

Sarah Zanon Agapito Tenfen

**DETECÇÃO DE ALTERAÇÕES NO PROTEOMA DE PLANTAS
GENETICAMENTE MODIFICADAS ORIUNDAS DE
INTERAÇÕES SINÉRGICAS E ANTAGONISTAS DA
INTEGRAÇÃO E EXPRESSÃO DE TRANSGENES**

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Orientador: Prof. Dr. Rubens Onofre
Nodari

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Se todos fossem no mundo iguais a vocês...

“However, not everything that can be counted counts, and not everything that counts can be counted.”

(William Bruce Cameron, 1963)

RESUMO

Organismo geneticamente modificado (OGM) é um organismo cujo material genético foi manipulado através da utilização de técnicas de DNA recombinante. Apesar da adoção generalizada de OGMs por muitos países, a necessidade de pesquisas em biossegurança continua sendo uma preocupação. As técnicas de transformação genética atualmente utilizadas para o desenvolvimento de plantas geneticamente modificadas inserem construções transgênicas em regiões aleatórias no genoma da planta hospedeira e são muitas vezes integradas perto de elementos genéticos importantes, como retrotransposons e sequências repetidas. Isto impõe riscos adicionais devido à introdução de novas sequências reguladoras, que podem conduzir a alterações espaciais e temporais na expressão de genes endógenos. Estas imprevisibilidades podem ter efeitos adversos sobre a estabilidade genética em longo prazo, bem como no valor nutricional, alergenicidade e toxicidade do OGM. Estes processos genéticos representam áreas de pesquisa omitida, bem como lacunas no conhecimento relacionado a potenciais efeitos na saúde e meio-ambiente. Além disso, a abordagem atual para a avaliação de possíveis efeitos indesejados de OGMs é baseada na suposição de que um OGM é composto por duas partes, a planta e a proteína transgênica, que funcionam de forma linear e aditiva. Esta abordagem, que se baseia no conceito de 'equivalência substancial', é altamente criticada pela comunidade científica e carece de hipóteses científicas bem fundamentadas. Assim, o objetivo deste trabalho foi testar dois novos modelos metodológicos para caracterizar os potenciais efeitos adversos dos OGMs em nível molecular. A primeira abordagem é baseada na análise comparativa do perfil proteico e níveis de transcrição transgênica de uma variedade de milho GM contendo duas inserções transgênicas, outra contendo apenas uma inserção sob o mesmo *background* genético e a variedade convencional correspondente. Este modelo biológico proporciona uma oportunidade única de rastreamento de potenciais alterações no proteoma que derivam da combinação dos dois transgenes. A segunda abordagem baseia-se na utilização da ferramenta de interferência por RNA para o silenciamento gênico. Esta ferramenta fornece um meio para estudar genótipos transgênicos sem a acumulação de proteínas transgênicas, isolando assim os efeitos da inserção *per se*. O desenvolvimento dessas metodologias também acarretou em uma extensa revisão da literatura sobre ensaios de interferência por RNA

expressas de maneira transiente e estável em plantas. Os resultados observados demonstram que as construções transgênicas não funcionam de forma linear e aditiva. Mas influenciam a expressão global de genes endógenos, principalmente relacionados ao metabolismo energético e de estresse, dependendo do número de cópias e da natureza do transgene inserido. A presença de mais de um inserto no genoma hospedeiro também altera os níveis de expressão do transgene. Ainda, a análise proteômica de plantas transgênicas silenciadas revelou um baixo número de proteínas endógenas alteradas, indicando que o acúmulo de proteína transgênica é um dos principais fatores que influenciam a modulação do proteoma da planta hospedeira. Portanto, conclui-se que as novas abordagens metodológicas descritas e testadas podem fornecer uma metodologia científica útil e robusta para avaliações de risco de OGMs. Por fim, sugerimos que as agências regulatórias de biossegurança de OGMs considerem que este tipo de estudo seja obrigatório e parte dos documentos produzidos pelos proponentes da tecnologia que visam a liberação comercial de novos eventos de transformação genética.

Palavras-chave: Organismo geneticamente modificado. Biossegurança. Análise de risco. RNA interferente. Expressão gênica.

ABSTRACT

Genetically modified organism (GMO) is an organism whose genetic material has been altered through the use of recombinant DNA techniques. Despite the widespread adoption of GMOs by many countries, the need for biosafety research remains a concern. Actual plant transformation methods include integration of transgenic constructs that take place at random locations in the recipient plant genome and are often close to important genetic elements, such as retrotransposons and repeated sequences. This poses additional risks due to the introduction of new promoter sequences, which may lead to altered spatial and temporal expression patterns of plant endogenous genes. All these events may have unpredictable effects on the long-term genetic stability of the GMO, as well as on their nutritional value, allergenicity and toxicant contents. These putative processes represent areas of omitted research with regard to health and environmental effects of GMOs. In addition, the current approach for the assessment of potential unintended effects of GM crops is based on the assumption that a GMO consists of two parts that function in a linear additive fashion, being that the crop and the novel GM transgene product. Based on the 'substantial equivalence' concept, this approach is highly disputed in the scientific community and lacks a well founded scientifically driven hypothesis testing. In this work, two different new methodological models were used to characterize potential adverse effects of GMOs at the molecular level. The first approach is based on the comparative proteomic analysis and transgenic transcript level quantification of a stacked GM maize variety containing two transgenic inserts *versus* the two single transgenic parental varieties. This biological model provides a unique opportunity to track potential changes in the host proteome that derived from the combination of two transgenes. The second approach is based on the use of RNA interference tool prior to the comparative analysis. By enabling transgene silencing, this tool provides a means to study transgenic genotypes without the accumulation of transgenic protein, thus isolating insertional effects. The development of all these methodologies lead to an extensive literature review on transient and stable RNAi experiment in plants, which then resulted in a review article on this subject. The obtained results showed that transgene constructs do not function in a linear additive fashion, but instead alter endogenous proteome profile.

These protein modulations are mainly related to energetic and stress metabolism, which then depended on the number of copies and the nature of inserted transgene sequences. The presence of more than one inserted sequences also affects the levels of transgene expression. In addition, the proteomic analysis of silenced transgenic plants showed a low number of altered endogenous proteins, indicating that the accumulation of transgenic protein is one of the main factors that influence the modulation of the host plant proteome. Therefore, it is concluded that the new methodological approaches described and tested can provide a useful and robust scientific methodology for risk assessment of GMOs. Finally, we suggest that GMO safety regulatory bodies take into account this kind of study and requires that it becomes part of the documentation produced by technology proponents intend to commercialize new genetic transformation events.

Keywords: Genetically modified organism. Biosafety. Risk assessment. RNA interference. Gene expression.

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LISTA DE ABREVIATURAS E SIGLAS

2D-DIGE- análise diferencial de eletroforese bidimensional
2-DE- eletroforese bidimensional
ANOVA- análise da variância
APX- ascorbato peroxidase
ATP- adenosina trifosfato
Bt- *Bacillus thuringiensis*
CaMV – vírus do mosaico da couve-flor
cDNA- DNA complementar
CHAPS-3-[(3-colamidopropil) dimetil-amônio]-1-propanesulfonato
CRY- proteínas cristais derivadas de *Bacillus thuringiensis*
CTNBio- Comissão Técnica Nacional de Biossegurança
DNA- ácido desoxirribonucléico
dsRNA- RNA de fita dupla
DTT- 1,4-ditio-DL-treitol
EDTA- ácido etilenodiamino tetra-acético
ELISA- teste imunoenzimático (*Enzyme Linked Immuno Sorbent Assay*)
g- gramas
GM- geneticamente modificado(a)
hpRNA- RNA grampo (*hairpin RNA*)
ID- identidade
IEF- focalização isoeletrica
IPG- tiras de gradients de pH imobilizado
kDa: kilodalton
M- molar
m/z- razão entre massa e carga
mA: miliampere
mMin- minuto
miRNA- micro RNA
mL- mililitros
mM- milimolar
MS- espectrometria de massa
nM- nano molar
OGM- organismo geneticamente modificado(a)
P35S- promotor gênico 35S do vírus do mosaico da couve flor
PC- componente principal
PCA- análise dos componentes principais
PCR- reação em cadeia da polimerase

PTGS- silenciamento gênico pós-transcricional

RT-qPCR- reação da transcriptase reversa, seguida de reação em cadeia da polimerase.

RR- Roundup Ready®

pI- ponto isoelétrico

RQ- quantificação relativa

RISC- complexo protéico de silenciamento baseado em RNA (*RNA-induced silencing complex*)

RNA- ácido ribonucléico

RNAi- interferência por RNA

ROS- espécie reativas ao oxigênio

SDS- dodecil sulfato de sódio

siRNA- pequeno RNA interferente

Tris- Tris-[hidroximetil] aminometano

UTR- região não traduzida (*untranslated region*)

Vh- volts por hora

µg- micrograma

µL -microlitro

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1. INTRODUÇÃO

A relação entre a regulação gênica e o desenvolvimento de um organismo é um dos tópicos mais efervescentes da biologia atualmente. Não apenas porque seu conceito e significado vêm se atualizando de acordo com as novas descobertas científicas e tecnológicas da biologia molecular, mas também porque muitos fenômenos genéticos e evolutivos ainda não são bem compreendidos.

O conceito de gene passou de uma noção abstrata, na qual era vista como unidade de herança, para ser mais tarde compreendida como uma noção de entidade física e real, essencialmente uma sequência de DNA herdável. Atualmente, o conceito de gene se torna ainda mais complexo no âmbito da biologia holística, na qual o gene não faz parte do centro das discussões sobre herança e evolução. Em relação à herança, muitas mudanças conceituais estão à tona; dentre estas, destacam-se: (i) a existência de outros fatores além dos genes na composição da herança, (ii) nem todas as variações na herança são aleatórias em sua origem, (iii) certas informações adquiridas são herdadas e (iv) mudanças evolutivas podem ser resultado de processos de seleção e também de *imprinting*. O *imprinting* genômico ocorre quando certos genes são expressos apenas por um alelo, enquanto o outro é inativado por metilação de DNA.

A epigenética é um exemplo destas mudanças conceituais que ocorreram na biologia, e é operacionalmente definida como mudanças herdáveis na expressão e função dos genes que não podem ser explicadas por alterações na sequência de DNA. Tal definição implica o conceito de ambiguidade do genoma (mais de um fenótipo a partir do mesmo genoma) e o aumento de informação durante o desenvolvimento.

No entanto, muitos cientistas relutam em reconhecer a importância destes fenômenos para a evolução dos organismos; além de se manterem fiéis aos princípios do Dogma Central da Biologia, proposto por Francis Crick em 1958. O Dogma Central da Biologia define que a informação é perpetuada através da replicação do DNA e é decodificada através de dois processos: a transcrição que converte a informação do DNA em uma forma mais acessível (RNA complementar) e através da tradução que converte a informação contida no RNA em proteínas. Apesar do Dogma ainda ser válido, ele representa apenas uma das formas de controle da regulação gênica e da herança e, tampouco, deveria ser excludente em relação a estas novas descobertas.

Tecnologias e intervenções que envolvem sistemas biológicos complexos implicam incertezas e, portanto, potenciais riscos e preocupações acerca de efeitos inesperados e desconhecidos. A utilização de organismos geneticamente modificados, ou transgênicos, na lavoura é um exemplo claro de produtos derivados da biotecnologia moderna. Além, existe uma carência por informações empíricas (evidências) e consenso científico, assim como em vários tipos de incertezas incorporadas nos processos biológicos dinâmicos. Tais lacunas limitam os recursos de conhecimento e informações com os quais as agências de regulamentação podem traçar efetivo acesso aos impactos na saúde humana e meio ambiente oriundos destas novas tecnologias.

Ainda, a resiliência dos agroecossistemas no qual os organismos geneticamente modificados são introduzidos também está relacionada com a plasticidade dos processos biológicos. De maneira similar, a dinâmica das redes biológicas depende da conexão de elementos e interações que seguem “regras de harmonia”, e são fixadas durante a evolução. As regras que existem nestas conexões, enquanto permitem uma transferência rápida de sinais e reações eficientes a mudanças internas e ambientais, tornam os sistemas vivos muito resistentes a estímulos aleatórios. Entretanto, tornam-as muito sensíveis a mudanças em “componentes-chave” que estão conectados a setores de significativa importância na rede. Esta é uma das principais razões da ocorrência de “efeitos adversos” relacionados à transferência de genes e sequências de DNA de um organismo para outro ancestral filogeneticamente distante, no que diz respeito à reação imprevisível que o organismo hospedeiro pode ter para preservar sua harmonia e plasticidade. A ausência de controle do processo de integração destes genes é, *per se*, uma fonte de variabilidade não desejada.

Portanto, a aleatoriedade inerente de sistemas biológicos e a ignorância decorrente de limitações conceituais geram profundas implicações na qualidade e produção de informações científicas. Consequentemente, a falta de conhecimento científico sobre determinados processos biológicos envolve e afeta o nível de incerteza sobre potenciais efeitos adversos relacionados à aplicação tecnológica dos mesmos.

No caso dos organismos geneticamente modificados, uma grande preocupação é a ocorrência de efeitos não intencionais causados, por exemplo, pela localização da integração do transgene (ex: interrupção de

importantes *open reading frames* ou sequências regulatórias), que poderia resultar em modificações no metabolismo, nova fusão de proteínas, ou outros efeitos pleiotrópicos. Dentre estes possíveis efeitos adversos, a possibilidade da produção de novos alergênicos ou toxinas poderia comprometer a segurança alimentar e ambiental destes produtos.

Estudos que comparam organismos geneticamente modificados e o seu correspondente convencional geralmente compreendem características agronômicas e composição nutricional, e são baseados no conceito de ‘equivalência substancial’. O conceito de ‘equivalência substancial’ nunca foi adequadamente determinado; o grau de diferença entre um alimento natural e sua alternativa transgênica, bem como o limite no qual suas “substâncias” deixam de ser aceitas como “equivalentes”, não está definido em nenhum lugar. Tampouco existe uma definição exata aceita por legisladores ou pela comunidade científica.

