EFEITO DAS UREASES DE SOJA (*Glycine max* (L.) Merr.) NA GERMINAÇÃO DE SEMENTES E DESENVOLVIMENTO DAS PLANTAS

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PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

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Dissertação 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 mestre em Genética e Biologia Molecular

Orientadora:
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Janeiro de 2012

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SUMÁRIO

LISTA DE ABREVIATURAS

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

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

GS1: Glutamina sintetase citosólica

HNO_3 – ácido nítrico.

JBU: urease majoritária da semente de *Canavalia ensiformis*

JBUREII: urease secundária de *Canavalia ensiformis*

hpt: gene marcador que codifica a higromicina fosfotransferase

kb: quilobases

kDa: kiloDaltons

N: nitrogênio

NH_4: amônia

NG: sementes que não germinaram

NO_3: nitrato

NT: não transgênica

ORF: do inglês *open reading frame*

PCR: do inglês *polymerase chain reaction*

pH: potencial de hidrogênio

P5CDH: pirrolina-5-carboxilato desidrogenase

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

UTR: região não-traduzida das bordas do mRNA

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

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

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

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

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

δOAT: ornitina aminotransferase

RESUMO

As ureases produzidas por plantas, fungos e bactérias, são metaloenzimas níquel-dependentes que catalisam a hidrólise da uréia em amônia e dióxido de carbono. Existem três proteínas de urease em soja. A urease ubíqua codificada pelo gene *Eu*4 é expressa em baixos níveis em todos os tecidos da planta e tem sido considerada responsável pela reciclagem de toda a uréia derivada metabolicamente. A urease embrião-específica codificada pelo gene *Eu*1 é expressa em níveis elevados em embriões em desenvolvimento e sementes maduras. Um terceiro gene codificando urease foi recentemente identificado. Este gene foi nomeado *Eu*5, e seu produto SBU-III. *Eu*5 é expresso nos primeiros estágios de desenvolvimento das raízes, mas o seu nível de expressão é menor se comparado ao de outras isoformas de urease. Plantas transgênicas com a supressão da expressão de *Eu*4 foram previamente obtidas por nossa equipe. Estas plantas transgênicas representam uma ferramenta poderosa para a genomica funcional, porque mutantes nulos nunca tinham sido obtidos para a urease ubíqua. Os objetivos do presente estudo foram caracterizar em nível molecular as progênies de plantas transgênicas com *Eu*4 co-suprimido e investigar o papel das ureases na capacidade de germinação de sementes e desenvolvimento das plantas. Sementes T_1 e T_2 obtidas de plantas transgênicas T_0 e T_1, respectivamente, foram selecionadas por expressão do gene repórter *gfp*. A presença de transgenes também foi confirmada por PCR. Níveis de expressão das três isoformas da urease (*Eu*4, *Eu*1 e *Eu*5) foram avaliados por RT-qPCR. Os resultados mostraram que co-supressão de *Eu*4 foi mantida na progênie transgênica. O silenciamento dos outros dois genes codificando ureases foi também demonstrado nessas plantas. A fim de investigar o papel individual dos genes que codificam urease nos processos de germinação e de desenvolvimento das plantas, mutantes simples *eu4*, *eu1* e duplo *eu4/eu1* foram também avaliados. Os resultados sugerem que a ausência de uma ou mais isoformas de urease não afetou a viabilidade das sementes, mesmo depois de armazenadas. Por outro lado, uma grande contribuição de *Eu*1 e *Eu*5 no padrão temporal de desenvolvimento das plantas foi demonstrada. A ausência de *Eu*1 determinou um atraso nos primeiros estágios de desenvolvimento, enquanto que nas plantas com silenciamento de todos os três genes que codificam ureases este fenótipo foi estendido para estágios de desenvolvimento subsequentes. Há sugestão na literatura de que

*Eu*5 não seja funcional uma vez que elevado número de mutações têm sido detectados neste gene. No entanto, de acordo com nossos resultados, o produto deste gene pode estar envolvido no desenvolvimento da planta.

ABSTRACT

Urease, produced by plants, fungi and bacteria, is a nickel-dependent metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide. There are three urease structural proteins in soybean. The ubiquitous urease encoded by *Eu4* gene is expressed at low levels in all plant tissues and has been considered to be responsible for recycling all metabolically-derived urea. The embryo-specific urease encoded by *Eu1* gene is expressed in high levels in developing embryos and ripe seeds. A third urease encoding gene was recently identified. This gene was named *Eu*5, and its product SBU-III. *Eu*5 is expressed in the first stages of root development, but its expression level is lower if compared to that of other urease isoforms. Transgenic plants with suppression of *Eu*4 expression were previously obtained by our team. These transgenic plants represent a powerful tool for functional genomics, because null mutants have never been obtained for the ubiquitous urease. The goals of the present study were to characterize at molecular level progenies of transgenic *Eu4* co-suppressed plants and investigate the role of ureases on seed germination capacity and plant development. T_1 and T_2 seeds obtained from T_0 and T_1 transgenic plants, respectively, were screened for *gfp*-reporter gene expression. Presence of transgenes was also confirmed by PCR. Expression levels of the three urease isoforms (*Eu4*, *Eu1* and *Eu5*) were evaluated by RT-qPCR. Results showed that *Eu4* co-suppression was maintained in the transgenic progeny. Silencing of the other two ureases encoding genes was also demonstrated in those plants. In order to investigate the role of individual urease encoding genes on germination and plant developmental processes, *eu4*, *eu1* single and *eu4/eu1* double mutants were also evaluated. Results suggest that lack of one or more ureases isoforms did not affect seed capacity even after stored. On the other hand, a great contribution of *Eu*1 and *Eu*5 on temporal plant developmental pattern was demonstrated. The lack of *Eu*1 determined a delay in the first developmental stages, whereas silencing of all three soybean ureases encoding genes distended this phenotype for further growth stages. *Eu*5 has been suggested not to be functional as it presents high number of mutations. However, according to our results the product of this gene might be involved on plant development.

Capítulo I

INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

1.1 SOJA

A soja [*Glycine max* (L.) Merri.] é uma espécie pertencente à família Fabaceae (Leguminosa) de grande interesse econômico em nível mundial por representar uma fonte importante de proteínas e de ácidos graxos. Atualmente, são produzidas, por ano, cerca de 200 milhões de toneladas deste grão. É uma planta diplóide (2n=40), de autofecundação e com ciclo de vida anual. Nativa da China, é cultivada há mais de 5 mil anos, sendo uma das mais antigas plantas cultivadas (Hymowitz 1976).

No Brasil, a cultura foi estabelecida a partir de 1960. De acordo com a CONAB (Companhia Nacional de Abastecimento), o Brasil aparece como o segundo maior produtor mundial de soja, atrás apenas dos EUA. Com a grande demanda de mercado, a área de cultivo de soja vem se expandido consideravelmente no País. A área plantada na safra 2010/2011 aumentou de 23.467,9 mil hectares (ha) para 24.158,1 mil ha em relação à safra de 2009/2010. No RS, o aumento foi de 2,7%, totalizando uma área plantada de 4.084,8 mil ha (www.conab.gov.br).

A produção no Brasil na safra 2010/2011 foi estimada em 75.039,3 milhões de toneladas, volume que é 9,2% maior que a safra 2009/2010. Quanto ao Estado do Rio Grande do Sul (RS), a produção aumentou 13,7%, passando de 10.218,8 mil toneladas para 11.621,3 mil toneladas no mesmo período (www.conab.gov.br).

Devido a sua importância econômica, 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 recentemente seu genoma quase completamente seqüenciado (Schmutz et al. 2010), o que tem permitido grandes avanços para estudos de genômica funcional e engenharia genética, que representam ferramentas importantes para estudos básicos,

visando contribuir para os programas de melhoramento desta espécie.

1.2 UREASES

A urease é uma enzima que ocupa lugar de destaque na história da ciência (Carlini e Polacco 2008). Foi isolada e cristalizada a partir de semente de feijão de porco (*Canavalia ensiformis*), por J.B. Sumner em 1926, provando definitivamente a origem protéica das enzimas. Foi a primeira a ser identificada como metaloenzima dependente de níquel (Dixon et al. 1975).

As ureases catalisam a hidrólise da uréia, 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) (ver Figura 1).

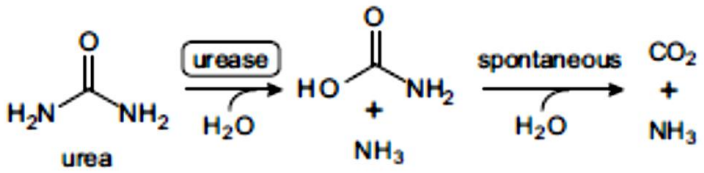

Figura 1. Reação de hidrólise da uréia catalisada pela enzima urease. Fonte: (Witte CP, 2011).

Vários grupos de organismos sintetizam a enzima, dentre eles bactérias, fungos e plantas (Mobley et al. 1995; Sirko e Brodzik 2000). Ureases de plantas e fungos são proteínas homo-oligoméricas, formadas por trímeros ou hexâmeros de uma única subunidade idêntica de aproximadamente 90-kDa. Já as ureases de bactérias são multímeros formados por duas ou três subunidades designadas α, β e γ (Figura 2) (Mobley et al. 1995; Follmer 2008, Krajewska 2009). O grau de similaridade é alto entre as seqüências de aminoácidos (é maior que 50% entre bactérias e plantas e maior que 70% de similaridade dentro de cada grupo), indicando que todas as ureases são variações de uma

mesma enzima ancestral e que, provavelmente, possuem similaridade também na estrutura terciária e no mecanismo catalítico (Follmer 2008).

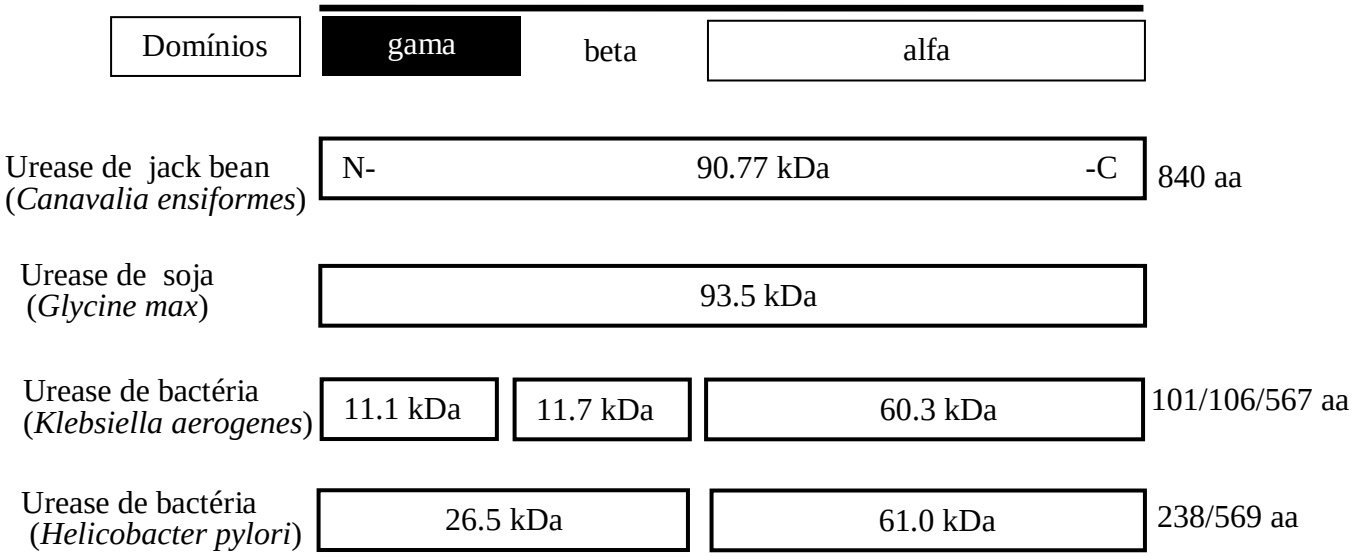

Figura 2. Comparação esquemática dos domínios das ureases de diferentes organismos. Ureases de plantas (*Glycine max* e *Canavalia ensiformes*) são formadas por subunidades idênticas. Ureases bacterianas podem ter duas (*Helicobacter pylori*) ou três subunidades (*Klebsiella aerogenes*). Fonte: Adaptado a partir de Krajewska (2009).

