Obtenção de plantas estavelmente transformadas pelo sistema integrado bombardeamento/*Agrobacterium* e análise funcional dos genes que codificam as ureases estruturais da soja

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"Se fui capaz de ver mais longe, é porque me apoiei em ombros de gigantes."

Isaac Newton

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SUMÁRIO

LISTA DE ABREVIATURAS

aa: aminoácidos

Amp^R: gene de resistência a ampicilina

attB: sítio de recombinação LR

BL: do inglês *left border*, borda esquerda do T-DNA

bp: pares de bases do DNA

BR: do inglês *right border*, borda direita do T-DNA

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

cDNA: DNA complementar

CeURE-IIb: equivalente a JBUREIIb, uma variação da urease estrutural secundária de *Canavalia ensiformis*

CTAB: brometo de cetiltrimetilamônio

cv: cultivar

D20: meio de proliferação

D40: meio de indução

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

Genes *R*: genes que codificam proteínas de resistência

GENOSOJA: Consórcio Nacional para Estudo do Genoma da Soja

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

Gm: *Glycine max*

GUS: β-glicuronidase

gus-A: gene repórter que codifica a β-glicuronidase

His: histidina

hpt: gene marcador que codifica a higromicina fosfotransferase

H_2O_2: peróxido de hidrogênio

JBU: urease estrutural majoritária da semente de *Canavalia ensiformis*

JBUREII: urease estrutural secundária de *Canavalia ensiformis*

kb: quilobases

kDa: kiloDaltons

LB: meio de cultura Luria Broth

MAMP: do inglês *microbe-associated molecular pattern*

MAPK: do inglês *Mithogen-activated Protein Kinase*

Mb: megabases

MS: meio de cultura Murashinge e Skoog

MSM6: meio de histodiferenciação

NaPB: fosfato de sódio

NCBI: do inglês *National Center for Biotechnology Information*

NH_4: amônia

nptII: gene marcador que codifica neomicina fosfotransferase II

NT: não transformada

olp: gene que codifica uma *osmotin like protein*

ORF: do inglês *open reading frame*

PAMP: do inglês *pathogen-associated molecular pattern*

pb: pares de base

PCR: do inglês *polymerase chain reaction*

PDB: meio de cultura *Potato Dextrose Broth*

pH: potencial de hidrogênio

PIG: do inglês *Particle Inflow Gun*

P*nos*: promoter do gene da nopalina sintase

P*rol*D: promoter raiz-específica de *Agrobacterium rhizogenes*

RB: do ingles *reddish-brown*, é um tipo de lesão vermelho-castanha sem ou com poucos esporos, observada em plantas resistentes quando infectadas pelo fungo *Phakopsora pachyrhizi*

PRR: do inglês *pattern recognition receptors*

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

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

RNAi: RNA de interferência

SAR: do ingles *systemic acquired resistance*

Tan: tipo de lesão contendo inúmeros esporos, causada pela interação suscetível entre o fungo *Phakopsora pachyrhizi* e a planta hospedeira

T-DNA: DNA de transferência de *Agrobacterium*

T*nos*: terminador do gene da nopalina sintase

T_0: plantas transgênicas recuperadas da cultura *in vitro*

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

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

UTR: região não-traduzida das bordas do mRNA

WRKY: superfamília de fatores de transcrição

V2: estágio fenológico da planta correspondente ao desenvolvimento vegetativo com dois nós foliares

X-Gluc: do inglês *5-bromo-4-chloro-3-indolyl-glucuronide*

2,4-D: ácido 2,4 diclorofenoxiacético

RESUMO

As urease de plantas catalisam a hidrólise da ureia e apresentam efeitos tóxicos a fungos patogênicos e insetos fitófagos. Em soja [*Glycine max* L. Merrill] foram descritas duas ureases estruturais: a embrião-específica, codificada pelo gene *Eu*1, e a ubíqua, codificada pelo gene *Eu*4. Sabe-se que a urease embrião-específica purificada apresenta efeito inibitório sobre o crescimento *in vitro* de fungos filamentosos e desenvolvimento de insetos. A urease ubíqua é responsável pela reciclagem de toda a ureia proveniente do metabolismo, mas não há informações sobre seu envolvimento no sistema de defesa das plantas. A transformação genética é uma ferramenta importante em estudos de genômica funcional e, portanto, a disponibilidade de sistemas eficientes é um pré-requisito essencial. O objetivo deste trabalho foi a obtenção de plantas estavelmente transformadas a partir de embriões somáticos de soja submetidos ao sistema integrado bombardeamento/*Agrobacterium*, bem como a identificação e caracterização funcional dos genes que codificam as ureases estruturais de soja, especialmente a urease ubíqua em relação aos processos de resposta a fungos patogênicos. Inicialmente, testamos a eficiência de transformação de embriões somáticos secundários por um método que combina o bombardeamento de partículas livres de DNA com o sistema *Agrobacterium*. Plantas transgênicas férteis foram regeneradas de vários experimentos independentes de transformação utilizando diferentes plasmídios. Posteriormente, foi realizada a caracterização dos genes que codificam ureases presentes no genoma da soja. O gene *Eu*4 apresentou um padrão de expressão diferencial para genótipos suscetível e resistente ao longo do período de infecção por *Phakopsora pachyrhizi*, o agente etiológico da ferrugem asiática. Plantas transgênicas foram geradas visando a superexpressão de *Eu*4. Contudo, apenas uma planta apresentou níveis aumentados de expressão desse gene, enquanto que as demais plantas apresentaram o fenômeno de co-supressão dos genes endógeno e transgene. Avaliou-se o crescimento vegetativo dos fungos *Rhizoctonia solani*, *Phomopsis sp.*, *Fusarium solani*, *Colletotrichum gossypii* e *Penicillium herguei* em meio de cultura contendo extrato protéico bruto de plantas transgênicas expressando maiores e menores níveis de urease e de plantas não-transgênicas. O crescimento dos fungos foi inversamente proporcional a quantidade da urease presente no extrato protéico das plantas. Quando infectadas por uredósporos de *P. pachyrhizi*, folhas destacadas das plantas co-suprimidas desenvolveram um número significativamente maior de lesões, pústulas e pústulas abetas do que folhas com níveis normais da enzima. Em conjunto estes resultados indicam um

importante envolvimento da urease ubíqua da soja na resposta à infecção da planta por fungos patogênicos. Além disso, um terceiro gene que codifica urease foi encontrado no banco de dados com a sequência completa do genoma da soja. O gene foi denominado *Eu*5 e seu produto SBU-III. A análise filogenética mostra que SBU-III está fortemente relacionada à isoforma embrião-específica. Apesar da grande similaridade na seqüência primária da proteína, SBU-III apresenta uma mutação em um aminoácido altamente conservado entre as ureases, sugerindo ausência da atividade ureolítica. O padrão de expressão do gene *Eu*5 em diferentes órgãos e estágios de desenvolvimento foi determinado por RT-qPCR. Transcritos foram detectados em sementes um dia após a quebra de dormência, em raízes de plantas jovens e em embriões em desenvolvimento. As evidências sugerem que SBU-III não está envolvida na disponibilização de nitrogênio para as plantas, mas esta pode ter função de defesa.

ABSTRACT

Plants ureases catalyze urea hydrolysis and display toxic effects against pathogenic fungi and phytophagous insects. For soybean [*Glycine max* L. Merrill] two structural ureases have been described: the embryo-specific, encoded by *Eu*1 gene, and the ubiquitous, encoded by *Eu*4 gene. The toxic property of purified embryo-specific urease against filamentous fungi and insects was demonstrated *in vitro*. The ubiquitous urease is responsible for recycling all metabolically-derived urea, but there were no information about its putative defense role. Plant genetic transformation offers significant advancement in functional genomics. Therefore an efficient transformation system is required. This study aims to obtain stable transformed plants derived from somatic embryos submitted to the integrated bombardment/ *Agrobacterium* system, as well as identify and functionally characterize the soybean structural urease-encoding genes, specially the ubiquitous urease gene response to fungi. First, the transformation of soybean proliferating somatic embryos by a procedure that combines DNA-free particle bombardment and *Agrobacterium* was evaluated. Transgenic fertile plants were recovered from many transformation experiments using different plasmids. After, a study of ureases enconding genes present in the soybean genome was carried out. In the present work, *Eu*4 gene showed a differential expression pattern in susceptible and resistant genotypes over the course of *Phakopsora pachyrhizi* infection, the Asian rust causal agent. Transgenic plants aiming *Eu*4 overexpression were obtained. However, a single transgenic plant exhibited *Eu*4 overexpression, whereas the other ones showed co-suppression of endogenous and transgenes urease genes. The growth of *Rhizoctonia solani*, *Phomopsis sp.*, *Fusarium solani*, *Colletotrichum gossypii* and *Penicillium herguei* in media containing crude protein extract from either transgenic or non-transgenic leaves was evaluated. Fugal growth was inversely proportional to ubiquitous urease amount in plant crude extracts. When infected by *P. pachyrhizi* uredospores, detached leaves of co-suppressed plants developed a significantly higher number of lesions, pustules and erupted pustules than leaves containing normal levels of the enzyme. These results suggested an important role of soybean ubiquitous urease in plant response against fungal infection. Furthermore, by searching the completed soybean genome sequence, a third urease-encoding locus was identified. The gene was designated *Eu*5 and its product, SBU-III. Phylogenetic analysis shows that SBU-III is closely related to the embryo-specific isoform. Although a high similarity in amino acid sequence was observed, a mutation in a highly conserved residue suggests absence of ureolytic activity.

Expression profile of *Eu*5 gene in different organs and developmental stages was determined by RT-qPCR. Transcripts were detected in seeds one day after dormancy break, roots of young plants and embryos of developing seeds. Evidences suggest that SBU-III may not be involved in nitrogen availability to plants, but a defense role was proposed.

Capítulo I

INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

1.1 Soja

O cultivo da soja [*Glycine max* (L.) Merrill] iniciou-se no noroeste da China durante o século XI a.C. e propagou-se pelo oriente nos séculos seguintes (Goellner et al. 2010). Hoje, o grão de soja é mundialmente utilizado para a alimentação humana e animal, além de servir de matéria prima para produção de biocombustíveis (Reetz et al. 2008; Varshney et al. 2009).

No Brasil, o plantio desta oleoginosa em larga escala só teve início em 1960 (Reetz et al. 2008) e, atualmente, é de grande importância para o agronegócio do país, sendo o principal responsável pelo crescente volume de exportações e o consequente avanço da economia nacional (Reetz et al. 2009). Somente em 2008 a soja gerou uma receita de aproximadamente US$ 17 bilhões (CONAB 2010; Tollefson 2010). A produção estimada na safra 2009/2010 foi de 68,69 milhões de toneladas, superando em 20,2% a do ano passado e em 12,7% a do ano retrasado (CONAB 2010).

No momento, o Brasil é o segundo maior produtor desta oleoginosa e já está competindo com os Estados Unidos pelo título de maior exportador em nível mundial (Reetz et al. 2008; Tollefson 2010). As previsões mostram que a produção agrícola no nosso país vai crescer mais durante a próxima década do que qualquer outra no mundo, aumentando em mais de 40% até o ano de 2019 (Tollefson 2010). O cenário positivo é uma consequência do alto investimento em pesquisa, tanto por parte de setores públicos quanto de privados. A ampliação da área agrícola, o incremento na produtividade, o aumento da qualidade do grão e a redução dos custos de produção são as contribuições mais importantes das pesquisas realizadas em nosso país (Reetz et al. 2008; Tollefson 2010).

O plantio de transgênicos também está aumentando marcadamente no Brasil, país que apresenta a segunda maior área cultivada com organismos geneticamente modificados no mundo, atrás apenas dos Estados Unidos (Tollefson 2010). Vinte e dois eventos de culturas transgênicas já foram aprovados pela Comissão Técnica Nacional de Biossegurança para plantio comercial no nosso país (CTNBio 2010). Dentre eles estão (1) a soja Roundap Ready (RR), que confere tolerância ao herbicida glifosato, (2) a soja Bt, que confere

resistência a insetos da ordem Lepidoptera, e (3) a soja Cultivance (BRCV), que confere tolerância a herbicidas do grupo das imidazolinonas. Este último evento, desenvolvido em conjunto pela EMBRAPA e pela empresa alemã BASF, representa um marco para o Brasil, que até então era totalmente dependente de tecnologias desenvolvidas no exterior (Tollefson 2010).

Embora o cenário geral da soja no mundo e, principalmente, no Brasil seja bastante otimista, diversos fatores bióticos (fungos, bactérias, nematóides, vírus, ervas daninhas, insetos) e abióticos (seca, alagamento, temperaturas extremas, salinidade, toxicidade de minerais) afetam severamente a produtividade desta cultura (Dita et al. 2006). Normalmente estes fatores não ocorrem isoladamente: plantas sob condições de estresse abiótico tornam-se ainda mais suscetíveis aos danos causados por ervas daninhas, insetos e doenças, aumentando consideravelmente as perdas (Dita et al. 2006). Em função das mudanças climáticas, muitos esforços têm sido dirigidos para o desenvolvimento de cultivares mais tolerantes às adversidades abióticas (Tollefson 2010).

Doenças e pragas são os principais limitadores bióticos. Muitas espécies de insetos atacam a soja, mas poucas causam grandes prejuízos. Lagartas e percevejos são as pragas principais (Vidor et al. 2004). As larvas de inseto de maior importância econômica são *Anticarsia gemmatalis* (Lagarta-da-soja), *Pseudoplusia includens* (Lagarta-mede-palmo) e *Epinotia aporema* (Broca-das-axilas), já entre os percevejos destacam-se *Nezara viridula* (Percevejo-verde, maria-fedida ou fede-fede), *Piezodorus guildinii* (Percevejo-pequeno ou percevejo-verde-pequeno) e *Euschistus heros* (Percevejo-marrom) (Vidor et al. 2004). A ocorrência da mosca-branca (*Bemisia tabaci*) também tem aumentado nas culturas de soja, especialmente em função de condições climáticas favoráveis e pelo controle inadequado. No futuro, esta praga pode causar prejuízos significativos (Reetz et al. 2008).

A suscetibilidade das plantas a inúmeras doenças aparece entre as causas mais importantes de redução da produtividade e vem aumentando a cada ano em função da expansão da área agrícola, da utilização de cultivares com pouca variabilidade genética e da monocultura empregada em algumas regiões (Barros e Borges 2007; Barros 2009). No Brasil já foram identificadas aproximadamente 40 doenças de soja causadas por fungos, bactérias, nematóides e vírus (Vidor et al. 2004). Uma ampla diversidade de fungos patogênicos ataca diferentes estruturas das plantas em diversos estádios de seu

desenvolvimento (Tabela 1). A importância econômica de cada doença fúngica varia de ano para ano e de região para região, dependendo principalmente da condição climática de cada safra (Vidor et al. 2004; Barros e Borges 2007; Barros 2009). A forma mais eficaz e econômica de controlar as doenças é através do uso de cultivares resistentes. Entretanto, para a maioria das doenças da soja, ainda não foram obtidas fontes de resistência (Camargo e Yuyama 2001).

As plantas podem ser infectadas por inúmeros patógenos com diferentes estilos de vida. Os patógenos biotróficos obtém sua nutrição a partir de tecidos vivos e, por isso, são altamente especializados e desenvolvem forte relação de dependência com seus hospedeiros. Os patógenos necrotróficos crescem em tecidos vegetais feridos, desnutridos ou senescentes e frequentemente produzem toxinas para matar o tecido hospedeiro e, posteriormente, colonizá-lo. Este tipo de patógeno é menos especializado e pode desenvolver hábito saprofítico (Wit 2007).

Atualmente, a maior ameaça ao cultivo da soja é a ferrugem asiática, causada pelo Basidiomiceto biotrófico *Phakopsora pachyrhizi*, preocupando tanto pesquisadores quanto produtores em função da extensão do prejuízo (Barros e Borges 2007; Goellner et al. 2010). O fungo foi identificado pela primeira vez na America do Sul em 2001 e disseminou-se consideravelmente nos anos seguintes (Freire et al. 2008). Na safra 2002/2003 a doença chegou ao Brasil, causando perdas estimadas de dois bilhões de reais (Goellner et al. 2010). Os esporos são disseminados pelo vento e sua germinação favorecida por chuvas bem distribuídas e longos períodos de umidade. Em condições ótimas, o patógeno é capaz de desfolhar os campos de soja em poucos dias, levando a perdas de produtividade que podem variar de 10% a 80% (Vidor et al. 2004; Goellner et al. 2010). Como não existem cultivares de soja resistentes a todos os isolados do patógeno, o controle da ferrugem é feito por aplicação de fungicidas (Goellner et al. 2010). Outros métodos foram empregados com sucesso no Brasil para conter sua disseminação. Dentre eles destacam-se (1) o vazio sanitário, período em que o cultivo de soja é proibido visando a redução de esporos viáveis, (2) o plantio simultâneo em macrorregiões, evitando a disseminação de esporos de lavouras mais adiantadas para outras mais atrasadas, e (3) o uso preferencial de cultivares precoces (Barros 2009).

Embora a ferrugem seja a doença mais importante da atualidade, outras doenças também podem causar reduções significativas na produtividade. O conhecimento sobre todas as doenças, mesmo as de importância secundária, assim como o desenvolvimento de estratégias para sua contenção, é importante, visto que estes patógenos poderão se tornar mais agressivos com o passar do tempo. Na safra 2007/2008, por exemplo, o mofo branco (*Sclerotinia sclerotiorum*) foi a doença que mais preocupou os estados do Paraná, Santa Catarina, Goiás, Minas Gerais e Bahia, mas Paraná e Santa Catarina também foram afetados pela podridão de carvão (*Macrophomina phaseolina*). Já no Maranhão e Tocantins, as maiores perdas foram em decorrência da mancha alvo (*Corynespora casiicola*), da mela (*Rhizoctonia solani*) e do crestamento foliar (*Cercospora kikuchii*) (Reetz et al. 2008).

R. solani, *Fusarium solani* e *Phomopsis* sp. são os fungos necrotróficos utilizados no decorrer deste trabalho. Em função disso, segue uma breve descrição da sua importância.

O fungo *R. solani* é um Basidiomiceto encontrado no solo, onde pode sobreviver por anos mesmo na ausência do hospedeiro. Possui esporos pesados que podem ser transportados a longas distâncias apenas através da água ou de material contaminado. O patógeno causa grandes epidemias em condições favoráveis de excesso de umidade e temperaturas elevadas. A mela é considerada como uma das doenças mais agressivas da soja nos Cerrados. Medidas de manejo cultural são consideradas alternativas importantes para controle antes do estabelecimento da doença (Camargo e Yuyama 2001; Vidor et al. 2004).

Phomopsis sp. é um Ascomiceto que causa a seca da haste e da vagem. Ele causa uma das doenças mais tradicionais da soja e, anualmente, junto à antracnose, é responsável pelo descarte de grande número de lotes de sementes. Pode-se observar maior dano em anos quentes e chuvosos. O armazenamento das sementes em temperatura ambiente reduz a viabilidade dos esporos, mas em temperaturas amenas ela é mantida. A germinação de sementes infectadas é prejudicada, contudo o tratamento com fungicida resolve o problema (Vidor et al. 2004).

Tabela 1. Principais doenças fúngicas identificadas na cultura de soja no Brasil.

Órgão afetado	Doença	Agente etiológico
Semente	Antracnose	*Colletotrichum dematium* var. truncata
	Mancha púrpura	*Cercospora kikuchii*
	Phomopsis	*Phomopsis* sp.
	Fusarium	*Fusarium semitectum*
	Aspergillus	*Aspergillus* sp.
	Penicilium	*Penicillium* sp.
Plântulas	Rhizoctonia	*Rhizoctonia solani*
	Tombamento	*Sclerotinium rolfsii*
	Pythium	*Phytium* sp.
Raízes	Rhizoctonia	*Rhizoctonia solani*
	Tombamento ou murcha	*Sclerotium rolfsii*
	Podridão vermelha da raiz ou síndrome da morte súbita	*Fusarium tucumaniae ou Fusarium solani*
	Roselínea	*Rosellinia necatrix*
	Macrophomina ou podridão de carvão	*Macrophomina phaseolina*
	Podridão radicular	*Corynespora casiicola*
	Podridão parda da haste	*Phialophora gregata*
	Podridão de Phytophthora	*Phytophthora megasperma*
	Podridão radicular de Cylindrocladium	*Cylindrocladium clavatum*
Folhas	Ferrugem asiática	*Phakopsora pachyrhizi*
	Ferrugem americana	*Phakopsora meibomiae*
	Cercospora ou crestamento foliar	*Cercospora kikuchii*
	Septoria ou mancha parda	*Septoria glycines*
	Míldio	*Peronospora manshurica*
	Antracnose	*Colletotrichum truncatum*
	Mancha-alvo	*Corynespora casiicola*
	Mancha olho de rã	*Cercospora sojina*
	Oídio	*Eryshiphe diffusa*
	Filosticta	*Phyllosticta sojicola*
	Mela ou requeima	*Rhizoctonia solani* (anamórfica) *Thanatephorus cucumeris* (teleomórfica)
	Ascoquita	*Ascochyta sojae*
	Mirotécio	*Myrothecium roridum*
	Alternaria	*Alternaia* sp.
Haste, vagem e pecíolo	Antracnose	*Colletotrichum truncatum*
	Cancro da haste	*Diaporthe phaseolorum* f.sp merdionalis (teleomórfica) *Phomopsis phaseoli* f.sp. meridionalis (anamórfica)
	Seca da haste e da vagem	*Phomopsis* sp.
	Podridão parda da haste	*Phialophora gregata*
	Seca da vagem	*Fusarium* sp.
	Mancha de levedura	*Nematospora corily*
	Podridão branca da haste	*Sclerotinia sclerotiorum*

* Modificado a partir de Vidor et al. (2004) e Barros e Borges (2007).

A podridão vermelha da raiz é causada por *F. solani* f.sp. *glycines*, outro Ascomiceto. Esta doença está disseminada por todo nosso país e é uma das mais preocupantes doenças da cultura de soja. Com exceção do uso de cultivares mais tolerantes ou parcialmente resistentes, nenhuma prática agronômica tem sido adequada para reduzir o impacto da doença. Além disso, safras chuvosas e semeadura direta favorecem a incidência da doença (Costamilan 2003; Vidor et al. 2004).

1.2 Genômica

De forma geral, a genômica pode ser dividida em três grandes áreas: estrutural, comparativa e funcional (Gutterson e Zhang 2004). A genômica estrutural estuda a estrutura do genoma, o que inclui programas de sequeniamento, estudos da estrutura e organização gênica, a contrução de mapas físicos, a resolução tridimensional das proteínas, dentre outros. A genômica comparativa consiste na análise e comparação de genomas de diferentes espécies com o propósito de entender a evolução e determinar, através de comparação, a função de regiões gênicas e intergênicas. O termo genômica funcional pode ter diversas interpretações e, no presente trabalho, foi utilizado na sua forma mais ampla, compreendendo o desenvolvimento e aplicação de técnicas que visam definir a função dos genes, englobando estudos de perfil de expressão, genética direta e reversa. Com a crescente disponibilidade de eficientes tecnologias para análise global de transcritos, proteínas e metabólitos, essa ferramenta tem sido amplamente utilizada para identificação e caracterização de genes envolvidos na resposta a estresses bióticos ou abióticos, naturais ou artificiais, bem como na análise funcional de rotas bioquímicas ou vias regulatórias (Gutterson e Zhang 2004; Dita et al. 2006). A genética reversa contribui para os estudos funcionais através da produção e análise de organismos transgênicos com alterações na expressão de genes, proporcionando o acesso cada vez mais amplo ao genoma das espécies e a identificação de genes e rotas metabólicas relacionadas aos mais variados tipos de estresse (Gutterson e Zhang 2004). A clonagem baseada em mapas genéticos é uma ferramenta da genética direta, sendo especialmente útil para espécies com genoma sequenciado e/ou com grande número de marcadores moleculares conhecidos (Gutterson e Zhang 2004).

Em conjunto estas abordagens permitem uma compreensão mais aprofundada da complexa biologia dos organismos e podem identificar genes-alvo interessantes para o

melhoramento de espécies de importância econômica (Gutterson e Zhang 2004; Varshney et al. 2009). Esforços consideráveis têm sido direcionados para determinar os mecanismos de proteção das plantas envolvidos na resistência ou tolerância a diferentes tipos de estresses. Como consequência de cada estresse específico, sofisticados mecanismos de percepção a condições desfavoráveis são ativados através de cascatas de transdução de sinais, as quais induzem ou reprimem genes e conduzem modificações fisiológicas e bioquímicas (Dita et al. 2006). O controle da expressão gênica ocorre em diferentes níveis e o estudo de como a expressão individual de cada gene contribui na resposta final em termos celulares, fisiológicos e agronômicos tem possibilitado o desenvolvimento de estratégias para a obtenção de plantas mais tolerantes.

Uma significativa contribuição da genômica de plantas é o alto nível de interferência e sobreposição entre diferentes rotas de transdução de sinais (Glazebrook et al. 2003; Gutterson e Zhang 2004; Mauch-Mani e Mauch 2005; Leon-Reyes et al. 2009). Portanto, a modificação nos níveis de expressão de um único gene pode interferir em múltiplas respostas, positivas por um lado, negativas por outro. O grande desafio para os pesquisadores é a identificação de genes que atuem em estágios específicos e estratégicos das vias de sinalização, cuja modificação ofereça o máximo de benefícios e o mínimo de efeitos colaterais, para que este possa ter utilidade na exploração comercial (Gutterson e Zhang 2004).

É importante que se tenha em mente que os estudos funcionais visam, em última análise, o melhoramento. Contudo, conforme ilustrado na Figura 1, diversos passos para evolução do conhecimento básico são necessários para se alcançar o sucesso desejado no produto final.

As leguminosas (família Fabaceae) constituem a terceira maior família entre as angiospermas. A importância agrícola de muitos membros desta família, juntamente com a existência de características únicas, como a capacidade de simbiose com rizóbio para fixação biológica de nitrogênio, justifica o número de estudos com estas plantas (Varshney et al. 2009; Sato et al. 2010). A maior parte do conhecimento genético e fisiológico da família refere-se às duas espécies modelo: *Lotus japonicus* e *Medicago truncatula*. Também foram obtidos muitos avanços em termos de conhecimentos moleculares e

genômicos destas espécies, incluindo o sequenciamento do genoma que está quase completo (Sato et al. 2010).

