it is possible that this interaction has a role in NND function, and additional analysis is needed to confirm that possibility.

Moreover, the addition and/or removal of lipid groups to certain amino acids have an important role in the subcellular trafficking, signaling and membrane association of proteins (Xie *et al.*, 2016). *N. tabacum* CYP82E3 presents the amino acid substitution W330C at the same position of the predicted lipid modification (S-Palmitoylation C330). It was demonstrated that the substitution of the aromatic residue tryptophan may impair the enzyme activity of CYP82E (Gavilano *et al.*, 2007). The S-Palmitoylation predicted for NtabCYP82E3 at the position C330 may also contribute for CYP82E3 non-activity, since PTM is related to alterations in the conformation of transmembrane domains (Blaskovic *et al.*, 2013). Furthermore, the homolog NtomCYP82E3 is a functional NND, and no PTM was predicted for this protein.

On the other hand, *N*-Myristoylation, as found at the position G10 of NsylCYP82E10, can influence the conformational stability of proteins and their ability to interact with membranes or other proteins (Podell and Gribskov, 2004). Although this PTM was already described in CYP, its relation with NND was not reported yet (Lamb and Waterman, 2013). No lipid modification was identified for the other CYP82E.

For transmembrane domain prediction, TMHMM and Phobius were applied, but the results obtained with each software were different. When compared with other software available for this prediction, as HMMTOP, TMpred and MEMSAT, TMHMM performs best especially at distinguishing between soluble and transmembrane proteins (Möller, Croning et al. 2001). However, it was showed that errors from cross prediction between transmembrane segments and signal peptides were reduced by Phobius in comparison to TMHMM and SignalP (Käll, Krogh et al. 2004, Käll, Krogh et al. 2007). Thus, the results of *in silico* predictions must be interpreted with care and, when possible, compared with *in vivo* analysis.

Regarding subcellular location, most of eukaryotic CYPs and all plant CYPs described so far are associated with microsomal membranes (Werck-Reichhart and Feyereisen, 2000; Bak *et al.*, 2011). Moreover, it was described that plant CYPs are usually anchored on the cytoplasmic surface of the ER, and it was already suggested

that NND P450 enzymes are ER localized (Lewis *et al.*, 2010; Bak *et al.*, 2011). Our analysis indicated the ER direction of CYP82E, and we also observed that the sequences we analyzed present a cluster of prolines after the hydrophobic amino-terminal segment. According to Werck-Reichhart et al. (Werck-Reichhart and Feyereisen, 2000), this is a common feature in CYPs associated with microsomal membranes (Supplementary Figure 2). Indeed, recently it was proved that NtomCYP82E4 and NtabCYP82E5 are located in the ER, corroborating with our predictions (Rajabi *et al.*, 2017).

Our data, together with CYP literature, indicates that CYP82E1, E2, E3, E4, E5, E8 and E21 are probably attached to the ER membrane, while CYP82E10 is probably a cytosolic protein not anchored to any membrane (Supplementary Figure2).

Phylogenetic analysis of the CYP82E family

Cytochrome P450 superfamily originated early in the evolution of life, before the emergence of the eukaryotes (Sezutsu *et al.*, 2013). CYPs are classified into clans, families and subfamilies. Each clan represents a group of proteins derived from a single ancestor, and families and subfamilies are grouped according to sequence similarity (Nelson and Werck-Reichhart, 2011).

There are 11 land plant clans, divided in single-family clans (CYP51, CYP74, CYP97, CYP710, CYP711, CYP727, CYP746) and multi-family clans (CYP71, CYP72, CYP85, CYP86). The evolution and diversification of the clans with multiple families was concomitant to the evolution of land plants and it is considered a key event for the adaptation in a total new environment (Nelson and Werck-Reichhart, 2011).

It was suggested that the CYP71 is one of the most ancient plant CYP clans, as it is also the largest clan in moss (Nelson, 2006). CYP71 clan represents by itself more than 50% of all plant CYPs, with a great diversity of functions (Hamberger and Bak, 2013). In the most ancient branches of this clan we can found CYPs involved in the hormone and biopolymers (lignin, cutin) biosynthesis (Nelson and Werck-Reichhart, 2011).

The subfamily CYP82E belongs to the CYP71 clan. The CYP82 family probably arose in dicots after their split from monocots (Nelson *et al.*, 2008). The CYP82 family is evolutionary very close to the CYP81 family, which is found in both monocots and

dicots. Thus, it was proposed that CYP81 and CYP82 ancestor split into one family that lies only in dicots and other family that lies in both (Nelson *et al.*, 2004).

All genes already known as belonging to the *CYP82* family clustered in the phylogenetic analysis. Moreover, we found uncharacterized genes of *N. benthamiana*, *N. sylvestris*, *N. tabacum* and *N. tomentosiformis* in the same group. Inside the *CYP82* group, we can clearly separate the *CYP82E* genes, being 23 yet uncharacterized genes. After the *in silico* analysis, the strongest candidate NND genes were identified and are highlighted in Figure 2.

The NND already described for *N. tabacum* clustered together with its ancestral parent homologs. In fact, it was suggested that *CYP82E3*, *CYP82E4*, *CYP82E5* and *CYP82E21* were inherited from the *N. tomentosiformis* ancestral parent, as they were not found in *N. sylvestris* genome (Gavilano *et al.*, 2007; Liedschulte *et al.*, 2016). In *N. tomentosiformis*, both CYP82E3 and CYP82E4 act in the conversion of nicotine to nornicotine in the green leaves, being CYP82E3 the main NND of this species, and CYP82E4 having a secondary role in the conversion process (Gavilano *et al.*, 2007). However, in the senescing leaves of *N. tomentosiformis* and *N. tabacum*, *CYP82E4* expression is strongly upregulated, playing a dominant role in nornicotine production (Siminszky *et al.*, 2005; Gavilano and Siminszky, 2007; Chakrabarti *et al.*, 2008) (Table 2).

On the other hand, *CYP82E5* is expressed in green leaves of *N. tomentosiformis* and *N. tabacum* (Table 2). *CYP82E5* is the main contributor to nornicotine production in the green leaves of *N. tabacum*, while in *N. tomentosiformis* has a discrete role in the conversion process (Gavilano and Siminszky, 2007). It was also demonstrated that *CYP82E5* is expressed in tobacco roots (Cai *et al.*, 2013).Otherwise, CYP82E21 was recently described as a functional NND expressed in *N. tabacum* ovary, but not in the leaves (Liedschulte *et al.*, 2016) (Table 2). The role of *CYP82E21* in *N. tomentosiformis* was not reported yet.

N. tabacum CYP82E2 and *CYP82E10* were inherited from the *N. sylvestris* ancestral parent (Chakrabarti *et al.*, 2007; Lewis *et al.*, 2010). It was showed that *CYP82E2* presents high NND activity and is expressed in the senescing leaves of *N. sylvestris* (Chakrabarti *et al.*, 2007) (Table 2). *CYP82E10* was identified in *N. tabacum* root-specific cDNA libraries, and it was thought to be preferentially expressed in root tissue (Lewis *et al.*, 2010). However, Cai et al. (Cai *et al.*, 2013) showed its expression also in the green leaves.

Table 2: Expression of *CYP82E* in *Nicotiana alata, N. langsdorffii, N. sylvestris*, *N. tabacum* and *N. tomentosiformis*.

Gene	Species	Expression Conditions	Mutation	Reference
CYP82E1	*N. tabacum*	Induced by pathogen		(Takemoto, Hayashi et al. 1999)
	N. tabacum	Elicitor-induced		(Ralston, Kwon et al. 2001)
CYP82E2	*N. sylvestris*	Senescing leaves		(Gavilano, Coleman et al. 2007)
	N. tabacum	Impaired by deleterious amino acid substitutions	E375K/ W422L	(Chakrabarti, Meekins et al. 2007)
	N. tomentosiformis	Not found		(Gavilano, Coleman et al. 2007)
CYP82E3	*N. sylvestris*	Not found		(Gavilano, Coleman et al. 2007)
	N. tabacum	Impaired by deleterious amino acid substitutions	W330C	(Chakrabarti, Meekins et al. 2007)
	N. tomentosiformis	Green leaves		(Gavilano, Coleman et al. 2007)
CYP82E4	*N. sylvestris*	Not found		(Chakrabarti, Bowen et al. 2008)
	N. tabacum	Senescing leaves/ ethylene induced		(Chakrabarti, Bowen et al. 2008)
	N. tomentosiformis	Green leaves/ senescence induced		(Gavilano, Coleman et al. 2007)
CYP82E5	*N. sylvestris*	Not found		(Gavilano and Siminszky 2007)
	N. tabacum	Green leaves		(Gavilano and Siminszky 2007)
	N. tabacum	Constitutive expression (leaves/roots)		(Cai, Jack et al. 2013)
	N. tomentosiformis	Green leaves		(Gavilano and Siminszky 2007)
CYP82E8	*N. tabacum*	No available data		-
CYP82E10	*N. sylvestris*	Donor to N. tabacum *CYP82E10*		(Lewis, Bowen et al. 2010)
	N. tabacum	Identified from root-specific cDNA libraries		(Lewis, Bowen et al. 2010)
	N. tabacum	Constitutive expression (leaves/roots)		(Cai, Jack et al. 2013)
	N. tomentosiformis	Not found		(Lewis, Bowen et al. 2010)
CYP82E15	*N. alata*	Leaves and roots		(Pakdeechanuan, Shoji et al. 2012)
CYP82E16	*N. alata*	Leaves and roots		(Pakdeechanuan, Shoji et al. 2012)
CYP82E17	*N. alata*	Leaves and roots		(Pakdeechanuan, Shoji et al. 2012)
CYP82E18	*N. alata*	Leaves and roots		(Pakdeechanuan, Shoji et al. 2012)
CYP82E19	*N. langsdorffii*	Impaired by transcriptional inactivation		(Pakdeechanuan, Teoh et al. 2012)
CYP82E20	*N. langsdorffii*	Impaired by premature stop-codon		(Pakdeechanuan, Teoh et al. 2012)
CYP82E21	*N. sylvestris*	Not found		(Liedschulte, Schwaar et al. 2016)
	N. tabacum	Flower ovary		(Liedschulte, Schwaar et al. 2016)
	N. tomentosiformis	Donor to N. tabacum *CYP82E21*		(Liedschulte, Schwaar et al. 2016)