A resolução da estrutura tridimensional da urease vegetal de feijão de porco (Jack bean urease - JBU) (Balasubramanian e Ponnuraj 2010) possibilitou a comparação estrutural com ureases já conhecidas das bactérias *Klebsiella aerogenes* (Jabri et al. 1995), *Bacillus pasteurii* (Benini et al. 1999) e *Helicobacter pylori* (Ha et al. 2001), permitindo a confirmação do alto grau de similaridade entre elas.

A ativação da urease é um processo complexo. Em plantas e bactérias é necessária a participação de várias proteínas acessórias para que ela se torne ativa através da incorporação seletiva do níquel. A ligação dos átomos de níquel é muito precisa e forte (Follmer 2008; Balasubramanian e Ponnuraj 2010). Para as bactéria *Klebsiella aerogenes* quatro proteínas acessórias (*UreD, UreF, UreG, UreE*) são requeridas para a ativação da urease (Mobley et al. 1995). Em plantas, três proteínas acessórias foram identificadas a partir de *Arabidopsis thaliana* (*At*UreD, *At*UreF, e *At*UreG), as quais não são apenas necessárias, mas, provavelmente, suficientes para ativar a enzima através da incorporação do níquel. Estas proteínas parecem atuar como chaperonas urease-específicas (Figura 3)

(Bacanamwo et al. 2002; Witte et al. 2005; Follmer 2008; Witte CP, 2011). Para soja, três proteínas acessórias (*UreD, UreF, UreG*) foram identificadas (Freyermuth et al. 2000; Bacanamwo et al. 2002; Polacco et al. 2011).

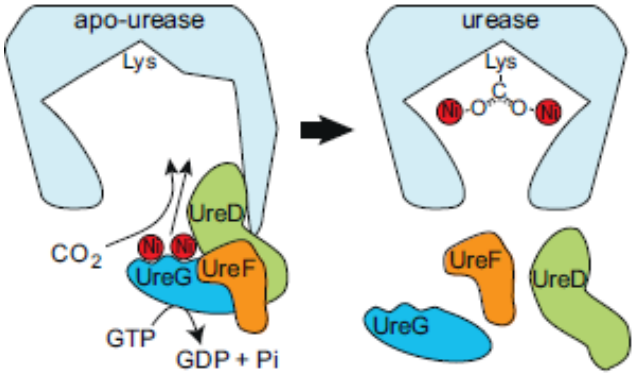

Figura 3. Modelo hipotético da ativação de urease de planta envolvendo a ligação das três proteínas acessórias (*UreD, UreF e UreG*) à apo-urease, modificação covalente de um sítio ativo de lisina e incorporação específica de dois íons de níquel por sítio ativo. As proteínas acessórias se dissociam da urease após a ativação. Fonte: (Witte CP, 2011).

1.3 UREASES EM PLANTAS

A urease vegetal mais bem caracterizada bioquimicamente é a de *Canavalia ensiformis* (feijão de porco) (Sirko e Brodzik 2000; Carlini e Polacco 2008; Follmer 2008) Nesta espécie são encontradas três isoformas estruturais: JBU, canatoxina e JBUREII. Apenas JBU e JBUREII têm as sequências completas de DNA/aminoácidos disponíveis (Pires-Alves et al. 2003; Mulinari et al. 2011).

A JBU foi a primeira enzima a ser cristalizada (Summer 1926). A canatoxina (CNTX) foi isolada e caracterizada em 1981 e, posteriormente, identificada como uma isoforma de urease (JBU), e é a menos abundante 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 dos brotos (Pires-Alves et al. 2003; Mulinari et al. 2011).

Atualmente, os melhores dados genéticos sobre ureases de plantas podem ser encontrados nos trabalhos com soja (Goldraij et al. 2003), para a qual são descritas três isoformas, codificadas por três genes estruturais independentes. A urease ubíqua, codificada pelo gene *Eu*4, expresso em níveis baixos em todos os tecidos vegetais, é responsável pela reciclagem da uréia derivada metabolicamente (Polacco et al. 1985; Torisky et al. 1994; Goldraij et al. 2003). A urease embrião-específica, codificada pelo gene *Eu*1, expresso em níveis elevados em embriões em desenvolvimento, é acumulada em sementes maduras e apresenta 1000 vezes mais atividade que a enzima urease ubíqua na semente (Polacco e Havir 1979; Polacco e Winkler 1984; Polacco e Holland 1993). Recentemente foi identificada uma terceira urease, codificada pelo gene denominado *Eu*5, tendo sido seu produto denominado SBU-III. Esta isoforma apresenta alta similaridade com as ureases ubíqua e embrião-específica e é transcrita nos primeiros estádios do desenvolvimento das raízes e em embriões em desenvolvimento (Wiebke-Strohm 2010).

Com o intuito de determinar a função das ureases de soja, esforços foram direcionados para obtenção de mutantes nulos por bombardeamento com nêutrons rápidos cuja coleção pertence à Universidade de Missouri, EUA. Foram obtidas com sucesso plantas mutantes nulos para a urease embrião-específica (*eu*1). A ausência da urease embrião específica não causou alterações nas condições fisiológicas das plantas (Polacco e Holland 1993). Por outro lado, mutantes nulos para a urease ubíqua nunca foram obtidos. Os mutantes denominados *eu4* acumulam a proteína, mas a mesma é deficiente na atividade ureolítica devido a uma mutação de ponto na região de inserção do níquel. As plantas *eu4* mostram uma substancial redução da atividade ureolítica nas folhas, raízes e hipocótilos. A partir da análise destas plantas, foi demonstrado que a urease ubíqua sozinha é responsável pela degradação da uréia em todos os tecidos da planta (Torisky e Polacco 1990; Stebbins et al. 1991; Witte et al. 2002). A outra classe, chamada de duplo mutante *eu1/eu4*, é praticamente desprovida de atividade ureolitica (Stebbins e Polacco 1995; Goldraij et al. 2003).

1.4 FUNÇÕES DAS UREASES

1.4.1 FUNÇÕES DEPENDENTES DA ATIVIDADE UREOLÍTICA

Em plantas, a primeira função atribuída à urease é a atividade ureolítica. Por sua vez, grande quantidade de nitrogênio pode ser aproveitada a partir da uréia devido a essa função (Follmer 2008). A maior parte do nitrogênio resultante do metabolismo dos organismos vivos é depositada sob forma de uréia. A arginina, o principal armazenador do nitrogênio, é hidrolisada pela arginase em ureia. Nas plantas, o nitrogênio encontrado na uréia só está disponível depois de ser hidrolisado pela urease. A amônia, produto da atividade da urease é incorporada em compostos orgânicos, principalmente pela atividade da glutamina sintetase (Figura 5) (Mobley et al. 1995; Sirko e Brodzik 2000). A importância da urease, portanto, é fornecer o nitrogênio necessário para o desenvolvimento dos seres vivos.

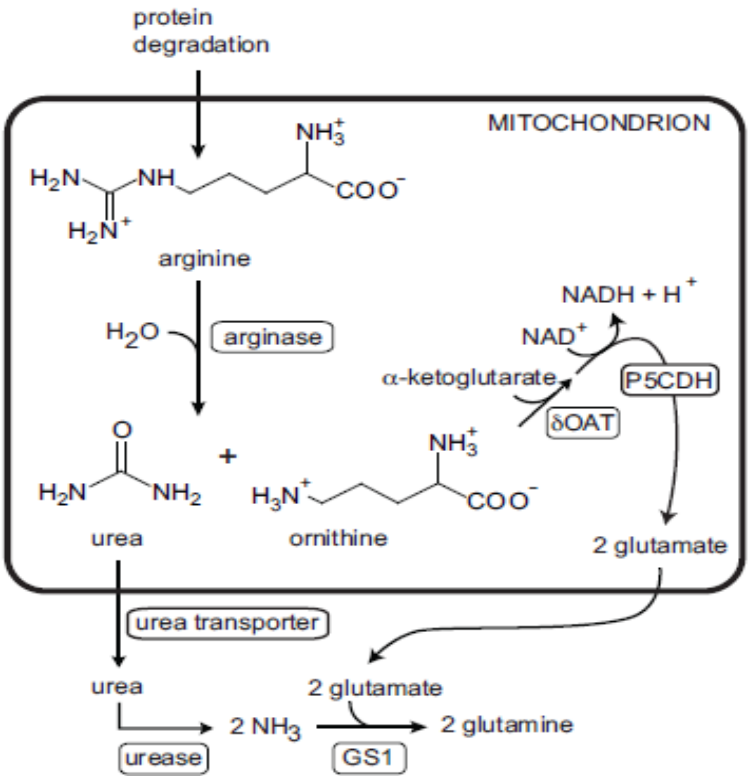

Figura 5 - Metabolismo da uréia em plantas. A arginina é hidrolisada na mitocôndria pela arginase, produzindo uréia e ornitina. A uréia deixa a mitocôndria e é hidrolisada pela urease. O produto da atividade da urease, a amônia, é liberado e re-assimilado pela glutamina sintetase citosólica (GS1). A ornitina aminotransferase (δOAT)

transfere o grupo amido da cadeia lateral da ornitina cetoglutarato, para gerar uma molécula de glutamato e pirrolina-5-carboxilato, que é oxidado a uma segunda molécula de glutamato por pirrolina-5-carboxilato desidrogenase (P5CDH). O glutamato pode ser exportado da mitocôndria e serve como substrato para a reaação citosólica (GS1). No total, todos os quatro átomos de nitrogênio da arginina são incorporados em glutamina. A urease é requerida para mobilizar metade do nitrogênio da arginina. Fonte: (Witte CP, 2011).

O nitrogênio é um nutriente importante para o crescimento da planta. Portanto, um mecanismo eficiente para capturar e realocar fontes de nitrogênio é necessário para a sua utilização otimizada nos processos essenciais, como a germinação de sementes e senescência (Witte CP, 2011). Como já mostrado acima, a urease está envolvida na remobilização do nitrogênio, bem como na assimilação de nitrogênio primário (Cao et al. 2010).

A importância da urease para reciclagem do nitrogênio é destacada pelo fato de que em sementes de *Arabidopsis thaliana* embebidas em água, na presença de um inibidor de urease, tiveram sua germinação atrasada por 36 horas e a germinação de sementes velhas foi inibida (Zonia et al. 1995). Além disto, na soja, a atividade da arginase é baixa durante o desenvolvimento do embrião e tem seus níveis aumentados durante a germinação (Goldraij e Polacco, 1999).

Por outro lado, plantas transgênicas de choupo (*Populus tremula*) que superexpressam a glutamina sintetase apresentaram aumento no crescimento, provavelmente por uma maior disponibilidade de nitrogênio (Gallardo et al. 1999).