Técnicas analíticas que avaliam o perfil molecular (*molecular profiling techniques*) de um organismo geneticamente modificado podem facilitar uma análise comparativa mais completa e holística. Essa abordagem envolve diversas tecnologias, tais como proteômica, transcriptômica e metabolômica; que estão sendo consideradas como ferramentas complementares para a avaliação de risco biológico. Tal abordagem metodológica não tem diretamente como alvo de investigação o transgene ou a transformação genética *per se*. Ao avaliar o organismo por inteiro e de maneira indiscriminada, proporciona oportunidades adicionais de se identificar potenciais efeitos adversos.

Recentemente, uma série de estudos publicados focaram na investigação de possíveis efeitos não intencionais da transformação genética e da expressão de transgenes em plantas. Muitos destes estudos são baseados em técnicas "ômicas". No entanto, os resultados de tais estudos não são consistentes ou coerentes, o que pode ser explicado pela utilização de plantas com diferentes *backgrounds* genéticos e/ou diferentes condições de crescimento, variações ambientais, variações nos métodos aplicados, entre outros. Ainda, nenhum destes estudos foi capaz de estabelecer por completo os processos biológicos que possam estar causando as alterações detectadas. Portanto, outras abordagens e técnicas devem ser conjuntamente aplicadas para investigações mais profundas sobre as possíveis causas de alterações relacionadas à introgressão de transgenes.

Desta forma, este trabalho utiliza duas novas estratégias metodológicas que se baseiam na aplicação de ferramentas da biologia molecular, a fim de contribuir na elucidação de possíveis alterações no proteoma oriundas da introgressão de transgenes em plantas. A primeira estratégia utiliza um evento de milho transgênico contendo dois insertos transgênicos que foram estaqueados, ou piramidados, por melhoramento convencional. Estes eventos são comparados com outras plantas de mesmo *background* genético que possuem apenas um dos insertos transgênicos. A comparação é feita em nível proteico através de análise proteômica comparativa com o objetivo de se compreender possíveis interações sinérgicas e antagônicas em plantas geneticamente modificadas quando mais de um transgene está presente. Na segunda estratégia, o transgene do evento de milho transgênico contendo dois insertos transgênicos estaqueados é silenciado pela técnica de interferência por RNA, a qual proporciona a análise do perfil proteico em plantas que possuem o constructo transgênico, mas não possuem a acumulação de proteínas transgênicas nas células. Assim, foi possível isolar os efeitos de cada um dos fatores envolvidos na regulação e expressão transgênica. Estas estratégias incluíram novas metodologias incluem avanços nas áreas de biologia molecular, expressão gênica, biotecnologia e biossegurança.

2. JUSTIFICATIVA

A elucidação dos processos genéticos e epigenéticos que regulam a expressão gênica de transgenes, bem como a expressão de genes endógenos em genomas transformados, ainda encontra lacunas no conhecimento científico (Traavik e Lim, 2009). Por outro lado, a adoção de organismos geneticamente modificados em larga escala, assim como o grande número de variedades comerciais híbridas de plantas geneticamente modificadas, apresentam uma oportunidade única para se estudar estes processos. Organismos geneticamente modificados podem ser considerados excelentes modelos biológicos no estudo de questões genéticas/epigenéticas e de fisiologia, pois possuem uma ampla gama de diferentes insertos, em diferentes *backgrounds* genéticos, além de serem fáceis de obter e propagar. Ainda tão importante quanto gerar conhecimento científico *per se*, ao estudar estes organismos que são comercializados também é gerado conhecimento prático sobre questões de biossegurança, de grande interesse sócio-ambiental.

O presente trabalho utilizou variedades de milho amplamente comercializadas no Estado de Santa Catarina e que possuem dois diferentes insertos transgênicos. Desses dois insertos, um deles produz duas toxinas inseticidas CRY (derivado do nome em inglês *crystalline*), comumente chamadas de Bt e que causam a morte de alguns insetos que se alimentam da planta transgênica (Gassmann *et al.*, 2014). O segundo inserto produz uma enzima chamada 5-enolpyruvylshikimate- 3-phosphate synthase isolada de *Agrobacterium* sp. linhagem CP4 (EPSPS) que participa na biossíntese de alguns aminoácidos aromáticos (Wang *et al.*, 2014). A tolerância ao herbicida à base de glifosato se dá pelos níveis elevados de expressão desta proteína na planta que, de maneira natural, teria sua atividade inibida pelo referido herbicida.

Alguns estudos já detectaram efeitos adversos da utilização de plantas geneticamente modificadas, tanto na forma de biomassa vegetal quanto na forma de grãos, em diversos organismos não-alvos, incluindo mamíferos (ex: Seralini *et al.*, 2014; Carman *et al.*, 2013; Hilbeck *et al.*, 2012; Meier *et al.*, 2012). Outros estudos que investigaram alterações bioquímicas e fisiológicas a nível celular e molecular também reportam efeitos não-esperados (ex: La Paz *et al.*, 2014; Agapito-Tenfen *et al.*, 2013; Balsamo *et al.*, 2011; Coll *et al.*, 2008; Zolla *et al.*, 2008). Os efeitos detectados estão relacionados com aumento ou diminuição da expressão do transgene e de genes endógenos que aparentemente não

participam da mesma rota metabólica do(s) transgene(s) inserido(s). Também foram observados efeitos no metabolismo de metabólitos secundários (Frank *et al.*, 2012; Barros *et al.*, 2010; Leon *et al.*, 2009; Manetti *et al.*, 2004), açúcares e outras moléculas de importância estrutural, como a lignina (Poerschmann *et al.*, 2005; Saxena *et al.*, 2001). Todavia, apesar desses estudos detectarem diversos efeitos adversos, os mesmos não conseguem gerar informação suficiente para que sejam elucidados os processos biológicos pelos quais as células transgênicas passam (Heinemann *et al.*, 2011; Batista e Oliveira, 2010).

A falta de elucidação destes processos está atrelada à falta de metodologias sensíveis e abordagens comparativas mais completas que consigam isolar os possíveis efeitos em cascata que acontecem simultaneamente na célula transgênica. Tais efeitos se estendem desde aqueles relacionados ao rompimento do genoma nativo e introdução de sequências de DNA exógenas, até aqueles oriundos da presença de grandes quantidades de proteína transgênica no ambiente celular e intracelular.

Dentro deste contexto, o presente trabalho buscou proporcionar e validar novas metodologias e abordagens que visam elucidar potenciais efeitos adversos a nível celular e molecular. A busca na literatura por protocolos pertinentes também levantou a discussão sobre como as técnicas de transformação de plantas é amplamente utilizada em modelos vegetais para o estudo da função gênica. No entanto, pouca atenção é dada aos possíveis efeitos adversos e alterações no metabolismo e fisiologia destas plantas.

3. HIPÓTESES E OBJETIVOS

3.1 GERAL

Hipótese Geral: Existem alterações no proteoma de plantas geneticamente modificadas oriundas de interações sinérgicas e antagonistas da integração e expressão de transgenes.

Objetivo Geral: Analisar possíveis alterações no proteoma de milho geneticamente modificado quando dois transgenes estão inseridos e quando a expressão do transgene é inibida.

3.2 ESPECÍFICOS

Hipótese 1: A combinação de dois ou mais transgenes na mesma planta, como em eventos transgênicos estaqueados, acarreta em alterações nos níveis de expressão de transgenes e genes endógenos que são distintas daquelas observadas em eventos transgênicos simples, com apenas um transgene sendo expresso.

Objetivo Específico 1: Quantificar a expressão relativa de transgenes em eventos transgênicos estaqueados e simples com o mesmo *background* genético e sob condições controladas de cultivo.

Objetivo Específico 2: Identificar proteínas diferencialmente expressas em milho transgênico estaqueado e simples com o mesmo *background* genético.

Hipótese 2: O rompimento e rearranjo do genoma hospedeiro pela transformação genética causam alterações no padrão de expressão de proteínas endógenas em milho geneticamente modificado.

Objetivo Específico 3: Isolar o potencial efeito da transformação genética *per se* no proteoma de milho geneticamente modificado através da inibição da tradução da proteína transgênica.

Objetivo Específico 4: Identificar proteínas endógenas diferencialmente expressas em milho geneticamente modificado com

níveis reduzidos de proteína transgênica, milho geneticamente modificado com expressão normal da proteína transgênica e milho convencional com o mesmo *background* genético e sob condições controladas de cultivo.

4. REVISÃO BIBLIOGRÁFICA

Organismo geneticamente modificado é definido no Brasil pela Lei de Biossegurança nº 11.105/2005 que descreve “um organismo geneticamente modificado é um organismo cujo material genético – ADN/ARN tenha sido modificado por qualquer técnica de engenharia genética”. E a engenharia genética é definida, por sua vez, como uma “atividade de produção e manipulação de moléculas de ADN/ARN recombinante” (Brasil, 2005).

As mudanças e avanços que ocorrem nas biotecnologias são rápidos e atingem diversos setores. Assim, a utilização de organismos geneticamente modificados (ou transgênicos) em larga escala e liberados no meio ambiente é um recente exemplo do desenvolvimento e adoção das biotecnologias modernas por diversos atores.

Segundo o relatório sobre a situação global das culturas biotecnológicas (geneticamente modificadas – GM) comercializadas em 2013, realizado pelo Serviço Internacional para Aquisição de Aplicações em Agrobiotecnologia (ISAAA), cerca de 175 milhões de hectares foram cultivados mundialmente com cultivos GM. O Brasil teria contribuído para esta soma com cerca de 40 milhões de hectares, sendo o segundo maior produtor de OGMs no mundo (ISAAA, 2013). Dentre as espécies mais cultivadas no Brasil, para as quais há disponibilidade de variedades transgênicas, destacam-se a soja, o milho e o algodão. Atualmente, 37 eventos de plantas geneticamente modificadas já possuem autorização para o plantio e comercialização em todo território nacional. Dentre elas, 19 são de milho geneticamente modificado (CTNBio, 2014). Em relação às características agrônomicas mais utilizadas em lavouras transgênicas, evidencia-se a resistência a insetos herbívoros e tolerância a herbicidas. Atualmente, a adoção por eventos transgênicos que combinam essas duas características por melhoramento convencional também tem crescido. A característica agrônômica de resistência a insetos da ordem Lepidóptera é obtida pela introdução de transgenes que expressam toxinas CRY, oriundos da bactéria *Bacillus thuringiensis* (assim também conhecidas como proteínas Bt) e transferidos para o milho (Sagstad *et al.*, 2007).

Organismos geneticamente modificados contendo transgenes que expressam esse tipo de toxina acabam expressando uma versão truncada da δ -endotoxina original derivada da bactéria *B. thuringiensis*. No caso do milho MON810, por exemplo, a proteína transgênica CRY1Ab é

encontrada na sua forma completa com peso molecular de 130kDa. Depois de ingerida pelo inseto susceptível, a pró-toxina sofre uma clivagem enzimática, perdendo sua parte N-terminal e gerando um produto de aproximadamente 70kDa, chamado de δ -endotoxina cuja maior parte é a C-terminal (Mekawi, 2010). A parte C-terminal é responsável pela formação de corpos de inclusão cristalinos, por isso o nome de proteínas CRY. A sequência de DNA do evento de milho transgênico MON810, que expressa a proteína CRY1Ab, está truncada e não possui a sequência terminadora deste transgene (Holk *et al.*, 2002). A transcrição do transgene além de sua sequência, fenômeno conhecido como *read-through*, foi detectado no milho MON810 em até 1 kbp na direção 5-3' de sequências endógenas do genoma hospedeiro, gerando transcritos poli-adenilados de diversos tamanhos (Rosati *et al.*, 2010).

As toxinas Bt são objetos de estudo de diversos grupos de pesquisa que visam investigar possíveis efeitos adversos em insetos não-alvos e em animais. Em um destes estudos, salmões foram alimentados contendo dieta com milho transgênico MON810 e, após análise, os pesquisadores verificaram que tais animais tinham maior proporção de granulócitos e monócitos, menor proporção de linfócitos, além de mudanças nas atividades das proteínas de estresse e alterações nas populações de leucócitos associados à resposta imune. Eles também concluíram que a toxina CRY é um adjuvante na mucosa e tão potente como a toxina do cólera, a qual aumenta principalmente as respostas de anticorpos IgG (em soro e intestino) (Vazquez-Padron *et al.*, 1999a, 1999b). Similarmente, dados analisados por Seralini *et al.* (2007), a partir do dossiê apresentado pela empresa proponente para a liberação do milho transgênico MON810, revelaram alterações significativas em órgãos e no sangue, tais como o aumento de basófilos, linfócitos e células brancas do sangue, diminuição do peso dos rins e aumento do açúcar no sangue. Ainda, camundongos jovens e adultos alimentados com uma dieta contendo o mesmo evento MON810 por 30 e 90 dias apresentaram alterações na porcentagem das células T e B e em CD4⁺, CD8⁺, $\gamma\delta$ T e $\alpha\beta$ T. Além disso, os autores verificaram um aumento de várias moléculas envolvidas em respostas alérgicas e inflamatórias (Finamore *et al.*, 2008).

Em relação aos efeitos adversos ambientais observados, um dos primeiros estudos reconhecidos cientificamente foi realizado por Hilbeck *et al.* (1998). Neste estudo, os autores demonstraram que 57% das larvas de *Chrysopa carnea*, um agente de controle biológico que

vive nos ecossistemas, morreram ao se alimentar de dieta contendo Bt oriunda do milho MON810. Os estudos de Ramirez-Romero *et al.* (2008) apresentaram resultados que indicam que altas concentrações (5000 ppb) da proteína CRY1Ab não causaram efeitos letais em abelhas produtoras de mel, entretanto, o seu comportamento foi afetado pois as abelhas expostas tiveram sua aprendizagem perturbada. Ainda, segundo os autores, as abelhas continuaram a responder a um odor condicionado, mesmo na ausência de uma recompensa do alimento. Os resultados deste trabalho indicam que as plantações transgênicas expressando a proteínas CRY podem afetar o consumo de alimentos ou de processos de aprendizagem e, assim, podem impactar a eficiência das abelhas no forrageamento.

Outros potenciais efeitos ecológicos relacionados a toxinas produzidas pelo milho Bt foram detectados. Rosi-Marshall *et al.* (2007) detectaram uma diminuição na taxa de crescimento ou mesmo mortalidade de espécies anfíbias aquáticas. Outro estudo realizado com *Daphnia magna*, um organismo indicador de ecotoxicologia, demonstrou que houve diminuição no valor adaptativo de *D. magna* alimentadas com milho MON810 (Bohn *et al.*, 2010).

