Universidade Estadual de Londrina

LEANDRO DATOLA TULLIO

ESTUDO DE GENES ENVOLVIDOS NA SÍNTESE DE FITORMÔNIOS E NA NODULAÇÃO DE *RHIZOBIUM TROPICI*

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Tese de Doutorado apresentada ao programa de Pós-Graduação em Biotecnologia da Universidade Estadual de Londrina, como requisito parcial para obtenção do título de Doutor em Biotecnologia.
Orientadora: Dra. Mariangela Hungria
Co-orientadora: Profª. Dra. Jesiane S. S. Batista

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TULLIO, Leandro Datola. **Estudo de genes envolvidos na síntese de fitormônios e na nodulação em *Rhizobium tropici***. 2019. 63f. Tese de Doutorado (doutorado em Biotecnologia) – Universidade Estadual de Londrina, Londrina, 2019.

RESUMO

O feijoeiro comum (*Phaseolus vulgaris* L.) é uma cultura de grande importância econômica e social, principalmente em países menos desenvolvidos. Embora o rendimento mundial do feijoeiro seja baixo, aumentos significativos têm sido obtidos por meio da inoculação com rizóbios, como a estirpe *Rhizobium tropici* CIAT 899, que é autorizada para o uso de inoculantes comerciais no Brasil. Este trabalho teve por objetivo estudar a relação do fitormônio ácido indol-3-acético (AIA) e de fatores de nodulação (fatores Nod), produzidos por *R. tropici* CIAT 899, em parâmetros simbióticos com o feijoeiro. Assim, procedeu-se à mutação dos genes *y4wF* e *tidC*, envolvidos com a síntese de AIA e genes responsáveis por modificações nos fatores Nod (*hsnT*, *nodF* e *nodE*). No Artigo A, estirpes mutadas nos genes *y4wF* e *tidC* aumentaram a síntese de exopolissacarídeos, atrasaram a formação dos nódulos, mas melhoraram a competitividade das estirpes. A presença de apigenina, flavonoide indutor dos genes de nodulação de *R. tropici*, foi necessária para ativar as vias da triptamina e do ácido indol-3-pirúvico, particularmente nas mutantes e uma forte indução dos genes *y4wF* e *tidC* foi observada, tanto na estirpe selvagem, quanto nas mutantes. Os resultados revelaram uma intrigante relação entre o metabolismo do AIA e a atividade indutora de genes de nodulação em CIAT 899. As vias biossintéticas de AIA foram discutidas e, com base nos resultados, foram atribuídas funções aos genes *y4wF* e *tidC*. No Artigo B, a expressão dos genes *hsnT*, *nodF* e *nodE* foi fortemente induzida na presença de apigenina e de sal e, em menor proporção, na ausência dos reguladores transcricionais *nodD*, sendo o NodD1 reconhecido como o principal regulador. Vinte e nove diferentes fatores Nod estruturalmente diferentes foram sintetizados por CIAT 899 induzida por apigenina e 36 quando induzida por sal, sendo drasticamente reduzidos por mutações em *hsnT*, *nodF* e *nodE*, em adição a mudanças estruturais específicas relacionadas a cada gene. Mutações nos três genes afetaram diferencialmente o desempenho simbiótico, de acordo com a planta hospedeira. Conclui-se que, embora não pertençam ao grupo principal de genes reguladores da nodulação, os genes *hsnT*, *nodF* e *nodE* contribuem para a síntese de fatores Nod, que impactam o desempenho simbiótico e a especificidade hospedeira.

Palavras-chave: Fixação Biológica de Nitrogênio. Exopolissacarídeos. AIA. Fatores de nodulação. Feijoeiro. *Phaseolus vulgaris*.

TULLIO, Leandro Datola. **Estudo de genes envolvidos na síntese de fitormônios e na nodulação em *Rhizobium tropici***. 2019. 63p. Tese de Doutorado (doutorado em Biotecnologia) – Universidade Estadual de Londrina, Londrina, 2019.

ABSTRACT

The common bean (*Phaseolus vulgaris* L.) is an important economical and social crop, mainly in less developed countries. Although the average yeld of this legume is low, significant improvement has been achieved with the inoculation with rhizobia, such as with *Rhizobium tropici* strain CIAT 899, used in commercial inoculants in Brazil. This study had the objective of studying the effects of indole-3-acetic acid (IAA) and of nodulation factors (Nod factors) produced by *R. tropici* CIAT 899 on symbiotic parameters with common bean. For that, the genes *y4wF* and *tidC*, involved in IAA biosynthesis, and *hsnT, nodF* and *nodE*, responsible for modifying the Nod factors were mutated. In Article A, strains mutated in the *y4wF* and *tidC* genes increased the synthesis of exopolysaccharide and delayed nodule formation, but strains competitiveness were increased. Apigenin, a flavonoid inducer of the nodulation genes of *R. tropici* was required for the activation of the tryptamine and indole-3-pyruvic acid pathways, particularly in the mutants, and a strong induction of *y4wF* and *tidC* genes was observed both in wild-type and the mutant CIAT 899 strains. The results revealed an intriguing relationship between IAA metabolism and the induction of nodulation genes in CIAT 899. The biosynthetic pathways of IAA were discussed, and based on the results obtained, we attributed functions to the *y4wF* and *tidC* genes. In Article B, the expression of *hsnT*, *nodF* and *nodE* genes was strongly induced in the presence of apigenin and salt, and at a lower level, in the absence of *nodD* transcriptional regulators, of which NodD1 is the main regulator. Twenty-nine Nod factors, structurally different, were synthesized by CIAT 899 when induced by apigenin, and 36 when induced by salt, being drastically decreased by mutations in *hsnT*, *nodF* and *nodE*, in addition to specific structural modifications related to each gene. Mutations in the three genes have resulted in different effects on the symbiotic performance, according to the host plant. We may conclude that although the *hsnT*, *nodF* and *nodE* genes are not included in the set of the main regulatory genes of nodulation, they contribute to the synthesis of Nod factors, impacting the symbiotic performance and host specificity.

Keywords: Biological Nitrogen Fixation. exopolysaccharides. IAA. nodulation factors. *Phaseolus vulgaris*.

LISTA DE ILUSTRAÇÕES

Figura 1 - Estrutura central de um fator de nodulação e posições de suas modificações mais comuns..14

Figura 2 - Processo de infecção, mediado por moléculas flavonoides e fatores Nod, durante a organogênese do nódulo, em leguminosa...15

Figura 3 - Representação esquemática dos genes *nodD* e sua vizinhaça no genoma de *Rhizobium tropici* CIAT 899. Estão representados os genes *nod* reguladores transcricionais (preto), sintetizadores de fatores Nod (cinza claro) e genes envolvidos com o controle dos níveis de fitormônios (cinza escuro); localizados no plasmídeo simbiótico B de *R. tropici* CIAT 899 (ORMEÑO-ORRILLO et al., 2012)..17

Figura 4 - Vias dependentes de triptofano para a biossíntese de AIA em *R. tropici* CIAT 899. Linhas pontilhadas representam possíveis etapas de acordo com genes identificados no genoma desta estirpe (ORMEÑO-ORRILLO et al., 2012)..20

SUMÁRIO

1. Introdução 10
2. Revisão de literatura 12
2.1. Fixação Biológica de Nitrogênio 12
2.2. Nodulação 13
2.3. O grupo "*Rhizobium tropici*" e a estirpe CIAT 899 15
2.4. Genes de nodulação em *Rhizobium tropici* 17
2.5. Fatores de nodulação de *Rhizobium tropici* 19
2.6. A síntese de AIA e a estirpe *R. tropici* CIAT 899 20
3. Referências 22
4. Objetivos 29
4.1. Objetivos específicos 29
5. Artigo A – Revelando os papéis dos genes *y4wF* e *tidC* na biossíntese de compostos indólicos e o impacto nas propriedades simbióticas de *Rhizobium tropici* CIAT 899 30
6. Artigo B – Regulação dos genes *hsnT*, *nodE* e *nodF* em *Rhizobium tropici* CIAT 899 e seus papéis na síntese de fatores Nod e na simbiose 48
8. Considerações finais 63

1. Introdução

A nutrição vegetal é um dos principais fatores que afetam a produção de alimentos, sendo o nitrogênio (N) o nutriente que, com maior frequência, limita a produção de grãos. O fornecimento de formas assimiláveis de N para a planta pode ser realizado por rizóbios, bactérias capazes de estabelecer simbiose mutualística com as plantas, majoritariamente da família Fabaceae (leguminosas), ocupando o interior de estruturas especializadas (os nódulos), onde realizam o processo de Fixação Biológica de Nitrogênio (FBN) e fornecem a amônia produzida diretamente aos tecidos vegetais.

A simbiose equivale a uma fertilização natural, sendo responsável por mais de 65% de todo o N fixado biologicamente em sistemas agrícolas. Dentre as leguminosas de interesse econômico, o feijoeiro comum (*Phaseolus vulgaris* L.) é o terceiro mais importante no mundo e desempenha papel ainda mais relevante em países sul-americanos, africanos, caribenhos e asiáticos onde, muitas vezes, representa a principal fonte de proteínas na dieta da população.

Embora o rendimento mundial do feijoeiro seja baixo, rendimentos superiores a 3.000 kg/ha têm sido obtidos por meio da inoculação com estirpes selecionadas de rizóbios. Uma dessas estirpes é a *Rhizobium tropici* CIAT 899, notável por diversas características desejáveis para inoculantes agrícolas, como sua alta eficiência na FBN com o feijoeiro, capacidade de sintetizar o fitormônio ácido indol-3-acético (AIA), tolerância a estresses abióticos, além de capacidade de nodular diversas outras leguminosas.

A nodulação é um processo que inicia com um diálogo molecular, com a liberação de moléculas indutoras pela planta hospedeira, especialmente flavonoides, na rizosfera, onde essas moléculas são reconhecidas por proteínas específicas (proteínas NodD) de rizóbios compatíveis. Essas proteínas desencadeiam a síntese e a secreção de fatores de nodulação (fatores Nod), que resultam em modificações na planta, levando à formação dos nódulos contendo bacteroides, formas diferenciadas de rizóbios, capazes de fixar N_2.

A estirpe *R. tropici* CIAT 899 apresenta um dos modelos mais complexos de nodulação já descritos, contendo cinco cópias dos genes *nodD*, reguladores transcricionais de um grande número de outros genes de nodulação, relacionados com a síntese e modificação de fatores Nod. Além disso, essa estirpe carrega genes envolvidos na síntese de fitormônios conhecidos por afetar o processo de nodulação.

Recentemente, um estudo de transcriptômica mostrou que genes relacionados à síntese de AIA (*y4wE*, *y4wF* e *tidC*) foram induzidos junto a um grande número de genes *nod* – incluindo *hsnT*, *nodF* e *nodE* - na presença de apigenina (flavonoide de feijoeiro) e de sal (300

mM de NaCl). Com a finalidade de explorar melhor a complexidade do processo de nodulação, principalmente do feijoeiro, por *R. tropici* CIAT 899, foram realizados dois estudos:

No primeiro estudo, foram analisados os genes *y4wF* e *tidC*, envolvidos com a síntese do fitormônio AIA, cuja expressão é significativamente induzida na presença do flavonoide apigenina e de sal. Estirpes mutadas nesses genes apresentaram alterações fenotípicas que afetam o processo de nodulação, a expressão de genes envolvidos na biossíntese de AIA e a produção diferencial de compostos intermediários entre o precursor L-triptofano e AIA. Tais perfis diferenciais permitiram atribuir função aos genes *y4wF* e *tidC*, na biossíntese de AIA e verificar o modo como a mutação desses genes afeta o processo de nodulação do feijoeiro, por CIAT 899.

No segundo estudo, os alvos foram os genes *hsnT*, *nodF* e *nodE* de *R. tropici* CIAT 899, que são expressos na presença do flavonoide apigenina e de sal. Esses genes têm sido relacionados com a especificidade rizóbio-hospedeiro, por modificarem a estrutura dos fatores Nod. A mutação dos genes *hsnT*, *nodF* e *nodE* afetou significativamente a nodulação e a produção de biomassa da maior parte das leguminosas avaliadas e alterou os perfis de fatores Nod produzidos por cada estirpe na presença de apigenina e de sal. A regulação da expressão desses genes também foi analisada em estirpes mutantes dos genes *nodD*, permitindo uma melhor elucidação sobre os principais indutores desses genes.

2. Revisão de literatura

2.1. Fixação Biológica de Nitrogênio em feijoeiro

A nutrição vegetal tem forte impacto na produção de alimentos, sendo o nitrogênio (N) o nutriente que, com maior frequência, limita a produção de grãos (HUNGRIA; VARGAS, 2000). Apesar do N ser o elemento mais abundante na atmosfera terrestre, sua forma molecular (N_2) é inerte para as plantas, que dependem da disponibilidade de formas passíveis de absorção e assimilação. Um dos processos em que o N_2 pode ser convertido em amônia é o da Fixação Biológica de Nitrogênio (FBN), um processo restrito a organismos procarióticos (TAIZ et al., 2017).

Os microrganismos capazes de realizar a FBN, também denominados como diazotróficos, podem estabelecer diferentes tipos de associação com espécies vegetais, podendo ser divididos, basicamente, em: extracelulares, que incluem microrganismos rizosféricos, epifíticos e endofíticos e; intracelulares, que habitam principalmente células no interior de nódulos - estruturas especializadas da raiz - onde estes microrganismos realizam o processo de FBN e estabelecem simbiose mutualística com a planta (PRASAD et al., 2019), fornecendo íons amônio diretamente aos tecidos vegetais que, em troca, fornecem açúcares à bactéria, principalmente para suprir a elevada demanda energética da FBN (JONES et al., 2007).

Os microrganismos diazotróficos capazes de estabelecer simbiose com plantas nos nódulos radiculares são denominados coletivamente de rizóbios, um termo que inclui uma ampla gama de gêneros bacterianos que se associam majoritariamente com plantas da família Fabaceae (=Leguminosae) (WEYENS et al., 2010). A simbiose fornece grande aporte de N à planta (HUNGRIA; CAMPO, 2007), sendo equivalente a uma fertilização natural, razão pela qual plantas da família Fabaceae exibem maior teor proteico do que as demais (BROUGHTON et al., 2003).

Estima-se que as associações rizóbios-leguminosas sejam responsáveis por mais de 65% de todo o N fixado biologicamente em sistemas agrícolas (HERRIDGE; PEOPLES; BODDEY, 2008). Dentre as leguminosas de interesse econômico, o feijoeiro comum (*Phaseolus vulgaris* L.) é o terceiro mais importante no mundo e desempenha papel ainda mais relevante em países sul-americanos, africanos, caribenhos e asiáticos onde, muitas vezes, representa a principal fonte de proteínas na dieta da população (MARTÍNEZ-ROMERO, 2003; FAGERIA; BALIGAR; JONES, 2010).

Apesar da importância econômica e social, o rendimento mundial do feijoeiro é baixo, mesmo em países desenvolvidos (FAGERIA; BALIGAR; JONES, 2010). No Brasil, o rendimento médio aumentou de 558 para 981 kg/ha entre as safras 1997/1998 e 2017/2018 (CONAB, 2018). Porém, rendimentos superiores a 3.000 kg/ha têm sido relatados em campos experimentais utilizando a inoculação com as estirpes de *Rhizobium tropici* CIAT 899 (=SEMIA 4077) e H 12 (=SEMIA 4088) e de *Rhizobium freirei* PRF 81 (=SEMIA 4080) (HUNGRIA et al., 2000; HUNGRIA; CAMPO; MENDES, 2003; PELEGRIN et al., 2009), atualmente autorizadas pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA - Brasil) para o uso em inoculantes comerciais para essa cultura (GOMES; ORMEÑO-ORRILLO; HUNGRIA, 2015). Assim, via FBN, o Brasil tem potencial para aumentar sobremaneira a produção de feijão, com baixo custo e sem a necessidade de avançar sobre áreas de preservação ambiental.

2.2. Nodulação

A nodulação é um processo complexo que, para a grande maioria das leguminosas, começa com a liberação de exsudatos pelas sementes ou raízes contendo, dentre outros compostos, moléculas sinalizadoras, a maioria flavonoides (OLDROYD, 2013; ANDREWS; ANDREWS, 2017). Os flavonoides atuam como moléculas sinalizadoras quimiotáticas, promotoras do crescimento de rizóbios e indutoras de genes de nodulação, cujo reconhecimento depende da compatibilidade com os rizóbios presentes no solo (COOPER, 2007).

No rizóbio, o flavonoide pode ser reconhecido por um regulador transcricional, a proteína NodD, que induz a expressão dos genes de nodulação *nod*, *nol* e *noe*, ligando-se a regiões promotoras conservadas, denominadas caixas de nodulação (*nod boxes*), localizadas próximas a esses genes. Mediante indução, os genes *nod* codificam enzimas responsáveis pela síntese e secreção de oligossacarídeos de lipoquitina (LCO) (Figura 1) pelos rizóbios, comumente denominados fatores de nodulação (fatores Nod) (JIMÉNEZ-GUERRERO et al., 2018). A síntese de fatores Nod, em *R. tropici* CIAT 899, também pode ser iniciada em condição de estresse salino (300 mM NaCl), sem a presença de flavonoides (ESTÉVEZ et al., 2009; GUASCH-VIDAL et al., 2013).

Os fatores Nod são moléculas sinalizadoras, compostas por um esqueleto de *N*-acetil-D-glicosamina (variando de 3 a 6 unidades) e uma cadeia de ácido graxo na posição C-2 do açúcar

não redutor (Figura 1). Os genes *nodC*, *nodB* e *nodA* codificam enzimas responsáveis pela síntese da estrutura básica dos fatores Nod: NodC catalisa a ligação de unidades de *N*-acetil-D-glicosamina; NodB remove o grupo acetil da posição C-2 do açúcar não redutor e; NodA adiciona uma cadeia de ácido graxo na mesma posição. Modificações na cadeia do ácido graxo e a adição de grupos (ex. fucose e sulfato) nos açúcares são comumente observadas (TAIZ et al., 2017).

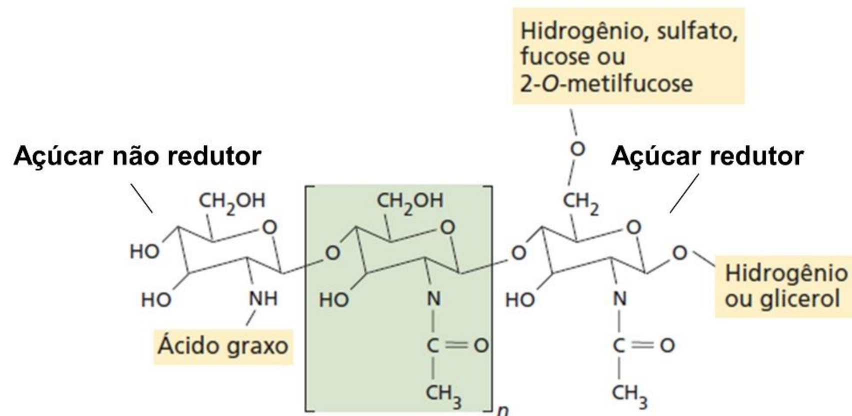

Figura 1 - Estrutura central de um fator de nodulação e posições de suas modificações mais comuns. **Fonte:** adaptado de TAIZ et al. (2017).