1.4.2 FUNÇÕES DE DEFESA

Além da atividade ureolítica, as ureases também estão envolvidas na defesa de plantas contra patógenos, apresentando função inseticida e propriedades antifúngicas. Os domínios da atividade fungicida e inseticida são independentes da capacidade de hidrólise da enzima, uma vez que as ureases continuam desempenhando suas funções de defesa

quando tratadas com inibidores da atividade ureolítica (Polacco e Holland et al. 1993; Follmer et al. 2004; Becker-Ritt et al. 2007).

Recentes 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 ambas as isoformas de *Canavalia ensiformis*, Canatoxina (CNTX), *Jack bean* urease (JBU) e a urease embrião específica de soja (SBU), (Follmer et al. 2004) são tóxicas para insetos que apresentam no trato digestivo enzimas proteolíticas do tipo catepsinas, que degradam a proteína e liberam um peptídeo tóxico de 10 kDa, não sendo tóxica para insetos com digestão baseada em enzimas serínicas do tipo tripsina.

O mecanismo de ação inseticida das ureases de plantas ainda precisa ser completamente elucidado, embora esteja claro que elas atuam como pró-toxina (Carlini e Polacco 2008). O efeito inseticida da urease em percevejos é estágio-dependente, afetando ninfas, mas não adultos, estando relacionado com o perfil de enzimas digestivas do inseto (Ferreira da Silva et al. 2002; Stanisçuaski et al. 2005). Observações do efeito da CNTX em *Rhodnius prolixus* sugeriram um comprometimento da diurese e balanço de eletrólitos (Carlini et al. 1997). Em abordagem mais recente, foi estudado o efeito das ureases de *C. ensiformis* e do peptídeo recombinante diretamente sobre túbulos de Malpighi isolados. Os resultados mostram que 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). Ninfas de *Oncopeltus fasciatus* alimentadas com urease de *Jack bean* mostraram uma taxa de mortalidade superior a 80% após duas semanas (Defferrari et al. 2011).

A função inseticida das ureases parece restrita à urease de plantas e pelo menos parte do peptídeo entomotóxico corresponde ao *gap* presente entre os domínios α e β das ureases bacterianas (Figura 2) (Follmer et al. 2004). A sequência de nucleotídeos correspondente ao peptídeo entomotóxico foi isolada e clonada a partir do gene da urease *jbureII* (Mulinari et al. 2007).

Alguns insetos que são importantes pragas para a agricultura, como o caruncho do feijão de corda (*Callosobruchus maculatus*), o percevejo manchador do algodão (*Dysdercus peruvianus*) e percevejo da soja (*Nezara viridula*) são sensíveis à CNTX. Já

insetos como as lagartas, *Manduca sexta* e *Anticarsia germmatalis* e o gafanhoto (*Schistocerca americana*) não são sensíveis a esta proteína (Carlini e Grossi de Sá 2002; Stanisçuaski et al. 2005).

Quanto à atividade antifúngica das ureases, sabe-se que em concentração sub-micromolar, as ureases purificadas de soja (embrião-específica), feijão de porco (JBU) e uma urease recombinante de *Helicobacter pylori* prejudicam o crescimento vegetativo *in vitro* de fungos fitopatogênicos (Becker-Ritt et al. 2007; Menegassi et al. 2008). O mecanismo de ação das ureases sobre os fungos ainda não é conhecido. A análise em microscopia eletrônica de varredura de fungos tratados com ureases de *Canavalia ensiformis* sugere plasmólise e lesões da parede celular (Becker-Ritt et al. 2007). Ainda não se sabe ao certo a localização da região do peptídeo responsável por essa função.

1.5 MANIPULAÇÃO DA EXPRESSÃO DE GENES QUE CODIFICAM UREASES

Apesar das ureases, serem estudadas desde 1926 sua função e seu modo de ação ainda não estão totalmente compreendidos e merecem atenção. A manipulação da expressão dos genes que codificam ureases pode auxiliar em uma melhor compreensão de suas funções.

Em nosso laboratório (Laboratório de Cultura de Tecidos e Transformação Genética de Plantas, do Departamento de Genética da UFRGS), foram realizados experimentos de transformação por bombardeamento com o objetivo inicial de gerar plantas de soja que superexpressassem a urease ubíqua, visando maior resistência a adversidades bióticas. Os resultados obtidos foram incluídos em um artigo recentemente aceito para publicação (Wiebke-Strohm et al. 2012). Resumidamente, 26 plantas transgênicas, portando de 10 a 14 cópias extras do gene *Eu*4, foram obtidas, representando quatro eventos independentes de transformação. Surpreendentemente, 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. Da mesma forma, uma atividade ureásica reduzida, como reflexo de menor acúmulo da enzima, foi observada nas plantas transgênicas. Estes resultados indicam que ao contrário da superexpressão

prevista, houve uma co-supressão do gene alvo. Algumas hipóteses foram lançadas para explicar a co-supressão, sendo que o grande número de cópias do transgene integrado ao genoma parece ser a causa mais provável (Wiebke-Strohm et al. 2012). Para confirmação desta hipótese é imprescindível a obtenção de plantas transformadas contendo menor número de cópias do transgene, que seriam mais facilmente obtidas através do método que integra bombardeamento e *Agrobacterium* (Wiebke-Strohm et al. 2011).

Tendo em vista que as ureases são potencialmente multifuncionais, as plantas co-suprimidas foram consideradas uma ferramenta importante para a determinação das funções adicionais à atividade ureásica. Resultados obtidos nos estudos com estas plantas mostraram que a falta da urease ubíqua torna as plantas mais susceptíveis a diversos fungos, comprovando que esta é uma das proteínas envolvidas no complexo de resistência da soja a estresses bióticos (Wiebke-Strohm et al. 2012). Novas análises das plantas transgênicas com o gene *Eu*4 co-suprimido poderá contribuir para elucidação do papel das ureases em outros processos biológicos, tais como o desenvolvimento de plantas.

1. Objetivos

1.1 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 (2) investigar o papel das ureases de soja durante a germinação de sementes e o desenvolvimento das plantas.

1.2 Objetivos específicos

a) Caracterizar molecularmente a progênie de plantas transgênicas com o gene *Eu*4 co-suprimido.

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 (*eu4, eu1*, *eu1/eu4*) e não mutantes.

c) Avaliar a capacidade de germinação de sementes transgênicas e não transgênicas; mutantes (*eu4*, *eu1*, *eu1/eu4*) e não mutantes.

d) Avaliar o padrão temporal de desenvolvimento de plantas transgênicas e não transgênicas, mutantes (*eu4*, *eu1*, *eu1/eu4*) e não mutantes.

Capítulo II

Effect of soybean ureases on seed germination and plant development

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Effect of soybean ureases on seed germination and plant development

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ABSTRACT

Ureases are nickel dependent enzymes that catalyze the hydrolysis of urea. In soybean three urease structural gene encoding urease isoforms have been described: ubiquitous urease encoded by *Eu*4 gene; embryo-specific urease encoded by *Eu*1 gene and a putative third urease encoded by the recently detected *Eu*5 gene. Transgenic plants with suppression of *Eu*4 expression were previously obtained by our team. The goals of the present study were to characterize at molecular level progenies of transgenic *Eu*4 silenced plants and investigate the role of ureases on seed germination capacity and plant development. Expression levels of the three urease isoforms were evaluated by RT-qPCR. Results showed that *Eu*4 silencing was maintained in the transgenic progeny. Silencing of the other two ureases encoding genes was also demonstrated in those plants. In order to investigate the role of individual urease encoding genes on germination and plant developmental processes, *eu4*, *eu1* single and *eu4/eu1* double mutants were also evaluated. Results suggest that lack of one or more ureases isoforms did not affect seed viability. On the other hand, a great contribution of *Eu*1 and *Eu*5 on temporal plant developmental pattern was demonstrated. The lack of *Eu1* determined a delay in the first developmental stages, whereas silencing of all three soybean ureases encoding genes distended this phenotype for further growth stages. *Eu*5 has been suggested not to be functional. However, our results indicate that the product of this gene might be involved on plant development.

Keywords: genetic transformation, *Glycine max*, plant development, urease silencing

Introduction

Nickel ion is an important micronutrient for plants and microorganisms because it plays an important role in the metalloenzymes activation (Jing Lv et al. 2011). Ureases (EC 3.5.1.5) are nickel-dependent metalloenzymes that have a nickel-containing active site. These enzymes catalyze the conversion of urea to ammonia and carbon dioxide. Ureases are synthesized by a wide variety of organisms including plants, algae, fungi and bacteria (Dixon et al. 1975; Krajewska 2009).

Nitrogen (N) is an important nutrient for plant growth. Therefore an efficient mechanism to capture and reallocate N sources is necessary for its optimal use in essential processes as germination and senescence of plants (Witte CP 2011). Arginine, the major N storage, is hydrolyzed by arginase to form urea. Nitrogen found in urea is only available after urea hydrolysis by urease. The product of urease activity – ammonia- is incorporated into organic compounds mainly by glutamine synthetase 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). The importance of urease for recycling N was highlighted by the fact that aged *Arabidopsis thaliana* seeds failed to germinate when urease was chemically inhibited, but could be rescued by an external N source (Zonia et al. 1995).

In soybean three urease isoforms have been described. Ubiquitous urease encoded by *Eu*4 gene is expressed at low levels in all plant tissues and it was described to be responsible for recycling metabolically-derived urea (Polacco et al. 1985; Torisky et al. 1994; Goldraij et al. 2003). Embryo-specific urease encoded by *Eu*1 gene is highly expressed in developing embryos and it is accumulated in mature seeds (Polacco and Havir 1979; Polacco and Winkler 1984; Polacco and Holland 1993). A third urease encoding gene was recently identified in soybean genome (Wiebke-Strohm 2010; Witte CP 2011). This gene was named *Eu*5, and its product SBU-III. *Eu*5 is expressed in the first stages of root development, but its expression level is lower if compared to that of other soybean urease isoforms (Wiebke-Strohm 2010).

In order to determine the role of soybean ureases, efforts have been directed to obtain null mutants. Embryo-specific urease (e*u1*) null mutant was successfully obtained (Polacco and Holland 1993). Unexpectedly, absence of embryo-specific urease did not

cause changes in physiological conditions of plants (Polacco and Holland 1993). On the other hand, null mutants for ubiquitous urease were never obtained. The e*u4* mutant accumulates the protein, but it displays a deficient ureolytic activity. These plants show a substantial reduced ureolytic activity in leaves, roots and hypocotyls (Torisky e Polacco 1990; Stebbins et al. 1991; Witte et al. 2002). Mutant plants deficient in the ureolytic activity, as well as wild-type plants grown under nickel-deprived conditions, were shown to exhibit necrotic leaf tips apparently due to urea burn (Polacco and Holland 1993). The *eu1/eu4* double mutants were considered virtually devoided of ureolytic activity (Stebbins and Polacco 1995; Goldraij et al. 2003). Besides the mutants for structural urease isoforms, plants containing a null allele for *Eu*3 gene (Freyermuth et al. 2000), which codifies a urease Ni-insertion apoprotein, are also available. Absence of *Eu3* is associated with a complete loss of urease activity. Recently, transgenic plants with suppression of *Eu*4 expression were obtained by our team (Wiebke-Strohm et al. 2012). Although the initial aim of this previous study was to overexpress *Eu*4, an unexpected transgene and endogenous gene co-suppression was observed in leaves of those plants. As null mutants have never been obtained for the ubiquitous urease, the co-supression transgenic plants represent a powerful tool for functional gene studies.

In the present study it was demonstrated that actually all soybean urease isoforms were silenced in the transgenic plants. Aiming to investigate soybean ureases role during seed germination and plant development, these processes were compared among transgenic and non-transgenic, mutants and non-mutant samples.