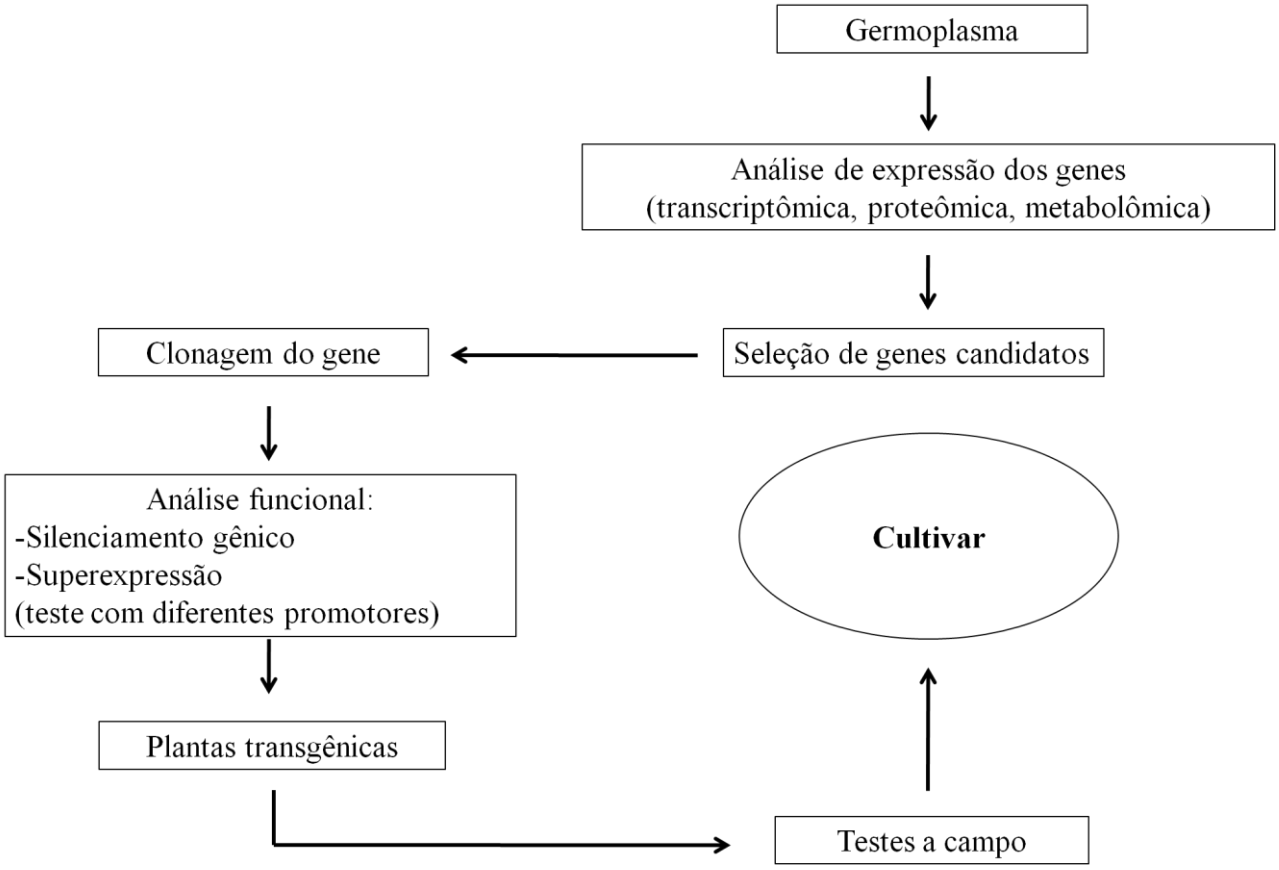

Figura 1. Esquema integrado mostrando os passos-chave para o melhoramento de plantas através de transformação genética. Adaptado a partir de Dita et al. (2006).

Recentemente, a soja foi recomendada pela comunidade científica internacional que trabalha com leguminosas como uma terceira planta modelo desta ampla família (Gepts et al. 2005). Em 2007 foi criado o Consórcio Internacional do Genoma da Soja (The International Soybean Genome Consortium - ISGC), visando um estudo aprofundado do seu genoma. Desse consórcio fazem parte os países Brasil, China, Japão, Coreia e Estados Unidos da América.

Um marco resultante dessas iniciativas é o sequenciamento *shotgun* do genoma da soja, cv. Williams 82 (Schmutz et al. 2010). Com 85% do sequenciamento concluído, os autores relataram as características do genoma, com tamanho estimado em 1.115 megabases (Mb). A maior parte do conteúdo genético da soja (57%) é composta de

heterocromatina e os 43% restantes representam a eucromatina. O genoma da soja foi considerado um paleopoliplóide com dois ciclos de duplicação. A primeira duplicação deve ter ocorrido há cerca de 59 milhões de anos, próxima a origem da subfamília Papilionoide, da qual a soja faz parte. A segunda aconteceu exclusivamente na espécie *G. max*, há aproximadamente 13 milhões de anos. Um total de 46.430 *loci* codificantes de proteínas foi identificado com alta confiabilidade. A maior parte dos genes, 34.073, distribuídos em 12.253 famílias gênicas, são ortólogos de uma ou mais sequências presentes em angiospermas. Outros 448 genes, distribuídos em 283 famílias, parecem próprios de leguminosas.

O sequenciamento do genoma destas três plantas (soja, *L. japonicus* e *M. truncatula*) serve como base para os estudos das demais leguminosas. Contudo, a coleção e avaliação de informações a respeito do fenótipo visível, transcriptoma, proteoma e metaboloma de um maior número de espécies são essenciais para estabelecer o significado dos processos biológicos das leguminosas (Sato et al. 2010). Desta forma, em um futuro não muito distante, o melhoramento assistido pela genômica, que combina as ferramentas genômicas com a seleção racional do germoplasma e a fenotipagem das características de interesse, pode representar grandes avanços no melhoramento das plantas cultiváveis (Varshney et al. 2009).

Assim como as tendências mundiais, no Brasil também foi proposta a união dos esforços para a formação de um Consórcio Nacional para Estudo do Genoma da Soja, que se consolidou através do projeto GENOSOJA-CNPq. A perspectiva é que a integração das informações nos diferentes níveis (estrutural, transcricional, proteico e funcional) permita a compreensão da função e dos mecanismos de controle da expressão de genes presentes na soja e envolvidos em diferentes processos de desenvolvimento e/ou defesa contra estresses ambientais (bióticos e abióticos). As seguintes instituições de pesquisa fazem parte do projeto: Embrapa Soja, Embrapa Recursos Genéticos e Biotecnologia, Universidade Federal de Viçosa (UFV), Universidade Estadual Paulista (UNESP), Universidade Federal do Rio Grande do Sul (UFRGS), Universidade Federal de Pernambuco (UFPE), Universidade Estadual de Campinas (UNICAMP), Universidade Federal do Paraná (UFPR) e Escola Superior de Agricultura Luiz de Queiroz (ESALQ-USP). O Laboratório de Cultura de Tecidos e Transformação Genética de Plantas, do Departamento de Genética da UFRGS, ficou responsável pela condução de experimentos relacionados ao estudo de

genes envolvidos na resposta a estresses bióticos, em especial a fungos, e abióticos, com ênfase em seca.

1.3 Transformação

O desenvolvimento de sistemas eficientes de transformação para cada espécie vegetal é o requisito básico para implantação de estudos funcionais e programas de melhoramento. Por sua vez, o sucesso nos sistemas de transformação depende de outros três pré-requisitos: (1) a obtenção de uma fonte de células totipotentes ou gametas que recebam o DNA, (2) o estabelecimento de uma metodologia para introduzir o DNA nas células alvo e (3) o desenvolvimento de um sistema de seleção ou identificação das células transformadas (Somers et al. 2003).

A embriogênese somática e a organogênese são os tecidos mais utilizados para introdução de genes em leguminosas (Somers et al. 2003). A regeneração *in vitro* das plantas desta família é um dos principais desafios para os programas de transformação, pois é lenta e altamente dependente do genótipo, além da recuperação de plantas adultas ocorrer em baixas frequências (Somers et al. 2003; Dita et al. 2006). Para soja foi conduzido um maior número de estudos e os resultados são bastante promissores (revidado por Trick et al. 1997; Somers et al. 2003; Dita et al. 2006; Mello-Farias e Chaves 2008).

A embriogênese somática consiste na obtenção de células com características embrionárias e totipotentes a partir de tecidos somáticos diferenciados. A vantagem na utilização deste sistema é a origem unicelular dos embriões somáticos secundários, o que permite a regeneração de plantas completamente transformadas (Finer 1988). Além disso, o desenvolvimento de protocolos que permitem a clonagem dos embriões secundários (Finer 1988; Finer e Nagasawa 1988; Wright et al. 1991) possibilita a obtenção de um grande número de plantas a partir de uma única célula transgênica (Sato et al. 1993). Trabalhos prévios permitiram o desenvolvimento de um protocolo eficiente para a regeneração *in vitro* de plantas de soja, via embriogênese somática, estabelecendo a base para a transformação genética de cultivares recomendadas para plantio comercial no Brasil (Santos et al. 1997; Droste et al. 2001; Körbes e Droste 2005; Weber et al. 2007; Droste et al. 2010).

Os métodos de transformação mais comumente utilizados para a transferência de genes para plantas são o bombardeamento de partículas (Sanford 1988) e o sistema *Agrobacterium* (Horsch et al. 1985). O bombardeamento ou biolística consiste em acelerar partículas de alta densidade (ouro ou tungstênio) cobertas com DNA em direção ao tecido alvo com uma força tal que as façam penetrar na célula vegetal. O método é de fácil manuseio, eficiente e permite a transformação de qualquer tipo de tecido, mesmo aqueles que apresentam resistência à transformação por *Agrobacterium* (Trick et al. 1997). Este sistema foi utilizado pela primeira vez para transformação de embriões somáticos de soja por Finer e McMullen (1991) e continua sendo amplamente empregado (Hernandez-Garcia et al. 2009; Li et al. 2009; Kita et al. 2010; Xing et al. 2010).

Atualmente, uma crescente tendência nos programas de melhoramento de plantas cultiváveis é a introdução de DNA via *Agrobacterium tumefaciens* (Somers et al. 2003). As principais vantagens da utilização do sistema *Agrobacterium* são a introdução de um menor número de cópias do T-DNA e formação de *loci* transgênicos menos complexos do que os observados para os métodos diretos de transformação (Gelvin 2003; Kohli et al. 2003). A obtenção da primeira planta de soja estavelmente transformada que fez uso desse sistema foi registrada em 1988, utilizando-se explantes cotiledonares (Hinchee et al. 1988). Um ano mais tarde, embriões somáticos primários também foram transformados por esse método e convertidos em plantas (Parrott et al. 1989). Hoje, sabe-se que a transferência de T-DNA depende de uma interação específica entre linhagem de *A. tumefaciens* e a cultivar de soja, portanto a combinação mais adequada de genótipos pode contribuir para maior eficiência de transformação. Além disso, o desenvolvimento de plasmídeos superbinários, com virulência aumentada, e a adição de acetosiringona durante a infecção da bactéria contribuem para maior eficiência da transformação (revisado por Somers et al. 2003). A partir destas pequenas melhorias, diversos explantes da soja foram transformados utilizando *Agrobacterium* como vetor de transferência do T-DNA (Trick e Finer 1998; Ko et al. 2004; Olhoft et al. 2006; Paz et al. 2006; Hong et al. 2007; Liu et al. 2008; Wang e Xu 2008).

A disponibilidade de um eficiente sistema de seleção de células ou tecidos transgênicos é crucial para garantir a confiabilidade nos protocolos de transformação. Em soja, o desenvolvimento de um sistema de seleção baseado em higromicina-B aumentou o número de plantas transgênicas e reduziu o número de escapes (plantas não

transformadas), bem como o tempo de cultura (Olhoft et al. 2003). Contudo, outros sistemas de seleção baseados em imazapir e glufosinato também são utilizados para seleção de transformantes da soja (Zhang et al. 1999; Aragão et al. 2000).

Em nosso laboratório (Laboratório de Cultura de Tecidos e Transformação Genética de Plantas, do Departamento de Genética da UFRGS) foi estabelecido um protocolo de transferência de genes para soja, via bombardeamento, utilizando como alvo o tecido embriogênico mantido em meio semi-sólido (Droste et al. 2002). Ao fazer uso desse protocolo, foram obtidas plantas transgênicas da cultivar IAS5, que expressam um gene modificado *cry1Ac* de *Bacillus thuringiensis* (Homrich et al. 2008), plantas das cultivares Bragg e IAS5, que expressam um gene que codifica uma osmotina de *Solanum nigrum* (Weber 2007), dentre outras (dados ainda não publicados). Também foi estabelecido, de forma pioneira, um protocolo combinando os métodos de bombardeamento e o sistema *Agrobacterium* (Droste et al. 2000). Neste primeiro trabalho, foram obtidos embriões somáticos transformados, embora não tenham sido regeneradas plantas transgênicas. Desde então, vários experimentos foram realizados visando à otimização desse protocolo. A identificação de uma combinação de antibióticos eficiente para suprimir a bactéria após o co-cultivo, com efeitos fitotóxicos mínimos sobre o tecido embriogênico alvo, foi proposta como uma alternativa para obtenção de plantas através desta metodologia (Wiebke et al. 2006).

1.4 Interação planta-patógeno

As plantas estão constantemente em contato com inúmeros patógenos, mas sua característica séssil não permite que as mesmas escapem de ataques. Por isso, os vegetais desenvolveram um sistema de defesa altamente especializado para proteger-se dos mais variados tipos de estresses. Graças à eficiência deste sistema a grande maioria das plantas é saudável e não sofre com a presença dos patógenos.

O primeiro obstáculo para invasão de microrganismos são as barreiras químicas e físicas presentes nas plantas, mesmo em condições ótimas de sobrevivência, como por exemplo a cutícula e a parede celular. Os organismos patogênicos possuem estratégias específicas para romper estas barreiras iniciais. Contudo, ao reconhecer invasores, as

plantas lançam mão de outro tipo de defesa, induzida pela presença do próprio patógeno (Göhre e Robatzek 2008).

O sistema de defesa induzido das plantas é bifásico e sua resposta ocorre no local da infecção (Bent e Mackey 2007; Wit 2007). A defesa primária é ativada através de um conjunto de receptores específicos e extremamente sensíveis presentes na superfície das células, os PRRs (*pattern recognition receptors*). Os PRRs identificam domínios conservados de estruturas microbianas, conhecidas por PAMPs ou MAMPs (*pathogen/microbe-associated molecular patterns*) (Bent e Mackey 2007; Boller e He 2009). Após o reconhecimento dos MAMPs, o mecanismo de defesa primário das plantas é induzido e desencadeia uma série de respostas, tais como alterações na parede celular, deposição de calose, ativação de cascatas kinases, expressão de RNAi ou acúmulo de proteínas relacionadas à defesa (quitinases, glucanases e proteases) (Figura 2a). Centenas de MAMPs e PRRs já foram identificados, mas acredita-se que existam muitos outros. Apesar da especificidade entre PRRs e MAMPs, as respostas de defesa desencadeadas pela interação destes componentes são muito similares (Bent e Mackey 2007; Wit 2007; Boller e He 2009). Juntas a resposta imune primária e as barreiras físicas da célula formam o sistema de defesa basal das plantas (Bent e Mackey 2007).

Alguns patógenos são capazes de suprimir o sistema de defesa primário dos vegetais através da secreção de múltiplos fatores de virulência (ou efetores) nas células hospedeiras (Figura 2b). Os efetores podem (1) romper as barreiras físicas preexistentes, (2) inibir diferentes alvos ao longo do processo de resposta imune primário (do reconhecimento dos MAMPs à modificações no perfil de expressão), (3) ativar rotas inadequadas de transdução de sinal ou (4) proteger o patógeno da ação das proteínas relacionadas à defesa (Bent e Mackey 2007; Wit 2007; Göhre e Robatzek 2008; Boller e He 2009). Os fatores de virulência são produzidos apenas por raças particulares de fitopatógenos e são efetivos apenas em linhagens específicas de plantas (Wit 2007; Boller e He 2009).

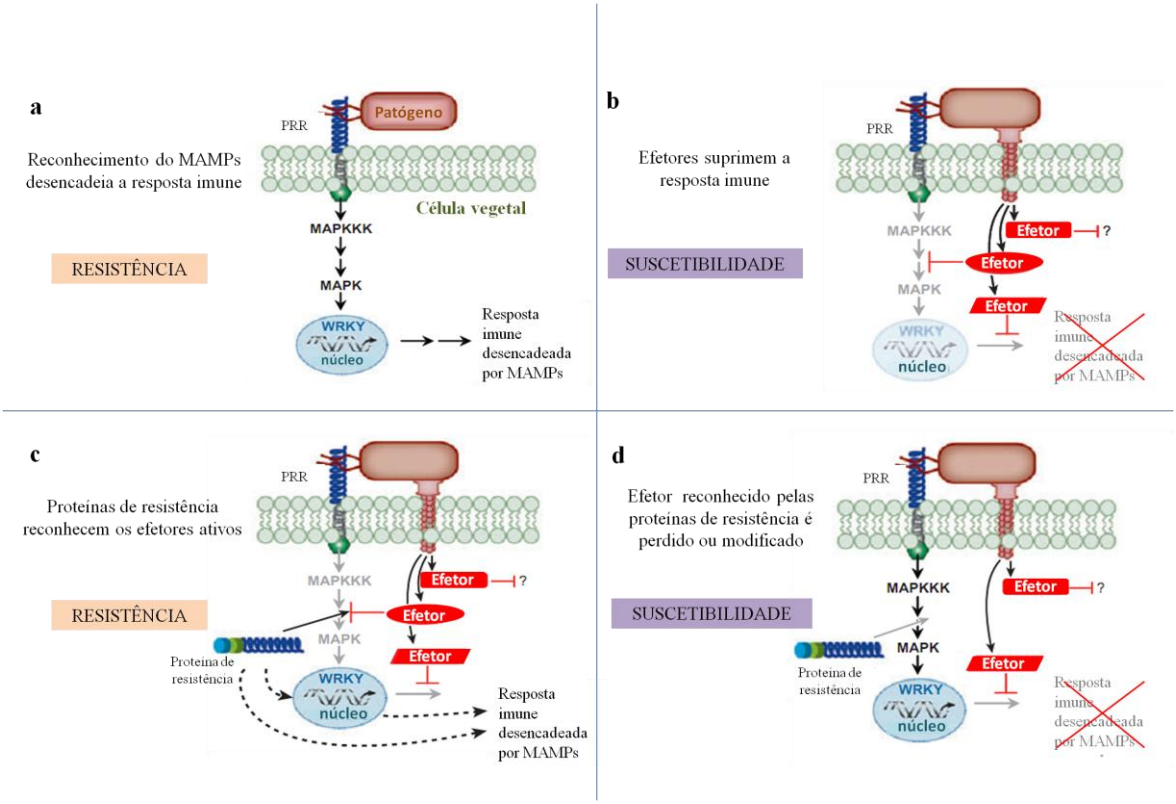

Figura 2. Modelo de indução e supressão da resposta imune primária e secundária em plantas infectadas por microorganismos patogênicos. a) O MAMP (*microbe-associated molecular pattern*) é reconhecido por um PRR (*pattern recognition receptor*) vegetal, desencadeando a resposta imune primária e impedindo a infecção. b) Patógenos verdadeiros secretam fatores de virulência (efetores) que interagem com componentes da célula vegetal e suprimem a defesa primária, permitindo a infecção das plantas. c) Plantas resistentes possuem proteínas de resistência capazes de reconhecer as perturbações na célula vegetal causadas pelos efetores, desencadeando a resposta imune secundária e impedindo a infecção. d) Os patógenos evitam o desencadeamento da defesa secundária através da eliminação ou modificação de seus efetores, impedindo seu reconhecimento pelas PRs e permitindo a infecção das plantas. Adaptado a partir de Bent e Mackey (2007).

O sistema de defesa secundário das plantas é ativado através do reconhecimento direto dos fatores de virulência ou das perturbações celulares causadas pela sua presença. As proteínas de resistência (codificadas pelos genes de resistência, genes R) são responsáveis pela detecção e subsequente desencadeamento da resposta imune secundária. Esta frequentemente culmina com a reação de hipersensibilidade, um tipo de morte celular programada, que bloqueia o desenvolvimento do patógeno (Figura 2c) (Wit 2007; Göhre e

Robatzek 2008). Novamente, embora os fatores de virulência/perturbações sejam reconhecidos por proteínas de resistência específicas que monitoram os diferentes estágios da resposta imune, a resposta induzida por este reconhecimento é a mesma, inibindo múltiplos patógenos de forma eficiente (Wit 2007). A resistência mediada por este tipo de proteínas é raça-específica e classicamente conhecida como resistência gene-a-gene (Göhre e Robatzek 2008; Boller e He 2009).

Ao longo da coevolução entre as plantas e seus patógenos, os fatores de virulência sofreram constantes modificações para passarem despercebidos pelo sistema de defesa do seu hospedeiro (Figura 2d). Os tipos de mutações dependem da vantagem competitiva que um dado efetor apresenta para a virulência e/ou outras situações da vida do microrganismo. Alguns genes que codificam efetores podem ser completamente removidos do genoma dos patógenos por deleção, enquanto outros sofrem apenas substituição pontual de nucleotídeos/aminoácidos (Bent e Mackey 2007; Wit 2007).

Além da resposta imune induzida primária e secundária, as plantas também contam com a resistência sistêmica adquirida (*systemic acquired resistance* - SAR). Como o próprio nome sugere, esta é uma resposta sistêmica e protege a planta de ataques subsequentes do patógeno (Wit 2007). A SAR é induzida juntamente com a resposta imune secundária e é dependente das rotas de sinalização de diferentes hormônios das plantas, incluindo ácido salicílico, ácido jasmônico, etileno, ácido abscísico ou até mesmo diversas combinações deles (Wit 2007; Göhre e Robatzek 2008). Esse tipo de defesa é efetivo contra um amplo grupo de microrganismos patogênicos e insetos. O nível, a eficiência e o tipo de resposta (ativação da rota de sinalização hormonal) são determinados pelo patógeno; por isso, microorganismos biotróficos e necrotróficos desencadeiam diferentes mecanismos de resistência (Wit 2007).

Os microrganismos fitopatogênicos precisam romper todos os sistemas de defesa da planta para estabelecer a infecção e obter sua nutrição. Por milhões de anos as plantas coevoluiram com seus patógenos, permitindo uma relação íntima entre determinadas raças de microrganismos e hospedeiros em ecossistemas naturais e agrícolas. Muitos genótipos de importância agrícola possuem características de resistência ou tolerância a estresses baseadas em genes R introduzidos por melhoramento. Por um lado, existem inúmeros exemplos em que esse tipo de resistência é rapidamente quebrado devido à evolução do

patógeno sob forte pressão de seleção, especialmente em sistemas de monocultura (Bent e Mackey 2007; Wit 2007). Por outro lado, a eficiência de muitos genes R permanece mesmo após décadas de uso intensivo. Tal eficiência deve-se ao reconhecimento de fatores de virulência altamente conservados e com forte vantagem adaptativa ao patógeno (Bent e Mackey 2007).

1.5 Ureases

As ureases apresentam importância histórica para biologia (Carlini e Polacco 2008). Em 1926, Sumner cristalizou pela primeira vez uma enzima, a urease majoritária da semente de feijão de porco (*Canavalia ensiformis*), provando definitivamente a origem protéica das enzimas (Sumner 1926). A uréia, principal substrato da urease, foi a primeira molécula orgânica a ser sintetizada em laboratório (Wöhler 1828). Além disso, a urease foi a primeira enzima identificada como dependente de níquel (Dixon et al. 1975). Hoje, sabe-se que as ureases são enzimas multifuncionais, com vários domínios de atividade, podendo ter importância muito mais ampla do que suposto inicialmente (Sirko e Brodzik 2000; Carlini e Polacco 2008; Follmer 2008; Balasubramanian e Ponnuraj 2010).

A urease é capaz de catalisar a hidrólise da uréia, dando origem a 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).

Vários grupos de organismos sintetizam a enzima, dentre eles bactérias, fungos e plantas (Mobley et al. 1995; Sirko e Brodzik 2000). As ureases de plantas e fungos são proteínas homo-oligoméricas, formadas por subunidades idênticas de aproximadamente 90-kDa, enquanto as ureases de bactérias são multímeros de duas ou três subunidades, designadas α, β e γ. Recentemente, foi demonstrado que as ureases de plantas em solução aquosa são hexâmeros, formados por um dímero de dois trímeros. As moléculas que compõe os trímeros constituem um triângulo fortemente ligado (Balasubramanian e Ponnuraj 2010). Independentemente do organismo, as ureases possuem grande similaridade na sequência de aminoácidos (acima de 50%) de forma que as subunidades das ureases bacterianas são alinhadas com a sequência única de plantas e fungos (Figura 3) (Sirko e Brodzik 2000; Balasubramanian e Ponnuraj 2010).

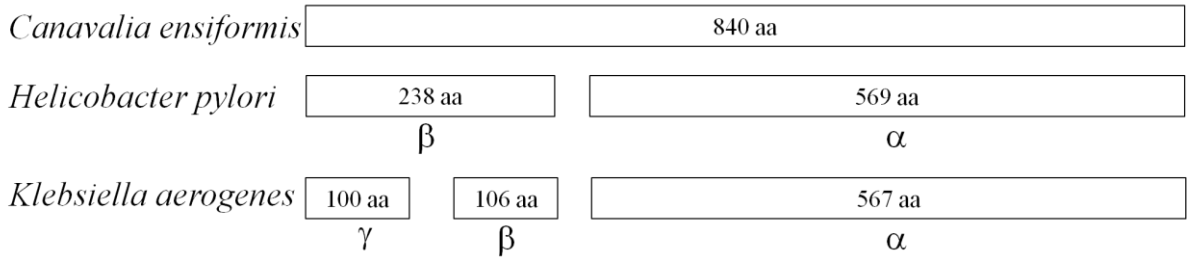

Figura 3. Comparação esquemática da estrutura das ureases de organismos selecionados. Adaptado a partir de Follmer (2008).

Tamanha similaridade entre as ureases de organismos tão distantes filogeneticamente sugere a existência de uma proteína ancestral comum, semelhança na estrutura tridimensional e conservação nos mecanismos catalíticos (Follmer 2008). Estudos aprofundados da filogenia destas enzimas ainda não foram realizados devido ao pequeno número de sequências de aminoácidos completas disponíveis nos bancos de dados. A estrutura tridimensional da primeira urease vegetal foi resolvida recentemente (Balasubramanian e Ponnuraj 2010), permitindo a confirmação do alto grau de similaridade com a estrutura das ureases já conhecidas das bactérias *Klebsiella aerogenes* (Jabri et al. 1995), *Bacillus pasteurii* (Benini et al. 1999) e *Helicobacter pylori* (Ha et al. 2001).

A comparação da estrutura primária das ureases de plantas e bactérias revelou a existência de resíduos de aminoácidos altamente conservados no sítio catalítico (Follmer 2008). A análise tridimensional mostrou que a região catalítica da enzima apresenta uma arquitetura muito similar, constituída de um centro bi-níquel com dois aminoácidos estabelecendo a ligação com o íon Ni1 e outros três com Ni2. Um sexto aminoácido forma a ligação dos dois íons de Ni entre si (Balasubramanian e Ponnuraj 2010). A ligação dos átomos de níquel à urease é muito precisa e forte. A presença do Ni2 mostrou-se essencial para estabilidade e funcionamento adequado do sítio catalítico, uma vez que a produção de ureases mutantes de *K. aerogenes*, contendo apenas o Ni1, apresentou deficiência na atividade ureolítica (Park e Hausinger 1993). Além disso, a participação de várias proteínas acessórias é necessária para incorporação do elemento químico. Estas proteínas parecem atuar como chaperonas urease-específicas (Mobley et al. 1995). Outra característica marcante é a presença de uma alça flexível que cobre o sítio catalítico das ureases. Através de mudanças na sua conformação, a alça regula o acesso do substrato ao sítio ativo das

ureases, bem como a liberação dos produtos da reação (Jabri et al. 1995; Balasubramanian e Ponnuraj 2010).