As proteínas transgênicas EPSPS, presentes em plantas tolerantes à aplicação de herbicida à base de glifosato, também foram alvo de investigações de biossegurança. O estudo de Seralini *et al.* (2014) demonstrou evidências de efeitos adversos na saúde de ratos alimentados com milho transgênico NK603 tolerante ao herbicida Roundup Ready[®] (a partir de 11% da dieta), cultivados com ou sem a pulverização com Roundup Ready[®] (a partir de 0,1 ppb na água). Os resultados deste estudo revelaram que animais de todos os grupos tratados morreram de 2 a 3 vezes mais em relação aos animais utilizados nos tratamentos controles, e mais rapidamente. Todos os resultados observados foram dependentes do sexo dos animais. Mais além, as fêmeas desenvolveram grandes tumores mamários quase sempre mais frequentemente do que os controles. Ainda, nos machos tratados, congestões hepáticas e necrose foram 2.5-5.5 vezes superior, entre outros efeitos detectados.

A análise de risco de OGMs é composta por três fases principais: (i) a avaliação de risco, que consiste na identificação e caracterização dos possíveis riscos e que está a cargo do proponente da atividade, (ii) gerenciamento ou administração dos riscos, nos quais tanto as autoridades governamentais quanto os proponentes da tecnologia são

envolvidos e, por fim, (iii) a comunicação dos riscos, a ser feito pelo poder público à sociedade, e em particular aos grupos de risco (Amman *et al.*, 2007).

As investigações científicas que são consideradas durante o processo de análise de risco de um OGM são várias. Técnicas de análise do perfil molecular de um OGM, tais como as descritas anteriormente, são amplamente utilizadas em estudos de ciência básica e podem preencher a falta de conhecimento prévio sobre OGM (Davies, 2010; Davies *et al.*, 2010; Fears, 2007; Van Aggelen *et al.*, 2010). Tal abordagem proporciona oportunidades adicionais de identificação de potenciais efeitos adversos, pois o alvo da investigação não é diretamente o transgene ou a transformação per se (Zolla *et al.*, 2008). No entanto, se a identificação do perigo não está devidamente orientada, ela pode também produzir informações irrelevantes, gerando informações e dados que são difíceis de interpretar (Kuiper *et al.*, 2003). Apesar dessa discussão, existe um número crescente de pesquisadores que usam ou apoiam a utilização dessas técnicas (Heinemann *et al.*, 2011).

Ainda, os potenciais efeitos adversos podem estar relacionados a fatores epigenéticos que influenciam a estabilidade e expressão de transgenes. Tais fatores epigenéticos são estudados pelo ramo da biologia conhecido como ‘epigenética’. A epigenética tem diferentes significados com raízes conceituais independentes. Segundo Conrad Waddington, o estudo da epigenética envolve a investigação sobre como genótipos dão origem aos fenótipos durante o desenvolvimento do organismo. Já para Arthur Riggs, ‘epigenética’ é definida como o estudo de mudanças no padrão de herança mitótica e meiótica que não podem ser explicadas pela alteração na sequência de DNA (Bird, 2002). Para Adrian Bird, uma definição mais moderna de ‘epigenética’ estaria relacionada com as adaptações estruturais de regiões cromossômicas para que sejam registradas, sinalizadas e perpetuadas condições específicas de atividade celular.

‘Epigenética’ abrange uma ampla gama de efeitos no metabolismo e desenvolvimento de plantas e animais, com consequências que podem ser herdadas por diversas gerações. Existem dois sistemas epigenéticos clássicos: (i) o complexo Polycomb e Trithorax e (ii) a metilação de DNA. Os grupos de proteínas Polycomb (PcG) e Trithorax (trxG) são reguladores essenciais de diversos genes responsáveis pelo desenvolvimento do organismo. Essas proteínas se

ligam a regiões específicas do DNA e direcionam as modificações pós-traducionais da maquinaria de histonas para silenciar ou ativar a expressão gênica (Schuettengruber *et al.*, 2007). Desta forma, são capazes de mediar a herança epigenética de condições ativadas ou silenciadas de cromatina durante o desenvolvimento do organismo. Estudos sugerem que o mecanismo de silenciamento de genes mediados pelas proteínas PcG também envolvem o mecanismo de RNA interferente (RNAi) (Grimaud *et al.*, 2006).

Interferência por RNA é um mecanismo altamente coordenado e relacionado com a regulação gênica transcricional e pós-transcricional. Neste último caso, as etapas iniciais do processo incluem a redução de moléculas de RNA de fita dupla (dsRNA) em moléculas pequenas de RNA interferente (siRNA) através de ribonucleases III (chamadas de *Dicer*). As moléculas de siRNA, também de fita dupla, degradam moléculas-alvo de RNA mensageiro (mRNA) e microRNAs (miRNA) impedindo a completa transcrição destes genes. Moléculas de siRNA são consideradas intermediárias no processo de RNAi e têm como alvo específico a clivagem de mRNA por pareamento. A clivagem da molécula-alvo de mRNA é catalizada por enzimas do complexo do silenciamento induzido por RNA (RISC) (Khvorova *et al.*, 2003). Os miRNAs são moléculas de RNA de fita simples, com tamanho entre 19 e 24 nucleotídeos e não codificadoras de proteínas. Estas moléculas são derivadas de precursores com forma de ‘grampo-de-cabelo’ (*hairpin precursors*) e atuam como potentes reguladores pós-transcricionais da expressão gênica em plantas e animais. Estima-se que 30% dos genes codificadores de proteínas em mamíferos são silenciados através do pareamento destas moléculas às sequências complementares nas regiões não-codificantes 3’ (Kim, 2005; Zhang *et al.*, 2009).

Apesar das barreiras entre as diversas classes de pequenos RNA estarem cada vez mais difíceis de serem discernidas, algumas distinções ainda persistem. Moléculas de miRNA e siRNA são as mais distribuídas em termos filogenéticos e fisiológicos, além de ambas serem caracterizadas pela natureza de fita dupla de seus precursores. Inicialmente, moléculas de miRNA e siRNA podem ser diferenciadas de duas maneiras. Primeiramente, miRNA são considerados como endógenos, expressos propositalmente pelo próprio genoma do organismo em questão. Ao contrário, siRNA são considerados ‘gatilhos’ exógenos (*exogenous triggers*) derivados de vírus, transposons ou transgenes. Em segundo, evidências indicam que os miRNA são

processados a partir de moléculas precursoras de sequência palindrômica arranjada em alça (*stem loop*), com a característica de uma incompleta fita dupla. Já os siRNAs foram descobertos por serem originários de moléculas longas e complementares de RNA (dsRNA) (Carthew e Sontheimer, 2009).

No entanto, fica claro que ambas as moléculas também compartilham semelhanças importantes que confirmam a biogênese e o mecanismo das mesmas. Elas possuem tamanhos similares e funções de inibição baseadas na especificidade da sequência nucleotídica. Ainda, miRNA e siRNA dependem das mesmas duas famílias de proteínas: enzimas *Dicer*, que as extirpam de seus precursores; e as proteínas *Argonaute*, que auxiliam no efetivo mecanismo de silenciamento. Mais além, a união destas duas proteínas associadas às moléculas de moléculas de RNA complementares (*duplex-derived RNA*) é reconhecida como a assinatura do mecanismo de silenciamento de genes por RNA (Carthew e Sontheimer, 2009).

Evidências do papel dos miRNAs vêm sendo observadas na modulação de vários processos biológicos, incluindo a diferenciação celular, apoptose, proliferação, resposta imune e manutenção da identidade de células e tecidos (Reinhart *et al.*, 2002; Ding *et al.*, 2009). Ainda, padrões de expressão de miRNAs específicos foram descobertos por estarem relacionados com diversas condições fisiológicas e patológicas (Li *et al.*, 2010). A desregulação da expressão de miRNAs, por sua vez, mostrou-se associada ao câncer e outras doenças (Ng *et al.*, 2009). Recentemente, foi descoberto que miRNAs são estáveis no soro e plasma de humanos e animais (Zhang *et al.*, 2012).

Em relação ao segundo sistema epigenético clássico, a metilação de DNA, foi observada que a introdução de transgenes no genoma de camundongos também pode alterar extensivamente o padrão de metilação de outros genes do genoma hospedeiro. As alterações observadas não tinham especificidade, sendo encontradas por todo o genoma e, dependendo o local da inserção do transgene, os padrões eram diferentes (Heller *et al.*, 1995; Remus *et al.*, 1999). Schumacher *et al.* (2000) descobriram um efeito genótipo-específico na manutenção da metilação ao longo de diversas gerações quando investigaram a estabilidade dos padrões de metilação de transgenes inseridos também em camundongos. Tais mudanças eram observadas no início da embriogênese, antes mesmo da estabilidade do padrão de desenvolvimento somático, durante a gastrulação.

Existem evidências diretas da influência de transgenes na herança epigenética em mamíferos. Garrick *et al.* (1998) verificou um aumento nos níveis de metilação e, conseqüentemente, o silenciamento dos transgenes, quando existia um maior número de cópias do transgene integradas em um mesmo loco. A eficiência na expressão do transgene foi aumentada assim que o número de cópias do transgene e a metilação destas sequências diminuiu.

A herança de padrões de miRNAs está muito menos esclarecida. Scheir (2007) e Stitzel e Seydoux (2007) realizaram os primeiros estudos abordando a questão da transferência de moléculas de RNA de células-mãe de camundongos para os oócitos. Tal transferência sugere a manutenção dos estádios primordiais da embiogênese antes mesmo da transcrição zigótica. Em plantas, muito pouco se sabe sobre a transferência de miRNAs e a manutenção dos padrões de expressão ao longo de gerações. Existe uma forte evidência relacionada à transferência de mRNAs com função específica para o óvulo recém fertilizado. Bayer *et al.* (2009) observaram a rota de sinalização e caracterização de proteínas ligadas ao suspensor durante os primeiros estádios da embriogênese de *Arabidopsis thaliana*. Esses autores descobriram que transcritos para as proteínas do suspensor são acumulados nas células germinativas e apenas após a fertilização eles são traduzidos. Recentemente, Borges *et al.* (2011) descobriram que o silenciamento pós-transcricional envolve miRNAs para eliminar transcritos presentes em estágios anteriores do desenvolvimento, modulando, portanto, novos processos fisiológicos em *A. thaliana*. Esses autores sugerem que os miRNAs também podem ser transferidos de células-mãe para células descendentes, tendo um papel importante como moléculas sinalizadoras e, possivelmente, atuando nos padrões de desenvolvimento do embrião. Desta forma, serviriam como peças-chave na reprogramação do zigoto em estágios primordiais, antes mesmo da expressão gênica do zigoto estar estabelecida.

5. CAPÍTULO I

Effect of stacking insecticidal CRY and herbicide tolerance EPSPS proteins on transgenic maize leaf proteome

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RESUMO

A mais recente comercialização de plantas transgênicas no Brasil e no mundo compreende eventos transgênicos estaqueados. Estes eventos são produzidos a partir do cruzamento convencional de outros eventos transgênicos. As plantas transgênicas estaqueadas são tratadas como novos organismos geneticamente modificados por órgãos regulatórios de diversos países e, portanto, requerem a avaliação dos potenciais riscos ambientais e riscos à saúde. Apesar de esta avaliação ser simplificada, ela deve garantir que efeitos adversos não intencionais devam ser detectados antes da liberação no meio ambiente. Técnicas de análise molecular podem ser consideradas ferramentas úteis para preencher as lacunas do conhecimento relacionadas à biossegurança de organismos geneticamente modificados. Este estudo apresenta os primeiros resultados da análise proteômica diferencial combinada com a análise da expressão dos transgenes estaqueados de híbridos comerciais de milho transgênico sob o mesmo background genético. O milho transgênico estaqueado contém transgenes inseticidas *cry* e tolerância ao herbicida à base de glifosato. Os resultados mostram que as proteínas expressas em ambos os eventos contendo transgenes *cry* foram a principal fonte de influência para o perfil proteico dos milhos transgênicos, seguida pela expressão de proteínas RR que conferem tolerância ao herbicida em questão. Vinte e duas proteínas mostraram-se

¹ Este capítulo trata do manuscrito submetido à publicação em revista científica.

diferencialmente moduladas em eventos transgênicos estaqueados e simples versus milho isogênico não-transgênico e uma variedade local de milho crioulo. Estas proteínas foram identificadas e a maioria delas esta relacionada ao metabolismo de energético. Além disso, os níveis de expressão dos transgenes estaqueados tiveram uma redução significativa de cerca de 30% quando comparado com as variedades híbridas de evento simples. Tais observações indicam que o estaqueamento de dois ou mais transgenes no genoma de apenas uma variedade de milho híbrido pode ter impacto na expressão total de genes endógenos. As alterações nos níveis de proteínas endógenas observadas foram além da variabilidade natural encontrada nas variedades de milho utilizadas neste estudo, incluindo a variedade crioula. Assim que bancos de dados globais sobre resultados de análises "omicas" tornam-se disponíveis, estes poderiam fornecer um referencial altamente desejável para a avaliação da segurança de eventos transgênicos estaqueados e simples. No entanto, mais estudos devem ser realizados a fim de abordar a relevância biológica e implicações de tais alterações.

5.1 ABSTRACT

The safe use of stacked transgenic crops in agriculture requires their environmental and health risk assessment, in which unintended adverse effects are expected to be assessed prior to their release in the environment. Molecular profiling techniques can be considered useful tools to address emerging biosafety gaps. Here we report the first results of a proteomic profiling coupled to transgene transcript expression analysis of this unique set of stacked commercial maize hybrid containing insecticidal and herbicide tolerant traits in comparison to the single event hybrids under the same genetic background. Our results show that CRY proteins expressed in both single and stacked event were the major source of influence to the expression pattern of genetically modified (GM) maize proteome followed by the expression of RR proteins also from both stacked and single events. Twenty-two proteins were shown to be differentially modulated in stacked and single GM events versus non-GM isogenic maize and a landrace variety with Brazilian genetic background. These proteins were mainly assigned to the energy/carbohydrate metabolism. Furthermore, stacked transgene expression levels had a significant reduction of about 30% when compared to single event hybrid varieties. Such observations indicate

that stacking two transgenic inserts into the genome of one GM maize hybrid variety may impact the overall expression of endogenous genes. Observed protein changes differ significantly from single event lines and conventional counterpart. Some of the protein modulation did not fall within the range of the natural variability found in the landrace used in this study. The identification of proteins related to the energy/carbohydrate metabolism suggests that the energetic homeostasis in stacked versus single event hybrid varieties differ and that this might be related to the higher demand of transgenic protein production by the stacked transgenic plant cell. As global databases on outputs from “omics” analysis become available, these could provide a highly desirable benchmark for the safety assessment of stacked transgenic crop events. Nevertheless, further studies should be conducted in order to address the biological relevance and implications of such changes.