Material and Methods

Plant material and growth conditions

In our laboratory transgenic plants of cv. IAS5 were obtained from embryogenic bombarded tissue with the initial aim of overexpressing *Eu*4 soybean gene. T-DNA region of binary vector used for soybean transformation is shown in Fig. 1. Unexpectedly transgenic plants exhibited transgene and endogenous gene co-suppression (Wiebke-Strohm et al. 2012). Plants derived from non-transgenic embryogenic tissues submitted to the same culture conditions were recovered and used as control.

Homozygous *eu4*, *eu1*, and *eu1/eu4* mutant seeds and non-mutant seeds were kindly provided by Dr. Joseph C. Polacco, Collection of the University of Missouri. These mutants were obtained in a Williams82 background.

T_1 and T_2 seeds obtained from To and T_1 transgenic plants, respectively, were placed in Petri dishes containing sterile filter paper moistened with sterile distilled water for 24 h. Seeds expressing *gfp*-reporter gene were selected under blue light using a fluorescence stereomicroscope Olympus®, equipped with a BP filter set containing a 488 ηm excitation filter and a 505-530 ηm emission filter. Images were captured using the software QCapture Pro™ 6 (QImaging®)

Transgenic IAS5 (T_1 and T_2), Williams82 mutants, IAS5 non-transgenic and Williams82 no-mutant seeds were sowed in pots containing vermiculite and maintained in a growth chamber at 26±1ºC with 16/8 h light/dark at a light intensity of 22.5 $\mu Em^{-2}s^{-1}$. After 21 days plantlets were transplanted to organic soil and transferred to a temperature-regulated (27± 5º C) greenhouse at FUNDACEP-CCGL, Cruz Alta, RS, Brazil until maturity. Plants were not supplemented with any nutrient solution containing NO_3 or NH_4 (as N source) during development.

DNA extraction and PCR-screening of transgenic plants

The total DNA extraction from plant leaves was performed according to Doyle and Doyle (1987) with modifications. Putative transgenic plants were PCR-screened for transgene presence of the hygromicin resistance gene (*hpt*) and the P*35S*-*Eu*4 chimeric gene formed by CaMV *35S* promoter (P*35S*) and the *Eu*4 ORF (Fig. 1). The following primer pairs were used in the PCR assays: 5'-GAGCCTGACCTATTGCATCTCC-3' and 5'-GGCCTCCAGAAGAAGATGTTGG-3' (*hpt*); 5'-CGCACAATCCCACTATCCTT-3' and 5'-ATGCTAGTTCAAGGTTTCCATTCT-3' (P*35S*-*Eu*4). The PCR mixture consisted of 200 ng of template DNA, 0.4 mM dNTP, 0.4 μM each *primer*, 2.5 mM $MgCl_2$ 50 mM, 1x Taq Buffer, 1 U Taq DNA Polymerase (Invitrogen, São Paulo, Brazil), and autoclaved distilled water to 25 μl. Reactions were hot-started (5 min at 94°C) and subjected to 30 cycles as follows: 45 s at 94°C; 45 s at 42°C and 45 s at 72°C. After electrophoresis in 1% agarose gel containing ethidium bromide (0.01 mg/L), PCR products were visualized under ultraviolet light.

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

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, USA) and further treated with DNAse I (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. First-strand cDNAs were obtained by using approximately 2 μg of DNA-free RNA, M-MLV Reverse Transcriptase System^{TM} (Invitrogen, Carlsbad, USA) using the DNA-free RNA and a polyT primer.

The RT-qPCR was performed using the StepOne Applied Biosystem Real-time Cycler^{TM}. PCR-cycling conditions were implemented as the following conditions: 5 min 94 °C, followed by 40 repetitions of 10 s at 94°C, 15 s at 60°C and 15 s at 72°C, by the end 2 min at 40°C. A melting curve analysis was performed at the end of the PCR run, over the range 55-99°C, increasing the temperature stepwise by 0.1°C every 1 s. Each 25 μL reaction comprised 12.5 μL diluted DNA template, 1x PCR buffer (Invitrogen, São Paulo, Brazil), 2.4 mM $MgCl_2$, 0.024 mM dNTP, 0.1 μM each primerM, 2.5 μL SYBR-Green (1:100,000, Molecular Probes Inc., Eugene, USA) and 0.3 U Platinum Taq DNA Polymerase (Invitrogen, São Paulo, Brazil). Two different templates were evaluated: first-strand cDNA-reaction product (1:100) in relative expression analyses and genomic DNA (1:100, 1:1,000 e 1: 10,000) for gene copy number estimation. All PCRs were carried out in technical quadruplicates. No-template reactions were used as negative controls.

PCR amplifications were performed using specific primer pairs (Table 1). Reference genes used for expression normalization were F-Box protein and a Metalloprotease (Jian et al. 2008; Libault et al. 2008). Lectin gene was used as reference for DNA amplification in gene copy number estimations (Schmidt and Parrott 2001). The expression data analyses were performed after comparative quantification of amplified products using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Transgene copy number was estimated using the relative quantification by standard curve analysis (Shou et al. 2004).

Ureolytic activity

Ureolytic activity in IAS5 transgenic urease co-suppressed plants, IAS5 non-transgenic plants, Williams82 *eu4* mutant plants and Williams82 non-mutant plants was

evaluated by determining the ammonia released by enzymatic activity in two independent assays.

In a first experiment, powdered leaves and roots (± 100 mg) of two-week old plants, as well as matured seed slices were incubated in a 1 mL urease indicator solution for 24 h at 60°C (Meyer-Bothling and Polacco, 1987). One L of urease indicator solution was prepared with 6g urea, 10 mL cresol red (1 mg/mL), 10 mL KH_2PO_4/K_2HPO_4/EDTA pH 7.0 and 1 mL azide 20% (w/v). As urea is hydrolyzed by urease, the ammonia released increases pH (due to consumption of H^+ in urea conversion to 2 NH_4^+ and HCO_3^-) and turns the yellow coloration to pink.

In the second experiment, protein crude extracts obtained from powdered leaves of two-weeks old plants were used for enzymatic activity quantification. Total protein crude content was determined by the method of Bradford (1976), using bovine serum albumin as standard. The ammonia released was measured colorimetrically using phenol-hypochlorite method (Weatherbur, 1967). Protein crude extracts were incubated with 10 mM urea in 10 mM sodium phosphate, pH 7.5, for 45 min at 37°C. One unit of urease releases 1 μmol of ammonia per minute.

Evaluation of germination capacity

Germination of IAS5 transgenic urease co-suppressed seeds (T_1 and T_2) was compared with that of IAS5 non-transgenic seeds. One- and eight-month stored T_1 seeds and eight-month stored T_2 seeds were evaluated. The *eu4, eu1, eu1/eu4* mutant germination capacity was compared with that of Williams82 non-mutant seeds. Mutant and Williams82 seeds have been stored for two years before the assays.

Evaluation of developmental capacity

Seven, 14 and 21 days after sowing, seedlings from all genotypes (IAS5 transgenic urease co-suppressed plants, IAS5 non-transgenic plants, Williams82 *eu4, eu1, eu1/eu4* mutant plants and Williams82 non-mutant plants) were classified according to their development stage following the categories proposed by Neumaier et al. (2000): VE = emergency of cotyledons, VC = completely opened cotyledons, V1 = first node of unifoliated leaves completely developed, V2 = second node of first trifoliated leaf

completely developed, V3 = third node of second trifoliated leaf completely developed. An additional category was adopted in the present work to classify the non-germinated seeds (= NG). Number of seeds produced per T_1 transgenic plant and non-transgenic plants were counted.

Statistical analysis

Germination capacity of transgenic and non-transgenic seeds was compared using a non-parametric *t* test. In order to compare developmental stages a score was attributed to each development category (NG = 1, VE = 2, VC = 3, V = 4, V2 = 5, V3 = 6). Analysis of Variance for repeated measures was applied to compare the seedling development among genotypes (IAS5 transgenic urease co-suppressed plants, IAS5 non-transgenic plants, Williams82 *eu4, eu1, eu1/eu4* mutant plants and Williams82 non-mutant plants) at different time-points. A non-parametric *t* test was carried out in order to compare the number of seeds produced per T_1 transgenic and per non-transgenic plants and ureolytic activity of T_1 transgenic, non-transgenic plants and, Williams82 *eu4* mutant plants and Williams82 non-mutant plants. The results with $p \leq 0.05$ were considered significant. Analyses were performed using SPSS 18.0 software.

Results

Characterization of progenies of transgenic urease co-suppressed plants

A total of 140 and 50 seeds from T_1 and T_2 generations, respectively, were screened for GFP expression under blue light (Fig. 2). Eighty-two seeds from T_1 and 33 from T_2 expressed GFP. Presence of transgenes was also confirmed by PCR using primers for *hpt* and *Eu*4 (data not shown). Transgenic and non-transgenic (control) seeds were planted in pots containing vermiculite and grown in the greenhouse under controlled conditions.

Number of transgene copies in transgenic progeny

T_1 transgenic plants and their T_0 respective progenitor were assayed for the number of *Eu*4 extra copies by RT-qPCR. Lectin gene was used as reference gene. This gene is present in two copies in soybean genome (representing four alleles in the diploid genome). Non-transgenic plants were used as control, as they contain one *Eu*4 copy (two alleles in the diploid genome). Number of *Eu*4 extra-copies in T_1 transgenic plant genomes and their

T_0 progenitor varied from 3 to 8 (Fig. 3). Variation in the number of *Eu*4 extra-copies among plants of the same line (A38, A32 and A33) indicates that transgenes are segregating in the progeny.

Gene expression

Transcript levels

Expression of *Eu*4 gene in leaves was evaluated by RT-qPCR (Fig. 4). As expected, the progeny of transgenic plants showed lower *Eu*4 expression than non-transgenic controls, suggesting that the co-suppressed phenotype was maintained in the progeny. Different degrees of *Eu*4 silencing were detected in T_1 plants.

Expression levels of the three urease isoforms was accessed in roots of two-week old transgenic plants and compared to that of non-transgenic plants. Roots were used because it has been shown that *Eu*5 is manly expressed in this organ (Wiebke-Strohm 2010). As observed in Fig. 5a, transgenic plants showed not only the expected co-suppression of *Eu*4, but also display silencing of *Eu*1 and *Eu*5 genes.

Ureolytic activity

Ureolytic activity in transgenic and non-transgenic plants was evaluated by determining the ammonia released by enzymatic activity (Meyer-Bothling and Polacco 1987). Leaves, roots and seeds were powdered and placed in a solution of urea containing cresol red as pH indicator. As expected for samples containing transgenic tissues, no color change was observed even after 24 h incubation (Fig. 6, upper panel). On the other hand, samples containing non-transgenic tissues showed pink coloration after the same time of incubation.

Decreased ureolytic activity in leaves, roots and seeds indicated absence of urease activity in all organs of transgenic plants. All together urease expression assays confirmed that transgenic plants have had all three urease isoforms silenced in all plant tissues, reinforcing the potential of these plants for functional studies.

Urease expression in mutant plants

Transcript levels

Transcript levels of *Eu*4, *Eu*1 and *Eu*5 in roots of two-week old e*u4*, e*u1* and *eu1/eu4* mutant plants, as well as of Williams82 non-mutant plants were determined by RT-qPCR (Fig. 5 b, c, d). Mutants e*u1* and *eu1/eu4* exhibited similar expression patterns: lower *Eu*1, but normal *Eu*4 and *Eu*5 expression levels. Mutant *eu4* showed normal transcript levels of all three urease-encoding genes.