1.5.1 Ureases de leguminosas

A maior parte dos estudos com ureases vegetais foi desenvolvido com as leguminosas soja e feijão de porco. As ureases de feijão de porco foram mais bem caracterizadas bioquimicamente e as de soja, geneticamente (Sirko e Brodzik 2000).

Em feijão de porco três isoformas estruturais foram encontradas: JBU, canatoxina e JBUREII. Apenas JBU e JBUREII têm as sequências completas de DNA/aminoácidos disponíveis (Pires-Alves et al. 2003). A urease majoritária, JBU, foi identificada em sementes desta espécie (Sumner 1926; Pires-Alves et al. 2003) e 83 anos mais tarde, sua estrutura tridimensional foi resolvida (Balasubramanian e Ponnuraj 2010). Esta foi a primeira descrição tridimensional de uma urease de origem vegetal e representa um avanço importante para a compreensão de suas propriedades biológicas. A canatoxina foi isolada e caracterizada em 1981 e, 20 anos mais tarde, identificada como uma isoforma de urease (Carlini e Guimaraes 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).

Para soja foram descritas até o momento duas isoenzimas estruturais, codificadas por dois genes independentes, que compartilham 87% de identidade na sequência de aminoácidos (Goldraij et al. 2003). A urease embrião-específica, codificada pelo gene *Eu*1, é uma proteína abundante nas sementes, enquanto que a urease ubíqua, codificada pelo gene *Eu*4, é encontrada em menor quantidade em todos os órgãos da planta (Torisky e Polacco 1990; Torisky et al. 1994; Goldraij et al. 2003). Além das proteínas estruturais, foram identificadas duas proteínas acessórias na soja, codificadas por genes não ligados (*Eu*2 e *Eu*3) (Freyermuth et al. 2000). Contudo, um maior número de proteínas acessórias deve existir nesta espécie, uma vez que três ortólogos estão presentes em *Arabidopsis* (Witte et al. 2005) e quatro em bactérias (Mobley et al. 1995).

Com o intuito de determinar a função das ureases de soja, esforços foram direcionados para obtenção de mutantes, cuja coleção pertence à Universidade de Missouri, EUA. Para urease embrião-específica, mutantes nulos, denominados *eu*1, foram

obtidos com sucesso, enquanto que os mutantes para a urease ubíqua (*eu*4) acumulam uma proteína deficiente na atividade ureolítica devido a uma mutação de ponto na região de inserção do níquel (Meyer-Bothling e Polacco 1987; Meyer-Bothling et al. 1987; Goldraij et al. 2003). Mutantes nulos para proteínas acessórias *eu*3 ou *eu*2 também foram obtidos e determinam a ausência total de atividade ureásica nas plantas (Freyermuth et al. 2000; J. C. Polacco. comunicação pessoal).

1.5.2 Funções das ureases

1.5.2.1 Funções dependentes da atividade ureolítica

Tanto ureases de bactérias quanto de plantas desempenham várias funções biológicas (Follmer et al. 2004; Carlini e Polacco 2008; Follmer 2008; Krajewska 2009). A primeira função atribuída a ureases é a atividade ureolítica. A maior parte do nitrogênio resultante do metabolismo dos organismos vivos é depositado sob forma de uréia. Enquanto em mamíferos a uréia funciona como veículo não-tóxico para eliminação da amônia, em plantas quantidades significativas do nitrogênio são aproveitadas a partir da uréia (Follmer 2008). A urease é a única enzima capaz de catalisar sua hidrólise e disponibilizar a amônia, que por sua vez pode ser aproveitada pelos organismos vivos (Mobley et al. 1995). A importância da urease, portanto, é fornecer o nitrogênio necessário para o desenvolvimento dos seres vivos.

A partir da análise dos mutantes de soja foi demonstrado que, nesta espécie, a urease ubíqua sozinha é responsável pela degradação da ureia em todos os tecidos da planta (Torisky e Polacco 1990; Torisky et al. 1994). Por sua vez, a ausência da urease embrião-específica não acarretou modificações nas condições fisiológicas das plantas, sugerindo seu envolvimento em outras funções (Stebbins e Polacco 1995). O fenótipo dos mutantes *eu*3/*eu*3 ou *eu*4/*eu*4 mimetiza a privação total da planta a nitrogênio e acarreta o acúmulo de ureia e manchas necróticas nas folhas (Polacco e Holland 1993; Goldraij et al. 2003).

Três enzimas são essências para o metabolismo da ureia nas plantas: a arginase, a urease e a glutamina sintetase (Sirko e Brodzik 2000). Conforme ilustrado na Figura 4, a ureia deriva principalmente da arginina através da atividade da arginase (Polacco e Holland

1993), enquanto a amônia, um dos produtos da reação catalisada pela urease, é incorporada nos compostos orgânicos especialmente pela glutamina sintetase (Sirko e Brodzik 2000).

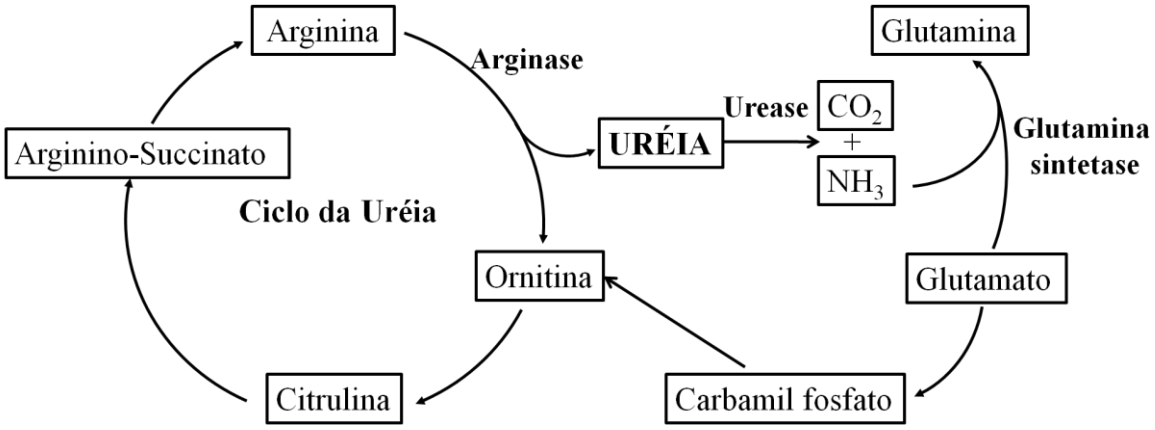

Figura 4. Metabolismo da ureia em plantas (Sirko e Brodzik 2000).

Acredita-se que a urease e a arginase atuem coordenadamente na disponibilização das substâncias de reserva da semente para nutrição do embrião durante a germinação (Polacco e Holland 1993). A importância da urease nesta etapa está comprovada, pois a germinação de sementes de *Arabidopsis* foi atrasada em 36 h na ausência de atividade ureolítica (Zonia et al. 1995). Além disso, maiores níveis de expressão da urease ubíqua de soja são encontrados em plantas nos estágios iniciais de desenvolvimento (Torisky e Polacco 1990) e sementes obtidas a partir das plantas *eu*3/*eu*3 tendem a perder a viabilidade de germinação mais rapidamente que as sementes com ureases ativas (Carlini e Polacco 2008).

Plantas transgênicas de poplar (*Populus tremula*) com níveis aumentados da glutamina sintetase apresentaram tamanho avantajado, provavelmente devido à maior disponibilidade de nitrogênio. Por outro lado, o aumento da atividade desta enzima em raízes de *L. japunicus* limitou a produção da biomassa. Estes resultados indicam a importância da enzima para o metabolismo do nitrogênio, mas também a complexidade do sistema, que provavelmente é controlado por um número muito maior de fatores do que a simples disponibilidade da enzima (revisado por Sirko e Brodzik 2000).

Além da ureia resultante dos processos metabólicos dos seres vivos, o composto também pode ser aplicado em plantas como adubo. Como este é um dos fertilizantes mais utilizados no mundo e a única forma de estar disponível para as plantas é através da

atividade da urease, este processo também tem grande importância agronômica (Witte et al. 2002; Follmer 2008).

A atividade enzimática da urease também tem impacto sobre o setor da saúde. As patologias causadas por *H. pylori*, agente causador das úlceras gástricas e câncer no estômago, devem-se a atividade ureolítica, que permite a sobrevivência e colonização das bactérias em condições muito ácidas (Mobley et al. 1995; Carlini e Polacco 2008; Follmer 2008).

1.5.2.2 Funções de defesa

Uma vez demonstrado que a abundante urease embrião-específica da soja não está envolvida no metabolismo da ureia (Stebbins e Polacco 1995), sugeriu-se que esta isoforma poderia desempenhar função de defesa do embrião. Polacco e Holland (1993) sugeriram que o ferimento ou a infecção de embriões imaturos levaria à ruptura das mitocôndrias e consequente à liberação de arginase, gerando uma grande quantidade de arginina. A ureia poderia, então, ser convertida em amônia, que teria um efeito deletério sobre os herbívoros ou patógenos. Embora não tenham sido conduzidos experimentos definitivos, as sementes dos mutantes *eu*3/*eu*3 parecem realmente mais suscetíveis a ataques por microorganismos (Carlini e Polacco 2008).

Trabalhos recentes comprovaram que ureases purificadas apresentam uma toxicidade *in vitro* contra insetos e fungos, mas que essa propriedade é independente da atividade catalítica (Follmer et al. 2004; Becker-Ritt et al. 2007). Além disso, também foi demonstrado que a região da proteína responsável pelo efeito entomotóxico não inibe o crescimento de fungos (Becker-Ritt et al. 2007). Estas evidências sugerem que domínios distintos das urease estejam envolvidos nas propriedades inseticida, fungicida e ureolítica (Carlini e Polacco 2008).

A urease embrião-específica de soja e as ureases de feijão de porco purificadas foram letais quando injetadas ou adicionadas à dieta de insetos das classes Hemiptera e Coleoptera, tais como *Rhodnius prolixus*, *Callosobruchus maculatus*, *Nezara viridula*, *Dysdercus peruvianus* e *Triatoma infestans* (revisado por Carlini e Polacco 2008). Ficou evidenciado que a atividade inseticida das ureases está restrita ao trato digestivo quando da presença de catepsinas (próprio das classes Hemiptera e Coleoptera), que degradam a

proteína e liberam um peptídeo tóxico de aproximadamente 10-13 kDa na hemolinfa (Carlini et al. 1997; Mulinari et al. 2007). A sequência de nucleotídeos correspondente ao peptídeo entomotóxico foi isolada e clonada a partir do gene *jbure*II (Mulinari et al. 2007). Curiosamente, o efeito inseticida das ureases parece restrito às isoformas vegetais e pelo menos parte do peptídeo entomotóxico corresponde ao *gap* presente entre as subunidades α e β das ureases bacterianas (Figura 3) (Follmer et al. 2004). O mecanismo de ação inseticida das ureases de plantas ainda precisa ser elucidado, embora esteja claro que ela atua como uma pró-toxina (Carlini e Polacco 2008).

A toxicidade das ureases de plantas é direcionada a organismos específicos. Insetos com digestão baseada em tripsina (próprio da classe Lepidoptera) não são afetados pela enzima em sua forma nativa, apenas pelo peptídeo. Por sua vez, em ratos não foram observados sintomas de toxicidade, mesmo quando o peptídeo foi adicionado à dieta (Mulinari et al. 2007).

Duas patentes foram depositadas para proteger a utilização das ureases quanto a sua atividade inseticida. A primeira visa a utilização de extratos purificados de canatoxina para o controle biológico (Carlini et al. 2000). Já a segunda protege a utilização do peptídeo inseticida em programas de transformação de plantas (Mulinari et al. 2004).

Até o momento, um menor número de estudos foi realizado visando compreender a atividade antifúngica das ureases. Sabe-se, no entanto, que, em concentração sub-micromolar, as ureases purificadas de soja (embrião-específica), feijão de porco (JBU) e algodão inibem o crescimento vegetativo *in vitro* de fungos filamentosos (Becker-Ritt et al. 2007; Menegassi et al. 2008). Embora menos expressiva, a atividade fungicida também foi encontrada para urease recombinante de *H. pylori* (Becker-Ritt et al. 2007). O efeito inibitório ou retardatário das ureases foi testado em fungos pertencentes aos Filos Ascomycota e Basidioycota (Tabela 2). Embora a urease embrião-específica da soja não tenha apresentado efeitos sobre o crescimento vegetativo de *F. solani* e *Trichoderma viride*, esta inibiu a germinação de esporos destes fungos (Becker-Ritt et al. 2007). Além disso, os resultados dos dois trabalhos mostram que a toxicidades da urease é dose dependente (Becker-Ritt et al. 2007; Menegassi et al. 2008).

Tabela 2. Efeito inibitório ou retardatário das ureases sobre o crescimento vegetativo de fungos patogênicos*.

Fungos testados	Feijão de porco (SBU)	Soja (embrião-específica)	Algodão	*H. pylori*
Colletotrichum musae Ascomiceto	+	+	+	
Curvularia lunata Ascomiceto	+	+	+	+
Fusarium solani Ascomiceto	+	-		
Fusarium oxisporum Ascomiceto	+	+		
Penicillium herguei Ascomiceto	+	+	+	+
Trichoderma sp. Basidiomiceto		+		
Trichoderma pseudokoningii Basidiomiceto		+		
Trichoderma viride Basidiomiceto		-		

*Urease desempenhou efeito inibitório (+) e não desempenhou efeito inibitório (-).

O mecanismo de ação das ureases sobre os fungos ainda não é conhecido. No entanto, a microscopia eletrônica de fungos tratados com a urease de feijão de porco sugere plasmólise e injúrias à parede celular (Becker-Ritt et al. 2007). Além disso, foi demonstrado que a atividade fungicida das ureases é independente das atividades inseticida e ureolítica, uma vez que ureases tratadas com inibidores irreversíveis da atividade ureolítica persistiram impedindo o crescimento de fungos, enquanto o peptídeo inseticida não desempenhou esse papel (Becker-Ritt et al. 2007).

Apesar de conhecidas e estudadas desde 1926, a função e o modo de ação das ureases ainda não foram totalmente compreendidos e merecem atenção. O estudo das ureases *in vivo* é de fundamental importância para o esclarecimento de seu papel real na resposta das plantas a diversas condições de estresses bióticos. A manipulação da expressão dos genes que codificam ureases pode auxiliar na determinação destas funções e, a longo prazo, no desenvolvimento de plantas mais resistentes. Além disso, todos os

estudos sobre a função de defesa das ureases de soja foram desenvolvidos com a isoforma embrião-específica. Considerando a alta similaridade na estrutura primária destas proteínas, postula-se que a urease ubíqua também desempenhe estas funções complementares.

1.6 Objetivos

1.6.1. Objetivos gerais

Os objetivos gerais deste trabalho foram (1) obter plantas estavelmente transformadas a partir de embriões somáticos de soja submetidos ao sistema integrado bombardeamento/*Agrobacterium* e (2) identificar e caracterizar funcionalmente os genes que codificam as ureases estruturais de soja, especialmente a urease ubíqua em relação aos processos de resposta a fungos patogênicos.

1.6.2 Objetivos específicos

a) Regeneração de plantas estavelmente transformadas pelo sistema integrado bombardeamento/*Agrobacterium*;

b) Identificação e caracterização de novos genes que codifiquem ureases de soja;

c) Determinação do perfil de expressão da urease ubíqua ao longo do processo de infecção da soja por fungos patogênicos;

d) Construção de vetores contendo o gene da urease ubíqua para transformação da soja;

e) Obtenção de plantas estavelmente transformadas com o gene *Eu*4, visando a superexpressão da urease ubíqua;

f) Avaliação das plantas transgênicas quanto à expressão do transgene;

g) Confirmação da transmissão e estabilidade do transgene na descendência;

h) Caracterização molecular das plantas transgênicas;

i) Caracterização fenotípica das plantas transgênicas, especialmente quanto à resistência a moléstias fúngicas.

Os capítulos seguintes desta tese incluem um artigo já publicado em periódico internacional, um encaminhamento de pedido de patente, um manuscrito submetido para

publicação e outro manuscrito a ser submetido em breve. Os resultados descritos ao longo deste trabalho também foram apresentados de forma parcial à comunidade científica em eventos locais, nacionais e internacionais (seis pôsteres e duas apresentações orais). A longo prazo, os resultados obtidos podem fornecer uma alternativa para a obtenção de plantas de soja mais resistentes a adversidades bióticas.

Capítulo II

Transgenic fertile soybean plants derived from somatic embryos transformed via the combined DNA-free particle bombardment and *Agrobacterium* system

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Transgenic fertile soybean plants derived from somatic embryos transformed via the combined DNA-free particle bombardment and *Agrobacterium* system

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Abstract An *Agrobacterium*-mediated transformation procedure for soybean [*Glycine max* L. Merrill] proliferating somatic embryos is here described. The A*grobacterium tumefaciens* LBA4404 strain harboring pTOK233, pCAMBIA1390-*olp* or pH7WG2D *wrky* plasmids was used to mediate gene transfer into the plant genome. Prior to *Agrobacterium* inoculation, proliferative soybean embryogenic clusters were microwounded by DNA-free tungsten particle bombardment. Three independent transformation experiments were performed. In Experiment I, 26 transgenic plants were obtained from a unique clone of cv Bragg, while 580 plants were recovered from 105 clones of cv IAS5. In Experiment II, a single hygromycin-resistant clone of cv BRSMG68 Vencedora was recovered and gave rise to five plants. In Experiment III, 19 plants of cv Bragg and 48 plants of IAS5 were recovered, representing five and 14 independent transformation events, respectively. PCR and Southern analyses confirmed the transgenes' integration into plant genomes. Transgenic plants were fertile. They flowered, set pods and seeds. Transgene segregation in two T_1 progenies fits the Mendelian pattern (3:1 transgenic:non-transgenic plants). This is the first report of transgenic fertile soybean plants obtained from somatic embryogenic tissues transformed by the system that combines DNA-free particle bombardment and *Agrobacterium*.

Keywords *Agrobacterium tumefaciens* · Embryogenic tissues · Genetic transformation · *Glycine max* · Somatic embryogenesis

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
cv	Cultivar
PCR	Polymerase chain reaction

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Introduction

Soybean [*Glycine max* (L.) Merr] is one of the most important oil- and protein-producing crops around the world. Genetic improvement of soybean by traditional breeding is limited due to the extremely narrow germplasm diversity. Thus, the majority of soybean cultivars in use are derived from very few parental lines (Priolli et al. 2002). Plant genetic transformation offers a significant advancement for soybean breeding programs allowing the production of novel and genetically diverse plant materials. Transgenic plants also represent a priceless tool for molecular, genetic, biochemical and physiological studies by, for instance, the overexpression or silencing of genes. However, genetic improvement and functional genomics in soybean depend heavily on an efficient transformation system.

Agrobacterium-mediated plant transformation (Horsch et al. 1985) and particle bombardment (Sanford 1988) are the two major extensively employed methods for genetic transformation of crop plants. The advantages of *Agrobacterium*-mediated gene transfer over particle bombardment include the possibility of transferring relatively large segments of DNA, lower number of transgene copies integration into plant genomes, rare transgene rearrangement, lower frequency of genomic DNA interspersion and reduced abnormal transgene expression (Gelvin 2003; Kohli et al. 2003).

Recovery of the first transgenic soybean plants using *Agrobacterium*-mediated transformation was reported by Hinchee et al. (1988) using cotyledonary nodes as target tissue. After this first work, advances in transformation techniques were achieved and transgenic fertile soybeans were obtained from different explants, such as cotyledonary nodes (Paz et al. 2004; Liu et al. 2008), immature zygotic cotyledons (Yan et al. 2000; Ko et al. 2003, 2004), hypocotyls (Aragão et al. 2000; Wang and Xu 2008), half-seed (Paz et al. 2006) and organogenic callus (Hong et al. 2007). Unfortunately, only a few viable transgenic lines could be generated, showing that more appropriate and effective methods need to be developed to improve the soybean transformation efficiency (Mello-Farias and Chaves 2008).

The unicellular origin of soybean secondary somatic embryos induced in vitro makes them an useful target tissue for genetic transformation, allowing the production of fully transformed plants (Sato et al. 1993). For this reason, proliferating somatic embryos remain one of the major target tissues for soybean transformation via particle bombardment in numerous laboratories (Finer and McMullen 1991; Sato et al. 1993; Stewart et al. 1996; Droste et al. 2002).

On the other hand, *Agrobacterium*-mediated transformation of proliferating somatic embryogenic cultures has proven to be challenging (Mello-Farias and Chaves 2008), and few works employing these target tissues have been published. The current literature reports the successful use of immature zygotic cotyledons and primary embryogenic tissue as targets for *Agrobacterium*-mediated transformation (Parrott et al. 1989; Yan et al. 2000; Ko et al. 2003, 2004). Attempts to use secondary embryogenic cultures for the production of transgenic plants via "Sonication-Assisted *Agrobacterium*-mediated Transformation" (SAAT) have also succeeded, but the recovered plants were not fertile (Trick and Finer 1997, 1998).

The generation of microwounds with DNA-free particles allowed *Agrobacterium* penetration and the effective transformation of sunflower meristems and tobacco leaves (Bidney et al. 1992), banana meristems (May et al. 1995), bean meristems (Brasileiro et al. 1996) and rapeseed microspores (Abdollahi et al. 2009). We have previously proposed the integrated bombardment and *Agrobacterium* transformation system as an alternative for soybean secondary somatic embryo transformation (Droste et al. 2000). In that study, transient GUS expression was not observed in tissues submitted to *Agrobacterium* infection without prior bombardment, whereas areas of intense blue staining containing many cells were detected in most of the embryogenic clusters submitted to the integrated transformation system. Although transient expression of the *gus*-intron gene was successfully achieved, stable transformants were not obtained. It was further demonstrated that the soybean somatic embryos employed were too sensitive to the antibiotics required for *Agrobacterium* suppression after gene transfer (Wiebke et al. 2006). A modification at the transformation protocol, regarding the antibiotic treatment, was suggested to maintain the viability of stable transformed cells and to allow their conversion into plants.

The goal of the present work was to recover soybean transgenic plants using the modified

integrated bombardment/*Agrobacterium* system. This work reports, for the first time, the successful recovery of a high number of soybean transgenic fertile plants obtained from the combination of DNA-free particle bombardment and *Agrobacterium*-mediated transformation using proliferating soybean somatic embryos as target.

Materials and methods

Plant material and culture conditions

Soybean genotypes Bragg, IAS5 and BRSMG 68 Vencedora were chosen for the transformation experiments due to their high sensitivity towards *A. tumefaciens* wild strains infection (Droste et al. 1994), as well as their responsiveness to in vitro culture conditions (Droste et al. 2001, 2002, 2010; Homrich et al. 2008). Bragg is a North American-adapted cultivar, commonly used in genetic improvement programs, while IAS5 and BRSMG 68 Vencedora are Brazilian cultivars indicated for commercial cropping Costamilan and Bertagnolli 2004).

Immature pods were harvested from field-grown plants and surface sterilized by immersion in 70% ethanol for 1 min, followed by 15 min in 4% sodium hypochlorite containing drops of Tween-20. Following three rinses in autoclaved distilled water, the embryonic axes of immature seeds (3–4 mm in length) were removed and the cotyledons used as explants for culture. Somatic embryogenesis was induced by placing each cotyledon with the abaxial side facing the modified D40 medium (Bailey et al. 1993), which contains MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 40 mg l^{-1} 2,4-D, 3% sucrose, 0.3% Phytagel®, pH 7.0 (prior to autoclaving). Twenty cotyledons were placed in each petri dish. After 40 days on D40 medium, cotyledons were transferred to D20 medium (D40 medium containing 20 mg l^{-1} 2,4-D, 3% sucrose, pH 6.4; Wright et al. 1991). Fourteen days later, clusters of secondary embryos were removed from cotyledons and transferred to fresh D20 medium. Embryogenic tissues were proliferated on this medium with subcultures every 14 days. Cultures were maintained at 26 ± 1°C with 16/8 h light/dark at a light intensity of 22.5 $\mu Em^{-2}s^{-1}$.

Agrobacterium strain, plasmids and culture conditions

The pTOK233 superbinary plasmid (Hiei et al. 1994) and the two vectors pCAMBIA1390-*olp* and pH7WG2D*wrky*, derived from pCAMBIA (Cambia.com) and pH7WG2D (Karimi et al. 2002) ordinary plasmids, respectively, were used to transfer marker and reporter genes into plant genome. As shown in Fig. 1, T-DNA region of all plasmids contain the hygromycin–phosphotransferase marker gene (*hpt*). The *β*-glucuronidase (*gus*A-intron) and green fluorescent protein (*gfp*) reporter genes are present in pTOK233 and pH7WG2D*wrky*, respectively. The plasmids were independently transformed into the strain LBA4404 of *Agrobacterium tumefaciens*.

Forty-eight hours prior to transformation, *Agrobacterium* was prepared according to Droste et al. (2000). Isolated bacterial colonies were grown 48 h in LB medium containing 50 mg l^{-1} rifampicin, 50 mg l^{-1} kanamycin and 100 µM acetosyringone under continuous shaking at 28°C. Cells were centrifuged and resuspended in D10 liquid medium (D20 medium with 10 mg l^{-1} 2,4-D) containing 100 µM acetosyringone to an optical density at 600 nm of 0.3.

Transformation procedure and selection of transgenic clones

Three independent transformation experiments were carried out as shown in Table 1. Forty-eight hours before transformation, 10–15 embryogenic clusters/dish, around 0.67 mg/cluster, were transferred to fresh D20 medium. In Experiments I and III, 10 dishes were prepared per cultivar, while in Experiment II, eight dishes were used. Fifteen minutes before bombardment, clusters were placed in the center of the dishes, which were maintained uncovered in a laminar flow hood for 15 min to reduce the turgor pressure of the plant material (Vain et al. 1993).

Transformation procedure followed the protocol described by Droste et al. (2000) with modifications. Bombardments were performed using a Particle Inflow Gun—PIG (Finer et al. 1992). The procedure is based on acceleration of DNA-free tungsten particles using low pressurized helium (60 PSI) in combination with a partial vacuum (28–30 Hg).