Na maioria das leguminosas, os fatores Nod produzidos pelos rizóbios são reconhecidos e induzem modificações nas raízes. Em diversas leguminosas, incluindo o feijoeiro, uma das primeiras modificações é a oscilação nos níveis de cálcio nas células epidérmicas das raízes (Figura 2A). Os pelos radiculares sofrem alongamento, seguido por encurvamento, envolvendo os rizóbios (Figura 2B) e a membrana plasmática do pelo sofre uma invaginação, formando uma cavidade, para o interior da qual os rizóbios crescem, formando o cordão de infecção (Figura 2C). O cordão de infecção se prolonga até as células do córtex da raiz, onde se irradia e os rizóbios são liberados nas células corticais por endocitose, circundados por uma membrana (Figura 2D) (HAAG et al., 2013; OLDROYD, 2013).

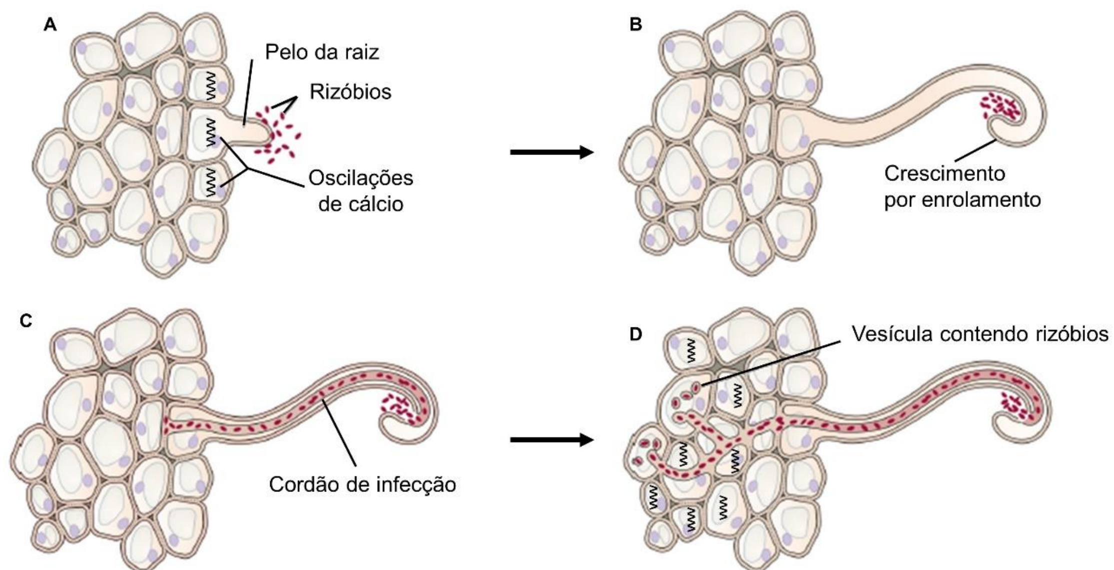

Figura 2 - Processo de infecção, mediado por moléculas flavonoides e fatores Nod, durante a organogênese do nódulo, em leguminosa. **Fonte:** adaptado de TAIZ et al. (2017).

As células do córtex da raiz também sofrem oscilações de cálcio (Figura 2D) e são reativadas à divisão celular, iniciando a formação do primórdio do nódulo. Sucessivas divisões dessas células levam ao desenvolvimento do nódulo, onde os rizóbios se diferenciam em bacteroides, formas capazes de fixar N_2 no nódulo maduro e ativo (HAAG et al., 2013; OLDROYD, 2013).

O processo de nodulação também é controlado por fitormônios, como as auxinas (GUINEL, 2015). Estudos indicam que, alterações em seus níveis, na raiz da planta hospedeira, são um pré-requisito para a formação do nódulo (MATHESIUS, 2008). Alguns rizóbios são capazes de controlar os níveis de fitormônios (PRASAD et al., 2019), como é o caso do ácido indol-3-acético (AIA), uma auxina sintetizada por *R. tropici* CIAT 899 (FIGUEIREDO et al., 2008; IMADA et al., 2017).

2.3. O grupo "*Rhizobium tropici*" e a estirpe CIAT 899

O grupo "*R. tropici*" encontra-se classificado, pela taxonomia atual, como pertencente a Domínio: Bacteria; Filo: Proteobacteria; Classe: Alfaproteobacteria*;* Ordem: Rhizobiales; Família: Rhizobiaceae; Gênero: *Rhizobium* (VELÁZQUEZ et al., 2017). Estirpes deste grupo apresentam a capacidade de metabolizar uma ampla variedade de fontes de carbono e de crescer

sob condições de estresses ambientais, como salinidade, acidez e temperatura elevada (DALL'AGNOL et al., 2014).

Dall’Agnol et al. (2014) indicaram uma grande diversidade metabólica do grupo *R. tropici*; dessa forma, a capacidade de ocupar ambientes nutricionalmente diferentes, além da tolerância a estresses ambientais (GOMES et al., 2012; GOMES; ORMEÑO-ORRILLO; HUNGRIA, 2015; DALL'AGNOL et al., 2014; TULLIO et al., 2019a), devem contribuir para a dominância de estirpes de *R. tropici* na nodulação de *P. vulgaris* nos solos brasileiros, particularmente ácidos (ANDRADE et al., 2002).

Dentre as estirpes mais efetivas em nodulação, FBN e tolerância a estresses ambientais, está a *R. tropici* CIAT 899, notável por diversas características desejáveis para inoculantes agrícolas, como sua alta eficiência na FBN com o feijoeiro, capacidade de sintetizar AIA, competitividade contra rizóbios indígenas, estabilidade genética e tolerância intrínseca a estresses abióticos, como altas temperaturas, acidez e salinidade no solo (FIGUEIREDO et al., 2008; ORMEÑO-ORRILLO et al., 2012; 2016; GUASCH-VIDAL et al., 2013; GOMES; ORMEÑO-ORRILLO; HUNGRIA, 2015; GUERRERO-CASTRO; LOZANO; SOHLENKAMP, 2018).

R. tropici CIAT 899 também apresenta a capacidade de nodular diversas outras leguminosas, como *Leucaena leucocephala* (Lam.) de Wit, *Lotus japonicus* (Regel) Larsen, *Macroptilium atropurpureum* (DC.) Urb., *Lotus burtii* Borsos (DEL CERRO et al., 2015a; 2015b; 2017; GOMES; ORMEÑO-ORRILLO; HUNGRIA, 2015). Tal habilidade pode estar associada ao elevado número de cópias de genes de nodulação, mais especificamente, cinco cópias de *nodD* e três de *nodA* (Figura 3) (ORMEÑO-ORRILLO et al., 2012), bem como à sua capacidade de sintetizar uma ampla variedade de fatores Nod, mesmo sob condições de acidez e salinidade no solo (MORÓN et al., 2005; ESTÉVEZ et al., 2009; GUASCH-VIDAL et al., 2013; DEL CERRO et al., 2015a; 2015b; GOMES et al., esta tese).

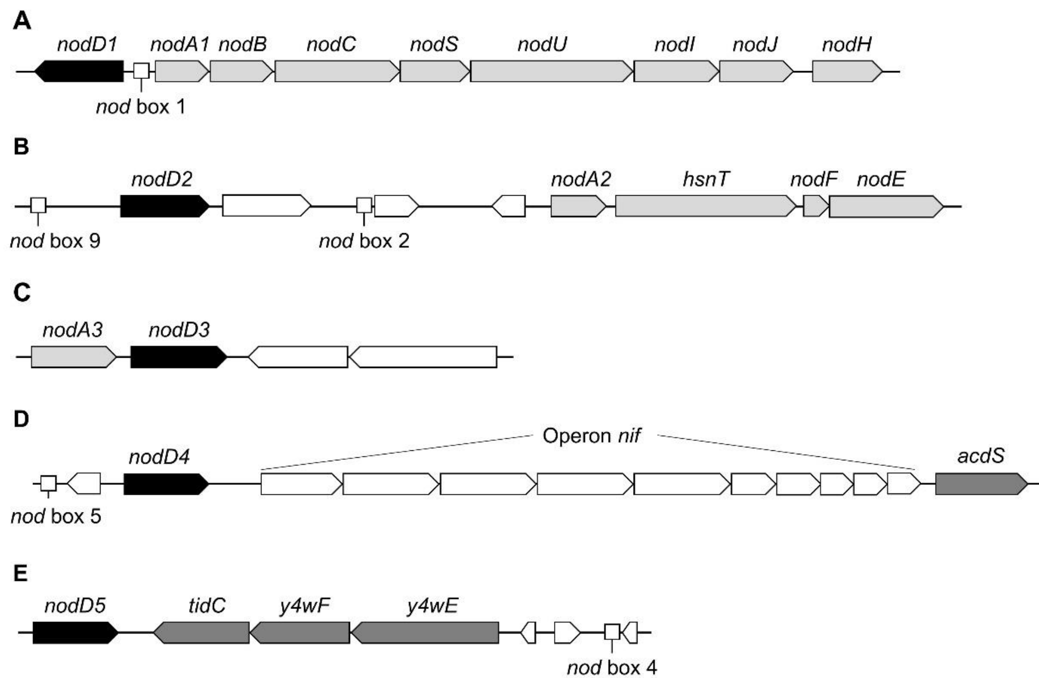

Figura 3 - Representação esquemática dos genes *nodD* e sua vizinhaça no genoma de *Rhizobium tropici* CIAT 899. Estão representados os genes *nod* reguladores transcricionais (preto), sintetizadores de fatores Nod (cinza claro) e genes envolvidos com o controle dos níveis de fitormônios (cinza escuro); localizados no plasmídeo simbiótico B de *R. tropici* CIAT 899 (ORMEÑO-ORRILLO et al., 2012). **Fonte:** o autor.

2.4. Genes de nodulação em *Rhizobium tropici*

Existem, basicamente, dois conjuntos de genes de nodulação, os que atuam como reguladores transcricionais e os que atuam na síntese dos fatores Nod. Vários reguladores transcricionais participam do processo de nodulação, por exemplo, NrcR (DEL CERRO et al., 2016), NolR (VINARDELL et al., 2004; LEE; KRISHNAN; JEZ, 2014), NodD (DEL CERRO et al., 2015a; 2015b; 2017), sendo este último o mais importante (DEL CERRO et al., 2016).

Os genes *nodD* apresentam expressão constitutiva (DUSHA; KONDOROSI, 1993), são passíveis de indução por NaCl ou flavonoides (como apigenina, exsudada pelas raízes do feijoeiro) e estão envolvidos não apenas com a síntese de fatores Nod, mas também determinam a especificidade bactéria/hospedeiro e podem afetar, direta ou indiretamente, características como a motilidade, a produção de exopolissacarídeos (EPS) e a síntese de fitormônios pela bactéria (DEL CERRO et al., 2015a; 2015b; 2017; PÉREZ-MONTAÑO et al., 2014; 2016).

No genoma de CIAT 899 foram encontradas cinco cópias do gene *nodD* (Figura 3), todas localizadas no plasmídeo simbiótico B (pRtrCIAT899b) (ORMEÑO-ORRILLO et al., 2012). Considerando que a indução por apigenina e NaCl é mediada por proteínas NodD, é importante ressaltar que existem nove *nod boxes* no genoma de CIAT 899 (ORMEÑO-ORRILLO et al., 2012), localizadas próximas a grupos gênicos envolvidos em diversos processos, como a síntese de fatores Nod, a síntese de fitormônios e o processo de FBN (ORMEÑO-ORRILLO et al., 2012; TULLIO et al., 2019b).

Estudos realizados em casa de vegetação com mutantes de CIAT 899 para cada um dos genes *nodD* mostraram que somente *nodD1* é essencial para a nodulação de *L. leucocephala, L. japonicus* e *M. atropurpureum*; no entanto, a simbiose com *P. vulgaris* e *L. burtii* não foi bloqueada pela mutação. Tanto a mutação do gene *nodD1*, quanto de *nodD2*, provocaram redução significativa no número de nódulos em *P. vulgaris*, mas somente a mutação nos dois genes aboliu a nodulação em todas as leguminosas, reforçando que existe um sistema complexo de regulação no estabelecimento da simbiose entre CIAT 899 e seus hospedeiros (DEL CERRO et al., 2015a) bem como que o papel dos genes *nodD3*, *nodD4* e *nodD5* pode ser considerado secundário (DEL CERRO et al., 2015a; 2015b; 2017; GOMES; ORMEÑO-ORRILLO; HUNGRIA, 2015),

Embora *nodD1* seja fundamental para a síntese de fatores Nod, sob indução por apigenina, del Cerro et al. (2017) confirmaram que a regulação da síntese de fatores Nod, na condição de estresse salino, é realizada pela proteína NodD2. Além disso, pela primeira vez, uma proteína NodD foi diretamente envolvida na ativação de genes simbióticos sob estresse abiótico.

Estudos também têm relatado que condições de crescimento diferentes afetam o conjunto de fatores Nod produzidos pela estirpe selvagem de *R. tropici* CIAT 899: Morón et al. (2005) observaram que 52 fatores Nod diferentes foram produzidos em pH 4,5 e somente 29 em pH neutro; já sob estresse salino (300 mM de NaCl) houve a síntese de 46 fatores Nod estruturalmente diferentes (ESTÉVEZ et al., 2009). Além disso, CIAT 899 sintetizou 29 e 36 fatores Nod distintos, sob indução por apigenina (3,7 µM) e sal (300 mM de NaCl), respectivamente (GOMES et al., esta tese).

A resposta de *R. tropici* CIAT 899 à indução por apigenina (3,7 µM) e sal (300 mM NaCl) também foi analisada a nível transcricional (PÉREZ-MONTAÑO et al., 2016) e o resultado confirmou os resultados obtidos por del Cerro et al. (2015a; 2017), de que *nodD1* e *nodD2* desempenham os papéis principais no processo de nodulação, sendo NodD1 o principal

regulador sob indução por apigenina (3,7 µM) e NodD2 sob condição de estresse salino (300 mM de NaCl).

O estudo de transcriptoma da estirpe CIAT 899 (PÉREZ-MONTAÑO et al., 2016) também indicou que, na presença de apigenina e NaCl, ao menos dois processos biológicos relevantes são ativados: a síntese de fatores Nod, por meio da indução dos grupos gênicos *nodA1BCSUIJHPQ1Q2* e *nodA2hsnTnodFnodE* e a síntese do fitormônio AIA, por indução dos genes *y4wEy4wFtidC* (ORMEÑO-ORRILLO et al., 2012; PÉREZ-MONTAÑO et al., 2016; TULLIO et al., 2019b).

2.5. Fatores de nodulação de *Rhizobium tropici*

Em *R. tropici* CIAT 899 o gene *nodD1* está localizado próximo ao operon *nodA1BC* (Figura 3A) (ORMEÑO-ORRILLO et al., 2012), responsável pela síntese do esqueleto básico dos fatores Nod (Figura 1) (JABBOURI et al., 1998; OLDROYD, 2013) que, nessa estirpe, é composto por três a cinco unidades de *N*-acetil-D-glicosamina, sendo os substituintes mais comuns o grupo sulfato, no açúcar redutor e os grupos carbamoil, metil e acil, no açúcar não-redutor. Contudo, outras estruturas menos comuns, como ligações insaturadas, presença de grupamentos de fucose, arabinose e sulfato podem aparecer no açúcar redutor (FOLCH-MALLOL et al., 1996; KRISHNAN; CHRONIS, 2008).

Os genes *hsnT, nodF e nodE* (Figura 3B), induzidos por apigenina e por NaCl (PÉREZ-MONTAÑO et al., 2016), devem estar relacionados à decoração dos fatores Nod em CIAT 899. HsnT adiciona ou substitui grupos funcionais ao açúcar redutor (LEROUGE et al., 1990), enquanto *nodE* e *nodF* codificam, respectivamente, enzimas envolvidas com a síntese e alongamento da cadeia de ácidos graxos que são adicionados ao açúcar não redutor do fator Nod (SPAINK et al., 1989; ARDOUREL et al., 1994).

Diferenças entre os fatores Nod têm sido relatadas como determinantes para a especificidade rizóbio-hospedeiro, como em estirpes de *S. meliloti* mutantes do gene *nodF*, que formaram menor número de cordões de infecção e não apresentaram nodulação efetiva com *Medicago sativa* L. (ARDOUREL et al., 1994; KRISHNAN; CHRONIS, 2008). Tendo em vista a importância dos genes *nod* para o processo de simbiose com leguminosas, é fundamental compreender seu funcionamento para alcançar o sucesso no processo de FBN.

2.6. A síntese de AIA e a estirpe *R. tropici* CIAT 899

Embora Figueiredo et al. (2008) tenham mostrado, pela primeira vez, a capacidade de *R. tropici* CIAT 899 sintetizar AIA, as vias de biossíntese utilizadas foram propostas somente por Imada et al. (2017). A importância do AIA para o processo de nodulação de feijoeiro, porém, já havia sido sugerida no estudo de Pérez-Montaño et al. (2016), em que, genes responsáveis pela síntese de AIA foram induzidos junto a uma série de genes de nodulação, tanto na presença de apigenina, quanto na condição de estresse salino.

As vias de biossíntese de AIA podem ser dependentes ou independentes de L-triptofano, ou seja, necessitam ou não da disponibilidade desse aminoácido como precursor. A síntese de AIA por vias independentes de triptofano foi observada em *Arabidopsis thaliana* (L.) Heynh, bem como em *Azospirillum brasilense*, porém, nenhuma enzima específica foi identificada (NONHEBEL, 2015; DI et al., 2016), sendo necessários mais estudos. Dentre as vias dependentes de triptofano (Figura 4), quatro foram previamente relatadas em rizóbios, as quais foram nomeadas de acordo com o intermediário principal: Indol-3-acetamida (IAM), Indol-3-acetonitrila (IAN), ácido Indol-3-pirúvico (IPyA) e Triptamina (TAM) (SPAEPEN; VANDERLEYDEN; REMANS, 2007).