5.2 BACKGROUND

The first decade of GM crop production has been dominated by genetically modified (GM) plants containing herbicide tolerance traits, mainly based on Roundup Ready® herbicide (Monsanto Company) spray, and by insect protection conferred by CRY proteins-related traits, also called ‘Bt toxins’. More recently, GM crop cultivation has been following a trend of products combining both traits by traditional breeding. In the existing literature, such combinations are referred to as “stacked” or “pyramided” traits or events (Taverniers et al., 2008). In recent years, an increasing number of GM plants that combine two or more transgenic traits reached about 47 million hectares equivalent to 27% of the 175 million hectares planted with transgenic crops worldwide in 2013, up from 43.7 million hectares or 26% of the 170 million hectares in 2012 (James, 2013).

According to current regulatory practice within the EU, stacked events are considered as new GM organism: prior to marketing they need regulatory approval, including an assessment of their safety, similar to single events (De Schrijver et al., 2007). In other countries, like Brazil, stacked events are also considered new GMOs but do not require full risk assessments if single parental events have been already approved. In other words, there is a simplified risk assessment procedure (provided by Normative Resolution n° 8/2009) that requires less safety studies than those under first time approval (CTNBio, 2009). In United

States, for example, this may not even be obligatory (Kuiper et al., 2001).

To accomplish the current international guidance on risk assessment of stacked GM events, additional information on the stability of transgene insertions, expression levels and potential antagonistic or synergistic interactions on transgenic proteins should be provided (EFSA, 2007; AHTEG, 2013).

Literature on molecular characterization of GM stacked events is scarce, and the comparison of their expression levels and potential cellular interaction to parental single GM lines is absent. Few recent studies about the possible ecological effects of stacked GM crops have been published, but many lack the comparison to the GM single lines or even the near-isogenic non-transgenic line (Schuppener et al., 2012; Hendriksma et al., 2013; Hardisty et al., 2013). In addition, the approach taken by these authors, which was to assess potential adverse effects of stacked transgenic crop products such as pollen and grain, does not establish the unique effects of stacking two or more transgenic inserts. Neither have they identified intended and unintended differences and equivalences between the GM plant and its comparator(s). Earlier literature also failed to recognize potential interaction between the events present and their stability. These genetically modified plants containing stacked events cannot be considered generally recognized as safe in general terms without specific supporting evidence (De Schrijver et al., 2007).

Profiling technologies, such as proteomics, allow the simultaneous measurement and comparison of thousands of plant components without prior knowledge of their identity (Heinemann et al., 2011). The combination of non-targeted methods facilitates a more comprehensive approach than targeted methods alone and thus provides additional opportunities to identify unintended effects of the genetic modification (Ruebelt et al., 2006).

Therefore, our novel approach uses proteomic as a molecular profiling technique to identify potential unintended effects resulting from the interbreeding of GM varieties (e.g. synergistic or antagonistic interactions of the transgenic proteins). The aim of this study was to evaluate the protein changes in stacked versus single event and control plants under a highly controlled condition, to examine the expression levels of transgenic transcripts under different transgene dosage and to provide insight into the formulation of specific guidelines for the risk

assessment of stacked events. We hypothesized that the combination of two transgenes could differentially modulate endogenous protein expression and that might have an effect in the plant metabolism and physiology. In addition, the expression of two transgenes may be altered in GM stacked events relative to single transformation events. To test these hypotheses, we have used GM stacked maize genotype containing cry1A.105/cry2Ab2 and epsps cassettes expressing both and insect resistance and herbicide tolerance as unlinked traits, as well as genotypes of each single transgene alone, being all maize hybrids under the same genetic background. This unique set of stacked and single maize events developed under the same genetic background, plus the conventional near-isogenic counterpart and a landrace variety enables the isolation of potential effects derived from stacking two transgenes. Finally, we have performed two dimensional differential gel electrophoresis analysis (2D-DIGE) and quantitative Real-Time PCR experiment (RT-qPCR) to determine differences in the proteome and transcription levels of transgene between stacked and single events.

5.3 METHODS

5.3.1 Plant material and growth chamber conditions

Five maize varieties were used in this study. Two of them are non-genetically modified maize seeds, the hybrid AG8025 (named here as ‘conventional’) from Sementes Agroceres and the open pollinated variety Pixurum 5 (named here as ‘landrace’). Pixurum 5 is an open pollinated variety (OPV) that has been developed and maintained by small farmers in South Brazil for around 16 years (Canci, 2004).

The other three varieties are genetically modified and have the same genetic background as the conventional variety since they are produced from the same endogamic parental lines. These are: AG8025RR2 (unique identifier MON-00603-6 from Monsanto Company, glyphosate herbicide tolerance, Sementes Agroceres); AG8025PRO (unique identifier MON-89034-3 from Monsanto Company, resistance to lepidopteran pests, Sementes Agroceres) and AG8025PRO2 (unique identifier MON-89034-3 x MON-00603-6 from Monsanto Company, stacked event resistance to lepidopteran pests and glyphosate herbicide tolerance, Sementes Agroceres). These are named in this study as RR, Bt and RRxBt, respectively. The AG8025

variety is the hybrid progeny of the single-cross between maternal endogamous line “A” with the paternal endogamous line “B”. Thus, the used commercial hybrid variety seeds have high genetic similarity (most seeds should be AB genotype).

The cultivation of MON-ØØ6Ø3-6, MON-89Ø34-3, and MON-89Ø34-3 x MON-ØØ6Ø3-6 has been approved in Brazil in 2007, 2008 and 2010, respectively (CTNBio, 2007, 2008 and 2010). The stacked hybrid MON-89Ø34-3 x MON-ØØ6Ø3-6 expresses two insecticidal proteins (Cry1A.105 and Cry2Ab2 proteins derived from *Bacillus thuringiensis*, which are active against certain lepidopteran insect species) and a protein providing tolerance to the herbicide glyphosate (CP4 EPSPS) (SCBD, 2014). The novel traits of each parent line have been combined through traditional plant breeding, to produce this new hybrid. The experimental approach currently applied to the comparative assessment requires the use of conventional counterpart and the single-event counterparts, all with genetic background as close as possible to the GM plant, as control (Codex, 2003; AHTEG, 2013; EFSA 2013).

After the confirmation by PCR of two transgenic events in GM seeds and the absence in the conventional and landrace ones (data not shown), the seeds from all the five varieties were grown side by side in growth chamber (EletrolabTM model 202/3) set to 16 h light period and 25°C (\pm 2°C). Seedlings were germinated and grown on Plantmax HT substrate (Buschle & Lepper S.A.) and watered daily. No pesticide or fertilizer was either applied. Fifteen plants were randomly sampled per maize variety (genotype). Out of these, three groups of five plants were pooled; these were considered biological replicates. Maize leaves were collected at V4 stage (20 days after seedling). Leaf pieces were cut, weighted and placed in 3.8 ml cryogenic tubes before immersion in liquid nitrogen. The samples were kept at -80°C until RNA and protein extraction.

5.3.2. RNA isolation and relative quantification analysis of transgene expression

RNA was extracted from approximately 100 mg of frozen leaf tissue using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. In brief, sample was homogenized with guanidine-isothiocyanate lysis buffer and further purified using silica-membrane. During purification, in-column DNA

digestion was performed using RNase-free DNase I supplied by Qiagen to eliminate any remaining DNA prior to reverse transcription and real-time PCR. The extracted RNA was quantified using NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, USA).

Reverse-transcription real-time PCR (RT-qPCR) assay was adapted from previously developed assays for the specific detection of MON-89Ø34-3 x MON-ØØ6Ø3-6 transgenes (CRL-GMFF, 2008) to hydrolysis ZEN - Iowa Black® Fluorescent Quencher (ZEN/ IBFQ) probe chemistry (Integrated DNA Technologies, INC Iowa, USA).

Following quantification, cDNA was synthesized and amplification of each target gene was performed using the QuantiTect Probe RT-PCR Kit (Qiagen) according to manufacturer instructions. RT-qPCR experiment was carried out in triplicates using StepOne™ Real-Time PCR System (Applied Biosystems, Singapore, Singapore). Each 20 µl reaction volume comprised 10 uM of each primer and probe and 50 ng of total RNA from each sample. The amplification efficiency was calculated from relative standard curves provided for each primer and calculated according to PffafI (2001).

The two most suitable endogenous reference genes out of five candidates (ubiquitin carrier protein, folylpolyglutamate synthase, leunig, cullin, and membrane protein PB1A10.07c) were selected as internal standard. The candidate genes were chosen based on the previous work of Manoli et al. (2012). The selection of the two best endogenous reference genes for this study was performed using NormFinder (Molecular Diagnostic Laboratory, Aarhus University Hospital Skejby, Denmark) statistical algorithms (Andersen et al., 2004). Multiple algorithms have been devised to process RT-qPCR quantification cycle (Cq). However, NormFinder algorithm has the capability to estimate both intragroup and intergroup variance, and the identification of the two reference genes as most stable normalizers (Latham et al., 2010). The leunig and membrane protein PB1A10.07c genes were used to normalize *epsps*, *cry1a.105* and *cry2ab2* mRNA data due to their best stability value (SV for best combination of two genes 0.025, data not shown). Conventional samples were also analyzed in order to check for PCR and/or seed contaminants. Primer and probe sequences used, as well as Genebank ID of target genes, are provided in Additional file 1. The primers and probes were assessed for their specificity with respect to known splice variants and single-nucleotide polymorphism positions documented in transcript and single-nucleotide

polymorphism databases.

The relative expression ratio value (RQ) was calculated for stacked transgenic event samples relative to one of the three-pooled samples correspondent to the single transgenic event according to the Pfaffl equation (Pfaffl, 2001).

5.3.3. Protein extraction and fluorescence hybridization

Approximately 100 mg of each sample was separately ground-up in a mortar with liquid nitrogen and protein extraction was subsequently carried out according to Carpentier et al. (2005) with some modification. Phenol extraction and subsequent methanol/ammonium acetate precipitation were performed and PMSF was used as protease inhibitor. Pellets were re-suspended in an urea/thiourea buffer compatible to further fluorescent labeling (4% w/v CHAPS, 5 mM PMSF, 7 M urea, 2 M thiourea and 30 mM Tris-base). Protein quantification was determined by means of copper-based method using 2-D Quant Kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Before sample storage in -80°C , 80 μg of each protein sample pool were labeled with 400 $\mu\text{mol}/\text{ul}$ of CyDye DIGE fluors (Cy3 and Cy5; GE Healthcare), according to the manufacturer's instructions. An internal standard was used in every run for normalization; this was labeled with Cy2. The internal standard is a mixture of equal amounts of each plant variety sample. After protein-fluor hybridization, samples were treated with lysine (10 mM) to stop the reaction and then mixed together for two-dimensional (2-D) DIGE gel electrophoresis separation. Samples pairs were randomly selected for bi-dimensional electrophoresis runs.

5.3.4. 2-D DIGE gel electrophoresis conditions

After protein labeling, samples were prepared for the isoelectric focusing (IEF) step. Strip gels of 24 cm and a linear pH range of 4-7 (GE Healthcare) were used. Strips were initially rehydrated with labeled protein samples (7 M urea, 2 M thiourea, 2% w/v CHAPS, 0.5% v/v IPG buffer (GE Healthcare), 2% DTT). Strips were then processed using an Ettan IPGPhor IEF system (GE Healthcare) in a total of 35000 $\text{Volts}\cdot\text{h}^{-1}$ and, subsequently, reduced and alkylated for 30 min under slow agitation in Tris-HCl solution (75 mM), pH 8.8, containing 2% w/v SDS, 29.3% v/v glycerol, 6 M urea, 1% w/v DTT and 2.5% w/v

iodocetamide. Strips were placed on top of SDS-PAGE gels (12%, homogeneous) and used in the second dimension run with a Hoefer DALT system (GE Healthcare). 2-D gel electrophoresis conditions were performed as described by Weiss and Görg (2008). Gels were immediately scanned with the FLA-9000 modular image scanner (Fujifilm Lifescience, Dusseldorf, Germany). To ensure maximum pixel intensity between 60 000 and 90 000 pixels for the three dyes, all gels were scanned at a 100 μm resolution and the photo multiplier tube (PMT) voltage was set between 500 and 700 V.

Preparative gels for each plant variety were also performed in order to extract relevant spots. These were performed with a 450 μg load of total protein pools in 24 cm gels from each variety, separately, and stained with coomassie brilliant blue G-250 colloidal (MS/MS compatible) as described by Agapito-Tenfen et al. (2013).

5.3.5. Image analysis

The scanned gel images were transferred to the ImageQuant V8.1 software package (GE Healthcare) for multiplexing colored DIGE images. After cropping, the images were exported to the software ImageMasterTM 2D Platinum 7.0, version 7.06 (GE Healthcare) for cross comparisons between gels. Automatic spots co-detection of each gel was performed followed by normalization with the corresponding internal standard and matching of biological replicates and varieties. Manual verification of matching spots was applied. This process results in highly accurate volume ratio calculations. Landmarks and other annotations were applied for determination of spot experimental mass and pI.