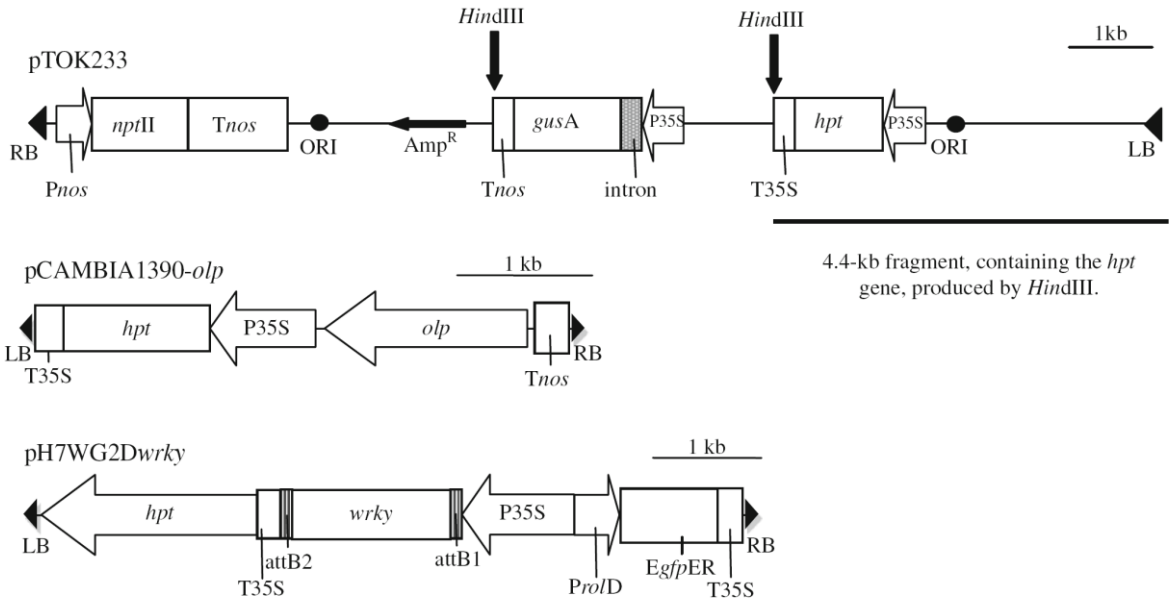

Fig. 1 Diagrams of the T-DNA regions of binary vectors pTOK233 (adapted from Hiei et al. 1994), pCAMBIA1390-*olp* and pH7WG2D*wrky* used for soybean transformation. The 4.4-kb fragment employed in Southern blot hybridizations is indicated. *BR* T-DNA right border, *BL* left border, nptII neomycin phosphotransferase gene, *gusA* β-glucuronidase gene, *hpt* hygromycin phosphotransferase gene, *Pnos* nopaline synthase gene promoter, *P35S* Cauliflower mosaic virus (CaMV) 35S promoter, *Tnos* nopaline synthase gene terminator, *T35S* CaMV 35S terminator, *EgfpER* enhanced green fluorescent protein, *ProlD* root loci D promoter, *wrky* a gene of WRKY family, *attB1* and *attB2* LR reaction site, *ORI* origin of replication of *Col*E1, *Amp*^{R} ampicillin-resistance gene active in *E. coli*, *HindIII* *Hin*dIII restriction enzyme, *kb* kilobase pairs (1,000 bp)

Table 1 Embryo histodifferentiation and conversion into plants of soybean embryogenic clusters submitted to the integrated bombardment/*Agrobacterium* transformation system

	Experiment I (pTOK233)		Experiment II (pCAMBIA1390-*olp*)	Experiment III (pH7WG2D*wrky*)	
	IAS5	Bragg	Vencedora	IAS5	Bragg
Culture time before transformation (months)	11	11	8	6	7
Number of clusters submitted to the transformation	120	120	80	150	150
Histodifferentiation					
Number of clones	232	48	1	20	19
Number of embryos	4,944	1,225	96	NR	475
Conversion					
Number of clones	105	1	1	14	5
Number of plants	580	26	5	48	19

NR non-recorded

Twenty-five µl tungsten particles resuspended in sterile distilled water were mixed with 25 µl 2.5 M $CaCl_2$ and 15 µl 0.1 M spermidin. After 5 min on ice, 45 µl of the supernatant were removed. Each dish was bombarded once with 2 µl of the pelleted mixture at a distance of 14 cm. A baffle, made of 500 µm nylon screen, was placed at a distance of 9 cm above the tissue (Finer et al. 1992). Following bombardment, clusters were inoculated and incubated for 20 min in the bacterial suspension. Then,

inoculated explants were blotted on sterile filter paper and co-cultured for 48 h on D20 medium supplemented with 100 μM acetosyringone. After this period, tissues were washed in sterile distilled water, blotted on sterile filter paper and transferred to D20 medium containing 250 mg l^{-1} cefotaxime (Claforan®, Hoechst Marion Roussel) and 250 mg l^{-1} vancomycin (Vanclomin®, Teuto Brasileiro). Explants were maintained on this medium for 10 days and then were transferred to fresh D20 medium supplemented with the same antibiotics plus 12.5 mg l^{-1} hygromycin-B (Sigma) for more 21 days. Thereafter, plant material was kept on D20 medium containing the same three antibiotics, but with a concentration of 25 mg l^{-1} hygromycin-B. After 18 days, embryogenic tissues were finally transferred to fresh medium containing only hygromycin-B for additional 51 days, with subcultures every 14 days. In summary, the potentially transformed tissues were submitted simultaneously to two antibiotic treatments: (1) cefotaxime + vancomycin for a total of 49 days and (2) hygromycin-B for 90 days. After the selection procedure, the pieces of green tissues were subcultivated individually in dishes containing fresh D20 medium without antibiotics for two additional months, with subcultures every 14 days.

GUS histochemical assay

Transient expression of *gus*A was assayed, as described by Jefferson (1987). Forty-eight hours after the end of the co-culture period, two embryogenic clusters were randomly picked from each dish and incubated overnight in 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) solution at 36°C, in the dark. After staining, explants were immersed in 70% ethanol for chlorophyll distaining and the number of blue foci was determined.

Embryo histodifferentiation and conversion into plants

To stimulate histodifferentiation, clusters of hygromycin-resistant embryogenic tissues were placed on modified MSM6 maturation medium (Finer and McMullen 1991), containing MS salts, B5 vitamins, 6% sucrose and 0.3% Phytagel® (pH 6.4 prior to autoclaving). After 30 days, somatic embryos were individualized and transferred to fresh maturation medium for additional 30 days. The histodifferentiated embryos were placed on empty sterile dishes without medium for 2 days in order to promote partial desiccation and thereby increase conversion velocity and frequency (Buchheim et al. 1989; Merkle et al. 1995).

Subsequently, partially dehydrated somatic embryos were placed on MS0 conversion medium containing MS salts, B5 vitamins, 3% sucrose and 0.3% Phytagel® (pH 6.4 prior to autoclaving). After the development of the primary root and the first leaf, plantlets were transferred to flasks containing the same medium. Conversion was recorded as the development of roots and at least one trifoliolate leaf. Conversion frequencies were calculated as the ratio of embryos converted into plantlets over the total number of histodifferentiated embryos. Regenerated plants were transferred to plastic pots containing vermiculite covered with plastic film to maintain humidity, being gradually exposed to environmental conditions. Finally, plants were transferred to pots containing organic soil and kept in greenhouse until their complete development.

GFP expression

GFP expression was detected under blue light using a fluorescence stereomicroscope Olympus®, equipped with a BP filter set containing a 488 nm excitation filter and a 505–530 nm emission filter. Untransformed tissues/plants submitted to the same growth conditions were used as negative control. Images were captured using the software QCapture Pro™ 6 (QImaging®).

Molecular analysis

Total genomic DNA was isolated from either embryogenic or leaf tissues of hygromycin-resistant mature embryos and plants according to the CTAB procedure described by Doyle and Doyle (1987) with modifications. Purified DNA samples were individually assayed for the presence of *hpt*. The PCR reaction mixture consisted of 100 ng of template DNA, 0.2 mM of each dGTP, dATP, dCTP and dTTP, 1.5 mM $MgCl_2$, 1× Taq Buffer, 2.5 units of Taq® DNA Polymerase (Invitrogen), 0.5 μM of each primer for the *hpt* gene (5′-GCGATTGCTGATCCCCATGTGTAT-3′

and 5′-GGTTTCCACTATCGGCGAGTACTT-3′) and autoclaved distilled water to 50 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 52°C and 45 s at 72°C. After electrophoresis in 1.5% agarose gel, PCR-generated fragments were transferred overnight onto Hybond® N^+ membrane (GE Healthcare) using Southern blot standard solutions and protocols (Sambrook and Russel 2001). DNA labeling and probe detection were conducted following the protocol of ECL® Direct Nucleic Acid Labelling and Detection Systems (GE Healthcare). The DNA blotting was probed with a PCR product containing the fragment of 512 bp of the *hpt* gene amplified from plasmid pTOK233, and purified from agarose gel using the GFX® kit (GE Healthcare). Hybridizing bands were detected by exposure of the nylon membrane to Kodak X-OMAT® autoradiography films for 10 min.

The Southern blot hybridization of digested genomic DNA was carried out using 20 µg of total genomic DNA from putative transgenic and non-transgenic (control) plants digested overnight at 37 °C with *Hin*dIII (Promega) restriction enzyme. Digested genomic DNA of each plant was separated by electrophoresis in 0.8% (w/v) agarose gel and transferred from the gel to a Hybond® N^+ nylon membrane as previously described. Probe labeling, hybridization, stringency, washes and detection were carried out as specified above. Hybridizing bands were detected after 5 h exposure.

Progeny obtainment and analysis

Transgene segregation in two T_1 progenies obtained from T_0 self-pollinated plants were screened by PCR or Southern blot hybridization of digested genomic DNA. The Fisher Exact Test (BioEstat 5.0) was used to confirm the expected Mendelian segregation pattern of 3:1 (transgenic:non-transgenic plants).

Results and discussion

In order to verify the success of the DNA transference into plant cells, reporter GUS activity was tested 48 h after the co-culture period in twenty randomly chosen embryogenic clusters (two clusters out of twelve per dish) from Experiment I. The mean number of blue *foci* per cluster was 7.3 ± 5.7 for cv Bragg and, 6.5 ± 2.1 for cv IAS5. As previously observed by Droste et al. (2000), areas of blue staining could be detected in samples of almost all dishes (19 out of 20 dishes), confirming the susceptibility of both cultivars to *A. tumefaciens*. The authors have also showed that previous wounding (via DNA-free particle bombardment) was absolutely essential for the *Agrobacterium* infection. The presence of the *gus*A-intron gene in the T-DNA of pTOK233 guaranteed a trustful transient result, since this gene can only be fully expressed in eukaryotic cells (Hiei et al. 1994).

After antibiotic selection, hygromycin-resistant embryogenic tissues could be visually selected and separately cultured for the proliferation of individual clones in hygromycin-free D20 medium. Each individual and spatially apart embryogenic clone was considered an independent transformation event. In Experiment I, the number of proliferative clones obtained was almost five-fold higher for cv IAS5 (232) when compared to cv Bragg (48) (Table 1). In Experiment II, only one hygromycin-resistant clone of cv BRSMG 68 Vencedora was recovered. In Experiment III, 19 and 20 independent hygromycin-resistant embryogenic tissues were selected for cv Bragg and for cv IAS5, respectively. Therefore, more than one hygromycin-resistant tissue were identified per cluster of cv IAS5 submitted to the transformation procedure in Experiment I, whereas for other cultivars/Experiments this was rarely observed.

The number of histodifferentiated embryos and recovered plants in each experiment are shown in Table 1. In Experiment I, the 26 plants regenerated from a single Bragg clone and the 580 plants recovered from 105 IAS5 clones, correspond to a conversion frequency of 2% and 12%, respectively. A conversion frequency of 5% was observed in Experiment II, at which five plants were regenerated from a unique BRSMG 68 Vencedora clone. In Experiment III, 19 plants were obtained from 5 Bragg clones, representing a 4% conversion frequency. In the same experiment, 48 plants from 14 IAS5 clones were regenerated. In this case, it was not possible to calculate the conversion frequency, since the number of histodifferentiated embryos was not recorded. Ten plants of cv Bragg and 100 of cv IAS5 from Experiment I, as well as all plants from Experiment II and III were gradually exposed to environmental conditions for acclimation and later placed into a greenhouse for further development.

Fig. 2 Transgenic soybean plants obtained from somatic embryogenic clusters submitted to the integrated bombardment/*Agrobacterium*-mediated transformation system. Plants were recovered from three independent genotypes, Bragg (**a**), IAS5 (**b**) and BRSMG 68 Vencedora (**c**)

Plants flowered and set seeds (Fig. 2). Seed set was recorded for 14 plants of cv IAS5 (9 independent events) and 5 plants of Bragg (one event) from Experiment I, two plants of BRSMG 68 Vencedora (one event) from Experiment II, three plants of cv IAS5 (three events) and two of cv Bragg (two events) from Experiment III (Table 2). A low number of seeds was obtained for most transgenic and non transgenic plants and may reflect the deleterious effect of in vitro culture conditions (Trick et al. 1997).

It has been reported that soybean genotype plays an important role at the regeneration capacity of somatic embryogenic tissue (Droste et al. 2001; Meurer et al. 2001; Yang et al. 2009). For instance, cv IAS5 is known to have a higher embryo-to-plant conversion frequency than cv Bragg (Droste et al. 2001; Körbes and Droste 2005). Although the final number of plants obtained in the present study was high, especially in Experiment I, the recovery frequency could be considered low when compared to previous works, in which conversion frequencies varied from 8 to 46% for cv Bragg and 30 to 51% for IAS5 (Droste et al. 2001; Körbes and Droste 2005).

Furthermore, it has been shown that long term-culture could interfere in transformation rates (Droste et al. 2000) and be harmful to plant recovery (Trick and Finer 1997, 1998; Droste et al. 2001). Although embryogenic cultures used in Experiment I were older (11 months) than those used in Experiments II (8 months) and III (6 and 7 months), the number of hygromycin-resistant clones was higher in the first experiment and minor effects were observed on the plant recovery frequency. On the one hand, our results suggest that the choice of a more efficient vector, such as the superbinary pTOK233, is a key point for a successful somatic embryos' transformation through the bombardment/*Agrobacterium* system. On the

Table 2 Seeds score of T_0 adult transgenic soybean plants obtained from the integrated bombardment/*Agrobacterium* transformation system

	Experiment I		Experiment II	Experiment III		Control[a]		
	IAS5	Bragg	BRSMG 68 Vencedora	IAS5	Bragg	IAS5	Bragg	BRSMG 68 Vencedora
Number of events	10	1	1	3	2			
Number of plants	14	5	2	3	2	2	2	2
Total number of seeds	76	40	76	41	29	43	16	53
Mean (variation) of seeds/plant	5.4 (2–11)	8.0 (3–17)	38 (6–70)	13.7 (10–16)	14.5 (6–26)	21.5 (13–30)	8.0 (2–14)	26.5 (15–38)

[a] Control plants were submitted to the same culture conditions of Experiments II and III

other hand, commercially available vectors such as pCAMBIA and pH7WG2D bring easiness of manipulation when the insertion of an interest gene is required.

When viewed under blue light, putative transgenic embryos and plants derived from Experiment III were green fluorescent, confirming the transgenes stable integration and expression (Fig. 3). GFP expression was better visualized in roots when compared to other plant tissues due to chlorophyll interference in GFP detection. Even with the appropriate filters, interference of chlorophyll with GFP detection could occur through an overabundance of red fluorescence or competition between GFP and chlorophyll for the same excitation wavelengths of light (Zhou et al. 2005). Therefore, GFP expression in a low-expressing clone can be of difficult observation.

A sample of histodifferentiated embryos and adult plants were PCR-screened for *hpt* presence. From Experiment I, two Bragg plants (from the same transformation event), 13 Bragg histodifferentiated embryos (13 independent events) and 24 IAS5 plants (21 independent events) were analyzed. From Experiment II, the two analyzed plants derived from the same event, while each plant from Experiment III (three IAS5 and two Bragg) represented independent events. As shown in Figs. 4A and 4B, no *hpt* amplification was detected in untransformed plants, whereas transformed materials and positive controls presented the expected 512 bp *hpt* fragment. PCR data confirmed that all Bragg embryo and plants from Experiment I and all plants from Experiments II and III were stably transformed. Twenty-one out of 24 IAS5 plants (Experiment I), representing 18 independent events, had the stable transformation condition confirmed. This result also attested that our selection system is very tight, since the presence of the transgene was detected in all analyzed samples from Experiments I (cv Bragg), II and III, as well as in 87.5% of IAS5 analyzed plants from Experiment I.

Genomic Southern blot hybridization was also performed in three T_0 and eight T_1 plants from Experiment I to confirm PCR results. As shown in Fig. 4C, plant genomic DNAs digested with *Hin*dIII and probed with an *hpt* fragment revealed at least three different hybridization bands for cv Bragg and one for IAS5. As expected all fragments were higher than the 4.4-kb fragment, since *Hin*dIII cuts the T-DNA once, keeping the fragment that contains the *hpt* gene intact (Fig. 1). The second cut occurs into the soybean genome (Hiei et al. 1994). Therefore, the number of hybridizing bands reflects the number of gene copies integrated into different *loci*.

Plants analyzed in this study presented one or three copies of the *hpt* gene. *Agrobacterium*-mediated transformation typically gives rise to lower transgene copy number, when compared to direct transformation methods (Kohli et al. 2003; Mello-Farias and Chaves 2008). However, it is not uncommon to find multiple transgene copies in the plant genome after

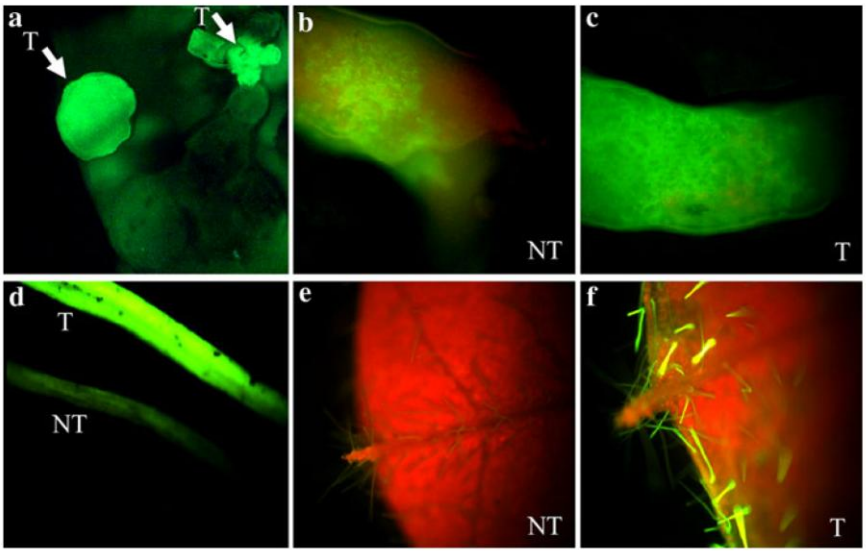

Fig. 3 GFP expression of transgenic soybean tissues obtained from the integrated bombardment/*Agrobacterium*-mediated transformation system using the vector pH7WG2D*wrky* (Experiment III). Secondary somatic embryos (**a**), histodifferentiated embryos (**b**, **c**), roots (**d**) and leaves (**e**, **f**) of transgenic (T) and non-transgenic (NT) plants were observed using a fluorescence microscope (40×)

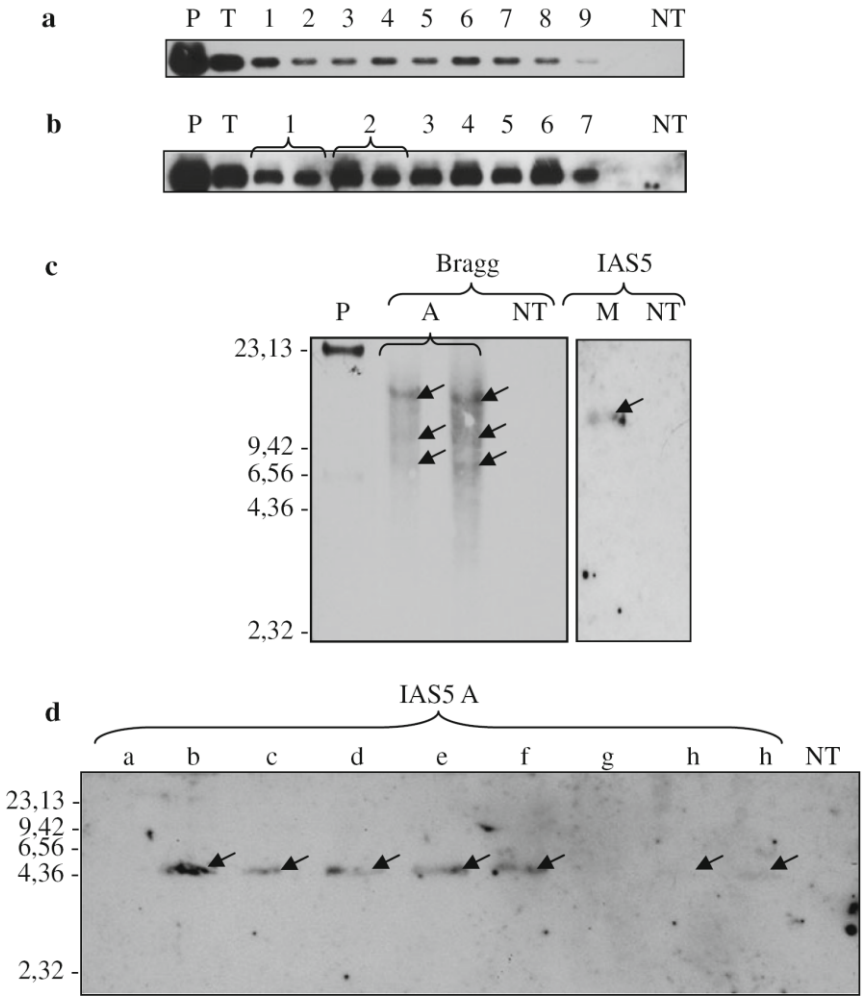

Fig. 4 Detection of the *hpt* gene in transgenic soybean embryos and plants. **A** PCR products amplified from DNA templates extracted from controls or transgenic histodifferentiated embryos from cv Bragg (Experiment I): pTOK233 (*P*); known-transgenic plant produced by particle bombardment (T, Homrich et al. 2009); embryos from nine independent transformation events (*1–9*); soybean untransformed plant of the cv Bragg (*NT*). **B** PCR products amplified from DNA templates extracted from controls or transgenic plants from cv IAS5 (Experiment I): pTOK233 (*P*); known-transgenic plant (*T*); plants from seven independent events (*1–7*); soybean untransformed plant of the cv IAS5 (*NT*). Events 1 and 2 are represented by two different plants. **C** Southern blot hybridization analysis of T_0 plants (Experiment I). Genomic DNAs were digested with *Hin*dIII and hybridized with a 4.4-kb probe derived from the *hpt* transgene present in pTOK233. Sizes of marker fragments are indicated in kb. Pattern of *hpt*–probe hybridization to pTOK233 (*P*); digested DNA from two Bragg plants derived from a single event (*A*); IAS5 plant representing event *M*; untransformed plants (*NT*) of cv Bragg and IAS5. *Arrows* show the different hybridization bands which might reflect the number of gene copies. **D** Southern blot hybridization analysis of T_1 plants derived from a single event (Experiment I). Genomic DNAs were digested with *Hin*dIII and hybridized with a 4.4-kb probe derived from the *hpt* transgene present in pTOK233. Sizes of marker fragments are indicated in kb. Pattern of *hpt*-probe hybridization digested DNA from eight IAS5 plants (*a–h*) corresponding to the progeny of a single plant (event A) and an untransformed plant (*NT*). Plant h was probed twice. Arrows show the different hybridization bands, which might reflect the number of gene copies

Agrobacterium-mediated transformation (Paz et al. 2004; Hong et al. 2007; Liu et al. 2008; Wang and Xu 2008).

Two T_1 progenies were used for transgene segregation analysis by PCR. As shown in Table 3, six out of eight T_1 IAS5 plants (Experiment I) and 12 out of

Table 3 Transgene segregation in two T_1 families

T_0 family			T_1 generation (number of plants)			
Experiment	Cultivar	Event	*hpt*+	*hpt*−	Expected ratio	p[a]
I	IAS5	A	6	2	3:1	1
II	BRASMG 68 Vencedora	A	12	8	3:1	0.5

[a] The segregation ratios for *hpt* was tested using the Fisher Exact Test

20 T_1 BRSMG 68 Vencedora plants (Experiment II) inherited the *hpt* gene, confirming the transgene stability in the progeny. For events with a single copy or with multiple copies in one locus, transgenes are expected to behave as dominant genes and to segregate in a 3:1 ratio for transgenic to non-transgenic progeny when plants are self-pollinated, because the transgene locus is considered to be hemizygous in the primary (T_0) transformant (Campbell et al. 2000). The transgene segregation observed in the present work fits the expected 3:1 segregation ratio. The eight T_1 IAS5 plants were also evaluated for transgene copy number by Southern blot hybridization (Fig. 4D). The result confirmed the PCR screening and segregation once the *hpt* sequence was present in a single copy in six T_1 plants.

In the last 15 years, several research teams reported *Agrobacterium*-mediated transformation of soybean using different target tissues. However, regeneration of a high number of *Agrobacterium* derived-transgenic plants was previously described only when proliferating somatic embryos were used as targets (Trick and Finer 1998). Indeed, stable soybean transformants were produced in the work of Trick and Finer (1998), but plants were fully sterile and progeny was not recovered. The difference between the method described by Trick and Finer (1998) and our procedure lies in the technique used to induce tissue wounding to provide an entry point for the bacteria: while the previous study used sonication, the present paper relied upon bombardment.

Considering advantages of *Agrobacterium*-mediated gene transfer, this is normally the method of choice when more than one transformation system is available to a plant species (Hiei et al. 1994; Gelvin 2003). In the last years, efforts have been made to achieve an efficient *Agrobacterium*-mediated transformation procedure for soybean somatic embryos (Trick and Finer 1997, 1998; Droste et al. 2000; Wiebke et al. 2006). The present work is the first report of transgenic fertile plants obtained from somatic embryogenic tissues transformed by the integrated free-DNA particle bombardment/*Agrobacterium* system. This method allowed us to consistently produce multiple plants through the proliferation of clones obtained from different transgene integration events. We obtained hundreds of transgenic fertile plants, especially for the soybean cv IAS5. Transgene insertion into the plant genome was confirmed in a high percentage of the screened samples. Therefore, the *Agrobacterium*-mediated gene transfer protocol is now in use in our laboratory as a routine procedure for the genetic transformation of proliferative soybean somatic embryos. Although tissue culture and transformation procedures are laborious and time-consuming, as demonstrated here, the efficiency and reliability of this method justify all efforts considering the limits imposed by soybean for its genetic breeding by conventional methods or by other transformation protocols.

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Capítulo III

Ubiquitous urease affects soybean susceptibility to filamentous fungi and *Phakopsora pachyrhizi* infection

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Ubiquitous urease affects soybean susceptibility to filamentous fungi and *Phakopsora pachyrhizi* infection

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Key words: co-suppression, functional genomics, genetic transformation, *Glycine max*, overexpression, plant defense system, plant resistance to fungus, urease.