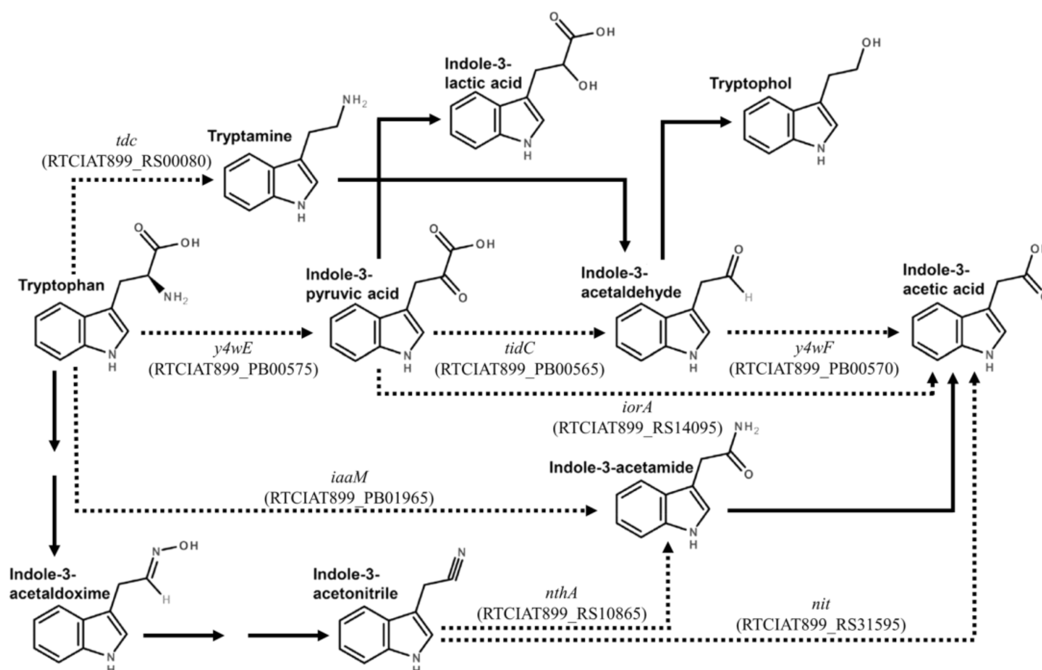

Figura 4 - Vias dependentes de triptofano para a biossíntese de AIA em *R. tropici* CIAT 899. Linhas pontilhadas representam possíveis etapas de acordo com genes identificados no genoma desta estirpe (ORMEÑO-ORRILLO et al., 2012). **Fonte:** IMADA et al. (2017); TULLIO et al. (2019b).

A via do IPyA pode ser encontrada em diversos microrganismos benéficos às plantas, como em representantes dos gêneros *Azospirillum*, *Bradyrhizobium* e *Rhizobium*. Nessa via, o L-triptofano é primeiro convertido em IPyA por uma enzima aminotransferase, sendo descarboxilado a indol-acetaldeído na etapa seguinte. Este, por fim, é oxidado por uma enzima desidrogenase, formando AIA (Figura 4) (SPAEPEN; VANDERLEYDEN, 2011).

Imada et al. (2017) mostraram que a síntese de AIA por *R. tropici* CIAT 899 aumenta significativamente na presença de triptofano, enquanto que, na presença de amônia, sua produção é drasticamente reduzida. A principal via de síntese de AIA utilizada por *R. tropici* CIAT 899 é a via do IPyA, para a qual foi sugerida a existência uma alternativa mais curta, em que a enzima indol piruvato ferredoxina oxidorredutase (IorA) converte IPyA diretamente em AIA (Figura 4). Essa alternativa foi, posteriormente, corroborada por Tullio et al. (2019b).

Enzimas da via do IPyA, em *Sinorhizobium* sp. NGR234, são codificadas pelos genes *y4wE* e *y4wF*, cuja expressão é regulada por flavonoides, envolvendo a proteína NodD e *nod boxes* (THEUNIS et al., 2004). Genes correspondentes foram encontrados no genoma de *R. tropici* CIAT 899, sendo os dois primeiros homólogos e um terceiro desconhecido (Figura 3E) (ORMEÑO-ORRILLO et al., 2012). Nesta tese, a função desses genes foi elucidada; assim, os genes *y4wE, y4wF* e *tidC* (renomeado) de CIAT 899 são responsáveis por cada uma das três etapas da conversão de L-triptofano em AIA (Figura 4) (TULLIO et al., 2019b).

Embora uma *nod box* e o gene *nodD5* estejam localizados na vizinhaça dos genes *y4wE, y4wF* e *tidC* em CIAT 899 (Figura 3E), del Cerro et al. (2015b) mostraram que uma mutação em *nodD5* não afetou a síntese de AIA e que NodD5 é um provável ativador da nodulação via *nodD1* e/ou *nodD2*.

Contudo, a relação entre síntese de fatores de nodulação, de fitormônios e o processo de nodulação do feijoeiro por *Rhizobium tropici* CIAT 899 não estão totalmente elucidados. Portanto, estudos aprofundados são de fundamental importância para melhorar a eficiência de inoculação do feijoeiro, alcançando níveis superiores de produtividade.

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4. Objetivos

Obter e caracterizar mutantes de *Rhizobium tropici* CIAT 899, visando determinar a função de genes envolvidos na nodulação e na biossíntese de fitormônios, verificando seus papéis no estabelecimento da simbiose com feijoeiro (*Phaseolus vulgaris*) e com outras leguminosas modelo.

4.1. Objetivos específicos:

- Determinar as funções dos genes *y4wF* e *tidC* de *R. tropici* CIAT 899 na biossíntese de ácido indol-3-acético (AIA) e em propriedades simbióticas com *P. vulgaris*.
- Analisar as funções dos genes *hsnT, nodF* e *nodE* de *R. tropici* CIAT 899 na síntese de fatores Nod e na especificidade com leguminosas hospedeiras.

5. Artigo A – Revelando os papéis dos genes *y4wF* e *tidC* na biossíntese de compostos indólicos e o impacto nas propriedades simbióticas de *Rhizobium tropici* CIAT 899

Resumo: A estirpe *Rhizobium tropici* CIAT 899 é conhecida por sua capacidade de nodular uma ampla gama de espécies leguminosas, por sintetizar grande variedade de fatores Nod, por sua tolerância a estresses abióticos e sua capacidade de fixar N_2 atmosférico, especialmente em simbiose com feijoeiro (*Phaseolus vulgaris* L.). Genes possivelmente relacionados à síntese de ácido indol-3-acético (AIA) foram encontrados no plasmídeo simbiótico de CIAT 899, próximos ao gene regulador *nodD5* e, neste estudo, foram obtidos mutantes de dois destes genes, *y4wF* e *tidC* (*R.* ***t****ropici* ácido **i**ndol-3-pirúvico **d**es**c**arboxilase), cuja expressão foi investigada na presença e na ausência de triptofano (TRP) e apigenina (API). Em geral, as mutações dos genes incrementaram a síntese de exopolissacarídeos (EPS), mas não a motilidade; houve atraso na formação dos nódulos, porém, melhoria na competitividade da estirpe. Foi observado que a via indol-3-acetamida (IAM) é ativa em CIAT 899 e não foi afetada pelas mutações; além disso, a apigenina foi necessária para ativar as vias da triptamina (TAM) e do ácido indol-3-pirúvico (IPyA), particularmente nas estirpes mutantes. Uma forte indução dos genes *y4wF* e *tidC* foi observada na presença de apigenina, tanto na estirpe selvagem, quanto nas mutantes. Os resultados revelaram uma intrigante relação entre o metabolismo de AIA e a atividade indutora de genes *nod* em *R. tropici* CIAT 899. As vias biossintéticas de AIA foram discutidas e, com base nos resultados, foram atribuídas funções aos genes *y4wF* e *tidC* de *R. tropici* CIAT 899.

Palavras-chave: Fixação Biológica de Nitrogênio, Genes de nodulação, Fitormônios, AIA, IPyA, Exopolissacarídeos.

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ORIGINAL PAPER

Revealing the roles of *y4wF* and *tidC* genes in *Rhizobium tropici* CIAT 899: biosynthesis of indolic compounds and impact on symbiotic properties

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Abstract
Rhizobium tropici CIAT 899 is a strain known by its ability to nodulate a broad range of legume species, to synthesize a variety of Nod factors, its tolerance of abiotic stresses, and its high capacity to fix atmospheric N_2, especially in symbiosis with common bean (*Phaseolus vulgaris* L.). Genes putatively related to the synthesis of indole acetic acid (IAA) have been found in the symbiotic plasmid of CIAT 899, in the vicinity of the regulatory nodulation gene *nodD5*, and, in this study, we obtained mutants for two of these genes, *y4wF* and *tidC* (*R*. ***t****ropici* **i**ndole-3-pyruvic acid **dec**arboxylase), and investigated their expression in the absence and presence of tryptophan (TRP) and apigenin (API). In general, mutations of both genes increased exopolysaccharide (EPS) synthesis and did not affect swimming or surface motility; mutations also delayed nodule formation, but increased competitiveness. We found that the indole-3-acetamide (IAM) pathway was active in CIAT 899 and not affected by the mutations, and—noteworthy—that API was required to activate the tryptamine (TAM) and the indol-3-pyruvic acid (IPyA) pathways in all strains, particularly in the mutants. High up-regulation of *y4wF* and *tidC* genes was observed in both the wild-type and the mutant strains in the presence of API. The results obtained revealed an intriguing relationship between IAA metabolism and *nod*-gene-inducing activity in *R. tropici* CIAT 899. We discuss the IAA pathways, and, based on our results, we attribute functions to the *y4wF* and *tidC* genes of *R. tropici*.

Keywords Biological nitrogen fixation · Nodulation genes · Phytohormones · IAA · IPyA · Exopolysaccharides

Abbreviations

API	Apigenin
EPS	Exopolysaccharides
IAA	Indole acetic acid
IAM	Indole-3-acetamide
IAN	Indole-3-acetonitrile
IPyA	Indol-3-pyruvic acid
LCOs	Lipochitooligosaccharides
TAM	Tryptamine
TRP	Tryptophan
YM	Yeast-extract mannitol medium

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Introduction

Rhizobia are a group of α- and β-proteobacteria able to establish symbioses with several leguminous species (Gopalakrishnan et al. 2015). The rhizobium–legume interaction is initiated with the exchange of molecular signals: the host plant secretes inducing molecules——mainly flavonoids——that are recognized by a LysR-type transcriptional regulator in the bacterial partner, the NodD protein. This protein binds to specific sequences, named *nod*-boxes, which are located upstream of the *nod* operon. The *nod*-encoded enzymes are implied in the production of lipochitooligosaccharides (LCOs), also known as Nod factors, which, in turn, induce the formation of root-nodule primordia and play essential roles in the legume root-infection by the rhizobia (Hungria et al. 1992; Hungria and Phillips 1993; Oldroyd 2013; Janczarek et al. 2015). With the formation of nodules, specific conditions are provided to the bacteria that allow the establishment of the N_2-fixation process (Haag et al. 2013).

Another important feature of many rhizobia relies on the synthesis of phytohormones (Mathesius 2008), with an emphasis on auxins, in particular indole acetic acid (IAA) (Spaepen et al. 2007a, b). Interestingly, in *Rhizobium leguminosarum* 248 (Prinsen et al. 1991), *Sinorhizobium* (= *Ensifer*) *meliloti* AK631, and in *Sinorhizobium fredii* strains NGR234 (Theunis et al. 2004) and HH103 (Vinardell et al. 2015; Pérez-Montaño et al. 2016b), the synthesis of IAA has been reported to be induced by flavonoids. Reports describe the involvement of IAA in nodulation, including nodule initiation and differentiation, vascular-bundle formation, and control of nodule number (Mathesius 2008). Moreover, auxin accumulation has been reported in early stages of nodule development in *Medicago truncatula* (van Noorden et al. 2007), *Lotus japonicus* (Suzaki et al. 2012), and soybean (*Glycine max*) (Turner et al. 2013). Interestingly, mutants of *S. meliloti* overproducing IAA are able to increase nodulation in *Medicago* (Pii et al. 2007), whereas, in *R. leguminosarum* bv viciae strain LPR1105, overproduction of IAA reduced nodule number, but the nodules were heavier, and with enlarged and more-active meristems; in addition, a significant increase in acetylene reduction activity was measured in nodules elicited in vetch (*Vicia sativa*) by the overproducing mutant (Camerini et al. 2008). Noteworthy, the results of Donoso et al. (2016) indicate interactions of IAA with other pathways, of acetyl-CoA carboxylase (ACCase) and homoserine lactones, altogether affecting plant colonization.

IAA biosynthesis in rhizobia can occur through different tryptophan (TRP)-dependent pathways, named accordingly to their intermediates molecules, four of them as follows: indole-3-acetamide (IAM), indole-3-pyruvic acid (IPyA), indole-3-acetonitrile (IAN), and tryptamine (TAM) (Spaepen and Vanderleyden 2011). The IPyA pathway represents the main route of IAA biosynthesis in plants, plant pathogens, and plant-growth-promoting bacteria (Spaepen and Vanderleyden 2011), including *Rhizobium tropici* strain CIAT 899, which has an alternative shorter IPyA pathway (Imada et al. 2017).

Rhizobium tropici CIAT 899 is a promiscuous strain, able to establish effective N_2-fixing nodules with several legume species, including common bean (*Phaseolus vulgaris*), *Leucaena* spp., *Lotus japonicus, Lotus burttii*, and *Gliricidia* spp. (Gomes et al. 2015; del Cerro et al. 2015a). The promiscuity of CIAT 899 might be associated with its carriage of five copies of the regulatory *nodD*, and of synthesizing a variety of Nod factors (del Cerro et al. 2015a, b). The complete sequencing of the genome of *R. tropici* CIAT 899 suggested that the genes of biosynthesis of IAA via the IPyA pathway are located in the symbiotic plasmid, composed by three genes (Supplementary Fig. S1), close to *nodD5* (Ormeño-Orrillo et al. 2012). In *R. tropici* CIAT 899, the genomic organization resembles that of *y4wEFG* of *S. fredii* NGR234, under the control of NodD protein (Theunis et al. 2004); *y4wE* and *y4wF* show 73% and 70% identities with the genes of NGR234, respectively, but with no identity in the third gene, which codes for a hypothetical protein containing the domain DUF4168 (unknown function), and that we will describe in this study as being *tidC* (*R.* ***t****ropici* **i**ndole-3-pyruvic acid **dec**arboxylase) gene.

Previously, we showed that IAA synthesis in CIAT 899 is not affected by a mutation in *nodD5*, but that mutations in *nodD1* and *nodD2* resulted in lower IAA production (del Cerro et al. 2015a, b). In addition, it has been shown that *nodD5* could be an activator of *nodD1* or *nodD2* in the presence of salt, promoting the expression of genes involved in IAA biosynthesis (del Cerro et al. 2015a, b). Noteworthy, increased IAA levels and of Nod factors were observed in CIAT 899 grown in cultures supplemented with API and salt (del Cerro et al. 2015b; Pérez-Montaño et al. 2016a), suggesting a linkage between IAA and the symbiosis.

Mutations in the *y4wEF* and in *tidC* genes of CIAT 899 and the characterization of the mutants may provide a better understanding of the relationship between the IAA metabolism and the symbiosis. Therefore, to improve our understanding of these genes and of how they affect the symbiotic properties of CIAT 899, we obtained and characterized mutants of *y4wF* and *tidC* genes. By mutating these two genes, we also confirmed the feasibility of the alternative route of IAA synthesis of CIAT 899 recently suggested by Imada et al. (2017), going straight from IPyA to IAA.

Materials and methods

Bacteria growth conditions, plasmids, and mutants

Rhizobium tropici CIAT 899 was pre-cultured in 10 mL of tryptone-yeast extract medium (TY) (Beringer 1974) at 28 °C and supplied with 100 µg/mL of rifampicin (Rif) and spectinomycin (Spc) for the derivative strains. Rhizobial strains were pre-cultured on an orbital shaker (180 rpm) for 24 h at 28 °C, washed twice in saline solution (0.85% NaCl), and re-suspended in the specific medium of each assay. Whenever necessary, culture media were supplemented with 1 mg/mL of tryptophan (TRP) or apigenin (API) (final concentration of 3.7 µM). *Escherichia coli* strains were cultured in Luria Bertani broth medium (LB) (Sambrook et al. 1989) at 37 °C with the addition of 30 µg/mL of kanamycin (Km) when necessary.

To obtain the mutants of *y4wF* (RTCIAT899_PB00570) and *tidC* (RTCIAT899_PB00565) genes, the following steps were used: Primers *y4wF*-like-F (5′–GGAGACAAT CGAATGGGAA) and *y4wF*-like-R (5′–CGTTTAGCA TCCAACTGGA) were used for the amplification of the *y4wF* gene, while primers *tidC*-F (5′–TATCATGATCGC CTCGCTA) and *tidC*-R (5′–CGTGCCAAGATTGTAGGA) were used for the amplification of the *tidC* gene. Each of the PCR products from *y4wF* (1,109-bp) and *tidC* (629-bp) genes were cloned into pGEM-T Easy (PROMEGA) (Amp^R 100 µg/mL). Fragment containing the gene *y4wF* was excised with the enzymes *Nco*I + *Sal*I and treated with the Klenow enzyme to convert the sticky ended restriction digest product to blunt ended. Fragment containing the *tidC* gene was excised with *Eco*RI. DNA fragments containing *y4wF* and *tidC* were cloned in the rhizobial suicide vector pK18mob (Schäfer et al. 1994), previously digested with *SmaI* or *Eco*RI to clone *y4wF* and *tidC* genes, respectively. This vector confers resistance to kanamycin (km^R 30 µg/mL). The resulting plasmids were digested with the enzymes *Ale*I and *Ssp*I, that cut in one site *y4wF* and *tidC*, respectively, and were ligated to a *Sma*I 2-kb fragment containing the Ω interposon (Spc^R, 100 µg/mL) (Prentki and Krisch 1984). The generated plasmids pK18mob containing *y4wF*::Ω (3.11-kb) and *tidC*::Ω (2.63-kb) were transferred from the *E. coli* strain DH5α to *R. tropici* CIAT 899 by conjugation, as described by Simon (1984), using the plasmid pRK2013 as helper (Figurski and Helinski 1979). The mutation events were confirmed by PCR.

Rhizobium tropici CIAT 899 and its derivative strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection # 1054), Londrina, Brazil, and also at the Department of Microbiology, University of Seville, Spain.

Total indolic compounds

The production of total indolic compounds by *R. tropici* CIAT 899 was evaluated in two experiments. In the first experiment, CIAT 899 and the mutant strains were assayed in YM + TRP, and in the second in YM + TRP + API media. Bacteria were incubated on an orbital shaker (180 rpm) at 28 °C until exponential growth phase (O.D. 0.8–0.85), or sampled after 48, 72, and 96 h for colorimetric and UPLC assays.

To perform quantification of indolic compounds by the colorimetric method, the supernatants were mixed with Salkowski reagent (Gordon and Weber 1951) in a 1:4 (v/v) ratio, followed by incubation for 30 min in the dark and determination of absorbance at 530 nm. A standard curve was constructed using synthetic indole-3-acetic acid (IAA) (Sigma® Aldrich), and the regression equation obtained was used to estimate the auxin concentration in the supernatants.

