O PAPEL DAS UREASES DE SOJA (*Glycine max* (L.) Merr.) NO DESENVOLVIMENTO DA PLANTA E NA PROTEÇÃO CONTRA NEMATOIDE CAUSADOR DE GALHA

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PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

CILIANA RECHENMACHER

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de doutor em Genética e Biologia Molecular

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"O que sabemos é uma gota; o que ignoramos é um oceano. Mas o que seria o oceano se não infinitas gotas?"

Isaac Newton

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Sumário

LISTA DE ABREVIATURAS

bar: gene marcador que confere resistência ao herbicida glufosinato de amônio

bp: pares de bases do DNA

CaMV: do inglês *Cauliflower mosaic vírus*, vírus do mosaico da couve-flor

cDNA: DNA complementar

cv: cultivar

DNA: Acido desoxirribonucléico

E*gfp*ER: gene repórter que codifica altos níveis da proteína fluorescente verde

FR: fator reprodutivo

GFP: do inglês *green fluorescent protein*, proteína fluorescente verde

GS1: Glutamina sintetase citosólica

HNO_3: ácido nítrico.

hpt: gene marcador que codifica a higromicina fosfotransferase

JBU: urease majoritária da semente de *Canavalia ensiformis*

JBUREII: urease secundária de *Canavalia ensiformis*

J2: segundo estádio juvenil

J3: terceiro estádio juvenil

J4: quarto estádio juvenil

kb: Kilobases

kDa: kiloDaltons

N: nitrogênio

NH_4: amônia

NG: sementes que não germinaram

NT: não transgênica

ORF: do inglês *open reading frame*

PCR: do inglês *polymerase chain reaction*

Pi: população inicial

pH: potencial de hidrogênio

P*35S*: promotor do gene *35S* do CaMV

RNA: ácido Desoxirribonucleico

RT-qPCR: transcrição reversa associada a PCR quantitativa em tempo real

T_0: plantas transgênicas recuperadas da cultura *in vitro*

T_1: progênie das plantas transgênicas recuperadas da cultura *in vitro*

T_2 progênie das plantas transgênicas da progênie T_1

T_3 progênie das plantas transgênicas da progênie T_2

T*35S*: terminador do gene *35S* do CaMV

UTR: região não-traduzida das bordas do mRNA

VE: estádio fenológico da planta correspondente ao desenvolvimento vegetativo com os cotilédones acima da superfície do solo

VC: estádio fenológico da planta correspondente ao desenvolvimento vegetativo com os cotilédones completamente abertos.

V1: estádio fenológico da planta correspondente ao desenvolvimento vegetativo com as folhas unifolioladas completamente desenvolvidas

V2: estádio fenológico da planta correspondente ao desenvolvimento vegetativo com a primeira folha trifoliolada completamente desenvolvida

V3: estádio fenológico da planta correspondente ao desenvolvimento vegetativo com a segunda folha trifoliolada completamente desenvolvida.

RESUMO

Ureases são tradicionalmente conhecidas por catalisar a hidrólise da ureia em amônia e dióxido de carbono. Em soja, três isoformas de urease foram descritas: 1) urease ubíqua, codificada pelo gene *Eu*4; 2) urease embrião específica, codificada pelo gene *Eu*1; 3) urease SBU-III, codificada por *Eu*5. O nitrogênio (N) é o nutriente mais limitante para o crescimento e desenvolvimento da planta. Portanto, mecanismos eficientes para capturar o N nas suas diversas formas, e realocá-lo, são necessários para otimizar o uso do nutriente pela planta. O produto N da atividade da urease - a amônia é incorporada em compostos orgânicos, principalmente, pela atividade da glutamina sintetase. Assim, a urease está envolvida na remobilização do N, bem como na assimilação do N primário. Um estudo anterior foi realizado por nossa equipe com o objetivo de superexpressar o gene *Eu*4 em plantas de soja. Inesperadamente, as plantas transgênicas exibiram co-supressão do transgene *Eu*4 e de todos os genes endógenos que codificam as isoformas de urease. Foi verificada também, uma diminuição da atividade ureolítica. Visando determinar o papel das ureases no desenvolvimento da soja, foram comparadas plantas transgênicas co-suprimidas, plantas mutantes e seus respectivos controles. O desenvolvimento das plantas foi avaliado 7, 14, 21 e 30 dias após a semeadura. As plantas co-suprimidas apresentaram um atraso no desenvolvimento durante o primeiro mês após a germinação. Um desenvolvimento mais lento foi observado para o duplo mutante *eu1-a/eu4-* e o mutante simples *eu3-a* (este gene codifica uma proteína acessória inativa). A absorção de N nas plantas transgênicas foi significativamente menor do que a das plantas não transgênicas. Entre os mutantes, *eu3-a* apresentou o menor e *eu1-a* o maior conteúdo de N. Um número significativamente menor de sementes foi obtido para as plantas transgênicas. Em conjunto estes resultados indicam que o aconteúdo da urease ou da atividade ureolítica desempenham um papel importante no desenvolvimento da planta.

A soja (*Glycine max*) é afetada por vários estresses bióticos e abióticos, que limitam a distribuição geográfica das culturas e levam a reduções significativas de crescimento e produtividade. No Brasil, as doenças causadas por nematoides são um dos estresses bióticos mais prejudiciais para a soja. As principais espécies encontradas no Brasil são *Meloidogyne spp*. (formadores de galhas), *Heterodera glycines* (cisto), *Pratylenchus brachyurus* (lesões radiculares) e *Rotylenchulus reniformis* (reniforme). Nematoides formadores de galhas e de cisto (nematóides sedentários), os patógenos mais prejudiciais à soja, são muito difíceis de controlar. Neste estudo, foi identificado um peptídeo derivado

da urease de soja (nomeado Soyuretox), que exerce efeito tóxico contra fitonematoides formadores de galhas (*M. javanica*). Soyuretox foi expresso em raízes de plantas compostas plantas transgênicas estáveis de soja. Raízes de plantas compostas e plantas transgênicas estáveis superexpressando Soyuretox exibiram uma redução significativa (50% e 37.5%, respectivamente) no número médio de nematoides e ovos, quando comparado com raízes não transformadas, 45 dias após a inoculação. Este é o primeiro relato de resistência a nematóides causada por um peptideo derivado de uma urease. Soyuretox pode representar uma ferramenta útil bem como uma nova e eficiente alternativa para o controle de pragas e doenças em culturas economicamente importantes.

ABSTRACT

Ureases are traditionally known for catalyzing the hydrolysis of urea to ammonia and carbon dioxide. In soybean, three urease isoforms have been described: 1) ubiquitous urease, encoded by the *Eu*4 gene; 2) embryo-specific urease, encoded by *Eu*1gene; 3) SBU-III urease, encoded by *Eu*5. Nitrogen (N) is the most limiting nutrient for plant growth and development. Therefore efficient mechanisms both to take up N in its various forms, and to reallocate it, are necessary for optimal N use efficiency. The N product of urease activity- ammonia- is incorporated into organic compounds mainly by glutamine synthase activity. Thus, urease is involved in N remobilization, as well as in primary N assimilation. A previous study was performed by our team aiming to overexpress *Eu*4 gene in soybean plants. Unexpectedly, the transgenic plants exhibited endogenous (for all three genes) and introduced *Eu*4 transgene co-suppression and decreased ureolytic activity. Here, we sought to determine urease roles in soybean development by silencing all urease isoforms. Analyses were performed using transgenic co-suppressed and mutant plants. Plant development was evaluated 7, 14, 21 and 30 days after sowing. The co-suppressed plants presented a developmental delay during the first month after germination when compared with control. A slower development was observed for the double *eu1-a/eu4-a* mutant and the *eu3-a* (this gene codify an inactive accessory protein) single mutant. The N uptake in transgenic plants was significantly lower than that captured by non-transgenic plants. Among mutants, *eu3-a* presented the lowest and *eu1-a* the highest N content. A significantly lower number of seeds was obtained for transgenic plants. Altogether, these results indicate that the urease content and/or ureolytic activity play an important role in plant development.

Soybeans (*Glycine max*) are affected by several abiotic and biotic stresses that limit the geographical distribution of cultures and lead to significant reductions in growth and productivity. In Brazil, the diseases caused by nematodes are one of the most damaging biotic stresses for soybeans.. The main species found in Brazil are *Meloidogyne spp*. (root-knot), *Heterodera glycines* (cyst), *Pratylenchus brachyurus* (root lesion) and *Rotylenchulus reniformis* (reniform). Root-knot and cyst nematodes (sedentary nematodes), the most damaging soybean pathogens, are very difficult to control. In this study, we identified a soybean urease-derived peptide (named Soyuretox) that exerts toxic effects against the root-knot phytonematode (*M. javanica*). Soyuretox was expressed in

soybean roots of composite plants and complete stable transgenic plants. Roots of composite plants and stable transgenic plants overexpressing Soyuretox exhibited a significant reduction (50% and 37.5%, respectively) in the average number of nematodes and eggs when compared with non-transformed roots, 45 days after inoculation. This is the first report of nematode resistance caused by a urease-derived peptide. Soyuretox may represent a useful tool as a new and efficient alternative to control pests and diseases in economically important crops.

Capítulo I

INTRODUÇÃO GERAL

1 Introdução Geral

1.1 Soja

A soja [*Glycine max* (L.) Merri.] é uma espécie de planta diploide (2n=40), pertencente à família Fabaceae (Leguminosa), de autofecundação e com ciclo de vida anual. O seu surgimento como cultura alimentar ocorreu no nordeste da China em torno de 1700 – 1100 a.C., sendo uma das mais antigas plantas cultivadas (Hartman et al. 2011). No Brasil o seu plantio em larga escala só teve início em 1960 (Reetz et al. 2008).

A semente da soja composta por 38% de proteína e 18% de óleo. Essas características fazem da soja um dos principais componentes da ração animal e uma importante matéria prima para produção de biocombustíveis. Seus produtos são também bastante utilizados na alimentação humana e na produção de cosméticos (Liu, 2008). O consumo mundial da soja vem aumentando gradativamente nos últimos anos (Liu, 2008; CONAB, 2016).

A oleaginosa é uma cultura de grande importância para o agronegócio do nosso país, sendo responsável por grande parte do aumento das exportações (Liu, 2008; CONAB, 2016). Segundo os dados do USDA (Departamento de Agricultura dos Estados Unidos), na safra mundial de soja em grãos 2015/2016 os Estados Unidos, o Brasil e a Argentina foram responsáveis por 31,95%, 31,82% e 17,61% respectivamente, o que representa 81,37% de toda a produção mundial do grão (CONAB, 2016). De acordo com os dados da CONAB e USDA (2016), na safra de 2015/16 foram produzidas 312.362 milhões de toneladas de soja no mundo. O Brasil aparece como o segundo maior produtor mundial de soja, com 95.631 milhões de toneladas na safra 2015/16, atrás apenas dos EUA. Nos últimos 10 anos obteve-se um incremento de 74,8% no nosso país, como resultado de um aumento de 40,4% na área plantada e de 24,5% na produtividade. Os estados que apresentaram maior produção no período de 2014/15 foram: Mato Grosso com 29,2%, Paraná com 17,8%, Rio Grande do Sul com 15,4% e Goiás com 9,1% da produção. Estima-se para a safra 2016/17, uma produção mundial aproximada de 324 milhões de toneladas e que o Brasil continue sendo o segundo maior produtor, com 103 milhões de toneladas de soja em grãos, ou seja, 6,19% maior que na safra 2015/16 (CONAB, 2016).

Devido a sua importância, a soja vem sendo alvo de constantes esforços para o seu melhoramento genético. Tal fato levou a comunidade científica internacional que trabalha com leguminosas a recomendar a soja como planta modelo para estudos genéticos e moleculares (Gepts et al. 2005). A soja teve seu genoma completamente sequenciado em 2010 (Schmutz et al. 2010), o que tem permitido grandes avanços em estudos de genômica funcional e engenharia genética, que representam ferramentas importantes para estudos básicos, visando contribuir para os programas de melhoramento desta cultura.

Apesar do cenário mundial da soja ser bastante positivo, principalmente para o Brasil, diversos estresses bióticos (fungos, bactérias, nematoides, vírus, ervas daninhas, insetos) e abióticos (seca, alagamento, temperaturas extremas, salinidade, toxicidade de minerais) limitam a distribuição geográfica das culturas e acarretam reduções significativas no desenvolvimento das plantas e na produtividade (Dita et al. 2006). Um dos principais limites impostos pelo ambiente é a suscetibilidade das plantas a inúmeras doenças causadas por fungos, bactérias, vírus e nematoides. A cada ano a severidade de certas doenças vem aumentando devido à expansão da área agrícola e a utilização de cultivares com pouca variabilidade genética (Barros & Borges, 2007; Barros, 2009).

Os nematoides são parasitas que representam uma ameaça significativa para a produção global de alimentos, com perdas anuais estimadas em mais de 80 bilhões de dólares (Nicol et al. 2011). No Brasil diferentes espécies de nematoides atacam as plantações de soja. Dentre estas espécies de fitonematoides destacam-se: formadores de galhas (*Meloidogyne spp*.), de cisto (*Heterodera glycines*), de lesões radiculares (*Pratylenchus brachyurus*) e o reniforme (*Rotylenculus reniformis*) (Pazhavarical, 2009; Dias et al. 2010). Os fitonematoides formadores de galhas apresentam vida sedentária e são de difícil controle pois estabelecem locais de alimentação complexos dentro de suas plantas hospedeiras (Jones et al. 2013). Plantas que são infestadas por esses parasitas apresentam drásticas mudanças morfológicas e fisiológicas, como atraso no crescimento, clorose e baixo rendimento, além do favorecimento da instalação de outros parasitas, como fungos, vírus e bactérias (Bird & Kaloshian, 2003; Williamson et al. 2003).

Os fitonematoides de galhas possuem ampla distribuição geográfica e uma grande disseminação, além de parasitarem quase todas as espécies de plantas vasculares (Sasser, 1979; Moens et al. 2009). Esses fitonematoides no seu estádio juvenil (J2, fase infectante)

são atraídos pelas raízes em crescimento. Através do aparelho bucal em forma de estilete os indivíduos J2 injetam enzimas celulolíticas e proteolíticas que quebram a parede celular, o que auxilia na sua penetração (Moens et al. 2009). Os fitonematoides migram através do cilindro vascular da raíz até encontrarem um local favorável para o seu sítio de alimentação, induzindo a formação de um conjunto de células gigantes, cuja função é a produção de alimentos para os parasitas. Devido à multiplicação dessas células, ocorre um aumento da estrutura das raízes, formando as galhas (Fuller et al. 2008). Os estádios subsequentes de desenvolvimento dos nematoides formadores de galhas são J3, J4 e, finalmente, adulto. Em condições ambientais favoráveis, ocorre a formação das fêmeas que apresentam vida sedentária e, em condições adversas, os machos são formados. Estes deixam a raíz em direção ao solo onde permanecem até sua morte. Os machos não são necessários para completar o ciclo de vida dos nematoides. Ovos viáveis podem ser produzidos pelas fêmeas sem que ocorra a fertilização, ou seja, a reprodução ocorre por partenogênese (Chitwood & Perry, 2009). As fêmeas podem depositar até 1.000 ovos, os quais permanecem em uma massa gelatinosa protetora incorporada nas galhas ou na superfície da raiz (Figura 1) (Moens et al. 2009).

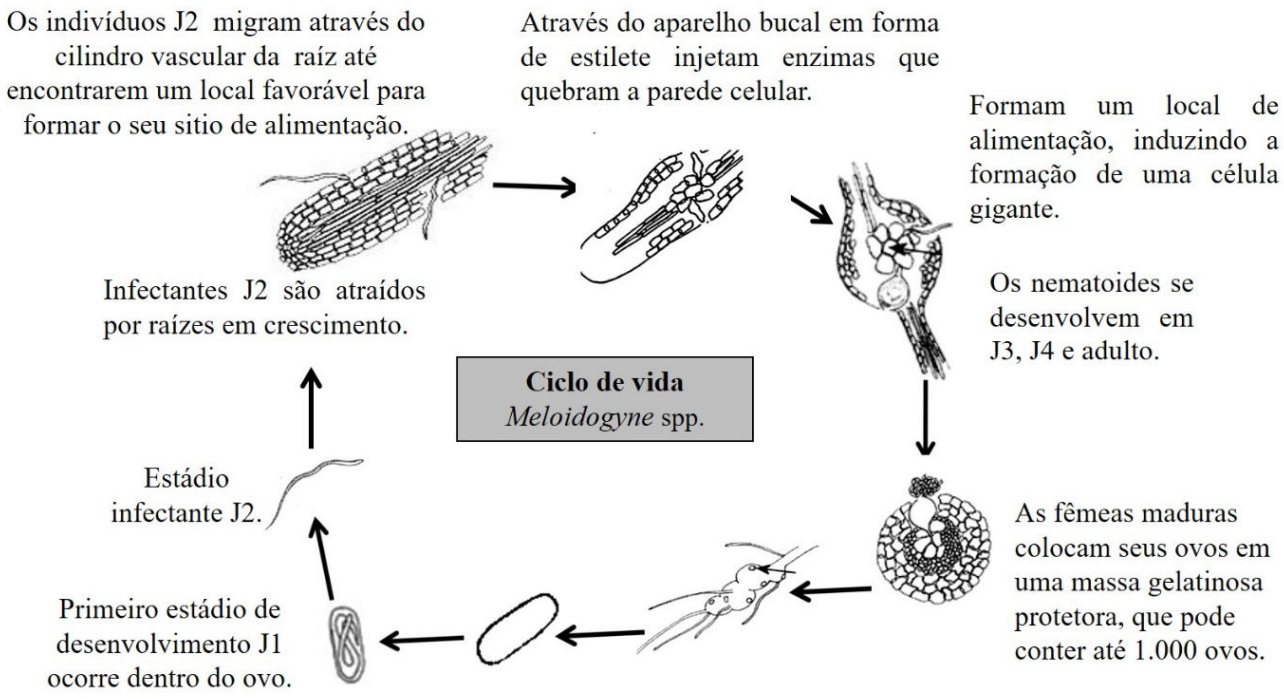

Figura 1. Ciclo de vida de *Meloidogyne* spp. Os estádios de desenvolvimento são divididos em ovo, juvenis: J1(dentro do ovo), J2, J3, J4 e fase adulta. A fêmea permanece na planta onde ovopositam em uma massa gelatinosa protetora, que pode conter até 1.000 ovos. Os ovos são liberados e o ciclo inicia-se novamente. As fêmeas produzem ovos por dois ou três meses e, após este período, podem viver por mais algum tempo naquele local. (Adaptado a partir de Proite, 2007).

Considerando que o prejuízo causado por nematoides em plantas de soja e que o uso dos nematicidas é extremamente prejudicial ao ambiente e não totalmente eficaz no controle desses patógenos (Jeyaratnam et al. 1990; Urwin et al. 2000; COODETEC, 2002; Embrapa Soja, 2016), destaca-se a importância da identificação e do desenvolvimento de cultivares resistentes. Nesse contexto, estratégias biotecnológicas apresentam-se como ferramentas promissoras, destacando-se a prospecção de genes que irão contribuir para a produção de variedades mais resistentes.

1.2 Transformação genética

A transformação genética é descrita como a introdução de um DNA recombinante na célula da planta, usando técnicas de engenharia genética. A condição básica para a

introdução de genes exógenos em plantas ou para estudos funcionais de genes é o desenvolvimento de sistemas eficientes de transformação, que atendam as características de cada espécie (Wiebke-Strohm et al. 2012). Dois diferentes sistemas têm sido desenvolvidos para regenerar plantas *in vitro*: a organogênese de tecido meristemático e a embriogênese somática (Somers et al. 2003). A organogênese é uma via de regeneração que utiliza tecidos oriundos de meristemas, nódulos cotiledonares ou folhas primárias imaturas (Thorpe, 1994). A embriogênese somática consiste na obtenção de células totipotentes a partir de tecidos somáticos diferenciados (Sato et al. 1993). Independentemente do método de transformação utilizado, embriões somáticos secundários (originários a partir de outros embriões somáticos que tendem a ter origem unicelular) são com frequência utilizados para a transformação de soja (Finer 1988; Finer & McMullen, 1991; Merkle et al. 1995).

Para a transformação estável de vegetais dois métodos têm sido utilizados com sucesso: bombardeamento de partículas (Sanford, 1988; Droste et al. 2002; Homrich et al. 2012; Wiebke-Strohm et al. 2012) e o sistema *Agrobacterium* (Horsch et al. 1985; Wang & Xu, 2008; Wiebke-Strohm et al. 2011). O método de bombardeamento de partículas consiste em introduzir o DNA adsorvido à superfície de micropartículas, que podem ser de ouro ou tungstênio (pesadas e inertes para a célula vegetal), num tecido alvo. As micropartículas são aceleradas a uma certa velocidade, atravessam a parede celular, as membranas plasmática e nuclear, e no núcleo o DNA é liberado, podendo ser integrado ao DNA cromossomal (Droste et al. 2002; Homrich et al. 2012; Wiebke-Strohm et al. 2012).

Agrobacterium é uma bactéria aeróbica, gram-negativa, fitopatogênica da família Rhizobiaceae, que naturalmente infecta diferentes plantas (DeCleene & DeLey, 1976). Esses fitopatógenos causam uma variedade de neoplasias incluindo a doença da galha-da-coroa (*crown-gall- A. tumefaciens*) e formação de raízes adventícias (*hairy root- A. rhizogenes*) nos locais de infecção (Gelvin, 2010). *A. tumefaciens* é amplamente utilizada para transferir de forma estável sequências de DNA desejadas para o genoma de células vegetais. Uma das vantagens deste método é o fato de que um menor número de cópias do T-DNA (parte do plasmídeo) são introduzidas no tecido vegetal (Kohli et al. 2003; Wiebke-Strohm et al. 2011).

A. rhizogenes também é capaz de co-transferir o T-DNA de vetores plasmidiais binários para as células hospedeiras, permitindo assim, integrar construções gênicas de interesse de forma estável no genoma vegetal (Christey, 2001; Broothaerts et al. 2005).

Este método tem sido bastante utilizado, pois permite a obtenção de plantas compostas, que apresentam somente raízes transformadas, reduzindo significativamente o tempo necessário para gerar eventos transgênicos, quando comparado aos métodos tradicionais de transformação de soja. As raízes transformadas apresentam crescimento rápido, são altamente ramificadas e capazes de crescer na ausência de reguladores de crescimento (Sevón et al. 1998; Collier et al. 2005). Construções gênicas contendo genes repórteres, tais como *gfp*, que codifica a proteína verde fluorescente (GFP-*green-fluorescent protein*), tem auxiliado bastante no uso desta técnica, para a seleção das raízes transformadas (Collier et al. 2005). O uso da *A. rhizogenes* tem sido bastante importante para aplicações biotecnológicas, tais como a identificação de genes, que quando superexpressos em raízes, apresentam potencial tóxico à patógenos que atacam este orgão (Guillon et al. 2006).

1.3 Ureases

São enzimas multifuncionais, com diferentes domínios de atividade (Carlini e Polacco, 2008). Em 1926, Sumner cristalizou pela primeira vez uma urease da semente de feijão de porco, *Canavalia ensiformis* (*Jack Bean urease*-JBU), provando definitivamente a origem protéica desta enzima. A urease foi a primeira molécula orgânica a ser sintetizada em laboratório (Wöhler, 1828). Estudos realizados com a proteína JBU demonstraram pela primeira vez que se trata de uma metaloenzima dependente de níquel (Dixon et al. 1975).

As ureases são conhecidas tradicionalmente por catalisar a hidrólise da ureia, produzindo amônia e carbamato. Em pH fisiológico, o carbamato é hidrolisado espontaneamente para formar gás carbônico e uma segunda molécula de amônia (Mobley et al. 1995) (Figura 2). A planta somente pode aproveitar o nitrogênio presente na ureia após ser catalisada pela urease (Follmer, 2008). Portanto, a urease está envolvida na remobilização do nitrogênio, bem como na assimilação de nitrogênio primário (Cao et al. 2010).

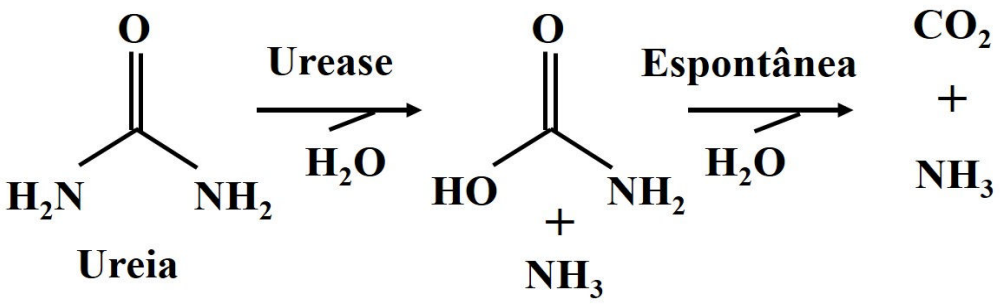

Figura 2. Reação de hidrólise da ureia catalisada pela enzima urease. (Fonte: Adaptado a partir de Witte CP, 2011).