Abstract

The soybean ubiquitous urease (encoded by *GmEu*4) is responsible for recycling metabolically-derived urea. Additional biological roles have been investigated for plant ureases, especially in defense. The relevance of *GmEu*4 in soybean response to fungi was investigated. A differential expression pattern was observed in susceptible and resistant genotypes over the course of *Phakopsora pachyrhizi* infection, especially 24 h after infection. Thirty adult transgenic plants, representing seven independent transformation lines, were obtained. Most transgenic plants showed *GmEu*4 co-suppression and decreased ureolytic activity. A single transgenic line exhibited overexpression and enhanced ureolytic activity. Progeny was obtained from co-suppressed plants. The growth of *Rhizoctonia solani*, *Phomopsis sp.*, *Fusarium solani*, *Colletotrichum gossypii* and *Penicillium herguei*

in media containing crude protein extract from either transgenic or non-transgenic leaves was evaluated. Protein extracts from the overexpressing plant inhibited fungal growth compared to extracts from non-transgenic plants, while extracts from co-suppressed plants were less inhibitory than extracts from non-transgenic controls. When infected by *P. pachyrhizi* uredospores, detached leaves of co-suppressed plants developed a significantly higher number of lesions, pustules and erupted pustules than leaves containing normal levels of the enzyme, thus suggesting importance of soybean ubiquitous urease in plant response against fungal infection.

INTRODUCTORY STATEMENTS

Soybean (*Glycine max*) is affected by several diseases that negatively affect plant yield, eventually resulting in significant crop losses (Sinclair and Hartmann 1999). Host genetic resistance is the most desirable and efficient control measure when genotypes are available. However, for some foliar fungal diseases, such as Asian soybean rust caused by *Phakopsora pachyrhizi*, fungicides are the only efficient measure to avoid crop losses under favorable conditions for disease development, but often result in increasing economic and environmental costs (Miles et al. 2007). Understanding the molecular basis of soybean defense against fungal infection and growth, identifying genes involved in hypersensitive or immune response, and characterizing their individual roles are key steps for engineering durable and quantitative disease resistance, contributing to minimize dependence on chemical control and crop losses.

Ureases (EC 3.5.1.5) are nickel-dependent metalloenzymes that catalyze the conversion of urea to ammonia and carbon dioxide, thus allowing organisms to use exogenous and internally generated urea as a nitrogen source (Dixon et al., 1975; Krajewska, 2009). These enzymes are synthesized by numerous organisms, including plants, fungi and bacteria (Follmer, 2008; Krajewska, 2009). Two isozymes, which share 87% amino acid identity, were described for soybean (Goldraij et al., 2003). The embryo-specific urease, encoded by the *GmEu*1 gene, is synthesized in the developing embryo and accumulated in mature seeds (Polacco and Havir, 1979; Polacco and Winkler, 1984;

Polacco and Holland, 1993), while the ubiquitous urease, encoded by the *GmEu*4 gene[1], is found in lower amounts in all plant tissues (Torisky et al., 1994). The ubiquitous urease is responsible for recycling all metabolically derived urea (Polacco et al., 1985; Stebbins and Polacco, 1995; Witte et al., 2002), whereas an assimilatory role for the abundant seed urease has not been demonstrated so far (Carlini and Polacco 2008).

In addition to providing organisms with nitrogen in the form of ammonia, other biological roles have been investigated for plant ureases, especially in defense. First, it was hypothesized that the defense function was related to ammonia released by ureolytic activity (Polacco and Holland, 1993). Later, it was confirmed that jackbean (*Canavalia ensiformis*) ureases and the soybean embryo-specific urease display entomotoxic effect (Carlini and Polacco, 2008; Carlini et al., 1997). More recently, it was demonstrated that *in vitro* purified ureases from jackbean, soybean and cotton seeds promote, at sub-micromolar concentrations, growth inhibition against a wide range of filamentous fungi (Becker-Ritt et al., 2007; Menegassi et al., 2008). Toxic activity against insects and fungi persisted after the urease treatment with irreversible inhibitors of ureolytic activity, demonstrating that other protein domain(s), without the participation of the ureolytic active site, are involved in defense mechanisms (Becker-Ritt et al., 2007; Follmer et al., 2004a; Follmer et al., 2004b). The entomotoxic sub-peptide has been identified and cloned from jackbean ureases (Mulinari et al., 2007). It is released by insects with cathepsin-based digestion (reviewed by Carlini and Polacco, 2008). However, the location of the antifungal domain of ureases has not yet been identified.

Previous studies on soybean urease defense properties were based on the embryo-specific isoform and *in vitro* toxic effects of the purified enzyme. In the present work, the role of the ubiquitous urease in the soybean defense response to fungi was investigated *in vivo* by transcript analyses and the manipulation of *GmEu*4 gene expression in transgenic soybean plants.

[1] GenBank accession AY230156

RESULTS

*GmEu*4 expression in response to *Phakopsora pachyrhizi* infection

In order to determine whether ubiquitous urease is involved in defense responses to the biotrophic fungus *P. pachyrhizi*, the transcript levels of *GmEu*4 over the course of *P. pachyrhizi* infection were determined by reverse transcription, quantitative (real-time) polymerase chain reaction (RT-qPCR). Two soybean genotypes exhibiting contrasting responses to fungal infection were assayed. Embrapa-48 is highly susceptible to *P. pachyrhizi* infection while PI561356 is known for its more resistant phenotype (van de Mortel et al., 2007; Abdelnoor R.V., personal communication). Interaction among genotypes, time-course and pathogen presence was highly significant ($p<0.0001$). Expression of non-infected plants was consistent for genotypes and time-course. As shown in Figure 1, in the susceptible host, Embrapa-48, *GmEu*4 transcripts were significantly upregulated (2.26-fold) at 1 h, followed by a strong downregulation (0.04-fold) at 24 h and a new upregulation peak at 192 h after pathogen inoculation. In contrast, in the resistant accession PI561356**,** *GmEu*4 expression was strongly upregulated (4.07-fold) at 24 h.

Response to infection was confirmed by *GmPR*4[2] expression, which encode a wound-induced protein, known for its accumulation in wounded and *Phytophthora sojae* elicitor-treated soybean tissues (Graham et al., 2003). As shown in Figure 1, PI561356 rapidly and consistently increased *GmPR*4 mRNA levels after infection while the susceptible Embrapa-48 increased mRNA levels only 192 h after inoculation.

Regeneration and characterization of transgenic plants

In order to determine whether *GmEu*4 functions in defense, soybean somatic embryos derived from cultivars IAS5 and Bragg were transformed with the pH7WG2D-*ure*U vector. The T-DNA region of the plasmid contains the ORF of *GmEu*4 gene under CaMV *35S* promoter control, the hygromycin-phosphotransferase marker gene (*hpt*) and the green fluorescent protein reporter gene (*gfp*). Thirty well-developed plants, representing seven independent transgenic lines, were regenerated from two transformation experiments (Table 1). All plants flowered and 22 set seeds.

[2] GenBank accession Z11977

Stable transgene integration was confirmed in all plants by PCR using primers for *hpt* and *GmEu*4 (one specific to the CaMV *35S* promoter and another specific to the *GmEu*4 ORF) (data not shown). Furthermore, transgenic embryogenic tissues and plants were green fluorescent under blue light (Supplementary Figure 1). GFP expression was quite low in *Agrobacterium* derived-tissues, but easily visualized in bombarded tissues, especially in roots and seeds (data not shown).

One plant from each independent transformed line was assayed for the number of *GmEu*4 extra copies by RT-qPCR. Lectin that is present in two copies in soybean (representing four alleles in the diploid genome) was used as reference gene to eliminate variations in template amounts between samples. PCR efficiencies were similar for both genes types: 104.6 and 106.2 for *GmEu*4 and lectin, respectively. The copy number was calculated based on DNA quantification of transgenic plants relative to that of non-transgenic plants, since the non-transgenic genome contains one *GmEu*4 copy in the soybean genome (two alleles in the diploid genome). The number of *GmEu*4 insertions in the soybean genome varied from 1 to 14 (Table 2). As expected bombardment-derived plants exhibited a higher number of extra *GmEu*4 copies (more than 10), while lower numbers were found in *Agrobacterium*-derived lines (one or two).

The expression of endogenous and introduced *GmEu*4 genes was evaluated by RT-qPCR, using two different primer pairs: one for endogenous plus transgenic transcripts (encoding region) and another for endogenous transcripts only (3' UTR region) (Figure 2). As expected, the same transcript levels were observed in non-transformed plants, which contain just the endogenous gene, independent of the primer pair used. Expression levels were equivalent in both IAS5 and Bragg soybean cultivars (data not shown). In transgenic plants, a drastic variation in *GmEu*4 mRNA levels was observed when different primer pairs were used in RT-qPCR, which may reflect transgene expression effects. Surprisingly, increased urease mRNA levels were observed in a single plant (line 5C) containing extra *GmEu*4 copies (Figure 2). In contrast, most transgenic plants (A3, A8, 4F and 7E lines) showed lower *GmEu*4 expressions than non-transformed controls. For two other transgenic plants (A1 and A2 lines), transcript accumulation was equivalent to wild-type plants. The expression pattern of endogenous *GmEu*4 was similar among plants of different transgenic lines: endogenous urease transcripts were downregulated in the presence of extra *GmEu*4 copy(ies). This phenomenon is termed ''gene co-suppression'' (Napoli et al., 1990) and, in

the present work, co-suppression refers to simultaneous endogenous and transgene silencing.

Although the initial aim of this study was to over-express *GmEu*4, the co-suppressed plants represent a powerful tool for functional genomics because null mutants have never been obtained for the ubiquitous urease (Carlini and Polacco, 2008). Thus, further experiments were carried out with co-suppressed plants of the A3 and A8 lines and the overexpressing 5C line.

Changes in the ureolytic activity confirmed the urease co-suppression and overexpression in transgenic soybean plants (Figure 3). Activity was determined by the semi-quantitative seed chip/leave peace assay (Meyer-Bothling and Polacco, 1987), in which either a leaf piece or a sliver taken non-destructively from a mature seed is placed in a solution of urea, weakly buffered at pH 7.0 and containing cresol red as pH indicator. As urea is hydrolyzed by urease in the tissue sample, increasing pH (due to consumption of H^+ in urea conversion to 2 NH_4^+ and HCO_3^-) converts the cresol red from yellow, at neutrality, to pink and finally to a deep vermilion or "red". For non-transformed seeds (positive control) the solution became pink in 1 min (Figure 3c and d). Leaves of non-transformed plants produced a light pink coloration after 8 h incubation (Figure 3b and f), in agreement with much lower urease levels in leaves compared to the seed levels (Torisky et al., 1994). At this time, pink coloration was more intense in solution containing leaf pieces derived from line 5C (Figure 3g) - the line accumulating higher than normal levels of urease transcripts (Figure 2). No color change was observed in solutions containing leaves of co-suppressed plants (Figure 3a), even after 24 h incubation.

Mutant plants (*eu*4/*eu*4) 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). Similar leaf tip necrosis was not observed in our transgenic co-suppressed plants, suggesting that the residual ureolytic activity was sufficient to recycle metabolically derived urea.

Transgenic progeny

Progenies were successfully obtained from three T_0 transgenic lines (A1, A3 and A8). Twenty plants, derived from each original line, were evaluated for transgene

segregation by PCR and GFP expression analyzes (Table 3). The data confirmed transgene stability and fit the expected 3:1 Mendelian segregation ratio for a single dominant locus. Fourteen T_1 PCR-positive plants from A3 and A8 families were analyzed by RT-qPCR for transgene expression and 10 plants maintained the co-suppressed phenotype (data not shown).

Bioassay of ubiquitous urease effects on filamentous fungi

The effects of the ubiquitous urease on filamentous fungal vegetative growth were demonstrated by a turbidimetric assay. Increasing turbidity of the medium, as the result of hyphal development, was observed over time (Figure 4). Protein extracts from two independent transgenic co-suppressed plants were significantly less effective in inhibiting *Penicillium herguei*, *Phomopsis sp*. and *Rhizoctonia solani* growth than those from non-transgenic plants (Figure 4A). On the other hand, protein extract from a single transgenic plant overexpressing ubiquitous urease significantly reduced growth of *Colletotrichum gossypii*, *P. herguei* and *Fusarium solani* when compared with extracts from non-transformed control (Figure 4B). The growth inhibition effect of urease-enriched plants was not significant for *Phomopsis sp*.

Furthermore, the presence of higher amounts of ubiquitous urease inhibited or delayed sporulation of some fungi. *C. gossypii* spores were not observed and a minimal number of *P. herguei* spores were identified in urease-enhanced plant extracts even after 10 days incubation, while in the presence of non-transformed plant extracts they could be visually detected (data not shown). Similar effects were not observed for the other fungi employed in this study.

Bioassays of ubiquitous urease effects on *P. pachyrhizi*

Susceptible interactions (Tan) between host and *P. pachyrhizi* are characterized by leaves and other plant organs with tan-colored lesions containing abundant sporulating uredia. Contrastingly, resistant plant genotypes generally develop reddish-brown (RB) lesions with little or no sporulation. The immune-reaction is an incompatible interaction without any visible disease symptoms on host leaves (Goellner et al., 2010). Tan lesions were observed in all detached leaves of both ubiquitous urease co-suppressed and non-transgenic samples 12 days after *P. pachyrhizi* inoculation (Figure 5A).

No visible difference was observed on the timing of lesion and pustules formation or pustule eruption (data not shown). However, considering the four disease parameters here evaluated (number of lesions, number of lesions with pustules, number of pustules and number of opened pustules), rust development was significantly higher in plants with lower levels of ubiquitous urease than in plants with normal levels of the enzyme (Figure 5A and B).

DISCUSSION

Generation of transgenic soybean plants with ubiquitous urease expression up- and down-regulated

The present work aimed to obtain transgenic soybean plants overexpressing the *GmEu*4 gene. However, most of the recovered transgenic plants exhibited simultaneous co-suppression of the endogenous *GmEu*4 gene and the transgene construct. The reduction in ureolytic activity, ascribable to lowered enzyme accumulation, confirmed the co-suppression. Co-suppressed plants derived from six independent transformation lines, into which one to 14 extra copies of recombinant *GmEu*4 were introduced. Only one adult plant (line 5C) among the seven transformation lines, into which two transgene copies were identified, overexpressed urease to moderate levels. Due to higher enzyme accumulation, an enhanced ureolytic activity was observed. Interestingly, GFP expression was stronger in plants with higher transgene copy numbers and lower exactly in 5C line tissues, which overexpressed *GmEu*4 (data not shown).

Co-suppression in transgenic plants was first reported over 20 years ago, when petunia with extra copies of the chalcone synthase gene exhibited repression of both the transgene and the corresponding endogenous gene (Napoli et al., 1990). For soybean, El-Shemy et al. (2006) reported similar results whereby a high percentage of transgenic plants lacked all subunits of glycinin, when an orthologous gene was introduced into the plant genomes. Actually, this phenomenon is not uncommon in transgenic plants (Francis and Spiker, 2005; Tang et al., 2007).

Several mechanisms have been proposed to explain the co-suppression. The effects of transgene copy number on the level of gene expression are known to be complex and could in part explain our results. Though the increase of transgene copy number is

expected to enhance the expression level (El-Shemy et al., 2006), there is evidence that gene co-suppression is frequently associated with multiple inserts (James et al., 2002; Lechtenberg et al., 2003; Tang et al., 2007). Tang et al. (2007) correlated transgene silencing in eastern white pine with the presence of two or three T-DNA insertions in the same chromosome. However, Lechtenberg et al. (2003) showed that neither tandemly repeated transgene arrangements nor inverted T-DNA structures were sufficient to trigger gene silencing in *Arabidopsis thaliana*. On the other hand, Francis and Spiker (2005) recovered high frequencies of *A. thaliana* silenced transgenic lines under selection-free conditions, suggesting that under selection bias such "down-regulated" transgenic lines may not be identified. The T-DNAs may have integrated into genomic regions that repress transgene expression. In agreement, El-Shemy et al. (2006) showed that the ratio of gene co-suppression was lower in transformants selected for antibiotic resistance plus GFP expression than in plants selected for antibiotic alone.

Through one or more mechanisms, silencing in plants takes place through one of the followings routes: transcriptional gene silencing or post transcriptional gene silencing (El-Shemy et al., 2006; Francis and Spiker, 2005; Tang et al., 2007). We were not able to ascribe the co-suppression observed in the present work to any of the mechanisms described above. However, the correlation between GFP expression level and *GmEu*4 co-suppression indicates that high expression may lead to *GmEu*4 co-suppression in soybean. Determining whether co-suppression will occur in a particular transgenic plant is still largely an empirical problem and requires analyses of individual plants.

James et al. (2002) showed that an additive effect of transgene expression can be observed in homozygous rice plants for some transformation events, but that in many other transformation events, the homozygous state appears to be disadvantageous, being associated with lower transgene expression levels, gene silencing or counter-selection of homozygous plants across generations. Our results showed that two-thirds of transgenic progeny shared the same *GmEu*4 co-suppression pattern with the T_0 progenitor plants. Homozygous condition might explain the occurrence of non-co-suppressed plants in PCR-positive T_1 progenies. However, additional analyses with a higher number of samples and generations are needed to confirm this preliminary observation.

The levels of ubiquitous urease in soybean affect the development of infecting filamentous fungi

Our results showed that soybean ubiquitous urease displays antifungal property, as previously demonstrated for purified seed ureases of soybean (embryo-specific urease), jackbean and cotton (Becker-Ritt et al., 2007; Menegassi et al., 2008). In addition, it was also confirmed that *in vivo* ureases have positive effects on plant resistance to the necrotrophic plant pathogens *P. herguei*, *Phomopsis sp*., *R. solani*, *C. gossypii*, and *F. solani*. Although bioassays were carried out *in vitro* with protein crude extracts, the results consistently demonstrated that urease co-suppressed plants yielded leave extracts that were less effective in inhibiting growth of the pathogens, whereas protein extracts from the urease overexpressing plant promoted significantly increased toxicity to most fungi analyzed. *Phomopsis sp*. growth was not affected by "high urease" protein extracts. However, on co-suppressed transgenic plants, these fungi developed faster than on non-transformed plants. Becker-Ritt et al. (2007) previously showed that for some fungi species the urease growth inhibition *in vitro* was dose dependent.

Urease interference in fungal osmotic balance was proposed by Becker-Ritt et al. (2007). Scanning electron microscopy demonstrated that *P. herguei*, a maize pathogen, exhibited cell wall damage when grown *in vitro* in the presence of purified soybean embryo-specific or jackbean urease (Becker-Ritt et al. 2007). Recently, it was shown that *in vitro* jackbean purified urease is able to permeabilize liposomes (Barros et al. 2009) and planar lipid bilayers (Piovesan A., personal communication). Further experiments comparing fungal growth in transgenic and non-transgenic plants will help to elucidate the urease fungitoxic effect.

Ubiquitous urease is involved in defense mechanisms against *P. pachyrhizi*

Two results indicated that *GmEu*4 is involved in the defense mechanisms of soybean against the biotrophic pathogen *P. pachyrhizi*. *GmEu*4 expression displayed contrasting expression patterns in resistant and susceptible soybean genotypes upon pathogen inoculation. The second experiment demonstrated that *GmEu*4 co-suppression significantly enhances susceptibility to *P. pachyrhizi*. *GmEu*4 overexpression in a susceptible genotype or silencing in a resistant genotype would be the most powerful tool

for functional analyses. Unfortunately, the efficiency of soybean transformation and regeneration via somatic embryogenesis is genotype-dependent (Droste et al., 2010; Meurer et al., 2001), and cvs. IAS5 and Bragg, both susceptible to *P. pachyrhizi*, have been currently used for somatic embryo transformation in Brazil (Droste et al., 2002; Homrich et al., 2008).

On leaves of both susceptible and resistant soybean varieties, as well as on leaves from non-host plants, uredospores are able to germinate and form an appressorium, which emits a penetration hypha that grows into an epidermal cell. From this point on, fungal growth differs among genotypes. In susceptible soybean cultivars the primary hypha expands, branches and originates haustoria in mesophyll cells. Thereafter, intense colonization of the mesophyll tissue occurs. The asexual reproduction of *P. pachyrhizi* is completed with uredia origination (Goellner et al., 2010; van de Mortel et al., 2007). In resistant soybean genotypes, secondary hyphae do not develop (Goellner et al., 2010). In *Arabidopsis*, a non-host plant, invasion is stopped immediately after penetration of the epidermal cell (Loehrer et al., 2008) and in barley, another non-host plant, some fungal growth was observed in the mesophyll, but cells died during or shortly after fungal penetration, and no haustoria or uredospores were apparent (Hoefle et al., 2009).

The most contrasting *GmEu*4 transcript expression between genotypes analyzed here was observed 24 h after *P. pachyrhizi* inoculation, when an upregulation peak was observed in the resistant host (PI561356) and a strongly downregulation occurred in the susceptible host (Embrapa-48). The initial infection process, common to resistant and susceptible hosts, occurs within the first 16-24 h (Goellner et al., 2010). At this point hyphal growth proceeds and haustoria become visible only in susceptible hosts, a stage in which a strong *GmEu*4 suppression was observed. In addition, in the present work the co-suppressed *GmEu*4 plants developed a significantly higher number of lesions, pustules and erupted pustules than the susceptible non-transformed plants, suggesting a more intensive colonization. Altogether our results suggest that ubiquitous urease may act directly or indirectly inhibiting fungal development and we hypothesize that urease interferes with the hyphae osmotic balance (Becker-Ritt et al., 2007).

Recently, large-scale transcript profiles of soybean under *P. pachyrhizi* infection were published (Choi et al., 2008; Panthee et al., 2007; Panthee et al., 2009; Soria-Guerra

et al., 2010a; Soria-Guerra et al., 2010b; van de Mortel et al., 2007). Studies were carried out with different resistant and susceptible genotypes of *G. max* and *Glycine tomentella*. Some groups of upregulated and downregulated genes were similar among hosts, although differences could also be observed. It remains unknown whether different resistant genotypes employ similar defense mechanisms (Goellner et al., 2010). Identification of multiple transcription factor family members suggests that a complex positive and negative regulation pattern is involved in defense to rust infection (Choi et al., 2008).

Soybean plants exhibited a biphasic gene expression in response to *P. pachyrhizi* infection; differences in gene expression between susceptible and resistant host peaked at 12 and 72 h post inoculation. The early transcriptional response observed in susceptible and resistant plants was suggested as a general response of soybean to the nonspecific recognition of any pathogen (van de Mortel et al., 2007). The second wave of gene expression occurred earlier in the resistant than in the susceptible plants and includes defense- and stress-related functions (Panthee et al., 2007; Soria-Guerra et al., 2010a; van de Mortel et al., 2007). Additionally, it has been demonstrated that host phenological stage during *P. pachyrhizi* inoculation affects gene expression pattern (Panthee et al., 2009). In fact, many data suggest that timing and the degree of induction of a defense pathway rather than the involvement of specific gene(s) determine the outcome of the soybean resistance to *P. pachyrhizi* (Choi et al., 2008; Goellner et al., 2010; Soria-Guerra et al., 2010a; Soria-Guerra et al., 2010b; van de Mortel et al., 2007).

Ureases were not identified in these large-scale transcript profile studies. It is likely that *GmEu*4 may have a less pronounced involvement in plant responses to fungal infection when compared to other genes. The host phenological stage during infection or the use of different genotypes may have contributed to our findings. Gutterson and Zhang (2004) explained that as global expression technologies cast a wide expression net, they provide only a provisional candidate gene set. In contrast, only few genes are considered suitable for transgenic approaches. In agreement with our results, urease upregulation was observed in *A. thaliana* leaves under 2 h salicylic acid treatment (available on http://www.ncbi.nlm.nih.gov/geo) (Thibaud-Nissen et al., 2006). Furthermore, in infected soybean plants, it has been observed a modification in the expression levels of the Ni-binding urease accessory protein UreG and glutamine synthase, an enzyme related to the urea metabolism (Panthee et al., 2009; Soria-Guerra et al., 2010a).

Soria-Guerra et al. (2010a) identified a high number of genes with metabolic-related functions upregulated in resistant *G. tomentella* genotype. Activities of many genes involved in metabolic processes are also affected upon pathogen infection and play essential roles in many plant defense responses (Soria-Guerra et al., 2010a). It is not clear yet whether the urease effect on soybean *P. pachyrhizi* infection is dependent or not on ureolytic activity.

Although there is no sufficient biochemical and molecular information currently available to predict the precise role of *GmEu*4 during fungal infection, urease contribution(s) to plant defense against invasion by a broad spectrum of fungal pathogens is here demonstrated. An important question that remains to be solved is how urease could be involved in resistance to both the biotrophic (*P. pachyrhizi*) and the necrotrophic fungi. Further bioassays with higher number of *GmEu*4 overexpressing plants or the transfer of *GmEu*4 co-suppression characteristic into resistant genotypes may provide new insights on the requirements of ubiquitous urease during the establishment of the biotrophic interaction between soybean and *P. pachyrhizi* or agronomicaly filamentous fungi.

MATERIALS AND METHODS

P. pachyrhizi bioassay

Soybean reaction to rust infection was assessed by the inoculation of *P. pachyrhizi* spores collected in the field in plants maintained under greenhouse conditions at Embrapa Soja, Londrina, PR, Brazil. Soybean plants were grown in a pot-based system and maintained in a greenhouse at 28±1ºC with 16/8 h light/dark at a light intensity of 22.5 $\mu Em^{-2}s^{-1}$. The Embrapa-48 genotype was used as a susceptible standard, which develops a Tan lesion (van de Mortel et al., 2007), and the PI561356 genotype was used as the resistant standard, which carries the resistance to soybean rust mapped on linkage group G (Abdelnoor R.V., personal communication). Uredospores were harvested from leaves exhibiting sporulating uredia and diluted in distilled water with 0.05% Tween-20 to a final concentration of 3×10^5 spores/mL. The spore suspension was sprayed onto plantlets at the V2 developmental stage. The same solution lacking spores was used for mock inoculations. Following fungal or mock inoculations, water-misted bags were placed over all plants for one day to promote infection and to prevent the cross-contamination of mock-

infected plants. One trifoliolate leaf from each plant was collected at 1, 12, 24, 48, 96 and 192 h after inoculation, frozen in liquid nitrogen, and stored at -80 °C. Three biological replicates from each genotype were analyzed for both treatments.