In *N. tabacum*, *CYP82E2* and *CYP82E3* were impaired by deleterious mutations (Siminszky *et al.*, 2005; Chakrabarti *et al.*, 2007; Gavilano *et al.*, 2007) (Table 2). Thus, commercial tobacco presents four functional NND genes identified so far (Lewis *et al.*, 2010). CYP82E4 is the main enzyme responsible for nicotine N-demethylation in tobacco leaves (Siminszky *et al.*, 2005; Xu *et al.*, 2007). Studies have pointed to a scenario where *CYP82E4* is expressed in very low levels in green healthy leaves and roots, and its expression is strongly induced during senescence, as well as by ethylene (Siminszky *et al.*, 2005; Gavilano *et al.*, 2006; Chakrabarti *et al.*, 2008; Cai *et al.*, 2013) (Table 2).

The role of *N. tabacum* CYP82E1 and E8 in the conversion process has not yet been evaluated. CYP82E1 may be involved in disease resistance in tobacco (Takemoto *et al.*, 1999), and it was also recovered from elicitor-treated cells (Ralston *et al.*, 2001) (Table 2). NtabCYP82E8 was named, but its function was not described so far.

Except for *N. tomentosiformis* and *N.* sylvestris, it is rare to find any information about *CYP82E* genes and the conversion process in wild species of *Nicotiana*. Some years ago, the role of *CYP82E* genes from *N. alata* and *N. langsdorffii* was described (Pakdeechanuan, Shoji*, et al.*, 2012; Pakdeechanuan, Teoh*, et al.*, 2012). It was demonstrated that the two *CYP82E* genes of *N. langsdorffii* do not present NND activity: one has a premature stop codon (for this reason was excluded from our analysis), and the other (*NlanCYP82E19*) is impaired by transcriptional inactivation, resulting in the absence of nornicotine in this species (Pakdeechanuan, Teoh*, et al.*, 2012). On the other hand, *N. alata* harbors four NND genes encoding functional enzymes. However, the alkaloid levels in its leaves are very low or even non-detectable (Saitoh *et al.*, 1985; Pakdeechanuan, Teoh*, et al.*, 2012). To explain that, it was demonstrated that this species produces alkaloids in the roots (including nicotine and nornicotine), but the alkaloid translocation to the leaves is not efficient (Saitoh *et al.*, 1985; Pakdeechanuan, Shoji*, et al.*, 2012).

Highlighting the importance of the theme, it is noted the increasing interest in the *CYP82E* genes and the conversion process. In a recent study, Moghbel et al. (Moghbel, 2016) identified *CYP82E* related genes in 24 wild species and subspecies of *Nicotiana* in Australia. Moreover, they revealed that nornicotine is the main alkaloid in 9 of the studied taxa: *Nicotiana cavicola*, *Nicotiana goodspeedii*, *Nicotiana megalosiphon* subsp. m*egalosiphon*, *Nicotiana megalosiphon* subsp. *sessifolia*,

Nicotiana monoschizocarpa, *Nicotiana occidentalis* subsp. *occidentalis*, *Nicotiana simulans*, *Nicotiana suaveolens* and *Nicotiana velutina* (Moghbel, 2016). Similarly, it has long been known that the main alkaloid of *Nicotiana plumbaginifolia* is also nornicotine, which implies that this wild species must have at least one functional NND as well (Manceau *et al.*, 1989).

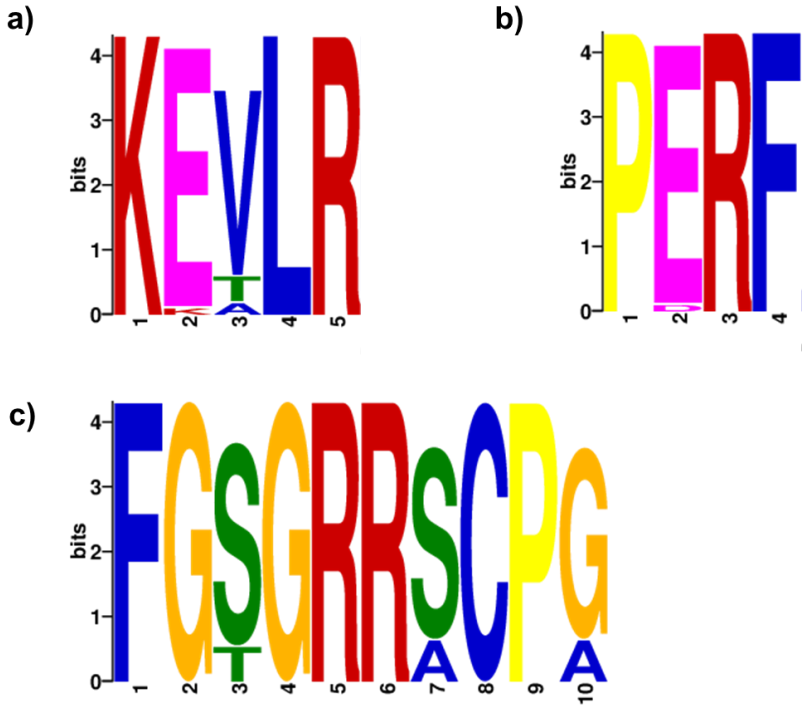
Here we present the first phylogenetic and *in silico* analysis of the CYP82E subfamily in tobacco and wild *Nicotiana* species. The phylogeny demonstrated that putative *CYP82E* genes form an independent cluster from other *CYP* families and *CYP82* subfamilies. We have identified a total of 23 uncharacterized putative *CYP82E* genes in *Nicotiana*, 2 for *N. benthamiana*, 7 for *N. sylvestris*, 10 for *N. tabacum* and 4 for *N. tomentosiformis*.

Additionally, we should consider that as the leaf is the main product of tobacco, the studies on NND activity have focused on leaves. However, different tobacco tissues may have been underestimated with respect to their contribution to the conversion process. *CYP82E21* was recently described as a new NND gene for *N. tabacum*, with expression restricted to ovaries (Liedschulte *et al.*, 2016). In this scenario, the identification of all potential NND could contribute to clarify the conversion process in *Nicotiana* species.

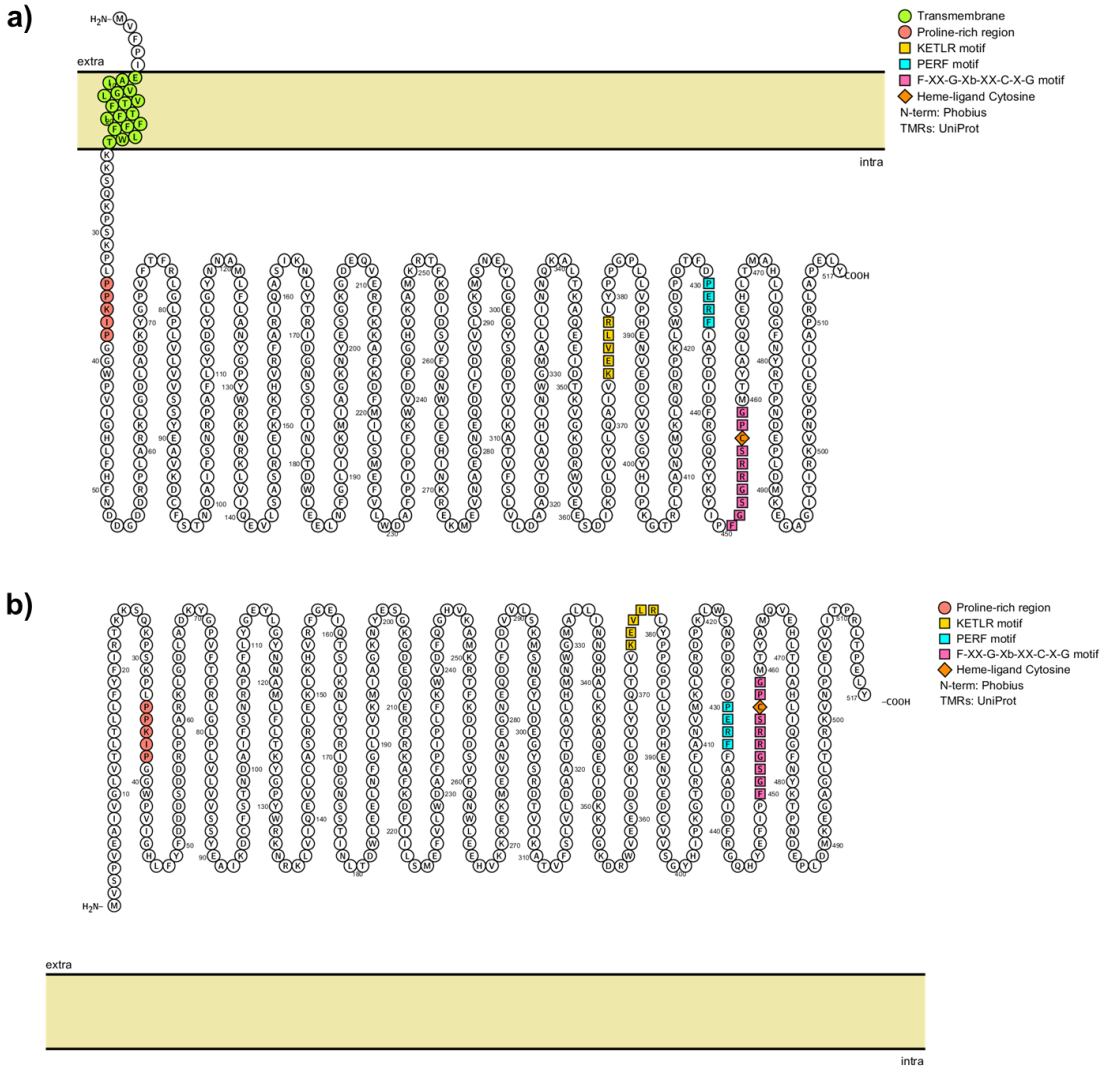
In silico characterization is a valuable tool as a first step in the identification of new genes, allowing to discard weak candidates before going through the *in vivo* analysis. Most of the new *CYP82E* genes here identified present the amino acid residues of important positions conserved, and may encode functional CYP enzymes. Therefore, further studies are needed to reveal the role of the newly identified genes in the nicotine conversion process, as well as to well-characterize the already known *CYP82E* genes.