5.3.6. In-gel digestion and protein identification by MS/MS

Spots from preparative gels were excised and sent to the Proteomic Platform Laboratory at the University of Tromsø - Norway for processing and analysis. These were subjected to in-gel reduction, alkylation, and tryptic digestion using 2–10 $\text{ng}/\mu\text{l}$ trypsin (V511A; Promega) (Shevchenko et al., 1996). Peptide mixtures containing 0.5% formic acid were loaded onto a nano ACQUITY Ultra Performance LC System (Waters Massachusetts, USA), containing a 5- μm Symmetry C18 Trap column (180 μm \times 20 mm; Waters) in front of a 1.7- μm

BEH130 C18 analytical column (100 μm \times 100 mm; Waters). Peptides were separated with a gradient of 5–95% acetonitrile, 0.1% formic acid, with a flow of 0.4 $\mu\text{l}/\text{min}$ eluted to a Q-TOF Ultima mass spectrometer (Micromass; Waters). The samples were run in data dependent tandem MS mode. Peak lists were generated from MS/MS by the Protein Lynx Global server software (version 2.2; Waters). The resulting ‘pkl’ files were searched against the NCBI nr 20140323 protein sequence databases using Mascot MS/MS ion search (Matrix Sciences; <http://matrixscience.com>). The taxonomy used was Viridiplantae (Green Plants) and ‘all entries’ and ‘contaminants’ for contamination verification. The following parameters were adopted for database searches: complete carbamidomethylation of cysteines and partial oxidation of methionines; peptide mass tolerance \pm 100 ppm; fragment mass tolerance \pm 0.1 Da; missed cleavages 1; and significance threshold level ($P < 0.05$) for Mascot scores ($-10 \text{ Log } (P)$). Even though high Mascot scores are obtained with significant values, a combination of automated database searches and manual interpretation of peptide fragmentation spectra were used to validate protein assignments. Molecular functions and cellular components of proteins were compared against ExPASy Bioinformatics Resource Portal (Swiss Institute for Bioinformatics; <http://expasy.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology system database release 69.0 2014 (<http://kegg.jp/kegg/ko.html>).

5.3.7. Statistical Analysis

Real-time relative quantification data were plotted and manually analyzed using Microsoft Excel (Microsoft, Redmond, WA). Normalized gene expression data was obtained using the Pfaffl method for efficiency correction (Pfaffl, 2001). C_q average from each technical replicate was calculated for each biological replicate and used to make a statistical comparison of the genotypes/treatment based on the standard deviation. Information on real-time data for this study has followed guidelines from the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Bustin et al., 2009).

The main sources of variation in the 2-D DIGE experiment dataset were evaluated by unsupervised multivariate PCA, using Euclidean distance for quantitative analysis. PCA analyses were performed by examining the correlation similarities between the

observed measures. The spot volume ratio was analyzed using covariance matrix on Multibase Excel Add-in software version 2013 (Numerical Dynamics, 2013). For the 2-D DIGE experiment, one-way ANOVA was used to investigate differences at individual protein levels. Tukey test at $P < 0.05$ was used to compare the multiple means in the dataset using Assisat software (Silva and Azeved, 2002). The calculations were performed on normalized spot volume ratios based on the total intensity of valid spots in a single gel. Differences at the level $P < 0.05$ were considered statistically significant. Statistical analyses were performed using ImageMasterTM 2D Platinum 7.0, version 7.06 (GE Healthcare).

5.4. RESULTS AND DISCUSSION

To examine potential unintended effects of combining transgenes by conventional breeding techniques, the protein expression profile, as well as transgenic mRNA expression, of stacked genetically modified maize leaves expressing insecticidal and herbicide tolerance proteins were evaluated in comparison to four other maize genotypes, being two single event GM hybrids with the same genetic background, the conventional counterpart non-GM hybrid AG8025 and a landrace variety (Pixurum 5) exposed to highly controlled growth conditions.

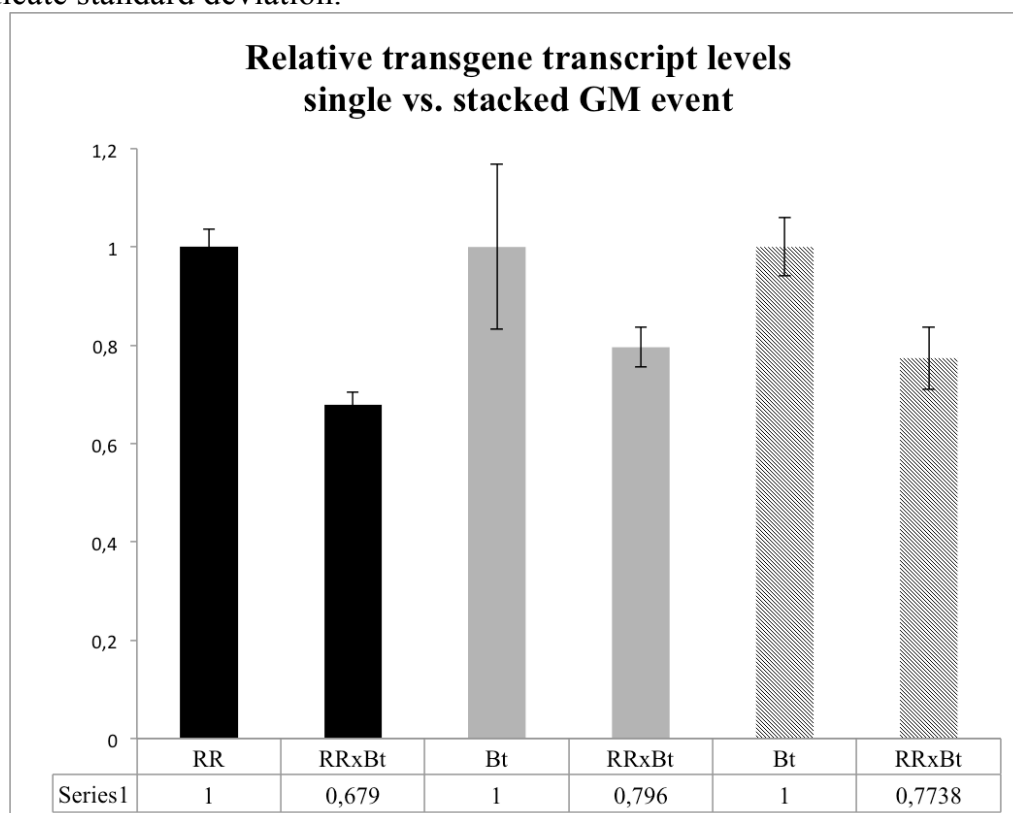
5.4.1. Transcription levels of *epsps*, *cry1A.105* and *cry2Ab2* in leaves of stacked GM

The levels of transgene mRNA accumulation were analyzed in leaves of stacked relative to single transgenic maize varieties containing *epsps* and/or *cry* genes by RT-qPCR. In order to minimize the influence of environmental conditions, we used leaves of V4-stage plants grown in parallel under controlled conditions. Figure 1 shows the *epsps*, *cry1A.105* and *cry2Ab2* mRNA relative values of three biological replicates (each being a pool of five plants) that were independently analyzed with respective primers (information on each primer pair and target gene is provided in Additional file 1 in *Apêndice*).

A clear reduction on transcript levels of all three transgenes is observed in stacked genetically modified maize plants (Figure 1). In the case of *epsps* transgene, the average reduction in transcript expression was approximately 32%. Both *cry* transgenes have similar decreased

levels of transcript accumulation. Transgene *cry1A.105* showed reduction of transgene expression to an average of 20%, whereas *cry2Ab2* transgene had 23%.

Figure 1. Transgene transcripts relative expression levels measured by delta-delta Cq method and Pffaf1 (2001) correction equation. The *epsps*, *cry1A.105* and *cry2Ab2* transgenes were quantified from stacked versus single transgenic maize events grown under controlled conditions at V3 stage were used in this analysis. Samples are means of three pools, each derived from five different plants. 'RR' samples are transgenic maize seedlings from MON-00603-6 event, 'Bt' samples are from MON-89034-3 event, and 'RRxBt' samples are transgenic maize seedlings from MON-89034-3 x MON-00603-6 event. Bars indicate standard deviation.



Maintenance patterns of gene repression or activation are governed by regulatory machinery acting at multiple levels: 1) transcription; 2) mRNA processing, export from the nucleus, translation, and degradation; 3) protein folding, modification, transport, and degradation. Thus, the control of gene expression is well coordinated and highly hierarchical, with transcription initiation control situated at the top of the regulatory sequence region. Various reasons have been

raised to explain variation in transgene expression levels among transformants that are not dependent on the insert copy number and imply that integrated transgenes cannot be regarded as independent transcription units (Stam et al., 1997). Nonetheless, the number of transgenes present in one genome can involve transgene/transgene interactions that might occur when homologous DNA sequences (e.g. expression controlling elements) are brought together (Fagard and Vaucheret, 2000). Homology-dependent gene silencing has been revealed in several organisms as a result of the introduction of transgenes (Park et al., 1996; Matzke and Matzke, 1998; Dong et al., 2001; Weld et al., 2001; Kohli et al., 2003). Gene silencing as a consequence of sequence duplications is particularly diffused among plant species. The introduction of transgenes in plants produces at least two different homology-dependent gene-silencing phenomena: post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) (Cogoni and Macino, 1999).

Typically one T-DNA exerts a dominant epigenetic silencing effect on another transgene on a second (unlinked) T-DNA in trans. Silencing is often correlated with hypermethylation of the silenced gene, which can persist after removal of the silencing insert. The results reported by Daxinger et al. (2008) imply that gene silencing mediated by 35S promoter homology between transgenes and T-DNAs used for insertional mutagenesis is a common problem and occurs in tagged lines from different collections.

Both transgenes *epsps* and *cryIA.105* present in the stacked line used in this study are controlled by homologous P35S. Whether silencing of 35S promoter in stacked events might be mediated by TGS or PTGS and the processes is not yet clear and requires further investigation. To the best of our knowledge this is the first report on reduced levels of transgenic transcripts in commercial stacked GM varieties.

In addition, the reduction of transgene expression might be also related to the high energetic demand of the cell. In this regard, increasing evidence supports the idea that constitutive promoters involve a high energetic cost and yield a penalty in transgenic plants (Rus et al. 2001; Grover et al. 2003; Pineda 2005; Muñoz-Mayor et al. 2008). In fact, results from research on salt tolerance suggest that the greater Na^+ exclusion ability of the homozygous transgenic line over-expressing *HAL1* induces a greater use of organic solutes, which seems to have an

energy cost and hence a growth penalty that reverts negatively on fruit yield (Muñoz-Mayor et al., 2008). These previous observations bring light to another possible explanation to the fact that stacked genetically modified events have reduced expression levels of transgenic proteins compared to their single event counterpart. The stacked event used in this study combines three constitutive promoter sequences, being two of P35S (derived from Cauliflower mosaic virus, CaMV) and one of FMV (derived from figwort mosaic virus).

It is important to note that changes in the level of expression of the introduced proteins in the GM stacked event compared to the single GM might affect its safety on a case-by-case basis. Higher levels of transgene expression might result in unintended adverse effects on non-target organisms, and lower levels might lead to increased risk for insect-resistance evolution of target organisms. Moreover, in cases which the expression level of an introduced/modified trait in GM stacked event falls outside the range of what was determined in the parental line, a re-evaluation of the environmental aspects might be necessary, if considered relevant (De Schijver et al., 2007).

Interestingly, there is a lack of published scientific literature on expression levels in stacked versus single genetically modified crops, being that on the market or not. For commercialized transgenic crop events that are subject to regulatory requirements, such as the Commission Implementation Regulation (EU) N° 503/ 2013, (i) stability of the inserts; (ii) expression of the introduced genes and their gene products; and (iii) potential synergistic or antagonistic effects analyses are mandatory (Kok et al., 2014). Although data on expression levels on such GM stacked events must be available for approved events, these are rarely disclosed or somewhat considered insufficient (Spök et al., 2007; Nielsen, 2013).

5.4.2. Proteomic profile of stacked Bt and RR transgenic maize

The proteomic profiles using 2-D DIGE were determined by the use of three biological replicates, each consisting of a pool of 5 plants, and fluorescent protein labeling. Quantitative protein differences between stacked GM hybrid and its single GM events, comparable near-isogenic non-transgenic hybrid and a landrace variety were investigated by comparison of twenty-three 2-D DIGE gel images. Experimental variations have been normalized by the use of a pooled internal standard

sample, which has run together in all gels.

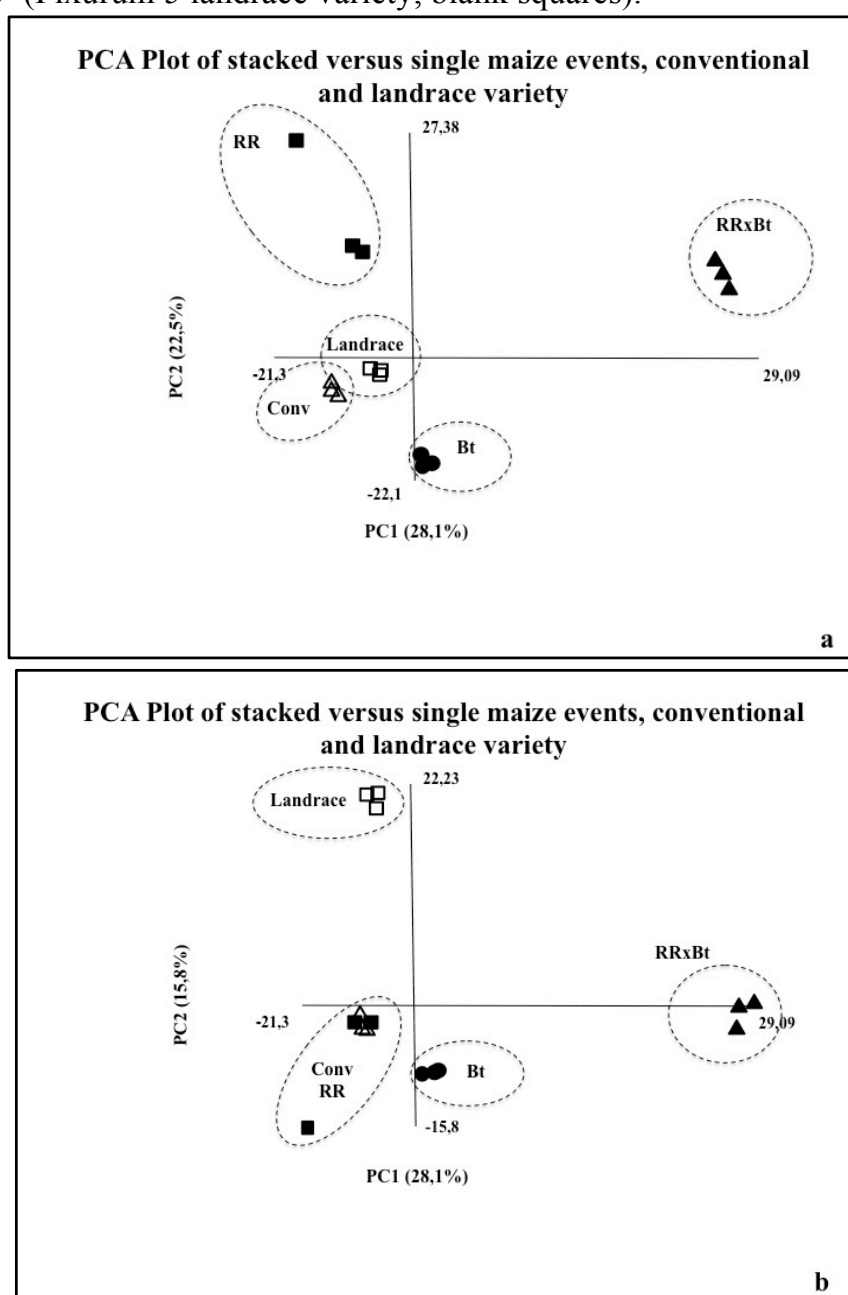
The total protein content mean was $1.43 \pm 0.6 \text{ mg.g}^{-1}$ of fresh weight. No statistically significant difference was found within replicates and treatments. The between genotype comparison showed divergence using one-way ANOVA, followed by Tukey ($P < 0.05$). Conventional, landrace and Bt samples had higher amounts of proteins extracted. Bt samples did not differ from RRxBt samples, which had higher amounts of protein extracted compared to RR (Tukey HSD = 0.76). The difference in the amount of protein extracted between plant genotypes did not affect the total number of spots resolved in the gel once sample loads were normalized to 80 ug per gel. The average number of spots detected (1123) on the 2-D DIGE gels showed similar pattern and they were considered well resolved for 24 cm fluorescent gel. No statistically significant differences ($P < 0.05$) were found between plant genotypes.