Plasmid construction

The plasmid pGPTV-JIT containing the ubiquitous urease cDNA was kindly provided by Dr. Mark Taylor (Scottish Crop Research Institute, Dundee, Scotland). Our group cloned the 2.514-kbp *GmEu*4 ORF into a pGEX-4T-2 glutathione S-transferase (GST) fusion vector (GE Healthcare, São Paulo, Brazil) and used this vector as template for PCR amplification. The PCR mixture consisted of 100 ng of template DNA, 0.2 mM dNTP, 0.5 μM each *primer* (5'-CACCTTAAAAATGAAACTG-3' and 5'-TAAAAGAGGAAGTAATTTCG-3'), 1x *Pfu* Buffer, 2.5 U *Pfu* DNA Polymerase (Fermentas, Glen Burnie, USA), and autoclaved distilled water to 50 μL. Reactions were hot-started (5 min at 94°C) and subjected to 35 cycles as follows: 1 min at 94°C; 1 min at 42°C and 3 min at 72°C. The Gateway® System (Invitrogen) was used to clone the PCR product into pH7WG2D vector (Karimi et al., 2002) for constitutive expression of *GmEu*4. The resulting pH7WG2D-*ure*U vector was transformed into *Agrobacterium tumefaciens* LBA4404 for plant transformation.

Plant transformation and regeneration

Seeds of soybean cultivars IAS5 and Bragg were supplied by Embrapa Soja, Londrina, PR, Brazil. Pods containing immature seeds 3-5 mm in length were harvested from field grown plants. Somatic embryogenesis was induced from immature cotyledons and proliferated as described by Droste et al. (2002).

Eight-month-old proliferating embryogenic tissues were submitted to transformation by particle bombardment using the particle inflow gun (PIG) (Finer et al., 1992) according to the procedure described by Droste et al. (2002) or by the combined DNA-free particle bombardment and *Agrobacterium* system as described by (Wiebke-Strohm et al., 2010). Seven dishes with 15 embryogenic clusters/dish, around 0.67 mg/cluster, were prepared for bombardment, while 10 dishes were used in the bombardment/*Agrobacterium* transformation experiment. After three months in

hygromicin-B selection medium, hygromycin-resistant embryogenic soybean tissues were visually selected, counted and separately cultured for the establishment of lines corresponding to putative independent transformation events.

Embryo histodifferentiation, conversion into plants and aclimatation were carried out as described by Droste et al. (2002). All plants derived from an independent piece of hygromycin-resistant tissue were noted as being cloned plants. Plants derived from non-transformed embryogenic tissues submitted to the same culture conditions were recovered and used as control in molecular characterization and bioassays.

For progeny analysis, seeds obtained from T_0 plants were planted in pots containing 1 kg of organic soil and grown in the greenhouse under the same conditions presented above.

PCR and GFP expression screening for transgenic plants

Total DNA was extracted from plant leaves (Doyle and Doyle, 1987). Putative transgenic plants were PCR-screened for the presence of the hygromicin resistance gene (*hpt*) and a chimeric gene (P*35S*-*GmEu*4) formed by CaMV *35S* promoter (P*35S*) and the ubiquitous urease ORF (*GmEu*4). 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*-*GmEu*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.

GFP expression was detected 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®).

Reverse transcription, quantitative (real-time) PCR (RT-qPCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, USA) and further treated with DNAse I (Promega, Madison, 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™ (Invitrogen, Carlsbad, USA) and 24-polyVT primer.

RT-qPCR was conducted in a StepOne Applied Biosystem Real-time Cycler™. PCR-cycling conditions were implemented as follows: 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 analyzes 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 gene specific primers (Table 4). Primer pairs designed to amplify an F-Box protein, a Metalloprotease and the Actin 11 sequences were used as internal controls to normalize the amount of mRNA present in each sample, whereas a primer pair for a Lectin gene was used as reference for DNA amplification in gene copy number estimations. These genes were confirmed as good house-keeping genes in previous reports (Jian et al., 2008; Libault et al., 2008; Schmidt and Parrott, 2001). All 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 transgenic and control plants was evaluated by determining the ammonia released by enzymatic activity. Five leaf discs (0.5 cm in diameter) per plant were incubated in a 1 mL urease indicator solution for 24 h at 60^{o}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).

Fungal bioassays

Powdered leaves (1 g) were resuspended in 5 mL of 20 mM sodium phosphate buffer (NaPB), 1 mM EDTA, and 2 mM β-mercaptoethanol, pH 7.5. Protein content in crude extracts was determined by the method of Bradford (1976), using bovine serum albumin as standard.

The growth of soybean fungal pathogens *R. solani*, *Phomopsis sp.* and *F. solani*, as well as the non pathogens *C. gossypii* and *P. herguei* was evaluated turbidimetrically according to (Becker-Ritt et al., 2007). Briefly, 10 μL spores suspension (100 spores/μL) were inoculated onto 96-well plates containing 110 μL of Potato Dextrose Broth (PDB, Becton Dickenson Co.) pH 7.0 and incubated at 28 °C. After 16 h, 15 μg of protein crude extract diluted in 50 μL NaPB buffer were added to the fungal suspension (0 h). Plates were incubated at 28°C and the absorbance (430 nm) was determined on a plate reader (Spectramax, Molecular Devices) at 0, 24, 36, 48 and 60 h after protein addition. Fungal growth at 24, 36, 48 and 60 h was calculated proportionally to the 0 h absorbance, which was considered one. NaPB buffer and 9.5% H_2O_2 were used as controls. Three sample/plant/fungus were prepared.

A detached leaf method was used to evaluate soybean infection by *P. pachyrhizi* (Twizeyimana et al., 2006). Fully expanded trifoliate leaves from 2-month-old plants were collected, rinsed in sterile distilled water and cut in 5 cm × 5 cm pieces. Each leaf piece was inoculated by dripping 1 mL uredospores suspension (10^5 spores /mL) and placed with the abaxial side upwards in a Petri dish covered with wet filter paper. Plates were incubated at 20°C with 12/12h light/dark cycle. Disease symptom development was recorded 12 days after inoculation.

Statistical analysis

*GmEu*4 relative expression levels in soybean under *P. pachyrhizi* infection were statistically compared by variance analysis with three factor factorial treatments: genotypes, time and pathogen presence. Data were transformed using the method of weighted least squares. Means were compared using Bonferroni multiple comparison test. The chi-square ($\chi 2$) test was used to confirm the Mendelian expected transgene segregation pattern in the progeny (3:1 transgenic: non-transgenic plants). Data on filamentous fungal bioassays were analyzed using one-way ANOVA. When necessary data were transformed using the method of weighted least squares. Bonferroni multiple comparison test was performed to compare treatments. A non-parametric *t* test was carried out in order to compare the effect of *P. pachyrhizi* on transgenic and non-transgenic plants. Results were considered significant at 0.05.

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Table 1. Number of transgenic soybean adult plants and independent lines obtained from two transformation experiments.

		Experiment I (bombardment)		Experiment II (bombardment/ *Agrobacterium*)	
		IAS5	Bragg	IAS5	Bragg
Adult plants	Independent lines	4	-	1	2
	Plants	26	-	1	3
Plants with seeds	Independent lines	3	-	-	1
	Plants	21	-	-	1

Table 2. Number of *GmEu*4 gene extra copies integrated into transgenic plant genome. Estimative was performed by RT-qPCR comparing DNA quantification of transgenic plants and non-transgenic plants.

	Cultivar	Transformation line	*GmEu*4 extra copies
Bombardment	IAS5	A1	11±2
		A2	ND*
		A3	13±3
		A8	14±2
Bombardment/ *Agrobacterium*	IAS5	4F	1±0.1
	Bragg	5C	2±0.3
		7E	1±0.1

* ND – not determined.

Tabela 3. Transgene segregation in three T_1 families of *GmEu*4 transgenic soybean plants.

T_0 line	Total of T_1 analyzed plants	T_1 plants PCR/GFP +	T_1 plants PCR/GFP -	Expected ratio	p*
IAS5 A1	20	14	6	3:1	0.72
IAS5 A3	20	11	9	3:1	0.18
IAS5 A8	20	12	8	3:1	0.31
IAS5 NT	20	0	20	-	-

*Transgene segregation ratios were tested by χ^2 non-parametric test.

Table 4. Primer set designed for RT-qPCR.

Target	Orientation	Tm*	Primer sequence	Efficiency of pimer (%)	PCR product size (bp)
Endogenous plus transgenic ubiquitous urease transcripts or DNA	Forward	59.52	5'- TGGTGATCAAAGGTGGTGAG -3'	104.6	121
	Reverse	60.25	5'- GAACTACCAGCCTTGCCAAA -3'		
Endogenous ubiquitous urease transcripts	Forward	60.13	5'- TCACTGTGGACCCAGAAACA -3'	ND**	160
	Reverse	59.58	5'- CTTGCTTATTGTTTTTTGCCAAT -3'		
Actin 11 transcripts	Forward	60.03	5'- CGGTGGTTCTAT CTTGGCATC -3'	98.04	142
	Reverse	59.07	5'- GTCTTTCGCTTCAA TAACCCTA -3'		
Metalloprotease transcripts	Forward	60.5	5'- ATGAATGACGGTTCCCATGTA -3'	99.35	114
	Reverse	60.17	5'- GGCATTAAGGCAGCTCACTCT -3'		
FBox transcripts	Forward	60.25	5'- AGATAGGGAAATGTTGCAGGT -3'	99.44	93
	Reverse	59.84	5'- CTAATGGCAATTGCAGCTCTC -3'		
PR4 transcripts	Forward	59.76	5'- AACCTTACTCATGGCGCAGT -3'	ND	150
	Reverse	59.04	5'- TGCTGCACTGATCTACGATTC -3'		
Lectin DNA	Forward	58.95	5'- TACCTATGATGCCTCCACCA -3'	106.2	129
	Reverse	58.94	5'- GAGAACCCTATCCTCACCCA -3'		

*Calculated Tm under PCR condition.

**ND – non-determined.

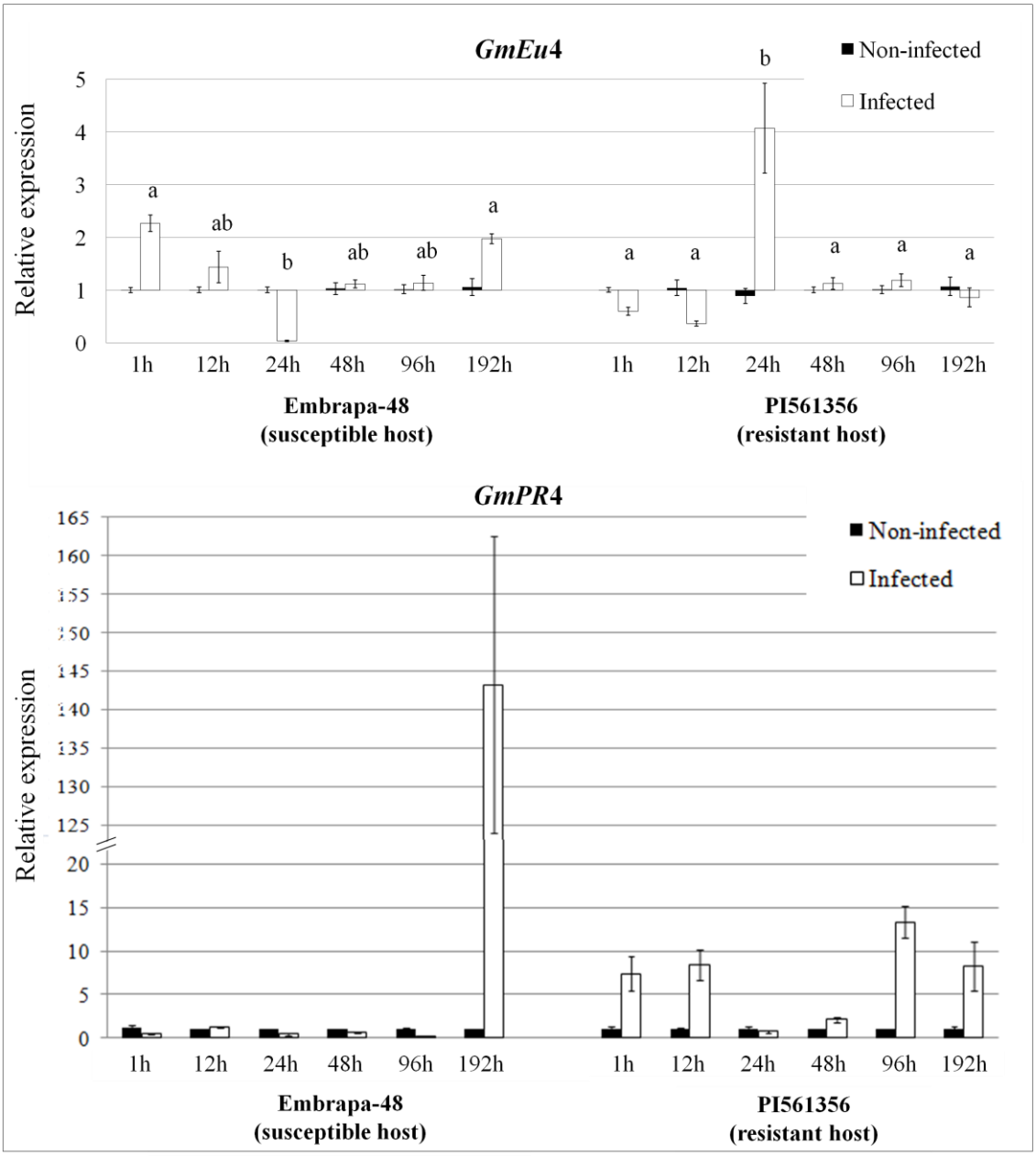


Figure 1. Response of *GmEu*4 e *GmPR*4 genes expression to *P. pachyrhizi* infection in susceptible and resistant soybean genotypes. Relative expression levels of *GmEu*4 e *GmPR*4 susceptible (Embrapa-48) and resistant (PI561356) in soybean genotypes were determined by RT-qPCR 1, 12, 24, 48, 92 and 196 h after *P. pachyrhizi* (infected) or mock (non-infected) inoculation. Values are means of three biological replicates and four technical replicates. Means followed by equal letters in the same cultivar do not differ significantly (Bonferroni multiple comparison test, $p<0.05$).

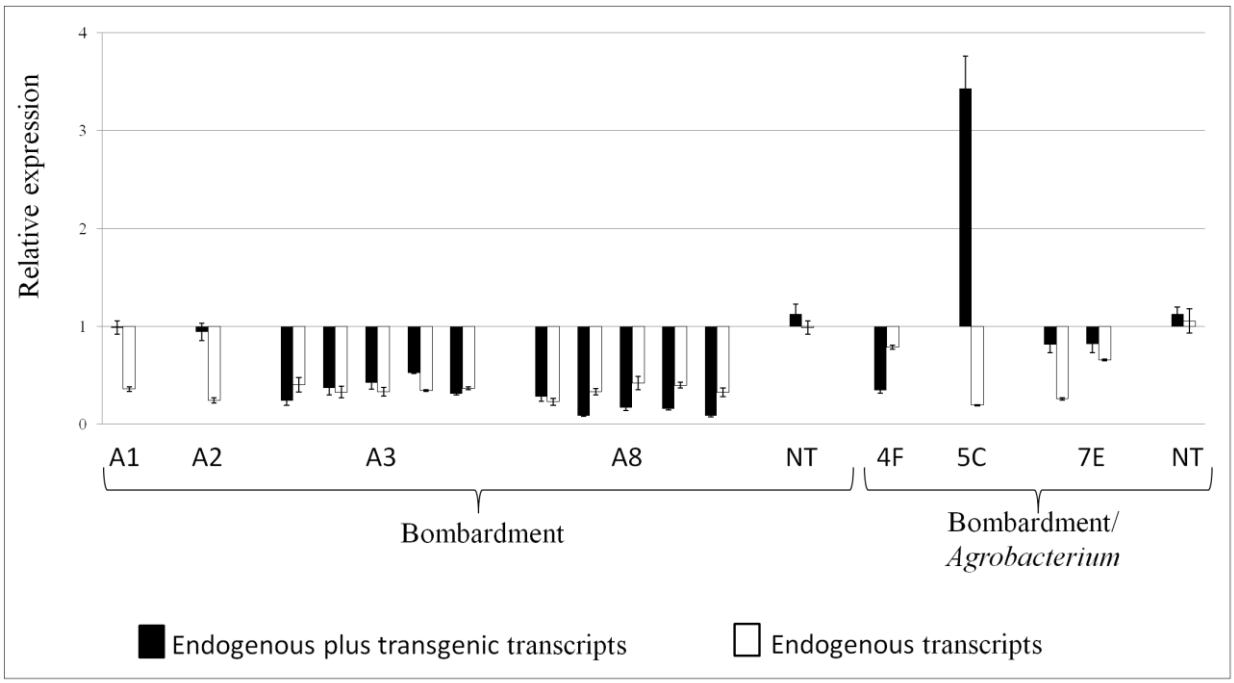


Figure 2. *GmEu*4 endogenous and transgene expression pattern in transgenic and non-transgenic (control) plants. Seven transgenic lines (A1, A2, A3, A8, 4F, 5C and 7E) were evaluated. Five plants derived from lines A3 or A8, two plants from line 7E and one plant from lines A1, A2, 4F or 5C were analyzed. NT represents the mean±SD of 4 non-transformed plants. Ubiquitous urease transcripts were detected by two different primer-pairs: one for endogenous plus transgenic transcripts (hybridizing to the encoding region) and another for endogenous transcripts (hybridizing to the 3' UTR region).

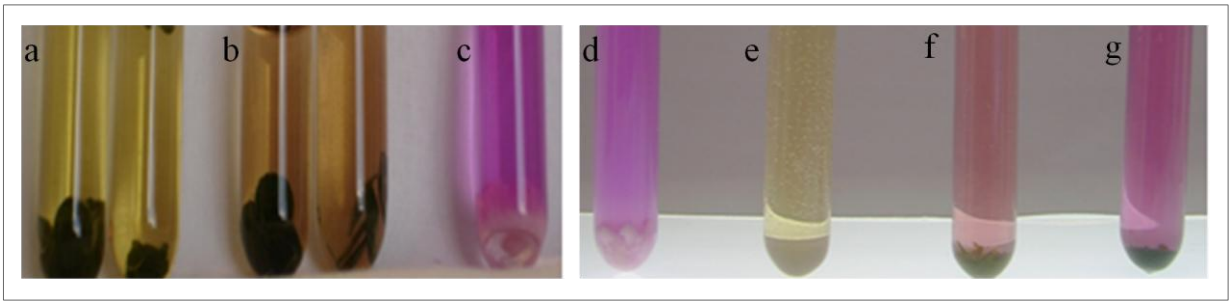


Figure 3. Ureolytic activity in urease co-suppressed and overexpressing plants. Leaf discs or seed chips were incubated in a pH-indicator reagent made of cresol red in the presence of weakly buffered 10 mM urea (Meyer-Bothling and Polacco, 1987). As ureolytic activity proceeds, released NH_4^+ increases the pH turning the solution from yellow to pinkish and finally to a deep vermillion. a) Leaves of IAS5 co-suppressed plants (A3 and A8 lines); b) leaves of IAS5 non-transformed plants; c) IAS5 non-transformed seeds; d) Bragg non-transformed seeds; e) solution without sample; f) leaves of Bragg non-transformed plant; g) leaves of Bragg overexpressing plant (5C line).

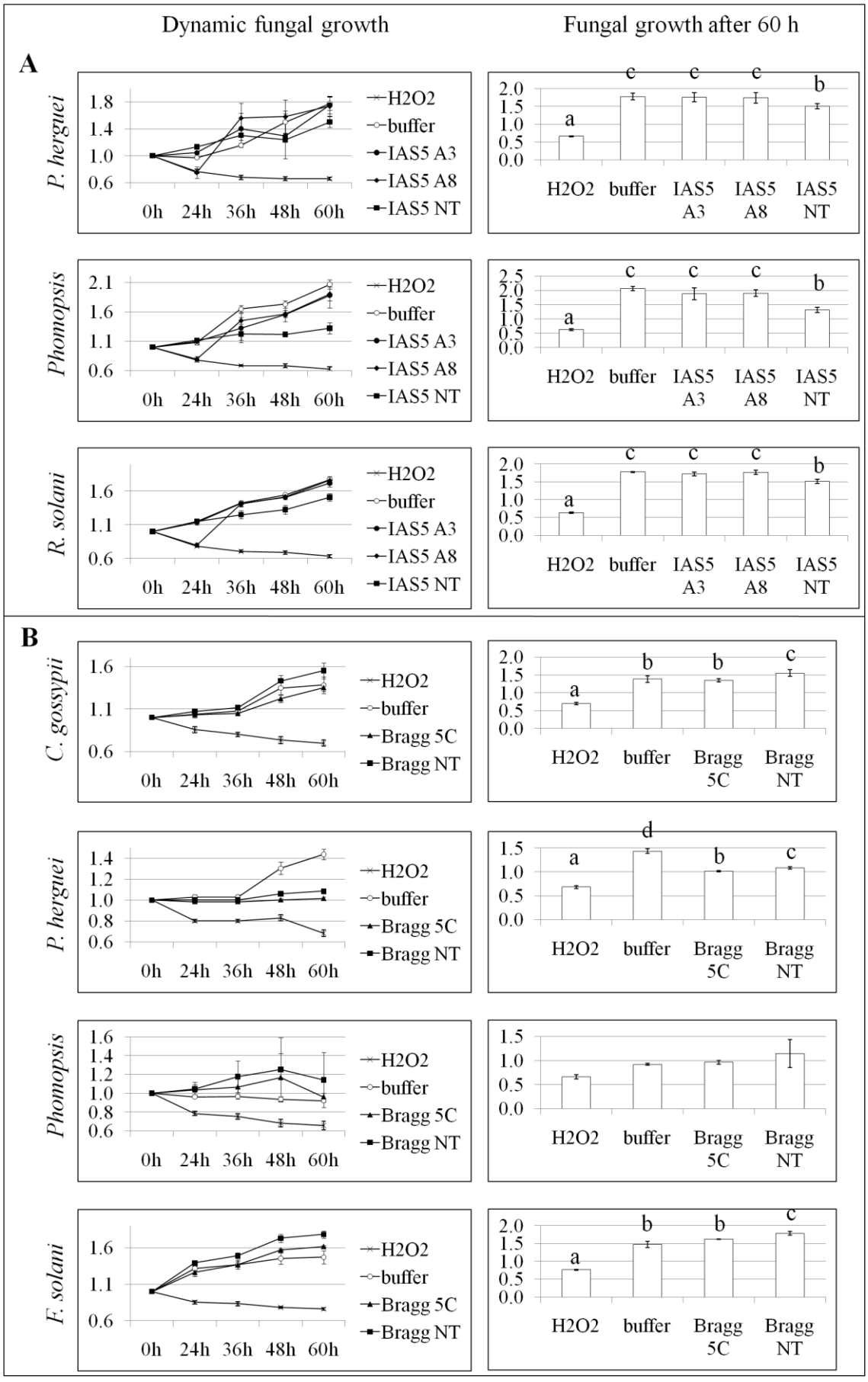

Dynamic fungal growth
Fungal growth after 60 h
A
P. herguei
Phomopsis
R. solani
H2O2
buffer
IAS5 A3
IAS5 A8
IAS5 NT
0h 24h 36h 48h 60h
H2O2 buffer IAS5 A3 IAS5 A8 IAS5 NT
B
C. gossypii
P. herguei
Phomopsis
F. solani
Bragg 5C
Bragg NT
H2O2 buffer Bragg 5C Bragg NT

Figure 4. Fungal growth after 60 h on soybean crude extracts of ubiquitous urease co-suppressed plants (A) and an overexpressing plant (B). Spores (1000 spores in 10 μL) were inoculated in 110 μL PDB, incubated at 28 °C for 16 h and subsequently leaf protein crude extract (15 μg in 50 μL) was added to the fungal culture. Samples were incubated at 28 °C and the absorbance (430 nm) was recorded every 12 h. The experiment was carried out with four T_0 co-suppressed plants (two from each A3 and A8 lines), one overexpressing plant (5C), three IAS5 non-transformed plants (IAS5 NT) and two Bragg non-transformed plants (Bragg NT). Hydrogen peroxide (H_2O_2) and protein extraction buffer were taken as positive and negative controls, respectively. Technical triplicates were analyzed per sample. Data (mean±SD) are proportional to the 0 h absorbance, which was considered one. Means followed by different letters in the same fungus/experiment are significantly different (ANOVA, Bonferroni multiple comparison test, $p<0.05$).