ACKNOWLEDGMENTS

We thank Paolo Donini for his review of this manuscript and helpful comments.



Supplementary Figure 1: Consensus sequence analysis of *Nicotiana* CYP82E gene subfamily. The height of each letter is proportional to the corresponding relative frequency. The amino acids are colored according to their chemical properties: blue for most hydrophobic residues (A, C, F, I, L, V, M, W); green for polar, noncharged, nonaliphatic residues (N, Q, S, T); red for positively charged residues (K and R); orange for glycine (G); rose for histidine (H); yellow for proline (P); magenta for acidic residues (D, E) and turquoise for tyrosine (Y). **a)** ExxR; **b)** PERF; **c)** F-xx-G-xb-xx-C-x-G.



Supplementary Figure 2: Representation of *N. tabacum* CYP82E4 (a) and CYP82E10 (b) proteins using Protter software. Transmembrane region, proline-rich region, and CYP's consensus sequences are highlighted with different colors. The Protter software uses the predictions of Phobius to load the images.

Supplementary Table 1: *Nicotiana* species organized in Sections according to Knapp et al. (2004).

Section	Species (with authorities)
Alatae	*Nicotiana alata* Link and Otto
	Nicotiana azambujae L.B. Smith and Downs*
	Nicotiana bonariensis Lehm.
	Nicotiana forgetiana Hemsl.
	Nicotiana langsdorffii Weinm.
	Nicotiana longiflora Cav.
	Nicotiana mutabilis Stehmann and Samir
	Nicotiana plumbaginifolia L.
Nicotiana	*Nicotiana tabacum* L.
Noctiflorae	*Nicotiana acaulis* Speg.
	Nicotiana ameghinoi Speg.*
	Nicotiana glauca Graham
	Nicotiana noctiflora Hook
	Nicotiana petuniodes (Griseb.) Millán
	Nicotiana paa Mart. Crov.*
Paniculatae	*Nicotiana benavidesii* Goodsp.
	Nicotiana cordifolia Phil.
	Nicotiana cutleri D Arcy
	Nicotiana knightiana Goodsp.
	Nicotiana paniculata L.
	Nicotiana raimondii J.F. Macbr.
	Nicotiana solanifolia Walp.
Petunioides	*Nicotiana attenuata* Torrey ex S. Watson
	Nicotiana acuminata (Graham) Hook.
	Nicotiana corymbosa J. Rémy
	Nicotiana linearis Phil
	Nicotiana longibracteata Phil.*
	Nicotiana miersii J. Rémy
	Nicotiana pauciflora J. Rémy
	Nicotiana spegazzinii Millán
Polydicliae	*Nicotiana clevelandii* A. Gray
	Nicotiana quadrivalvis Pursh
Repandae	*Nicotiana nudicaulis* S. Watson
	Nicotiana nesophila I.M. Johnston
	Nicotiana repanda Willd.
	Nicotiana stocktonii Brandegee
Rusticae	*Nicotiana rustica* L.
Suaveolentes	*Nicotiana africana* Merxm.
	Nicotiana amplexicaulis N.T.Burb.
	Nicotiana benthamiana Domin
	Nicotiana burbidgeae Symon*
	Nicotiana cavicola N.T.Burb.
	Nicotiana debneyi Domin

	Nicotiana excelsior (J.M.Black) J.M.Black
	Nicotiana exigua H.-M.Wheeler
	Nicotiana fragrans Hooker
	Nicotiana gossei Domin
	Nicotiana goodspeedii H.-M.Wheeler
	Nicotiana hesperis N.T.Burb.
	Nicotiana heterantha Kenneally and Symon
	Nicotiana ingulba J.M.Black
	Nicotiana megalosiphon Van Huerck and Müll.Arg.
	Nicotiana maritima H.-M.Wheeler
	Nicotiana occidentalis H.-M.Wheeler;
	Nicotiana rosulata (S. Moore) Domin
	Nicotiana rotundifolia Lindl.
	Nicotiana suaveolens Lehm
	Nicotiana simulans N.T.Burb.
	Nicotiana stenocarpa H.-M.Wheeler*
	Nicotiana truncata D.E. Symon
	Nicotiana umbratica N.T.Burb.
	Nicotiana velutina H.-M.Wheeler
	Nicotiana wuttkei Clarkson and Symon*
Sylvestres	*Nicotiana sylvestris* Speg. and Comes
Tomentosae	*Nicotiana kawakamii* Y. Ohashi
	Nicotiana otophora Griseb.
	Nicotiana setchellii Goodsp.
	Nicotiana tomentosiformis Goodsp.
	Nicotiana tomentosa Ruiz and Pav.
Trigonophyllae	*Nicotiana obtusifolia* M. Martens and Galeotti
	Nicotiana palmeri A. Gray
Undulatae	*Nicotiana arentsii* Goodsp.
	Nicotiana glutinosa L.
	Nicotiana thrysiflora Bitter ex Goodsp.
	Nicotiana undulata Ruiz and Pav
	Nicotiana wigandioides Koch and Fintelm.

*Species that were not found in NCBI database.

Supplementary Table 2: Sequences used in the phylogenetic analysis and respective GenBank accession numbers.

Specie	Sequence	Accession Number
N. alata	NalaCYP82E15	BAM36723.1
N. alata	NalaCYP82E16	BAM36724.1
N. alata	NalaCYP82E17	BAM36725.1
N. alata	NalaCYP82E18	BAM36726.1
N. benthamiana	Nben1	Niben101Scf00270g05017.1
N. benthamiana	Nben2	Niben101Scf08430g01003.1
N. benthamiana	Nben3	Niben101Scf01569g04006.1
N. benthamiana	Nben4	Niben101Scf02322g03009.1
N. benthamiana	Nben5	Niben101Scf00113g05009.1
N. benthamiana	Nben6	Niben101Scf00219g04015.1
N. benthamiana	Nben7	Niben101Scf02749g01008.1
N. benthamiana	Nben8	Niben101Scf07242g07006.1
N. benthamiana	Nben9	Niben101Scf04995g11016.1
N. benthamiana	Nben10	Niben101Scf01035g03002.1
N. benthamiana	Nben11	Niben101Scf02427g00009.1
N. benthamiana	Nben12	Niben101Scf10708g00002.1
N. benthamiana	Nben13	Niben101Scf10999g00004.1
N. benthamiana	Nben14	Niben101Scf01065g08009.1
N. benthamiana	Nben15	Niben101Scf00922g00009.1
N. benthamiana	Nben16	Niben101Scf01748g02002.1
N. benthamiana	Nben17	Niben101Scf04011g04001.1
N. langsdorffii	NlanCYP82E19	BAM36727.1
N. sylvestris	NsylCYP82E2	ABR57311.1
N. sylvestris	NsylCYP82E10	ADP65810.1
N. sylvestris	Nsyl1	XP_009757628.1
N. sylvestris	Nsyl2	XP_009778755.1
N. sylvestris	Nsyl3	XP_009758062.1
N. sylvestris	Nsyl4	XP_009768110.1
N. sylvestris	Nsyl5	XP_009770601.1
N. sylvestris	Nsyl6	XP_009792748.1
N. sylvestris	Nsyl7	XP_009761500.1
N. sylvestris	Nsyl8	XP_009801176.1
N. sylvestris	Nsyl9	XP_009780811.1
N. sylvestris	Nsyl10	XP_009795251.1
N. sylvestris	Nsyl11	XP_009804510.1
N. sylvestris	Nsyl12	XP_009801068.1
N. sylvestris	Nsyl13	XP_009771564.1
N. sylvestris	Nsyl14	XP_009771565.1
N. sylvestris	Nsyl15	XP_009796225.1
N. sylvestris	Nsyl16	XP_009795889.1
N. sylvestris	Nsyl17	XP_009796226.1
N. sylvestris	Nsyl18	XP_009796227.1
N. sylvestris	Nsyl19	XP_009791128.1