These experiments, as well as all others here described, were performed with three biological replicates, each with three technical replicates. For all evaluations except for RT-qPCR, data were analyzed statistically using the ANOVA test ($p < 0.05$), using the software Statistica 7.0.

Auxin production evaluated by UPLC analysis

For the determination and the quantifications of indolic compounds, aliquots of 20 mL of bacterial cultures grown in YM + TRP and YM + TRP + API media until the exponential phase (O.D. 0.8–0.85) were collected and centrifuged for 10 min at 10,000 rpm, to obtain the supernatants. Metabolites were extracted using the solid-phase extraction (SPE) cartridge Strata-X® (500 mg, 6 mL, Phenomenex), according to the following procedure: the cartridge was activated with 20 mL methanol and equilibrated with 1 mL of HCl 0.05 M. After loading 20 mL of supernatant, the cartridge was washed with 10 mL of HCl 0.05 M, and the metabolites were eluted with 5 mL methanol and stored at − 80 °C.

Separation of indolic compounds was carried out by UPLC with photodiode array (PDA) detection on a Waters Acquity UPLC H-Class instrument, consisting of a quaternary solvent manager, FTN sample manager, and PDA detector. The separations were performed using a Waters Acquity 1.7 µm Ethylene Bridged Hybrid (BEH) C18 column (50 × 2.1 mm), in an isocratic system for 8 min, with a mobile phase of 50% solvent A (12.5% methanol, pH 5.3), and 50% solvent B (50% methanol, pH 4.9) to separate tryptophan (TRP) and tryptamine (TAM). To separate indole-3-acetic acid (IAA), indole-3-pyruvic acid (IPyA), indole-3-acetamide (IAM), indole-3-acetonitrile (IAN), indole-3-butyric acid (IBA), and indole-3-lactic acid (ILA), an isocratic system with a mobile phase of 80% solvent A and 20% solvent C (50% methanol pH 2.8) was employed

for 7 min. Both methods were performed with an injection volume of 0.1 µL of the sample at a flow rate of 0.4 mL/min at 30 °C. The system was calibrated using external standards of each indolic compound (98% purity or superior). The spectra were obtained between 200 and 400 nm. The Empower 3 software from Waters was used for data acquisition and processing.

Analysis of exopolysaccharides

The standard anthrone-H_2SO_4 assay was used to determine the total carbohydrate of exopolysaccharides (EPS) contained in the supernatants of the bacterial cultures, evaluated as glucose (Morris 1948). Pre-cultured strains were adjusted to 0.40 to 0.46 (O.D.$_{600\ nm}$) in 5 mL of liquid B^- medium (control) (mannitol, 10 g/L; $MgSO_4$•$7H_2O$, 0.55 g/L; KNO_3, 0.55 g/L; $Ca(NO_3)_2$•$4H_2O$, 1.3 g/L; Fe(III)-NaEDTA, 33 mg/L; biotin, 0.2 mg/L; thiamine•HCl, 5 mg/L; $MnSO_4$•H_2O, 0.609 g/L; $ZnSO_4$•$7H_2O$, 0.1 g/L; H_3BO_3, 1.27 g/L; $NaMoO_4$•$2H_2O$, 0.4 g/L; $CuSO_4$, 0.04 g/L; K_2HPO_4 pH 7.2, 0.1742 g/L) (Spaink et al. 1992), B^- + TRP medium, B^- + API and B^- + TRP + API medium. Cultures were incubated at same conditions for 48 h (O.D. 0.4–0.45). At 48 h, 1-mL samples were centrifuged (14,000 rpm, 5 min), and the supernatants were assayed for EPS content via sulfuric acid hydrolysis in the presence of the colorimetric indicator anthrone (Tomlinson et al. 2010).

Motility assays

Swimming and surface motility phenotypes were assayed in TY medium without antibiotics. Aliquots of 2 mL from each culture were washed twice and re-suspended in 500 µL of TY medium, from which 5 µL was used as inoculum in swimming assays (0.28% agar), or drop-inoculated on the surface for the surface motility assays (0.4% agar) into Petri dishes. The motility of strains was evaluated in TY (control), TY + TRP, TY + API, and TY + TRP + API media. The inoculated plates were wrapped with parafilm, incubated for 96 h at 28 °C in up-right position, and the halo diameters were measured after 24 h.

Nodulation assays

For the evaluation of the symbiotic phenotypes, rhizobial strains were cultured in modified yeast-extract mannitol medium (YM) (Hungria et al. 2016), and incubated on a shaker (150 rpm), at 28 °C, until the concentration of 10^9 cells/mL was reached. *Phaseolus vulgaris* seeds were surface-sterilized (Vincent 1970), and pre-germinated for 3 days at 25 °C. Pre-germinated seeds were then transferred to sterilized pouch bags containing Fåhraeus N-free solution and inoculated with 1 mL of bacterial culture. Common beans have the determinate-type nodule, and in our study nodulation kinetics was evaluated by the presence of nodule primordia (nodules not fully developed, with growth starting from the root segments), and nodules fully developed. Nodulation was verified from the 4th to 10th day after inoculation. Growth conditions were of 16 h at 26 °C in the light and 8 h and 18 °C in the dark, with 70% of humidity.

The same procedures of germination and growth were taken for the competitiveness experiment, except for that the inoculation was performed with 1 mL of each bacterial culture, or with 1 mL of a mix (1/1, v/v, at the concentration of 10^5 cells/mL for each strain) of two bacterial cultures. Nodule occupancy in the competitiveness assays between CIAT 899 and the mutant strains was verified in 80 nodules from eight plants (10 nodules per plant), 40 from plants inoculated with *y4wF*::Ω, and 40 with plants inoculated with the *tidC*::Ω strain. Nodules were surface-sterilized (Vincent 1970) and placed on TY plates to confirm that they were free of surface contaminants. The nodules were crushed, streaked and grown in TY with and without antibiotic (Spc, 100 µg/mL) at 28 °C, for 2–3 days. Isolated colonies were independently picked on TY and TY supplemented with Spc to discriminate the wild-type (Spc^S) and the *y4wF*::Ω or *tidC*::Ω mutant strains (Spc^R).

RNA isolation, cDNA synthesis, and RT-qPCR

Rhizobium tropici CIAT 899 and its derivative strains were cultured in YM + TRP and YM + TRP + API on an orbital shaker (180 rpm), at 28 °C, until exponential growth phase (O.D. 0.8–0.85). Total RNA was extracted, as described before (Gomes et al. 2014). The total concentration of the purified RNA was estimated in a NanoDrop ND 1000 spectrophotometer (NanoDrop-Technologies, Inc.), and the integrity was assessed in a 1% (*w*/*v*) agarose gel. Extracted RNA samples were treated with DNAseI (Invitrogen/Life Technologies), and the first strand of cDNA was synthesized using SuperscriptIII™ reverse transcriptase (Invitrogen/ Life Technologies), according to the manufacturer's protocol. The candidates for transcriptional analysis were chosen based on previous reports of involvement in IAA biosynthesis pathways. Primers for RT-qPCR assays (listed in Supplementary Table S1) were designed based on the *R. tropici* CIAT 899 genome (Ormeño-Orrillo et al. 2012) (Accession numbers NC_020059, NC_020060, NC_020061, NC_020062, for chromosome, pRtrCIAT899a, pRtrCIAT899b, and pRtrCIAT899c, respectively), and were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/), to obtain amplicons of 50–150 bp. A pair of primers for the 16S rRNA was also obtained and applied to normalize the relative expression of the targets. To avoid unspecific alignments, the primers sequences were searched against the *R. tropici* CIAT 899 genome.

After determining the primers efficiency rate, the selected genes were amplified by RT-qPCR using a 7500 RT-qPCR Thermocycler (Applied Biosystems/Life Technologies, Grand Island, NY, USA), with the following conditions: 50 °C for 2 min, 95 °C for 10 min, 45 cycles at 95 °C for 2 min, 60 °C for 30 s, and 72 °C for 30 s, in 45 cycles. The 16S rRNA gene was used as endogenous gene.

The data analysis was performed using the Rest2009 software package (Pfaffl et al. 2002). The normalization of cycle threshold (C_t) of RT-qPCR amplifications was performed based on the selected endogenous gene (16S rRNA).

Results and discussion

Genome context of *R. tropici* CIAT 899, and mutants obtained

Rhizobium tropici CIAT 899 is a very effective strain in promoting common bean growth, therefore, used in commercial inoculants in several countries of South America and Africa (Gomes et al. 2015; Hungria et al. 2000). Symbiosis genes of *R. tropici* CIAT 899 are located in the plasmid pRtrCIAT899b (0.55 Mb), which encompasses five copies of the regulatory *nodD* gene (Ormeño-Orrillo et al. 2012); in the neighborhood of *nodD5*, there is an operon carrying *y4wEF* + *tidC* genes (Supplementary Fig. S1). It is worth mentioning that there are nine *nod*-boxes in the genome of CIAT 899, and that one of them, NB4, is located 1792 bp before *y4wE* (Pérez-Montaño et al. 2016a). In this study we were able to obtain mutants for the *y4wF* and *tidC* genes. By using our mutation strategy, the mutation could have a polar effect and a mutation on *y4wF* could also affect the *tidC* gene. Thus, the *tidC*::Ω, would be a single mutant and the *y4wF*::Ω would be a double *y4wF* and *tidC* mutant. Most important, these mutations could help to confirm the alternative pathway of synthesis of IAA——from IPyA to IAA——suggested by Imada et al. (2017) (Fig. 1).

Quantification of indolic compounds

The total indolic content was always significantly higher ($p < 0.05$) in CIAT 899 and its derivative strains cultured with API in comparison to the control (Fig. 2). Increased IAA synthesis has already been reported in *S. fredii* NGR234 cultured with the flavonoid-inducers daidzein and luteolin (Theunis et al. 2004). In general, no differences were observed between the CIAT 899 and its derivative strains at 48 h; however, at 96 h, both mutant strains accumulated lower levels of IAA when grown with API (Fig. 2).

We should mention that the spectrophotometric method provides only the total indole content (Glickmann and

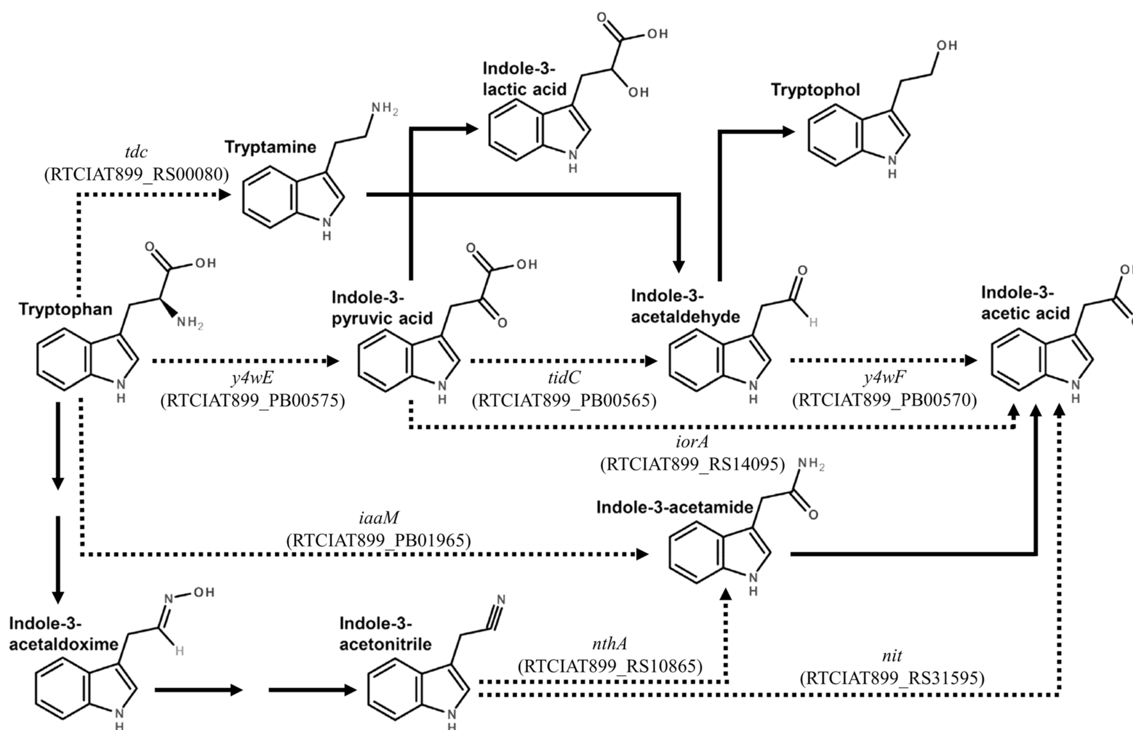

Fig. 1 TRP-dependent IAA biosynthesis pathways. Dotted lines represent possible steps in *R. tropici* CIAT 899, according to the genes suggested in our study, and in the studies by Ormeño-Orrillo et al. (2012) and Imada et al. (2017)

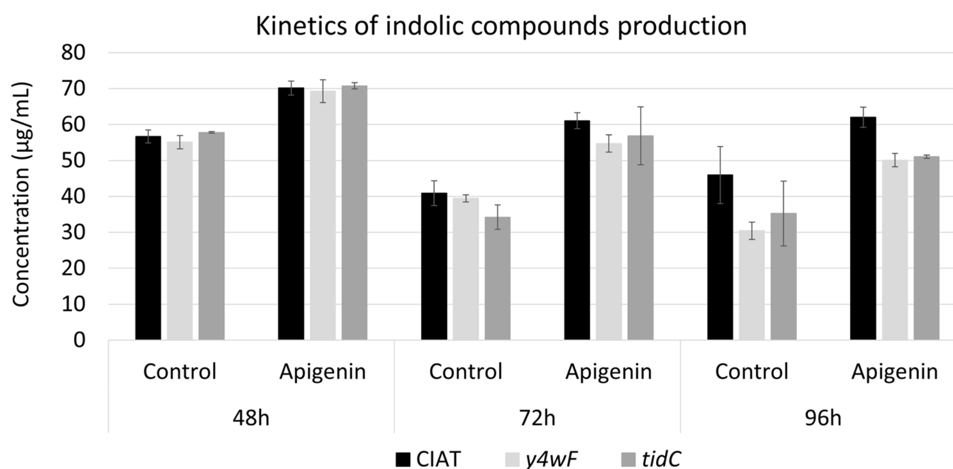

Fig. 2 Production of total indolic compounds after 48, 72, and 96 h. *Rhizobium tropici* wild-type CIAT 899 and the derivative mutants for genes *y4wF* and *tidC* are represented by black, light gray, and dark gray bars, respectively. Data represent the means of three biological replicates, each with three replicates and bars indicate the standard deviation (SD)

Dessaux 1995). In addition, limitations in the correlation between the colorimetric and the HPLC quantifications for *Azospirillum* spp. have been pointed out (Crozier et al. 1988). Therefore, to elucidate the differences among the wild-type and its derivative strains, we proceeded with a more detailed analysis by UPLC.

UPLC analysis of indolic compounds

Among the eight indolic compounds evaluated, indole-3-butyric acid (IBA) and IAN (indole-3-acetonitrile) were not detected in any sample, suggesting that the IAN pathway is inactive under these conditions. Tryptophan (TRP) and indole-3-acetamide (IAM) were found in all samples, and traces of the indole-3-pyruvic acid (IPyA) pathway intermediates (indole acetic acid-IAA, IPyA, and indole-3-lactic acid-ILA) were found only in the wild-type CIAT 899 (Table 1). Interestingly, although not synthesizing the IPyA intermediates, the mutant strains consumed more TRP. This indicates differences from *S. fredii* NGR 234, in which, in the absence of TRP, the IpyA pathway is activated to produce IAA (Theunis et al. 2004).

Although Ormeño-Orrillo et al. (2012) have suggested that the IAM pathway is inoperative in CIAT 899, we have now shown that, in the presence of TRP and in the absence of flavonoids, the pathway is partially active. However, IAA content does not follow proportionally the large IAM accumulation, and none of the IPyA pathway intermediates were produced by CIAT mutants (Table 1). Therefore, IAM could be converted into another compound through non-elucidated ways, including that it could be converted into IPyA following this pathway to IAA; the IAA level could be affected by the consumption of the IAA synthesized. In addition, IAM pathway was not affected by mutations in *y4wF* or *tidC*, as

Table 1 Quantification by UPLC of indolic compounds in *Rhizobium tropici* CIAT 899 and the mutants *y4wF*::Ω and *tidC*::Ω under control and apigenin (API) conditions

Strains	Trp[a] (µg/ml)	IAM (µg/ml)	TAM (µg/ml)	ILA (µg/ml)	IAA (µg/ml)	IPyA (µg/ml)
Without apigenin						
CIAT 899	882.24 ± 30.33 Aa[b]	175.29 ± 8.00 Ab	–	3.00 ± 1.02	1.73 ± 0.25	3.07 ± 1.01
*y4w*F::Ω	701.08 ± 22.61 Ba	171.98 ± 13.10 Ab	–	–	–	–
*tid*C::Ω	636.47 ± 25.54 Ba	176.42 ± 9.64 Ab	–	–	–	–
With apigenin						
CIAT 899	746.37 ± 33.39 Ab	294.31 ± 1.22 Ba	37.65 ± 2.22 B	26.80 ± 1.95 C	12.99 ± 2.48 B	41.30 ± 2.64 B
y4wF::Ω	250.90 ± 35.66 Cb	348.14 ± 15.03 Aa	43.52 ± 2.11 B	51.27 ± 5.16 A	19.69 ± 6.07 A	56.91 ± 8.41 A
tidC::Ω	552.45 ± 49.40 Bb	382.40 ± 7.42 Aa	52.33 ± 3.97 A	37.53 ± 2.94 B	19.21 ± 1.50 A	42.96 ± 2.90 AB

Evaluation performed in the supernatant at the exponential growth stage

[a]Tryptophan (Trp), indole-3-acetamide (IAM), tryptamine (TAM), indole-3-lactic acid (ILA), indole acetic acid (IAA), indole-3-pyruvic acid (IPyA). IBA (indole-3-butyric acid), and IAN (indole-3-acetonitrile) were not detected in any case

[b]Data represent the means of three biological replicates, each with three replicates. Values followed by the same capital letters do not show statistical difference (Tukey's test, $p \leq 0.05$) between strains for each compound, and values followed by the same small letter do not show statistical difference (Tukey's test, $p \leq 0.05$) in the comparison between compounds for each strain

IAM was equally produced by the wild-type and the mutant strains; however, CIAT 899 cultured in YM with lower TRP concentration (0.1 mg/mL rather than 1 mg/mL) did not produce detectable IAM levels (Imada et al. 2017).