Vários grupos de organismos sintetizam ureases, dentre eles bactérias, fungos e plantas. Estas enzimas são ubiquamente presentes em todos os tecidos das plantas, mas não são encontradas nos animais (Mobley & Hausinger, 1989; Ligabue-Braun et al. 2013). Nas plantas e fungos as ureases são proteínas homo-oligoméricas, formadas por trímeros ou hexâmeros de subunidades idênticas de aproximadamente 90-kDa. No entanto, as ureases de bactérias são multímeros formados por duas ou três subunidades designadas α, β e γ. Independente do organismo as ureases apresentam alto grau de similaridade entre as sequências de aminoácidos (maior que 50% entre bactérias e plantas e maior que 70% dentro de cada grupo) (Figura 3) (Mobley et al. 1995; Follmer, 2008, Krajewska, 2009, Ligabue-Braun et al. 2013). A comparação estrutural das ureases de plantas com as já conhecidas das bactérias *Klebsiella aerogenes* (Jabri et al. 1995), *Bacillus pasteurii* (Benini et al. 1999) e *Helicobacter pylori* (Ha et al. 2001) foi possível devido à resolução da estrutura tridimensional da urease de *C. ensiformis* (JBU) (Balasubramanian & Ponnuraj, 2010), permitindo assim a confirmação do alto grau de similaridade entre as ureases de diferentes organismos.

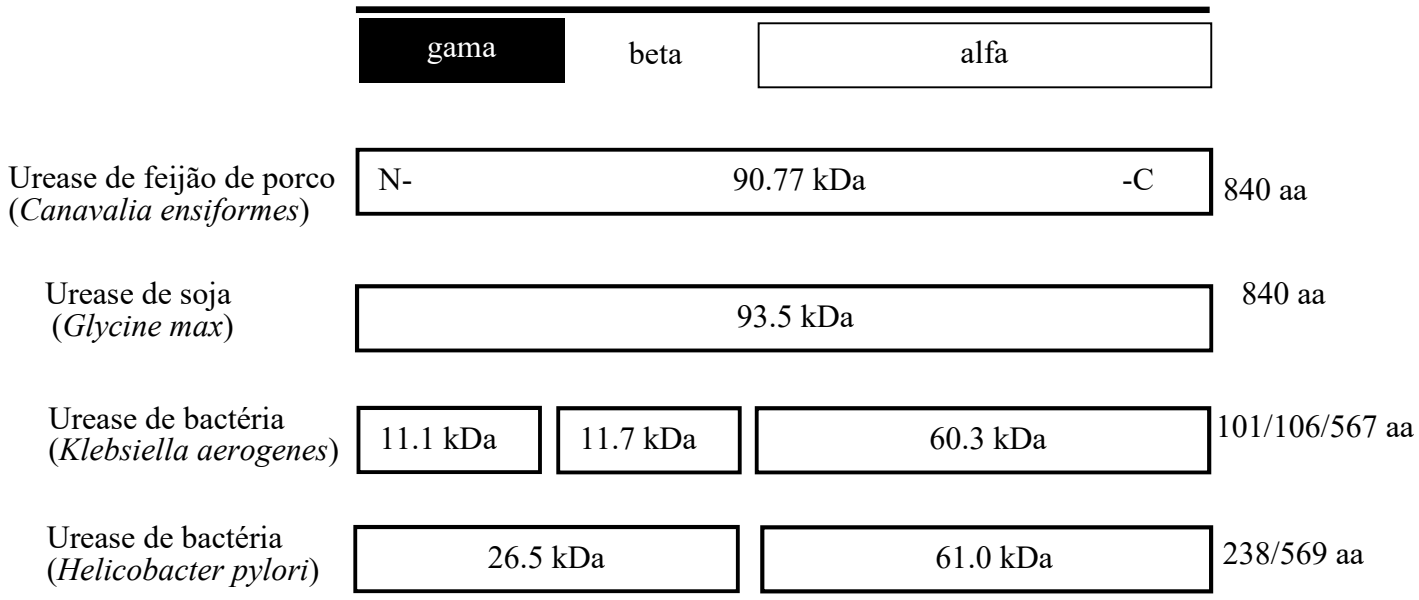

Figura 3. Comparação esquemática dos domínios das ureases de diferentes organismos. Ureases de plantas (*Canavalia ensiformes* e *Glycine max*) são formadas por subunidades idênticas. Ureases bacterianas podem ter três subunidades (*Klebsiella aerogenes*) ou duas (*Helicobacter pylori*). (Fonte: Adaptado a partir de Krajewska, 2009).

A comparação entre as sequências de aminoácidos de 41 ureases sugere que todas as ureases divergiram de uma proteína ancestral comum em Viridiplantae, contudo as ureases de monocotiledôneas e eudicotiledôneas tem evoluído independentemente ao longo do tempo (Figura 4) (Wiebke-Strohm et al. 2016).

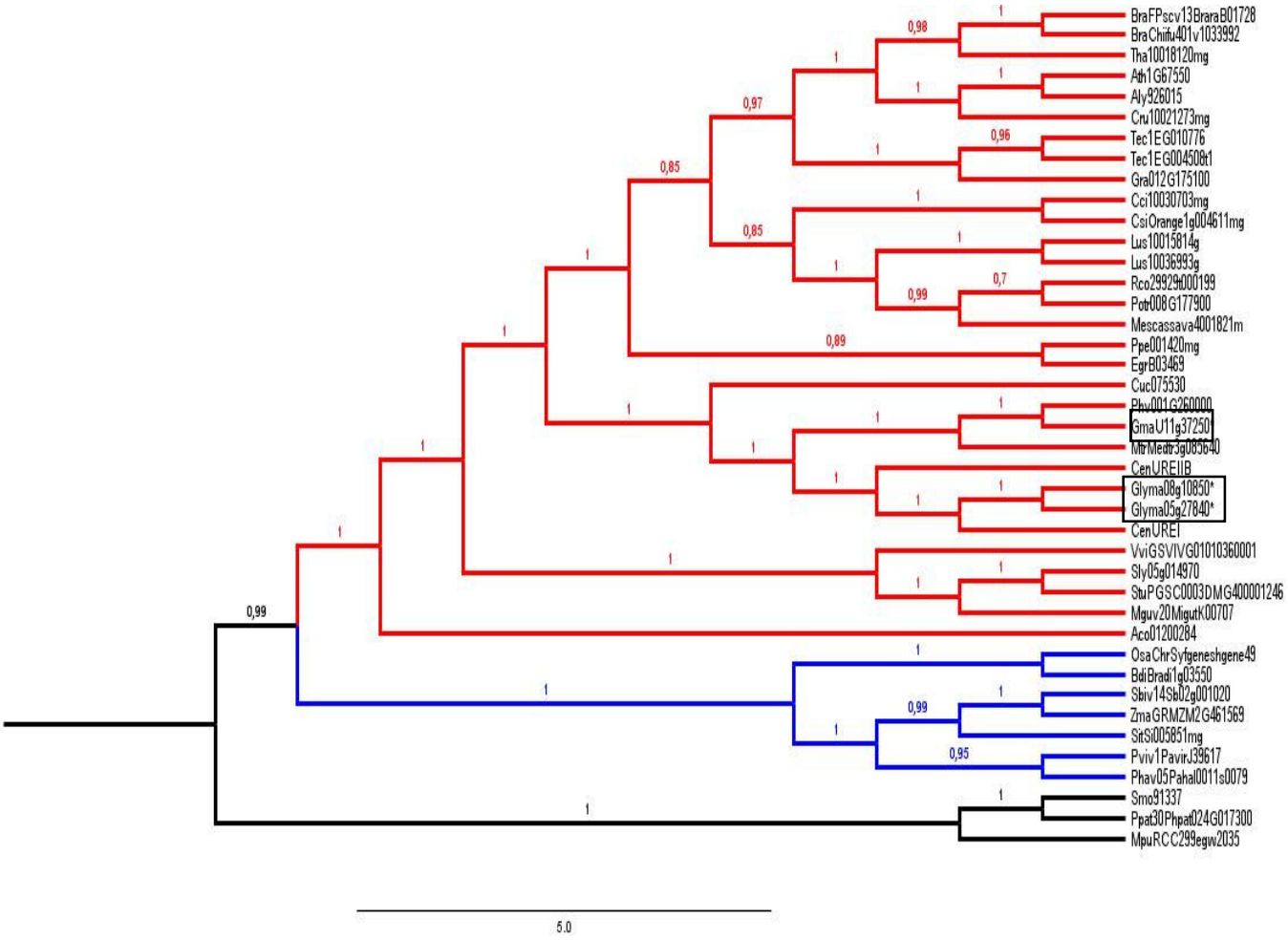

Figura 4. Análise filogenética de ureases de plantas, musgos e algas. Clado em azul consiste em ureases de monocotiledôneas, em vermelho de plantas eudicotiledôneas e em preto clado basal. Asterisco e as caixas representam as ureases de soja. (Fonte: Wiebke-Strohm et al. 2016).

A ativação das ureases é um processo complexo. Nas plantas e bactérias requer a participação de chaperonas chamadas de proteínas acessórias das ureases, para que ocorra a incorporação de dois íons de níquel (Ni) à enzima. Essa ligação é precisa e forte (Follmer, 2008; Balasubramanian & Ponnuraj, 2010). Nas bactérias, quatro proteínas acessórias são necessárias: UreD, UreF, UreG, UreE, que formam um complexo que se liga ao metalocentro, sendo UreE uma chaperona de Ni que facilita a entrega desse metal para o sítio ativo da urease (Mobley et al. 1995; Zambelli et al. 2011). Em plantas e fungos o processo de incorporação do níquel é pouco conhecido. Foram identificadas para soja

três proteínas acessórias (UreD, UreF, UreG) (Figura 5) (Freyermuth et al. 2000; Bacanamwo et al. 2002; Polacco et al. 2011).

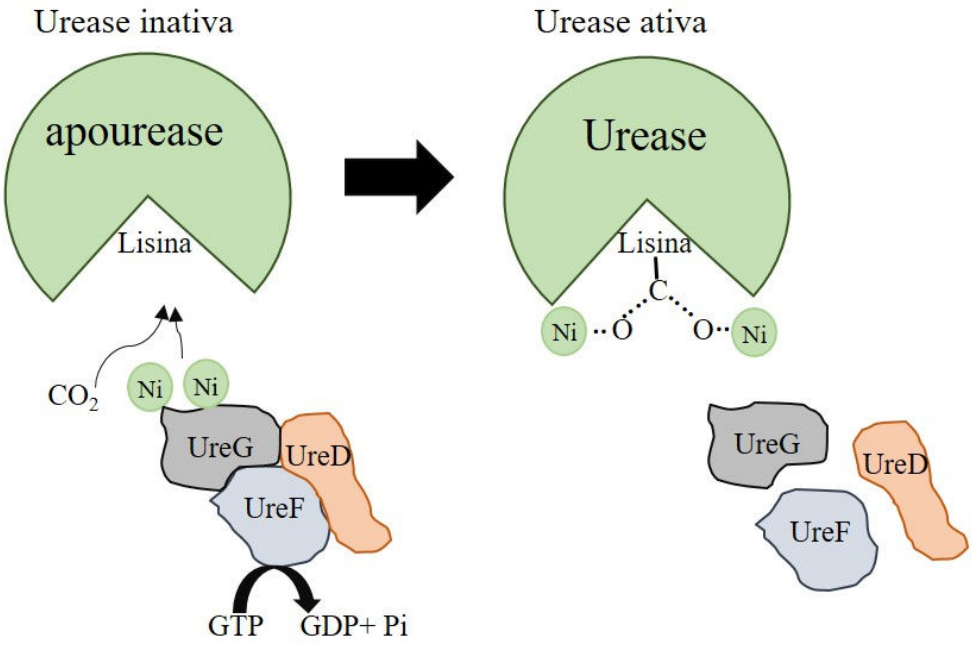

Figura 5. Modelo da ativação de urease de planta envolvendo a ligação das três proteínas acessórias (UreD, UreF e UreG) à apourease: modificação covalente de um sítio ativo de lisina e incorporação específica de dois íons de níquel por sítio ativo. As proteínas acessórias se dissociam da urease após a ativação. (Fonte: Adaptado a partir de Witte CP, 2011).

1.4 Ureases em Plantas

Grande parte dos estudos com ureases vegetais foram realizados com *C. ensiformis* e *G. max*. As ureases de *C. ensiformis* (feijão de porco) são melhor caracterizadas em nível bioquímico (Sirko & Brodzik, 2000; Carlini e Polacco, 2008; Follmer, 2008).

Em *C. ensiformis* são encontradas três isoformas estruturais da urease: JBU foi a primeira enzima a ser cristalizado por Summer em 1926. A segunda isoforma foi isolada e caracterizada em 1981 e denominada canatoxina (CNTX), sendo menos abundante na semente do que JBU (Carlini e Guimarães, 1981; Follmer et al. 2001). Uma terceira proteína desta família (JBUREII) foi encontrada em diferentes etapas de desenvolvimento das flores, dos embriões e da plântula (Pires-Alves et al. 2003; Demartini et al. 2011;

Mulinari et al. 2011). Até o presente momento apenas JBU e JBUREII têm as sequências completas de DNA/aminoácidos elucidadas (Mamiya et al. 1985; Riddles et al. 1991; Pires-Alves et al. 2003; Mulinari et al. 2011).

Em soja são descritas três isoformas, codificadas por três genes estruturais independentes. A urease ubíqua, codificada pelo gene *Eu*4 (Glyma11g248700; GenBank acesso AJ276866), expresso em níveis baixos em todos os tecidos vegetais, é responsável pela reciclagem da ureia derivada do metabolismo (Polacco et al. 1985; Torisky et al. 1994; Goldraij et al. 2003). A urease embrião-específica, codificada pelo gene *Eu*1 (Glyma05g146000; GenBank acesso NM001249869), é altamente expressa em embriões em desenvolvimento e acumulada em sementes maduras (Polacco & Havir, 1979; Polacco & Winkler, 1984; Polacco & Holland, 1993). Um terceiro gene codificando uma urease foi recentemente identificado no genoma da soja (Polacco et al. 2011; Polacco et al. 2013). Este gene foi denominado *Eu*5 (Glyma08g103000) e seu produto SBU-III, apresenta uma maior similaridade com a urease embrião-especifica (Figura 4). Baixos níveis de transcritos foram detectados em sementes após um dia da quebra da dormência, raízes jovens e sementes em desenvolvimento (Wiebke-Strohm et al. 2016).

Afim de definir o papel das ureases de soja, experimentos foram direcionados para a obtenção de mutantes nulos por bombardeamento com nêutrons rápidos cuja coleção pertence à Universidade de Missouri, EUA. Mutantes nulos para a urease embrião-específica (*eu1*-a) foram obtidos com sucesso. Esses mutantes não exibem nenhuma alteração fisiológica na planta (Polacco et al. 2011). Mutantes *missense* para urease ubíqua foram denominados *eu4-a* e *eu4-b*, produzem uma proteína inativa e exibem substancial redução na atividade ureolítica em folhas, raízes e hipocótilos, devido a mutação ser na região de inserção do níquel (Torisky & Polacco, 1990; Stebbins et al. 1991; Witte et al. 2002; Goldraij et al. 2003; Polacco et al. 2011). Por outro lado, mutantes nulos para a urease ubíqua nunca foram obtidos. Os duplos mutantes (*eu1-a/eu4-a*), obtidos através do cruzamento entre os mutantes para urease ubíqua e embrião-específica, são praticamente desprovidos de atividade ureolítica (Stebbins e Polacco, 1995; Goldraij et al. 2003). Mutante nulo para o gene *Eu*3 que codifica a proteína acessória UreG, necessária para a incorporação do níquel e ativação da urease, também foi obtido e não apresenta atividade ureolítica (Freyermuth et al. 2000).

1.5 Papel das Ureases

1.5.1 Papel das Ureases em relação à atividade ureolitica

As ureases desempenham diferentes funções biológicas nas plantas (Follmer et al. 2004; Carlini e Polacco, 2008; Carlini e Ligabue-Braun, 2016). A primeira função atribuída é a atividade ureolítica. Grande parte do nitrogênio (N) utilizado pela planta para seu crescimento e desenvolvimento só pode ser aproveitada a partir da ureia, que é uma importante fonte interna e externa de N (Follmer, 2008; Wang et al. 2008). A arginina, o principal armazenador de N, é convertida pela enzima mitocondrial arginase em ureia e ornitina. A ureia é transportada para o citoplasma e pela ação da urease é convertida a dióxido de carbono e amônia, que é incorporada em compostos orgânicos, principalmente pela atividade da glutamina sintetase (Figura 6) (Mobley et al. 1995; Goldraij e Polaccoc, 1999, 2000; Sirko e Brodzik, 2000). Portanto, a urease exerce um papel importante na remobilização e assimilação do N necessário para o desenvolvimento (Cao et al. 2010).

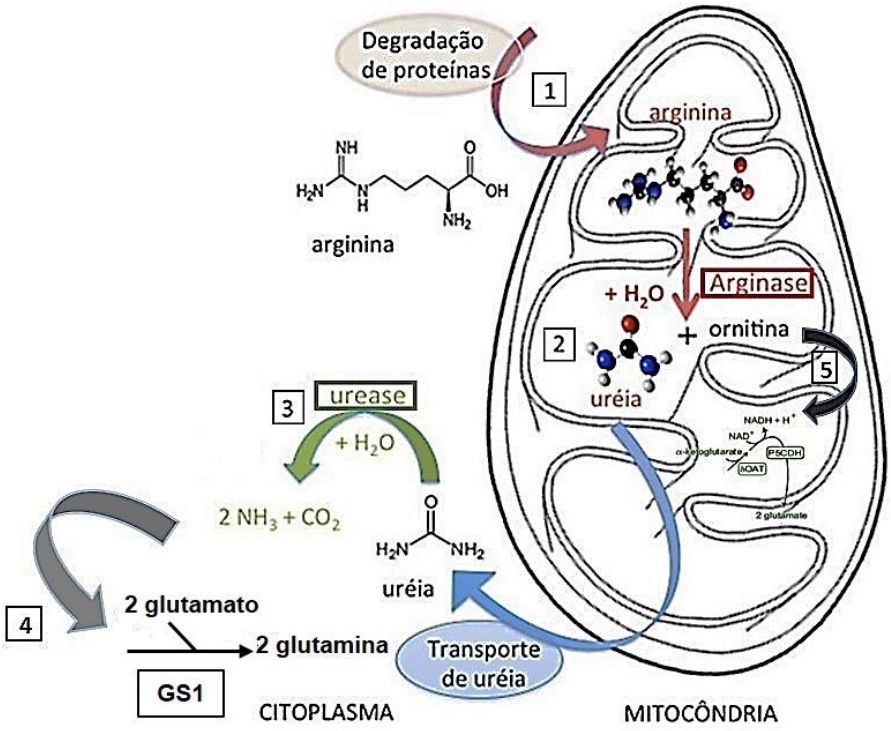

Figura 6. Metabolismo da uréia em plantas. O aminoácido arginina proveniente da degradação de proteínas **(1)**. A arginina é hidrolisada na mitocôndria pela arginase,

produzindo ureia e ornitina **(2)**. A ureia deixa a mitocôndria e é hidrolisada pela urease **(3)**. O produto da atividade da urease, a amônia, é liberado e re-assimilado pela glutamina sintetase citosólica (GS1) **(4)**. O glutamato gerado a partir da ornitina é exportado da mitocôndria e serve como substrato para a reação citosólica (GS1) **(5)**. A urease é requerida para mobilizar metade do nitrogênio da arginina. (Fonte: Adaptado a partir de Witte CP, 2011 e Laprotox: Disponível em http://www.ufrgs.br/laprotox/>Acesso em setembro de 2016).

Devido à importância do nutriente N para o crescimento da planta, torna-se necessário um mecanismo eficiente para capturar e realocar fontes de N para sua utilização em processos essenciais da planta como a germinação de sementes e senescência (Witte CP, 2011). Em *Arabidopsis thaliana* a importância da urease é destacada pelo fato de que quando suas sementes são tratadas com um inibidor para esta enzima sua germinação é atrasada (Zonia et al. 1995). Os mutantes de soja *eu3-a eu2*-a, *eu4-a* e *eu4-b*, que exibem atividade ureolítica nula, apresentam acúmulo de ureia e a presença de manchas necróticas na ponta das folhas (Polacco & Holland, 1993; Goldraij et al. 2003).

1.5.2 Papel de defesa das ureases

As ureases não estão envolvidas apenas na remobilização e assimilação do N necessário para o desenvolvimento da planta, mas também na defesa de plantas contra patógenos tais como fungos e insetos (Carlini e Polacco, 2008; Stanisçuaski e Carlini, 2012). Os domínios da atividade fungicida e inseticida são independentes da capacidade de hidrólise da enzima, devido as funções de defesa continuarem agindo mesmo após as ureases serem tratadas quimicamente com um inibidor da atividade ureolítica (Polacco e Holland, 1993; Follmer et al. 2004; Becker-Ritt et al. 2007).

Estudos evidenciaram que as ureases purificadas a partir de plantas apresentam uma toxicidade contra insetos e fungos, com atividade independente da catalítica. Alguns trabalhos (Carlini et al. 1997; Ferreira da Silva et al. 2002) mostraram que duas isoformas de *Canavalia ensiformis*, Canatoxina (CNTX) e *Jack bean* urease (JBU), e a urease embrião específica de soja (Follmer et al. 2004) são tóxicas para insetos que apresentam no seu trato digestivo enzimas proteolíticas do tipo catepsinas, que degradam a proteína e liberam um peptídeo tóxico. Para JBU o peptideo liberado apresenta aproximadamente ~10 kDa, não sendo tóxico para insetos com digestão baseada em enzimas serínicas do

tipo tripsina (Carlini et al. 1997; Stanisçuaski et al. 2005; Piovesan et al. 2008; Stanisçuaski e Carlini, 2012).

A função inseticida das ureases parece restrita à urease de plantas, o que provavelmente se deve ao fato de pelo menos parte do peptídeo entomotóxico corresponder ao gap presente entre os domínios α e β das ureases bacterianas (Figura 3) (Follmer et al. 2004). O peptídeo entomotóxico de ~10 kDa isolado a partir da canatoxina é denominado pepcanatox (Carlini et al. 1997; Carlini et al. 2000). Posteriormente, a região correspondente a um outro peptideo tóxico foi identificada, isolada e clonada a partir do gene *jbureII*, outra isoforma de urease da *C. ensiformis*. O peptídeo resultante foi denominado jaburetox-2Ec (Mulinari et al. 2007) ou jaburetox (Postal et al. 2012).

O jaburetox recombinante mostrou atividade inseticida contra ninfas de *Dysdercus peruvianus* e *Rhodnius prolixus* (Stanisçuaski et al 2005, 2009). Curiosamente, o peptídeo não mostrou somente toxicidade a esses insetos, mas também a insetos que não eram afetados por ingestão da urease intacta como a lagarta *Spodoptera frugiperda*, que apresenta digestão baseada em enzimas serínicas do tipo tripsina (Mulinari et al. 2007). Por outro lado, mostrou-se inócuo se injetado intraperitonealmente ou administrado por via oral em ratos neonatos e camundongos (Mulinari et al. 2004, 2007; Tomazetto et al. 2007). O peptídeo tóxico age diretamente sobre túbulos de Malpighi isolados do inseto. Os polipeptídeos inibem a diurese em doses abaixo do nanomolar, sendo que as rotas de sinalização recrutadas pela urease e pelo peptídeo para produzir o efeito antidiurético são diferenciadas (Stanisçuaski et al. 2009).

Quanto à atividade antifúngica, as ureases e jaburetox têm mostrado exercer forte propriedades contra fungos fitopatogênicos filamentosos e leveduras na concentração de 10^{-5} M (peptideo) a 10^{-8} M (ureases), inibindo o crescimento vegetativo *in vitro* de fungos filamentosos (Becker-Ritt et al. 2007; Postal et al. 2012; Wiebke-Strohm et al. 2012). O polipeptídio afeta a permeabilidade da membrana e interfere no metabolismo energético de leveduras (Becker-Ritt et al. 2007; Postal et al. 2012).

1.6 Efeito da superexpressão de genes que codificam ureases de soja

Embora investigações com ureases venham sendo realizadas há quase um século, estudos relacionados a sua função e seu modo de ação merecem ainda atenção, pois não

estão totalmente compreendidos. O estudo da superexpressão dos genes que codificam ureases é uma forma de auxiliar em uma melhor compreensão de suas funções.

Experimentos de transformação de soja por bombardeamento com o objetivo inicial de gerar plantas que superexpressassem a urease ubíqua (*Eu*4) e possivelmente resistentes a adversidades bióticas foram conduzidos em nosso laboratório (Laboratório de Cultura de Tecidos e Transformação Genética de Plantas, do Departamento de Genética da UFRGS). Vários eventos foram obtidos, mas ao contrário da superexpressão prevista, houve uma co-supressão do gene alvo na maioria das plantas. Os níveis de mRNA da urease ubíqua de todas as plantas transgênicas eram menores que os observados em plantas não transgênicas, submetidas às mesmas condições de cultura. O mesmo foi observado para a atividade ureásica, indicando um menor acúmulo da enzima nas plantas transgênicas. Bioensaios realizados no estudo com estas plantas mostraram que a falta da urease ubíqua torna as plantas mais susceptíveis a diversos fungos (Wiebke-Strohm et al. 2012).

Algumas hipóteses foram levantadas para explicar o ocorrido: o grande número de cópias integradas ao genoma na transformação por bombardeamento, a intolerância das plantas a altos níveis da urease ubíqua, a existência de um mecanismo endógeno de auto-regulação dos níveis de mRNA ou a toxicidade da proteína (Wiebke-Strohm et al. 2011). Após inúmeras tentativas de transformação através do método que integra bombardeamento e *Agrobacterium* (Wiebke-Strohm et al. 2011) foi obtida uma planta transformada contendo um menor número de cópias do transgene e com níveis de expressão do gene aumentados. Contudo, está planta apresentou desenvolvimento lento e acabou morrendo (dados não publicados). Tal fato, motivou a busca de outras alternativas para superexpressão das ureases em soja, sendo a opção mais oportuna, em função do número de estudos prévios, a expressão de partes dos genes que codificam os peptídeos tóxicos.