N. sylvestris	Nsyl20	XP_009796228.1
N. sylvestris	Nsyl21	XP_009782203.1
N. sylvestris	Nsyl22	XP_009797406.1
N. sylvestris	Nsyl23	XP_009766074.1
N. sylvestris	Nsyl24	XP_009802012.1
N. sylvestris	Nsyl25	XP_009773047.1
N. sylvestris	Nsyl26	XP_009770873.1
N. sylvestris	Nsyl27	XP_009760136.1
N. sylvestris	Nsyl28	XP_009760134.1
N. sylvestris	Nsyl29	XP_009769777.1
N. sylvestris	Nsyl30	XP_009789828.1
N. sylvestris	Nsyl31	XP_009767392.1
N. sylvestris	Nsyl32	XP_009782058.1
N. sylvestris	Nsyl33	XP_009757705.1
N. sylvestris	Nsyl34	XP_009765717.1
N. sylvestris	Nsyl35	XP_009787639.1
N. sylvestris	Nsyl36	XP_009770332.1
N. sylvestris	Nsyl37	XP_009770888.1
N. sylvestris	Nsyl38	XP_009770013.1
N. sylvestris	Nsyl39	XP_009770887.1
N. sylvestris	Nsyl40	XP_009796229.1
N. sylvestris	Nsyl41	XP_009770688.1
N. sylvestris	Nsyl42	XP_009785012.1
N. sylvestris	Nsyl43	XP_009764952.1
N. sylvestris	Nsyl44	XP_009759043.1
N. sylvestris	Nsyl45	XP_009788494.1
N. sylvestris	Nsyl46	XP_009762893.1
N. sylvestris	Nsyl47	XP_009771410.1
N. sylvestris	Nsyl48	XP_009762891.1
N. sylvestris	Nsyl49	XP_009763889.1
N. sylvestris	Nsyl50	XP_009780649.1
N. sylvestris	Nsyl51	XP_009782145.1
N. sylvestris	Nsyl52	XP_009794145.1
N. sylvestris	Nsyl53	XP_009780215.1
N. sylvestris	Nsyl54	XP_009802554.1
N. sylvestris	Nsyl55	XP_009775980.1
N. sylvestris	Nsyl56	XP_009789924.1
N. sylvestris	Nsyl57	XP_009766832.1
N. sylvestris	Nsyl58	XP_009772507.1
N. sylvestris	Nsyl59	XP_009801864.1
N. sylvestris	Nsyl60	XP_009757704.1
N. sylvestris	Nsyl61	XP_009771988.1
N. sylvestris	Nsyl62	XP_009779413.1
N. sylvestris	Nsyl63	XP_009780363.1
N. sylvestris	Nsyl64	XP_009794456.1
N. sylvestris	Nsyl65	XP_009774708.1

N. sylvestris	Nsyl66	XP_009777846.1
N. sylvestris	Nsyl67	XP_009757123.1
N. sylvestris	Nsyl68	XP_009785657.1
N. sylvestris	Nsyl69	XP_009793679.1
N. sylvestris	Nsyl70	XP_009758268.1
N. sylvestris	Nsyl71	XP_009757342.1
N. sylvestris	Nsyl72	XP_009803463.1
N. sylvestris	Nsyl73	XP_009777857.1
N. sylvestris	Nsyl74	XP_009765039.1
N. sylvestris	Nsyl75	XP_009796910.1
N. sylvestris	Nsyl76	XP_009794425.1
N. sylvestris	Nsyl77	XP_009776868.1
N. sylvestris	Nsyl78	XP_009769208.1
N. sylvestris	Nsyl79	XP_009803037.1
N. sylvestris	Nsyl80	XP_009788755.1
N. sylvestris	Nsyl81	XP_009786696.1
N. sylvestris	Nsyl82	XP_009794009.1
N. sylvestris	Nsyl83	XP_009764618.1
N. sylvestris	Nsyl84	XP_009780029.1
N. sylvestris	Nsyl85	XP_009785656.1
N. sylvestris	Nsyl86	XP_009793271.1
N. sylvestris	Nsyl87	XP_009794007.1
N. sylvestris	Nsyl88	XP_009757934.1
N. sylvestris	Nsyl89	XP_009772938.1
N. sylvestris	Nsyl90	XP_009780149.1
N. sylvestris	Nsyl91	XP_009787971.1
N. sylvestris	Nsyl92	XP_009775574.1
N. sylvestris	Nsyl93	XP_009774376.1
N. sylvestris	Nsyl94	XP_009794426.1
N. sylvestris	Nsyl95	XP_009765480.1
N. sylvestris	Nsyl96	XP_009792560.1
N. sylvestris	Nsyl97	XP_009763140.1
N. tabacum	NtabCYP82E1	AAK62347.1
N. tabacum	NtabCYP82E2	ABA07806.2
N. tabacum	NtabCYP82E3	ABA07807.1
N. tabacum	NtabCYP82E4	ABA07805.1
N. tabacum	NtabCYP82E5	ABX56037.1
N. tabacum	NtabCYP82E8	ABC69420.1
N. tabacum	NtabCYP82E10	ADP65809.1
N. tabacum	NtabCYP82E21	XP_016500333.1
N. tabacum	Ntab1	XP_016490207.1
N. tabacum	Ntab2	XP_016456159.1
N. tabacum	Ntab3	XP_016481198.1
N. tabacum	Ntab4	XP_016487175.1
N. tabacum	Ntab5	XP_016492039.1
N. tabacum	Ntab6	XP_016489590.1

N. tabacum	Ntab7	XP_016446571.1
N. tabacum	Ntab8	XP_016507136.1
N. tabacum	Ntab9	XP_016492423.1
N. tabacum	Ntab10	XP_016441406.1
N. tabacum	Ntab11	XP_016505040.1
N. tabacum	Ntab12	XP_016478189.1
N. tabacum	Ntab13	XP_016458765.1
N. tabacum	Ntab14	XP_016460361.1
N. tabacum	Ntab15	XP_016507832.1
N. tabacum	Ntab16	XP_016468278.1
N. tabacum	Ntab17	XP_016459371.1
N. tabacum	Ntab18	XP_016471585.1
N. tabacum	Ntab19	XP_016483932.1
N. tabacum	Ntab20	XP_016482953.1
N. tabacum	Ntab21	XP_016457209.1
N. tabacum	Ntab22	XP_016503274.1
N. tabacum	Ntab23	XP_016452444.1
N. tabacum	Ntab24	XP_016477062.1
N. tabacum	Ntab25	XP_016457208.1
N. tabacum	Ntab26	XP_016503270.1
N. tabacum	Ntab27	XP_016503271.1
N. tabacum	Ntab28	XP_016503272.1
N. tabacum	Ntab29	CAA65580.1
N. tabacum	Ntab30	XP_016506428.1
N. tabacum	Ntab31	XP_016447078.1
N. tabacum	Ntab32	XP_016459206.1
N. tabacum	Ntab33	XP_016488782.1
N. tabacum	Ntab34	XP_016450159.1
N. tabacum	Ntab35	XP_016450066.1
N. tabacum	Ntab36	XP_016510777.1
N. tabacum	Ntab37	XP_016479430.1
N. tabacum	Ntab38	XP_016463151.1
N. tabacum	Ntab39	XP_016512891.1
N. tabacum	Ntab40	XP_016486582.1
N. tabacum	Ntab41	XP_016432435.1
N. tabacum	Ntab42	XP_016479719.1
N. tabacum	Ntab43	BAH84783.1
N. tabacum	Ntab44	XP_016476830.1
N. tabacum	Ntab45	XP_016506427.1
N. tabacum	Ntab46	XP_016495342.1
N. tabacum	Ntab47	XP_016485779.1
N. tabacum	Ntab48	XP_016495496.1
N. tabacum	Ntab49	XP_016463150.1
N. tabacum	Ntab50	XP_016507825.1
N. tabacum	Ntab51	XP_016466159.1
N. tabacum	Ntab52	CAA64635.1

N. tabacum	Ntab53	XP_016470640.1
N. tabacum	Ntab54	XP_016446949.1
N. tabacum	Ntab55	XP_016497448.1
N. tomentosiformis	NtomCYP82E3	ABM46919.1
N. tomentosiformis	NtomCYP82E4	ABM46920.1
N. tomentosiformis	NtomCYP82E5	XP_009619218.1
N. tomentosiformis	NtomCYP82E21	XP_009627979.1
N. tomentosiformis	Ntom1	XP_009609539.1
N. tomentosiformis	Ntom2	XP_009609537.1
N. tomentosiformis	Ntom3	XP_009595933.1
N. tomentosiformis	Ntom4	XP_009606611.1
N. tomentosiformis	Ntom5	XP_009616319.1
N. tomentosiformis	Ntom6	XP_009599169.1
N. tomentosiformis	Ntom7	XP_009606708.1
N. tomentosiformis	Ntom8	XP_009624056.1
N. tomentosiformis	Ntom9	XP_009630633.1
N. tomentosiformis	Ntom10	XP_009622945.1
N. tomentosiformis	Ntom11	XP_009627914.1
N. tomentosiformis	Ntom12	XP_009607330.1
N. tomentosiformis	Ntom13	XP_009626729.1
N. tomentosiformis	Ntom14	XP_009620153.1
N. tomentosiformis	Ntom15	XP_009591954.1
N. tomentosiformis	Ntom16	XP_009620154.1
N. tomentosiformis	Ntom17	XP_009594272.1
N. tomentosiformis	Ntom18	XP_009615258.1
N. tomentosiformis	Ntom19	XP_009586899.1
N. tomentosiformis	Ntom20	XP_009619291.1
N. tomentosiformis	Ntom21	XP_009603863.1
N. tomentosiformis	Ntom22	XP_009597427.1
N. tomentosiformis	Ntom23	XP_009588170.1
N. tomentosiformis	Ntom24	XP_009608339.1
N. tomentosiformis	Ntom25	XP_009605293.1
N. tomentosiformis	Ntom26	XP_009600860.1
N. tomentosiformis	Ntom27	XP_009620580.1
N. tomentosiformis	Ntom28	XP_009595838.1
N. tomentosiformis	Ntom29	XP_009618794.1
N. tomentosiformis	Ntom30	XP_009621248.1
N. tomentosiformis	Ntom31	XP_009607201.1
N. tomentosiformis	Ntom32	XP_009619292.1
N. tomentosiformis	Ntom33	XP_009587545.1
N. tomentosiformis	Ntom34	XP_009597267.1
N. tomentosiformis	Ntom35	XP_009601421.1
N. tomentosiformis	Ntom36	XP_009628880.1
N. tomentosiformis	Ntom37	XP_009596523.1
N. tomentosiformis	Ntom38	XP_009624917.1
N. tomentosiformis	Ntom39	XP_009616607.1