The IAM pathway corresponds to an important virulence factor for pathogens such as *Agrobacterium tumefaciens* (Juhas et al. 2008), and its activation seems to be related to higher TRP levels. The synthesis of TRP is energetically costly (Akashi and Gojobori 2002), and the first enzyme of the IAM pathway, the tryptophan 2-mooxygenase, converts TRP only when it is available in excess (Patten et al. 2013). One possibility is that TRP might be recognized by soil microbes as a signal of plant-host vicinity, as seed and root exudates are known to contain TRP (Kamilova et al. 2006). The tryptamine (TAM) pathway also does not seem to be active in CIAT 899, as we did not detect the compound either in the wild-type or in the mutant strains in the presence of TRP. The TAM synthesis might depend on the *tdc* gene—that codes for the tryptophan decarboxylase enzyme—induction by a flavonoid, as it was detected in the presence of API (Table 1).

The presence of API increased the synthesis of indolic compounds by all strains (Table 1). Noteworthy, in the presence of API, TAM was detected in all strains, and ILA, IAA, and IPyA, which have not been detected in the mutants grown without API, were then synthesized. In addition, in the presence of API, *y4wF*::Ω and *tidC*::Ω strains increased the synthesis of IAM, ILA, and IAA in comparison to CIAT 899, and *y4wF*::Ω accumulated more IPyA than CIAT 899, whereas *tidC*::Ω accumulated more TAM than CIAT 899 and *y4wF*::Ω. In addition, we may hypothesize that activation could be mediated by a *nod*-box (B4) of *nodD1* gene that precedes the genes *y4wE, y4wF*, and *tidC*.

In the case of the *y4wF* mutant, the IPyA accumulated could be converted into ILA. In the *tidC* mutant, TAM would be accumulated because the flux through TAM pathway could be enhanced to bypass the blockage on IPyA pathway. The increase in the IAA concentration in both mutants, especially in the *y4wF*::Ω, in comparison to the wild-type strain could be explained by the use of an alternative IAA biosynthesis pathway by CIAT 899.

Considering the position of the genes in the IAA operon (Supplementary Fig. S1), we may hypothesize that, in the presence of API, TAM and IPyA pathways would be impaired in *y4wF*::Ω, whereas IPyA would be truncated in the *tidC*::Ω strain. Carreno-Lopez et al. (2000), using HPLC analyses, verified that IPyA is an unstable compound that does not accumulate in bacterial cultures; however, we found a significant accumulation of IPyA in the presence of API. Our results suggest that the *tidC* gene might be involved in the conversion of IPyA into indole-3-acetaldehyde (IAld), acting as an IPyA decarboxylase; moreover, higher amounts of ILA are possibly a product from IPyA non-converted into IAld. Based on these results, we have changed the nomenclature of this gene, previously labelled as hypothetical (Ormeño-Orrillo et al. 2012), to *tidC*, meaning "*R.* ***t****ropici* **i**ndole-3-pyruvic acid **dec**arboxylase".

Noteworthy, our results indicate that CIAT 899 activates pathways of IAA synthesis only in the presence of *nod*-gene-inducing molecules, probably for the purpose of maximizing nodulation. Thus, in the presence of API, *y4wF*::Ω and *tidC*::Ω strains seem to bypass the blockages for IAA synthesis by increasing the flux of non-affected pathways, as the alternative IPyA (Imada et al. 2017) for both *tidC*::Ω and *y4wF*::Ω, or TAM only for *tidC*::Ω (Table 1). Interestingly, in *Gluconacetobacter diazotrophicus* (Rodrigues et al. 2016) and *Azospirillum brasilense* Sp245 (Prinsen et al. 1993), the mutation in genes related to IAA synthesis resulted in impairment in the synthesis of IAA, while in our study the derivative strains of CIAT 899 accumulated the same amount of indolic compounds, highlighting the great versatility of CIAT 899 to ensure IAA biosynthesis and the nodulation process, taking advantage of alternative pathways.

Exopolysaccharide production

Bacterial exopolysaccharides (EPSs) have several functions, such as nutrient gathering, protection against environmental stresses, and attachment to surfaces; in rhizobia, EPS are also critical for the successful infection of the host plant, as demonstrated in EPS-deficient mutants of *R. leguminosarum* and *S. meliloti*, in which nodule-cell invasion and N_2 fixation were impaired (Janczarek 2011). Although in our previous studies we have not observed changes in EPS production due to mutations on each one of the five copies of *nodD* genes of *R. tropici* CIAT 899 (del Cerro et al. 2015a, b), as IAA synthesis was affected by mutations on *nodD1* and *nodD2* (del Cerro et al. 2015b), it was important to evaluate EPS production in our mutants.

In the procedure that we have used, all sugars found in the supernatant of cell cultures were measured; although the method may be limited, as it can also detect other surface polysaccharides such as cyclic glucans, it allows a valuable preliminary comparison. In CIAT 899 the EPS production was not affected by the presence of TRP or API (Fig. 3). The *tidC* and *y4wF* genes seemed to negatively affect the EPS synthesis because the mutant of the first one showed higher EPS than CIAT 899 in the absence of any inducer molecule and with TRP, and the *y4wF* mutant produced the highest amount of EPS in the presence of apigenin compared to CIAT 899 and *tidC* mutant (Fig. 3).

The EPS production by *S. fredii* HH103 has been reported to be repressed by nod-gene inducers flavonoids such as genistein, in a NodD1-dependent manner, and enhanced by the NolR transcriptional regulator (Vinardell et al. 2004;

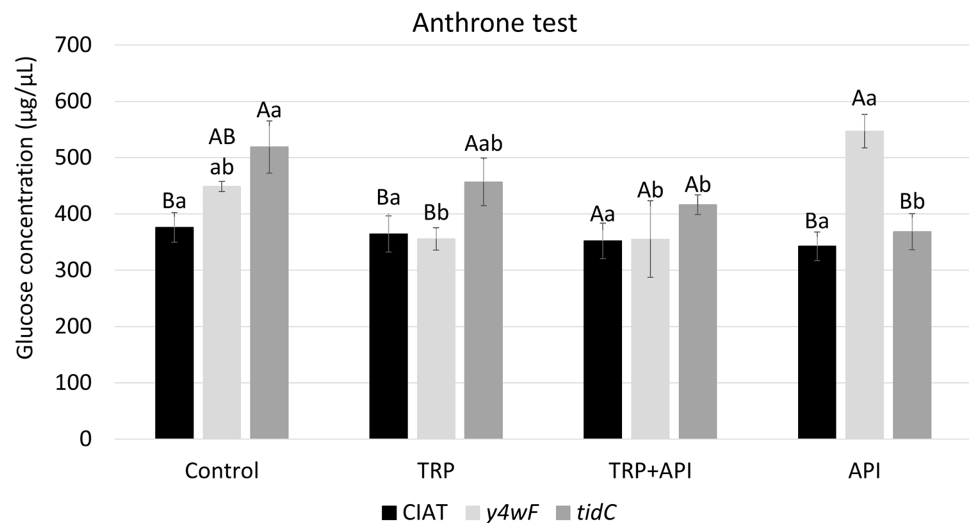

Fig. 3 EPS production in *Rhizobium tropici*. Strains CIAT 899 and the derivative mutants for genes *y4wF* and *tidC* are represented by black, light gray, and dark gray bars, respectively. Data represent the mean of three biological replicates, each with three replicates. Capital letters correspond to the statistical comparison among strains in each treatment, while lowercase letters correspond to the statistical comparison of each strain among treatments (ANOVA, $p < 0.05$)

Acosta-Jurado et al. 2016); similar results were observed in CIAT 899 by the NrcR-a NolR like protein—in the presence of apigenin (del Cerro et al. 2016). In our study, the EPS repression observed in both control and with inducing molecules (Fig. 3) might be a result from the NodD1 repression (Acosta-Jurado et al. 2016), as it is constitutively expressed (Dusha and Kondorosi 1993) and induced by apigenin (del Cerro et al. 2015a); and now, for the first time, an EPS repression effect is suggested for *y4wF* and *tidC*.

Swimming and surface motility

Motility is critical for bacterial survival, allowing movement towards favorable conditions, such as to colonize niches and access new sources of nutrients, enhancing resistance to antibiotics, increasing competitiveness (Swiecicki et al. 2013; Kearns 2010), and providing ecological advantage in the rhizosphere, including colonization of plant hosts (Tambalo et al. 2013).

In general, swimming was not stimulated by TRP or API in CIAT 899 or its derivative strains (Supplementary Fig. S2). Therefore, swimming motility appears to be independent of EPS or IAA under the conditions evaluated.

The methodology employed in our study has been broadly used to evaluate surface motility, including studies with CIAT 899 (del Cerro et al. 2015a, b, 2016). However, we may consider that it might not distinguish swarming from other types of motility, such as twitching, gliding, surfing, etc. In some rhizobia, surface motility can be influenced by exudates from the host-legume root that are rich in flavonoids (Tambalo et al. 2013), and in CIAT 899 it is apparently constitutively suppressed by NodD1 (del Cerro et al. 2015b). However, in our study, in general, we found no differences between the wild-type and the mutant strains (Fig. 4). However, statistically significant differences were attributed to API. Bacteria growth was not affected by the addition of API (data not shown); however, the strains cultured in the control medium and in the medium enriched with TRP presented higher surface motility levels, whereas significantly lower rates were observed when API was added, both alone and combined with TRP (Fig. 4). We may conclude that, apparently, API represses surface motility, possibly mediated by NodD1 (del Cerro et al. 2015b, 2016).

Nodulation kinetics and competitiveness

Early nodulation can be critical for successful N_2 fixation, as it has been shown for several legumes including common bean (Barradas and Hungria 1989). Competitiveness is another critical property for achieving a successful contribution of N_2 fixation, and several traits affect competitiveness, including the ability to respond to flavonoid induction (Wielbo et al. 2010), motility, and EPS production (del Cerro et al. 2016). For example, in *S. fredii* HH103, EPS levels were associated with higher competitiveness (Rodríguez-Navarro et al. 2014), whereas, in CIAT 899, a mutation in the regulatory *nrcR* gene (*nolR*-like plasmid c Regulator) resulted in a negative effect on the competitiveness due to EPS overproduction (del Cerro et al. 2016).

In our study, *tidC*::Ω and *y4wF*::Ω strains showed delays of 1- and 2-days in the nodulation of common bean. Nodule primordia (as defined in the methods section) were detected in all cases on the fourth day after inoculation, and mature nodules were detected at 8, 9, and 10 days (Tukey, $p < 0.05$) after inoculation in the parental, the *tidC*::Ω and the *y4wF*::Ω strains, respectively (Supplementary Fig. S3). In the co-inoculation experiment, both mutant strains were more competitive than the wild-type CIAT 899, occupying 90% and 70% of nodules for *y4wF*::Ω and *tidC*::Ω, respectively (Tukey, $p < 0.05$). Taking into account the importance

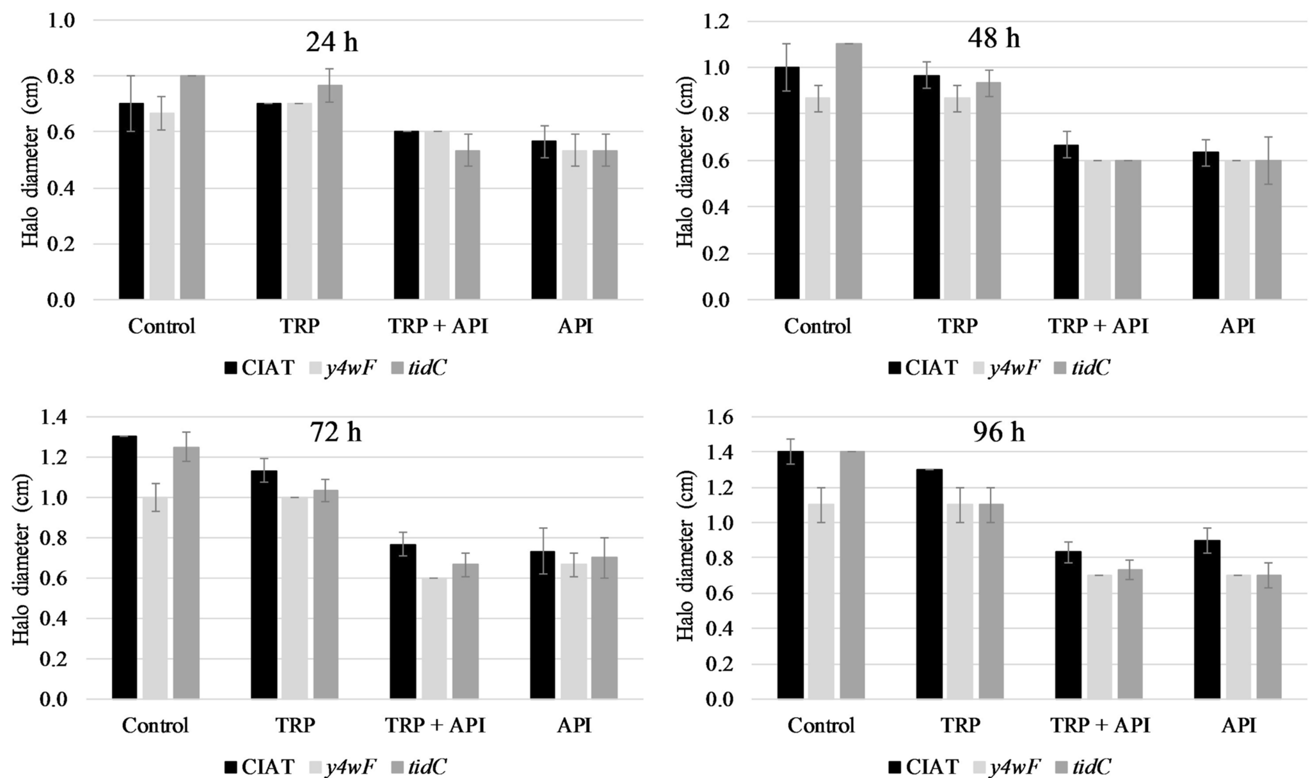

Fig. 4 Surface motility phenotype of *Rhizobium tropici* wild-type CIAT 899, and of the derivative mutants for genes *y4wF* and *tidC* after incubation for 24, 48, 72, and 96 h. Data represent the means of three biological replicates, each with three replicates and bars indicate the standard deviation (SD)

of IAA to cell division and, consequently, to nodule development (Mathesius 2008), we may suggest that nodulation and competitiveness could be related to the differences observed in IAA and/or EPS.

Gene expression evaluated by RT-qPCR

The selection of candidates for gene-expression analysis was based on literature about IAA biosynthesis pathways (*y4wE, y4wF, tidC, nthA, tdc, iaaM*) (Theunis et al. 2004; Imada et al. 2017; Patten et al. 2013), and on studies of rhizobial roles in the nodulation process (*acdS*) (Ding and Oldroyd 2009).

Nitrile hydratase protein, coded by *nthA* (RTCIAT899_RS10865), converts IAN into IAM (Liu et al. 2017); the *tdc* gene (RTCIAT899_RS00080) encodes a homologue to the key protein TRP-decarboxylase, involved in the conversion of TRP to tryptamine (Imada et al. 2017); a TRP-2-monooxygenase encoded by *iaaM* (RTCIAT899_PB01965) is responsible for the conversion of TRP into IAM (Cerboneschi et al. 2016); *y4wE* (RTCIAT899_PB00575) may convert TRP into IPyA; and *y4wF* (RTCIAT899_PB00570) may oxidize IAld forming IAA (Theunis et al. 2004). Although *tidC* (RTCIAT899_PB00565) is adjacent to *y4wE* and *y4wF* (Ormeño-Orrillo et al. 2012; Jijón-Moreno et al. 2015), its involvement in IAA biosynthesis has never been demonstrated.

When CIAT 899 was grown in the presence of API, the expression of all seven genes was significantly higher in comparison to its growth in the absence of API (Fig. 5). The expressions of *y4wE, y4wF*, and *tidC* were significantly lower in the mutant strains in comparison to the expression in wild-type CIAT 899 (Fig. 5). The lower expression of the *tidC* gene in the *y4wF*::Ω background could indicate that this mutant is actually a double *y4wF-tidC* mutant (Fig. 5). The expressions of *iaaM* and *nthA* were significantly lower only in the *tidC* mutant in comparison with the parental strain; with this, we may hypothesize that in the *tidC* mutant an excessive accumulation of IAM in the cells would be avoided. The *tdc* gene was down-regulated in both mutant strains, what could represent a strategy to avoid the excessive accumulation of TAM. On the contrary, the expression of *acdS* was not significantly different between the mutant strains. Although an increased expression of the IAA operon in the presence of flavonoids has already been reported by Theunis et al. (2004), del Cerro et al. (2015b), and Pérez-Montaño et al. (2016a), this is the first time that the induction was reported for the *y4wE-y4wF-tidC* operon of *R. tropici*, indicating that IAA metabolism is related

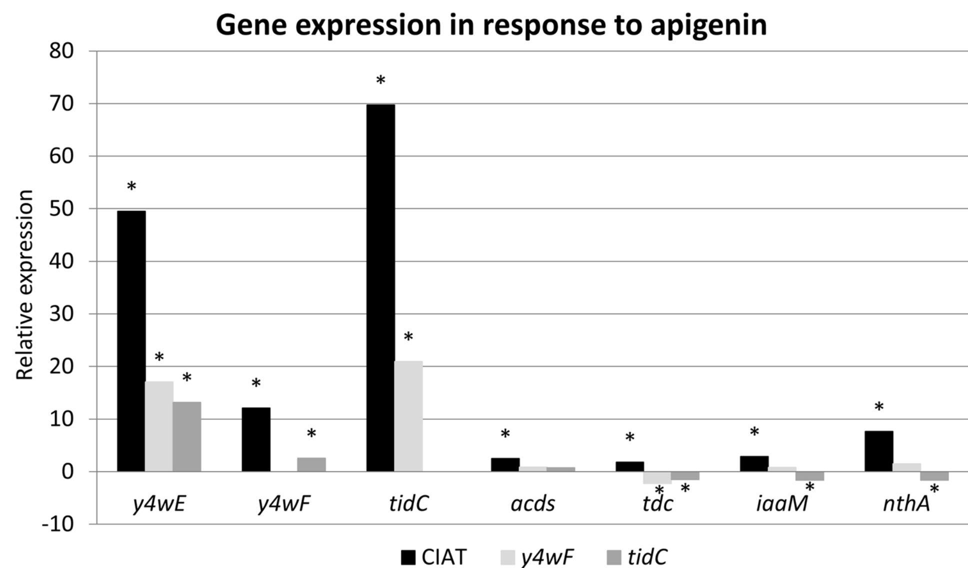

Fig. 5 Induction of genes related to IAA metabolism by the *nod*-gene inducer apigenin (API) in comparison to the control in medium without API for *Rhizobium tropici* CIAT 899 and the derivate mutants for genes *y4wF* and *tidC* (represented by black, light gray, and dark gray bars, respectively). Data represent the means of three biological replicates, each with three technical replicates, and asterisks denote statistically significant differences (Rest2009 software, $p < 0.05$)

to the interaction with the host plant, being up-regulated in the presence of the flavonoid *nod*-gene inducer apigenin.