In two dimensional gel electrophoresis, the lack of reproducibility between gels leads to significant system variability making it difficult to distinguish between technical variation and induced biological change. On the other hand, the methodological approach used in the present work, called 2D-DIGE, provides a platform for controlling variation due to sample preparation, protein separation and difference detection due to the fluorescent labeling and the co-migration of treatment and control samples in the same gel (Lilley and Friedman, 2004; Marouga et al., 2005; Minden et al., 2009). Nonetheless, each 2-D DIGE run consisted of three samples, two of which were randomly selected from all plant variety samples and one being an internal standard used in all runs for normalization purposes.

5.4.3. Principal Component Analysis

Principal Component Analysis was used to demonstrate similarities in protein quantity between different gels and to gain insight into possible proteome x transgene interactions in the dataset. In the analysis of the PCA, the first four eigenvalues corresponded to approximately 80% of accumulated contribution. All fifteen samples were represented 2-dimensionally using their PC1, PC2 and PC3 scores (in two separated plots), revealing groups of samples based on around 66% of all variability (Figure 2a and 2b). This analysis showed a

Figure 2. PCA score plots of proteome data of genetically modified stacked and single events, non-genetically modified near-isogenic variety, and landrace maize variety. Proteome data was obtained by 2D-DIGE analysis from leaf tissue of maize plants grown under controlled conditions. PC1 and PC2 (a) and PC1 and PC3 (b) show the results of ‘RR’ samples (transgenic maize seedlings from MON-00603-6 event, filled squares), ‘Bt’ samples (MON-89034-3 event, filled circles), ‘RRxBt’ samples (transgenic maize seedlings from MON-89034-3 x MON-00603-6 event, filled triangles), ‘CONV’ samples (conventional non-transgenic near isogenic maize variety, blank triangles), and ‘landrace’ (Pixurum 5 landrace variety, blank squares).



complete separation in the first plot (PC1 x PC2) between the transgenic events containing insecticidal CRY proteins and other maize varieties that do not express those (the conventional, the landrace and the RR transgenic event), which explained 28.1% of the total variation (F1 values below -21.3 and above +29.9, respectively). The Principal Component 2 explained 22.5% of the variation and showed a separation of plant genotypes containing RR transgene.

Our previous investigation using another Bt event (MON-ØØ81Ø-6) grown under two different agroecosystems showed that the environment was the major source and accounted for 20% of the total variation; however, the different genotypes (Bt and comparable conventional) accounted for the second major variation source, about 9% (Agapito-Tenfen et al., 2013).

Barros et al. (2010) used the same RR transgenic event utilized in the present study and a different Bt event (MON-ØØ81Ø-6) under the same genetic background and found an interesting proteomic pattern that accounted to 31% of the total variation in their dataset. RR maize samples were grouped separately from Bt and conventional samples grown at field conditions. This pattern was also observed in their microarray and gas chromatographic / mass spectrometric metabolite profile analysis. Even when the environment or the plant genetic background accounts for the majority of the quantitative data variation, transgenic and their conventional near-isogenic varieties are frequently observed in separated groups by PCA (Coll et al., 2010).

In our second plot (PC1 x PC3) another clear separation was observed for landrace samples, thus explaining 15.6% of the variation in the full dataset (Figure 2b). Differently from expected, the landrace variety did not account for the majority of the variation in dataset. There was virtually no variation between biological replicates within each plant variety, but pool 2 from RR samples seems to deviate from other replicates. Although 66.2% of the variation might represent the majority of the total variation, careful must be taken when interpreting these results because other sources of variation might be present in the next factors.

The inclusion of a landrace variety in this study aimed at considering the extent of protein variation in the proteome of different maize genetic backgrounds, as well as to possibly detect differences in GM lines that might fit or not within the variation observed in non-modified germplasm. It should be highlighted that this is not a

requirement of international guidelines addressing the issue of comparative assessments for the environmental and health risk analysis of GM plants (AHTEG 2013), mainly because the presence of a biological relevant difference unique to the GMO being evaluated is not dependent of the overall variation observed in particular environment \times gene scenarios or breeding conditions (Heinemann et al., 2011).

A landrace variety was also included in the comparative analysis of potato tuber proteomes of genetically modified potato varieties (Lehesranta et al., 2005). These authors have found an extensive genotypic variation when analyzing around 25 genetically modified, non-modified and landrace varieties, with most of the proteins detected showing significant quantitative and qualitative differences between one or more varieties and landraces. Unfortunately, these authors have not plotted all the varieties in the same PCA.

Taken together, these results showed the relevance of detecting major sources of variation in the experiment dataset. Thus, for benchmarking and comparative analysis approaches, the deployment of broader scale, less biased analytical approaches for GM safety assessment should also embrace the issues of sources and extents of variation (Davies, 2012).

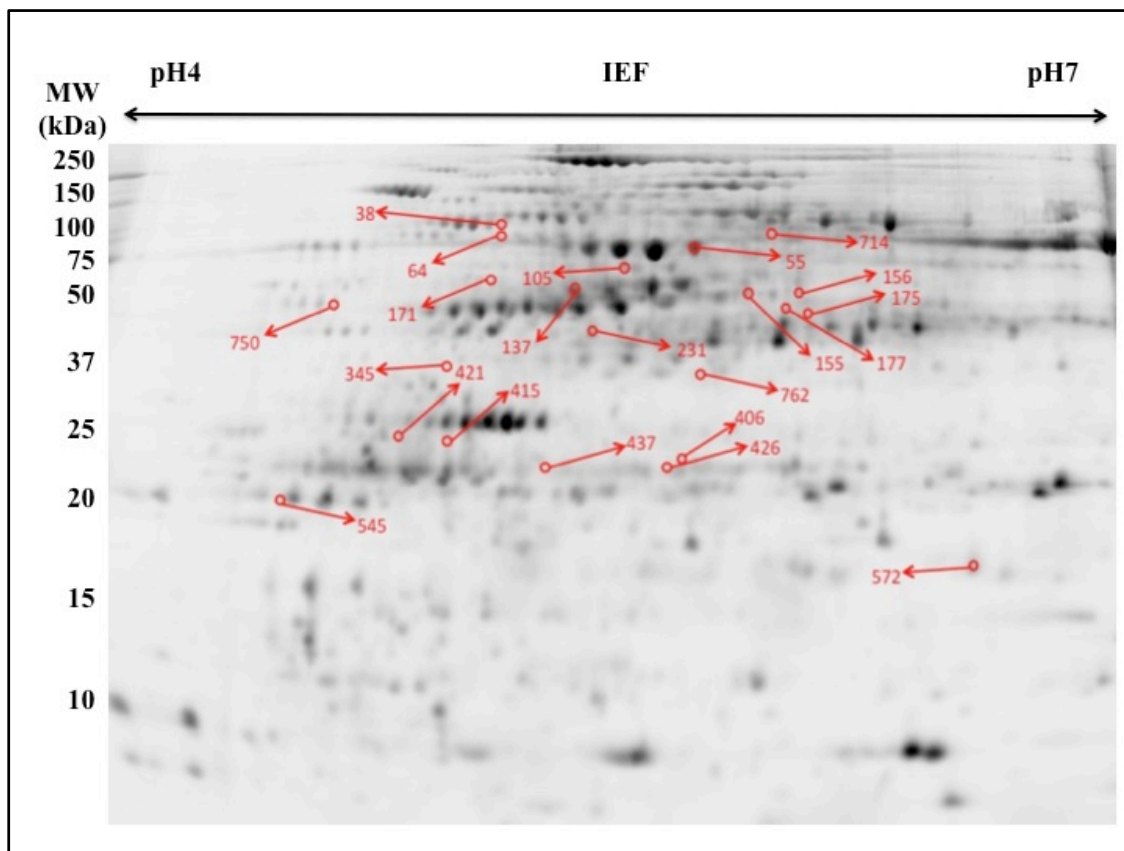
5.4.4. Mass spectral identification of differentially expressed proteins

Comparison of stacked and single GM varieties, both under the same genetic background, and non-GM varieties (the near-isogenic conventional counterpart and a landrace) revealed a total of 22 different proteins that were either present, absent, up- or down-regulated in one of the hybrids, at a statistically significant level ($P < 0.05$) (Table 1). Proteins that were not detected in this study, they were either not present or below the detection limit of approximately 1 ng, and were then considered absent in the sample.

All 22 proteins were identified with Mascot scores value greater than 202 using Quadrupole Time-of-Flight (Q-TOF) tandem mass spectrometry analysis (MS/MS) ($P < 0.05$). These proteins were all identified in *Zea mays* species. Table 1 presents the MS/MS parameters and protein identification characteristics for this experiment and is provided in the Appendix, Figure 3 shows their location in a representative gel. It was found that 17 proteins differed in their

expression levels between genotypes and 5 were found to be present only at one or two specific genotypes. Normalized quantitative values for each of these proteins and statics analysis are present in Table 2.

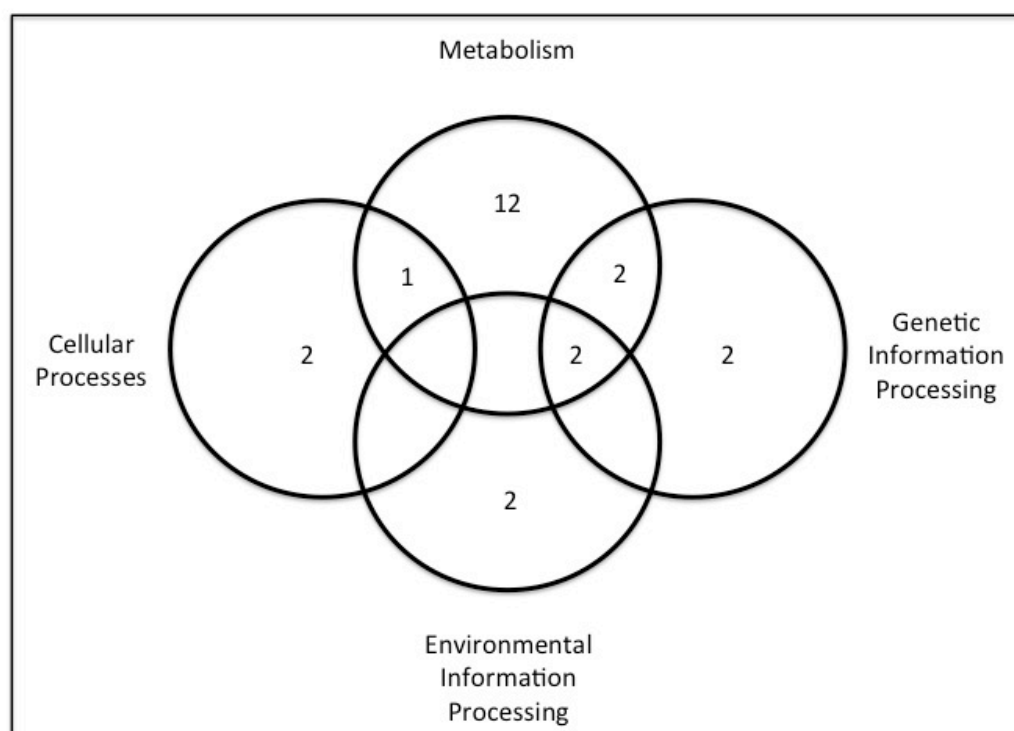
Figure 3. Representative 24 cm two-dimensional gel electrophoresis (2-D DIGE) image of the proteome of genetically modified maize plants AG8025 hybrid varieties MON-89Ø34-3 and MON-ØØ6Ø3-6 single events, and MON-89Ø34-3 x MON-ØØ6Ø3-6 stacked event, and non-modified maize (conventional counterpart AG8025 hybrid variety and landrace Pixurum 5 variety) grown under controlled conditions. Two random replicate samples were run together with an internal standard sample, each labeled with a different fluorescence. Individual gel images were obtained and were plotted together using ImageQuant TL software from GE healthcare. Linear isoelectric focusing pH 4–7 for the first dimension and 12% SDS–PAGE gels in the second dimension were used. Molecular mass standard range from 250 to 10 kDa are given on the left side. Red arrows point to differentially expressed protein spots selected for mass spectrometry identification. ID of identified proteins from Table 1 are indicated in red numbers.