N. tomentosiformis	Ntom40	XP_009619846.1
N. tomentosiformis	Ntom41	XP_009626597.1
N. tomentosiformis	Ntom42	XP_009595836.1
N. tomentosiformis	Ntom43	XP_009618532.1
N. tomentosiformis	Ntom44	XP_009610077.2
N. tomentosiformis	Ntom45	XP_009608153.1
N. tomentosiformis	Ntom46	XP_009609786.1
N. tomentosiformis	Ntom47	XP_009588689.1
N. tomentosiformis	Ntom48	XP_009597287.1
N. tomentosiformis	Ntom49	XP_009615716.1
N. tomentosiformis	Ntom50	XP_009629564.2
N. tomentosiformis	Ntom51	XP_009631554.1
N. tomentosiformis	Ntom52	XP_009623936.1
N. tomentosiformis	Ntom53	XP_009622691.1
N. tomentosiformis	Ntom54	XP_009591952.1
N. tomentosiformis	Ntom55	XP_009595834.1
N. tomentosiformis	Ntom56	XP_009612725.1
N. tomentosiformis	Ntom57	XP_009625417.1
N. tomentosiformis	Ntom58	XP_009592484.1
N. tomentosiformis	Ntom59	XP_009591248.1
N. tomentosiformis	Ntom60	XP_009616520.1
N. tomentosiformis	Ntom61	XP_009595835.1
N. tomentosiformis	Ntom62	XP_009623934.1
N. tomentosiformis	Ntom63	XP_009610421.1
N. tomentosiformis	Ntom64	XP_009619325.1
N. tomentosiformis	Ntom65	XP_009626636.1
N. tomentosiformis	Ntom66	XP_009626802.1
N. tomentosiformis	Ntom67	XP_009606204.2
N. tomentosiformis	Ntom68	XP_009608635.1
N. tomentosiformis	Ntom69	XP_009612942.1
N. tomentosiformis	Ntom70	XP_009622900.1
N. tomentosiformis	Ntom71	XP_009626574.1
N. tomentosiformis	Ntom72	XP_009587552.1
N. tomentosiformis	Ntom73	XP_009603940.1
N. tomentosiformis	Ntom74	XP_009598320.1
N. tomentosiformis	Ntom75	XP_009626046.1
N. tomentosiformis	Ntom76	XP_009612417.1
N. tomentosiformis	Ntom77	XP_009589554.1
N. tomentosiformis	Ntom78	XP_009612418.1
N. tomentosiformis	Ntom79	XP_009616474.1
N. tomentosiformis	Ntom80	XP_009627815.1
N. tomentosiformis	Ntom81	XP_009602679.1
N. tomentosiformis	Ntom82	XP_009617877.1
N. tomentosiformis	Ntom83	XP_009613834.1
N. tomentosiformis	Ntom84	XP_009624010.1
N. tomentosiformis	Ntom85	XP_009611155.1

N. tomentosiformis	Ntom86	XP_009631781.2
N. tomentosiformis	Ntom87	XP_009624011.1
N. tomentosiformis	Ntom88	XP_009596429.1
N. tomentosiformis	Ntom89	XP_009613392.1
N. tomentosiformis	Ntom90	XP_009607443.1
N. tomentosiformis	Ntom91	XP_009609252.1
N. tomentosiformis	Ntom92	XP_009619866.1
Ammi majus	AmajCYP82H1	AAS90126.1
Arabidopsis thaliana	AthaCYP82F1	NP_180088.1
Arabidopsis thaliana	AthaCYP82G1	NP_189154.1
Arabidopsis thaliana	AthaCYP82C4	NP_194922.1
Arabidopsis thaliana	AthaCYP82C3	NP_194923.1
Arabidopsis thaliana	AthaCYP82C2	NP_194925.1
Eschscholzia californica	EcalCYP82B1	AAC39454.1
Eschscholzia californica	EcalCYP82N2	BAK20464.1
Glycine max	GmaxCYP82A1	CAA71515.1
Glycine max	GmaxCYP82A3	CAA71876.1
Glycine max	GmaxCYP82A4	CAA71877.1
Gossypium hirsutum	GhirCYP82D1	AII31758.1
Gossypium hirsutum	GhirCYP82D2	AII31759.1
Gossypium hirsutum	GhirCYP82D3	AII31760.1
Mentha x piperita	MpipCYP82D62	AGF30366.1
N. tabacum	NtabCYP92A2	ABC69381.1
N. tabacum	NtabCYP92B2	ABC69386.1
N. tabacum	NtabCYP81B2	ABC69377.1
N. tabacum	NtabCYP81C6	ABC69379.1
N. tabacum	NtabCYP82M1	ABC69372.1
Ocimum basilicum	ObasCYP82D33	AGF30364.1
Papaver somniferum	PsomCYP82X1	AFB74614.1
Papaver somniferum	PsomCYP82Y1	AFB74617.1
Pisum sativum	PsatCYP82A1	AAG09208.1
Populus trichocarpa	PtriCYP82L1	XP_002305292.2
Populus trichocarpa	PtriCYP82K1	EEF04891
Solanum lycopersicum	SlycCYP85A3	BAD98244.1
Solanum lycopersicum	SlycCYP82E11	XP_004236938.1
Solanum tuberosum	StubCYP82E11	XP_015167656.1
Solanum tuberosum	StubCYP82E13	XP_015165235.1
Stevia rebaudiana	SrebCYP82Q1	ABB20912.1

Supplementary Table 3: *CYP82E* genes in the *Nicotiana* genus.

Specie	Sequence	Reference	Accession Number
N. alata	NalaCYP82E15	(Pakdeechanuan, Shoji et al. 2012)	BAM36723.1
N. alata	NalaCYP82E16	(Pakdeechanuan, Shoji et al. 2012)	BAM36724.1
N. alata	NalaCYP82E17	(Pakdeechanuan, Shoji et al. 2012)	BAM36725.1
N. alata	NalaCYP82E18	(Pakdeechanuan, Shoji et al. 2012)	BAM36726.1
N. benthamiana	Nben1	*	Niben101Scf00270g05017.1
N. benthamiana	Nben2	*	Niben101Scf08430g01003.1
N. langsdorffii	NlanCYP82E19	(Pakdeechanuan, Teoh et al. 2012)	BAM36727.1
N. langsdorffii	NlanCYP82E20 #	(Pakdeechanuan, Teoh et al. 2012)	AB709937.1
N. sylvestris	NsylCYP82E2	(Chakrabarti, Meekins et al. 2007)	ABR57311.1
N. sylvestris	NsylCYP82E10	(Lewis, Bowen et al. 2010)	ADP65810.1
N. sylvestris	Nsyl1	*	XP_009757628.1
N. sylvestris	Nsyl2	*	XP_009778755.1
N. sylvestris	Nsyl3	*	XP_009758062.1
N. sylvestris	Nsyl4	*	XP_009768110.1
N. sylvestris	Nsyl5	*	XP_009770601.1
N. sylvestris	Nsyl6	*	XP_009792748.1
N. sylvestris	Nsyl7	*	XP_009761500.1
N. tabacum	NtabCYP82E1	(Takemoto, Hayashi et al. 1999)	AAK62347.1
N. tabacum	NtabCYP82E2	(Siminszky, Gavilano et al. 2005)	ABA07806.2
N. tabacum	NtabCYP82E3	(Siminszky, Gavilano et al. 2005)	ABA07807.1
N. tabacum	NtabCYP82E4	(Siminszky, Gavilano et al. 2005)	ABA07805.1
N. tabacum	NtabCYP82E5	(Gavilano and Siminszky 2007)	ABX56037.1
N. tabacum	NtabCYP82E8	-	ABC69420.1
N. tabacum	NtabCYP82E9 #	(Siminszky, Gavilano et al. 2005)	DQ131890
N. tabacum	NtabCYP82E10	(Lewis, Bowen et al. 2010)	ADP65809.1
N. tabacum	NtabCYP82E21	(Liedschulte, Schwaar et al. 2016)	XP_016500333.1
N. tabacum	Ntab1	*	XP_016490207.1
N. tabacum	Ntab2	*	XP_016456159.1
N. tabacum	Ntab3	*	XP_016481198.1
N. tabacum	Ntab4	*	XP_016487175.1
N. tabacum	Ntab5	*	XP_016492039.1
N. tabacum	Ntab6	*	XP_016489590.1
N. tabacum	Ntab7	*	XP_016446571.1
N. tabacum	Ntab8	*	XP_016507136.1
N. tabacum	Ntab9	*	XP_016492423.1

N. tabacum	Ntab10	*	XP_016441406.1
N. tomentosiformis	NtomCYP82E3	(Gavilano, Coleman et al. 2007)	ABM46919.1
N. tomentosiformis	NtomCYP82E4	(Gavilano, Coleman et al. 2007)	ABM46920.1
N. tomentosiformis	NtomCYP82E5	(Gavilano and Siminszky 2007)	XP_009619218.1
N. tomentosiformis	NtomCYP82E21	(Liedschulte, Schwaar et al. 2016)	XP_009627979.1
N. tomentosiformis	Ntom1	*	XP_009609539.1
N. tomentosiformis	Ntom2	*	XP_009609537.1
N. tomentosiformis	Ntom3	*	XP_009595933.1
N. tomentosiformis	Ntom4	*	XP_009606611.1

* new putative *CYP82E* genes

described as pseudogenes

REFERENCES

BAK, S. et al. Cytochromes p450. **Arabidopsis Book,** v. 9, p. e0144, 2011. ISSN 1543-8120. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22303269 >.