Up-regulation of 2.5-fold of *acdS* in response to API was observed only in CIAT 899 (Fig. 5). This gene encodes an ACC deaminase which decreases the ethylene levels by cleaving its immediate precursor (Pramanik et al. 2017), potentially reducing the inhibitory impact on rhizobial infection during nodulation (Ding and Oldroyd 2009), and representing an additional mechanism to ensure nodulation success (Checcucci et al. 2017). In addition, overexpressed ACC deaminase has been reported to promote enhanced symbiotic proficiency (Tittabutr et al. 2008; Conforte et al. 2010), and perhaps contributes to the higher competitiveness of the mutant strains.

Interactions of Nod factors and auxins in *M. truncatula* have been demonstrated, with genes split into groups in which auxin-regulated were enhanced or antagonized by Nod factors (Herrbach et al. 2017). Flavonoids and Nod factors act as IAA-transport inhibitors in plants (Mathesius et al. 1998), which may lead to local shifts, as has been suggested for nodule development, when high IAA content is observed during the early stages, and at later stages when lower IAA levels can impair nodulation success (Chan and Gresshoff 2009). In CIAT 899, API via *nodD1* but not via *nodD5*, activated both the Nod factor biosynthesis genes and the IAA synthesis genes (del Cerro et al. 2015a, b). Maybe in this rhizobium strain the Nod factors could modulate the IAA production.

Concluding remarks

Genes and metabolites involved in IAA pathways in *R. tropici* CIAT 899 were shown to be affected in the comparison of the wild type and its derivatives *y4wF*::Ω and *tidC*::Ω mutant strains. Such differences have allowed us to elucidate the probable function of the *tidC* gene in the IPyA pathway and its possible influence on the IAA synthesis through several pathways. The results obtained with the mutants of *y4wF* and *tidC* genes in the presence of API suggest that the pathway from indole-3-pyruvic (IPyA) acid to indole-3-acetic acid is functional in CIAT 899. Based on probable functions, the *y4wF* gene would correspond to the same gene of NGR234, with an activity of monooxigenase or dehydrogenase, which would also find support in the study performed by Spaepen et al. (2007a, b) with *A. brasilense*. The *tidC* gene did not show similarity with the *y4wG* gene of NGR234; we suggest that it would code for a protein with a decarboxylase function. Our results suggest that the *tidC* gene, previously described as a hypothetical protein with a DUF4168 domain of unknown function present in many rhizobia strains, could be implicated in the biosynthesis of IAA. From our results, we hypothesize that the biosynthesis of IAA induced by flavonoids in *R. tropici* CIAT 899 is very important to the symbiosis, such that when the main genes of the synthesis of IAA are mutated, the bacterium uses alternative routes, to ensure synthesis of IAA.

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Authors contributions Conceived and designed the experiments: All authors. Performed the experiments: LDT, ASN, DFG. Analyzed the data: All authors. Contributed reagents/materials/analysis tools: FJO,

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Data Availability The strains are freely available for distribution for research from our culture collection, on upon filling the forms required by the legislation. All results were informed in the manuscript and as supplementary material. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Ethics approval and consent to participate The authors declare no ethical conflicts; authors declare that they have consented to contribute to the manuscript.

Consent to publish The authors declare consent to publish the manuscript.

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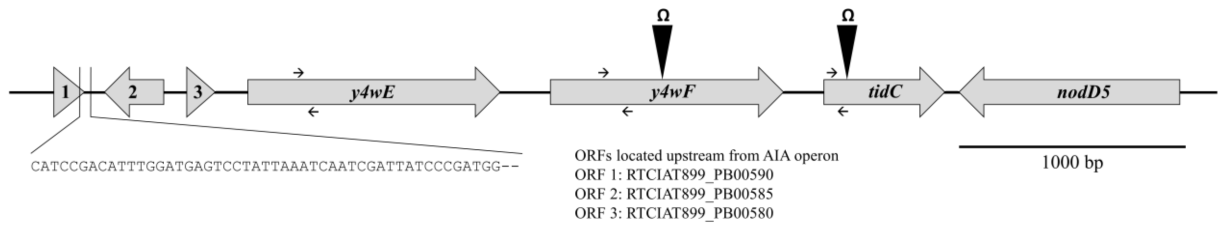
Supplementary Table S1. Sequences of the primers used in the RT-qPCR and sizes of the PCR products obtained.

Name	**Sequence**	**Amplicon**
tdc-F	5'- ATCCGCAATCATGTCGCCTG -3'	82 bp
tdc-R	5'- AACGGCTCGGTGACAACTTC -3'	
iaaM-F	5'- CCGCTGAGCCTTTCCGAAAT -3'	145 bp
iaaM-R	5'- GGTCGTCCACTTCCTTGCTG -3'	
y4wE-F	5'- CCCGTCTCATGCAAATTTCT -3'	106 bp
y4wE-R	5'- GGGAAAGACGGCAACAAGTA -3'	
y4wF-F	5'- TGATCAATCCTCGTTTCGTG -3'	147 bp
y4wF-R	5'- GGTGGAGTCTGCAGGTCATT -3'	
tidC-F	5'- TCAGTGGACGAATACAACGAA -3'	89 bp
tidC-R	5'- GCGGATTTCTGCAGTTTGTC -3'	
acdS-F	5'- CGAACTCGGAAGAGAAATCG -3'	89 bp
acdS-R	5'- TTGTTTCCTCGGAAGGAATG -3'	
nthA-F	5'- CCACTATTCGGATATGCAGGCG -3'	103 bp
nthA-R	5'- ATAGGTCTCGACGATGCGGT -3'	
16S rRNA-F	5'- ACACACGTGCTACAATGGTG -3'	129 bp
16S rRNA-R	5'- GCGATTACTAGCGATTCCAA -3'	



Supplementary Fig. S1. Neighborhood of IAA operon on plasmid B of *R. tropici* CIAT 899. Genes represented are located on the 3'-5' direction. Highlighted sequence corresponds to the *nod*-box 4. Small arrows show the fragment amplified on RT-qPCR assays. The Ω-interposon insertion is indicated by the Ω symbol.

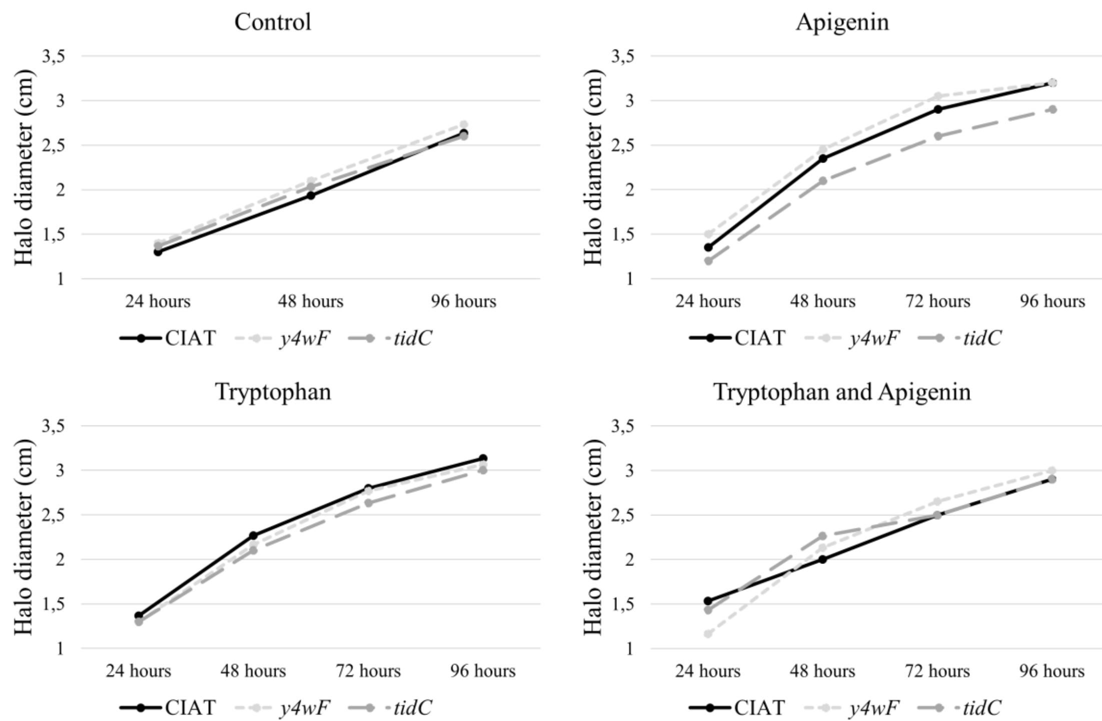
Additional information:

Location: pRtrCIAT899b on the complement strand

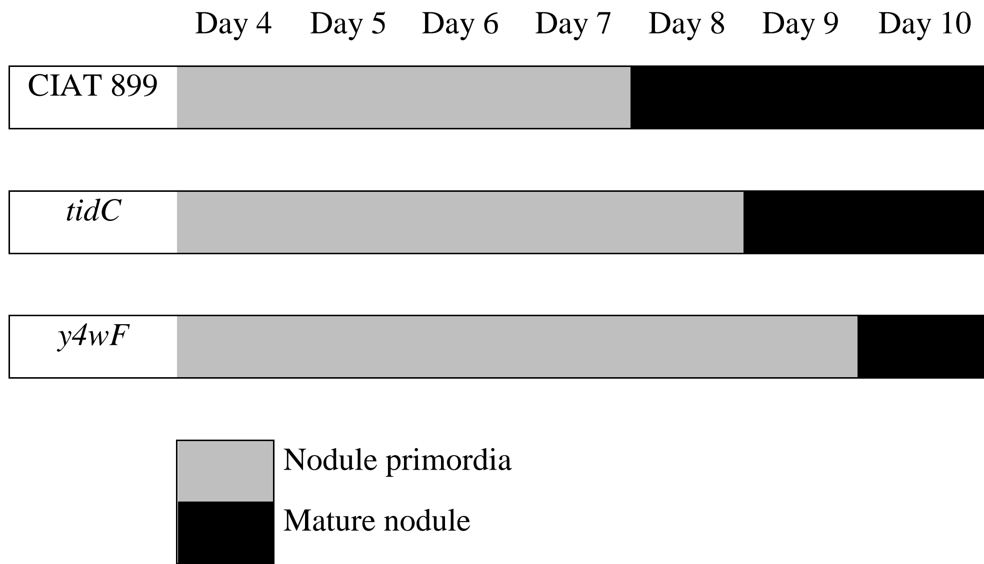
y4wE: from 97717 to 98829; RT-qPCR amplicon (97840-97945)

y4wF: from 96476 to 97504; RT-qPCR amplicon (96687-96833); Ω-interposon (97050)

tidC: from 95773 to 96306; RT-qPCR amplicon (95789-95877); Ω-interposon (95953)



Supplementary Fig. S2. Swimming phenotype of *Rhizobium tropici* CIAT 899, and of the derivative mutants for genes *y4wF* and *tidC* after incubation of 24, 48, 72 and 96 h. Words before strain names mean: C (control), T (presence of TRP), A (presence of apigenin) and AT (presence of TRP and apigenin).



Supplementary Fig. S3. Nodulation kinetics in days after the inoculation with *Rhizobium tropici* strain CIAT 899 and with the derivative mutants for genes *y4wF*::Ω and *tidC*::Ω.

6. Artigo B – Regulação dos genes *hsnT*, *nodE* e *nodF* em *Rhizobium tropici* CIAT 899 e seus papéis na síntese de fatores Nod e na simbiose

Resumo: *Rhizobium tropici* CIAT 899 é uma estirpe com propriedades agronômicas excepcionais, incluindo tolerância a estresses ambientais, amplo espectro de nodulação de diversos hospedeiros e alta capacidade de fixação de nitrogênio com o feijoeiro (*Phaseolus vulgaris* L.); além disso, a estirpe possui características intrigantes, tais como cinco cópias do gene regulatório *nodD* e a capacidade de sintetizar uma variedade de fatores de nodulação (FN), mesmo de uma maneira independente de flavonoides, quando submetida a estresses abióticos. Contudo os papéis de diversos genes *nod* de CIAT 899 ainda não foram determinados. Foram investigadas a regulação dos genes *hsnT*, *nodE* e *nodF* de CIAT 899, seus papéis na síntese de fatores Nod e nas propriedades simbióticas. A expressão dos três genes foi fortemente induzida na presença de apigenina e de sal e, em menor proporção, na ausência de cada um dos cinco genes *nodD*, sendo o NodD1 reconhecido como o principal regulador. Vinte e nove fatores Nod estruturalmente diferentes foram sintetizados por CIAT 899 induzida por apigenina e 36 quando induzida por sal, números drasticamente reduzidos por mutações em *hsnT*, *nodF* e *nodE*, especialmente sob estresse osmótico, com mudanças estruturais específicas relacionadas a cada gene. Mutações nos três genes afetaram diferencialmente o desempenho simbiótico, de acordo com a planta hospedeira. Os resultados indicam que, embora *hsnT*, *nodF* e *nodE* não pertençam ao grupo principal de genes reguladores da nodulação, eles contribuem para a síntese de fatores Nod, que impactará o desempenho simbiótico e a especificidade hospedeira.

Palavras-chave: Nodulação, Genes *nod*, Genes de especificidade hospedeira, Fixação Biológica de Nitrogênio, Fatores Nod.

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Regulation of *hsnT*, *nodF* and *nodE* genes in *Rhizobium tropici* CIAT 899 and their roles in the synthesis of Nod factors and in the symbiosis

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Abstract

Rhizobium tropici strain CIAT 899 possesses outstanding agronomic properties as it displays tolerance to environmental stresses, a broad host range and high effectiveness in fixing nitrogen with the common bean (*Phaseolus vulgaris* L.); in addition, it carries intriguing features such as five copies of the regulatory *nodD* gene, and the capacity to synthesize a variety of nodulation factors (NFs), even in a flavonoid-independent manner, when submitted to abiotic stresses. However, the roles of several *nod* genes of the repertoire of CIAT 899 remain to be determined. In this study, we obtained mutants for the *hsnT*, *nodF* and *nodE* genes of CIAT 899 and investigated their expression, NF structures and symbiotic properties. Either in the presence of the flavonoid apigenin, or of salt the expression of *hsnT*, *nodF* and *nodE* in wild-type CIAT 899 was highly up-regulated in comparison to the mutants of all five copies of *nodD*, indicating the roles that regulatory *nodD* genes play in the activation of *hsnT*, *nodF* and *nodE*; however, NodD1 was recognized as the main inducer. In total, 29 different NF structures were synthesized by wild-type CIAT 899 induced by apigenin, and 36 when induced by salt, being drastically reduced by mutations in *hsnT*, *nodF* and *nodE*, especially under osmotic stress, with specific changes related to each gene, indicating that the three genes participate in the synthesis of NFs. Mutations in *hsnT*, *nodF* and *nodE* affected differently symbiotic performance (nodule number and shoot dry weight), according to the host plant. Our results indicate that the expression of *hsnT*, *nodF* and *nodE* genes of CIAT 899 is mediated by *nodD* genes, and although these three genes do not belong to the main set of genes controlling nodulation, they contribute to the synthesis of NFs that will impact symbiotic performance and host specificity.

INTRODUCTION

Rhizobium tropici is recognized by its ability to overcome environmental stressful conditions, mainly low pH, high temperature and salinity, all commonly limiting biological nitrogen fixation (BNF) in the tropics [1–5]. Notable is also the capacity of *R. tropici* in establishing effective N_2-fixing nodules with a broad range of host legumes, with an emphasis on the common bean (*Phaseolus vulgaris* L.) [1, 4, 6, 7]. Due to their tolerance of environmental stresses and the stability of the symbiotic plasmid, strains belonging to the '*R. tropici* group' are the only ones authorized for the use in commercial inoculants for the common bean crop in Brazil [4]; however, understanding the genetic basis of the *R. tropici*–common bean symbiosis is important to improve their success in the BNF process.

The rhizobium–legume symbiosis begins with the exudation of molecules – mainly flavonoids – from the host legume, which are recognized by the compatible bacteria. In response to the molecules, the transcriptional regulatory NodD – which belongs to the LysR-type transcriptional-regulatory family – of the compatible rhizobia is activated, promoting the transcription of a main set of nodulation genes (*nod* genes, in general, *nodABC*) implicated in the synthesis of lipochitooligosaccharides (LCOs), also known as nodulation factors (NF) [8, 9].

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Abbreviations: LC, liquid chromatography; RT-q, Reverse Transcriptase-quantitative.
One supplementary figure and two supplementary figures are available with the online version of this article.

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The NF backbone varies in length from three to five *N*-acetyl glucosamine residues and substituted by an *N*-acyl chain at the non-reducing end. However, different decorations are present depending on the *nod* genes present in the genome of the rhizobium. The main differences among the NFs rely on the length of the acyl chains, the presence or absence of unsaturated bonds, and the decoration of the reducing ends with substituents such as fucose, arabinose or sulphate [10, 11]. It has been shown that several of the NF structural modifications are involved in host-specificity determination. For example, *Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. viciae, *R. leguminosarum* bv. trifolii, *R. galegae* and *Mesorhizobium huakuii* synthesize NFs with α,β-unsaturated fatty acids, which have been selected in co-evolution with the specific host legumes [12]. However, the role of NodD proteins seems to go far beyond nodulation and host specificity, including, for example, motility, biofilm formation and synthesis of indole acetic acid [6, 7].

R. tropici is particularly remarkable by carrying in its genome five copies of the regulatory *nodD* gene [13], as well as by its ability to synthesize NFs under abiotic stresses such as acidity [2] and osmotic stress [3, 6, 7, 14–16] in a flavonoid-independent manner. Transcriptomic studies revealed that regulation by flavonoid and salt (osmotic stress) in CIAT 899 is mediated by NodD1 and NodD2, respectively [16, 17]. Mutations in the *nodD1* of CIAT 899 results in the absence of nodules in some host legumes [6], while a double mutation in *nodD1* and *nodD2* abolishes nodulation in all legumes [16].

Rhizobia also carry a second set of *nod* genes implied in host specificity [8], but the main roles of some of these genes remain to be determined. Therefore, seeking a better understanding of host-specificity determinants in *R. tropici* CIAT 899, we performed studies with the wild-type and mutants for their *hsnT, nodF* and *nodE* genes, as these genes might be implicated in the structure of the NFs [8, 18–20].