Tendo em vista que as ureases são potencialmente multifuncionais, as plantas co-suprimidas são consideradas uma ferramenta importante para a determinação das funções adicionais à atividade ureásica. Considerando que mutantes nulos para a urease ubíqua (*Eu*4) nunca foram obtidos até o presente momento, as plantas transgênicas co-suprimidas representam uma poderosa ferramenta para estudos funcionais que poderão contribuir para a elucidação do papel das ureases de soja em outros processos biológicos, tais como o desenvolvimento de plantas.

1.7 Peptídeo recombinante derivado de urease de soja

Mais recentemente a equipe da doutora Célia Carlini do Laboratório de Proteínas Tóxicas (Laprotox) da UFRGS, identificou a região que corresponde ao peptídeo tóxico da urease ubíqua de soja, que foi denominado Soyuretox (Kappaun, 2014). A identificação da sequência interna da urease ubíqua de soja colinear com a sequência interna do Jaburetox peptídeo derivado de uma urease de *C. ensiformis*, foi realizada através do alinhamento entre os aminoácidos componentes do Jaburetox e a sequência de aminoácidos correspondente na urease ubíqua. O peptídeo da urease ubíqua da soja foi chamado de Soyuretox e apresenta 72% de identidade com o Jaburetox (figura 7) (Kappaun, 2014).

Figura 7. (A) Alinhamento da sequência de amino ácidos de Soyuretox e Jaburetox. (*) correspondem em ambos os peptídeos. (:) forte similaridade; (.) fraca similaridade. **(B)** Sequência de aminoácidos da urease ubíqua traduzida do gene *Eu*4 de

Glycine max (Glyma11g37250;AJ276866), com sequência do peptídeo interno Soyuretox marcada em cinza. Fonte: (Kappaun, 2014).

1.8 Justificativa

Assim como os insetos, os nematóides também apresentam no seu trato digestório enzimas proteolíticas das classes cisteína e aspártico proteases, o que sugere um possível potencial tóxico das ureases vegetais também contra nematóides. Até o presente momento nenhum trabalho foi realizado para compreender a resposta das ureases frente ao ataque desse patógenos.

O presente trabalho foi desenvolvido com o objetivo de testar o possível efeito tóxico das ureases contra nematoides. Como mencionado acima, em nosso laboratório foram realizados experimentos de transformação por bombardeamento com o objetivo inicial de gerar plantas de soja que superexpressassem a urease ubíqua de soja (Wiebke-Strohm et al. 2012). Tendo em vista a dificuldade de superexpressar a urease intacta, utilizou-se a alternativa de isolar o peptídeo tóxico denominado Soyuretox, derivado da urease ubiqua da soja e superexpressá-lo na planta, visando maior resistência a adversidades bióticas.

1.9 Objetivos

1.9.1 Objetivos gerais

Os objetivos gerais deste trabalho foram (1) caracterizar em nível molecular a progênie de plantas transgênicas com o gene *Eu*4 co-suprimido e investigar o papel das ureases de soja durante o desenvolvimento das plantas. (2) caracterizar *in vivo* a toxicidade do peptídeo Soyuretox, codificado por parte do gene *Eu4* da soja, contra nematoides, usando como modelo o fitonematoide formador de galhas *Meloidogyne javanica*.

1.9.2 Objetivos específicos

a) Caracterizar molecularmente a progênie de plantas transgênicas com os genes que codificam ureases co-suprimidos.

b) Determinar o perfil de expressão das ureases codificadas pelos genes *Eu*1*,* *Eu*4 e *Eu*5 em plantas transgênicas e não transgênicas; mutantes (*eu1-a, eu4-a, eu1-a/eu4-a*, *eu3-a*) e não mutantes.

c) Avaliar o padrão temporal de desenvolvimento de plantas transgênicas e não transgênicas, mutantes (*eu1-a, eu4-a, eu1-a/eu4-a*, *eu3-a*) e não mutantes.

d) Obter plantas compostas através da transformação por *Agrobacterium rhizogenes* superexpressando nas raízes o peptídeo Soyuretox.

e) Obter e regenerar plantas estavelmente transformadas, por bombardeamento de partículas, superexpressando o Soyuretox.

f) Caracterizar em nível molecular as plantas transgênicas obtidas pela transformação de raízes e por transformação estável.

g) Desafiar as raízes transgênicas das plantas compostas e das plantas completamente transformadas com o fitonematoide formador de galhas, *M. javanica*;

h) Comparar o fator reprodutivo do nematoide *M. javanica* nas raízes transgênicas das plantas compostas, completas e raízes controles (plantas com raízes não-transformadas).

Os capítulos seguintes desta tese incluem um artigo já aceito para a publicação na revista Genetics and Molecular Biology, da Sociedade Brasileira de Genética, um manuscrito submetido a revista Transgenic Research e o de Termo de Adição encaminhado a um pedido de patente previamente depositado no INPI. Os resultados destes trabalhos também foram apresentados de forma parcial à comunidade científica em eventos nacional e internacional. Os resultados obtidos neste trabalho podem fornecer uma alternativa para a obtenção de plantas de soja mais resistentes a adversidades bióticas.

Capítulo II

Effect of soybean ureases on seed germination and plant development

Manuscrito aceito para a publicação na revista Genetics and Molecular Biology

RESUMO

Ureases são tradicionalmente conhecidas por catalisar a hidrólise da ureia para amônia e dióxido de carbono. Três isoformas são descritas para a soja: urease ubiqua, urease embrião especifica e urease nova. Um estudo anterior realizado pelo nosso grupo, teve como objetivo superexpressar a urease ubiqua em plantas de soja. Inesperadamente, as plantas transgênicas exibiram co-supressão do transgene e dos genes endógenos. O objetivo deste trabalho foi determinar o papel das ureases no desenvolvimento da soja. As análises foram realizadas com plantas transgênicas, plantas mutantes e seus respectivos controles.

Effect of soybean ureases on seed germination and plant development

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Short running title: Role of ureases in plant development

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Abstract

Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide. The ammonia (nitrogen (N) product of urease activity) is incorporated into organic compounds. Thus, urease is involved in N remobilization, as well as in primary N assimilation. Two urease isoforms have been described for soybean: the embryo-specific, encoded by the *Eu*1 gene, and the ubiquitous urease, encoded by *Eu*4. A third urease-encoding gene was recently identified, designated *Eu*5, which encodes the putative protein product SBU-III. The present study aimed to evaluate the contribution of soybean ureases to seed germination and plant development. Analyses were performed using *Eu*1/*Eu*4/*Eu*5-co-suppressed transgenic plants and mutants of the *Eu*1 and *Eu*4 urease structural genes, as well as a urease-null mutant (*eu3-a*) that activates neither the ubiquitous nor embryo-specific ureases. The co-suppressed plants presented a developmental delay during the first month after germination; shoots and roots were significantly smaller and lighter. Slower development was observed for the double *eu*1-*a*/*eu*4-*a* mutant and the *eu*3-*a* single mutant. The N content in transgenic plants was significantly lower than in non-transgenic plants. Among the mutants, *eu*3-*a* presented the lowest and *eu*1-a the highest N content. Altogether, these results indicate that increased ureolytic activity plays an important role in plant development.

Keywords: Plant development, functional study, transgenic plants, mutants, urease

Introduction

Nitrogen (N) is the most limiting plant nutrient, possibly after fixed carbon, for plant growth and development (Marschner, 2012). Therefore, efficient mechanisms both to take up N in its various forms and to reallocate it are necessary for optimal N use efficiency (Witte, 2011). In plant cells, urea is an important internal and external source of N that must be converted to ammonia for N assimilation (Wang *et al.,* 2008). In nature, two major biochemical processes lead to urea production: (1) arginase-catalyzed production of urea (and ornithine) from arginine (a major N storage form); (2) purine degradation to glyoxylate and urea. While arginases are active in plants, purine degradation exclusively through urea, though occurring in many bacteria, fungi and algae, does not occur in soybean and Arabidopsis. Rather, in these dicotyledonous plants, the purine degradation product allantoin, which contains the four ring N atoms of purine, is degraded to four ammonia molecules, bypassing a urea intermediate (summarized in Witte, 2011).

Nonetheless, plant assimilation of arginine-derived urea is important for efficient N use, both in mobilization of seed N reserves during germination, as well as in remobilization of N in senescing tissues. In soybean, arginine is the major amino acid repository of seed N (Micallef and Shelp, 1989), and one of the predominant amino acids in angiosperm seed protein in general (Van Etten *et al.,* 1963). In soybean, arginase action during germination releases much urea, which is hydrolyzed by urease action (Goldraij and Polacco, 1999, 2000)

The importance of urease for recycling N was highlighted in aged *Arabidopsis thaliana* seeds that failed to germinate when urease was chemically inhibited, but could be rescued by an external N source (Zonia *et al.,* 1995). And, according to Bohner *et al.,* (2015) 13% of N exported out of senescing leaves of *A. thaliana* via the petiole is urea.

Urease-negative soybean (mutants and nickel-deprived wildtype) accumulate urea in necrotic leaf tips (Stebbins *et al.,* 1991) to levels approaching 2.5% dry weight (Eskew *et al.,* 1983). We note that in Stebbins, (1991) and Eskew *et al.* (1983) available N was not limiting.

Urea can be hydrolyzed by two different enzymes: urease and an ATP (and biotin)-dependent urea carboxylase/allophanate hydrolase. The latter, found in some fungi, algae and at least one bacterium (Kanamori *et al.,* 2004), has never been reported in plants. Rather, all plants appear to have a urease (Hogan *et al.,* 1983 and our own observations). In soybean and Arabidopsis, urea nitrogen is only available after urea hydrolysis by urease (Goldraij *et al.,* 2003; Witte*,* 2011; Polacco *et al.,* 2013). Urease (EC 3.5.1.5) was the first identified nickel-dependent metalloenzyme (Dixon *et al.,* 1975), and much has been learned of the construction and function of its metallocenter (Carter *et al.,* 2009). Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide. In addition to plants, ureases are synthesized by bacteria, fungi and algae (Krajewska, 2009). The N product of urease activity – ammonia - is incorporated into organic compounds mainly by glutamine synthase activity (Mobley *et al.,* 1995; Sirko and Brodzik, 2000). Thus, urease is involved in N remobilization, as well as in primary N assimilation (Cao *et al.,* 2010).

In addition to the N assimilatory function of urease, plant ureases appear to have defensive roles against herbivore and fungal attack (Carlini and Ligabue-Braun, 2016). In soybean, three urease isoforms have been described. The ubiquitous urease, encoded by the *Eu*4 gene, is expressed at low levels in all tissues and is responsible for recycling both metabolically-derived and exogenous urea (Polacco *et al.,* 1985; Torisky *et al.,* 1994; Goldraij *et al.,* 2003). The embryo-specific urease, encoded by *Eu*1, is highly expressed in developing embryos and accumulates in mature seeds (Polacco and Havir, 1979;

Polacco and Winkler, 1984; Polacco and Holland, 1993). A third urease-encoding gene was recently identified in the soybean genome (Polacco et al. 2011, 2013). This gene was designated *Eu*5, and its putative protein product was named SBU-III. *Eu*5 is expressed in the first stages of root development and during seed maturation. Its transcript levels are lower than those of the other two soybean urease isoforms (Wiebke-Strohm et al 2016).

Urease-negative mutant soybean plants were examined to ascertain the role(s) of the urease isoforms. An embryo-specific urease null mutant (*eu1-a*) seems not to exhibit an altered physiology (Polacco *et al.,* 2011). Ubiquitous urease missense mutants (e*u4-a* and e*u4-b*) produce an inactive protein and display no ureolytic activity in leaves, roots and hypocotyls (Torisky and Polacco, 1990; Stebbins *et al.,* 1991; Witte *et al.,* 2002, Goldraij *et al.,* 2003; Polacco *et al.,* 2011). e*u4* callus cultures cannot use 5 mM urea as N source, but are resistant to 50 mM urea in the presence of a standard $NH_4^+ + NO_3^-$ N source, and show growth responses contrary to those of *Eu4* cultures (Goldraij *et al.,* 2003). The *eu*1-*a*/*eu*4-*a* double mutants were considered virtually devoid of ureolytic activity (Stebbins and Polacco, 1995; Goldraij *et al.,* 2003).

A null mutant for the *Eu*3 gene, which encodes an accessory protein, UreG, necessary for urease activation, has also been characterized (Freyermuth *et al.,* 2000). There is only a single copy of this gene in the soybean genome, and the deletion mutant *eu*3-*a* exhibits a complete loss of urease activity (Stebbins and Polacco, 1995; Polacco *et al.,* 2011; Tezotto *et al.,* 2016).

A previous study was performed by our team aiming to overexpress *Eu*4 in soybean plants. Unexpectedly, the transgenic plants exhibited co-suppression of the endogenous and the introduced *Eu4* transgene, resulting in decreased ureolytic activity (Wiebke-Strohm *et al.,* 2012). As null mutants for the ubiquitous urease have not been obtained to date, the co-suppressed transgenic plants represent a powerful tool for

functional gene studies. Here, we sought to determine the roles of urease in soybean development by elimination of all urease isoforms.

Material and Methods

Plant material and growth conditions

Homozygous *eu1-a, eu4-a*, *eu*1-*a*/*eu*4-*a* and *eu3-a* mutants have been described previously. All, except *eu*1-*a*, were recovered from EMS (ethyl methane sulfonate) mutagenesis of cv. Williams, and were subsequently outcrossed to Wiliams 82. The original *eu*1-*a* mutation was recovered from the 'Itachi' landrace and introgressed into Williams by Dr Dick Bernard (University of Illinois-Champaign-Urbana) by five crosses. Thus, the genetic background of these mutants is the Williams (*eu*1-*a)* and Williams82 (*eu*4-*a*, *eu*1-*a*/*eu*4-*a* and *eu*3-*a*) cultivars. Williams and Williams82 are supposedly isogenic, except for a fungal resistance gene introgressed into Williams 82 (Bernard and Cremeens, 1988).

Two independent transgenic events (A3 and A8) of soybean cultivar IAS5 that presented co-suppression of *eu4* were obtained from bombarded embryogenic tissue. The vector used for transformation contained the *Eu*4 and the *gfp*-encoding sequences (Wiebke-Strohm *et al.,* 2012). Plants derived from non-transgenic embryogenic tissues submitted to the same culture conditions were recovered and used as a control. Subsequent generations were obtained by self-fertilization of plants.

Transgenic seeds of A3 and A8 events (from T_1, T_2 and T_3) were placed in Petri dishes containing sterile filter paper moistened with sterile distilled water for 24 h. Seeds expressing the *gfp* reporter were selected under blue light using a fluorescence stereomicroscope (Olympus®), equipped with a BP filter set with a 488 nm excitation

filter and a 505-530 nm emission filter. GFP-positive and negative plants were also PCR-screened to confirm presence/absence of the transgene using the protocol described by Wiebke-Strohm *et al.* (2012). Positive transgenic, as well as non-transgenic seeds, were sown in organic soil and maintained in a greenhouse until maturity at FUNDACEP-CCGL (Cruz Alta, RS, Brazil) and supplemented with a nutrient solution containing either NO_3 or NH_4 (as N source).

For seed germination and developmental evaluation, GFP-positive transgenic (T_2), mutants, IAS5 non-transgenic and Williams82 seeds were sown in pots containing vermiculite and maintained for one month in a growth chamber at 26 ± 1º C with a 16/8 h light/dark cycle at a light intensity of 22.5 $\mu Em^{-2}s^{-1}$. Plants were not supplemented with any nutrient solution during the first 30 days of development**.**

RNA extraction, cDNA synthesis and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from roots with Trizol reagent (Invitrogen) and treated with DNase I (Invitrogen) according to the manufacturers' instructions. First-strand cDNAs were obtained using 1 µg of DNA-free RNA, M-MLV Reverse Transcriptase SystemTM (Invitrogen) and oligo(dT) primers.

RT-qPCR was performed on a StepOne Real-time Cycler™ (Applied Biosystems). PCR-cycling conditions were implemented as described: 5 min at 94 °C, followed by 40 cycles of 10 s at 94 °C, 15 s at 60 °C and 15 s at 72 °C. A melting curve analysis was performed at the end of the PCR run, over the range of 55-99 °C, increasing the temperature stepwise by 0.1 °C every 1 s. Each 25-µL reaction comprised 12.5 µL cDNA (1:50 dilution), 1x PCR buffer (Invitrogen), 2.4 mM $MgCl_2$, 0.024 mM dNTPs, 0.1 µM of each primer, 2.5 µL of SYBR-Green (1:100,000, Molecular Probes) and 0.03

U of Platinum Taq DNA Polymerase (5 U/µl, Invitrogen). All PCR assays were performed in technical quadruplicates and 10 biological samples. Reactions lacking cDNA were used as negative controls.

Transcript levels of the three urease-encoding genes were evaluated. The F-box protein and a metalloprotease were used as references for gene expression normalization (Jian *et al,.* 2008; Libault *et al.,* 2008). Primer sequences are presented in Table 1. The expression data analyses were performed after comparative quantification of amplified products using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Ureolytic activity

Urease activity in transgenic (A3 and A8) and non-transgenic plants was determined with a urease indicator solution: 6 g urea, 10 mL cresol red (1 mg/mL ethanol), 10 mL KH_2PO_4/K_2HPO_4/EDTA (10 mM KPi/1 mM EDTA pH 7.0) and 1 mL sodium azide 20% (w/v) per liter (Meyer-Bothling and Polacco, 1987). Powdered leaves and roots (± 100 mg) of two-week old plants, were incubated in a 1 mL urease indicator solution for 24 h at 60 ºC, as well as mature seed slices that were incubated for 20 min at room temperature. The *eu3-a* mutant was used as negative control. As urea is hydrolyzed by urease, the ammonia released increases the pH and turns to pink the initial yellow coloration of the cresol red pH indicator.

Germination

Germination capacity of seeds (T_2) from two transgenic events (A3 and A8) was compared with that of IAS5 non-transgenic seeds. For *eu1-a, eu*4-*a, eu*1-*a*/*eu*4-*a* and *eu*3-*a* mutant seeds germination capacity was compared with that of Williams82 non-mutant seeds. Seed germination, defined as radicle protrusion, was recorded over one month.

Developmental pattern

Seven, 14, 21 and 30 days after sowing, seedlings from all genotypes were classified according to their developmental stage following the categories proposed by Neumaier *et al.* (2000): VE = emergence of cotyledons; VC = completely opened cotyledons; V1 = completely developed unifoliate leaf pair; V2 = completely developed first trifoliate leaf; V3 = completely developed second trifoliate leaf. Eighteen plants derived from each event (A3 and A8), 10 non-transgenic plants, 18 plants from each mutant and 10 non-mutant plants were observed. In addition, the dry matter and length of roots and shoots were also determined one month after sowing.

Grain yield

The number of seeds produced per plant in three generations of transgenic (T_1, T_2 and T_3) and non-transgenic plants were compared.

Nitrogen content

Shoot nitrogen content (N mobilized from the cotyledons**)** was measured by the Kjeldahl method according to the methodology described by Tedesco *et al.* (1995) one month after sowing**.** This analysis evaluated 18 plants derived from each line A3 and A8 and 10 non-transgenic ones. Eighteen plants from each mutant (*eu*1-*a, eu*4-*a, eu*1-*a*/*eu*4-*a* and *eu*3-*a*) and non-mutant plants were also evaluated.

Statistical analysis

A Student's *t* test was used to compare the expression levels (RT-qPCR) of urease-encoding genes (*Eu*4*, Eu*1*, Eu*5) in roots of transgenic vs. non-transgenic plants and mutant vs. non-mutant plants.

In order to compare developmental stages, a score was attributed to each developmental category (VE = 1, VC = 2, V1 = 3, V2 = 4, V3 = 5). A generalized linear model for repeated measures was used to compare plant development among genotypes (IAS5 transgenic vs. IAS5 non-transgenic plants; *eu1-a* or *eu4-a* or *eu3-a* or *eu*1-*a*/*eu*4-*a* mutant vs. Williams82 non-mutant plants). ANOVA followed by Bonferroni's post hoc test were performed on dry matter, length and weight of roots and shoots data. A Student's *t* test was carried out in order to compare the number of seeds produced per transgenic and non-transgenic plant in different generations (T_1, T_2 and T_3). Data on shoot nitrogen content was compared among genotypes by ANOVA followed by Tukey's post hoc test. Analyses were performed using SPSS 18.0 software.

Results

Gene expression

Roots were chosen for gene expression analysis by RT-qPCR because *Eu*5 transcripts are mainly detected in this organ (Wiebke-Strohm *et al.,* 2016). As expected, the progeny of transgenic plants showed lower *Eu*4 transcript levels than non-transgenic controls, suggesting that the co-suppressed phenotype was maintained. Additionally, it was observed that the other urease-encoding genes, *Eu*1 and *Eu*5, were also down-regulated (Figure 1A).

The transcript levels of all three urease-encoding genes in the mutants plants followed the predicted pattern: *eu*4-*a* and *eu3-a* displayed normal mRNA levels of the three genes; *eu*1-*a* and *eu*1-*a*/*eu*4-*a* presented lower levels of *Eu*1, but normal *Eu*4 and

*Eu*5 expression levels (Figure1B). It is worth noting that although not affecting the mRNA expression levels, the *eu*4-*a* (and *eu*4-*b*) mutant produces a non-functional enzyme with a single amino acid replacement (Goldraij *et al.,* 2003).

Ureolytic activity

Ureolytic activity in transgenic and non-transgenic plants was evaluated with cresol red pH indicator by the seed chip assay of dried samples of leaves, roots and seeds. As expected, samples containing non-transgenic tissues showed pink coloration, indicative of urea hydrolysis. On the other hand, leaf and root samples of transgenic plants showed no observable color change even after 24 h incubation at 60 ºC, indicating absence or drastic reduction of urease activity. Slices of mature seeds exhibited little or no activity. Very low urease activity was confirmed comparing transgenic with *eu3-a* seeds (used as a negative control) (Supplemental Figure S1).

Taken together, urease expression and activity assays indicate that transgenic plants have had all three urease-encoding genes silenced in all tissues, reinforcing the potential of these plants for functional studies.

Germination

The germination rate of T_2 seeds from the two independent transgenic events was evaluated and compared to that of non-transgenic seeds. No differences were detected, suggesting that the absence of all three ureases did not affect germination. The same result was observed on germination of the *eu1-a*, *eu4-a, eu1-a/eu4-a* and *eu3-a* mutants and non-mutant Williams82. Germination rates were higher than 90% for all genotypes (data not shown).

Developmental pattern

Plant development was evaluated 7, 14, 21 and 30 days after sowing. T_2 transgenic plants and non-transgenic plants, as well as *eu1-a*, *eu4-a, eu1-a/eu4-a* and *eu3-a* mutant and non-mutant plants were classified into developmental categories. Interaction among genotype, developmental categories and time-course was highly significant ($p<0.01$). The two independent transgenic events showed a significant delay in development when compared with non-transgenic plants (Figure 2A and Figure 3). Size and dry weight of shoots and roots were significantly lower in transgenic plants when compared with non-transgenic (Table 2).

The developmental pattern of mutant plants was compared to non-mutants. The *eu1-a* single mutant showed a pattern similar to that of the non-mutant. The *eu4-a* mutant developed faster than control plants. Slower development was observed for the *eu1-a/eu4-a* double mutant and the *eu3-a* single mutant when compared to the non-mutant plants (Figure 2B). One month after germination, no differences were observed among the shoot and root sizes of *eu1-a* and *eu4-a* mutants and non-mutant plants. The shoot sizes of *eu1-a/eu4-a* double mutants and *eu3-a* single mutants were significantly smaller than those of the other genotypes. Regarding shoot and root weight, the *eu1-a/eu4-a* and *eu3-a* mutants were lighter than the other two mutants, but did not differ significantly from control. Total root length analysis showed that mutant *eu3-a* had the smallest root system (Table 2).

Nitrogen content

The nitrogen content was measured in shoots of one-month-old plants. The N content in transgenic plants of the two independent events was significantly lower than

that present in non-transgenic plants (Figure 4A). The comparison among mutants showed that *eu*3-*a* presented the lowest and *eu*1-*a* the highest N content (Figure 4B)

Grain yield

The number of seeds produced by three generations of transgenic plants was compared with those produced by non-transgenic plants. A significantly lower number of seeds was obtained for transgenic plants (Figure 5).

Discussion

The present study aimed to evaluate the contribution of soybean ureases to seed germination and plant development. Analyses were performed using co-suppressed transgenic plants and plants with mutations in urease-related genes. The transgenic plants were the progeny (T_2) of two independent events in which the *Eu*4 gene was down-regulated as previously described (Wiebke-Strohm *et al.,* 2012). Molecular analyses showed that *Eu*4 co-suppression was maintained in the transgenic progeny. In addition it was verified that transgenic plants exhibited very low transcript levels of the other two ureases encoded by the *Eu*1 and *Eu*5 genes. The phenomenon of co-suppression by transgenic DNA has been observed in many organisms, with the introduction of transgenic copies of a gene resulting in reduced expression of the transgene, as well as of the endogenous gene. This effect depends on the sequence identity between transgene and endogenous gene (Ketting and Plasterk, 2000).