Ureolytic activity

Ureolytic activity in *eu4* mutant and in Williams82 non-mutant plants were evaluated as described above for transgenic plants. For samples containing leaves and roots of mutant *eu4* no color change was observed (Fig. 6, lower panel), even after 24 h. Samples containing tissues from non-mutant plants presented a light pink coloration after the same time of incubation. Both, mutant and non-mutant seeds turn the yellow solution to pink. Thus, mutant *eu4* presents decreased ureolytic activity in both leaves and roots, but normal activity in seeds.

In the order to compare the residual ureolytic activity present in leaves of transgenic urease co-suppressed plants and *eu*4 mutants, ureolytic activity was measured colorimetrically in phenol-hypochlorite (Weatherburn 1967). Ureolytic activity in leaves of transgenic and *eu4* mutant plants was lower when compared with non-transgenic or non-mutant plants of the same background (Fig. 7). Interestingly, decreased in urease activity was deeper in transgenic plants that in mutant *eu4*.

Germination capacity

Germination rate of young (one month) and middle-aged (eight month) transgenic T_1 and T_2 seeds was evaluated and compared to that of non-transgenic seeds (Fig. 8a). Significant lower germination rate was observed for transgenic T_1 middle-aged seeds. However, T_2 transgenic middle-aged seeds did not show significant decrease on germination rate, suggesting that germination capacity was recovered. On the other hand, differences on germination percentage were not detected among *eu4*, *eu1*, *eu1/eu4* mutants and Williams82 non-mutant seeds stored for two years (Fig. 8b).

Development pattern

Development of plants was evaluated seven, 14 and 21 days after sowing. T_1 and T_2 transgenic plants and non-transgenic plants, as well as *eu4*, *eu1*, *eu4/eu1* mutants and non-mutants plants were classified into development categories according to Neumaier et al. (2000). Interaction among genotype, developmental stage and time-course was highly significant ($p<0.001$). Transgenic plants showed a significant delay in development when compared with non-transgenic plants (Fig. 9a). On the 7th day, while most non-transgenic plants were classified as VC (completely opened cotyledons), most T_1 transgenic plants were between NG (non-germinated) and VE (emergency of cotyledons above of soil surface) stages (Fig.10). The same behavior was observed in T_2 generation, in which most transgenic plants were at VE stage. On the 14th day, T_1 and T_2 transgenic plants were at VC and V1 (first node of unifoliated leaves completely developed) stages, whereas non-transgenic plants were at V1 and V2 (second node of first trifoliated leaf completely developed). On the last day of observation, transgenic plants were at V1 and V2 stages and non-transgenic plants at V3 (third node of second trifoliated leaf completely developed). Even after one month, transgenic plants still showed delayed development.

When mutants and non-mutants were compared a significant interaction among time, developmental stage and urease presence was also observed ($p<0.001$). During the first 14 days, *eu1* and *eu1/eu4* mutants showed a delay in development when compared with non-mutant plants (Fig. 9b, Fig. 11). However, on the 21th day *eu1* and *eu1/eu4* mutant and non-mutant plants were classified on the same developmental stage. Surprisingly, most *eu4* mutants displayed a faster development up to 7th day. Subsequently, *eu4* mutant and non-mutant plants were at the same developmental stages.

Number of seeds produced by T_1 transgenic plants was compared with those produced by non-transgenic plants. Statistically significant lower number of seeds was obtained for transgenic plants (Fig.12).

Discussion

The present study aimed to characterize at molecular level the progeny of transgenic *Eu*4 co-suppressed plants obtained in our laboratory (Wiebke-Strohm et al. 2012). T_1 and T_2 transgenic seeds and plants were also evaluated for their germination capacity and temporal developmental pattern in comparison with non-transgenic plants of the same background. Molecular analyses showed that *Eu*4 co-suppression was maintained

in the transgenic progeny. In addition it was verified that transgenic plants display very low levels of the other two ureases isoforms encoded by *Eu*1 and *Eu*5 genes. In light of these results *eu4*, *eu1* single and *eu4/eu1* double mutants were also evaluated. Mutants e*u1* and *eu1/eu4* exhibited similar expression patterns: lower *Eu*1, but normal *Eu*4 and *Eu*5 expression levels. As *eu1* is a null mutant (Polacco et al. 1993), the lower level of *Eu*1 is expected. Mutant *eu4* showed normal transcript levels of all three urease-encoding genes. This result could be anticipated since it has been shown that *eu4* plants produce a truncated ubiquitous urease (Torisky et al. 1990; Stebbins et al. 1991; Witte et al. 2002).

The reaction catalyzed by urease is essential to allow the organisms to use external or internally generated urea as a nitrogen source (Mobley and Hausinger 1989; Mobley et al. 1995). Nevertheless, the physiological relevance of ureases is still unclear (Follmer 2008).

It has been demonstrated that *A. thaliana* seeds failed to germinate when urease was chemically inhibited, but seed viability could be rescued by an external N source (Zonia et al. 1995). In addition, the soybean *eu3* mutant, which did not produce the urease Ni-insertion apoprotein, presents a complete loss of urease activity. It has been observed that *eu3* seeds tend to lose germination ability more rapidly than urease-positive seeds. However, this preliminary observation was not further explored in order to determine if that difference can be attributed to the loss of ureolytic activity. Results obtained in the present study did not corroborate the previous idea that ureolytic activity affects seed viability**.** Significant differences were not detected on germination rates when two-years aged seeds of *eu4*, *eu1, eu1/eu4* mutants and non-mutants were compared. Transgenic T_1 middle-aged seeds (with lower levels of all three ureases) exhibited lower germination rate when compared to non-transgenic ones, but the difference was not observed for T_2 generation. We assume that the disparity detected in T_1 generation was an isolated phenomenon, not genetically determined. Altogether our results suggest that lack of one or more ureases isoforms did not affect seed viability even after storage.

In *A. thaliana* both urease transcripts and ureolytic activity increased after germination, especially in eight/nine old-day seedlings (Zonia et al. 1995). Urease is also present in the early developmental stages (up five days) of *Canavalia ensiformis* in proteomic level (Demartini et al. 2011). 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 2010). Embryo-specific urease activity in young plants was also observed by Torisky and Polacco (1990). Increased urease content and ureolytic activity during seedling growth indicate an important role of these enzymes in earlier stages of plant development. An additional evidence of significance of ureases on developmental process was observed for *Brassica napus*, which present a substantial growth repression under Ni-deprived environment (Gerendás and Sattelmacher 1999; Gerendás et al. 1999). Ni-free conditions result in no urease activation. These evidences support a direct association between urease activity and plant development.

Importance of urease activity for urea hydrolysis can be measured by urea accumulation: the higher ureolytic activity, the lower urea amount. Accumulation of urea was not observed in *eu1* mutants. In contrast, an increased amount of urea was detected in *eu4* seedlings. The *eu1/eu4* double mutant seedling accumulated a similar amount of urea than the *eu4* single mutant (Stebbins et al. 1991). Considering these evidences, N availability during plant developmental has been attributed to soybean ubiquitous urease.

A goal of the present study was to associate ureases role with temporal plant developmental pattern. Surprisingly, our results indicate that *Eu*1 and *Eu*5 have a great contribution on this process. This is evidenced by the fact that *eu1* and *eu1/eu4* mutants showed a delay in the first developmental stages, but the subsequent growth was not affected. Silencing of all three soybean ureases encoding genes in transgenic plants, especially *Eu*5, forwarded this phenotype to further developmental stages. Delay in development was maintained even in adult transgenic plants and may be the cause of lower production of seeds. These results suggest a synergistic effect of *Eu*1 and *Eu*5 on plant growth. Based on bioinformatics analyses, *Eu*5 has been suggested not to be functional as enzyme as it presents high number of mutations (Witte CP 2011). However, according our results the product of this gene might be involved on plant development.

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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'
Lectin DNA	Forward	5'- TACCTATGATGCCTCCACCA -3'
	Reverse	5'- GAGAACCCTATCCTCACCCA -3'

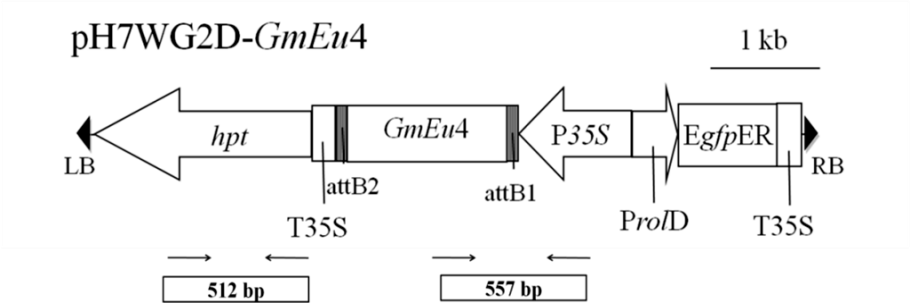

Fig. 1 T-DNA region of binary vector pH7WG2D-*Eu*4 used for soybean transformation (Adapted from Wiebke-Strohm, 2010). RB – T-DNA right border, LB – left border, *hpt* – hygromycin phosphotransferase gene, P35S – Cauliflower mosaic virus (CaMV) 35S promoter, T35S – CaMV 35S terminator, **Egfp*ER*** – enhanced green fluorescent protein, ***P*rolD** – root loci D promoter, *GmEu*4 – soybean ubiquitous urease encoding gene, **attB1** and **attB2** – LR reaction site, kb – kilobase pairs (1,000 bp). Arrows and boxes represent primers and PCR reaction product used for transgene screening.

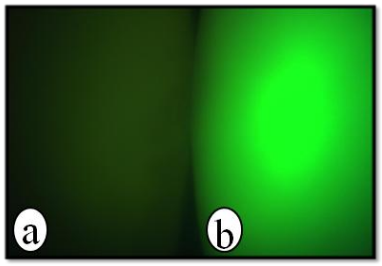

Fig. 2 GFP expression analysis. (a) Non-transgenic and (b) green fluorescent transgenic seed one day after dormancy break. GFP expression was detected under blue light using a fluorescence stereomicroscope Olympus® (40X), equipped with a BP filter set containing a 488 ηm excitation filter and a 505-530 ηm emission filter. Images were captured using the software QCapture Pro™ 6 (QImaging®).

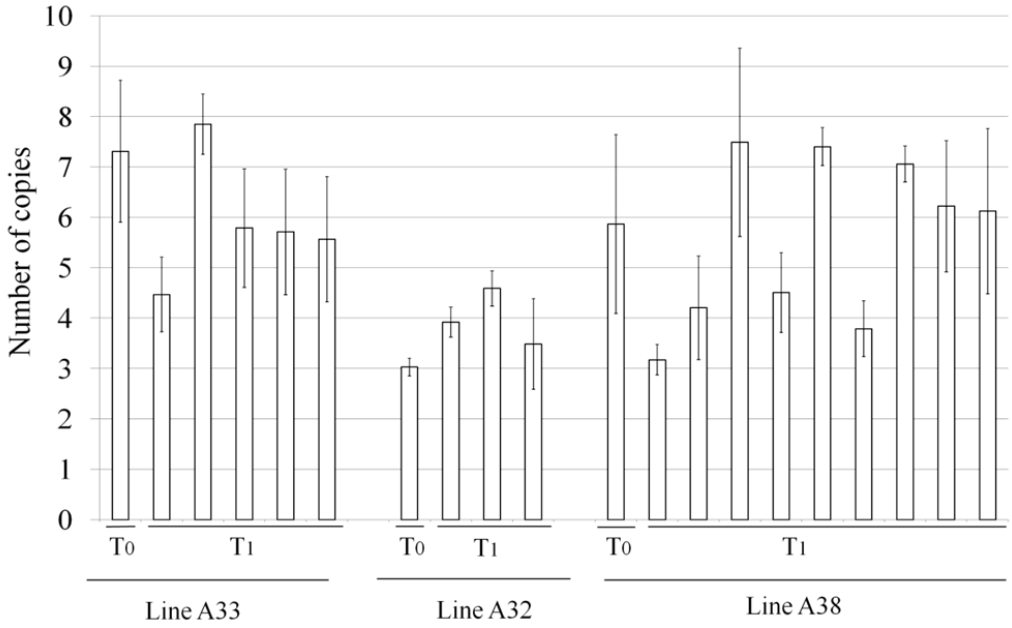

Fig. 3 Number of *Eu*4 extra copies in the genome of T_0 transgenic plants and their respective T_1 transgenic progeny. Estimative was performed by RT-qPCR. The number of *Eu*4 extra-copies varied among plants of the same line (A38, A32 and A33). five plants derived from line A33, three from line A32 and eight from line A38. T_0 and T_1 represents the mean±SD of the relative quantification. Transgenic plants are from cv. IAS5.