METHODS

Bacteria growth conditions, plasmids and mutants

R. tropici CIAT 899 and derivative mutant strains of *nodD1* [3], *nodD2* [6], *nodD3*, *nodD4*, *nodD5* [7], *hsnT*, *nodF* and *nodE* (this study) were grown at 28 °C on tryptone yeast (TY) [21], B^- minimal [22] or modified yeast extract mannitol (YM) [23] media, supplemented when necessary with apigenin 3.7 µM or with 300 mM of NaCl. *Escherichia coli* strains were cultured on Luria–Bertani (LB) medium [24] at 37 °C. When required, the media were supplemented with the appropriate antibiotics, as described by Lamrabet *et al.* [25].

To obtain the mutants of *hsnT* (RTCIAT899_PB01100), *nodF* (RTCIAT899_PB01105) and *nodE* (RTCIAT899_PB01110) genes, the interposon Ω was inserted disrupting each target gene. Primers used for the amplification of *hsnT, nodF* and *nodE* are listed in Table S1 (available in the online version of this article); each of the PCR products from the target genes was cloned into pGEM-T Easy (Promega) (Amp^R 100 µg ml^{-1}). The resulting plasmids were digested with the enzymes *Sma*I, *Hin*dIII and *Mfe*I, that cut in one site *hsnT, nodF* and *nodE* genes, respectively. The *hsnT* and *nodF* genes were directly ligated to the 2 kb fragment containing the Ω interposon (Spc^R, 100 µg ml^{-1}), previously digested with the same enzymes that digested each gene, except for *nodE*, that was first treated with the Klenow enzyme to convert sticky ends into blunt ends and then ligated to a *Sma*I-digested Ω interposon [26]. All three plasmids obtained were transformed into *E. coli* strain DH5α.

The following step consisted of extracting the genes with Ω fragments from pGEM-T Easy and cloning them in the rhizobial suicide vector pK18mob [27], which confers resistance to kanamycin (Km^R 30 µg ml^{-1}). For cloning *hsnT, nodF* and *nodE* mutated genes into pk18mob, both genes and the vector were restricted with *Eco*RI. Plasmids were transferred from *E. coli* to *Rhizobium* strains by conjugation as described by Simon [28], using the plasmid pRK2013 [29] as the helper. The plasmids generated were used for the homogenization of the mutants of the *nodF*, *nodE* and *hsnT* genes by using the methodology previously described by López-Baena *et al.* [30]. The mutation events were confirmed by PCR.

The parental and mutant strains were deposited at the culture collection of the Department of Microbiology of the Universidad de Sevilla and at the ‘Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja’ (WFCC Collection No. 1213, WDCC Collection No. 1054).

Identification of NFs

Purification and LC-MS/MS analyses of the NFs produced by *R. tropici* CIAT 899 and derivative strains grown in B^- minimal medium [22] supplemented when required with NaCl 300 mM or apigenin 3.7 µM were performed as described before [3].

RNA isolation, cDNA synthesis and quantitative RT-qPCR

Wild-type strain CIAT 899 and the derivative *nodD1*, *nodD2, nodD3*, *nodD4* and *nodD5, hsnT* and *nodF* mutant strains were pre-cultured in 10 ml aliquots of TY medium [21] at 100 r.p.m. and 28 °C in the dark. After 48 h, the pre-inoculated strains were transferred to a new medium and subjected to the following treatments: control (without induction), supplied with apigenin 3.7 µM, and supplied with 300 mM NaCl. In addition, the *hsnT* and *nodF* mutant strains were grown in the presence of apigenin (3.7 µM). The cultures were grown in triplicate under the same conditions as for the pre-cultures, at 100 r.p.m., 28 °C, in the dark, except that they were grown until the exponential phase (OD at 600 nm of 0.5 to 0.6).

Total RNA was extracted using Trizol reagent (Life Technologies), as described before [31]. Total concentrations were estimated in a NanoDrop ND 1000 spectrophotometer (NanoDrop-Technologies) and the integrity was assessed by gel electrophoresis. Extracted RNA samples were submitted to DNAse I treatment (Invitrogen/Life Technologies, Grand Island, NY, USA) and the first strand of cDNA was synthesized

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using SuperscriptIII reverse transcriptase (Invitrogen), according to the manufacturer's protocol.

Primers for the RT-qPCR targets genes *hsnT, nodF* and *nodE* were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/), aiming to obtain amplicons of 50–150 bp. With the same software, primers for the 16S rRNA were obtained and used to normalize the relative expression of the targets, as described for the evaluation of other *nod* genes of *R. tropici* CIAT 899 [6, 7, 32]. To avoid unspecific alignments, the primer sequences were searched against the *R. tropici* CIAT 899 genome (http://www.ncbi.nlm.nih.gov/nuccore/440224888?report=genbank). The primer sequences and amplicon sizes are shown in Table S2.

RT-qPCR reactions were performed in a 7500 RT-qPCR Thermocycler (Applied Biosystems/Life Technologies, Grand Island, NY, USA). The Platinum SYBR Green Master Mix kit (Applied Biosystems) was used according to the manufacturer's instructions. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 45 cycles at 95 °C for 2 min, 60 °C for 30 s and 72 °C for 30 s, in 45 cycles. The data analysis was performed using the Rest2009 software package [33]. The normalization of cycle threshold (Ct) of RT-qPCR amplifications was performed based on the selected endogenous gene (16S rRNA), as described before [32].

Nodulation assays

For the evaluation of the symbiotic phenotypes, wild-type and *hsnT, nodF* and *nodE* mutants of *R. tropici* strain CIAT 899 were grown in modified YM medium [23] until a concentration of 10^9 cells ml^{-1} so it could be used as the inoculum. Surface-sterilized seeds [23] were used for the assays with the common bean (*Phaseolus vulgaris* L.), leucaena (*Leucaena leucocephala* (Lam.) de Wit), siratro [*Macroptilium atropurpureum* (Benth.) Urb.] and lotus [*Lotus japonicus* (Regel) K.Larsen, =*Lotus corniculatus* var. japonicus Regel]. Pre-germinated seeds (2 days after germination) were placed on sterilized pouch bags or Leonard jars containing N-free nutrient solution [34], and were inoculated with 1 ml of the inoculum of each strain. Plants were grown with a 16 h photoperiod at 26 °C in light and 18 °C in the dark with 70 % humidity. Plants were evaluated after 40 (siratro), 42 (leucaena), 50 (lotus) or 25 days (common bean) for nodule number (NN) and shoot dry weight (SDW). For SDW, shoots were dried at 70 °C for 48 h and weighed. The experiment was performed with three biological replicates, each with six replicates, and the data were analysed statistically using one-way ANOVA followed by Duncan's test ($P<0.05$), using the software Statistica 12.0.

RESULTS AND DISCUSSION

Genome context of *hsnT*, *nodF* and *nodE*

In a first analysis of the genome of *R. tropici* CIAT 899, Ormeño-Orrillo *et al.* [13] suggested that the *nodA2hsnTnodFnodE* genes might comprise an operon. Following, it has been shown that these genes are transcriptionally induced in the presence of both the *nod*-gene inducer flavonoid apigenin, and of 300 mM of NaCl [17]. It is worth mentioning the presence of a conserved *nod*-box (NB2) sequence located about 250 bp upstream of *nodA2* (Fig. S1), that could be responsible for the regulation of this operon [16, 17]. Genome and transcriptome data from previous studies [13, 17] suggest that *nodA2* is in the same operon as *hsnTnodFnodE*; in any case, all genes (*nodA2, hsnT, nodF, nodE*) should be under the control of NB2, as we found no other *nod*-box in the vicinity of these genes. To confirm the nature of the mutants, we performed the analysis by RT-PCR. The RT-qPCR data revealed that the mutations in *hsnT* and *nodF* genes have a polar effect. In comparison to the CIAT 899 wild-type strain (1.000), the relative expression of *nodF* (0.059±0.028) and *nodE* (0.002±0.001) were drastically decreased in *hsnT*::Ω, as well as of *nodE* (0.009±0.005) in *nodF* mutant strain.

Expression of hsnT, nodF and *nodE* under different backgrounds of the regulatory *nodD* genes

Nodulation genes are usually regulated by NodD transcriptional regulators, and our research group has proposed roles for all five copies of *nodD* genes in CIAT 899 [6, 7]. In this study we investigated the relative expression of *hsnT*, *nodF* and *nodE* genes by RT-qPCR in all five *nodD* mutant backgrounds of CIAT 899. In the presence of either the *nod*-gene inducer flavonoid apigenin, or of salt, the expression of *hsnt*, *nodF* and *nodE* in wild-type CIAT 899 was highly up-regulated (Fig. 1), in agreement with Pérez-Montaño *et al.* [17]. However, the expression was drastically reduced in all five *nodD* backgrounds (Fig. 1), indicating that all copies of the *nodD* genes should be involved in the regulation of *hsnT*, *nodF* and *nodE* genes.

In the absence of flavonoid, but under osmotic stress, all three genes were also highly up-regulated, and again, with drastic decreases with mutations in all five *nodD* mutants (Fig. 1). When induced by apigenin, the most drastic decrease in the expression of all three genes was related to a mutation in *nodD1*. Indeed, in *R. tropici* CIAT 899 *nodD1* has been recognized as the main regulatory gene of nodulation when induced by flavonoid [6, 7], and our results confirm that the expression of *hsnT*, *nodE* and *nodF* depends on the activation of *nodD1*. However, under osmotic stress, the gene expression of *hsnT*, *nodE* and *nodF* in the *nodD2* mutant was remarkably lower than in the wild-type strain (Fig. 1), confirming the role of NodD2 as the main regulatory gene under osmotic conditions [16, 17]. It is worth mentioning that *nodD2* has been described as a repressor of *nod* genes in *Sinorhizobium* strain NGR234 [35]. However, in *R. tropici* CIAT 899 the gene was found to be a positive regulator of *nodC* [6], and our results confirm, in agreement with the RNAseq results of del Cerro *et al.* [16], the important role of *nodD2* in up-regulation of *hsnT*, *nodF* and *nodE*.

Despite the drastic decrease in the expression of *hsnT*, *nodF* and *nodE* in the *nodD2, nodD3, nodD4 and nodD5* mutants, the three genes remained significantly up-regulated mainly in the presence of apigenin. This indicates that the mutants of

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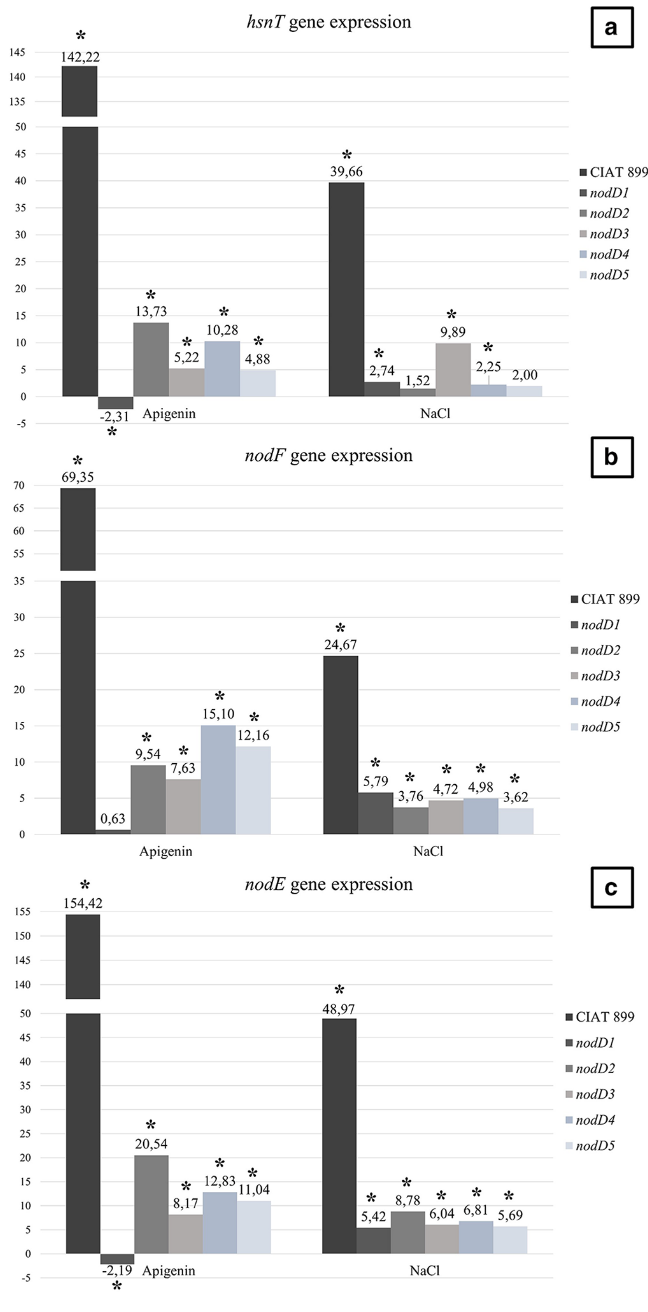

Fig. 1. RT-qPCR analysis of the expression of *hsnT, nodF* and *nodE* genes from *R. tropici* CIAT 899 and derivative mutants grown under control (TY medium), in the absence and in the presence of apigenin (3.7 µM) or NaCl (300 mM). Expression data shown are the mean of three biological replicates, each with three replicates. Data were normalized in relation to the endogenous control (16S rRNA). The asterisks indicate a statistically significant expression at 5 %, determined by REST2009 software. Relative expression of *hsnT, nodF* and *nodE* genes are represented in (a), (b) and (c), respectively.

these four *nodD* genes are still able to guarantee *hsnT*, *nodF* and *nodE* up-regulated expression; unlike the *nodD1* mutant, in which *hsnT* and *nodE* were down-regulated with apigenin (Fig. 1).

Table 1. Number of NFs produced by the wild-type *R. tropici* strain CIAT 899 and the *hsnT, nodF and nodE* mutants when grown in B$^-$ medium with 3.7 µM of apigenin or salt (NaCl 300 mM)

The structures of NFs under each condition are shown in Tables 2 and 3.

	Apigenin	Salt
CIAT 899 – WT	29	36
hsnT mutant	21	9
nodF mutant	14	10
nodE mutant	18	2

NF profiles

NFs are molecules synthesized by rhizobia as a result of the transcription of *nod* genes, and in CIAT 899 they are synthesized in response to both *nod*-gene plant inducers and to abiotic stresses in the absence of plant inducers [2, 3, 6, 7, 14, 15]. *R. tropici* CIAT 899 is well known for producing a variety of NFs [2, 3, 6, 7, 10, 14], and in our study 29 different structures were detected when the wild-type strain was induced by apigenin, and 36 when induced by salt (Table 1). Although the synthesis of NFs was still observed when *hsnT*, *nodF* and *nodE* were mutated, the number of NFs synthesized was drastically decreased, especially when induced by salt, such that only two structures were synthesized by the *nodE* mutant under osmotic stress (Table 1). Therefore, we may conclude that all three genes play a role in the synthesis of NFs. In addition, we must consider that, as we have shown, the polar effect of the mutations did not completely shut off the expression of the dowstream genes in the operon, which could be an explanation of the unique *in planta* behaviour of the mutants, as we will discuss in the phenotype and symbiotic item.

Considering the order of the genes (Fig. S1), a mutation in *hsnT* would affect both *nodF* and *nodE*; and a mutation in *nodF* would affect *nodE*. Thus, in theory, if the mutation was to affect the downstream genes, the *hsnT* and *nodE* mutants would present the lowest and the highest variety of NFs, respectively. However, as shown in Table 1, we did not verify that, indicating that the regulation of NF synthesis is complex and deserves further studies.

Host-specificity nodulation (*hsn*) genes are important for nodulation of several legumes, expanding the rhizobial host range. The *hsn* genes participate in the NF decoration, by the addition or by the replacement of functional groups in their reducing ends [36]. Fucosylated NFs have been related to salt tolerance in *R. tropici* CIAT 899 [14, 37]. Interestingly, in our study a mutation in *hsnT* of CIAT 899 resulted in the synthesis of NFs carrying methylfucose (MeFuc) only in the presence of apigenin [V (C14 : 0, MeFuc), V (C16 : 1, MeFuc), V (C18 : 0, MeFuc) and V (C18 : 1, MeFuc)] (Table 2). The mechanisms involved in the synthesis of fucosylated NFs in CIAT 899 deserve a better investigation, as the genes described for the fucosyl synthesis, transport or attachment

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Table 2. NF structure biosynthesized in B^- minimal medium in the presence of 3.7 µM of apigenin by wild-type CIAT 899 and the *hsnT*, *nodF* and *nodE* mutants

$[M+H]^+$ (*m/z*)	B_i ions	Structure*	CIAT899†	*hsnT*†	*nodF*†	*nodE*†
824	400, 603	III ($C_{16:0}$)	–	–	+	–
838	414, 617	III ($C_{16:0}$, NMe)	+	–	–	–
850	426, 629	III ($C_{18:1}$)	+	–	–	+
852	428, 631	III ($C_{18:0}$)	+	–	+	–
864	440, 643	III ($C_{18:1}$, NMe)	+	–	–	–
999	372, 575, 778	IV ($C_{14:0}$)	+	–	–	–
1011	384, 597, 790	IV ($C_{14:1}$, NMe)	+	–	+	–
1013	386, 589, 792	IV ($C_{14:0}$, NMe)	+	–	–	+
1025	398, 601, 804	IV ($C_{16:1}$)	+	–	–	+
1027	400, 603, 806	IV ($C_{16:0}$)	+	–	+	+
1039	412, 615, 818	IV ($C_{16:1}$, NMe)	+	+	–	+
1041	414, 617, 820	IV ($C_{16:0}$, NMe)	+	+	+	+
1053	426, 629, 832	IV ($C_{18:1}$)	+	+	+	+
1055	428, 631, 834	IV ($C_{18:0}$)	+	+	+	–
1067	440, 643, 846	IV ($C_{18:1}$, NMe)	+	+	+	+
1069	442, 645, 848	IV ($C_{18:0}$, NMe)	+	+	–	–
1081	454, 657, 860	IV ($C_{20:1}$)	+	–	–	–
1147	440, 643, 846	IV ($C_{18:1}$, NMe, S)	–	+	–	–
1202	372, 575, 778, 981	V ($C_{14:0}$)	+	+	–	+
1213	426, 629, 832	IV($C_{18:1}$, MeFuc)	–	+	–	–
1214	426, 629, 790, 832, 993	V ($C_{18:1}$, dNAc)	+	–	+	–
1216	386, 589, 792, 995	V ($C_{14:0}$, NMe)	+	+	–	+
1228	440, 643, 846, 1007	V ($C_{18:1}$, NMe, dNAc)	+	+	–	+
1230	400, 603, 806, 1009	V ($C_{16:0}$)	+	–	–	–
1242	412, 615, 818, 1021	V ($C_{16:1}$, NMe)	+	–	–	+
1244	414, 617, 820, 1023	V ($C_{16:0}$, NMe)	+	+	–	+
1256	426, 629, 832, 1035	V ($C_{18:1}$)	+	+	+	+
1270	440, 643, 846, 1049	V ($C_{18:1}$, NMe)	+	+	+	+

Continued

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Table 2. Continued

$[M+H]^+$ (*m/z*)	B_i ions	Structure*	CIAT899†	*hsnT*†	*nodF*†	*nodE*†
1272	442, 645, 848, 1051	V ($C_{18:0}$, NMe)	+	+	+	+
1324	414, 617, 820, 1023	V ($C_{16:0}$, NMe, S)	+	–	–	–
1336	426, 629, 832, 1035	V ($C_{18:1}$, S)	+	–	–	+
1350	440, 643, 846, 1049, $[M-80]^{+‡}$= 1270	V ($C_{18:1}$, NMe, S)	+	+	+	+
1352	442, -, 848, 1051, $[M-80]^{+‡}$=1272	V($C_{18:0}$, NMe, S)	–	+	–	–
1362	372, 575, 778, 981	V($C_{14:0}$, MeFuc)	–	+	–	–
1388	398, 601, 804, 1007	V($C_{16:1}$, MeFuc)	–	+	–	–
1416	426,629, 832, 1035	V($C_{18:1}$, MeFuc)	–	+	–	–
1418	631, 834, 1037	V($C_{18:0}$, MeFuc)	–	+	–	–

*NF structures are represented following the convention [22] that indicates the number of GlcNAc residues in the backbone (Roman numeral), the length and degree of unsaturation of the fatty acyl chain, and the other substituents, which are listed in the order in which they appear, moving clockwise from the fatty acid. NMe, *N*-methyl group at glucosamine non-reducing residue; S, sulfate group at reducing glucosamine residue; MeFuc, methylfucose group at the reducing end; dNAc, deacetylated.