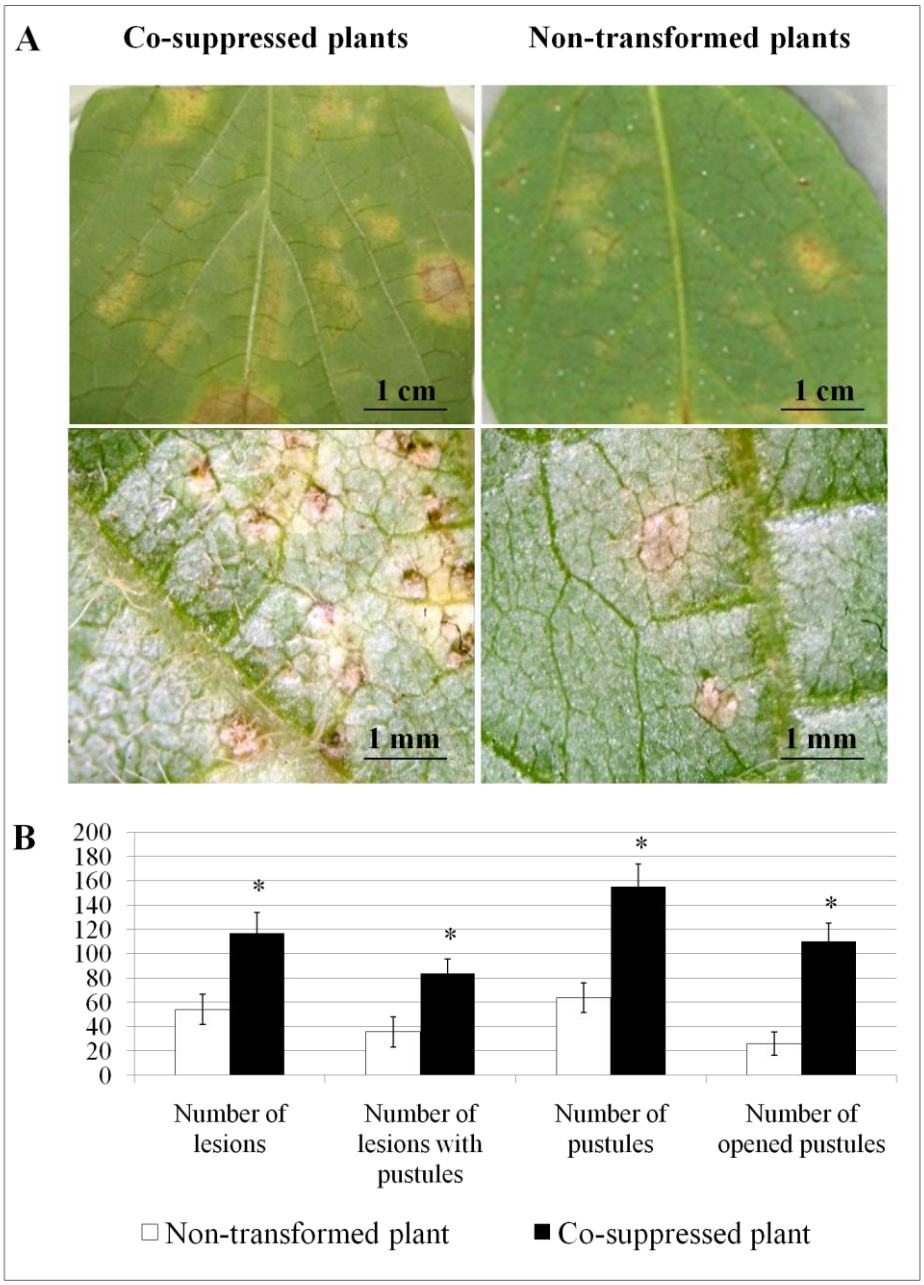
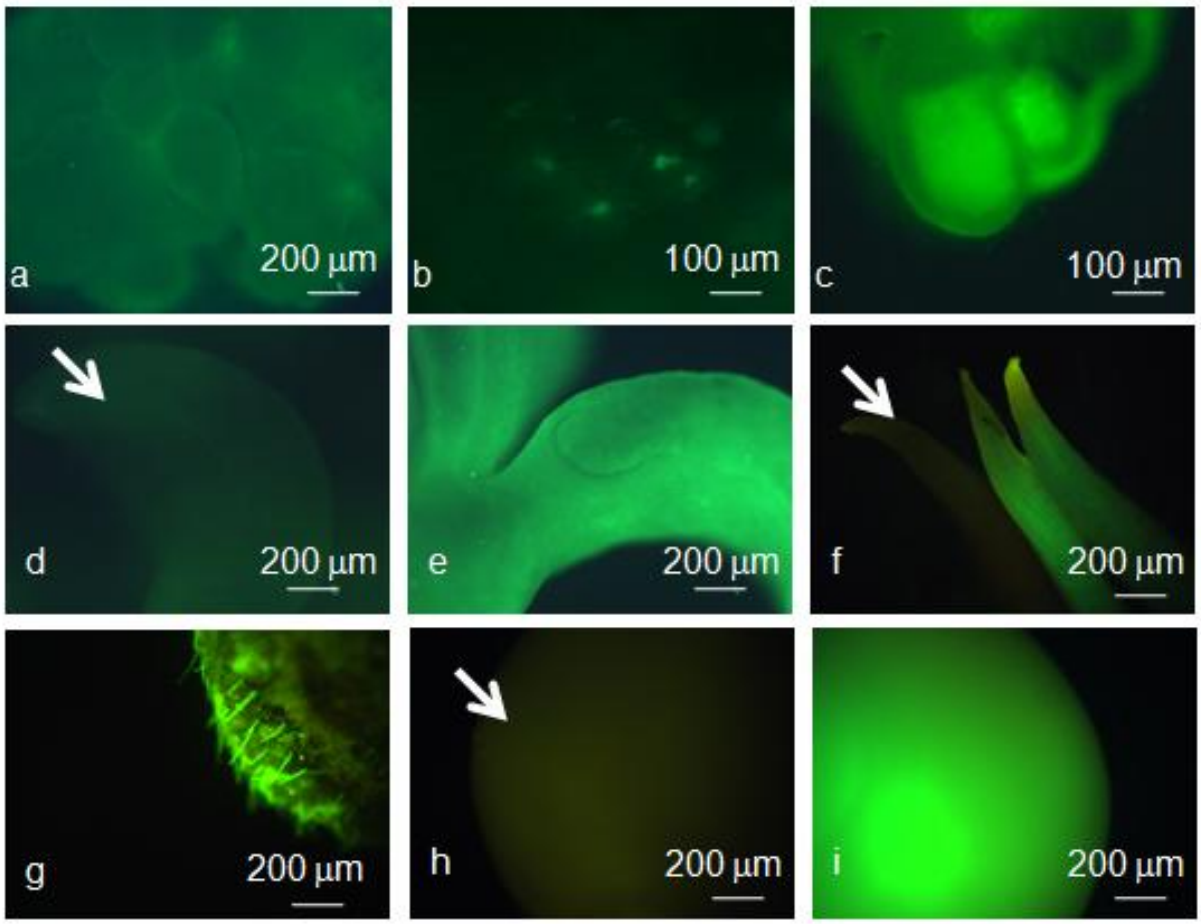


Figure 5. Soybean rust (*Phakopsora Pachyrhizi*) development on detached soybean leaves of ubiquitous urease co-suppressed plants 12 days after inoculation. Detached leaves were inoculated with 10^5/mL uredospore suspension and incubated at 20°C. A. Tan-coloured lesions and pustules under stereomicroscope. B. Four infection parameters were evaluated in seven T_1 co-suppressed plants (four from A3 line and three from A8 line) and three non-transgenic plants. *Means are statistically different in leaves of transformed and non-transformed plants (Student's *t* -test, $p<0.05$).



Supplementary Figure 1. GFP expression analyses in transgenic embryogenic tissues and plants. a) Non-transformed somatic embryos; b) green fluorescent areas one week after transformation; c) green fluorescent proliferative somatic embryos four weeks after transformation; d) non-transformed histodifferentiated embryo (arrow); e) transgenic histodifferentiated embryo; f) roots of transgenic and non-transgenic (arrow) plants; g) leaf of a transgenic plant; h) non-transgenic seed (arrow) and i) transgenic seed one day after dormancy break. GFP expression was detected 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®).

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2. Natureza:

◉ Invenção ○ Modelo de Utilidade ○ Certificado de Adição

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3. Título da Invenção ou Modelo de Utilidade ou Certificado de Adição (54):

Célula Transformada, Material Vegetal Transgênico, Método de Proteção Celular, Uso e Composição Compreendendo Ureases

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Data de Depósito:

5. Prioridade:

○ interna ○ unionista

O depositante reinvindica a(s) seguintes(s):

País ou organização de origem	Número do depósito	Data do depósito

6. Inventor (72):

☐ Asinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)

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6.2 Qualificação: DOCENTE DE ENSINO SUPERIOR 6.3 CPF: 183275590-87

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6.8 E-mail:

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7. Declaração na forma do item 3.2 do Ato Normativo nº 127/97:

☐ 7.1 Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

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8. Declaração de divulgação anterior não prejudicial: (Período de graça):
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9. Procurador (74)

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9.2 CPF/CNPJ: 9.3 API/OAB:

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9.8 E-Mail:

10. Listagem de sequências Biológicas (documentos anexados) (se houver):

☐ Listagem de sequências em arquivo eletrônico: nº de CDs ou DVDs (original e cópia).

☐ Código de controle alfanumérico no formato de código de barras: fl.

☐ Listagem de sequências em formato impresso: fls.

☐ Declaração de acordo com o artigo da Resolução INPI nº 228/09: fls.

11. Documentos anexados (assinale e indique também o número de folhas):
(Deverá ser indicado o nº total de somente uma das vias de cada documento)

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	11.2 Procuração	fls.	X	11.6 Reivindicações	2 fls.
	11.3 Documentos de Prioridade	fls.	X	11.7 Desenhos	1 fls.
	11.4 Doc. de contrato de trabalho	fls.	X	11.8 Resumo	1 fls.
X	11.9 Outros que não aqueles definidos no campo 11(especificar): Termo de Cessão, Portaria Competência, Cópia DOU				12 fls.

12. Total de folhas anexadas (referentes aos campos 10 e 11): 44 **fls.**

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Porto Alegre, 06/08/2010
Local e Data

Assinatura e Carimbo
Profª Raquel S. Mauler
Secretária de Desenvolvimento Tecnológico
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Capítulo IV

Characterization of a novel member of the soybean urease gene family

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Manuscrito a ser submetido.

Characterization of a novel member of the soybean urease gene family

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*These authors contributed equally to the work.

Abstract

In plants, ureases have been related to urea degradation, as well as to defense against pathogenic fungi and phytophagous insects. Two urease structural isoforms have been described for soybean: the embryo-specific, encoded by *Eu*1 gene, and the ubiquitous, encoded by *Eu*4 gene. By searching the completed soybean genome sequence, a third urease-encoding locus was identified. The gene was designated *Eu*5 and its product SBU-III. Phylogenetic analysis shows that the third urease is closely related to the embryo-specific isoform. 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. Expression profile of urease-encoding genes in different organs and developmental stages was determined by RT-qPCR. *Eu*5 transcripts were detected in seeds one day after dormancy break, roots of young plants and developing seeds. *Eu*1 and *Eu*4 transcripts were found in all analyzed organs, but *Eu*4 expression was more prominent in seeds one day after dormancy break and *Eu*1 in developing seeds.

Evidences suggest that SBU-III may not be involved in nitrogen availability to plants, having a putative defensive role instead.

Key words: *Glycine max*, urea amidohydrolase, expression profile, 3D-structure.

Introduction

Ureases (EC 3.5.1.5), also referred to as urea amidohydrolases, are nickel dependent enzymes that catalyze the hydrolysis of urea to form ammonia and carbamate. The later compound spontaneously hydrolyzes at physiological pH to form carbonic acid and a second molecule of ammonia (Mobley et al. 1995). This enzyme has been isolated from a variety of natural sources including plants and microorganisms (Krajewska 2009). The high similarity among all ureases suggests that they share a common ancestral gene and catalytic mechanisms (Follmer 2008; Mobley et al. 1995; Sirko and Brodzik 2000). Ureases are present virtually in all plants, but are especially abundant in leguminous seeds (Krajewska 2009). Actually, among plants, soybean (*Glycine max*) and jackbean (*Canavalia ensiformis*) have the best characterized ureases in genetic and biochemical levels (Carlini and Polacco 2008; Follmer 2008; Sirko and Brodzik 2000).

So far, two isozymes were described for soybean (Goldraij et al. 2003; Torisky et al. 1994) and three for jackbean (Follmer et al. 2001; Pires-Alves et al. 2003; Sumner 1926). In soybean, the ubiquitous urease, encoded by the *Eu*4 gene, is found in all plant tissues, in which it catalyzes urea hydrolysis and thereby allow organisms to use exogenous and internally generated urea as a nitrogen source (Torisky et al. 1994; Witte et al. 2002). The embryo-specific urease, encoded by the *Eu*1 gene, was identified in the developing embryos and mature seeds (Polacco and Holland 1993). The physiological or biological functions of soybean embryo-specific and jackbean ureases remain widely unclear, but their toxicity *in vitro* against some insects and fungi have been demonstrated (Carlini and Polacco 2008). The toxic properties were shown to be independent from the ureolytic activity, although these enzymes have completely active enzymatic sites (Becker-Ritt et al. 2007; Follmer et al. 2004).

Soybean and jackbean belong to the family Fabaceae, subfamily Papilionoideae (Sato et al. 2010; USDA 2009), thus they are closely related. Although a family of three urease-related genes was described for jackbean, embryo-specific and ubiquitous ureases

were believed to be the only functional structural genes in soybean (Carlini and Polacco 2008), since *eu*1/*eu*4 double mutants are virtually devoid of ureolytic activity (Goldraij et al. 2003; Stebbins and Polacco 1995). However, the soybean whole-genome sequence was recently reported (Schmutz et al. 2010) and brought new insights by allowing accurate study of its genome. Here we described a novel soybean urease-encoding gene.

Materials and methods

Bioinformatics analyses

A search for identify urease structural isoforms in soybean genome was carried out using BLAST analysis in Phytozome (http://www.phytozome.net/cgi-bin/gbrowse/soybean). Genomic, cDNA and protein sequences from the soybean urease-encoding genes were identified and downloaded from the data base. The protein sequence similarity analysis was performed with the BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). Multiple alignments of soybean and jackbean ureases were carried out using the CLUSTALW software (http://www.ebi.ac.uk/clustalw).

The phylogenetic tree was constructed by the neighbor-joining method (10,000 bootstrap replicates) using the MEGA (Molecular Evolutionary Genetics Analysis) program version 4.1 (Tamura et al. 2007). A total of 11 plant ureases were selected from National Center for Biotechnology Information and Phytozome or other databases for leguminous species (Sato et al. 2010).

The construction of three-dimensional (3D)-structural models of the soybean ureases was performed using homology modeling techniques, employing MODELLER 9v8 (Sanchez et al. 2000). The template used for modeling was the jackbean urease crystal structure (PDB id 3LA4, 2.05 Å resolution) (Balasubramanian and Ponnuraj 2010). Ten models were built for each protein. These models were stereochemically evaluated with PROCHECK (Laskowski et al. 1993) and had their 1D-3D profile theoretically validated with Verify3D (Luthy et al. 1992). The best model for each urease was selected based on these assessments.

Plant growth conditions and treatment

The Brazilian cv. MGBR-46 Conquista was chosen for expression analyses. A first pool of seeds was placed on dishes containing wetted germination paper and maintained in the dark for one day. A second pool of seeds was sowed in vermiculite and plants were grown for two weeks. A third pool of seeds was planted in pots containing organic soil and plants were grown until completely development in a green house at 28±1ºC with 16/8 h light/dark at a light intensity of 22.5 $\mu Em^{-2}s^{-1}$. Different plant organs were collected in four phenological stages as shown in Table 1. For each organ, four biological replications were collected; each replication was represented by material collected from 4 different plants. All samples were quickly frozen in liquid nitrogen and stored at -80 °C.

Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, USA) and further treated with DNAse (Promega, Madison, USA) according to the manufacturer's instruction. The first-strand cDNAs were obtained by using approximately 2 μg of DNA-free RNA, M-MLV reverse transcriptase system (Invitrogen, Carlsbad, USA) and 24-polyTV primer.

RT-qPCR was conducted using a StepOne Applied Biosystem real-time cycler™, based on SYBR fluorescence. Each 25 μl reaction comprised 12.5 μl cDNA (1:100), 2.5 μl 10x PCR buffer (Invitrogen, São Paulo, Brazil), 1.2 μl $MgCl_2$ 50 mM, 0.06 μl dNTP mixture 10 mM, 0.25 μl each primer pair 10 μM, 5.31 μl ultra pure water, 2.5 μl SYBR-Green (1:100000 - Molecular Probes Inc., Eugene, USA) and 0.06 μl de Platinum Taq DNA polimerase (5 U/μl – Invitrogen, São Paulo, Brazil). PCR-cycling conditions were implemented as follows: 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. All PCR reactions were carried out in technical quadruplicate. No-template reactions were used as negative controls. Using the StepOne Software v2.0, baseline was automatically determined and threshold was stablished at 2.

A set of four candidate reference genes was selected from previous reports (Table 2) (Jian et al. 2008; Libault et al. 2008). Specific primer pairs were projected for each urease-encoding gene using Primer3 (v. 0.4.0) software (Table 2). Expression data analyses were performed after comparative quantification of amplified products using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

Bioinformatic analyses

Based on the soybean whole-genome sequence (Schmutz et al. 2010), a new putative urease structural isoform was identified. This third urease encoding-gene was designated *Eu*5 (Glyma08g10850.1) and its deduced peptide, SBU-III (soybean urease III). A comparison among the three urease-encoding genes is shown in Table 3. The 11 plant urease complete amino acids sequences available in databases were aligned to build a phylogenetic tree (Figure 1), which revealed that all leguminous plant ureases have diverged from a common ancestor protein. It was found that SBU-III is closely related to embryo-specific urease. Curiously, soybean ubiquitous urease was grouped in a different branch than the other soybean ureases.

Alignment of SBU-III amino acid sequence with other leguminous urease sequences revealed a high similarity (Figure 2). However, SBU-III presented a mutation in one of the highly conserved residues of the active site (Balasubramanian and Ponnuraj 2010; Jabri et al. 1995), in which a histidine residue (His409 in CeURE-IIb) was substituted by a tyrosine. Long gaps were also observed in SBU-III, including part of the entomotoxic peptide region (residues 247-264 in CeURE-IIb). This entomotoxic peptide gap do not comprise the amphipathic β-hairpin region (Balasubramanian and Ponnuraj 2010).

The 3D-structure of the three soybean ureases was predicted in order to compare SBU-III with its counterparts. As observed in the crystal structure of jackbean and bacterial ureases (Balasubramanian and Ponnuraj 2010; Jabri et al. 1995), soybean ureases also formed the hammer or T-shaped molecule (Figure 3). The ureolytic active site cavity and the mobile flap of SBU-III exhibited an overall architecture very similar to embryo-specific and ubiquitous ureases. Nevertheless, the SBU-III structure seemed to have a more

flexible conformation when compared to the other soybean ureases, especially in domains connection. The absence of part of the entomotoxic peptide and of a helix motif near the active site was also observed. Apart from these major differences, there are also some other small variations.

Urease expression in different plant organs

A set of four reference genes previously tested for other finalities was selected (Table 2). The specificity of the amplicons was confirmed by the presence of a single peak in the melting curve and no amplification in the absence of template. The expression stability of the genes was examined by geNorm software v3.5 (Vandesompele et al. 2001). Fbox, Metalloprotease and Actin 11 genes had an M value below 1.5 recommended by geNorm. Results indicated FBox protein and Metalloprotease genes as the most stable reference genes for expression normalization under our experimental conditions. The same result was found when data were analyzed by Normfinder software v0.953.

The specificity of the primer pairs for the different ureases-encoding genes was confirmed by (1) the presence of a single peak in the melting curve, (2) a single fragment of the expected size in agarose gel electrophoresis and (3) sequencing amplicons (data not shown). As expected, ubiquitous urease transcripts were identified in all analyzed samples (Figure 5A), but a higher level was detected in seeds one day after the dormancy break. Surprisingly, embryo-specific urease transcripts were also present in all organs (Figure 4A), but, as supposed, the higher levels were observed in samples containing embryogenic tissues: the pods with seeds and seeds. Contrastingly to the other ureases expression pattern, SBU-III mRNA was identified in specific organs and developmental stages: seeds one day after the dormancy break, roots of young plants, pods with seeds and developing seeds (Figure 4A). Transcripts observed in pods with seeds and developing seeds may be derived from developing embryos or seed coat, since there was no amplification in pods without seeds. Nevertheless, when expression levels of the three ureases were compared in specific organs, the lower amount of SBU-III transcripts become evident (Figure 4B), justifying the complete lack of identification of the enzyme by traditional techniques.

Discussion

In the present study, the *Eu*5 gene was identified (Glyma08g10850.1) in the soybean genome. *Eu*5 gene shows high sequence similarity with the urease-encoding genes. The *Eu*5 predicted product, SBU-III, was shown to be more closely related to the soybean embryo-specific isoform than to the ubiquitous isoform (Figure 1). Although a lower level of *Eu*5 transcripts was detected, its expression pattern was also similar to those of the embryo-specific urease (Figure 5). Interestingly, *Eu*5 transcripts were observed only on specific organs under development: seeds one day after dormancy break, young roots and developing seeds. The *in vivo* identification and characterization of the new urease isoform was not achieved so far and must be a challenge due to its low expression level (Figure 5). However, it is noteworthy that SBU-III predicted molecular size is 78 kDa (Table 3) and that Torisky and Polacco (1990) identified a smaller species of urease with approximately 80 kDa on denaturing gels. This 80 kDa-urease was reported in 800-fold purified protein extract derived from root tissues of young plants, hypocotyl/radicle of seedlings and embryo root axes.

The presence of a third urease-encoding gene in soybean genome was surprising, because double mutants for ubiquitous and embryo-specific urease are virtually devoid of ureolytic activity (Goldraij et al. 2003; Stebbins and Polacco 1995). In agreement with these previous works, our results indicate that SBU-III may not catalyze urea hydrolysis. The SBU-III structural model exhibited conserved ureolytic active site and mobile flap (Figure 3). However, a mutation was identified in an important residue of the active site (Figure 2), which may impair the ureolytic activity. The mutation determined substitution of an amino acid that is highly conserved not only among leguminous plants, but also among other plants and microorganisms (Balasubramanian and Ponnuraj 2010; Follmer 2008; Mobley et al. 1995). The ureases catalytic site consists of a bi-nickel center and the mutated residue is one of the six ligands of Ni atoms, binding to Ni-2 (Balasubramanian and Ponnuraj 2010). A mutation (H134A) produced in *Klebsiella aerogenes* urease exhibited only one (Ni-1) of the two nickel ions (Park and Hausinger 1993). The mutant urease was inactive and binds to Ni-2 inefficiently, showing that Ni-2 is required for an active urease site formation (Follmer 2008). Additionally, a α-helix is absent in the α domain. This domain comprises the active site and is traditionally described as a distorted

TIM-barrel (Jabri et al. 1995). These barrels, also known as $(\alpha\beta)_8$-barrels, are formed by alternating 8 α-helixes and 8 β-strands and the absence of a constitutive helix may further impair the SBU-III ureolytic activity. Together these data highly suggest that SBU-III must not be involved in urea degradation function.

On the other hand, it is known that both bacterial and plant ureases display several biological roles that are independent of their ureolytic activity (Becker-Ritt et al. 2007; Follmer et al. 2004). In plants, ureases have been related to antifungal and insecticidal properties (Becker-Ritt et al. 2007; Carlini and Polacco 2008; Follmer et al. 2004). The urease entomotoxic effect relies on an internal peptide, released by cathepsins activity in the digestive system of susceptible insects (Figure 2). A β-hairpin motif within the peptide has been related to the toxicity against insects (Balasubramanian and Ponnuraj 2010; Mulinari et al. 2007). The sequence comparison of various plant ureases revealed that this motif is highly conserved and exhibits amphipathic character (Balasubramanian and Ponnuraj 2010). Recently, it was reported that the entomotoxic peptide exhibited membrane-disruptive ability on acidic lipid bilayers (Barros et al. 2009). As previously shown, a long internal region is absent in the predicted SBU-III entomotoxic peptide, but the amphipathic β-hairpin motif is highly conserved (Figure 2).

As expected, ubiquitous urease transcripts were identified in all analyzed samples (Figure 5A). Significantly higher expression levels were detected in seeds one day after the dormancy break, indicating an important role of this enzyme in earlier stages of plant development. This indication reinforces previous observations that urease has an important role in *Arabidopsis* seed germination and seedling (Zonia et al. 1995).

Embryo-specific urease is supposed to derive mainly from embryogenic tissues (Torisky et al. 1994; Torisky and Polacco 1990). Surprisingly, in our study its transcripts were also identified in all organs examined (Figure 4A). Embryo-specific urease activity in root and shoot of young plants was previously observed by Torisky and Polacco (1990). However, this activity was accounted to a remnant of protein laid down during embryo development and not made *de novo* after dormancy break, because no amplification was detect by conventional PCR-reverse transcriptase approach (Torisky et al. 1994). Our findings, which complement the previous studies, were based on real time PCR approach, a technology with high sensitivity, specificity and throughput capacity (Walker 2002).

Nevertheless, as expected, consistently higher levels were observed in samples containing embryos: the pods with seeds and seeds. Embryo-specific urease expression must achieve even higher levels along embryo maturation, since its activity was shown to increase during this period (Torisky et al. 1994). Thus, the embryo-specific urease continuous aptly named.

In the present work we described a new soybean urease-encoding gene and compare its expression and putative product to the already known isoforms. Evidences suggest that SBU-III must not be involved in nitrogen availability to plants, but it can display a defense function.

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Table 1. Different plant organs in four phenological stages collected for gene expression analyses.

	Plant phenological stage	Plant Organ
1	1 day after dormancy break	Seed
2	Young plants[a]	Root
		Stem
		Leaf
3	Adult plants – flowering[b]	Stem
		Leaf
		Flower before fertilization
		Flower at fertilization
		Flower after fertilization
4	Adult plants – seed development[c]	Immature pod without seeds
		Immature pod
		Immature seed

[a]Two week-old plants after expansion of the first trifoliolate leaf.

[b]Organs were collected at the moment the first flowers opened, about three month after sowing. An association between soybean flower bud size and development stage was previously reported (Lauxen et al. 2003). Flower buds with 4-5 mm in length, presenting immature anthers, were considered not fertilized. Flower buds with 6-7 mm in length, with mature anthers and pollen, were determined at fertilization. The opened flowers were considered fertilized.

[c]Organs were harvested about three weeks after flowering, at the moment the seeds achieved 3-4mm in length.

Table 2. Primers set 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'
Actin 11	Forward	5'-CGGTGGTTCTATCTTGGCATC-3'
	Reverse	5'-GTCTTTCGCTTCAATAACCCTA-3'
Cyclophilin	Forward	5'-ACGACGAAGACGGAGTGG-3'
	Reverse	5'-CGACGACGACAGGCTTGG-3'

Table 3. Urease encoding-genes characteristics

Urease	Encoding-gene (ID)	Chromosome	Gene size (bp)	Number of introns	ORF[a] size	Number of amino acids	Molecular mass (kDa)
Embryo-specific	*Eu*1 (Glyma05g27840.1)	5	7736	17	2520	840	94
Ubiquitous	*Eu*4 (Glyma11g37250.1)	11	7287	18	2514	837	94
SBU-III	*Eu*5 (Glyma08g10850.1)	8	5849	18	2142	714	78[b]

[a] Open reading frame.

[b] Predicted molecular size of the putative protein was obtained by submitting the sequence to the ProtParam tool available at the Expasy site (http://ca.expasy.org/tools/protparam.html).

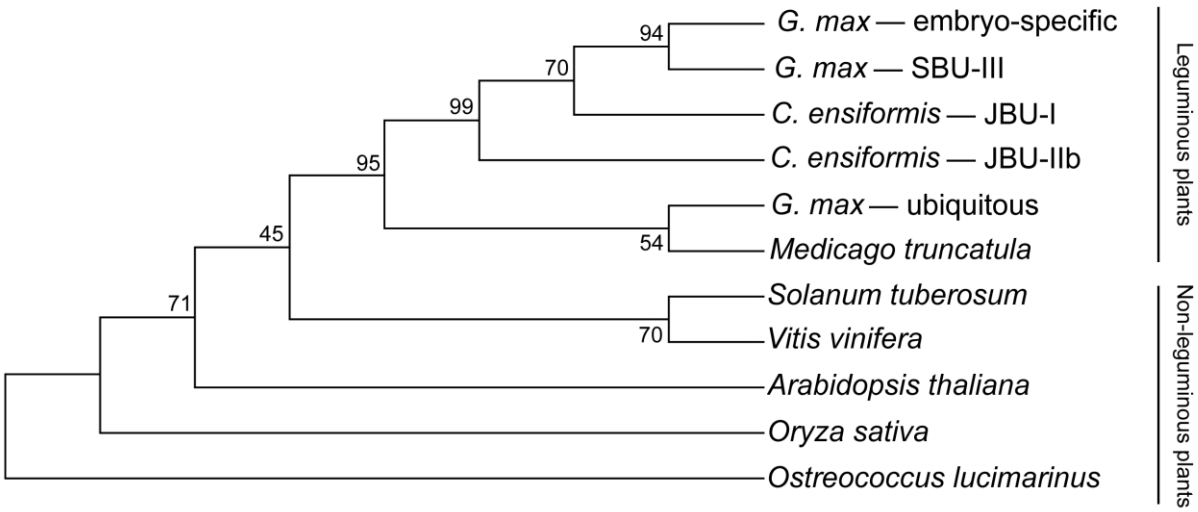


Figure 1. Phylogenetic analysis of plant ureases. The phylogenetic tree was constructed based on the alignment of the full length amino acid sequences of plant ureases. The percentage of replicate trees in which the associated sequences clustered in the bootstrap test (10,000 replicates) is shown next to the branches.