Soybean plants with mutations in urease genes were also evaluated confirming the expected expression pattern. Normal transcript levels of all three urease-encoding genes were detected for *eu4-a* and *eu3-a* mutants. This result is consistent with the *eu*4-*a* allele encoding G468E missense-altered ubiquitous urease (Goldraij *et al.,* 2003). *Eu*3 is the

only UreG-encoding gene in the soybean genome. UreG is essential for urease activation, and the *eu*3-*a* mutant presents a complete loss of urease activity (Freyermuth *et al.,* 2000), consistent with a >90% deletion of the UreG ORF (Tezotto *et al.,* 2016). The *eu*3-*a* mutation did not alter *Eu*4, *Eu*1 and *Eu*5 expression levels, indicating a lack of feedback control on urease structural gene transcription by apo-urease(s). The mutants *eu1-a* and *eu1-a/eu4-a* exhibited similar expression patterns: lower *Eu*1, but normal *Eu*4 and *Eu*5 transcript levels. Since *eu1-a* is a null mutant (Polacco *et al.,* 1993), low levels of *Eu*1 transcripts are expected, and indeed, Torisky *et al.* (1994) employing *Eu*1-specific PCR primers, recovered no detectable product from *eu*1-*a* embryo cDNA.

Ureolytic activity in transgenic and non-transgenic plants was evaluated by the seed chip assay. As expected, leaf and root samples of transgenic plants showed no color change even after 24 h incubation. The urease activity level can be inferred based on the time change "yellow to pink" on the seed chip assay (Polacco *et al.,* 2011). According to the authors, 0.2% normal urease specific activity requires 10 hours for changing the solution color. When the activity decreases to 0.15%, the time required for solution color change is 48 hours. Based on these data, we conclude that the urease activity in leaves and roots of transgenic plants was absent or less than 0.2%, since no change in color was observed after a 24 hours incubation. The reaction catalyzed by urease is essential to allow most organisms (those lacking urea carboxylase) to use external or internally generated urea as a nitrogen source (Mobley and Hausinger, 1989; Mobley *et al*., 1995).

It has been demonstrated that aged *A. thaliana* seeds fail to germinate when urease was chemically inhibited, but seed viability could be rescued by an external N source (Zonia *et al*., 1995). In the present study no differences were detected in germination rates of transgenic and mutant seeds. However, it is important to highlight that soybean seeds were not aged and have a much higher protein content than *A. thaliana* seeds.

An association between urease activity and developmental pattern was observed. Transgenic plants, as well as *eu3-a* and the double *eu1-a/eu4-a* mutants, showed a delay over the first month after sowing. The delay in development was maintained even in adult transgenic plants and may be the cause of lower seed production. In *A. thaliana,* both urease transcripts and ureolytic activity increased after germination, especially in 8/9-day-old wild-type seedlings (Zonia *et al.,* 1995). Embryo-specific urease (*Eu*1) activity in young soybean plants was also observed by Torisky and Polacco, (1990). Similarly, high transcript level of *Eu*4 and moderate transcript levels of *Eu*1 and *Eu*5 were detected in soybean seeds one day after dormancy break (Wiebke-Strohm *et al.,* 2016). Taken together, these results indicate that increased urease content and/or ureolytic activity play a role in early stages of plant development.

A role in making nitrogen available during plant development has been attributed to soybean ubiquitous urease due to its catalytic activity and tissue distribution (Stebbins *et al.,* 1991). However, our results indicate that *Eu*1 and *Eu*5 have a contribution in the developmental process as well. This is supported by the finding that transgenic A3 and A8, mutant *eu1-a/eu4-a* and *eu3-a* plants showed a delay in the first developmental stages. In transgenic plants, the impairment in development was confirmed by the significant reduction in size and weight of roots and shoots one month after germination. These data are consistent with significantly lower nitrogen content detected in transgenic plants. Regarding mutant plants, *eu3-a* tends to be smaller and lighter than the other genotypes, although significant differences were only detected for roots and shoots size. The reduction in dry matter is also reflected in the lowest nitrogen content. An unexpected result was the significantly higher nitrogen content present in *eu1-a*. The differences in nitrogen content might be due to differences in mutant genetic backgrounds. Williams is

the background for *eu*1-*a,* while Williams82 is the one for the other mutants. In our experiment, non-mutant Williams82 was used as control.

Based on bioinformatics analyses, *Eu*5 has been suggested not to be a functional ureolytic enzyme due to a number of mutations, including deletions (Witte, 2011). However, according to our results the product of this gene might be involved in plant development. Further studies are necessary to elucidate whether the ureolytic activity and/or other non-enzymatic property of ureases are involved on plant development.

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References

Bernard RL and Cremeens CR (1988) Registration of Williams 82 soybean. Crop Sci 28:1027-1028.

Bohner A, Kojima S, Hajirezaei M, Melzer M and Von Wiren N (2015) Urea retranslocation from senescing Arabidopsis leaves is promoted by DUR3-mediated urea retrieval from leaf apoplast. Plant J 81:377-387.

Cao F-Q, Werner AK, Dahncke K, Romeis T, Liu L-H and Witte CP (2010) Identification and characterization of proteins involved in rice urea and arginine catabolism. Plant Physiol 15:98-108.

Carlini C and Ligabue-Braun R (2016) Ureases as multifunctional toxic proteins: a review. Toxicon 110:90-109.

Carter EL, Flugga N, Boer JL, Mulrooney SB and Hausinger RP (2009) Interplay of metal ions and urease. Metallomics 1:207-221.

Dixon NE, Gazzola TC, Blakeley RL and Zermer B (1975) Jack bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? J Am Chem 97:4131-4133.

Eskew DL, Welch RM and Cary EE (1983) Nickel: an essential micronutrient for legumes and possibly all higher plants. Science 222:621-623.

Freyermuth SK, Bacanamwo M and Polacco JC (2000) The soybean *Eu3-a* gene encodes an Ni-binding protein necessary for urease activity. Plant J 21:53-60.

Goldraij A and Polacco JC (1999) Arginase is inoperative in developing soybean seeds. Plant Physiol 119:297-304.

Goldraij A and Polacco JC (2000) Arginine degradation by arginase in mitochondria of soybean seedling cotyledons. Planta 210:652-658.

Goldraij A, Beamer LJ and Polacco JC (2003) Interallelic complementation at the ubiquitous urease coding locus of soybean. Plant Physiol 132:1801-1810.

Hogan ME, Swift IE and Done J (1983) Urease assay and ammonia release from leaf tissues. Phytochemistry 22:663-700.

Jian B, Liu B, Bi Y, Hou W, Wu C and Han T (2008) Validation of internal control for gene expression study in soybean by quantitative real-time PCR. BMC Mol Biol 9:59.

Kanamori T, Kanou N, Atomi H and Imanaka T (2004) Enzymatic characterization of a prokaryotic urea carboxylase. J Bacteriol 186:2532-2539.

Ketting, RF and Plasterk, RH (2000) A genetic link between co-suppression and RNA interference in C. elegans. Nature 404:296-298.

Krajewska B (2009) Ureases I. Functional, catalytic and kinetic properties: a review. J Mol Catal B Enzymatic 59:9-21.

Libault MS, Thibivilliers DD, Bilgin O, Radwan M, Benitez SJ, Clough and Stacey G (2008) Identification of four soybean reference genes for gene expression normalization. Plant Genome 1:44-54.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\text{Delta Delta C(T)})}$ method. Methods 25:402-408.

Marschner P (2012) Marschner's Mineral Nutrition of Higher Plants. 3rd edition Academic Press, London, Waltham, MA, 651p.

Meyer-Bothling LE and Polacco JC (1987) Mutational analysis of the embryo-specific urease locus of soybean. Mol Gen Genet 209:439-444.

Micallef BJ and Shelp BJ (1989) Arginine metabolism in developing soybean cotyledons. I. Relationship to nitrogen nutrition. Plant Physiol 90:624-630.

Mobley HL and Hausinger RP (1989) Microbial ureases: significance, regulation and molecular characterization. Microbiol Rev 53:85-108.

Mobley HL, Island MD and Hausinger RP (1995) Molecular biology of microbial ureases. Microbiol Rev 59:451-480.

Neumaier N, Nepomuceno AL and Farias JRB (2000) Estádios de desenvolvimento da cultura de soja. In: Bonato ER (ed). Estresses em soja. EMBRAPA CNPT, Passo Fundo, RS, pp 19-44.

Polacco JC and Havir EA (1979) Comparisons of soybean urease isolated from seed and tissue culture. J Biol Chem 254:1707-1715.

Polacco JC and Holland MA (1993) Roles of urease in plant cells. Int Rev Cytol 47: 1189-1195.

Polacco JC and Winkler RG (1984) Soybean leaf urease: a seed enzyme? Plant Physiol 74:800-803.

Polacco JC, Krueger RW and Winkler RG (1985) Structure and possible ureide degrading function of the ubiquitous urease of soybean. Plant Physiol 79:794-800.

Polacco JC, Hyten DL, Medeiros-Silva M, Sleper DA and Bilyeu KD (2011). Mutational analysis of the major soybean UreF paralogue involved in urease activation. J Exp Bot 62:3599-3608.

Polacco JC, Mazzafera P and Tezotto T (2013) Opinion - Nickel and urease in plants: still many knowledge gaps. Plant Science 199-200:79-90.

Sirko A and Brodzik R (2000) Plant ureases: roles and regulation. Acta Biochim Pol 47:1189-1195,

Stebbins N and Polacco JC (1995) Urease is not essential for ureide degradation in soybean. Plant Physiol 109:169-175.

Stebbins N, Holland MA, Cianzio SR and Polacco JC (1991) Genetic tests of the roles of the embryonic Uureases of soybean. Plant Physiol 97:1004-1010.

Tedesco MJ, Gianello C, Bissani CA, Bohnen H and Vokweiss SJ (1995) Análise de solo, plantas e outros materiais. 3rd edition (Boletim Técnico, 5). UFRGS, Porto Alegre, RS, 174 p.

Tezotto T, Souza SCR, Mihail J, Favarin JL, Mazzafera P, Bilyeu K and Polacco JC (2016) Deletion of the single UreG urease activation gene in soybean NIL lines: characterization and pleiotropic effects. Theor Exp Plant Physiol 28:307-320.

Torisky RS and Polacco JC (1990) Soybean roots retain the seed urease isozyme synthesized during embryo development. Plant Physiol 94:681-689.

Torisky RS, Griffin JD, Yenofsky RL and Polacco JC (1994) A single gene (*Eu*4) encodes the tissue-ubiquitous urease of soybean. Mol Gen Genet 242:404-414.

Van Etten CH, Miller RW, Wulf IA and Jones Q (1963) Amino acid composition of seeds from 200 angiospermous plant species. J Agric Food Chem 11:399-410.

Wang WH, Kohler B, Cao FQ and Liu LH (2008) Molecular and physiological aspects of urea transport in higher plants. Plant Sci 175:467–477.

Wiebke-Strohm B, Pasquali G, Margis-Pinheiro M, Bencke M, Bücker-Neto L, Becker-Ritt AB, Martinelli AHS, Rechenmacher C, Polacco JC, Stolf R, *et al.* (2012) Ubiquitous urease affects soybean susceptibility to fungi. Plant Mol Biol 78:1-13.

Wiebke-Strohm B, Ligabue-Braun R, Rechenmacher C, de Oliveira-BUsatto LA, Carlini CR and Bodanese-Zanettini MH (2016) Structural and transcriptional characterization of a novel member of the soybean urease gene family. Plant Physiol Biochem 101:96-104.

Witte CP (2011) Urea metabolism in plants. Plant Sci 180:431–438.

Witte CP, Tiller SA, Taylor MA and Davies HV (2002) Leaf urea metabolism in potato. Urease activity profile and patterns of recovery and distribution of (15) N after foliar urea application in wild-type and urease-antisense transgenics. Plant Physiol 128:1129-1136.

Zonia LE, Stebbins N and Polacco JC (1995) Essential role of urease in germination of nitrogen-limited *Arabidopsis thaliana* seeds. Plant Physiol 107:1097-1103.

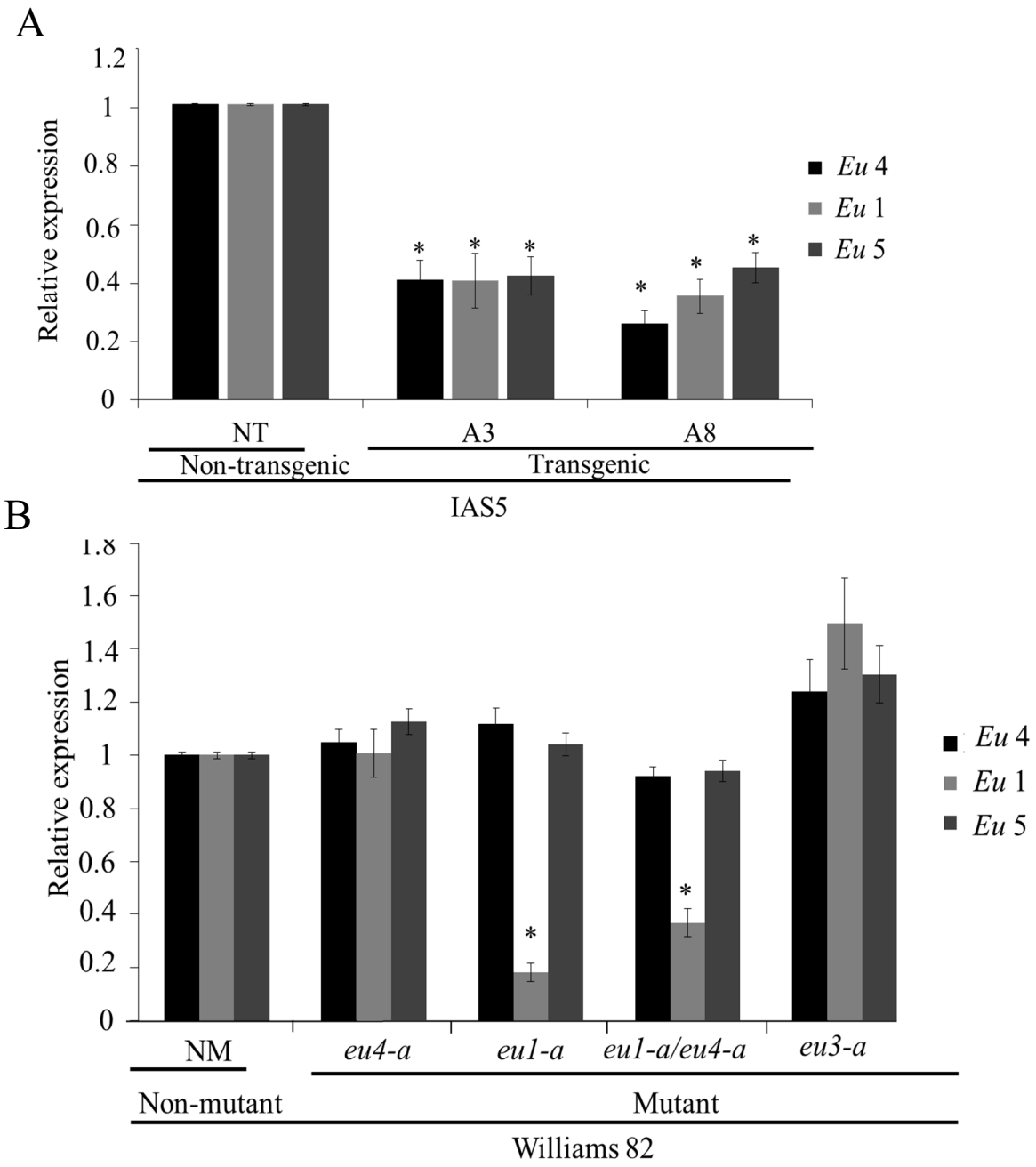

Figure 1 Transcript levels (RT-qPCR) of urease-encoding genes (*Eu*4, *Eu*1, *Eu*5) in roots of two-week-old plants. (A) Two independent transgenic events (A3 and A8) and non-transgenic plants (NT, control) from cv. IAS5. (B) Williams82 non-mutant (NM, control) and *eu4-a*, *eu1-a*, *eu1-a/eu4-a* and *eu3-a* mutants. The bars represent mean±SD of two non-transgenic plants, 10 transgenic plants from each event, two non-mutant plants and 10 plants from each mutant. Transcripts level of *Eu*4, *Eu*1 and *Eu*5 detected in non-transgenic or non-mutant plants were used to normalize transcript accumulation in

transgenic or mutant plants, respectively. F-Box and Metalloprotease reference genes were used as internal controls to normalize the amount of mRNA present in each sample. * indicates that the mean of each gene is significantly different from the control (*t* -test, $p<0.05$).

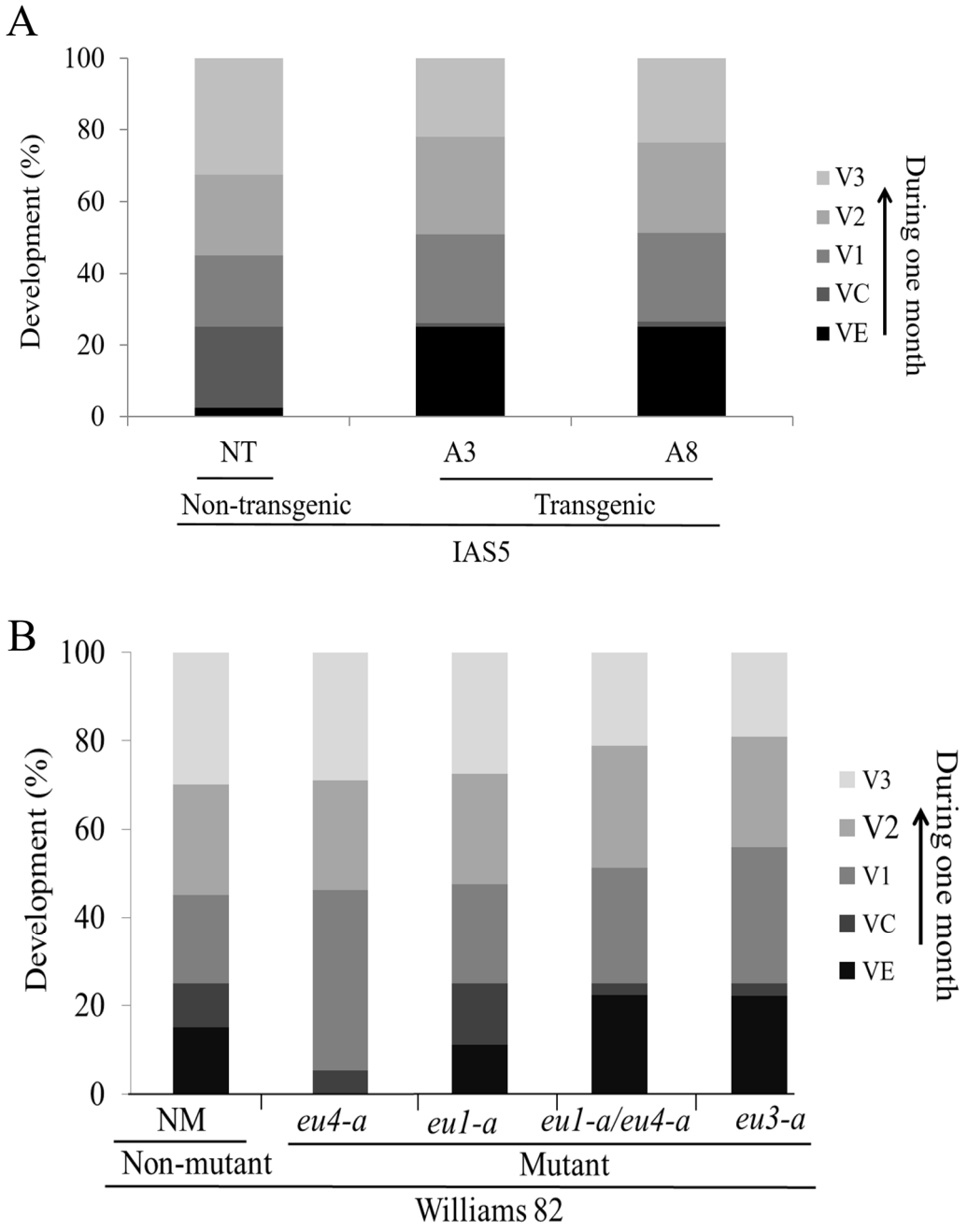

Figure 2 - Plant developmental pattern over the first month after germination. (A) Transgenic and non-transgenic plants were evaluated: 18 plants derived from each event (A3 and A8) and 10 non-transgenic plants. (B) *eu4-a, eu1-a, eu1-a/eu4-a* and *eu3-a* mutant and non-mutant plants were evaluated: 18 plants from each mutant and 10 non-mutant plants. Plants were classified according categories: VE= emergency of cotyledons; VC= completely opened cotyledons; V1 = completely developed unifoliate

leaf pair; V2= completely developed first trifoliate leaf; V3= completely developed second trifoliate leaf. A generalized linear model for repeated measures was used to compare the plant development among genotypes ($p<0.01$).

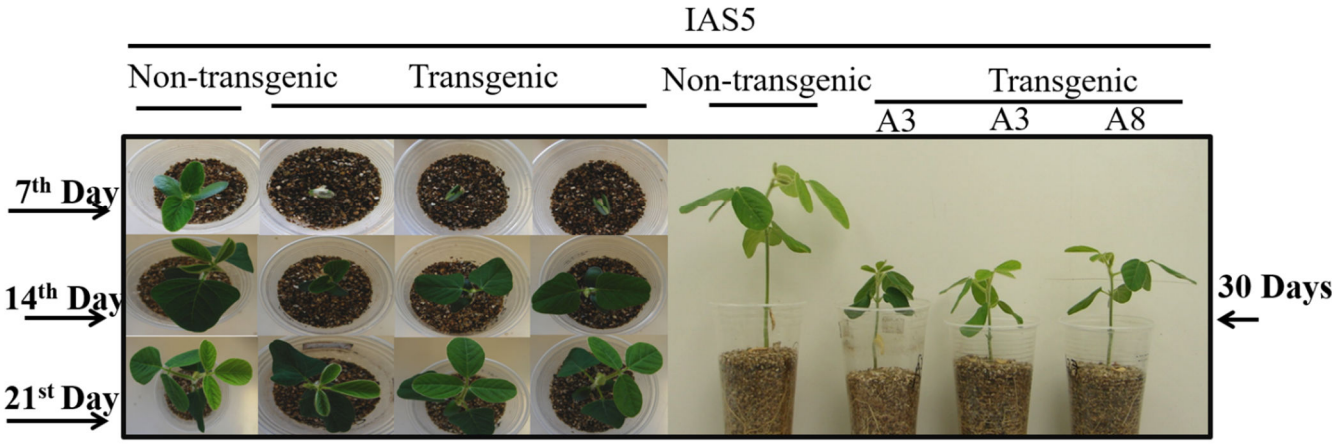

Figure 3 - Transgenic and non-transgenic plants seven, 14, 21 and 30 days after germination. Transgenic plants and non-transgenic plants are from cv. IAS5.

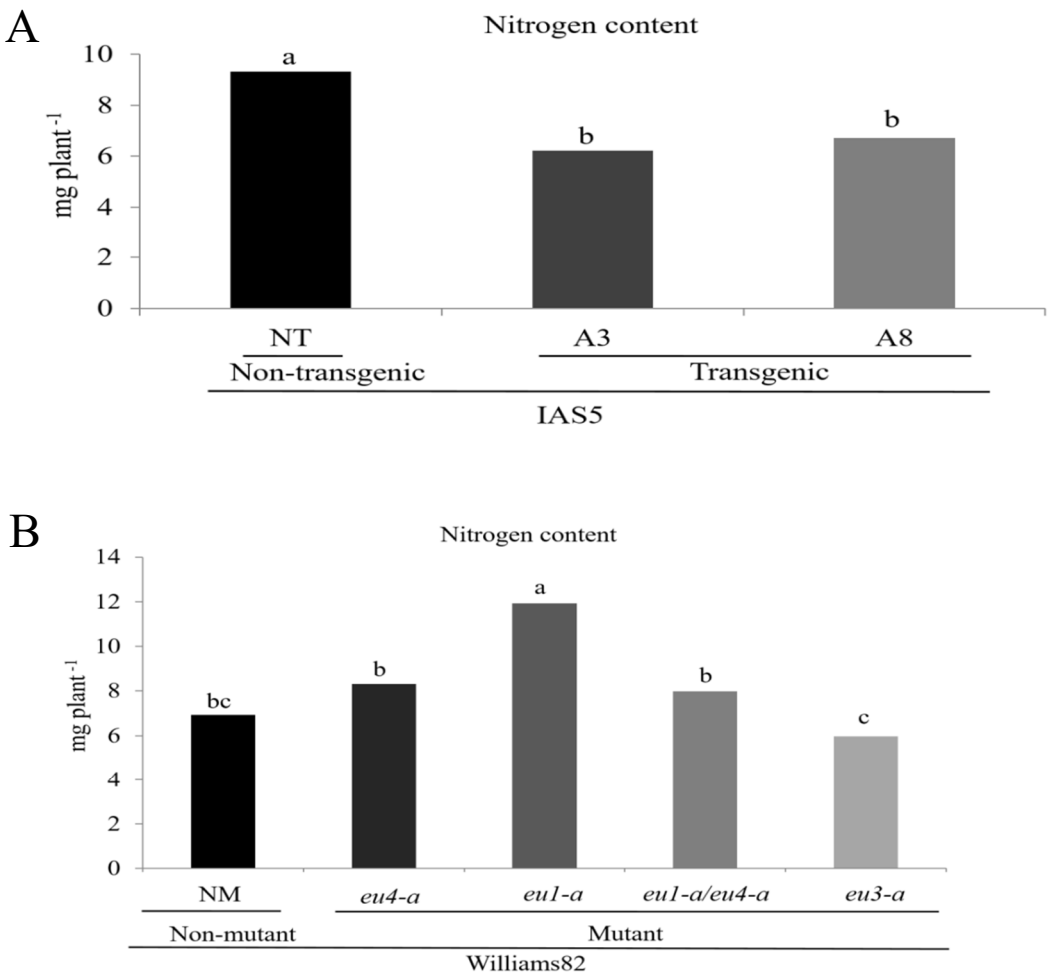

Figure 4 - Nitrogen content rate in shoot of soybean at one month after germination. (A) Transgenic and non-transgenic plants were evaluated: 18 plants derived from each event (A3 and A8) and 10 non-transgenic plants. (B) *eu4-a, eu1-a, eu1-a/eu4-a* and *eu3-a* mutant and non-mutant plants were evaluated: 18 plants from each mutant and 10 non-mutant plants. ANOVA, $p<0.0001$. Means followed by the same letter did not differ by Tukey's post hoc test.