Functional classification of the identified proteins, carried out in accordance with the KEGG Orthology system database, showed that

they were assigned to one out of these four main ortholog groups: (a) Metabolism (Energy, Carbohydrate and biosynthesis of amino acid, Fatty acid, Cofactors and vitamins, Secondary metabolites), (b) Cellular Processes (Transport and catabolism, Cell growth and death), (c) Genetic Information Processing (Folding, sorting and degradation, Transfer RNA biogenesis), and (d) Environmental Information Processing (Signal transduction). As represented in Figure 4, the 'Metabolism' group constituted the major category for all proteomes (77% of all identified proteins), although represented by different proteins.

Figure 4. Schematic representation of proteins categories, and the corresponding number of proteins, that were found to be differentially modulated by means of 2D-DIGE analysis of stacked versus single transgenic maize and corresponding non-transgenic counterpart. Protein categories were assigned according to KEGG Orthology (www.genome.jp/kegg/ko.html) categories.



The five exclusive proteins belong to different protein families. These are: cupin family (uncharacterized protein LOC100272933 precursor - PRO and PRORR samples; carbohydrate metabolism), esterase and lipase family (gibberellin receptor GID1L2 - PRO and

Table 2. Relative protein expression levels analysis of differentially modulated ($P < 0.05$) proteins measured by 2D-DIGE analysis. Modulations are reported as normalized spot volume in stacked vs. single GM event plants and control samples. Tukey Test was applied at $P < 0.05$ for means separation and statistical significance. The different letters represents statistically significant mean values. For the last 5 spots (345, 545, 572, 38 and 750) missed values in protein abundance is not reported because these proteins were not detected in these respective plant varieties. Protein identities are provided in Table 1 according to their Match ID number.

Protein ID	Conventional	Landrace	RR	Bt	RRxBt
55	0.713 a	0.511 b	0.804 a	0.621 ab	0.731 a
64	0.934 b	0.920 b	0.831 b	1.161 a	1.097 a
105	0.865 abc	0.647 c	0.994 a	0.948 ab	0.704 bc
137	0.934 ab	0.646 c	1.174 a	0.816 bc	0.974 ab
155	0.696 b	0.939 a	0.782 b	0.775 b	0.694 b
156	0.709 b	0.949 a	0.778 b	0.837 ab	0.725 b
171	1.375 a	1.181 abc	0.954 bc	1.272 ab	0.921 c
175	0.928 ab	0.659 b	0.807 ab	0.981 a	0.926 ab
177	1.035 a	0.555 b	0.857 ab	0.898 a	0.815 ab
231	0.891 b	1.090 a	0.793 b	0.860 b	0.905 b
406	1.157 a	0.696 b	1.169 a	1.074 a	1.027 a
415	0.862 a	0.330 b	1.192 a	0.947 a	1.032 a
421	0.739 b	0.652 b	0.750 b	0.997 a	0.847 ab
426	0.993 ab	0.780 c	0.851 bc	1.077 a	0.902 abc
437	1.055 ab	1.077 a	0.887 bc	0.977 abc	0.812 c
714	0.910 ab	0.954 a	0.650 b	0.880 ab	0.765 ab
762	0.880 ab	0.467 b	1.228 a	0.850 ab	0.914 ab
345	-	-	-	1.119a	0.676b
545	-	-	-	0.709b	0.806a
572	-	-	-	0.945a	0.688b
38	-	-	0.920	-	-
750	-	-	1.248	-	-

PRORR samples; environmental information processing), peroxiredoxin family (2-cys peroxiredoxin BAS1 - PRO and PRORR samples; transport and catabolism), chaperonin family (LOC100281701 - RR samples; genetic information processing), and ankyrin repeat family (ankyrin repeat domain-containing protein 2 - RR samples; genetic information processing).

Six proteins were found to be differentially expressed in landrace only. These are ATP synthase CF1 beta subunit (Match ID 55), hypothetical protein ZEAMMB73_661450 (Match ID 155), glutamate-oxaloacetate transaminase2 (Match ID 156), fructose-bisphosphate aldolase (Match ID 231), APx2-cytosolic ascorbate peroxidase (Match ID 406) and 6-phosphogluconolactonase isoform 1 (Match ID 415).

Enolase proteins were also assigned to other two spots (Match ID 105 and 714) the latter having higher expression levels in single GM events, as well as ATP synthase, which were identified in spots ID 55 and 64, the latter having higher expression levels in the vacuole of GM expressing Bt toxin only. These proteins are considered to represent different protein isoforms resulting from posttranslational modifications that introduce changes of molecular weight (MW) and/or isoelectric point (pI).

5.4.5. Proteins related to energetic homeostasis

The identity of proteins related to the energetic metabolism can be found at Table 1. They belong to the protein families of ATP synthases, NADH dehydrogenases, aminotransferases, fructose-bisphosphate aldolases, peroxidases, isopropylmalate dehydrogenases, enolases and the cupin family. Except for the cupin protein that was only detected in PRO and PRORR samples, all the other proteins were present in all samples at different levels of expression.

Enzymes that catalyze chemical reactions involved in carbohydrate/energetic pathways (i.e. oxidative phosphorylation, glycolysis and tricarboxylic acid cycle-TCA) were already observed in other comparative proteomic studies of transgenic versus non-transgenic crops. In fact, the energetic metabolism, including the carbohydrate metabolism, has been the most frequent observed protein category within comparative analysis of transgenic versus non-transgenic crops (see compilation at Table 3 from Agapito-Tenfen et al., 2013).

We have observed that enolase enzymes that participate in the

glycolysis pathway were found to be differentially modulated among single versus stacked GM events (Match ID 105 and 714). At spot 105, RRxBt samples showed reduced levels of expression compared to single GM events and the conventional variety. Differently, spot 714 was less abundant in RR samples. Barros et al. (2010) also found differential modulation of enzymes related to the glycolysis. According to the authors, the glyceraldehyde 3-phosphate dehydrogenase had higher expression levels in transgenic plants expressing CRY proteins compared to non-transgenic and RR samples by analyzing gene expression mean levels (3 years) obtained by microarray profiling of maize grown in South Africa. In addition, Coll et al. (2011) observed reduced levels of triose-phosphate isomerase protein, a glycolysis enzyme too, in transgenic plants expressing CRY insecticidal protein compared to their non-transgenic counterpart. Indeed, the flux through of the glycolysis metabolic pathway can be regulated in several ways: availability of substrate, concentration of enzymes responsible for rate-limiting steps, allosteric regulation of enzymes and covalent modification of enzymes (e.g. phosphorylation) (Mathews et al., 2012). Currently, there is a poor understanding of the transcriptional control of plant glycolysis (Ferne et al., 2004). Studies on transgenic potato plants exhibiting enhanced sucrose cycling revealed a general upregulation of the glycolytic pathway that is most probably mediated at the level of transcription (Ferne et al., 2008).

Higher levels of sucrose and fructose were observed in transgenic maize plants expressing CRY proteins in comparison to RR transgenic maize and non-transgenic samples obtained by H-NMR-based metabolite fingerprinting (Barros et al., 2010).

Intense nuclear functions, such as transgenic DNA transcription and transport of macromolecules across the nuclear envelope require efficient energy supply, yet principles governing nuclear energetics and energy support for nucleus-cytoplasmic communication are still poorly understood (Mattaj and Englmeier, 1998; Dzeja et al., 2002). Dzeja et al., (2002) have suggested that ATP supplied by mitochondrial oxidative phosphorylation, not by glycolysis, supplies the energy demand of the nuclear compartment.

Higher expression levels of ATP synthase, an enzyme that participates in the oxidative phosphorylation pathway, were observed in Bt and RRxBt plants compared to Bt and conventional (Match ID 64). Regarding proteins related to the TCA cycle, the 3-isopropylmalate

dehydrogenase (Match ID 171) was differentially modulated in all GM events. Plants expressing the stacked event had lower levels compared to Bt single GM event, and RR samples had intermediate levels.

5.4.6. Proteins related to other cellular metabolisms and processes

Proteins assigned to other metabolic pathways other than those related to the energetic metabolism were grouped in this section. These proteins are enzymes related to fatty acid, vitamins and secondary metabolites metabolism; transport and catabolism and cell growth and death; folding, sorting and degradation of nucleic acids; and signal transduction. Table 2 shows expression levels obtained by 2D-DIGE experiment.

Coproporphyrinogen III oxidase and S-adenosyl methionine (SAM) (Match ID 177 and 437) are important enzyme and co-factor, respectively, which act on the metabolism of vitamins in plants. These were modulated in similar matters in each maize variety with greater expression in conventional variety. The first enzyme plays an important role in the tetrapyrrole biosynthesis that is highly regulated, in part to avoid the accumulation of intermediates that can be photoactively oxidized, leading to the generation of highly reactive oxygen intermediates (ROI) and subsequent photodynamic damage (Ishikawa et al., 2001). SAM plays a critical role in the transfer of methyl groups to various biomolecules, including DNA, proteins and small-molecule secondary metabolites (Chiang et al., 1996). SAM also serves as a precursor of the plant hormone ethylene, implicated in the control of numerous developmental processes (Wang, et al. 2002).

Two other proteins related to the synthesis of secondary metabolites were expressed at statistically different levels. These are Match ID 137 and 762.

Interestingly, both enzymes have been observed to be expressed at higher levels in all hybrid plants (GM and non-GM) compared to the landrace samples. DIMBOA UDP-glucosyltransferase BX9 is an enzyme that participates in the synthesis of 2,4-Dihydroxy-7-methoxy-1,4-benzoxazine-3-one (DIMBOA) compound that plays an important role in imparting resistance in gramineous plants against disease and insect pests (Klun and Robinson, 1969) and herbicide tolerance (Hamilton, 1964). DIMBOA decreases *in vivo* endoprotease activity in the larval midgut of the European corn borer (*Ostrinia nubilalis*)

limiting the availability of amino acids and reducing larval growth (Houseman et al. 1989, 1992). But the protection against insect attack that DIMBOA confers to the plant is restricted to early stages of plant development because DIMBOA concentration decreases with plant age (Morse et al. 1991; Barry et al. 1994; Cambier et al. 2000). The other enzyme related to the metabolism of secondary metabolites follows exactly the same trend in expression. Dihydroflavonol-4-reductase catalyzes a key step late in the biosynthesis of anthocyanins, condensed tannins (proanthocyanidins), and other flavonoids, important to plant survival, including defense against herbivores (Peters and Constabel, 2002).

Two enzymes related to genetic information processing were observed in RR samples only. Match ID 750 was identified to contain an ankyrin repeat domain. The ankyrin repeats are degenerate 33-amino acid repeats found in numerous proteins, and serve as domains for protein-protein interactions (Michaely and Bennett, 1992). By using antisense technique, Yan et al. (2002) were able to reduce the expression levels of an ankyrin repeat-containing protein, which resulted in small necrotic areas in leaves accompanied by higher production of H_2O_2 . These results were found to be similar to the hypersensitive response to pathogen infection in plant disease resistance (Yan et al., 2002). Although we were not able to identify an annotated protein to Match ID 38, blast results show that this protein belong to the chaperonin protein family. Chaperones are proteins that assist the non-covalent folding or unfolding and the assembly or disassembly of other macromolecular structures. Therefore, cells require a chaperone function to prevent and/or to reverse incorrect interactions that might occur when potentially interactive surfaces of macromolecules are exposed to the crowded intracellular environment (Ellis, 2006). A large fraction of newly synthesized proteins require assistance by molecular chaperones to reach their folded states efficiently and on a biologically relevant timescale (Hartl and Hayer-Hartl, 2009).

Another relevant class of enzymes is linked to plant perception and response to environmental conditions (environmental information processing). An important protein of this category is gibberellin receptor *GID1L2* (Match ID 345). Gibberellins (GAs) are plant hormones that are essential for many developmental processes in plants, including seed germination, stem elongation, leaf expansion, trichome development, pollen maturation and the induction of flowering (Davière and Achard,

2013). This protein was only detected in transgenic plant samples expressing CRY proteins, both Bt and RRxBt samples).

5.4.7. Contributions to the risk assessment of stacked transgenic crop events

Recent discussions about risk assessment of stacked events and the respective opinion delivered by the European Food Safety Authority (EFSA) have highlighted the controversy among risk assessors about the particular risk assessment requirements for applications for this type of GMO. These are mainly related additional data submission rather than previous data from parental GM single events only (Spök et al., 2007). Similar debate has taken place in the Comissão Técnica Nacional de Biossegurança (CTNBio) in Brazil while approving stacked GM events under a simplified risk assessment procedure provided by Normative Resolution n° 8 from 2009 (CTNBio, 2009).

Consensus issues related to such requirements consider the evaluation of the expression levels of transgenes between parental GM events (single events) and the stacked event, and the need to consider any potential interaction of combined GM traits in the stacked events (Spök et al., 2007; Kok et al., 2013).

It is clear, and for reasons discussed previously in this paper, that expression levels of stacked GM events are of major concern. On the other hand, testing potential interaction of stacking transgenic proteins, and other genetic elements involved in its expression, is an obscure issue and simple compositional analysis and/or evaluation of agronomic characteristics might not be effective to further clarification.

Molecular profiling at the hazard identification step can fill the biosafety gap emerging from the development of new types of GMOs that have particular assessment challenges (Heinemann et al., 2011).

A number of published studies have focused on the investigation of possible unintended effects of the transformation event and expression of transgenes in plants based on general “omics” technologies over the past few years (Ruebelt et al., 2006; Coll et al., 2009; Balsamo et al., 2011; Ricrick et al., 2011). These have focused in the comparison of single events versus non-transgenic near-isogenic conventional counterpart.

Therefore, the aim of the present study was to broaden the state of knowledge about the inherent natural variability in GM crop

composition induced by stacking genetic modified events and its modulation by the genetic background. To date, no other study was able to characterize differentially expressed proteins from stacked GM maize events compared to their parental single event hybrids and non-transgenic varieties; therefore, there is a lack of literature that applies this approach to the assessment of stacked GM events.

The evaluation of GM maize proteomes under different transgene dosage conditions resulted in reduced levels of transgenic proteins expression in stacked events (average of 25% reduction). In addition, we have also detected different modulation of several proteins related to a diverse range of physiological metabolic pathways that could be grouped into three major categories: general metabolism, cellular processes, genetic information processing and environmental information processing. Within these, around 77% of all detected proteins were assigned to participate in energy-related metabolic pathways. Nevertheless, many of these proteins have also been detected in other studies. The compilation of these literatures with the results obtained from the present work reveals protein families that are involved in similar metabolic pathways. It is interesting to note that each of these studies was performed with a different plant hybrid expressing the same transgene cassette but grown under distinct environmental conditions (Heinemann et al., 2011; Agapito-Tenfen et al., 2013).