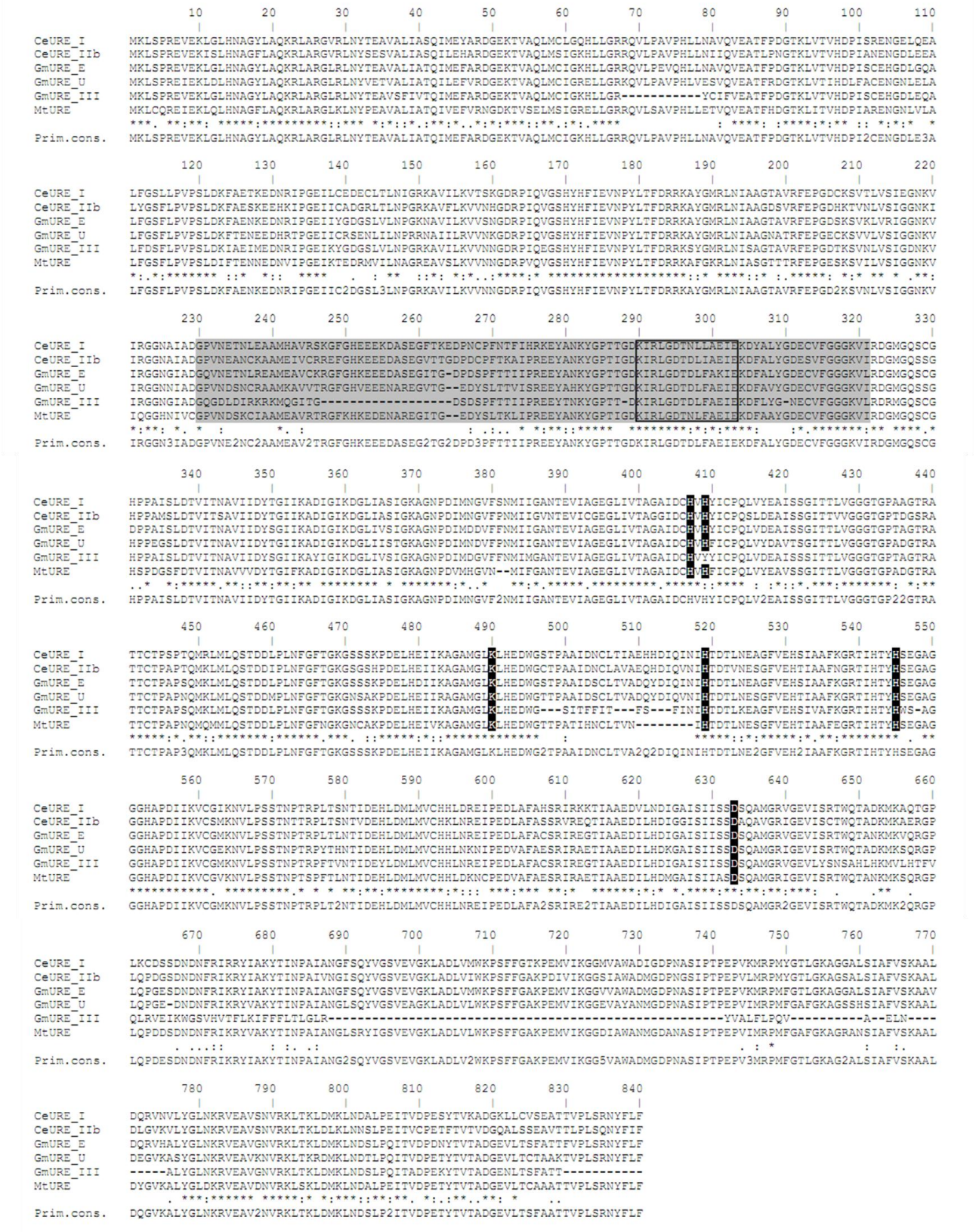
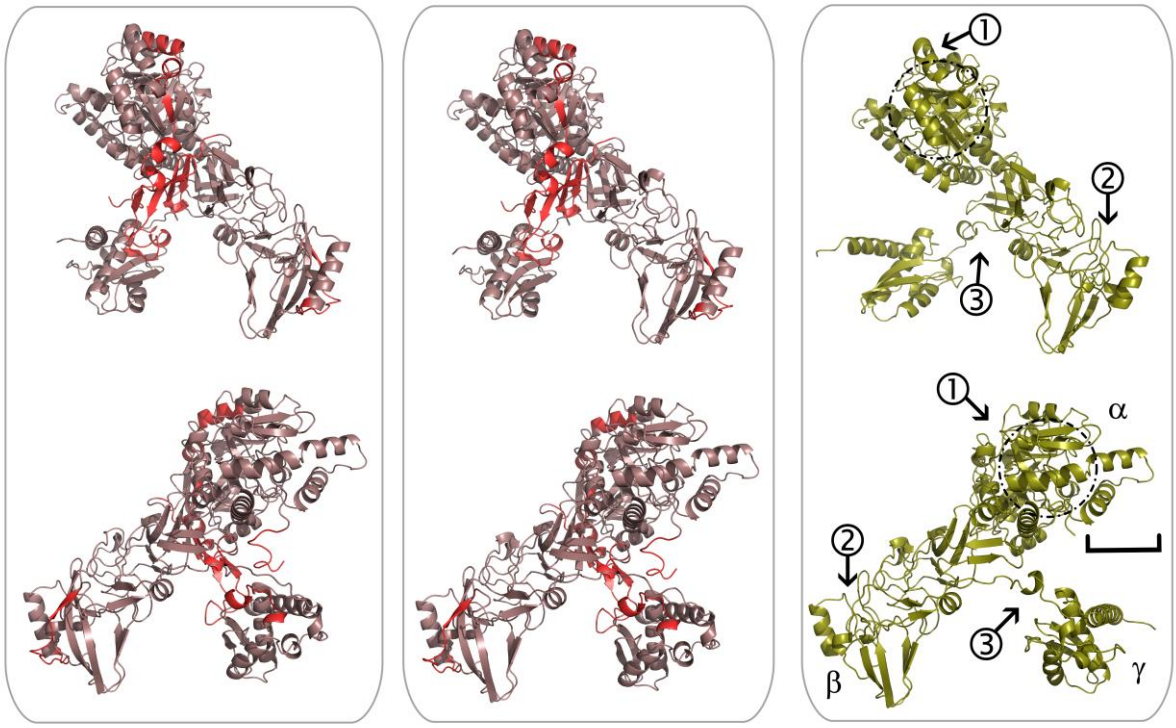

CeURE_I
CeURE_IIb
GmURE_E
GmURE_U
GmURE_III
MtURE
Prim.cons.

Figure 2. Alignment of the amino acid sequences of leguminous ureases using CLUSTALW. The sequences are from *C. ensiformis* urease-I (CeURE-I), *C. ensiformis* urease-IIb (CeURE-IIb), *G. max* embryo-specific urease (GmURE-E), *G. max* ubiquitous urease (GmURE-U), *G. max* urease-III (GmURE-III), *M. truncatula* (MtURE). Highly conserved residues in leguminous plants are indicated by (*), strongly similar by (:) and weakly similar by (.). Residues from the active site, conserved among all ureases, are shaded in black. The 10-kDa entomotoxic peptide region is shaded in gray and its amphipathic β-hairpin region is boxed in black.



SBU-U
Ubiquitous

SBU-E
Embryo-specific

SBU-III

Figure 3. Comparison of the three-dimensional (3D)-structural models of the three soybean ureases. Molecular molding was predicted based on JBURE-I X-ray crystallographic structure. The gaps observed in SBU-III primary structure are colored by red in ubiquitous and embryo-specific ureases. α (TIM barrel), β and γ are the different structural domains. The ureolytic active site region is indicated by a circle and the mobile flap by a bracket. The main differences in the SBU-III structure are pointed by arrows. Arrow 1 shows the absence of a helix segment near the active site. Arrow 2 indicates the lack of part of the entomotoxic peptide region. Arrow 3 point out a relaxed connection among domains.

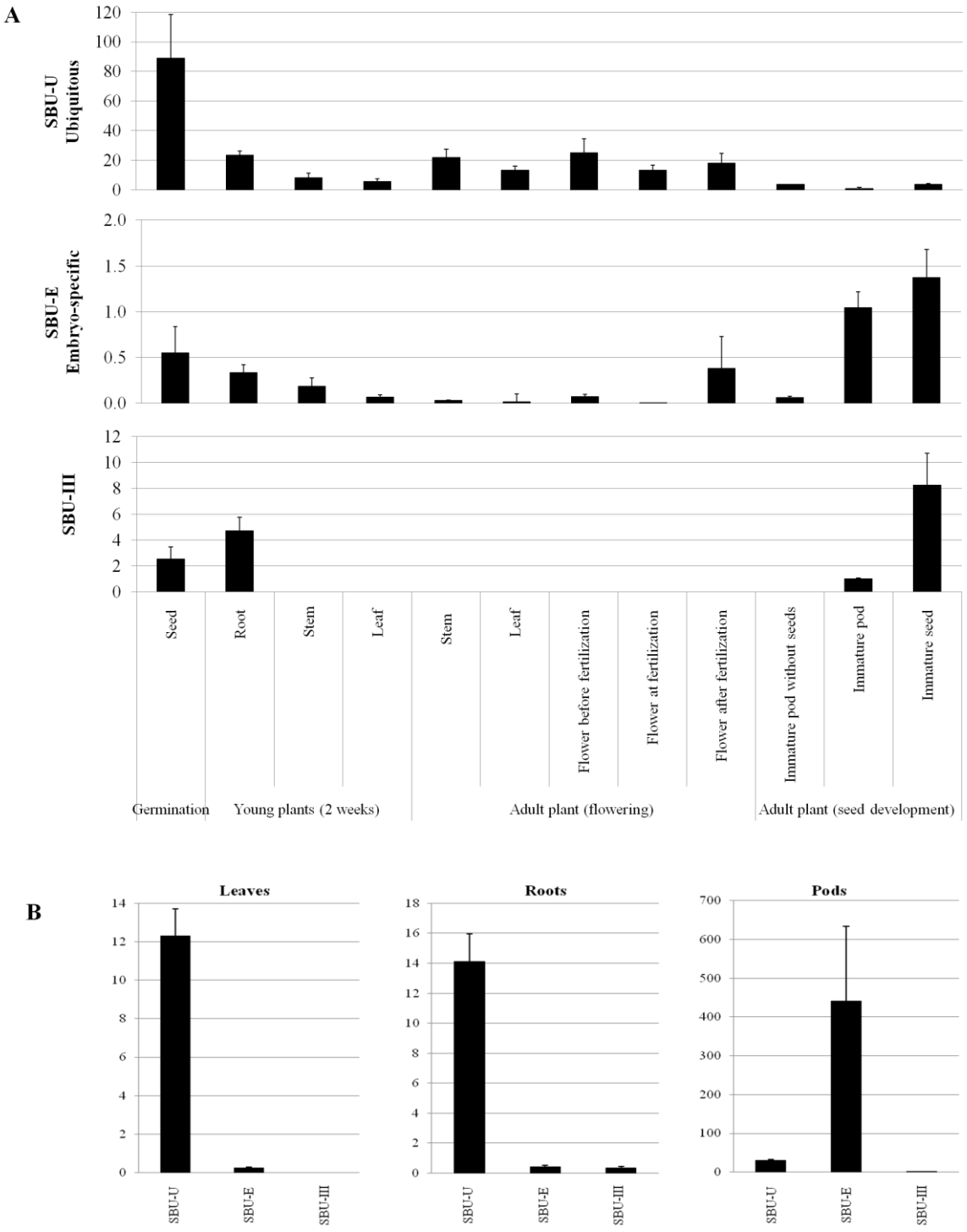


Figure 4. Relative expression levels (RT-qPCR) of the three urease-encoding genes in different soybean organs and developmental stages. A. Expression pattern of each urease-encoding gene. B. Comparison of expression levels among the urease-encoding genes in three different organs: leaves and roots of young plants and immasture pods. Values are means of four biological replicates and four technical replicates.

Capítulo V

DISCUSSÃO GERAL

5. DISCUSSÃO

5.1 Transformação de soja pelo sistema integrado bombardeamento/ *Agrobacterium*

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). Esse potencial deve-se à disponibilidade de um maior número de genes para o melhoramento de plantas, que até então estava limitado a espécies sexualmente compatíveis, e aos estudos de genômica funcional, que permitem a identificação e validação de novos genes com potencial biotecnológico (Somers et al. 2003). Portanto, o desenvolvimento de sistemas eficientes de transformação para cada espécie vegetal é o requisito básico para implementação de estudos funcionais e, a longo prazo, para fornecer uma nova estratégia para o melhoramento.

Tanto o bombardeamento quanto o sistema *Agrobacterium* têm sido utilizados para introdução de genes em diversos tecidos da soja (Somers et al. 2003; Mello-Farias e Chaves 2008). Entretanto, o bombardeamento de embriões somáticos aparece em um número expressivamente maior de trabalhos que empregam a transformação em sua metodologia (Hernandez-Garcia et al. 2009; Li et al. 2009; Kita et al. 2010; Xing et al. 2010). Por outro lado, por introduzir *loci* transgênicos menos complexos, há uma inclinação crescente para a utilização do sistema *Agrobacterium*, principalmente em programas de melhoramento (Somers et al. 2003; Mello-Farias e Chaves 2008).

Embriões somáticos são alvos altamente promissores para transformação por apresentarem origem unicelular e capacidade de multiplicação (Trick et al. 1997). A transformação do tecido embriogênico pelo sistema *Agrobacterium* foi inicialmente um desafio (Mello-Farias e Chaves 2008). Contudo, a abertura de microferimentos através da sonicação ou do bombardeamento de partículas livres de DNA permitiu a infecção do tecido por *Agrobacterium* e sua consequente transformação (Trick e Finer 1997, 1998; Droste et al. 2000). Conforme resultados apresentados nos Capítulos 2 e 3, foram regenerados com sucesso plantas transgênicas de soja a partir de quatro experimentos independentes de transformação. Dentre eles obtivemos plantas que superexpressam a urease ubíqua de soja (Capítulo 3), um fator de transcrição da família WRKY da própria

soja (Osorio 2010) e uma osmotina de *Solanum nigrum* (dados não publicados). Todas estas plantas encontram-se em fase de análise funcional dos genes.

Embora o sistema integrado bombardeamento/*Agrobacterium* já esteja sendo utilizado rotineiramente em nosso laboratório, uma maior eficiência de transformação ainda é desejada. Estudos complementares serão necessários visando aumentar o número de eventos independentes de transformação e a taxa de regeneração das plantas. Uma alternativa seria a adição de compostos tiol durante e após a infecção por *Agrobacterium*. Esses compostos amenizam a resposta de defesa das plantas a ferimentos e patógenos e, consequentemente, aumentam a viabilidade das células transformadas. A adição de tiol aumentou a frequência de transformação por *Agrobacterium* em outro tecido alvo (Olhoft et al. 2003; Somers et al. 2003; Olhoft et al. 2006). Outra alternativa seria a utilização de linhagens mais virulentas de *Agrobacterium*, como a KAT23 (Yukawa et al. 2008).

Se comparados os dois métodos disponíveis em nosso laboratório quanto à eficiência de transformação e à praticidade, o bombardeamento continua sendo o método mais conveniente (dados não publicados). Contudo, de acordo com a finalidade da transformação, a utilização do sistema integrado justifica os esforços. Por exemplo, a possibilidade de regenerarmos plantas superexpressando um gene determinado a partir de dois sistemas de transformação é bastante interessante, uma vez que o número de cópias integradas ao genoma vegetal pode interferir na sua capacidade de expressão (James et al. 2002; Lechtenberg et al. 2003; Tang et al. 2007). Os resultados obtidos no Capítulo 3 constituem um exemplo real para esta afirmação, quando através do bombardeamento foram obtidas plantas co-suprimidas para o gene da urease ubíqua que apresentavam10-14 cópias do *Eu*4, enquanto o sistema integrado permitiu a obtenção de plantas com 1-2 cópias extras, sendo que uma delas superexpressou o transgene. Por outro lado, em experimentos que visam silenciamento de genes, nos quais o padrão de integração e o número de cópias parece não apresentar consequências tão impactantes, a utilização do bombardeamento, que é mais eficiente e menos laborioso, seria a escolha mais acertada.

Apesar da existência de cultivares transgênicas de soja liberadas comercialmente em vários países do mundo e do número crescente de estudos visando sua transformação, o sucesso na obtenção de plantas transformadas tem sido limitado a poucos grupos mesmo em nível mundial. Como relatado anteriormente, em nosso laboratório foram regeneradas

plantas totalmente transformadas e férteis a partir de vários eventos e métodos de transformação. Segundo Trick et al. (1997) e Somers et al. (2003), a "arte" de transformar e regenerar plantas a partir da cultura de tecidos é um ponto chave para o sucesso dos programas de transformação, pois requer treino considerável dos pesquisadores no desenvolvimento de habilidades para gerar plantas suficientes para trabalhos promissores.

5.2 Superexpressão e co-supressão da urease ubíqua da soja

Enquanto um grande número de trabalhos relata as propriedades de defesa da urease embrião-específica da soja (Carlini e Polacco 2008; Follmer 2008; Krajewska 2009), estudos funcionais da urease ubíqua estão restritos à atividade ureolítica (Torisky e Polacco 1990; Torisky et al. 1994). A principal limitação para os estudos da urease ubíqua é a dificuldade de purificar quantidades satisfatórias da enzima para bioensaios *in vitro*. Além disso, os estudos de expressão da enzima em sistemas homólogos e heterólogos têm sido outro grande desafio. Martinelli (2007) tentou expressar a enzima em *Escherichia coli*, mas a fusão com a glutationa transferase resultou em várias proteínas truncadas. Os experimentos de transformação de soja descritos no Capítulo 3 foram realizados com o objetivo inicial de gerar plantas que superexpressassem a urease ubíqua, visando estudos funcionais *in vivo* e *in vitro*. Embora um número considerável de eventos tenha sido obtido, apenas uma planta foi capaz de superexpressar o gene moderadamente (4 vezes), enquanto as demais plantas transgênicas apresentaram co-supressão. 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. Infelizmente, ainda não há indícios suficientes para comprovar qualquer hipótese. Contudo, há grande possibilidade de que níveis elevados de expressão conduzam à co-supressão. A mesma dificuldade de superexpressão da urease ubíqua foi relatada em trabalhos prévios com calos transgênicos de soja e com plantas de tabaco transformadas (Torisky et al. 1994; Becker-Ritt 2005). Torisky et al. (1994) buscavam a identificação do gene através de complementação de mutantes e só conseguiram a expressão do transgene em 20% dos calos transformados.

Em conjunto estes resultados mostram a dificuldade de aumentar os níveis da urease ubíqua na soja. Desta forma, um número muito grande de experimentos de

transformação é necessário para obtenção de um conjunto razoável de plantas que efetivamente superexpressem a enzima. Outra alternativa seria a utilização de vetores contendo promotores (1) mais fracos do que o 35S, (2) induzíveis ou (3) tecido-específicos. Uma última opção seria a expressão dos vários peptídeos que compõem urease ubíqua separadamente, mas essa alternativa prejudicaria os estudos funcionais.

Uma vez que todas estas tentativas de expressão foram conduzidas com a urease ubíqua, permanece a dúvida do que aconteceria em um ensaio para superexpressar outras ureases sob o controle de um promotor constitutivo forte. Como as ureases apresentam alto grau de similaridade (Follmer 2008; Krajewska 2009), postula-se que o fenótipo seja o mesmo.

Mesmo apresentando co-supressão, as plantas obtidas durante o desenvolvimento deste trabalho representam um avanço no estudo funcional do gene, uma vez que mutantes nulos para urease ubíqua nunca foram obtidos (Carlini e Polacco 2008). Tendo em vista que as ureases são potencialmente multifuncionais (Carlini e Polacco 2008; Follmer 2008; Krajewska 2009), o conjunto das plantas apresentando superexpressão ou co-supressão representa uma ferramenta importante para a determinação das funções adicionais à atividade ureolítica.

5.3 Função de defesa da urease ubíqua da soja

Por muito tempo os estudos funcionais das ureases estiveram restritos a sua atividade catalítica, que envolve a reciclagem do nitrogênio derivado da ureia (Polacco e Havir 1979; Torisky e Polacco 1990; Polacco e Holland 1993; Torisky et al. 1994; Mobley et al. 1995; Sirko e Brodzik 2000). Esta função é particularmente importante durante a germinação, quando as proteínas de reserva são mobilizadas para a nutrição do embrião (Goldraij et al. 2003). No entanto, em soja a função de reciclagem da ureia parece envolver apenas a urease ubíqua (Torisky e Polacco 1990; Carlini e Polacco 2008). Há muito se sabe que a urease embrião-específica da soja está presente em altos níveis nas sementes, mas sua ausência não foi relacionada a nenhum fenótipo específico (Meyer-Bothling e Polacco 1987; Sirko e Brodzik 2000). A descoberta de que as ureases apresentam toxicidade a insetos (Follmer et al. 2004; Carlini e Polacco 2008) e fungos (Becker-Ritt et al. 2007; Carlini e Polacco 2008; Menegassi et al. 2008) redirecionou o foco dos estudos desta

enzima. Os ensaios de atividade inseticida e fungicida foram realizados *in vitro* com ureases purificadas a partir de sementes de soja, feijão-de-porco e algodão. Nossos estudos do padrão de expressão do gene *Eu*4 mostraram que a urease ubíqua de soja está envolvida no mecanismo de resistência a *P. pachyrizy*. Quando o fungo é inoculado em folhas destacadas, observa-se maior número de lesões e pústulas em plantas com a expressão do gene co-suprimida do que em plantas selvagens. Além disso, bioensaios *in vitro* foram realizados para comparar o crescimento de fungos filamentosos na presença de extratos protéicos totais de plantas transgênicas, com maiores e menores níveis da urease ubíqua, com o crescimento observado em extratos de plantas selvagens, com níveis normais da enzima. Concluiu-se que a falta da urease ubíqua torna as plantas mais suscetíveis e seu aumento as torna mais resistentes. Esse trabalho tem fundamental importância para o avanço do conhecimento das propriedades tóxicas das ureases sobre fungos patogênicos por dois motivos principais: (1) este é o primeiro relato de que a urease ubíqua também apresenta atividade antifúngica e (2) comprova que a urease contribui para função de defesa da planta *in vivo*, mesmo na presença de outras proteínas de defesa.

A obtenção das plantas transgênicas com maiores e menores níveis de expressão da urease ubíqua possibilitará, no futuro, estudos mais aprofundados sobre a função fungicida das ureases. Bioensaios para avaliar a capacidade de infecção e do crescimento dos fungos filamentosos diretamente sobre as plantas podem ajudar a elucidar o momento exato do efeito inibitório e/ou retardatário da urease.

Uma questão intrigante da atividade fungicida da urease ubíqua é a toxicidade direcionada tanto a fungos necrotróficos quanto a um fungo biotrófico. Estudos complementares são necessários para verificar se o princípio inibitório é o mesmo sobre estes grupos tão distintos de fungos. Sabe-se que o efeito das ureases sobre fungos filamentosos é independente da atividade ureolítica (Becker-Ritt et al. 2007), mas seu efeito sobre *P. pachyrizy* pode envolver essa atividade catalítica. Uma possibilidade é comparar a resposta do fungo em plantas transgênicas (co-suprimidas e superexpressas) com a de plantas mutantes *eu*4, nas quais a proteína é expressa, mas sua atividade catalítica está comprometida.

Até o momento, o efeito da urease ubíqua sobre a infecção por *P. pachyrizy* só pode ser testado em plantas co-suprimidas, que apresentaram maior número de lesões e pústulas

do que plantas selvagens. Esforços estão sendo direcionados para análises da progênie da planta T_0 que superexpressou o transgene, na expectativa de obter maior número de plantas para realização dos bioensaios. Por outro lado, a co-supressão foi obtida em cultivares naturalmente suscetíveis ao fungo. Para obtenção de resultados mais consistentes, seria desejável repetir os testes em genótipos naturalmente resistentes ao patógeno e com menores níveis da enzima. Uma estratégia viável é a transferência, por cruzamento convencional, dos transgenes que determinaram a co-supressão para um genótipo resistente, na expectativa de obtenção de quebra da resistência.

Estudos prévios, realizados *in vitro* com proteínas purificadas, mostraram que as ureases têm propriedade inseticida contra sugadores com trato digestivo baseado em catepsinas (revisão de Carlini e Polacco 2008). As plantas obtidas durante o desenvolvimento deste trabalho também podem servir para estudos *in vivo* da propriedade inseticida da urease ubíqua.

5.4 A identificação de uma nova urease estrutural e perfis de expressão

Por um lado, a existência de três isoformas estruturais de urease em feijão de porco (Sumner 1926; Follmer et al. 2001; Pires-Alves et al. 2003) apontava para a presença de uma terceira urease em soja. Por outro lado, a perda da atividade ureolítica em mutantes duplos para as ureases conhecidas de soja indicava inexistência de outros genes desta família (Carlini e Polacco 2008). A identificação do gene que codifica a terceira urease, *Eu*5, foi possível apenas após o sequenciamento completo do genoma da soja (Schmutz et al. 2010). A perda da atividade enzimática da SBU-III, aliada aos seus baixíssimos níveis de expressão, justifica o tempo que a mesma passou despercebida. Até o momento, foi possível caracterizar a estrutura da proteína *in silico* e determinar seu perfil de expressão em diferentes órgãos e estágios de desenvolvimento. Para a conclusão do artigo apresentado no Capítulo 4, experimentos adicionais encontram-se em andamento, incluindo o isolamento e a clonagem do cDNA, além da purificação e caracterização da proteína em espectrofotômetro de massas. Contudo, a grande similaridade entre as três isoformas de urease, juntamente os baixos níveis de expressão, tem dificultado consideravelmente as análises. Os estudos *in silico* mostraram que SBU-III, assim como as ureases ubíqua e a embrião-específica, pode estar envolvida na função de defesa das plantas; contudo, uma comprovação prática ainda é necessária.

A confirmação da existência de SBU-III e da expressão da urease embrião específica nos diversos órgãos da soja têm implicações importantes sobre os trabalhos realizados pelo nosso grupo até o momento. Investigações futuras são necessárias para avaliar a situação destas ureases nas plantas transgênicas, assim como uma possível interação das três proteínas durante o ataque de fungos.

5.5 Conclusão

A existência de metodologias de transformação genética e regeneração *in vitro*, somada à caracterização funcional dos genes que codificam ureases e a compreensão do seu mecanismo de ação durante situações de estresse permitirão, a longo prazo, a obtenção de plantas transgênicas de soja com maior resistência ao ataque de fungos patogênicos e insetos sugadores.

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DOS CAPÍTULOS I E V

REFERÊNCIAS BIBLIOGRÁFICAS

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