†Symbol: +=detected; –=non-detected.

‡These ions arise by loss of a neutral with mass 80 Da, corresponding to the loss of SO_3.

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Table 3. NF structure biosynthesized in B⁻ minimal medium in the presence of 300 mM NaCl by wild-type CIAT 899 and the *hsnT*, *nodF* and *nodE* mutants

$[M+H]^+$ (*m/z*)	B_i ions	Structure*	CIAT899†	*hsnT*†	*nodF*†	*nodE*†
824	400, 603	III ($C_{16:0}$)	+	–	–	–
838	414, 617	III ($C_{16:0}$, NMe)	+	–	–	–
850	426, 629	III ($C_{18:1}$)	+	–	–	–
864	440, 643	III ($C_{18:1}$, NMe)	+	–	–	–
999	372, 575, 778	IV ($C_{14:0}$)	+	–	–	–
1013	386, 589, 792	IV ($C_{14:0}$, NMe)	+	–	–	–
1025	398, 601, 804	IV ($C_{16:1}$)	+	–	–	–
1027	400, 603, 806	IV ($C_{16:0}$)	+	+	–	–
1041	414, 617, 820	IV ($C_{16:0}$, NMe)	+	–	–	–
1053	426, 629, 832	IV ($C_{18:1}$)	+	+	–	+
1055	428, 631, 834	IV ($C_{18:0}$)	+	+	+	–
1067	440, 643, 846	IV ($C_{18:1}$, NMe)	+	–	–	–
1069	442, 645, 848	IV ($C_{18:0}$, NMe)	+	–	–	–
1147	440, 643, 846	IV ($C_{18:1}$, NMe, S)	+	–	+	–
1149	442, 645, 848	IV ($C_{18:0}$, NMe, S)	+	–	–	–
1203	414, 617, 820, 1023	IV Hex ($C_{16:0}$, NMe)	+	–	–	–
1205	414, 617, 820, 1023	IV Hex-ol ($C_{16:0}$, NMe)	+	–	–	–
1215	426, 629, 832, 1035	IV Hex ($C_{18:1}$)	+	–	–	–
1216	386, 589, 792, 995	V ($C_{14:0}$, NMe)	+	–	–	–
1229	440, 643, 846, 1049	IV Hex ($C_{18:1}$, NMe)	+	–	+	–
1230	400, 603, 806, 1009	V ($C_{16:0}$)	+	–	–	–
1231	440, 643, 846, 1049	IV Hex-ol ($C_{18:1}$, NMe)	+	–	–	–
1233	442, 645, 848, 1051	IV Hex-ol ($C_{18:0}$, NMe)	+	–	–	–
1242	412, 615, 818, 1021	V ($C_{16:1}$, NMe)	+	–	–	–
1244	414, 617, 820, 1023	V ($C_{16:0}$, NMe)	+	–	–	–
1256	426, 629, 832, 1035	V ($C_{18:1}$)	+	+	+	+
1258	428, 631, 834, 1037	V ($C_{18:0}$)	+	–	–	–
1270	440, 643, 846, 1049	V ($C_{18:1}$, NMe)	+	+	+	–
1272	442, 645, 848, 1051	V ($C_{18:0}$, NMe)	+	+	+	–
1298	468, 671, 874, 1077	V ($C_{20:1}$, NMe)	+	–	–	–
1324	414, 617, 820, 1023	V ($C_{16:0}$, NMe, S)	+	–	+	–
1336	426, 629, 832, 1035	V ($C_{18:1}$, S)	+	+	+	–
1350	440, 643, 846, 1049, $[M-80]^{+c}$= 1270	V ($C_{18:1}$, NMe, S)	+	+	+	–
1352	442, 645, 848, 1051	V ($C_{18:0}$, NMe, S)	+	+	+	–
1378	468, 671, 874, 1077	V ($C_{20:1}$, NMe, S)	+	–	–	–
1380	470, 673, 876, 1079	V ($C_{20:0}$, NMe, S)	+	–	–	–

Continued

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Table 3. Continued

$[M+H]^+$ (*m/z*)	B_i ions	Structure*	CIAT899†	*hsnT*†	*nodF*†	*nodE*†

*NF structures are represented following the convention [22] that indicates the number of GlcNAc residues in the backbone (Roman numeral), the length and degree of unsaturation of the fatty acyl chain, and the other substituents, which are listed in the order in which they appear, moving clockwise from the fatty acid. NMe, *N*-methyl group at glucosamine non-reducing residue; S, sulfate group at reducing glucosamine residue.
†Symbol: +=detected; −=non-detected.
‡These ions arise by loss of a neutral with mass 80 Da, corresponding to the loss of SO_3.

(i.e. *nodZ* and *noeJ*) [25, 37] are not present in the genome of *R. tropici* CIAT 899 [13].

The gene *nodF* encodes an acyl-carrier protein related to the elongation of fatty acid chains, which are incorporated to the NF [38]. Although CIAT 899 has synthesized NFs with C20 when induced by both apigenin and salt, no C20 acyl chain was synthesized by the *nodF* mutant (Table 2). The CIAT 899 *nodD1* mutant was also defective in the synthesis of NF with C20 acyl chains [6], which could be a consequence of the remarkable decrease in the expression of *nodF* (Fig. 1b). This may have contributed to the symbiotic phenotype presented by the CIAT 899 *nodD1* mutant, which significantly reduced the number of nodules on the common bean, and lost the capacity of nodulating leucaena (*Leucaena leucocephala*) and siratro (*Macroptilium atropurpurem*) [6]. In *Sinorhizobium meliloti*, a *nodF* mutant strain defective in the synthesis of C16 polyunsaturated LCOs [18] presented a very low number of infection threads and defective nodulation on the host plant alfalfa (*Medicago sativa* L.) [11, 39]. *nodE* codes for a β-acetoacetylsynthase related to the biosynthesis of unsaturated fatty acids that are incorporated at the non-reducing end of NF [38]. In *R. leguminosarum*, NodE is required for the synthesis of the polyunsaturated acyl chains of the LCO, which does not occur in CIAT 899, that presents only one unsaturated bond in some fatty acids of its NFs [6, 7]. Therefore, in CIAT 899, *nodE* should play a different role in the synthesis of NFs that goes beyond the inclusion of unsaturated bonds on the acyl chains, once the lack of a functional *nodE* gene in our mutant strain was not sufficient to abolish the synthesis of LCOs composed by unsaturated fatty acids (Tables 2 and 3).

The wild-type CIAT 899 produced NF type [IV($C_{20:1}$)] in the presence of apigenin, or types [V ($C_{20:1}$, NMe), V ($C_{20:0}$, NMe, S) and V ($C_{20:1}$, NMe, S)] with salt (Tables 2 and 3). However, such long acyl-chain NFs were not synthesized by *hsnT, nodF* and *nodE* mutants, indicating that these genes are responsible for the synthesis and incorporation of fatty acids C20 as decoration to the NFs; consequently, they should play a role on host-specificity determination. It is worth mentioning that *hsnT* codes for an acyltransferase unrelated to the NodA2 one [13]. As apigenin and salt induce the expression of *nodA2*, *hsnT*, *nodF* and *nodE* [17], it is possible that both HsnT and NodA2 enzymes couple the C20 acyl chain to the NF.

Interestingly, the *nodD1* mutant was also unable to produce the same NF type IV($C_{20:1}$) when induced by apigenin, while the synthesis by *nodD2* mutant was not affected; in addition, the three NFs [V ($C_{20:1}$, NMe), V ($C_{20:0}$, NMe, S) and V ($C_{20:1}$, NMe, S)] synthesized with salt were not produced by *nodD1* and *nodD2* mutants [6]. The synthesis of long acyl-chain NFs was probably not affected in the *nodD2* mutant because the main inducer of *hsnT, nodF* and *nodE*, NodD1, was present; while the abolished NF synthesis both in *nodD1* and *nodD2* mutants deserves further studies, as they might be needed under salt stress.

In *S. meliloti*, the replacement of the N-linked C16 : 2 acyl group by a C18 : 1 significantly reduced rhizobial infection and nodule development [39]. In this case, the C18 : 1 makes

Table 4. NN (n°/plant) and SDW (g/plant) of siratro (*Macroptilium atropurpureum*), *leucaena (Leucaena leucocephala*), lotus (*Lotus japonicus)* and the common bean (*Phaseolus vulgaris*) inoculated with wild-type CIAT 899 and the *hsnT*, *nodF* and *nodE* mutants

Plants evaluated after 40 (siratro), 42 (leucaena), 50 (lotus) and 25 days (common bean) of growth under controlled conditions. Means followed by different letters differ from each other by Duncan's test at 5 %. Experiment performed three times, each with six replicates.

CIAT 899 and mutants	*M. atropurpureum*		*L. leucacephala*		*L. japonicus*		*P. vulgaris*	
	NN	SDW	NN	SDW	NN	SDW	NN	SDW
Wild-type	15.7 a	0.03 a	16.0 a	0.29 b	37.3 a	0.08 a	250.3 a	1.39 a
hsnT	14.0 a	0.03 a	12.0 ab	0.35 a	21.5 b	0.03 bc	221.0 bc	0.88 b
nodF	14.0 a	0.04 a	7.0 b	0.25 bc	19.5 b	0.05 b	238.0 ab	1.21 ab
nodE	18.0 a	0.03 a	10.0 ab	0.19 c	20.5 b	0.06 ab	211.5 c	1.29 a
None	–	0.04 a	–	0.09 d	–	0.01 c	–	0.44 c

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the NF 100-times less active for calcium spiking induction, an essential process during the early stages of nodulation [40]. It is possible that the substitution of fatty acids C20 in CIAT 899 affects the nodulation at different levels, depending on the host legume.

Altogether, the results corroborate that NodD1 is the main inducer of *hsnT, nodF* and *nodE* genes, suggested here as being responsible for synthesizing long acyl-chain NFs. In contrast, the *nodD3*, *nodD4* and *nodD5* mutants synthesize some types of NFs with long acyl chain in both the presence of apigenin and salt [7], suggesting these genes might modulate *hsnT, nodF* and *nodE* expression.

Phenotypes *in vitro* and in symbiotic properties

Phenotypes were investigated in *R. tropici* CIAT 899 wild-type and in the mutants for the *hsnT, nodE* and *nodF* genes. Although the mutated genes are preceded by a *nod*-box, properties previously described to be regulated via NodD proteins such as cell motility (swimming and surface motility), biofilm formation and exopolysaccharide (EPS) synthesis were analysed as described before [6, 7], but no differences were observed between the wild-type and the mutant strains (data not shown).

Symbiotic phenotypes (NN and SDW) of CIAT 899 and the mutant strains were verified in four host legumes. Inoculation of CIAT 899 improved biomass production in all host plants, except for siratro, where no statistical differences were found both in NN and SDW (Table 4).

Interestingly, in leucaena, a mutation in *hsnT* increased SDW (Table 4), which might result from the effect of the fucosylated NFs present in this mutant strain in response to plant flavonoids (Table 2). The lack of NFs containing fucose and methylfucose substituent in a *S. fredii* mutant resulted in lower nodulation of *Cajanus cajan*, and also affected strain competitiveness [25]. In contrast, mutations in *nodF* and *nodE* decreased NN and SDW, respectively, in leucaena. As previously commented, *nodF* and *nodE* products are involved with the elongation of fatty acid chains and incorporation into NF [38]; in addition, as the *nodF* mutant has lost the ability to synthesize NF with C20 (Table 2); this could have affected nodulation. Strikingly, the positive effect of fucosylated NFs in leucaena may be negative for the common bean, as the inoculation of the *hsnT* mutant has decreased both NN and SDW. In addition, the result from inoculation with the *nodE* mutant was even worse than with *hsnT* for NN formation. Previously, in a study by Spaink *et al.* [41], NodE product was determined as the main factor distinguishing the host range of nodulation of *R. trifolii* (now *R. leguminosarum* bv. trifolii) and *R. leguminosarum* (now *R. leguminosarum* bv. viciae).

The significant decreases in NN of lotus by mutations in *hsnT, nodE* and *nodF*, and in SDW by mutations in *hsnT* and *nodF* (Table 4) may be related with the lack of NFs containing C16:1 and C20 acyl chains. NFs with unsaturated C16 acyl chains were also absent in a *nodF* mutant of *S. meliloti*, resulting in a decrease on NN [11, 18, 39].

CONCLUSIONS

Our results show that NodD1 is the main inducer of *hsnT, nodF* and *nodE* genes in CIAT 899, and that although the three genes are not the main determinants of nodulation, they contribute to the nodulation process. In addition, the three genes are important for the biosynthesis and specific decoration of the NFs, impacting host specificity and symbiotic performance in some leguminous species.

Availability of data and materials

The strains are freely available for distribution for research from our culture collection, after filling the forms required by legislation. All results were informed in the manuscript and as supplementary material. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
Conceived and designed the experiments: All authors.
Performed the experiments: D. F. Gomes, L.D. Tullio, P. del Cerro, A. S. Nakatani, A. A. P. Rolla Santos.
Analyzed the data: All authors.
Contributed reagents/materials/analysis tools: F. J Ollero, M. Hungria
Wrote the paper: All authors.
All authors read and approved the final manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The authors declare no ethical conflicts; authors declare that they have consented to participate in the manuscript.

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Table S1. Primers sequences applied for mutagenesis of *hsnT*, *nodF* and *nodE genes.*

Gene	Primer Sequence	Amplicon
hsnT	Foward: 5' – GCCGCTCATCTGGGACTGCTA – 3' Reverse: 5' – AGCGAGCTCAGTTCGGTCTCA – 3'	2,714 bp
nodF	Foward: 5' – GAGGAAGGCGAGGGGAAGCA – 3' Reverse: 5' – CCGATGATCGGCCAAACCTCA – 3'	2,021 bp
nodE	Foward: 5' – GAGGAAGGCGAGGGGAAGCA – 3' Reverse: 5' – CCGATGATCGGCCAAACCTCA – 3'	2,021 bp

Table S2. Sequences of the primers used in the RT-qPCR analyses and sizes of the PCR products obtained.

Gene	Primer Sequence	Amplicon
hsnT	Foward: 5' – GGCCCGTGCTTGTCTATATG – 3' Reverse: 5' – CGGAGACGAGACATGACGTA – 3'	153 bp
nodF	Foward: 5' – AGGGAGGCTTGAATGGACAG – 3' Reverse: 5' – GATAGCTGAACGCCCTTCG – 3'	114 bp
nodE	Foward: 5' – GGCGGTGGATCAGATTAGAC – 3' Reverse: 5' – GCTTCCCAAGACTTCAGCAC – 3'	96 bp
16S rRNA	Foward: 5'- ACACACGTGCTACAATGGTG – 3' Reverse:5'- GCGATTACTAGCGATTCCAA – 3'	129 bp

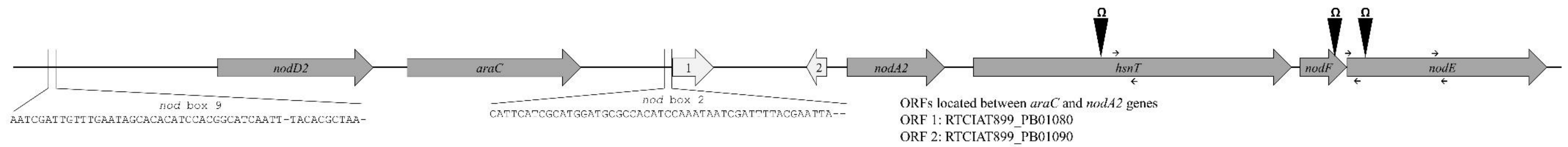

Fig. S1. Gene neighborhood of *hsnT*, *nodF*, and *nodE* genes and representation of the mutations. Gene location in the symbiotic plasmid (pRtCIAT899b) of *R. tropici* strain CIAT 899 and primers used to perform RT-qPCR experiments (dark arrows).

8. Considerações finais

Foram obtidas e caracterizadas estirpes de *Rhizobium tropici* CIAT 899 mutadas em cinco genes envolvidos com a biossíntese de fitormônios e de fatores de nodulação (fatores Nod). As prováveis funções dos genes *y4wF* e *tidC* foram determinadas, os quais fazem parte de uma via de biossíntese de ácido indol-3-acético (AIA), além de corroborar a viabilidade de uma via alternativa à do IPyA, com menos etapas, recém proposta.

O estudo dos genes *hsnT, nodF* e *nodE* revelou que NodD1 é o principal regulador da expressão destes genes e que, embora não sejam os principais determinantes da nodulação, as modificações estruturais que seus produtos realizam são importantes para o processo de nodulação e afetam o desempenho simbiótico e a especificidade com o hospedeiro.

Considerando a importância da síntese de fatores Nod e do AIA, em etapas cruciais para a nodulação e a fixação biológica do nitrogênio com o feijoeiro, os conhecimentos obtidos neste estudo permitem traçar estratégias para melhorar a eficiência do processo biológico com essa cultura.