BENOWITZ, N. L. Pharmacology of nicotine: addiction, smoking-induced disease, and therapeutics. **Annu Rev Pharmacol Toxicol,** v. 49, p. 57-71, 2009. ISSN 0362-1642 (Print)
0362-1642 (Linking). Available at: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18834313 >.

BERNHARDT, R. Cytochromes P450 as versatile biocatalysts. **J Biotechnol,** v. 124, n. 1, p. 128-45, Jun 2006. ISSN 0168-1656. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/16516322 >.

BLASKOVIC, S.; BLANC, M.; VAN DER GOOT, F. G. What does S-palmitoylation do to membrane proteins? **FEBS J,** v. 280, n. 12, p. 2766-74, Jun 2013. ISSN 1742-4658. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/23551889 >.

CAI, B. et al. (R)-nicotine biosynthesis, metabolism and translocation in tobacco as determined by nicotine demethylase mutants. **Phytochemistry,** v. 95, p. 188-96, Nov 2013. ISSN 1873-3700. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23849545 >.

CHAKRABARTI, M. et al. CYP82E4-mediated nicotine to nornicotine conversion in tobacco is regulated by a senescence-specific signaling pathway. **Plant Mol Biol,** v. 66, n. 4, p. 415-27, Mar 2008. ISSN 0167-4412. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18196465 >.

_____. Inactivation of the cytochrome P450 gene CYP82E2 by degenerative mutations was a key event in the evolution of the alkaloid profile of modern tobacco. **New Phytol,** v. 175, n. 3, p. 565-74, 2007. ISSN 0028-646X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17635231 >.

CLARKSON, J. J. et al. Phylogenetic relationships in Nicotiana (Solanaceae) inferred from multiple plastid DNA regions. **Mol Phylogenet Evol,** v. 33, n. 1, p. 75-90, Oct 2004. ISSN 1055-7903. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15324840 >.

DEWEY, R. E.; XIE, J. Molecular genetics of alkaloid biosynthesis in Nicotiana tabacum. **Phytochemistry,** v. 94, p. 10-27, Oct 2013. ISSN 1873-3700 (Electronic)
0031-9422 (Linking). Available at: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23953973 >.

DICKE, M.; BALDWIN, I. T. The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. **Trends Plant Sci,** v. 15, n. 3, p. 167-75, Mar 2010. ISSN 1878-4372. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/20047849 >.

DRUMMOND, A. J.; RAMBAUT, A. BEAST: Bayesian evolutionary analysis by sampling trees. **BMC Evol Biol,** v. 7, p. 214, 2007. ISSN 1471-2148. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17996036 >.

EMANUELSSON, O. et al. Locating proteins in the cell using TargetP, SignalP and related tools. **Nat Protoc,** v. 2, n. 4, p. 953-71, 2007. ISSN 1750-2799. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17446895 >.

GAVILANO, L. B. et al. Functional analysis of nicotine demethylase genes reveals insights into the evolution of modern tobacco. **J Biol Chem,** v. 282, n. 1, p. 249-56, Jan 2007. ISSN 0021-9258. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17102129 >.

_____. Genetic engineering of Nicotiana tabacum for reduced nornicotine content. **J Agric Food Chem,** v. 54, n. 24, p. 9071-8, Nov 29 2006. ISSN 0021-8561 (Print) 0021-8561 (Linking). Available at: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17117792 >.

GAVILANO, L. B.; SIMINSZKY, B. Isolation and characterization of the cytochrome P450 gene CYP82E5v2 that mediates nicotine to nornicotine conversion in the green leaves of tobacco. **Plant Cell Physiol,** v. 48, n. 11, p. 1567-74, Nov 2007. ISSN 0032-0781. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/17923451 >.

HAMBERGER, B.; BAK, S. Plant P450s as versatile drivers for evolution of species-specific chemical diversity. **Philos Trans R Soc Lond B Biol Sci,** v. 368, n. 1612, p. 20120426, Feb 2013. ISSN 1471-2970. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23297350 >.

KNAPP, S. et al. Solanaceae--a model for linking genomics with biodiversity. **Comp Funct Genomics,** v. 5, n. 3, p. 285-91, 2004. ISSN 1531-6912. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18629162 >.

KNAPP, S.; CHASE, M. W.; CLARKSON, J. J. Nomenclatural Changes and a New Sectional Classification in Nicotiana (Solanaceae). **Taxon,** v. 53, n. 1, p. 10, 2004.

KROGH, A. et al. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. **J Mol Biol,** v. 305, n. 3, p. 567-80, Jan 2001. ISSN 0022-2836. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11152613 >.

KÄLL, L.; KROGH, A.; SONNHAMMER, E. L. A combined transmembrane topology and signal peptide prediction method. **J Mol Biol,** v. 338, n. 5, p. 1027-36, May 2004. ISSN 0022-2836. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15111065 >.

KÄLL, L.; KROGH, A.; SONNHAMMER, E. L. Advantages of combined transmembrane topology and signal peptide prediction - the Phobius web server. **Nucleic Acids Res,** v. 35, n. Web Server issue, p. W429-32, Jul 2007. ISSN 1362-4962. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17483518 >.

LAMB, D. C.; WATERMAN, M. R. Unusual properties of the cytochrome P450 superfamily. **Philos Trans R Soc Lond B Biol Sci,** v. 368, n. 1612, p. 20120434, Feb 2013. ISSN 1471-2970. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/23297356 >.

LEE, S. et al. Herbivore-induced and floral homoterpene volatiles are biosynthesized by a single P450 enzyme (CYP82G1) in Arabidopsis. **Proc Natl Acad Sci U S A,** v. 107, n. 49, p. 21205-10, Dec 2010. ISSN 1091-6490. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21088219 >.

LEWIS, R. S. et al. Three nicotine demethylase genes mediate nornicotine biosynthesis in Nicotiana tabacum L.: functional characterization of the CYP82E10 gene. **Phytochemistry,** v. 71, n. 17-18, p. 1988-98, Dec 2010. ISSN 1873-3700. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20977974 >.

_____. RNA interference (RNAi)-induced suppression of nicotine demethylase activity reduces levels of a key carcinogen in cured tobacco leaves. **Plant Biotechnol J,** v. 6, n. 4, p. 346-54, May 2008. ISSN 1467-7652. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/18282175 >.

LIEDSCHULTE, V. et al. Identification of CYP82E21 as a functional nicotine N-demethylase in tobacco flowers. **Phytochemistry**, Sep 2016. ISSN 1873-3700. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/27596288 >.

LIU, F. et al. The Arabidopsis P450 protein CYP82C2 modulates jasmonate-induced root growth inhibition, defense gene expression and indole glucosinolate biosynthesis. **Cell Res,** v. 20, n. 5, p. 539-52, May 2010. ISSN 1748-7838. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/20354503 >.

MANCEAU, F.; FLINIAUX, M.-A.; JACQUIN-DUBREUIL, A. Ability of a *Nicotiana plumbaginifolia* cell suspension to demethylate nicotine into nornicotine. **Phytochemistry,** v. 28, n. 10, p. 2671-2674, 1989.

MOGHBEL, N. **Investigations into the pituri plant: nicotine content, nicotine conversion to nornicotine, nicotine release and cytotoxicity of Australian native *Nicotiana* spp.** 2016. 135 (Doctor of Philosophy). School of Pharmacy, The University of Queensland, Queensland, Australia.

MÖLLER, S.; CRONING, M. D.; APWEILER, R. Evaluation of methods for the prediction of membrane spanning regions. **Bioinformatics**, v. 17, n. 7, p. 646-53, Jul 2001. ISSN 1367-4803. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11448883 >.

NELSON, D.; WERCK-REICHHART, D. A P450-centric view of plant evolution. **Plant J,** v. 66, n. 1, p. 194-211, Apr 2011. ISSN 1365-313X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21443632 >.

NELSON, D. R. **Plant cytochrome P450s from moss to poplar**. Phytochem Rev. 5**:** 193-204 p. 2006.

NELSON, D. R. et al. **Comparison of Cytochrome P450 Genes from Six Plant Genomes**. Tropical Plant Biology. 1**:** 216-235 p. 2008.

_____. Comparative genomics of rice and Arabidopsis. Analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and a dicot. **Plant Physiol,** v. 135, n. 2, p. 756-72, Jun 2004. ISSN 0032-0889. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15208422 >.

NOMURA, T. et al. The last reaction producing brassinolide is catalyzed by cytochrome P-450s, CYP85A3 in tomato and CYP85A2 in Arabidopsis. **J Biol Chem,** v. 280, n. 18, p. 17873-9, May 2005. ISSN 0021-9258. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/15710611 >.

PAKDEECHANUAN, P.; SHOJI, T.; HASHIMOTO, T. Root-to-shoot translocation of alkaloids is dominantly suppressed in Nicotiana alata. **Plant Cell Physiol,** v. 53, n. 7, p. 1247-54, Jul 2012. ISSN 1471-9053. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/22555816 >.

PAKDEECHANUAN, P. et al. Non-functionalization of two CYP82E nicotine N-demethylase genes abolishes nornicotine formation in Nicotiana langsdorffii. **Plant Cell Physiol,** v. 53, n. 12, p. 2038-46, Dec 2012. ISSN 1471-9053. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23034878 >.