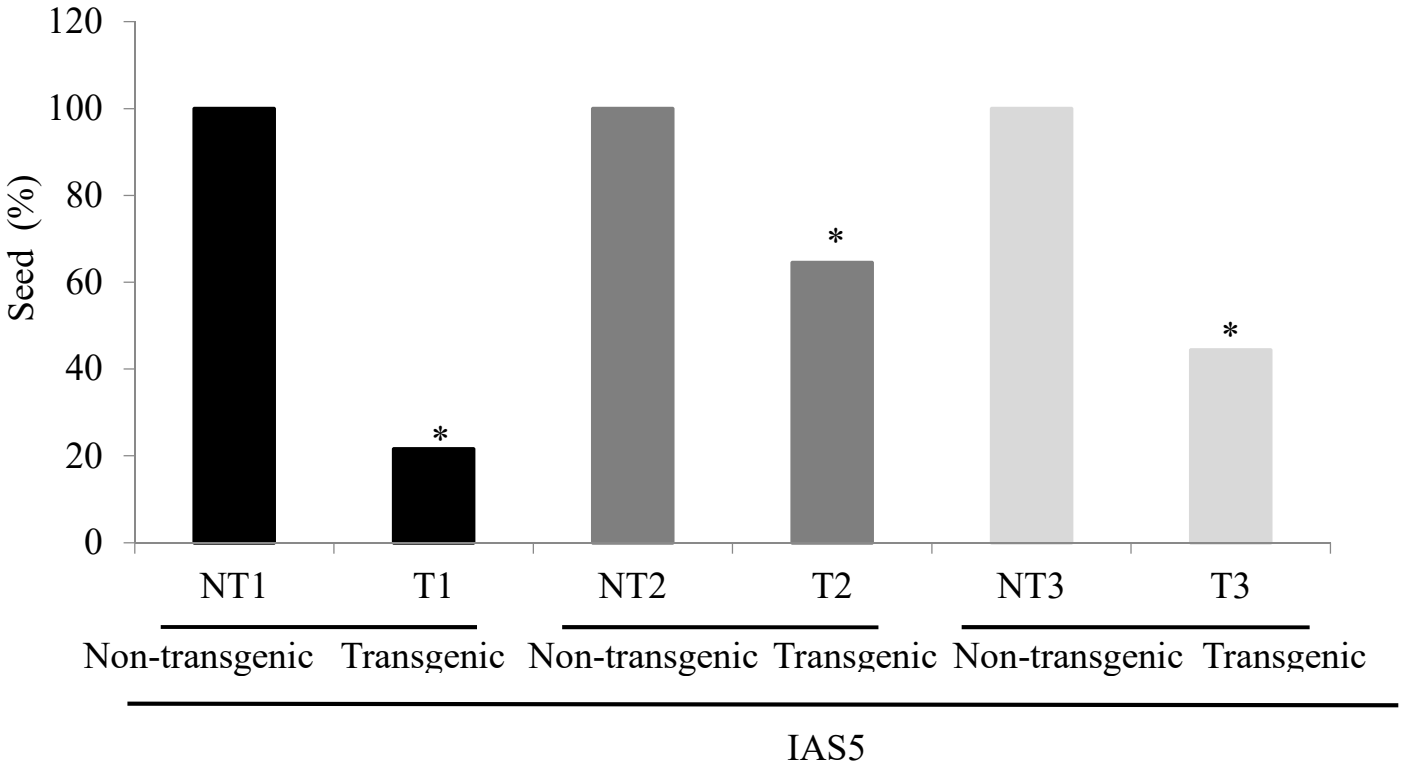

Figure 5 - Relative percentage of seeds produced by transgenic plants in three generations (T_1, T_2 and T_3). The mean number of seeds produced by non-transgenic plant was considered 100%. Transgenic plants and non-transgenic plants are from cv. IAS5. * indicates that the mean of transgenic seeds is significantly different from non-transgenic in each generation (*t* -test, $p<0.05$).

Table 1 - Primer set designed for RT-qPCR

Target gene	Orientation	Primer sequence
***Eu*1 (embryo-specific urease)**	Forward	5'-ACCAGTTTTGCAACCACCTT-3'
	Reverse	5'-AAGAACAAGAGCAGGGGAACT-3'
***Eu*4 (ubiquitous urease)**	Forward	5'-TCACTGTGGACCCAGAAACA-3'
	Reverse	5'-CTTGCTTATTGTTTTTTGCCAAT-3'
***Eu*5 (urease III)**	Forward	5'-GTCGAGTTGGAGAGGTCCTTTAT-3'
	Reverse	5'-GAGAAATGTCACATGCACACTG-3'
Metalloprotease	Forward	5'-ATGAATGACGGTTCCCATGTA-3'
	Reverse	5'-GGCATTAAGGCAGCTCACTCT-3'
FBox protein	Forward	5'-AGATAGGGAAATGTTGCAGGT-3'
	Reverse	5'-CTAATGGCAATTGCAGCTCTC-3'

Table 2 - Dry matter and length of roots and shoots one month after germination.

		Shoot		Root	
		Lengtht(cm)	**weight(g)**	**Lengtht(cm)**	**weight(g)**
IAS5	**NT**	12.58 a	0.63a	21.46a	0.37[a]
	A3	7.70b	0.29b	13.68b	0.21b
	A8	9.61b	0.39b	14.66b	0.28b
Williams 82	**NM**	12.57A	0.59 AB	55.65A	0.45AB
	eu4-a	13.65A	0.69A	47.97A	0.58[a]
	eu1-a	13.62 A	0.71A	52.37A	0.62[a]
	eu1-a/eu4-a	10.02B	0.51B	45.30AB	0.31B
	eu3-a	10.61B	0.47B	22.58B	0.23B

ANOVA, $p<0.05$. Means followed by the same letter did not differ by Bonferroni's test.

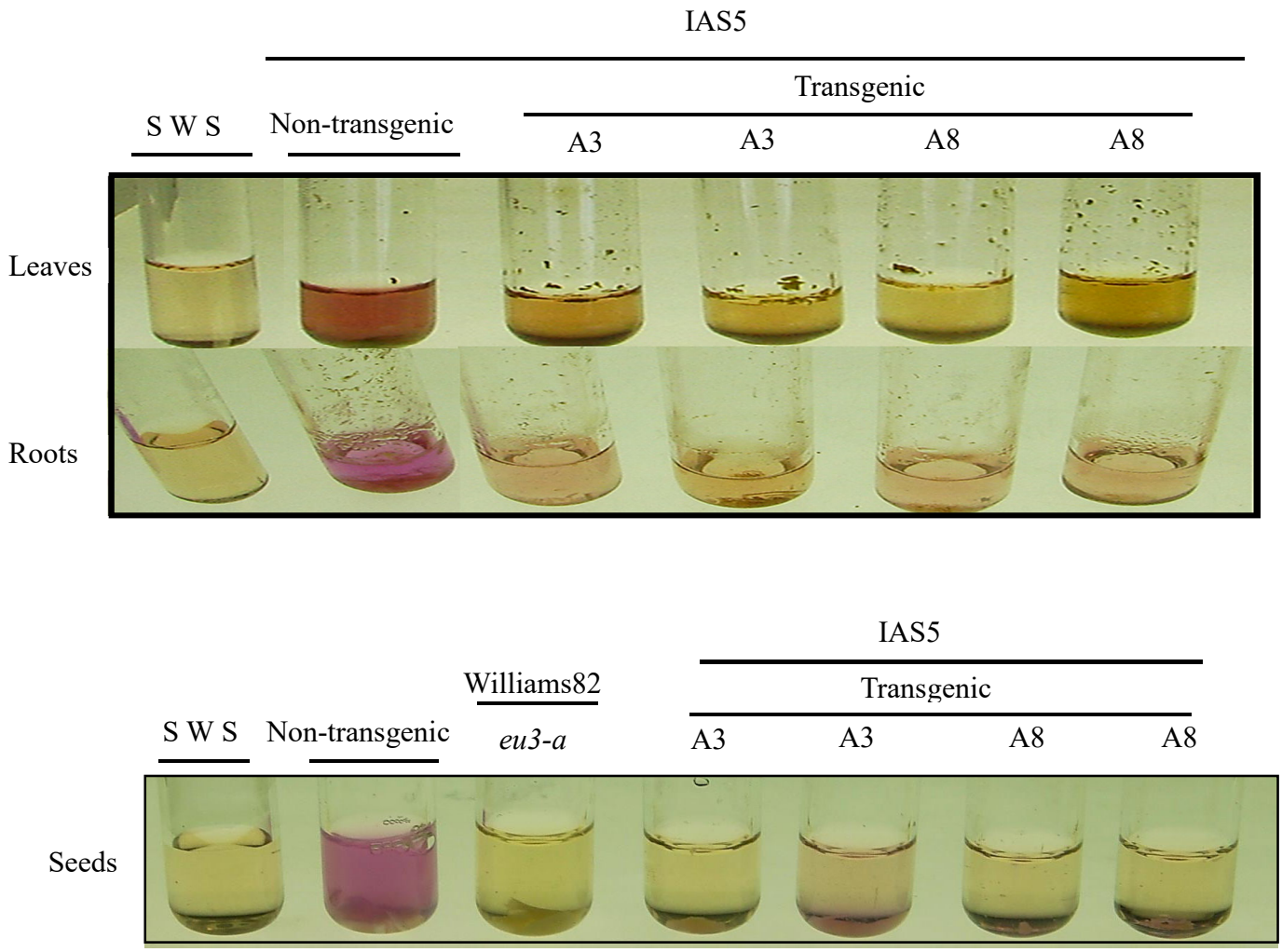

Figure S1- Ureolytic activity in transgenic and non-transgenic plants. (A) powdered leaves and roots of two-week old plants (B) slices of mature seeds were incubated in a pH-indicator reagent containing cresol red and weakly buffered 10 mM urea. As the ureolytic activity proceeds, the released NH_4^+ increases the pH, turning the solution from yellow to pinkish. SWS = solution without sample; non-transgenic plant from cv. IAS5; *eu3-a* mutant (used as negative control) and two transgenic plants from each event (A3 and A8).

Capítulo III

Soyuretox, a Urease-derived Peptide, for Plant Protection against Root-knot Nematodes

Manuscrito submetido para a publicação na revista Transgenic Research

O conteúdo deste trabalho faz parte do Termo de Adição encaminhado a um pedido de patente previamente depositado no INPI

RESUMO

Nematoides formadores de galhas, são um dos patógenos mais prejudiciais à soja, sendo de difícil controle. Neste trabalho, foi identificado um peptideo derivado de uma urease de soja, denominado Soyuretox, que exerce efeito tóxico contra o fitonematoide formador de galha *Meloidogyne javanica*. O Soyuretox foi expresso em raízes de plantas compostas e em plantas transgênicas estáveis de soja. O fator reprodutivo do nematoide foi avaliado tanto em raízes de plantas compostas como em plantas estavelmente transformadas.

Soyuretox, a Urease-derived Peptide, for Plant Protection against Root-knot Nematodes

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Abstract

Nematodes are pathogens of many important agricultural crops, including soybeans. The main species found in Brazil are root-knot (*Meloidogyne spp.*), cyst (*Heterodera glycines*), root lesion (*Pratylenchus brachyurus*) and reniform (*Rotylenculus reniformis*) nematodes. Ureases are traditionally known for catalyzing the hydrolysis of urea to ammonia and carbon dioxide. Besides the main function they present other independent biologically active domains, which ascribe among other activities toxicity against insects and fungi. It has been shown that toxicity against insects involves the proteolytic activity of digestive enzymes resulting in the release of toxic peptides. In the current study, we produced soybean stable transgenic plants and soybean composite plants with hairy roots overexpressing a soybean urease-derived peptide (named Soyuretox) and characterized their response to *Meloidogyne javanica*. This is the first report to demonstrate the potential of Soyuretox to confer resistance against nematodes. Thus, Soyuretox may represents a new and efficient alternative to control pests and diseases in economically important crops.

Keywords *Agrobacterium rhizogenes,* Genetic transformation, *Glycine max*, Nematode resistance, Soyuretox

Introduction

Soybeans (*Glycine max*) are affected by several abiotic and biotic stresses that limit the geographical distribution of cultures and lead to significant reductions in growth and productivity. Nematodes represent a significant threat to global food production, causing losses greater than 80 billion dollars annually due to lower yields of economic crops worldwide (Nicol et al. 2011). In Brazil, the diseases caused by nematodes are one of the most damaging biotic stresses for soybeans (Dias et al. 2010).

Various nematode genera infect and parasitize soybeans. The main species found in Brazil are *Meloidogyne spp*. (root-knot), *Heterodera glycines* (cyst), *Pratylenchus brachyurus* (root lesion) and *Rotylenchulus reniformis* (reniform) (Dias et al. 2010). Root-knot and cyst nematodes are obligate biotrophs sedentary nematodes. This group of nematodes are the most damaging, because they induce the formation of complex feeding sites within the roots hence they are very difficult to control (Jones et al. 2013).

The root-knot nematode life cycle, which can be completed in approximately 30 days under optimal conditions, includes six stages: the egg, four juvenile stages and the adult (Moens eta al. 2010). Upon infection, pre-parasitic second-stage juveniles (J2) penetrate the roots and migrate toward the vascular cylinder where they induce the growth of a multinuclear feeding site, which consists of several giant cells (Moens eta al. 2010). The expansion and proliferation of neighboring cortical and pericycle cells leads to gall formation (Fuller et al. 2008). After feeding, the larvae develop into third-stage juveniles (J3) and fourth-stage juveniles (J4) and finally into adults. Adult males are vermiform and leave the root. Females continue to feed and grow, and they become pear-shaped (Chitwood and Perry 2009). Mature females lay eggs in a protective gelatinous matrix, forming an egg mass, which are found on the root surface or are incorporated into galls, and can contain up to 1,000 eggs (Moens eta al. 2010).

Plants infested by root-knot nematodes exhibit drastic morphological and physiological changes such as retarded growth, chlorosis and low yields. In addition, the infection facilitates attack by other pests, such as fungi, viruses and bacteria (Bird and Kaloshian 2003; Williamson and Gleason 2003).

Genetic engineering has the advantage of incorporating genes from any species for any crop that could provide resistance against pathogens. Chitinase and glucanase-encoding genes have been used to produce fungal-resistant transgenic plants (Ceasar and Ignacimuthu 2012). Studies on the effects of

candidate molecules against nematodes are scarce. Only a few reports have shown toxicity of some *Bacillus thuringiensis* isolates against several species of nematodes. Genes encoding Cry proteins efficient against Lepidoptera are quite common, whereas those with nematicidal properties are rare (Gonçalves et al. 2014).

Plant ureases are multifunctional enzymes (Carlini and Ligabue-Braun 2016) that are traditionally known to catalyze the conversion of urea to ammonia and carbon dioxide (Mobley et al. 1995). More recently, it has been demonstrated that ureases are toxic to fungi and insects (Carlini and Polacco 2008; Stanisçuaski and Carlini 2012). The domains conferring the insecticidal and fungicidal properties are independent of the enzyme activity (Follmer et al. 2004; Becker-Ritt et al. 2007).

Insecticidal activity is mostly mediated through a proteolytic activation process that depends on the profile of the insect's digestive enzymes, which release toxic peptides (Carlini et al. 1997; Ferreira-DaSilva et al. 2000). *Canavalia ensiformis* (jack bean) and soybean ureases are particularly toxic to Coleoptera and Hemiptera, whose digestive tracts rely on proteolytic cathepsin-like enzymes (cysteine and aspartic proteases) (Stanisçuaski and Carlini 2012).

An insecticidal peptide of ~10 (kDa) was isolated from canatoxin, one of the isoforms of jack bean urease (Carlini and Ferreira-Da-Silva 2000; Ferreira-DaSilva et al. 2000). Subsequently, the corresponding DNA sequence was identified for another *C. ensiformis* urease-encoding gene (*jbure-II*) and the encoded peptide, called jaburetox-2Ec (Mulinari et al. 2007) or simply jaburetox (Postal et al. 2012), was expressed in *Escherichia coli.* The recombinant jaburetox exhibited toxicity against insects such as *Dysdercus peruvianus* and *Rhodnius prolixus*, including some species that were not affected by native ureases, such as the fall armyworm *Spodoptera frugiperda* (Stanisçuaski and Carlini 2012).

In soybeans, two urease isoforms have been described: the ubiquitous urease encoded by the *Eu*4 gene (Glyma11g37250; GenBank accession AJ276866) and the embryo-specific urease encoded by the *Eu*1 gene (Glyma05g27840; GenBank accession NM001249869) (Holland et al. 1987; Goldraij et al. 2003). A third urease-encoding gene was recently identified, designated *Eu*5 (Glyma08g103000), and its putative protein product SBU-III (Wiebke-Strohm et al. 2016).

In the present study, the DNA sequence corresponding to jaburetox was identified as part of the soybean *Eu*4 gene, and the resulting peptide was named Soyuretox. The effect of Soyuretox against the root-knot nematode *Meloidogyne javanica* was tested. This is the first report providing evidence that the overexpression of Soyuretox in hairy roots and stable transgenic plants can be used to increase resistance against nematodes.

Materials and Methods

Identification of Soyuretox

The internal sequence of the soybean ubiquitous urease (Glyma11g37250; AJ276866) collinear with jaburetox was identified by amino acid alignment using ClustalW (Larkin et al. 2007) (Fig. 1).

Construction of transformation vector

The 242 bp Soyuretox-encoding sequence was amplified from a previously cloned pET23a::*Eu*4 construct using a specific primer pair (Table 1). Start (ATG) and termination (TGA) codons and the CACC sequence (necessary for directional GateWay cloning) were added to the primer sequences. The PCR mixture was prepared with 10 ng of recombinant plasmid, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 0.4 µM of each primer, 1 U Taq DNA polymerase (5 U/µl - Invitrogen) and autoclaved distilled water in a final volume of 25 µL. Reactions were hot-started (3 min at 94 °C) and subjected to 32 cycles as follows: 1 min at 95 °C, 1 min at 52 °C and 2 min at 72 °C.

The Gateway® system (Invitrogen) was used to recombine the PCR product into the pH7WG2D plant overexpression vector (Karimi et al. 2002). The T-DNA region of the resulting pH7WG2D::Soyuretox vector contained the Soyuretox-encoding sequence under the control of the CaMV 35S promoter (P35S), the hygromycin-phosphotransferase marker gene (*hpt*) and the green fluorescent protein reporter gene (*gfp*) (supplementary Fig. S1). The amplicon identity was confirmed by sequencing using the P35S forward primer and Soyuretox reverse primer (Table 1). The pH7WG2D::Soyuretox and empty-pH7WG2D (control) plasmids were independently transformed into the *Agrobacterium rhizogenes* K599 strain.

Soybean root transformation by *A. rhizogenes*

Single colonies of *A. rhizogenes* K599 harboring pH7WG2D::Soyuretox or empty-pH7WG2D were inoculated in 10 mL of Yeast Extract Peptone (YEP) liquid medium containing 100 mg/L rifampicin and 100 mg/L spectinomycin and incubated overnight with shaking at 28 °C. The pre-inocula were inoculated in 500 mL of the same medium and incubated for an additional 24 h. The cultures were centrifuged at 500 rpm at 4 °C for 10 minutes, and the pellets were resuspended in 8 mL of autoclaved Milli-Q water.

The soybean composite plants presenting transgenic roots were obtained as previously described (Kuma et al. 2015) with minor modifications. Briefly, the radicle of plants (Williams82 cultivar) were inoculated with 0.1 mL of *A. rhizogenes* culture using a syringe. The seedlings were transferred to a medium containing 15 µg/mL of hygromycin-B. After 10 days, the first selection of transformed roots was carried out by observing GFP expression under a fluorescence stereomicroscope Leica MZIII (Leica Microsystems GmbH, Wetzlar, Germany). GFP-negative roots were removed. Plants were recovered in a hydroponic system for 10 days, follow by a new procedure of GFP-positive selection under microscope. Composite plants that presented only transformed roots were transplanted into tubes containing autoclaved sand for bioassays with root-knot nematodes.

Transformation by particle bombardment

Somatic embryogenesis was induced from IAS5 cultivar immature cotyledons. Proliferation, transformed embryo selection, maturation and plant regeneration were carry out according to protocol previously described (Droste et al. 2002; Wiebke-Strohm et al. 2012). After three months in hygromycin-B selection medium, resistant embryogenic soybean tissues were visually selected and individually cultured for the establishment of transgenic lines.

Molecular analysis of transgenic hairy roots and plants

The extraction of total DNA from roots and leaves was performed (Doyle and Doyle 1987). Putative transgenic roots (composite plants) and leaves (stable transgenic plants) were PCR-screened for the presence of the P*35S*-Soyuretox chimeric gene using the primer pair described in Table 1. The PCR mixture contained 200-300 ng DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 0.4 µM each primer, 1 U Taq DNA Polymerase (Invitrogen), and autoclaved distilled water to a final volume of 25 µL. Reactions were hot-started for 3 min at 94 °C and subjected to 32 cycles as follows: 1 min at 95 °C; 1 min at 52 °C and 2 min at 72 °C.

Total RNA was extracted from roots and leaves using Trizol (Invitrogen) and treated with DNAse I (Invitrogen) according to the manufacturer's instructions. First-strand cDNAs were obtained using 1 µg of DNA-free RNA, the M-MLV Reverse Transcriptase System™ (Invitrogen) and oligo (dT) primers.

Real Time quantitative PCR (qPCR) was performed on a StepOne Applied Biosystem Real-time Cycler™. PCR-cycling conditions were implemented as described: 5 min at 94 °C, followed by 40

repetitions of 10 s at 94 °C, 15 s at 60 °C and 15 s at 72 °C. A melting curve analysis was performed at the end of the PCR run, over the range of 55-99 °C, increasing the temperature stepwise by 0.1 °C every 1 s. Each 25-µL reaction comprised 12.5 µL of cDNA (1:50 dilution), 1x PCR buffer (Invitrogen), 2.4 mM $MgCl_2$, 0.024 mM dNTPs, 0.1 µM each primer, 2.5 µL of SYBR-Green (1:100,000, Molecular Probes) and 0.03 U of Platinum Taq DNA Polymerase (5 U/µL, Invitrogen). All PCR reactions were performed in technical quadruplicates. Reactions lacking cDNA were used as negative controls. Primers for the Soyuretox transcript detection were designed using Primer3 software (Table 1). The F-box protein and a metalloprotease were used as references for gene expression normalization (Jian et al. 2008; Libault et al. 2008). The expression data analyses were performed after comparative quantification of amplified products using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Root-knot nematode assays

Eggs from *M. javanica* were placed in a hatching chamber to obtain the infective juvenile stage (J2). After 2 days, J2 larvae were separated from unhatched eggs and adjusted to a final concentration of 500 J2s/mL. In the assay using composite soybean plants, 500 nematodes were inoculated in the root system of each plant through a hole in the sand. Eleven composite plants transformed with pH7WG2D::Soyuretox, ten with empty-pH7WG2D and nine non-transformed plants were inoculated. In the bioassay using stable transformed events T_2 transgenic plants, derived from the same transformation event, were inoculated with 5000 J2/plant. Ten T_2 plants transformed with pH7WG2D::Soyuretox and six non-transformed plants were used in the test.

Forty-five days after inoculation, all roots of individual plants were collected, washed and divided into two sets: one was used for molecular analyses and the second to estimate the nematode reproduction factor.

Confirmation of *M. javanica* infection

Primers that amplify a partial sequence of the nematode 18S rRNA encoding-gene (Table 1) were used to confirm the nematode presence in root tissue. Female nematode DNA was used as a positive control. The PCR mixture was prepared as described above in the “Molecular analysis of transgenic hairy roots and plants” section. The conditions used were a hot-start for 1 min at 94 °C and 30 cycles as follows: 30 s at 95 °C, 30 s at 64 °C and 1 min at 72 °C.

M. javanica reproduction factor

To determine the nematode final populations, roots were processed as previously described (Boneti and Ferraz 1981). The infected roots were weighted, cut in pieces, placed in a blender, immersed in a 0.5% sodium hypochlorite solution and crushed. The resulting suspension was filtered through 60 and 500-mesh sieves, successively. The material caught in the 500-mesh sieve (which included eggs and J2 larvae) was washed with distilled water to remove the excess of hypochlorite and resuspended in 80 mL of distilled water.

To count the numbers of eggs and J2 individuals, 1 mL of the suspension was placed on a Peters slide and analyzed under an optical microscope (40x). The reproduction factor [RF = number of eggs and J2 in roots/Pi (initial population)] was obtained from the average of three counts/plant. The resulting value was divided by the root fresh weight of each plant (Wilcken et al. 2010).