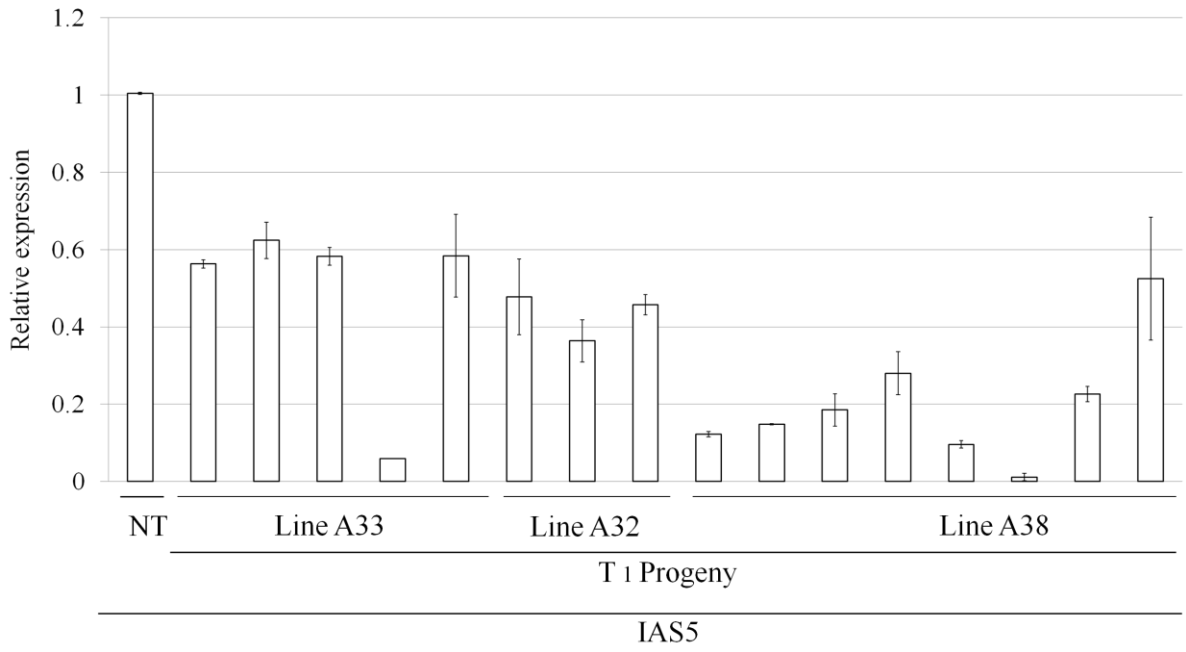

Fig. 4 *Eu*4 expression pattern in transgenic and non-transgenic (control) plants. Three transgenic lines (A33, A32, and A38) were evaluated: five plants derived from line A33, three from line A32 and eight from line A38. NT represent non-transgenic plant. Transgenic plants and non-transgenic plants are from cv. IAS5. NT represents the mean±SD of 2 non-transformed plants. F-Box protein and Metalloprotease reference genes were used as internal controls to normalize the amount of mRNA present in each sample. Transcript level of *Eu*4 present in non-transgenic plants was used to normalize transcript accumulation in transgenic plants.

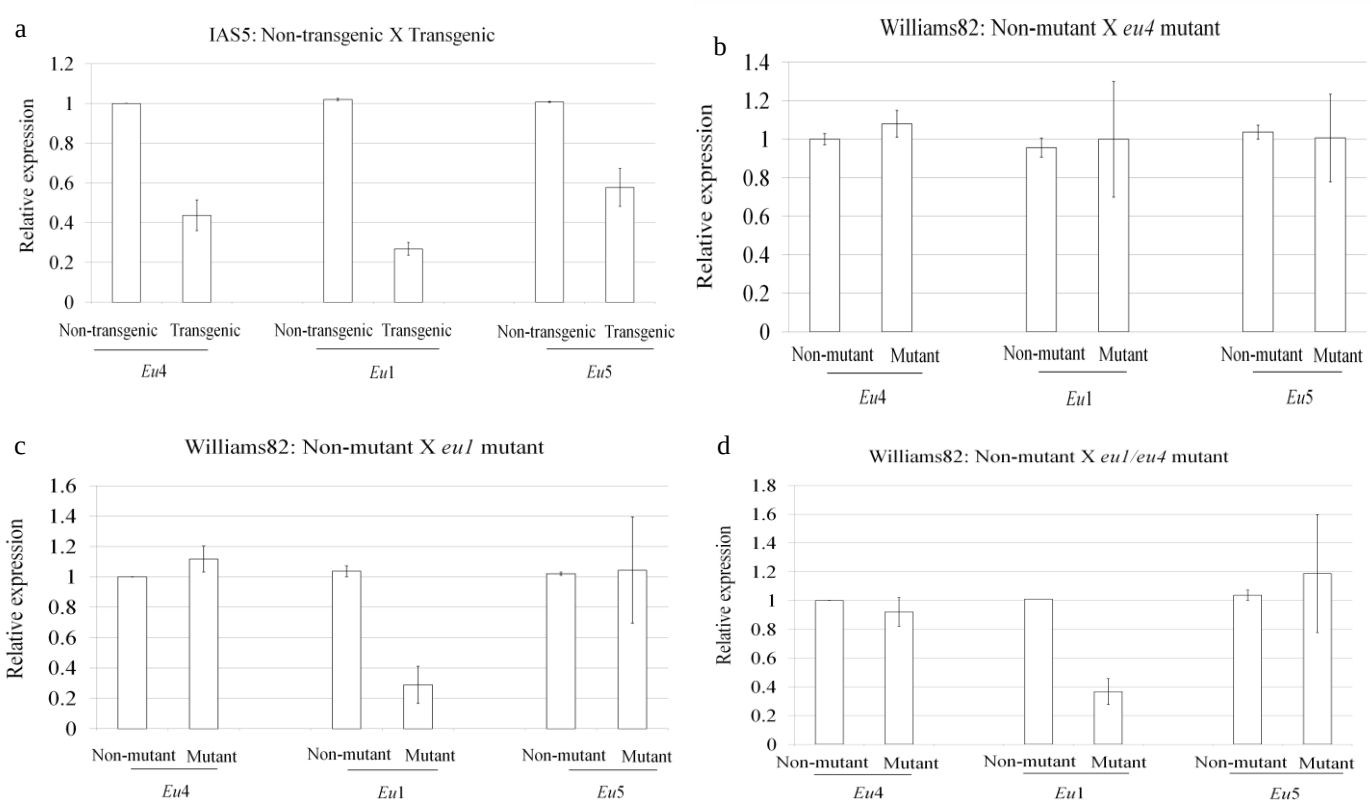

Fig. 5 Expression levels (RT-qPCR) of three urease encoding genes (*Eu*4, *Eu*1, *Eu*5) in roots of two-week old IAS5 transgenic and non-transgenic plants. (a), Williams82 non-mutants and mutants *eu4*, *eu1*, *eu1/eu4* (b, c, d respectively) plants. F-Box protein and Metalloprotease reference genes were used as internal controls to normalize the amount of mRNA present in each sample. The bars represent mean±SD carried out with two non-transgenic, 10 transgenic, two non-mutant and 10 mutant *eu4*, *eu1*, *eu1/eu4* roots. Transcripts level of *Eu*4 *Eu*1, *Eu*5 present in non-transgenic or non-mutant plants were used to normalize transcript accumulation in transgenic or mutant plants, respectively. Transgenic plants and non-transgenic plants are from cv. IAS5, non-mutants and mutant *eu4* are from cv. Williams82.

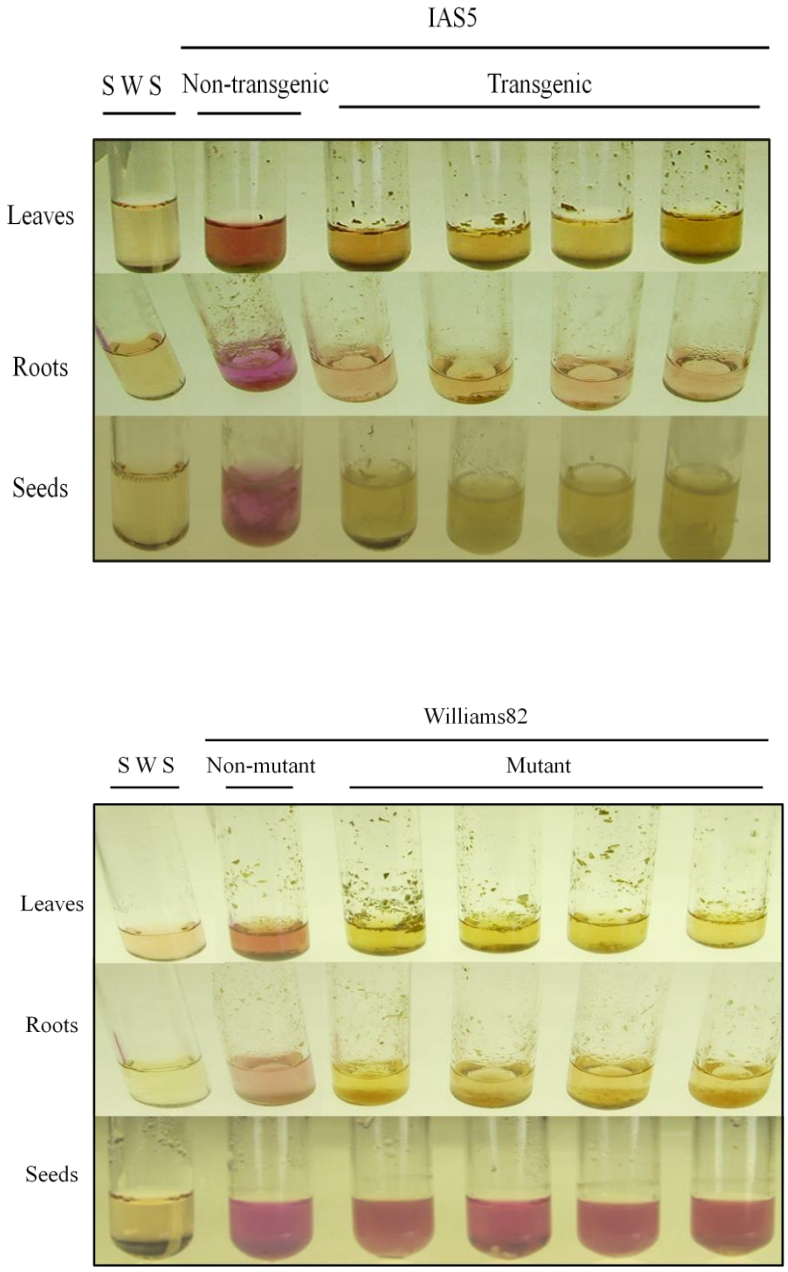

Fig. 6 Ureolytic activity in transgenic and non-transgenic plants (upper panel), mutant *eu4* and Williams82 non-mutant plants (lower panel). Powdered leaves and roots of two-week old plants and slice of mature seeds were incubated in a pH-indicator reagent containing cresol red and weakly buffered 10 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. Transgenic plants and non-transgenic plants are from cv. IAS5, non-mutants and mutant *eu4* are from cv. Williams82.