PAQUETTE, S. M.; BAK, S.; FEYEREISEN, R. Intron-exon organization and phylogeny in a large superfamily, the paralogous cytochrome P450 genes of Arabidopsis thaliana. **DNA Cell Biol,** v. 19, n. 5, p. 307-17, May 2000. ISSN 1044-5498. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10855798 >.

PODELL, S.; GRIBSKOV, M. Predicting N-terminal myristoylation sites in plant proteins. **BMC Genomics,** v. 5, n. 1, p. 37, Jun 2004. ISSN 1471-2164. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/15202951 >.

RAJABI, F. et al. Combination of Plant Metabolic Modules Yields Synthetic Synergies. **PLoS One,** v. 12, n. 1, p. e0169778, 2017. ISSN 1932-6203. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/28081182 >.

RALSTON, L. et al. Cloning, heterologous expression, and functional characterization of 5-ep*i*-Aristolochene-1,3-Dihydroxylase from tobacco (Ni*cotiana tabacum)*. **Archives of Biochemistry and Biophysics,** v. 393, n. 2, p. 14, 2001.

SAITOH, F.; NOMA, M.; KAWASHIMA, N. The alkaloid contents of sixty Nicotiana species. **Phytochemistry,** v. 24, n. 3, p. 477-480, 1985. ISSN **0031~9422/8**.

SEZUTSU, H.; LE GOFF, G.; FEYEREISEN, R. Origins of P450 diversity. **Philos Trans R Soc Lond B Biol Sci,** v. 368, n. 1612, p. 20120428, Feb 2013. ISSN 1471-2970. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/23297351 >.

SHI, H. et al. Changes in TSNA contents during tobacco storage and the effect of temperature and nitrate level on TSNA formation. **J Agric Food Chem,** v. 61, n. 47,

p. 11588-94, Nov 2013. ISSN 1520-5118. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24188376 >.

SHITAN, N. et al. Involvement of the leaf-specific multidrug and toxic compound extrusion (MATE) transporter Nt-JAT2 in vacuolar sequestration of nicotine in Nicotiana tabacum. **PLoS One,** v. 9, n. 9, p. e108789, 2014. ISSN 1932-6203. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25268729 >.

SIMINSZKY, B. et al. Conversion of nicotine to nornicotine in Nicotiana tabacum is mediated by CYP82E4, a cytochrome P450 monooxygenase. **Proc Natl Acad Sci U S A,** v. 102, n. 41, p. 14919-24, Oct 2005. ISSN 0027-8424. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/16192354 >.

SIMMONS, M. P.; OCHOTERENA, H. Gaps as characters in sequence-based phylogenetic analyses. **Syst Biol,** v. 49, n. 2, p. 369-81, Jun 2000. ISSN 1063-5157. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12118412 >.

STEPPUHN, A. et al. Nicotine's defensive function in nature. **PLoS Biol,** v. 2, n. 8, p. E217, Aug 2004. ISSN 1545-7885. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/15314646 >.

SUN, B. et al. Genetic variation in alkaloid accumulation in leaves of Nicotiana. **J Zhejiang Univ Sci B,** v. 14, n. 12, p. 1100-9, Dec 2013. ISSN 1862-1783. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/24302710 >.

SUN, L. et al. Cotton cytochrome P450 CYP82D regulates systemic cell death by modulating the octadecanoid pathway. **Nat Commun,** v. 5, p. 5372, 2014. ISSN 2041-1723. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/25371113 >.

TAKEMOTO, D. et al. Molecular cloning of a defense-response-related cytochrome P450 gene from tobacco. **Plant Cell Physiol,** v. 40, n. 12, p. 1232-42, Dec 1999. ISSN 0032-0781. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10682345 >.

TAKEMURA, T. et al. Molecular cloning and characterization of a cytochrome P450 in sanguinarine biosynthesis from Eschscholzia californica cells. **Phytochemistry,** v. 91, p. 100-8, Jul 2013. ISSN 1873-3700. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/22421633 >.

TAMURA, K. et al. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. **Mol Biol Evol,** v. 28, n. 10, p. 2731-9, Oct 2011. ISSN 1537-1719. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/21546353 >.

TSO, T.-C. **Production, Physiology and Biochemistry of Tobacco Plant**. Beltsville: Ideals, 1990. 785.

WERCK-REICHHART, D.; FEYEREISEN, R. Cytochromes P450: a success story. **Genome Biol,** v. 1, n. 6, p. REVIEWS3003, 2000. ISSN 1474-760X. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/11178272 >.

WHITBRED, J. M.; SCHULER, M. A. Molecular characterization of CYP73A9 and CYP82A1 P450 genes involved in plant defense in pea. **Plant Physiol,** v. 124, n. 1, p. 47-58, Sep 2000. ISSN 0032-0889. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/10982421 >.

XIE, Y. et al. GPS-Lipid: a robust tool for the prediction of multiple lipid modification sites. **Sci Rep,** v. 6, p. 28249, 2016. ISSN 2045-2322. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/27306108 >.

WORLD HEALTH ORGANIZATION. WHO STUDY GROUP ON TOBACCO PRODUCT REGULATION. Report on the Scientific Basis of Tobacco Product Regulations: 1st report of a WHO study group. **World Health Organ Tech Rep Ser**, n. 945, p. 1-120, back cover, 2007. ISSN 0512-3054.

XU, D. et al. Biochemical and molecular characterizations of nicotine demethylase in tobacco. **Physiologia Plantarum,** v. 129, p. 307-319, 2007. ISSN 0031-9317.

YTTERBERG, A. J.; JENSEN, O. N. Modification-specific proteomics in plant biology. **J Proteomics,** v. 73, n. 11, p. 2249-66, Oct 2010. ISSN 1876-7737. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/20541636 >.

ZHAO, Q. et al. GPS-SUMO: a tool for the prediction of sumoylation sites and SUMO-interaction motifs. **Nucleic Acids Res,** v. 42, n. Web Server issue, p. W325-30, Jul 2014. ISSN 1362-4962. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/24880689 >.

4 CONCLUSÕES

O tabaco (*Nicotiana tabacum*) destaca-se como uma cultura economicamente importante para o Brasil, visto que o país é o segundo maior produtor de tabaco em folha do mundo e lidera o ranking mundial como maior exportador desse produto (Sinditabaco, 2017). A nicotina é o alcaloide mais conhecido em plantas do gênero *Nicotiana*, podendo representar até 5% do peso seco da folha (Hung et al., 2013). Do ponto de vista ecológico, a nicotina é essencial na defesa das plantas contra herbívoros (Shitan et al., 2014).

No primeiro capítulo desse trabalho, foram abordadas as principais questões sobre a biossíntese, o transporte e o metabolismo da nicotina em *Nicotiana*. Através da revisão realizada, foi possível obter um panorama geral com as informações mais importantes desses processos. Ainda, foram identificados e listados os pontos que necessitam de maiores esclarecimentos: a) as reações finais da síntese de nicotina, envolvendo as enzimas A622 e BBL, e b) quais transportadores atuam na translocação de nicotina, especificamente da raiz para o xilema, e do xilema para as células das folhas.

Além da nicotina, outros alcaloides também estão presentes nas plantas desse gênero, sendo a nornicotina o segundo mais encontrado (Shi et al., 2013). A desmetilação da nicotina, reação catalisada por enzimas nicotina *N*-desmetilase (NND) dá origem à nornicotina (Lewis et al., 2010). Essa, por sua vez, pode reagir comóxidos de nitrogênio presentes nas folhas de tabaco, formando *N*-nitrosonornicotina (NNN), substância cancerígena do grupo das Nitrosaminas Específicas do Tabaco (TSNA – *Tobacco Specific Nitrosamines*) (Hecht, 1998).

O primeiro passo na formação de NNN é enzimático, mas o segundo passo ocorre de maneira espontânea. Assim, o conhecimento sobre a subfamília gênica *CYP82E*, responsável pela expressão de enzimas NND no gênero *Nicotiana*, é essencial para compreender a formação de NNN, bem como para buscar a diminuição dos níveis dessa substância nos produtos de tabaco. Alguns genes da subfamília *CYP82E* já foram descritos para *N. tabacum* (Lewis et al., 2010; Liedschulte et al., 2016). Entretanto, a regulação da expressão desses genes ainda não está totalmente elucidada.

No segundo capítulo deste trabalho apresentamos a identificação de novos genes putativos da subfamília *CYP82E* em diferentes espécies do gênero *Nicotiana*.

Além disso, foi proposta uma filogenia incluindo os novos genes identificados, o que pode auxiliar no entendimento da organização subfamília *CYP82E* em *Nicotiana*. Utilizamos, ainda, ferramentas *in silico* na caracterização das sequências encontradas, e através dessa análise, observamos algumas predições em comum para a maioria das sequências, tanto as já descritas como as novas: a) a localização subcelular das proteínas no retículo endoplasmático, e b) a interação das proteínas com pequenos modificadores semelhantes à ubiquitina (*SUMOs – small ubiquitine-like modifiers*). Através desse estudo buscamos contribuir para a compreensão da organização da subfamília gênica *CYP82E* em *Nicotiana*. Esses dados podem auxiliar no entendimento do processo de conversão da nicotina em nornicotina, e assim, podem ser úteis no desenvolvimento de novas soluções com o objetivo de reduzir substâncias cancerígenas em produtos de tabaco, especialmente as TSNA.

REFERÊNCIAS

ALBUQUERQUE, L. B.; VELÁZQUEZ, A.; MAYORGA-SAUCEDO, R. Solanaceae composition, pollination and seed dispersal syndromes in Mexican Mountain Cloud Forest. Acta Botanica Brasilica, v. 20, n. 3, p. 599-613, 2006.