Statistical analysis

A non-parametric *t* test was carried out to compare the Soyuretox transcript levels in transformed *vs*. non-transformed hairy roots and transformed *vs.* non-transformed plants.

The nematode RF values were compared among: (1) pH7WG2D*::*Soyuretox, empty-pH7WG2D transformed roots and non-transformed hairy roots, and (2) transformed vs. non-transformed plants. One-way ANOVA followed by Tukey's test was used. Analyses were performed using SPSS 18.0 software. Results with $p \leq 0.05$ were considered statistically significant.

Results

Generation and characterization of composite and transgenic plants

In order to access whether Soyuretox overexpression would enhance resistance to nematodes, composite plants and stable transgenic plants were obtained by *A. rhizogenes* and bombardment transformation, respectively.

After the selection period, hairy roots transformed with pH7WG2D*::*Soyuretox (expressing Soyuretox and GFP) and empty-pH7WG2D vector (expressing only GFP) were screened for the presence of GFP. Strong green fluorescence was evident in roots of the 21 obtained composite plants (11 carrying pH7WG2D*::*Soyuretox and 10 empty-pH7WG2D), indicating the insertion and expression of the *gfp* gene harbored by the vector (Fig. 2). The transformation efficiency (number of positive composite plants/number

of seedlings subjected to transformation) obtained was 54 and 47% for seedlings transformed with pH7WG2D::Soyuretox and empty-pH7WG2D, respectively.

The presence of 35S promoter and Soyuretox-enconding sequences in the genome of the ten transgenic plants and in the hairy roots of 11 composite plants was confirmed by PCR (data not shown). The Soyuretox relative expression varied from 2 to 42-fold in transformed hairy roots, comparing to the non-transformed roots (Fig. 3a). Soyuretox transcript levels were also significantly higher in transgenic plants (2 to 21-fold) when compared with non-transformed plants (Fig. 3b). Basal transcript levels were detected in non-transformed hairy roots and plants, corresponding to endogenous *Eu*4 gene expression.

Soyuretox effects on nematode infection

Two bioassays were carried out to evaluate the potential of Soyuretox in conferring resistance against nematodes. Composite plants containing only GFP-positive roots were inoculated with *M. javanica* J2. After 45 days, 80 and 70% of roots transformed with Soyuretox or empty-pH7WG2D, respectively, still exhibited GFP fluorescence. Roots overexpressing Soyuretox exhibited a significant reduction (50%; $p<0.05$) in the average number of nematodes and eggs when compared with non-transformed roots (Fig. 4a). Transgenic plants overexpressing Soyuretox also exhibited significant reduction (37.5%; $p<0.05$) on the number of eggs and nematodes when compared with non-transformed plants (Fig. 4b) 45 days after inoculation. These results indicate that the increased resistance is attributable to Soyuretox overexpression.

All developmental stages of nematodes were found inside both Soyuretox-overexpressing and non-transformed roots (supplementary Fig. S2). The nematodes were almost exclusively detected in the root cortex.

Discussion

Characterization of Soyuretox

Although ureases are highly conserved molecules, one of the regions that exhibit remarkable variation in amino acid sequence corresponds to the N-terminal domain of jaburetox (Mulinari et al. 2007). This portion of the polypeptide was later identified as the jaburetox insecticidal domain (Martinelli et al. 2014). Jaburetox has also been described as fungitoxic to filamentous fungi and yeasts (Postal et al. 2012), although the structure-activity relationships implicated in this biological activity are not yet known. It is reasonable to expect that peptides collinear to jaburetox that are derived from other plant ureases might

possess different biological properties due to differences in the primary sequences of their N-terminal domains; for this reason, this is a topic worth investigating. Of 101 amino acid residues, 74 (71.8%) are identical in Jaburetox and Soyuretox, and out of the 27 that are different, 20-21 are in the N-terminal region.

Resistance of transformed soybean against M. javanica

Given the very specialized feeding habits of root-knot nematodes, bioassays aimed at identifying compounds with nematicidal properties often require a transformed plant expressing this compound in its roots. In the present study, Soyuretox-expressing roots were challenged with *M. javanica*.

The *A. rhizogenes*-mediated transformation method allows the high-throughput production of transgenic roots. This system has been widely used because it significantly reduces the time required to generate transgenic events compared with traditional methods of soybean transformation (Weber and Bodanese-Zanettini 2011; Lin et al. 2013). This characteristic has facilitated the use of composite plants for biotechnological applications (Guillon et al. 2006). The transformation efficiency obtained (54 and 47% for seedlings transformed with pH7WG2D*::*Soyuretox and empty-pH7WG2D, respectively) is similar to those obtained in other studies using *A. rhizogenes* transformation (Cho et al. 2000; Weber and Bodanese-Zanettini 2011).

The increased resistance exhibited by Soyuretox-expressing hairy roots was validated using a stable transgenic line. T_2 Soyuretox plants with a range of transgene expression also showed highest resistance when confronted with *M. javanica*.

Studies of soybean-nematode interactions have focused mainly on the number of galls and cysts (Ibrahim et al. 2011; Youssef et al. 2013a; Youssef et al. 2013b: Matthews et al. 2014). In the current study, the reproduction factor of *M. javanica* was determined by counting the number of eggs and nematodes after one cycle of replication (45 days). This approach is widely used in traditional nematological studies (Carneiro et al. 2000; Wilcken et al. 2010). Because the number of eggs inside a gall varies, the reproduction factor provides a more precise result. A 50% and 37.5% reduction in the reproduction factor was observed in hairy roots and roots of stable transgenic plants overexpressing Soyuretox respectively. Interestingly, the reduction did not exhibit a direct correlation with transgene transcript levels, indicating that a low amount of Soyuretox is sufficient to hinder nematodes.

Considering the injury caused in soybeans by nematodes and the occurrence of resistance breakage in current varieties, the identification of genes with the potential to confer resistance and the development

of biotechnological strategies that will contribute to the production of highly resistant varieties are mandatory. It is well documented that ureases and their derived peptides exhibit toxic activity against insects and fungi (Carlini et al. 1997; Stanisçuaski et al. 2005; Becker-Ritt et al. 2007; Defferrari et al. 2011; Wiebke-Strohm et al. 2012; Martinelli et al. 2014). This is the first study to investigate the nematicidal activity of a urease-derived peptide.

In conclusion, the overexpression of Soyuretox in hairy roots and solid transgenic plants was found to increase the soybean resistance to root-knot nematodes. The use of Soyuretox-like urease-derived peptides may represent a useful tool as a new and efficient alternative to control pests and diseases in economically important crops, thus reducing the use of chemical pesticides and consequently contributing to a reduction in environmental impact. In addition, the employment of a peptide derived from a soybean protein, as Soyuretox, would facilitated to access GM crop safety prior commercialization.

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Author contribution

Conceived and performed the experiments: CR, BW-S, LAO-B, FCM-G, KK, MCMC, VSLC, SMHS, WPD, CRC, MHB-Z. Wrote and revised the paper: CR, BW-S, LAO, FCM-G, CRC, MHB-Z. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Becker-Ritt AB, Martinelli AH, Mitidieri S, Feder V, Wassermann GE, Santi L, Vainstein MH, Oliveira JT, Fiuza LM, Pasquali G, Carlini CR (2007) Antifungal activity of plant and bacterial ureases. Toxicon 50:971-983. doi:10.1016/j.toxicon.2007.07.008

Bird DM, Kaloshian I (2003) Are roots special? Nematodes have their say. Physiol. Mol Plant Path 62:115-123. doi:10.1016/S0885-5765(03)00045-6

Boneti JIS, Ferraz S (1981) Modificações do método e Hussey e Baker para a extração de Meloidogyne exigua de cafeeiro. Fitopatol, Brasil, Brasilia, pp 553

Carlini CR, Oliveira AE, Azambuja P, Xavier-Filho J, Wells MA (1997) Biological effects of canatoxin in different insect models, evidence for a proteolytic activation of the toxin by insect cathepsin like enzymes. J Econ Entomol 90:340-348. doi: http://dx.doi.org/10.1093/jee/90.2.340

Carlini CR, Ferreira-Da-Silva CT: Gombarovits MEC Peptídeo Entomotóxico da Canatoxina. Processo de Produção. Patente de invenção, PI0003334-0, DEINPI/RS País, Brasil. Data de depósito 06/06/2000.

Carlini CR, Polacco JC (2008) Toxic Properties of Urease. Crop Sci 48:1665-1672. doi:10.2135/cropsci2007.10.0576

Carlini CR, Ligabue-Braun R (2016) Ureases as multifunctional toxic proteins: A review, Toxicon 110:90-109. doi: 10.1016/j.toxicon.2015.11.020

Carneiro R MDG, Rading O, Almeida MRA, Campos AD (2000) Resistence of vegatable crops to Meloidogyne spp., Suggestion for a crop rotation system. Nematol bras 24:49-54.

Ceasar AS, Ignacimuthu S (2012) Genetic engineering of crop plants for fungal resistance, role of antifungal genes. Biotechnol Lett 34:995-1002. doi: 10.1007/s10529-012-0871-1

Chitwood DJ, Perry RN (2009) Reproduction, physiology and biochemistry. In Perry M, Moens JL Starr. Root-knot Nematodes. (ed) CABI, Wallingford, UK, pp 182-200

Cho HJ, Farrand SK, Noel GR (2000) High-efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. Planta 210:195-204

Defferrari MS, Demartini DR, Marcelino TB, Pinto PM, Carlini CR (2011) Insecticidal effect of Canavalia ensiformis major urease on nymphs of the milkweed bug Oncopeltus fasciatus and characterization of digestive peptidases. Insect. Biochem Mol Biol 41:388-399. doi: 10.1016/j.ibmb.2011.02.008

Dias WP, Garcia A, Silva JFV, Carneiro GE de S (2010) Nematóides de soja: identificação e controle. (ed) Embrapa: Circular Tecnica 76

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11-15

Droste A, Pasquali G, Bodanese-Zanettini MH (2002) Transgenic fertile plants of soybean [Glycine max (L) Merrill] obtained from bombarded embryogenic tissue. Euphytica 127:367-376

Ferreira-DaSilva CT, Gombarovits MEC, Masuda H, Oliveira CM, Carlini CR (2000) Proteolytic activation of canatoxin, a plant toxic protein, by insect cathepsin-like enzymes. Arch Insect Biochem Physiol 44:162-171. doi: 10.1002/1520-6327

Follmer C, Real-Guerra R, Wasserman GE, Olivera-Severo D, Carlini CR (2004) Jackbean, soybean and Bacillus pasteurii ureases, biological effects unrelated to ureolytic activity. Eur J Biochem 271:1357-1363. doi: 10.1111/j.1432-1033.2004.04046

Fuller VL, Lilley CJ, Urwin PE (2008) Nematode resistance. New Phytol 180:27-44. doi: 10.1111/j.1469-8137.2008.02508

Goldraij A, Beamer LJ, Polacco JC (2003) Interallelic complementation at the ubiquitous urease coding locus of soybean. Plant Physiol 132:1801-1810. doi: 10.1104/pp.103.022699

Gonçalves JF, Laia ML, Barbosa BFF, Bergamasco VB, Santos JM, Costa AL, Lemos MVF (2014) Cloning and expression of a novel cry gene that is potentially active against nematodes. Afr J Microbiol Res 8:1017-1025. doi: 10.5897/AJMR2013.6282

Guillon S, Tremouillaux-Guiller J, Pati PK, Rideau M, Gantet PH (2006) The potential of hairy roots, dawn of a new era. Trends Biotechnol 24:403-409. doi: http://dx.doi.org/10.1016/j.tibtech.2006.07.002

Holland MA, Griffin JD, Meyer-Bothling LE, Polacco JC (1987) Developmental genetics of the soybean urease isozymes. Dev Genet 8:375-387. doi: 10.1002/dvg.1020080508

Ibrahim HMM, Hosseini P, Alkharouf NW, Hussein EHA, El-Din ABD, El Kader Y, Gamal AM, Matthews BF (2011) Analysis of Gene expression in soybean (Glycine max) roots in response to the root knot nematode Meloidogyne incognitausing microarrays and KEGG pathways. BMC Genomics 12: 2-16. doi:10.1186/1471-2164-12-220

Jian B, Liu B, Bi Y, Hou W, Wu C, Han T (2008) Validation of internal control for gene expression study in soybean by quantitative real-time PCR. BMC Mol Biol 9:1-14. doi: 10.1186/1471-2199-9-59

Jones JT, Haegeman A, Danchin EGJ, Gaur HS, Helder J, Jones MGH, Kikuchi T, Manzanilla-Lópe R, Palomares-Rius JE, Wesemael WML, Perry RN (2013) Top 10 plant-parasitic nematodes in molecular plant pathology. Molecular plant pathology 14: 946-961. doi: 10.1111/mpp.12057

Karimi M, Inze D, Depicker A (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci 7:193-195. doi: 10.1016/S1360-1385(02)02251-3

Kuma KM, Lopes-Caitar V S, Romero CCT, Silva SMH, Kuwahara MK, Carvalho MCCG, Abdelnoor RV, Dias WP, Marcelino-Guimarães FC (2015) A high efficient protocol for soybean root

transformation by Agrobacterium rhizogenes and most stable reference genes for RT-qPCR analysis. Plant Cell Rep 1-14. doi:10.1007/s00299-015-1845-2

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 21:2947–2948. doi:10.1093/bioinformatics/btm404

Libault MS, Thibivillier DD, Bilgin O, Radwan M, Benitez SJ, Clough SG (2008) Identification of four soybean reference genes for gene expression normalization. Plant Genome 1:44-54. doi:10.3835/plantgenome2008.02.0091

Lin JY, Mazarei M, Zhao N, Zhu JWJ, Zhuang XF, Liu WS, Pantalone VR, Arelli PR, Stewart CN, Chen F (2013) Overexpression of a soybean salicylic acid methyltransferase gene confers resistance to soybean cyst nematode. Plant Biotech. J. 11:1135-1145. doi: 10.1111/pbi.12108

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\text{Delta Delta C(T)})}$. Method Methods 25:402-408. doi:10.1006/meth.2001.1262

Martinelli AHS, Kappaun K, Ligabue-Braun R, Defferrari MS, Piovesan AR, Stanisçuaski F, Demartini DR, Verli H, Dal Belo CA, Almeida CGM, Follmer C, Carlini CR, Pasquali G (2014) Structure-function studies on Jaburetox, a recombinant insecticidal and antifungal peptide derived from jack bean Canavalia ensiformis) urease. Biochim Biophys Acta - General Subjects 1840:935-944. doi: 10.1016/j.bbagen.2013.11.010

Matthews BF, Beard H, Brewer E, Kabir S, MacDonald MH, Youssef RM (2014) Arabidopsis genes, AtNPR1, AtTGA2 and AtPR-5, confer partial resistance to soybean cyst nematode (Heterodera glycines) when overexpressed in transgenic soybean roots. BMC Plant Biol 14:2-19. doi:10.1186/1471-2229-14-96

Mobley HL, Island MD, Hausinger RP (1995) Molecular biology of microbial ureases. Microbiol Rev 59:451-480

Moens M, Perry RN, Star JL (2010) Meloidogyne species – a diverse group of novel and important plant parasites. In: Moens M, Perry RN, Star JL (ed) Root-knot nematodes. Wallingford: CABI International, pp1-13

Mulinari F, Stanisçuaski F, Bertholdo-Vargas LR, Postal M, Oliveira-Neto OB, Ridgen DJ, Grossi-de-Sá MF, Carlini CR (2007) Jaburetox-2Ec, an insecticidal peptide derived from an isoform of urease from the Canavalia ensiformis plant. Peptides 28:2042-2050. doi:10.1016/j.peptides.2007.08.009

Nicol JM, Turner SJ, Coyne DL, Den Nijs L, Hockland S, Maafi ZT (2011) Current nematode threats to world agriculture. In Jones JT, Gheysen G, Fenoll C (ed) Genomics and Molecular Genetics of Plant–Nematode Interactions. Heidelberg: Springe pp 21-44

Postal M, Martinelli AHS, Becker-Ritt AB, Ligabue-Braun R, Demartini DR, Ribeiro SFF, Pasquali G, Gomes VM, Carlini CR (2012) Antifungal properties of Canavalia ensiformis urease and derived peptides. Peptides 38:22-32. doi:10.1016/j.peptides.2012.08.010

Stanisçuaski F, Ferreira-DaSilva CT, Mulinari F, Pires-Alves M, Carlini CR (2005) Insecticidal effects of canatoxin on the cotton stainer bug Dysdercus peruvianus (Hemiptera, Pyrrhocoridae). Toxicon 45:753-760. doi:10.1016/j.toxicon.2005.01.014

Stanisçuaski F, Carlini CR (2012) Plant Ureases and Related Peptides: Understanding their entomotoxic properties. Toxins 4:55-67. doi:10.3390/toxins4020055

Weber RLM, Bodanese-Zanettini MH (2011) Induction of transgenic hairy roots in soybean genotypes by Agrobacterium rhizogenes-mediated transformation. Pesq Agropec Bras Brasília 46:1070-1075. doi:10.1590/S0100-204X2011000900014

Wiebke-Strohm B, Ligabue-Braun R, Rechenmacher C, Abruzzide Oliveira-Busatto L, Carlini CR, Bodanese-Znettini MH (2016) Structural and transcriptional characterization of a novel member of the soybean urease gene Family, Plant Physiol Bioch 101:96-104. doi: 10.1016/j

Wiebke-Strohm B, Pasquali G, Margis-Pinheiro M, Bencke M, Bücker-Neto L, Becker-Ritt AB, Martinelli AHS, Rechenmacher C, Polacco JC, Stolf R, Marcelino FC, Abdelnoor RV, Homrich MS, Del Ponte EM, Carlin CR, De Carvalho MCCG, Bodanese-Zanettini MH (2012) Ubiquitous urease affects soybean susceptibility to fungi. Plant Mol Biol 78:1-13. doi: 10.1007/s11103-012-9894-1

Wilcken SRS, Rosa JMO, Higuti ARO, Garcia MJM, Cardoso AII (2010) Reprodução de Meloidogyne spp. em porta-enxertos e híbridos de pepino. Hortic bras 28:120-123. doi:10.1590/S0102-05362010000100023

Williamson VM, Gleason CA (2003) Plant-nematode interactions. Curr Opin Plant Biol 6:327-333. doi:10.1016/S1369-5266(03)00059-1

Youssef RM, Margaret HM, Eric PB, Gary RB, Kim Kyung-Hwan, Matthews BF (2013a) Ectopic expression ofAtPAD4 broadens resistance of soybean to soybean cyst and root-knot nematodes. BMC Plant Biol 13:2-11. doi:10.1186/1471-2229-13-67

Youssef RM, Kim Kyung-Hwan, Haroon SA, Matthews BF (2013b) Post-transcriptional gene silencing of the gene encoding aldolase from soybean cyst nematode by transformed soybean roots. Exp Parasitol 134:266-274. doi:10.1016/j.exppara.2013.03.009

a

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Soyuretox       MGPVNDSNCRAAMKAVVTRGFGHVEEENAREGVTG--EDYSLTTVISREEYAHKYGPTTG
Jaburetox       MGPVNEANCKAAMEIVCRREFGHKEEEDASEGVTTGDPDCPFTKAIPREEYANKYGPTIG
                *****::**:***: *  * *** ***:* ****    * .:*..*.*****:***** *

Soyuretox       DKIRLGDTDLFAEIEKDFAVYGDECVFGGGKVAAALEHHHHHH
Jaburetox       DKIRLGDTDLIAEIEKDFALYGDESVFGGGKV--ILEHHHHHH
                **********:********:****.*******   ********
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b

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  1 MKLSPREIEK LDLHNAGYLA QKRLARGLRL NYVETVALIA TQILEFVRDG EKTVAQLMCI
 61 GRELLGRKQV LPAVPHLVES VQVEATFRDG TKLVTIHDLF ACENGNLELA LFGSFLPVPS
121 LDKFTENEED HRTPGEIICR SENLILNPRR NAIILRVVNK GDRPIQVGSH YHFIEVNPYL
181 TFDRRKAYGM RLNIAAGNAT RFEPGECKSV VLVSIGGNKV IRGGNNIADG PVNDSNCRAA
241 MKAVVTRGFG HVEEENAREG VTGEDYSLTT VISREEYAHK YGPTTGDKIR LGDTDLFAEI
301 EKDFAVYGDE CVFGGGKVIR DGMGQSSGHP PEGSLDTVIT NAVIIDYTGI IKADIGIKDG
361 LIISTGKAGN PDIMNDVFPN MIIGANTEVI AGEGLIVTAG AIDCHVHFIC PQLVYDAVTS
421 GITTLVGGGT GPADGTRATT CTPAPNQMKL MLQSTDDMPL NFGFTGKGNS AKPDELHEII
481 RAGAMGLKLH EDWGTTPAAI DSCLTVADQY DIQVNIHTDT LNESGFVEHT IAAFKGRTIH
541 TYHSEGAGGG HAPDIIKVCG EKNVLPSSTN PTRPYTHNTI DEHLDMLMVC HHLNKNIPED
601 VAFAESRIRA ETIAAEDILH DKGAISIISS DSQAMGRIGE VISRTWQTAD KMKSQRGPLQ
661 PGEDNDNFRI KRYVAKYTIN PAIANGLSQY VGSVEAGKLA DLVLWKPSFF GAKPEMVIKG
721 GEVAYANMGD PNASIPTPEP VIMRPMFGAF GKAGSSHSIA FVSKAALDEG VKASYGLNKR
781 VEAVKNVRKL TKRDMKLNDT LPQITVDPET YTVTADGEVL TCTAAKTVPL SRNYFLFStop
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Fig. 1 Soyuretox amino acid sequence. (a) Alignment of the amino acid sequences of Soyuretox and jaburetox using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). (*) identity in both peptides. (:) strongly similar; (.) weakly similar. (b) Amino acid sequence of ubiquitous urease translated from the *G. max Eu*4 gene (Glyma11g37250; AJ276866) with the internal Soyuretox sequence shown in gray.

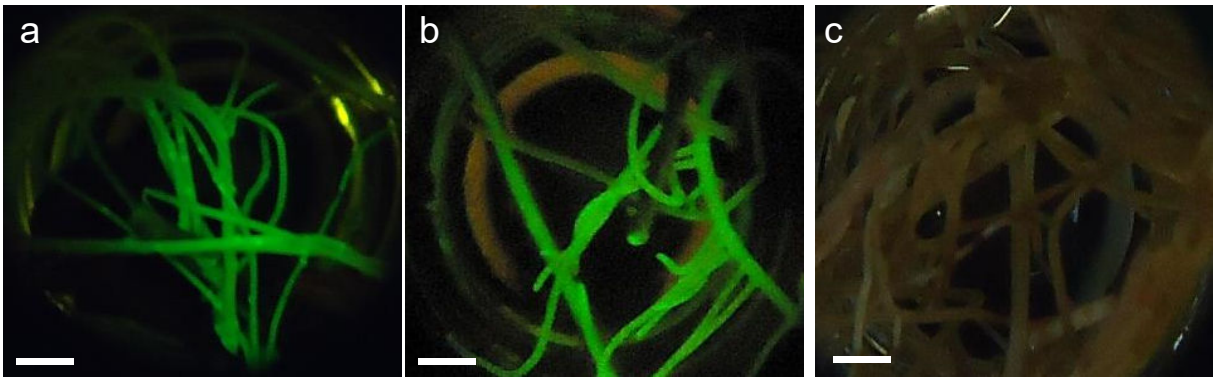


Fig. 2 Detection of GFP in transformed and non-transformed roots. (a) Soybean roots transformed with pH7WG2D::Soyuretox. (b) Soybean roots transformed with empty-pH7WG2D. (c) Non-transformed roots. GFP expression was detected under blue light using a fluorescence stereomicroscope Leica MZIII (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a BP filter set containing a 488-ηm excitation filter and a 505-530 ηm emission filter. Bar = 1mm.