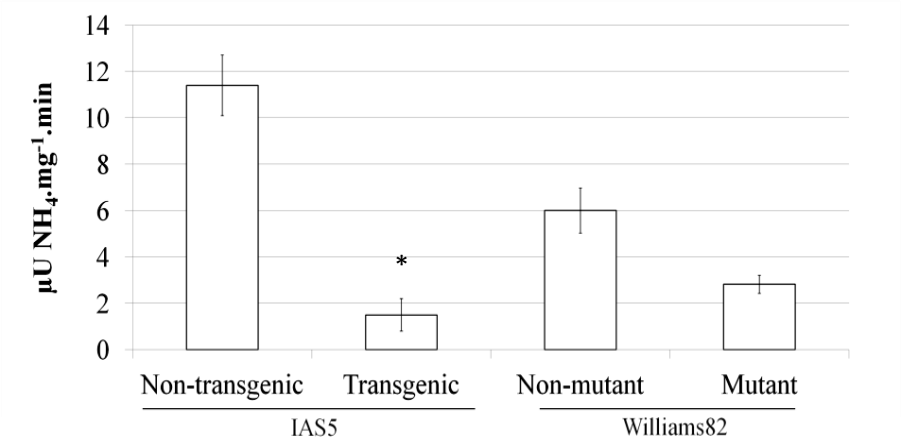

Fig. 7 Comparison of the ureolytic activity in leaves of transgenic and non-transgenic plants, *eu4* mutant and non-mutant plants. Phenol-hypochlorite was used to determine the amount of liberated ammonium catalyzed by the protein crude extracts of leaves. One unit of urease releases 1 µmol of ammonia per minute at 37 °C, pH 7.5. The bars represent mean±SD of two independent experiments carried out with two non-transgenic, eight transgenic, two non-mutant and six mutant plants. Transgenic plants and non-transgenic plants are from cv. IAS5, while non-mutants and mutants are from cv. Williams82. Ureolytic activity was compared using a non-parametric *t* test. *Difference statistically significant.

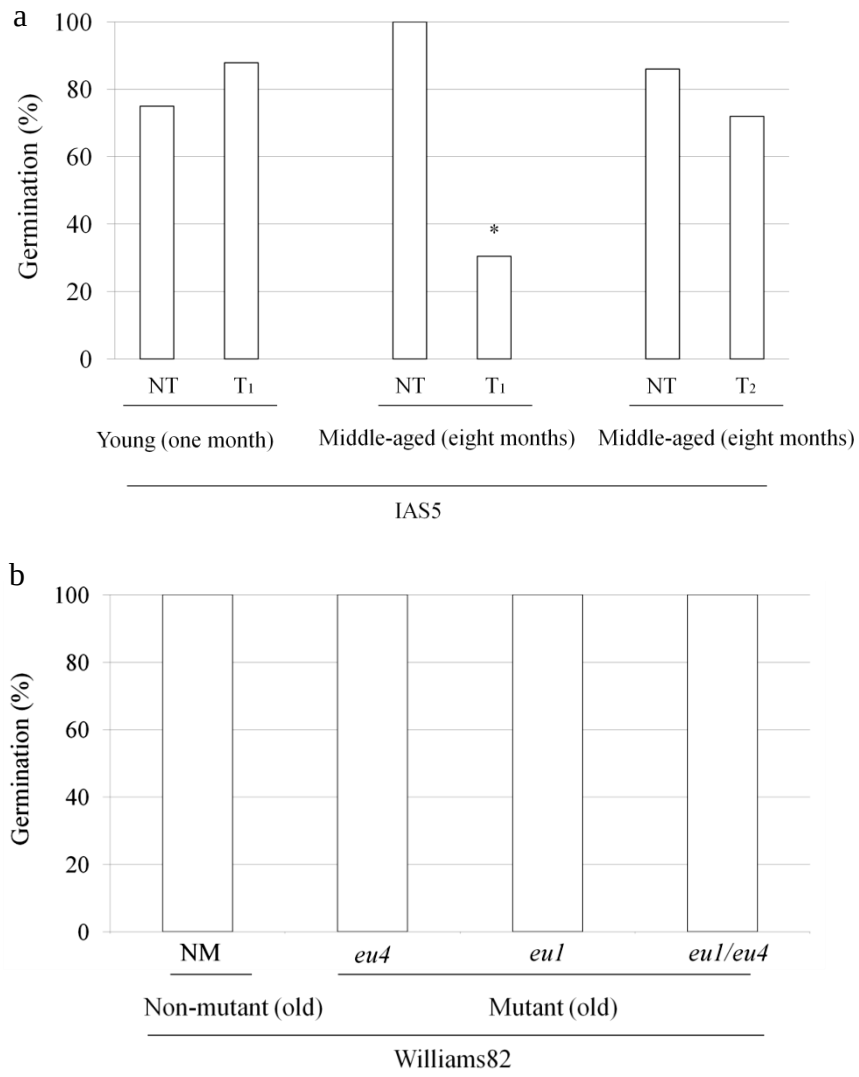

Fig. 8 Seed germination. Germination rates of transgenic and non-transgenic young (one month) and middle-aged (eight months) seeds, e*u4*, e*u1* and *eu1/eu4* mutants and non-mutant seeds. The experiment was carried out with 16 T_1 transgenic and 34 non-transgenic (NT IAS5) young seeds, 82 T_1, 33 T_2 transgenic and ten non-transgenic middle aged seeds, 10 *eu4*, 10 *eu1*, 10 *eu1/eu4* mutants and 10 non-mutant seeds. Transgenic plants and non-transgenic plants are from cv. IAS5 (a). While non-mutants and mutants are from cv. Williams82 (b). Germination capacity of transgenic and non-transgenic seeds was compared using a non-parametric *t* test. *Difference statistically significant.

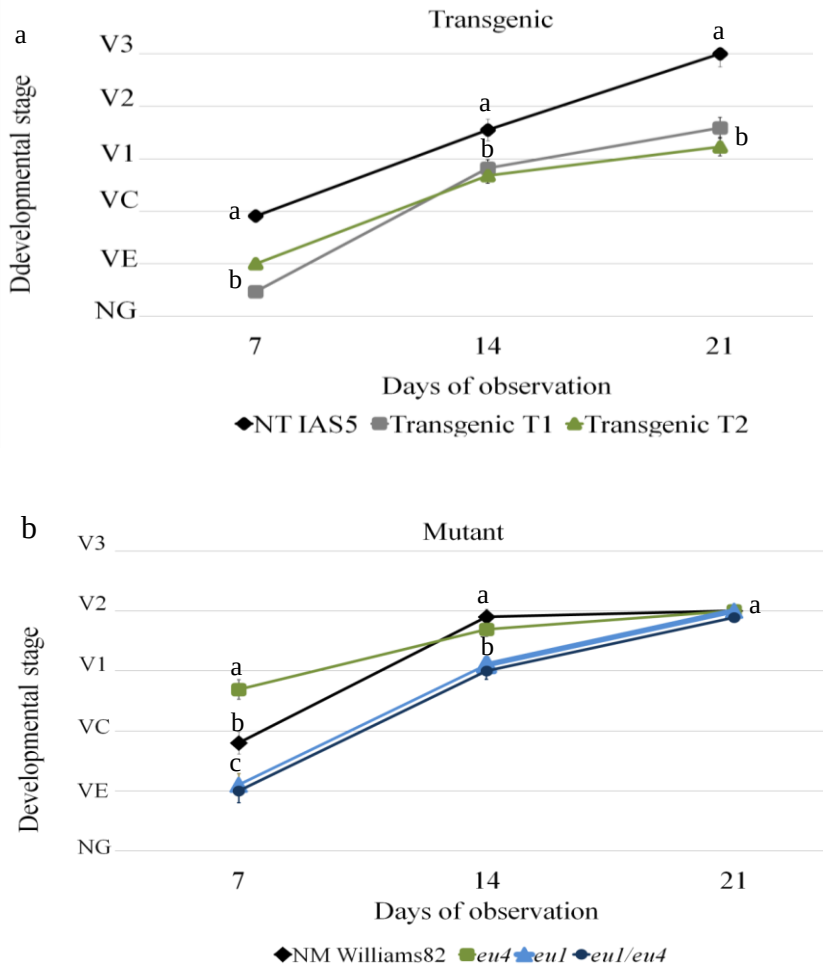

Fig. 9 Plant development of transgenic and non-transgenic plants (a), *eu4*, *eu1*, *eu1/eu4* mutant and non-mutant plants (b). Plants were classified according to their developmental stage seven, 14 and 21 days after sowing. Categories were proposed by Neumaier *et al.* (2000): VE = emergency of cotyledons, VC = completely opened cotyledons, V1 = first node of unifoliated leaves completely developed, V2 = second node of first trifoliated leaf completely developed, V3 = third node of second trifoliated leaf completely developed. An additional category was adopted in the present work to classify the non-germinated seeds (= NG). To compare developmental stages a score was attributed to each developmental category (NG = 1, VE = 2, VC = 3, V = 4, V2 = 5, V3 = 6). The experiment was carried out with 17 T_1 and 22 T_2 transgenic plants, 10 non-transgenic plants (NT IAS5), 10 *eu4*, 10 *eu1*, 10 *eu1/eu4* mutant plants and 10 non-mutant plants. Transgenic plants and transgenic plants are from cv. IAS5, while non-mutants and mutants are from cv. Williams82. Analysis of Variance for repeated measures was applied to compare the seedling development among genotypes at different time-points. Means followed by different letters in the same time-point are significantly different.

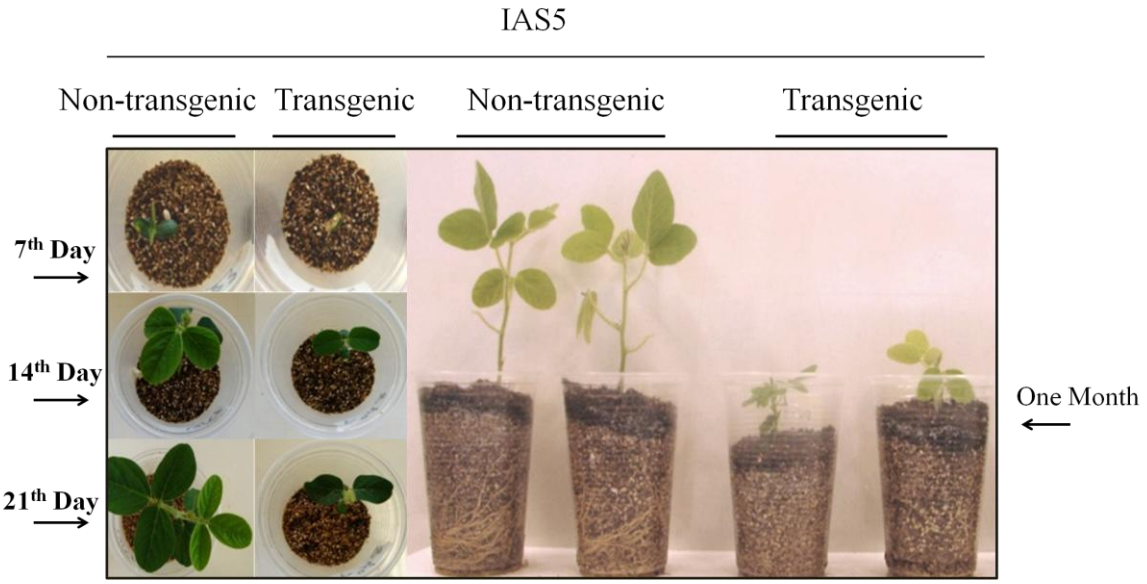

Fig. 10 Development of non-transgenic plants with seven, 14, 21 and 30 days, and transgenic plants with seven, 14, 21 and 30 days. Transgenic plants and transgenic plants are from cv. IAS5.