BALDWIN, I. T.; KARB, M. J.; OHNMEISS, T. E. Allocation of 15N from nitrate to nicotine: production and turnover of a damage-induced mobile defense. Ecology, v. 75, n. 6, p. 1703-1713, 1994.

BONATO, A. A. A fumicultura no Brasil e a convenção-quadro para controle do tabaco. Departamento de Estudos Sócio-Econômicos Rurais - DESER. 2007

BROGAN, A. P. et al. Altered retinoid homeostasis catalyzed by a nicotine metabolite: implications in macular degeneration and normal development. Proc Natl Acad Sci U S A, v. 102, n. 30, p. 10433-8, Jul 2005. ISSN 0027-8424. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/16014706>.

BROWN, B. G.; BORSCHKE, A. J.; DOOLITTLE, D. J. An analysis of the role of tobacco-specific nitrosamines in the carcinogenicity of tobacco smoke. Nonlinearity Biol Toxicol Med, v. 1, n. 2, p. 179-98, Apr 2003. ISSN 1540-1421. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/19330121>.

CAI, B. et al. (R)-nicotine biosynthesis, metabolism and translocation in tobacco as determined by nicotine demethylase mutants. Phytochemistry, v. 95, p. 188-96, Nov 2013. ISSN 1873-3700. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23849545>.

CARVALHO, B. L. et al. Genetic parameters estimates associated to conversion of nicotine to nornicotine in Burley tobacco. American Journal of Plant Sciences, v. 5, p. 3380-3388, 2014.

CHAKRABARTI, M. et al. CYP82E4-mediated nicotine to nornicotine conversion in tobacco is regulated by a senescence-specific signaling pathway. Plant Mol Biol, v. 66, n. 4, p. 415-27, Mar 2008. ISSN 0167-4412. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/18196465>.

CHANDRASEKHARARAO, C. et al. Effect of nitrogen levels and leaf position on carbohydrate and nitrogen metabolismo in FVC tobacco (Nicotiana tabacum L.). Indian Journal of Plant Physiology, v. 19, n. 3, p. 244-249, 2014.

CLARKSON, J. J. et al. Phylogenetic relationships in Nicotiana (Solanaceae) inferred from multiple plastid DNA regions. Mol Phylogenet Evol, v. 33, n. 1, p. 75-90, Oct 2004. ISSN 1055-7903. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15324840>.

COLLINS, W. K.; HAWKS JR., S. N. Principles of flue-cured tobacco production. First edition. North Carolina, EUA: North Carolina State University, 1993. 301.

COLLINS, W. K.; HAWKS, S. N. Principles of flue-cured tobacco production. Madison: University of Wisconsin, 1983. 358.

DICKERSON, T. J.; JANDA, K. D. A previously undescribed chemical link between smoking and metabolic disease. Proc Natl Acad Sci U S A, v. 99, n. 23, p. 15084-8, Nov 2002. ISSN 0027-8424. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/12403823>.

EL-BAYOUMY, K. et al. Increased expression of cyclooxygenase-2 in rat lung tumors induced by the tobacco-specific nitrosamine 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanone: the impact of a high-fat diet. Cancer Res, v. 59, n. 7, p. 1400-3, Apr 1999. ISSN 0008-5472. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/10197601>.

GAVILANO, L. B. et al. Genetic engineering of Nicotiana tabacum for reduced nornicotine content. J Agric Food Chem, v. 54, n. 24, p. 9071-8, Nov 2006. ISSN 0021-8561. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17117792>.

GAVILANO, L. B.; SIMINSZKY, B. Isolation and characterization of the cytochrome P450 gene CYP82E5v2 that mediates nicotine to nornicotine conversion in the green leaves of tobacco. Plant Cell Physiol, v. 48, n. 11, p. 1567-74, Nov 2007. ISSN 0032-0781. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17923451>.

HECHT, S. S. Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. Chem Res Toxicol, v. 11, n. 6, p. 559-603, Jun 1998. ISSN 0893-228X. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/9625726>.

HIBI, N. et al. Gene expression in tobacco low-nicotine mutants. Plant Cell, v. 6, n. 5, p. 723-35, May 1994. ISSN 1040-4651. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/8038607>.

HUNG, C. Y. et al. Alteration of the alkaloid profile in genetically modified tobacco reveals a role of methylenetetrahydrofolate reductase in nicotine N-demethylation. Plant Physiol, v. 161, n. 2, p. 1049-60, Feb 2013. ISSN 1532-2548. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/23221678>.

IARC. IARC monographs on the evaluation of carcinogenic risks to humans. Lyon, France: International Agency for Research on Cancer, 2007. 641 ISBN 1017-1606 0250-9555.

LEWIS, R. S. et al. Three nicotine demethylase genes mediate nornicotine biosynthesis in Nicotiana tabacum L.: functional characterization of the CYP82E10 gene. Phytochemistry, v. 71, n. 17-18, p. 1988-98, Dec 2010. ISSN 1873-3700. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/20977974>.

_____. RNA interference (RNAi)-induced suppression of nicotine demethylase activity reduces levels of a key carcinogen in cured tobacco leaves. Plant Biotechnol J, v. 6, n. 4, p. 346-54, May 2008. ISSN 1467-7652. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/18282175>.

_____. Impact of alleles at the Yellow Burley (Yb) loci and nitrogen fertilization rate on nitrogen utilization efficiency and tobacco-specific nitrosamine (TSNA) formation in air-cured tobacco. J Agric Food Chem, v. 60, n. 25, p. 6454-61, Jun 2012. ISSN 1520-5118. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/22676549>.

LIEDSCHULTE, V. et al. Identification of CYP82E21 as a functional nicotine N-demethylase in tobacco flowers. Phytochemistry, Sep 2016. ISSN 1873-3700. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/27596288>.

LIM, P. O.; KIM, H. J.; NAM, H. G. Leaf senescence. Annu Rev Plant Biol, v. 58, p. 115-36, 2007. ISSN 1543-5008. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/17177638>.

LOCHMANN, H. et al. Determination of tobacco alkaloids in single plant cells by capillary electrophoresis. J Chromatogr A, v. 917, n. 1-2, p. 311-7, May 2001. ISSN 0021-9673. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/11403483>.

PEEK, D. R. et al. Burley tobacco production guide. Harvesting, curing, stripping, and marketing. TECH, V. Petersburg: Virginia Polythechnic Institute and State University 2008.

REED, T. D. Flue-cured tobacco production guide. Curing tobacco. TECH, V. Petersburg: Virginia Polytechnic Institute and State University: 61-64 p. 2008.

ROTON, C. de; WIERNIK, A.; WAHLBERG, I.; VIDAL, B. Factors influencing the formation of Tobacco-Specific Nitrosamines in French air-cured tobaccos in trials and at the farm level. Contributions to Tobacco Research, v. 21, n. 6, p. 305-320, Jul 2005.

SHI, H. et al. Changes in TSNA contents during tobacco storage and the effect of temperature and nitrate level on TSNA formation. J Agric Food Chem, v. 61, n. 47, p. 11588-94, Nov 2013. ISSN 1520-5118. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/24188376>.

SHI, Q.; LI, C.; ZHANG, F. Nicotine synthesis in Nicotiana tabacum L. induced by mechanical wounding is regulated by auxin. J Exp Bot, v. 57, n. 11, p. 2899-907, 2006. ISSN 0022-0957. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/16868042>.

SHIBATA, F. et al. Tobacco karyotyping by accurate centromere identification and novel repetitive DNA localization. Chromosome Res, v. 21, n. 4, p. 375-81, Jul 2013. ISSN 1573-6849. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/23700277>.

SHITAN, N. et al. Involvement of the leaf-specific multidrug and toxic compound extrusion (MATE) transporter Nt-JAT2 in vacuolar sequestration of nicotine in Nicotiana tabacum. PLoS One, v. 9, n. 9, p. e108789, 2014. ISSN 1932-6203. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/25268729>.

SIERRO, N. et al. Reference genomes and transcriptomes of Nicotiana sylvestris and Nicotiana tomentosiformis. Genome Biol, v. 14, n. 6, p. R60, 2013. ISSN 1474-760X (Electronic)
1474-7596 (Linking). Disponível em: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23773524>.

SIMINSZKY, B. et al. Conversion of nicotine to nornicotine in Nicotiana tabacum is mediated by CYP82E4, a cytochrome P450 monooxygenase. Proc Natl Acad Sci U S A, v. 102, n. 41, p. 14919-24, Oct 2005. ISSN 0027-8424. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/16192354>.

SINDITABACO. Tobacco in Southern Brazil. Santa Cruz do Sul, p.17. 2017

SUN, B. et al. A Quantitative Real-Time PCR-Based Strategy for Molecular Evaluation of Nicotine Conversion in Burley Tobacco. Int J Mol Sci, v. 16, n. 11, p. 27422-32, Nov 2015. ISSN 1422-0067. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/26593897>.

TSO, T.-C. Production, Physiology and Biochemistry of Tobacco Plant. Beltsville: Ideals, 1990. 785.

VIEGAS, C. A. Noncigarette forms of tobacco use. J Bras Pneumol, v. 34, n. 12, p. 1069-73, Dec 2008. ISSN 1806-3756. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/19180343>.

XI, X. Y.; LI, C. J.; ZHANG, F. S. Nitrogen supply after removing the shoot apex increases the nicotine concentration and nitrogen content of tobacco plants. Ann Bot, v. 96, n. 5, p. 793-7, Oct 2005. ISSN 0305-7364. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/16100227>.

XU, D. et al. Biochemical and molecular characterizations of nicotine demethylase in tobacco. Physiologia Plantarum, v. 129, p. 307-319, 2007. ISSN 0031-9317.