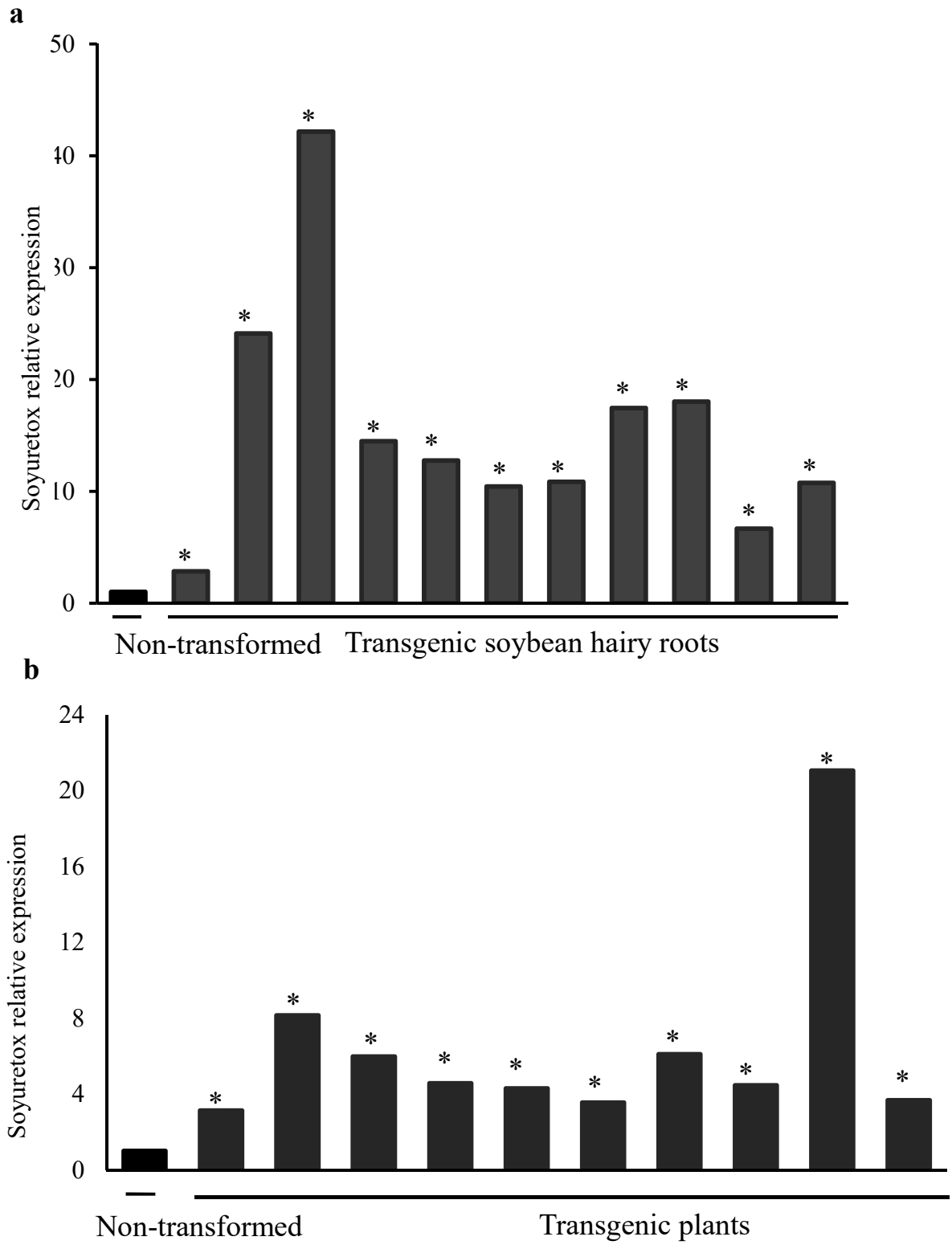


Fig. 3 Soyuretox transcript levels (RT-qPCR). (a) Hairy roots of eleven composite plants and two non-transformed plants (NT) from cv Williams82. (b) Ten transgenic plants and two non-transformed plants (NT) from cv IAS5. F-Box and Metalloprotease reference genes were used as internal control. Basal *Eu*4

transcript levels detected in non-transformed hairy roots and leaves were used to normalize transcript accumulation in transgenic samples. The bars represent the means of four technical replicates. A non-parametric t test ($p<0.05$) was carried out to compare the Soyuretox transcript levels in transformed vs. non-transformed hairy roots and transformed vs. non-transformed plants. The symbol * indicates that values are significantly different from control sample (NT).

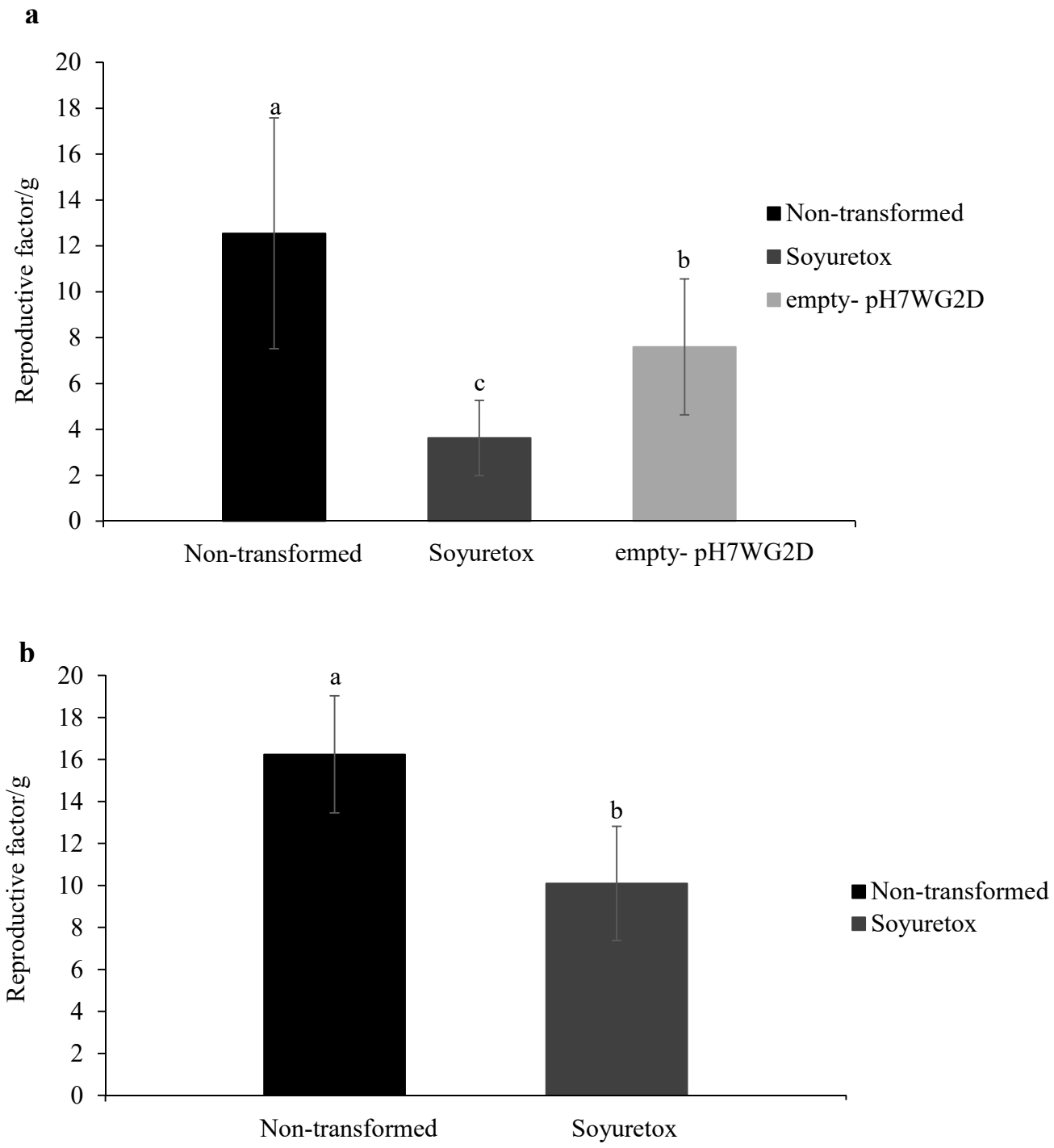
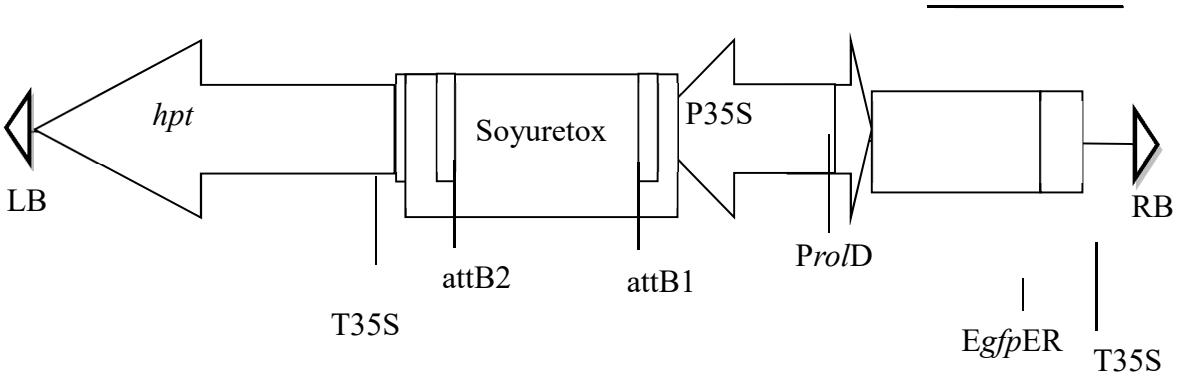


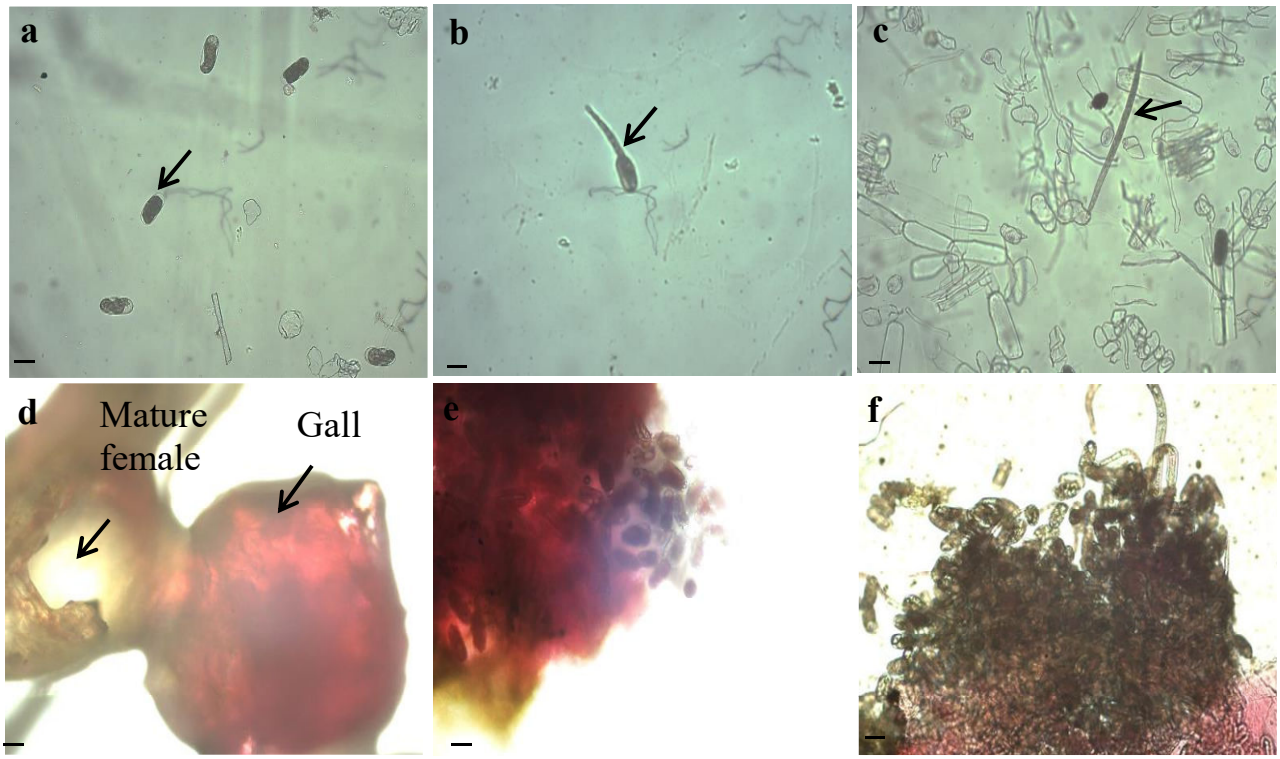
Fig. 4 Reproduction factor of *M. javanica* per gram of roots 45 days after inoculation. The bars represent the average number of eggs and nematodes ±SD present in: (a) hairy roots of 11 composite plants transformed with pH7WG2D::Soyuretox (overexpressing Soyuretox), 10 composite plants transformed with empty-pH7WG2D and 9 non-transformed (NT) plants from cv Williams82. (b) Ten transgenic plants transformed with pH7WG2D::Soyuretox (overexpressing Soyuretox) and six non-transformed plants (NT) from cv IAS5. Bars with different letters differ according to ANOVA and Tukey's test at $p<0.05$.

Table 1 Primer set designed for PCR and sequencing

Target gene	Orientation	Sequence	Reference
Isolation of the coding sequence of Soyuretox (PCR)	Forward Reverse	5'-CACCATGGGTCCAGTTAATGAT-3' 5'-TCAGACTTTCCCACCTCCAAA-3'	This study
Confirmation of the condition of transgenic roots (PCR)	Forward Reverse	5'-CGCACAATCCCACTATCCTT-3' 5'-TCAGACTTTCCCACCTCCAAA-3'	This study
Confirmation of the presence *M. javanica* in roots (PCR)	Forward Reverse	5'-CAAAACCACGCGGCTTCGGC-3' 5'-TGGGGGTGCCCTTCCGTCAA-3'	This study
Soyuretox (RT-qPCR)	Forward Reverse	5'-ATTGCAGAGCAGCCATGAA-3' 5'-TGGCCCTACAACTGGTGAC-3'	This study
Actin II (RT-qPCR)	Forward Reverse	5'-CGGTGGTTCTAT CTTGGCATC-3' 5'-GTCTTTCGCTTCAA TAACCCTA-3'	Jian et al. 2008
Metalloprotease (RT-qPCR)	Forward Reverse	5'-ATGAATGACGGTTCCCATGTA-3' 5'- GGCATTAAGGCAGCTCACTCT -3'	Libault et al. 2008
Fbox protein (RT-qPCR)	Forward Reverse	5'-AGATAGGGAAATGTTGCAGGT-3' 5'-CTAATGGCAATTGCAGCTCTC-3'	Libault et al. 2008



Supplementary Fig. S1 T-DNA region of the binary vector pH7WG2D::Soyuretox used for soybean transformation. RB – T-DNA right border, LB – left border, *hpt* – hygromycin phosphotransferase gene, P35S – cauliflower mosaic virus (CaMV) 35S promoter, T35S – CaMV 35S terminator, *Egfp*ER – enhanced green fluorescent protein, P*rolD* – root loci D promoter of Soyuretox, *attB1* and *attB2* – LR reaction site, kb – kilobase pairs (1,000 bp). Arrows and boxes represent primers and PCR reaction products used for transgene screening.



Supplementary Fig. S2 Developmental stages of *M. javanica* in infected roots. (a) Eggs of nematodes. Arrow indicates one egg. (b) Second-stage juvenile (J2) hatching from an egg. Arrow indicates one J2. (c) Infective J2. Arrow indicates one J2 (d). Mature female with white-pearly color and globular body. Egg mass is pink stained with acid fuchsin. Arrows indicate mature female and egg mass (gall). (e) Gall releasing the eggs. (f) Eggs outside the gall. Images were captured by an optical microscope (40x). Bar = 1mm.

Relatório Descritivo de Patente de Invenção

TOXINA PRAGUICIDA, CONSTRUÇÃO GÊNICA, PROCESSO DE PRODUÇÃO DE PRAGUICIDA, COMPOSIÇÃO PRAGUICIDA, MÉTODO DE CONTROLE DE PRAGAS

Pedidos de Patentes Correlatos

[1] O presente é um texto com o objeto de certificado de adição ao pedido de patente PI 0403435-0, depositado em 08/04/2004.

Campo da Invenção/Adição

[2] A presente invenção refere-se genericamente a praguicidas. Mais especificamente, a presente invenção se refere a uma toxina praguicida derivada de um polipeptídeo não natural obtido através da truncagem da(s) sequência(s) gênica(s) da família da urease. A presente Adição, a qual ora se busca certificação, é à Invenção objeto do pedido de patente PI 0403435-0, que proporciona uma toxina praguicida, uma construção gênica, um processo de produção de praguicida, uma composição praguicida e um método de controle de pragas.

[3] Na presente adição são providos dados adicionais sobre a toxicidade das ureases e peptídeos derivados, para a proteção de plantas contra nematóides. As plantas modificadas geneticamente com a construção gênica da invenção comprovam que há efetivo controle de pragas com o uso da invenção.

Capítulo IV

CONCLUSÕES E PERSPECTIVAS

4. Conclusões e Perspectivas

4.1 Análise das plantas transgênicas com o gene *Eu*4 co-suprimido

A análise das plantas transgênicas obtidas por Wiebke-Strohm et al. (2012), confirmou que a co-supressão do gene *Eu*4 (urease ubíqua) foi mantida ao longo das gerações. Além disto, a redução dos níveis de mRNA foi também observada para os genes *Eu*1 (urease embrião especifica) e *Eu*5 (SBU-III). Portanto, a co-supressão ocorreu para todas as ureases da soja, resultado que pode ser atribuído ao fato de existir um alto grau de similaridade entre as sequências (Follmer, 2008; Krajewska, 2009). Mesmo apresentando o silenciamento de todas as ureases, as plantas co-suprimidas representam variantes genéticas importantes para o avanço no estudo funcional das ureases, uma vez que mutantes nulos para urease ubíqua nunca haviam sido obtidos.

O papel das ureases quanto ao padrão de desenvolvimento da planta de soja foi analisado no presente trabalho. Esse processo biológico foi investigado em plantas transgênicas e comparados com plantas não transgênicas. Para fins de estudo funcional de genes individuais os mutantes simples *eu1-a, eu4-a* e *eu3-a*, duplo *eu1-a/eu4-a*, foram utilizados e comparados com plantas não mutantes. As plantas mutantes foram também caracterizadas quanto ao nível de expressão das três ureases. Para os mutantes *eu4-a* e *eu3-a*, foram observados níveis normais de transcritos de mRNA para todas as ureases. Estes resultados eram esperados já que *eu4-a* produz uma proteína inativa (Torisky e Polacco, 1990; Stebbins et al. 1991; Witte et al. 2002; Goldraij et al. 2003; Polacco et al. 2011) e *eu3-a* codifica a proteína acessória UreG (Freyermuth et al. 2000). Estas mutações, portanto, não alteram o nível de expressão dos genes *E*u4, *Eu*1e *Eu*5, quando estimado pelo perfil de transcritos. Os outros mutantes, *eu1-a* e *eu1-a/eu4-a*, exibiram apenas níveis menores para *Eu*1, uma vez que *eu1* é um mutante nulo para urease embrião especifica (Polacco et al. 1993).

Os resultados obtidos indicaram que as plantas com co-supressão dos genes que codificam ureases apresentaram um comprometimento no seu desenvolvimento, com redução do peso, do tamanho da parte aérea e do sistema radicular, além de menor conteúdo de nitrogênio, se comparadas com as plantas controles. O mutante simples *eu3-a* e o duplo mutante *eu1-a/eu4-a* apresentaram um pequeno atraso no desenvolvimento durante o primeiro mês. O conjunto de dados resultante deste trabalho indicam que a

urease embrião especifica e urease SBU-III possam também estar contribuindo em processos que estejam relacionadas ao desenvolvimento.

Esse conjunto de plantas apresentando as urease co-suprimidos representam, também, uma ferramenta importante para a elucidação das funções adicionais à atividade ureolítica. No trabalho realizado por Wiebke-Strohm et al. (2012) utilizando essas mesmas plantas, foi demonstrado que a urease ubíqua de soja está envolvida no mecanismo de resistência ao fungo *Phakopsora pachyrhizi* (causador da ferrugem asiática), pois mostraram maior número de lesões e pústulas, quando comparadas com plantas não transformadas. Essas plantas também poderão ser utilizadas em futuros bioensaios *in vivo* utilizando nematoides de importância agronômica, a fim de comparar a resposta das plantas silenciadas com plantas superexpressando o peptídeo soyuretox. Além disto teste nos quais as referidas plantas serão desafiadas com insetos alvo e não-alvo para a soja, poderão servir como prova de conceito para a capacidade inseticida desta enzima.

4.2 Transformação de soja mediada por *Agrobacterium rhizogenes* e bombardeamento de partículas

A transformação genética é uma ferramenta potencial para o aumento da qualidade das culturas e para a superação dos limites impostos pelo ambiente ao aumento da produção (Popelka et al. 2004). Portanto, torna-se necessário o desenvolvimento de sistemas eficientes de transformação e regeneração de plantas para cada espécie vegetal, bem como a realização de estudos funcionais, visando identificar genes que possam conferir características de interesse.

Tanto o bombardeamento de partículas quanto o sistema *Agrobacterium* têm sido utilizados para introdução de genes em diversos tecidos da soja e regeneração de plantas completamente transformadas (Somers et al. 2003; Mello-Farias e Chaves, 2008; Wiebke-Strohm et al. 2011; Kuma et al. 2015). Cabe ressaltar que o método de obtenção de raízes transformadas por *A. rhizogenes* é também uma ferramenta bastante útil para testar o efeito de genes de interesse em um curto espaço de tempo. Isso é especialmente importante em estudos envolvendo características do sistema radicular, tais como doenças causadas por patógenos de raiz (Cho et al. 2000).

Conforme resultados apresentados no Capítulo 3, foram regeneradas com sucesso plantas de soja totalmente transgênicas e férteis expressando o peptídeo soyuretox e

plantas compostas expressando o peptídeo somente na raiz. As plantas compostas foram obtidas em algumas semanas, enquanto que as plantas completamente transformadas, após pelo menos um ano (contando a obtenção de embriões somáticos, etapas de transformação, seleção e regeneração dos transformantes primários).

É importante salientar que independentemente do método utilizado, a transformação de soja não é um trabalho trivial, exigindo muita experiência e habilidade do pesquisador. A "arte" de regenerar plantas a partir da cultura de tecidos transformados é um ponto crucial para o sucesso dos programas de transformação genética. Por isso, torna-se importante o treinamento e desenvolvimento de habilidades dos pesquisadores para gerar plantas suficientes, tanto em trabalhos de estudos funcionais de genes como para a obtenção de plantas de interesse agronômico (Trick et al. 1997; Somers et al. 2003).

4.3 Análise da toxicidade do peptídeo Soyuretox contra o ataque do fitonematóide *M. javanica*.

Em nosso laboratório, como já mencionado anteriormente, foram realizados experimento de transformação visando a superexpressão da urease ubíqua. Embora um número considerável de eventos tenha sido obtido, apenas uma planta foi capaz de superexpressar o gene moderadamente (4 vezes mais do que a controle), enquanto as demais plantas transgênicas apresentaram co-supressão (Wiebke-Strohm et al. 2012). Entretanto, a planta que superexpressava o gene não deixou descentes. Com o intuito de obter plantas transgênicas superexpressando a urease ubíqua, novos experimentos de transformação foram conduzidos em nosso laboratório, porém novamente não foram obtidas plantas com esta característica. Devido à dificuldade de superexpressar a urease ubíqua completa, a alternativa mais promissora e viável no momento foi estudar a expressão de partes do gene que codifica esta urease de soja, correspondente ao peptídeo tóxico.

Recentemente, a equipe da doutora Célia Carlini do Laboratório de Proteínas Tóxicas (Laprotox) da UFRGS, identificou a região que corresponde ao peptídeo tóxico da urease ubíqua de soja, que foi denominado Soyuretox (Kappaun, 2014). Este resultado possibilitou investigar as propriedades de defesa da urease ubíqua, adicionais à atividade ureolítica.

Os resultados obtidos no padrão de expressão do peptídeo Soyuretox demonstraram ser possível superexpressar o peptideo tanto nas raízes obtidas através da

transformação por *A. rhizogenes*, quanto nas plantas totalmente transformadas, obtidas por bombardeamento. Em bioensaios as plantas compostas e completamente transformadas revelaram ser mais resistentes a *M. javanica* do que as plantas controle, mostrando uma redução na reprodução deste nematóide em torno de 50% e 37.5%, respectivamente.

As plantas transgênicas superexpressando o Soyuretox representam uma alternativa promissora para fornecer um controle eficaz contra esse tipo de patógeno, diminuindo assim o gasto com nematicida e, consequentemente, contribuindo com uma melhor qualidade do meio ambiente.

Esse trabalho tem fundamental importância para o avanço do conhecimento das propriedades tóxicas do Soyuretox sobre nematóides por dois motivos principais: (1) este é o primeiro relato de um peptídeo derivado de uma urease que apresenta atividade nematicida e (2) comprova que esse peptídeo contribui para função de defesa da planta *in vivo*, mesmo na presença de outras proteínas de defesa. Assim, os dados obtidos destacam o potencial do Soyuretox para uma aplicação biotecnológica.

Novos experimentos de transformação visando a superexpressão do Soyuretox pela técnica de bombardeamento foram realizadas, usando um novo vetor de superexpressão, o pEarleyGate100, que tem o promotor CaMV 35S controlando o gene de interesse, além do gene marcador *bar*, que confere resistência ao herbicida glufosinato de amônio. Do ponto de vista de estudos básicos, este vetor facilita a seleção das plantas transgênicas em populações segregantes. As análises moleculares destas plantas confirmarão o estado transgênico, o grau de expressão e a estabilidade do transgene nas gerações descendentes.

Os resultados obtidos até o presente momento são altamente promissores, pois demostraram expressiva resistência das plantas transgênicas ao ataque do nematóide *M. javanica*. As plantas resultantes desses dois experimentos de transformação poderão ser utilizadas não apenas em bioensaios *in vivo* com outros nematóides de importância agronômica, mas também testadas contra insetos alvos e não-alvos e fungos como por exemplo o *P. pachyrhiz*i (causador da ferrugem asiática). Esses dados servirão como prova de conceito para a capacidade tóxica do peptídeo e confirmarão seu potencial para geração de um futuro produto biotecnológico.

REFERÊNCIAS BIBLIOGRÁFICAS DOS CAPÍTULOS I E V

REFERÊNCIAS BIBLIOGRÁFICAS

Bacanamwo M, Witte CP, Lubbers MW, Polacco JC (2002) Activation of the urease of *Schizosaccharomyces pombe* by the UreF accessory protein from soybean. Mol Genet Genomics 268:525–534.

Balasubramanian A, Ponnuraj K (2010) Crystal structure of the first plant urease from jack bean: 83 years of journey from its first crystal to molecular structure. J Mol Biol 400:274-283.