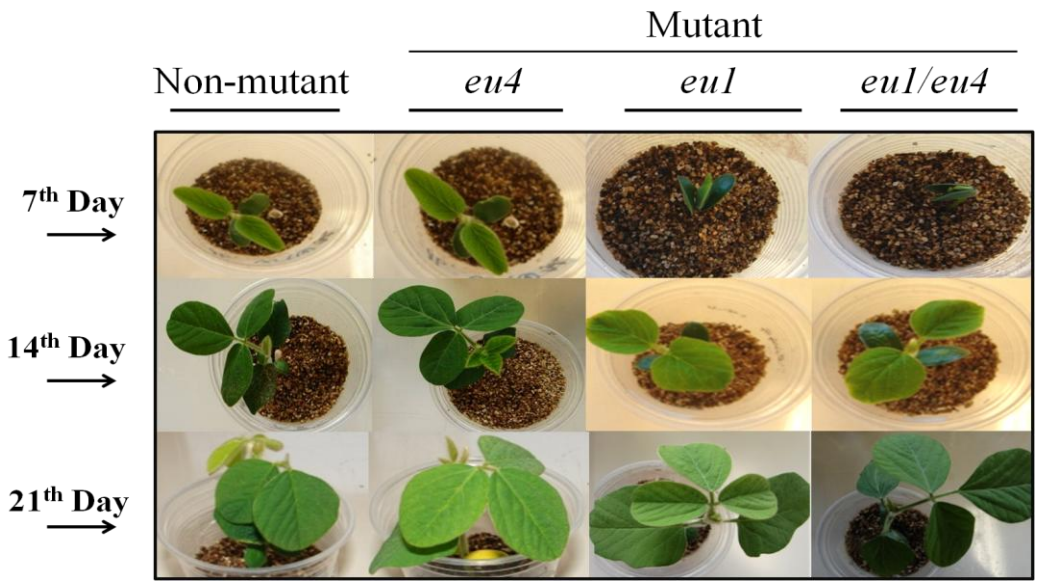

Fig. 11 Development of non-mutants plants with seven, 14 and 21 days, and *eu4*, *eu1*, *eu1/eu4* mutant plants with seven, 14 and 21 days. Non-mutants and mutants are from cv. Williams82.

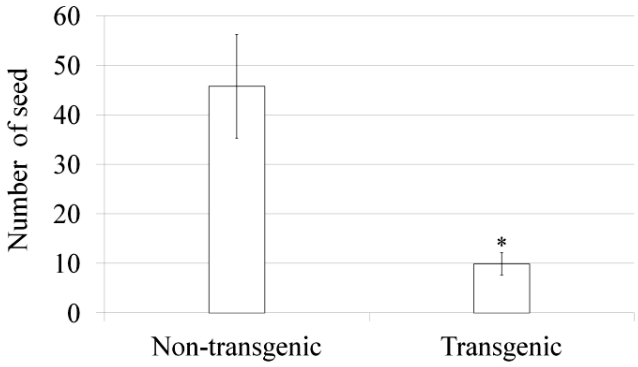

Fig. 12 Number of seeds produced by transgenic plants of T_1 generation and non-transgenic plants. *Means are statistically different in seeds of transgenic and non-transgenic plants (*t* -test, $p<0.05$). Transgenic plants and transgenic plants are from cv. IAS5.

Capítulo III

CONCLUSÕES E PERSPECTIVAS

3. Conclusões e perspectivas

3.1 Análise da progenie das plantas transgênicas de soja a urease ubíqua co-suprimida

De acordo com os dados obtidos em estudo anterior (Wiebke-Strohm et al. 2012) e no presente trabalho a progênie das plantas transgênicas co-suprimidas apresenta padrão de herança mendeliana. Uma vez que as plantas transgênicas T_0 apresentavam múltiplas cópias extras do gene *Eu*4 (10 a 14), com base nos estudos de padrão de herança sugeriu-se que estas teriam sido integradas *in tandem*. No entanto, os resultados obtidos para as plantas transgênicas da geração T_1, mostraram um número menor de cópias do transgene: 3 a 8. A redução no número de cópias dos transgenes poderia ser explicada pela ocorrência de recombinação intercromossomal associada à presença de fragmentos de DNA genômico intercalando os transgenes presentes em um mesmo locus (Kohli et al. 2003).

A caracterização molecular da progênie das plantas transgênicas confirmou que a co-supressão de *Eu*4 (urease ubíqua) foi mantida ao longo das gerações. Essas plantas transgênicas foram também caracterizadas quanto à expressão dos genes *Eu*1 (urease embrião especifica) e *Eu*5 (nova urease). As plantas transgênicas mostraram silenciamento de todos os genes que codificam ureases.

No presente estudo, o papel das ureases de soja foi investigado durante a germinação e o desenvolvimento. Estes processos foram avaliados em plantas transgênicas T_1 e T_2 e comparados com os de plantas não transgênicas, mutantes *eu4*, *eu1* e *eu1/eu4* e não mutantes. As plantas mutantes *eu1* e *eu1/eu4* apresentaram níveis normais de transcritos para os genes *Eu*4 e *Eu*5 e menores para *Eu*1. O mutante *eu4* mostrou níveis normais dos transcritos para todos os genes que codificam ureases. Esse resultado já era esperado uma vez que *eu1* é um mutante nulo para Eu1 (Polacco et al. 1993) e que *eu4* produz uma urease ubíqua truncada (Torisky et al. 1990; Stebbins et al. 1991; Witte et al. 2002). Para fins de estudo funcional de genes individuais os mutantes simples *eu4* e *eu1* e duplo *eu1/eu4* foram utilizados.

Tem sido proposto que a função da atividade ureolítica é particularmente importante durante a germinação, quando as proteínas de reserva são mobilizadas para a nutrição do embrião (Goldraij et al. 2003). Alguns autores relataram que quando a urease é inibida quimicamente, há um atraso na germinação ou perda na viabilidade das sementes. Contudo, os resultados apresentados no presente estudo mostraram que sementes transgênicas T_1 apresentaram menor germinação quando comparadas com plantas não transgênicas, mas sementes transgênicas T_2 não mostraram a mesma diferença. Quanto às sementes mutantes *eu4*, *eu1* e *eu1/eu4*, todas germinaram. Portanto, não há indicação de que a ausência de uma ou mais ureases interfira na capacidade de germinação das sementes.

Muitos estudos relatam que as ureases são importantes nos estágios iniciais de desenvolvimento das plantas. No capítulo 2 foi descrito que as plantas transgênicas com silenciamento dos genes que codificam as três isoformas de ureases apresentaram atraso no desenvolvimento quando comparadas com plantas não transgênicas. As mutantes *eu1* e *eu1/eu4*, exibiram atraso nos primeiros 14 dias de observação. Mas após este período todas as plantas estavam na mesma classificação. Os resultados obtidos sugerem que as ureases *Eu*1 e *Eu*5 estejam sendo importantes para o crescimento das plantas. Os resultados encontrados parecem ser contrastantes com o que a literatura tem apresentado (Stebbins et al. 1991). Em vista disso, nova análise do desenvolvimento das plantas mutantes será realizada.

3.2 Superexpressão da urease ubíqua da soja

3.2.1 Estudos prévios

Os experimentos de transformação de soja conduzidos por Wiebke-Strohm (2010) foram realizados com o objetivo inicial de gerar plantas que superexpressassem a urease ubíqua (*Eu*4). Mas ao contrário da superexpressão prevista, houve uma co-supressão do gene alvo na maioria das plantas. 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. Foi obtida apenas uma planta contendo duas cópias de transgene e com maiores níveis de expressão. Contudo, esta planta não deixou descendentes com a característica, fazendo-se necessários esforços adicionais para obtenção de um maior número de linhagens com o fenótipo desejado.

Com o intuito de obter plantas transgênicas com uma ou poucas cópias extras do gene *Eu*4 e aumentar a possibilidade de superexpressão do gene, novos experimentos de transformação foram realizados.

3.2.2 Experimentos de transformação em andamento

Para a transformação via bombardeamento, foi utilizado o procedimento descrito por Finer & McMullen (1991) com modificações (Droste et al. 2002). A transformação via sistema integrado bombardeamento/*Agrobacterium* seguiu o protocolo descrito por Wiebke-Strohm et al. (2011). Em todos os testes, conjuntos de embriões somáticos globulares das cultivares IAS5 e Bragg foram utilizados como tecido alvo e pH7WG2D-ureU, descrito anteriormente, como vetor de transferência.

A seleção dos embriões transformados foi realizada através de meio de cultura contendo higromicina por três meses. Tecidos higromicina resistentes foram transferidos para meios de cultura que permitem a histodiferenciação, maturação e regeneração das plantas. Na tabela 1 estão representados os dados obtidos até o momento. As plântulas encontram-se em fase final de regeneração e aclimatação.

Tabela 1: Conjuntos embriogênicos resistentes a higromicina, embriões histodiferenciados e plantas convertidas obtidos a partir de experimentos de transformação.

	Método				
	Experimento I **Bombardeamento**		**Experimento II** **Bombardeamento/** *Agrobacterium*		**Experimento III** **Bombardeamento**
Cultivar	**Bragg**	**IAS5**	**Bragg**	**IAS5**	**IAS5**
Número de clusters embriogênicos	120	120	120	120	120
Número de clusters higromicina-resistentes	12	17	3	-	11
Número de embriões histodiferenciados	40	11	-	7	21
Número de plântulas	20	-	-	-	-
Plântulas em aclimatação	10	-	-	-	-
Plântulas confirmadas por PCR	2	-	-	-	-

As análises moleculares visando a confirmação da integração dos transgenes nas plantas obtidas estão em andamento. A presença dos transgenes será confirmada por PCR usando primers para *hpt* e *Eu*4. A expressão dos transgenes em nível de mRNA e proteína será avaliada por RT-qPCR e testes bioquímicos para atividade ureolítica.

Um grande esforço tem sido direcionado para a obtenção de plantas de soja que superexpressem genes que codificam ureases. A geração de plantas com essa característica poderia contribuir para os trabalhos do grupo, confirmando o envolvimento das ureases em processos biológicos importantes tais como desenvolvimento e defesa contra insetos praga e fungos patogênicos. Além dos estudos de base, o crescente número de publicações demonstrando o potencial das ureases nos processos de defesa (Ferreira da Silva et al. 2002; Stanisçuaski et al. 2005; Stanisçuaski et al. 2009; Defferrari et al. 2011) reforça a expectativa de obtenção de plantas com maior resistência a adversidades bióticas.

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