Barros R, Borges EP (2007) Doenças da cultura da soja Soja Tecnologia e Produção: Soja e Milho 2006/2007 Disponível em <wwwfundacaomsorgbr/requestphp?65>

Barros R (2009) Doenças da Cultura da Soja Tecnologia e Produção: Soja e Milho 2008/2009, pp 109-122 Disponível em wwwfundacaomsorgbr/requestphp?97.

Becker-Ritt AB, Martinelli AH, Mitidieri S, Feder V, Wassermann GE, Santi L, Vainstein MH, Oliveira JT, Fiuza LM, Pasquali G, Carlini CR (2007) Antifungal activity of plant and bacterial ureases. Toxicon 50:971-983.

Bird DM, Kaloshian I (2003) Are roots special? Nematodes have their say. Physiological and Molecular Plant Pathology 62:115–123.

Broothaerts W, Mitchell HJ, Weir B, Kaines S, Smith LMA, Yang W, Mayer JE, Roa-Rodriguez C, Jefferson RA (2005) Gene transfer to plants by diverse species of bacteria. Nature 43:(3)629-633.

Cao F-Q, Werner AK, Dahncke K, Romeis T, Liu L-H, Witte CP (2010) Identification and characterization of proteins involved in rice urea and arginine catabolism. Plant Physiol 15:98-108.

Carlini CR, Oliveira AE, Azambuja P, Xavier-Filho J, Wells MA (1997) Biological effects of canatoxin in different insect models: evidence for a proteolytic activation of the toxin by insect cathepsin like enzymes. J Econ Entomol 90:340-348.

Carlini CR, Guimarães JA (1981) Isolation and characterization of a toxic protein from *Canavalia ensiformis* (jack bean) seeds, distinct from concanavalin A. Toxicon 19:667-675.

Carlini CR, Ferreira-DaSilva CT, Gombarovits, ME C Peptídeo Entomotóxico da Canatoxina: Processo de Produção Patente de invenção: PI0003334-0, DEINPI/RS País: Brasil Data de depósito: 06/06/2000.

Carlini CR, Polacco JC (2008) Toxic Properties of Urease. Crop Sci 48:1665-1672.

Carlini C, Liagabue- Braun R (2016) Ureases as multifunctional toxic proteins: A review Toxicon 110:90-109.

Chitwood DJ, Perry RN (2009) Reproduction, physiology and biochemistry Chapter 8, In RN Perry, M Moens and JL Starr, (eds) Root-knot Nematodes CABI, Wallingford, UK, pp 488.

Cho HJ, Farrand SK, Noel GR (2000) High-efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. Planta 210:195-204.

Christey, MC (2001) Use of Ri-mediated transformation for production of transgenic plants in Vitro. Cell Dev Biol Plant 37:687–700.

Collier R, Fuchs B, Walter N, Kevin LW, Taylor CG (2005) Ex vitro composite plants: an inexpensive, rapid method for root biology. Plant J 43:449-457.

Companhia Nacional De Abastecimento (CONAB) Acompanhamento da safra brasileira de grãos, 2016 Disponível em: <http://wwwconabgovbr> Acesso em julho 2016.

Coodetec (2002) Doenças emergentes em soja Cascavel: Coodetec: Bayer. Crop Science p51.

DeCleene, M, DeLey, J (1976) The host range of crown gall. *Botanical Gazette* 42: 389-466.

Demartini DR, Carlini CR, Jay JT (2011) Global and targeted proteomics in developing jack bean (*Canavalia ensiformis*) seedlings: an investigation of urease isoforms mobilization in early stages of development. Plant Mol Biol 75:53-65.

Dias, W P, Garcia, A, Silva, JFV, Carneiro, GE de S (2010) Nematoides de soja: identificação e controle Embrapa: Circular Tecnica, 76 eds.

Dita MA, Rispail N, Prats E, Rubiales D, Singh KB (2006) Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. Euphytica 147:1-24.

Dixon NE, Gazzola TC, Blakeley RL, Zermer B (1975) Letter: Jack bean urease (EC 3515) A metalloenzyme A simple biological role for nickel? J Am Chem Soc 97:4131-4133.

Droste A, Pasquali G, Bodanese-Zanettini MH (2002) Transgenic fertile plants of soybean [*Glycine max* (L) Merrill] obtained from bombarded embryogenic tissue. *Euphytica*, 127:367-376.

Embrapa Soja, disponível em: <http://wwwcnpsoembrapabr> Acesso em outubro de 2016.

Ferreira Da Silva CT, Gombarovits MEC, Masuda H, Oliveira CM, Carlini CR (2002) Proteolytic activation of canatoxin, a plant toxic protein, by insect cathepsin-like enzymes Arch Insec.t Biochem Physiol 44:62-71.

Finer JJ (1988) Apical proliferation of embryogenic tissue of soybean [*Glycine max*(L) Merrill]. Plant Cell Rep 7:238-241.

Finer JJ, McMullen MD (1991) Transformation of soybean via particle bombardment of embryogenic suspension culture tissues. In Vitro Cell Dev Biol 27:175-182.

Follmer C (2008) Insights into the role and structure of plant ureases. Phytochemistry 69:18-28.

Follmer C, Barcellos GB, Zingali RB, Machado OL, Alves EW, Barja-Fidalgo C, Guimaraes JA, Carlini CR (2001) Canatoxin, a toxic protein from jack beans (Canavalia

ensiformis), is a variant form of urease (EC 3.5.1.5): biological effects of urease independent of its ureolytic activity. Biochem J 360:217-224.

Follmer C, Real-Guerra R, Wasserman GE, Olivera-Severo D, Carlini CR (2004) Jackbean, soybean and *Bacillus pasteurii* ureases: biological effects unrelated to ureolytic activity. Eur J Biochem 271:1357-1363.

Freyermuth SK, Bacanamwo M, Polacco JC (2000) The soybean *Eu*3 gene encodes an Ni-binding protein necessary for urease activity. Plant J 21:53-60.

Fuller VL, Lilley CJ, Urwin PE (2008) Nematode resistance New Phytologist 180:27-44.

Gelvin SB (2010) Plant proteins involved in *Agrobacterium*-mediated genetic Transformation. Annual Review Phytopathology l48:45-68.

Gepts P, Beavis WD, Brummer EC, Shoemaker RC, Stalker HT, Weeden NF, Young ND (2005) Legumes as a model plant family: genomics for food and feed report of the cross-legume advances through genomics conference. Plant Physiol 137:1228-1235.

Goldraij A, Polacco JC (1999) Arginase is inoperative in developing soybean seeds. Plant Physiol 119:297-304.

Goldraij A, Polacco JC (2000) Arginine degradation by arginase in mitochondria of soybean seedling cotyledons. Planta 210:652-658.

Goldraij ALJ, Beamer Polacco JC (2003) Interallelic complementation at the ubiquitous urease coding locus of soybean. Plant Physiol 132:1801-1810.

Guillon S, Tremouillaux-Guiller J, Pati, PK, Rideau M, Gantet PH (2006) The potential of hairy roots: dawn of a new era. Trends Biotechnol 24:403-409.

Ha NC, Oh ST, Sung JY, Cha KA, Lee MH, Oh BH (2001) Supramolecular assembly and acid resistance of *Helicobacter pylori* urease. Nat Struct Biol 8:505-509.

Hartman GL, West ED, Herman TK (2011) Crops that feed the World 2. Soybean-worldwide production, use, and constraints caused by pathogens and pests. Food Sec 3:5-17.

Homrich MS, Wiebke-Strohm B, Weber RLM, Bodanese-Zanettini MH (2012) Soybean genetic transformation: a valuable tool for the functional study of genes and the production of agronomically improved plants. Genetics and Molecular Biology 35:998-1010.

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229-1231.

Kappaun, K (2014) Clonagem e expressão da urease da bactéria diazotrófica Azospirillum brasilense FP2 e do peptídeo soyuretox, derivado da urease ubíqua de soja (Glycine max) Dissertação (Biologia Celular e Molecular) Universidade Federal do Rio Grande do Sul, Porto Alegre, RS Brasil pp 88.

Kohli A, Twyman RM, Abranches R, Weget E, Stoger E, Christou P (2003) Transgene integration, organization and interaction in plants. Plant Molecular Biology l52:247-258.

Krajewska B (2009) Ureases I Functional, catalytic and kinetic properties: A review J Mol Catal B Enzymatic 59:9-21.

Kuma KM, Lopes-Caitar VS, Romero CCT, Silva SMH, Kuwahara MK, Carvalho MCCG, Abdelnoor RV, Dias WP, Marcelino-Guimarães FC (2015) A high efficient protocol for soybean root transformation by Agrobacterium rhizogenes and most stable reference genes for RT-qPCR analysis. Plant Cell Rep 34(11):1987-2000.

Jabri E, Carr MB, Hausinger RP, Karplus PA (1995) The crystal structure of urease from *Klebsiella aerogenes*. Science 268: 998-1004.

Jeyaratnam J (1990) Acute pesticide poisoning: a major global health problem. World Health Stat Quaterly 43(3):139-144.

Jones JT, Haegeman A, Danchin EGJ, Gaur HS, Helder J, Jones MGH, Kikuchi T, Manzanilla-Lópe R, Palomares-Rius JE, Wesemael WML, Perry RN (2013) Top 10 plant-parasitic nematodes in molecular plant pathology. Molecular plant pathology 14(9): 946-961.

Laboratório de Proteínas Tóxicas/Universidade Federal do Rio Grande do Sul Disponível em: http://wwwufrgsbr/laprotox/>Acesso em setembro 2016.

Ligabue-Braun R, Andreis FC, Verli H, Carlini CR (2013) 3-to-1: unraveling structural transitions in ureases. Naturwissenschaften 100:459-467.

Liu K (2008) Food use of whole soybeans In Johnson PJW, Galloway R, (eds) Soybeans: chemistry, production, processing, and utilization. Urbana IL: AOCS Press pp441-482.

Mamiya G, Takishima K, Masakuni M, Kayumi T, Ogawa K, Sekita T (1985) Complete Amino-Acid Sequence of Jack Bean Urease Proceedings of the Japan. Academy Series B-Physical and Biological Sciences 61:395-398.

Mello-Farias PC, Chaves ALS (2008) Advances in *Agrobacterium*-mediated plant transformation with enphasys on soybean. Sci Agric 65:95-106.

Merkle SA, Parrott WA, Flinn BS (1995) Morphogenic aspects of somatic embryogenesis In: In vitro embryogenesis in plants, TA Thorpe, (eds) Kluwer Academic, Dordrecht, Netherlands pp155-203.

Mobley HLT, Hausinger RP (1989) Microbial ureases-significance, regulation, and molecular characterization. Microbiological Reviews 53:85-108.

Mobley HL, Island MD, Hausinger RP (1995) Molecular biology of microbial ureases. Microbiol Rev 59:451-480.

Moens M, Perry RN, Starr JL (2009) Meloidogyne species-a diverse group of novel and important plant parasites In: Perry RN, Moens M, Starr JL (eds) Root-Knot Nematodes Wallingford, Oxfordshire: CAB International, p 1-17

Mulinari F, Staniscuaski F, Bertholdo-Vargas LR, Postal M, Oliveira-Neto OB, Rigden DJ, Grossi-de-Sa MF, Carlini CR (2007) Jaburetox-2Ec: an insecticidal peptide derived from an isoform of urease from the plant *Canavalia ensiformis*. Peptides 28:2042-2050.

Mulinari F, Becker-Ritt AB, Demartini, DR, Ligabue-Braun R, Stanisçuaski F, Verli H, Fragoso RR, Schroeder EK, Carlini CR, Grossi-de-Sá MF (2011) Characterization of JBURE-IIb Isoform of *Canavalia ensiformis* (L) DC Urease. Biochimica et Biophysica Acta Proteins and Proteomics 1814:1758-1768.

Mulinari F, Freitas-Silva MA, Grossi-de-Sa MF, Moraes MG, Kurtenbach E, Carlini CR (2004) Toxina Praguicida, Construção Gênica e Método de Controle de Pragas (INPI-RS 001120, Brazil) pp 8-4.

Nicol JM, Turner SJ, Coyne DL, den Nijs L, Hockland, S, Maafi ZT (2011) Current nematode threats to world agriculture In: Jones JT, Gheysen G and Fenoll C (eds) Genomics and Molecular Genetics of Plant–Nematode Interactions Heidelberg: Springer, p 21-44.

Pazhavarical S (2009) Investigation on the early stages of interactions between the nematodes *Meloidogyne javanica* and *Pratylenchus thornei* and two of their plant hosts Thesis (PhD) University of Western Sydney, Sydney.

Piovesan AR, Staniscuaski F, Marco-Salvadori J, Real-Guerra R, Defferrari MS, Carlini CR (2008) Stage-specific gut proteinases of the cotton stainer bug Dysdercus peruvianus: role in the release of entomotoxic peptides from Canavalia ensiformis urease Insect. Biochem Mol Biol 38:1023-1032.

Pires-Alves M, Grossi-de-Sa MF, Barcellos GB, Carlini CR, Moraes MG (2003) Characterization and expression of a novel member (JBURE-II) of the urease gene family from jackbean [*Canavalia ensiformis* (L) DC]. Plant Cell Physiol 44:139-145.

Polacco JC, Havir EA (1979) Comparisons of soybean urease isolated from seed and tissue culture. J Biol Chem 254:1707-1715.

Polacco JC, Winkler RG (1984) Soybean leaf urease: a seed enzyme? Plant Physiology 74:800-803.

Polacco JC, Krueger RW, Winkler RG (1985) Structure and possible ureide degrading function of the ubiquitous urease of soybean. Plant Physiol Heidelberg: Springer 79:794-800.

Polacco JC, Holland MA (1993) Roles of urease in plant cells. International Review of Cytology 14:(5)65-103.

Polacco JC, Hyten DL, Medeiros-Silva Ma, Sleper DA, Bilyeu KD (2011) Mutational analysis of the major soybean UreF paralogue involved in urease activation. J Exp Bot 62:1-10.

Polacco JC, Mazzafera P, Tezotto T (2013) Opinion - Nickel and urease in plants: still many knowledge gaps. Plant Science 199-200:79-90.

Popelka JC, Terryn N, Higgins TJV (2004) Gene technology for grain legumes: can it contribute to the food challenge in developing countries? Plant Sci 167:195-206.

Postal M, Martinelli AHS, Becker-Ritt AB, Ligabue-Braun R, Demartini DR, Ribeiro SFF, Pasquali G, Gomes VM, Carlini CR (2012) Antifungal properties of Canavalia ensiformis urease and derived peptides. Peptides 38:22-32.

Proite K (2007) Busca de genes envolvidos na resistência de amendoim silvestre ao nematóide das galhas (Meloidogyne arenaria) Tese (Doutorado em Biologia Molecular) - Universidade de Brasília, Brasília p 220.

Reetz ER, Snatos C, Corrêa S, Silveira Dd, Carvalho Cd, Beling RR (2008) Anuário

Brasileiro da Soja In: Beling RR (ed) Anuário Brasileiro da Soja p 136.

Riddles PW, Whan V, Blakeley RL, Zerner B (1991) Cloning and sequencing of a jack bean urease-encoding cDNA .Gene 108:265-267.

Sanford JC (1988) The biolistic process. Trends Biotechnology 6(12):299-302.

Sasser JN (1979) Economic importance of Meloidogyne in tropical countries In: Lamberti, F, Taylor, CE (eds) Root-knot nematodes (Meloidogyne species): systematics, biology and control. London: Academic Press, p 256-268.

Sato S, Newell C, Kolacz K, Tredo L, Finer J, Hinchee M (1993) Stable transformation via particle bombardment in two different soybean regeneration systems. Plant Cell Reports p 408-413.

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, et al (2010) Genome sequence of the palaeopolyploid soybean. Nature 463:178-183.

Sevón N, Hiltunem R, Oksman-Caldentey K-M (1998) Somaclonal variation in Agrobacterium transformed roots and protoplast-derived hairy root clones. Hyoscyamus muticusPlant Med 64:37-41.

Sirko A, Brodzik R (2000) Plant ureases: roles and regulation. Acta Biochim Pol 47:1189-1195.

Somers DA, Samac DA, Olhoft PM (2003) Recent advances in legume transformation. Plant Physiol 131:892-899.

Stanisçuaski F, Ferreira-DaSilva CT, Mulinari F, Pires-Alves M, Carlini CR (2005) Insecticidal eff ects of canatoxin on the cotton stainer bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). Toxicon 45:753-760.

Stanisçuaski F, Brugge TEV, Carlini CR, Orchard I (2009) *In vitro* effect of *Canavalia ensiformis* urease and the derived peptide Jaburetox-2Ec on *Rhodnius prolixus* Malpighian tubules. J Insect Physiol 55:255-263.

Stanisçuaski F, Carlini CR (2012) Plant Ureases and Related Peptides: Understanding Their Entomotoxic Properties. Toxins 4**:**55-67.

Stebbins N, Holland MA, Cianzio SR, Polacco JC (1991) Genetic Tests of the Roles of the Embryonic Ureases of Soybean. Plant Physiol 97:1004-1010.

Stebbins N, Polacco JC (1995) Urease is not essential for ureide degradation in soybean. Plant Physiol 109:169-175.

Sumner JB (1926) The isolation and crystallization of the enzyme urease .J Biol Chem 69:435-444.

Thorpe TA (1994) Morphogenesis and regeneration In: Vasil, KI, Thorpe, TA (eds) Plant cell and tissue culture. Kluwer Academic: Netherland, p17-36.

Tomazetto G, Mulinari F, Stanisçuaski F, Settembrini BP, Carlini CR, Ayub MAZ (2007) Expression kinetics and plasmid stability of recombinant E coli encoding urease-derived peptide with bioinsecticide activity. Enzyme and Microbial Technology 41:821-827.

Torisky RS, Polacco JC (1990) Soybean roots retain the seed urease isozyme synthesized during embryo development. Plant Physiol 94:681-689.

Torisky RS, Griffin JD, Yenofsky RL, Polacco JC (1994) A single gene (*Eu*4) encodes the tissue-ubiquitous urease of soybean. Mol Gen Genet 242:404-414.

Trick HN, Dinkins RD, Santarém ER, Di R, Samoylov V, Meurer CA, Walker DR, Parrott WA, Finer JJ, Collins GB (1997) Recent advances in soybean transformation Plan.t Tiss Cult Biotechnol 3:9-26.

UNITED STATES Department of Agriculture Foreign Agricultural Service (FSA-USDA) PSD Online - Custom Query Disponível em: <http://appsfasusdagov/psdonline/psdQueryaspx> Acesso em: maio de 2016.

Urwin PE, Levesley A, McPherson MJ, Atkinson HJ (2000) Transgenic resistance to the nematode *Rotylenchulus reniformis* conferred by *Arabidopsis thaliana* plants expressing proteinase inhibitors. Molecular Breeding 6(8):257-264.

Zambelli B, Musiani F, Benini S, Ciurli S (2011) Chemistry of Ni (2+) in Urease: Sensing,Trafficking, and Catalysis Acc. Chem Res 44:520-530.

Zonia LE, Stebbins N, Polacco JC (1995) Essential role of urease in germination of nitrogen-limited *Arabidopsis thaliana* seeds. Plant Physiol 107:1097-1103.

Wang G, Xu Y (2008) Hypocotyl-based Agrobacterium-mediated transformation of soybean (Glycine max) and application for RNA interference. Plant Cell Rep 27:1177-1184.

Wang WH, Kohler B, Cao FQ, Liu LH (2008) Molecular and physiological aspects of urea transport in higher plants. Plant Sci 175:467-477.

Wiebke-Strohm B, Droste A, Pasquali G, Osório MB, Bücker-Neto L, Passaglia LMP, Bencke M, Homrich MS, Margis-Pinheiro M, Bodanese-Zanettini MH (2011) Transgenic

fertile soybean plants derived from somatic embryos transformed via the combined DNA-free particle bombardment and Agrobacterium system. Euphytica (Wageningen) 177:343-354.

Wiebke-Strohm B, Pasquali G, Margis-Pinheiro M, Bencke M, Bücker-Neto L, Becker-Ritt AB, Martinelli AHS, Rechenmacher C, Polacco JC, Stolf R, Marcelino FC, Abdelnoor RV, Homrich MS, Del Ponte EM, Carlin CR, De Carvalho MCCG, Bodanese-Zanettini MH (2012) Ubiquitous urease affects soybean susceptibility to fungi .Plant Mol Biol 78:1-13.

Wiebke-Strohm B, Ligabue-Braun R, Rechenmacher C, De Oliveira-Busatto LA Carlini CR, Bodanese-Zanettini MH (2016) Structural and transcriptional characterization of a novel member of the soybean urease gene family. Plant Physiology and Biochemistry (Paris) 101:96-104.

Williamson VM, Gleason CA (2003) Plant-nematode interactions. Current Opinion in Plant Biology 6:327-333.

Witte CP (2011) Urea metabolism in plants. Plant Science 180:431-438.

Witte CP, Tiller SA, Taylor MA, Davies HV (2002) Leaf urea metabolism in potato Urease activity profile and patterns of recovery and distribution of (15) N after foliar urea application in wild-type and urease-antisense transgenics. Plant Physiol 128:1129-1136.

Wöhler F (1828) Über künstliche bildung des harnstoffs. Ann Phys Chem 253-256.

Anexos

Artigos completos publicados em periódicos

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RESEARCH ARTICLE Open Access

Expression of an osmotin-like protein from *Solanum nigrum* confers drought tolerance in transgenic soybean

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Abstract

Background: Drought is by far the most important environmental factor contributing to yield losses in crops, including soybeans (*Glycine max* (L.) Merr.). To address this problem, a gene that encodes an osmotin like protein isolated from *Solanum nigrum* var. *americanum* (*SnOLP*) driven by the UBQ3 promoter from *Arabidopsis thaliana* was transferred into the soybean genome by particle bombardment.

Results: Two independently transformed soybean lines expressing *SnOLP* were produced. Segregation analyses indicated single locus insertions for both lines. qPCR analysis suggested a single insertion of *SnOLP* in the genomes of both transgenic lines, but one copy of the *hpt* gene was inserted in the first line and two in the second line. Transgenic plants exhibited no remarkable phenotypic alterations in the seven analyzed generations. When subjected to water deficit, transgenic plants performed better than the control ones. Leaf physiological measurements revealed that transgenic soybean plants maintained higher leaf water potential at predawn, higher net CO_2 assimilation rate, higher stomatal conductance and higher transpiration rate than non transgenic plants. Grain production and 100 grain weight were affected by water supply. Decrease in grain productivity and 100 grain weight were observed for both transgenic and non transgenic plants under water deficit; however, it was more pronounced for non transgenic plants. Moreover, transgenic lines showed significantly higher 100 grain weight than non transgenic plants under water shortage.

Conclusions: This is the first report showing that expression of *SnOLP* in transgenic soybeans improved physiological responses and yield components of plants when subjected to water deficit, highlighting the potential of this gene for biotechnological applications.

Keywords: Abiotic stress, Bombardment, Drought tolerance, Genetic transformation, *Glycine max*, Osmotin, Water deficit

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Phakopsora pachyrhizi Infection Bioassay in Detached Soybean Transgenic Leaves for Candidate Gene Validation

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[Abstract] Soybean (*Glycine max*) is one of the most important crops in the world. *Phakopsora pachyrhizi* is a plant pathogenic basidiomycete fungus that infects soybean, causing Asian Soybean Rust (ASR) disease and affecting production. Here, we describe how to prepare the plant material and the uredospore suspension (from spores harvested from leaves exhibiting sporulating uredinia) for *in vitro* leaf infection. Plant material is sprayed with the uredospore suspension and incubated for 12 days. During the incubation period, the presence of lesions and pustules is visually verified. After this incubation period, the leaves are classified according to the lesion type. The number of uredospores per cm^2 of leaf was also estimated. The detached-leaf assay is routinely used to test fungicide efficiency (Scherb and Mehl, 2006). Detached-leaf, greenhouse and field results have been shown to be significantly correlated (Twizeyimana *et al.*, 2007). The present protocol was adapted from the two publications cited above. The usefulness of this approach for studying *P. pachyrhyzi* infection on transgenic soybean was previously demonstrated by our research team (Wiebke-Strohm *et al.*, 2012; Bencke-Malato *et al.*, 2014).

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Research article

Structural and transcriptional characterization of a novel member of the soybean urease gene family

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ABSTRACT

In plants, ureases have been related to urea degradation, to defense against pathogenic fungi and phytophagous insects, and to the soybean–*Bradyrhizobium japonicum* symbiosis. Two urease isoforms have been described for soybean: the embryo-specific, encoded by *Eu1* gene, and the ubiquitous urease, encoded by *Eu4*. A third urease-encoding locus exists in the completed soybean genome. The gene was designated Eu5 and the putative product of its ORF as SBU-III. Phylogenetic analysis shows that 41 plant, moss and algal ureases have diverged from a common ancestor protein, but ureases from monocots, eudicots and ancient species have evolved independently. Genomes of ancient organisms present a single urease-encoding gene and urease-encoding gene duplication has occurred independently along the evolution of some eudicot species. SBU-III has a shorter amino acid sequence, since many gaps are found when compared to other sequences. A mutation in a highly conserved amino acid residue suggests absence of ureolytic activity, but the overall protein architecture remains very similar to the other ureases. The expression profile of urease-encoding genes in different organs and developmental stages was determined by RT-qPCR. *Eu5* transcripts were detected in seeds one day after dormancy break, roots of young plants and embryos of developing seeds. *Eu1* and *Eu4* transcripts were found in all analyzed organs, but *Eu4* expression was more prominent in seeds one day after dormancy break whereas *Eu1* predominated in developing seeds. The evidence suggests that SBU-III may not be involved in nitrogen availability to plants, but it could be involved in other biological role(s).