It has been already demonstrated that major changes in the proteome profile of GM crops are driven by genotypic, environmental (geographical and seasonal) and crop management influences (and combinations thereof) than by genetic engineering. However, it has been also observed that the genetic engineering does have an influence in the modulation of certain proteins and pathways thereby (Prescott et al., 2005). Nonetheless, off-target effects of GM crops have been also evidenced at different levels and some do not directly correspond to the levels of transgenic protein expression (Ramirez-Romero et al., 2008). In some cases, beneficial effects of the transgene might be influenced by pleiotropic effects derived from the use of strong promoters and new proteins (Romero et al., 1997; Capell et al., 1998; Kasuga et al., 1999).

5.5. CONCLUSIONS

In conclusion, our results showed that CRY insecticidal and RR herbicide tolerant proteins expressed in both single and stacked event

were the major source of influence to the expression of GM maize proteome. It should be highlighted that stacked GM genotype was clustered together and distant from other genotypes analyzed by PCA. In addition, we have observed evidence of possible synergistic and antagonistic interactions mediated by stacking transgenes into GM maize genome by conventional breeding due to the observation of twenty-two proteins that were statistically differentially modulated. These proteins were mainly assigned to the energy/carbohydrate metabolism (77% of all identified proteins). Moreover, transcript expression levels of transgene had a significant reduction of about 25% when compared to parental single event varieties. Such observations indicate that the genome changes in stacked GM maize may have specific impact on the overall gene expression that might be relevant to safety assessments. Some of these proteins modulation did not fall within the range of the natural variability found in a commonly used landrace. Thus, the inclusion of a non-counterpart genotype, in addition to be useful in risk assessment, could also be useful in cases where the non-GM counterpart is available. To the best of our knowledge, this was the first report on comparative proteomic analysis of stacked versus single event transgenic crops. Nevertheless, the detection of changes in protein profiles does not present a safety issue per se; therefore, further studies should be conducted in order to address the biological relevance and implications of such changes.

5.6. REFERENCES

- Agapito-Tenfen SZ, Guerra MP, Wikmark OG, Nodari RO: Comparative proteomic analysis of genetically modified maize grown under different agroecosystems conditions in Brazil. *Proteome Sci* 2013, 11:46.
- Ad Hoc Technical Expert Group, United Nations Environment Programme, Convention on Biological Diversity: Guidance document on risk assessment of living modified organisms. [<http://www.cbd.int/doc/meetings/bs/mop-05/official/mop-05-12-en.pdf>]
- Andersen CL, Jensen JL, Ørntoft TF: Normalization of real-time quantitative reverse transcription-pcr data: a model-based variance estimation approach to identify genes suited for

- normalization, applied to bladder and colon cancer data sets. *Cancer Res* 2004, 64:5245-5250.
- Balsamo GM, Cangahuala-Inocente GC, Bertoldo JB, Terenzi H, Arisi AC: Proteomic analysis of four Brazilian MON810 maize varieties and their four non-genetically-modified isogenic varieties. *J Agr Food Chem* 2011, 59:11553-11559.
- Barros E, Lezar S, Anttonen MJ, van Dijk JP, Röhlig RM, Kok EJ, Engel KH: Comparison of two GM maize varieties with a near-isogenic non-GM variety using transcriptomics, proteomics and metabolomics. *Plant Biotechnol J* 2010, 8:436-451.
- Barry D, Alfaro D, Darrah LL: Relation of European corn borer (Lepidoptera: Pyralidae) leaf feeding resistance and DIMBOA content in maize. *Environ Entomol* 1994, 23:177-182.
- Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009, 55:611-622.
- Bustin SA, Benes V, Garson J, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley G, Wittwer CT, Schjerling P, Day PJ, Abreu M, Aguado B, Beaulieu JF, Beckers A, Bogaert S, Browne JA, Carrasco-Ramiro F, Ceelen L, Ciborowski K, Cornillie P, Coulon S, Cuypers A, De Brouwer S, De Ceuninck L, De Craene J, De Naeyer H, De Spiegelaeere W, et al.: The need for transparency and good practices in the qPCR literature. *Nat Methods* 2013, 10:1063-1067.
- Cambier V, Hance T, de Hoffman E: Variation of DIMBOA and related compounds content in relation to the age and plant organ in maize. *Phytochem* 2000, 53:223-229.
- Canci IJ, Brassiani IA: *Anchieta História, Memória e Experiência: Uma caminhada construída pelo povo*. São Miguel do Oeste: Mclee; 2004.
- Capell T, Escobar C, Liu H, Burtin D, Lepri O, Christou P: Overexpression of the oat arginine decarboxylase cDNA in transgenic rice (*Oryza sativa* L.) affects normal development patterns *in vitro* and results in putrescine accumulation in transgenic plants. *Theor Appl Genet* 1998, 97:246-254.
- Carpentier SC, Witters E, Laukens K, Deckers P, Swennen R, Panis B: Preparation of protein extracts from recalcitrant plant tissues: an

- evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics* 2005, 5:2497-2507.
- Chiang PK, Gordon RK, Tal J, Zeng GC, Doctor BP, Pardhasaradhi K, McCann PP: S-adenosylmethionine and methylation. *FASEB J* 1996, 10:471-480.
- Codex Alimentarius Commission: Guideline for the conduct of food safety assessment of food derived from recombinant DNA plants. Rome CAC/GL 45-2003. [http://www.fao.org/fileadmin/user_upload/gmfp/docs/CAC.GL_45_2003.pdf]
- Cogoni C, Macino G: Homology-dependent gene silencing in plants and fungi: a number of variations on the same theme. *Curr Opin in Microbiol* 1999, 2:657-662.
- Coll A, Nadal A, Collado R, Capellades G, Messeguer J, Melé E, Palau-del-màs M, Pla M: Gene expression profiles of MON810 and comparable non-GM maize varieties cultured in the field are more similar than are those of conventional lines. *Transgenic Res* 2009, 18:801-808.
- Coll A, Nadal A, Collado R, Capellades G, Kubista M, Messeguer J, Pla M: Natural variation explains most transcriptomic changes among maize plants of MON810 and comparable non-GM varieties subjected to two N-fertilization farming practices. *Plant Mol Biol* 2010, 73:349-362.
- Coll A, Nadal A, Rossignol M, Puigdomènech P, Pla M: Proteomic analysis of MON810 and comparable non-GM maize varieties grown in agricultural fields. *Transgenic Res* 2011, 4:939-949.
- Community Reference Laboratory for GM Food and Feed: Event-specific Method for the Quantification of Maize Line MON 89034 Using Real-time PCR, Protocol CRLVL06/06VP. [http://gmo-crl.jrc.ec.europa.eu/summaries/MON89034_validated_Method.pdf]
- Comissão Técnica Nacional de Biossegurança: Parecer Técnico nº 1596/2008 - Liberação Comercial de Milho Geneticamente Modificado Tolerante ao Glifosato, Milho Roundup Ready 2, Evento NK603 - Processo nº 01200.002293/2004-16. [<http://www.ctnbio.gov.br/index.php/content/view/12341.html>].

- Comissão Técnica Nacional de Biossegurança: Normative Resolution No. 08 of July 3th, 2009. [<http://www.ctnbio.gov.br/index.php/content/view/13863.html>].
- Comissão Técnica Nacional de Biossegurança: Parecer Técnico nº 2052/2009 - Liberação Comercial de Milho Resistente a Insetos, Milho MON 89034 - Processo nº 01200.003326/2008-61. [<http://www.ctnbio.gov.br/index.php/content/view/14098.html>].
- Comissão Técnica Nacional de Biossegurança: Parecer Técnico nº 2725/2010 - Liberação Comercial de Milho Geneticamente Modificado Resistente a Insetos e Tolerante a Herbicidas, Milho MON 89034 x NK 603 - Processo nº 01200.003952/2009-38. [<http://www.ctnbio.gov.br/index.php/content/view/15704.html>].
- Davière JM, Achard P: Gibberellin signaling in plants. *Development* 2013, 140:1147-1151.
- Davies H: A role for “omics” technologies in food safety assessment. *Food Control* 2012, 21:1601-1610.
- Daxinger L, Hunter B, Sheikh M, Jauvion V, Gascioli V, Vaucheret H, Matzke M, Furner I: Unexpected silencing effects from T-DNA tags in Arabidopsis. *Trends Plant Sci* 2008, 13:1360-85.
- De Schrijver A, Devos Y, Van den Bulcke M, Cadot P, De Loosed M, Reheul D, Sneyers M: Risk assessment of GM stacked events obtained from crosses between GM events. *Trends Food Sci Tech* 2007, 18:101-109.
- Dzeja PP, Bortolon R, Perez-Terzic C, Holmuhamedov EL, Terzic A: Energetic communication between mitochondria and nucleus directed by catalyzed phosphotransfer. *Proc Natl Acad Sci USA* 2002, 99:10156-10161.
- Ellis, J: Molecular chaperones: assisting assembly in addition to folding. *Trends Biochem Sci* 2006, 31:395-401.
- European Food Safety Authority: Guidance Document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants containing stacked transformation events. *EFSA J* 2007, 512:1-5.
- Fagard M, Vaucheret H: (Trans)Gene Silencing in Plants: How many mechanisms? *Annu Rev Plant Physiol Plant Mol Biol* 2000, 51:167-194.
- Fernie AR, Carrari F, Sweetlove LJ: Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Curr Opin Plant Biol* 2004, 7:254-261.

- Grover A, Aggarwal PK, Kapoor A, Katiyar-Agarwal S, Agarwal M, Chandramouli A: Addressing abiotic stresses in agriculture through transgenic technology. *Curr Sci India* 2003, 84:355-367.
- Hamilton RH: Tolerance of several grass species to 2-chloro-s-triazine herbicides in relation to degradation and content of benzoxazinone derivatives. *J Agric Food Chem* 1964, 12:14-17.
- Hardisty JF, Banas DA, Gopinath C, Hall WC, Hard GC, Takahashi M: Spontaneous renal tumors in two rats from a thirteen week rodent feeding study with grain from molecular stacked trait lepidopteran and coleopteran resistant (DP-004114-3) maize. *Food Chem Toxicol* 2013, 53:428-31.
- Hartl FU, Hayer-Hartl M: Converging concepts of protein folding *in vitro* and *in vivo*. *Nat Struct Mol Biol* 2009, 16:574-581.
- Heinemann JA, Kurenbach B, Quist D: Molecular profiling - a tool for addressing emerging gaps in the comparative risk assessment of GMOs. *Environ Int* 2011, 37:1285-1293.
- Hendriksma HP, Küting M, Härtel S, Näther A, Dohrmann AB, Steffan-Dewenter I, Tebbe CC: Effect of stacked insecticidal Cry proteins from maize pollen on nurse bees (*Apis mellifera carnica*) and their gut bacteria. *PLoS One* 2013, 8:e59589.
- Houseman JG, Philogene BJR, Downe AER: Partial characterization of proteinase activity in the larval midgut of the European corn borer, *Ostrinia nubilalis* Hubner (Lepidoptera: Pyralidae). *Can J Zool* 1989, 67:864-868.
- Houseman JG, Campos F, Thie NMR, Philogene BJR, Atkinson J, Morand P, Arnason JT (1992) Effect of the maize-derived compounds DIMBOA and MBOA on growth and digestive processes of European corn borer (Lepidoptera: Pyralidae). *J Econ Entomol* 1992, 85:669-674
- Ishikawa A, Okamoto H, Iwasaki Y, Asahi T: A deficiency of coproporphyrinogen III oxidase causes lesion formation in Arabidopsis. *Plant J* 2001, 27:89-99.
- James C: *Global Status of Commercialized Biotech/GM Crops: 2012*. ISAAA Brief No. 44. Ithaca: ISAAA Press; 2012.
- Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K: Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat Biotechnol* 1999, 17:287-291.

- Klun JA, Robinson JF: Concentration of two 1,4-benzox-azinones in dent corn at various stages of development of the plant and its relation to resistance of the host plant to the European corn borer. *J Econ Entomol* 1969, 62:214-220
- Kohli A, Twyman RM, Abranches R, Wegel E, Stoger E, Christou P: Transgene integration, organization and interaction in plants. *Plant Mol Biol* 2003, 52:247-58.
- Kok EJ, Pedersen J, Onori R, Sowa S, Schauzu M, De Schrijver A, Teeri TH: Plants with stacked genetically modified events: to assess or not to assess? *Trends Biotech* 2014, 32:70-73.
- Kuiper HA, Kleter GA, Noteborn HP, Kok EJ: Assessment of the food safety issues related to genetically modified foods. *Plant J* 2011, 27:503-528.
- Latham GJ: Normalization of microRNA quantitative RT-PCR data in reduced scale experimental designs. *Methods Mol Biol* 2010, 667:19-31.
- Lehesranta SJ, Davies HV, Shepherd LVT, Nunan N, McNicol JW, Auriola S, Koistinen KM, Suomalainen S, Kokko HI, Kärenlampi SO: Comparison of Tuber Proteomes of Potato Varieties, Landraces, and Genetically Modified Lines. *Plant Physiol* 2005, 138:1690-1699.
- Lilley KS, Friedman DB: All about DIGE: quantification technology for differential-display 2D-gel proteomics. *Expert Rev Proteomics* 2004, 1:401-409.
- Manoli A, Sturaro A, Trevisan S, Quaggiotti S, Nonis A: Evaluation of candidate reference genes for qPCR in maize. *J Plant Physiol* 2012, 169:807-815.
- Marouga R, David S, Hawkins E: The development of the DIGE system: 2D fluorescence difference gel analysis technology. *Anal Bioanal Chem* 2005, 382:669-678.
- Mathews CK, van Holde KE, Appling DR, Antony-Cahill SR: *Biochemistry*. New Jersey: Prentice Hall; 2012.
- Mattaj JW, Englmeier L: Nucleocytoplasmic transport: the soluble phase. *Annu Rev Biochem* 1998, 67:265-306.
- Matzke M, Matzke A: Gene silencing in plants: relevance for genome evolution and the acquisition of genomic methylation patterns. *Novartis Foundation Symposium on Epigenetics* 1998, 214:168-186.

- Michaely P, Bennett V: The ANK repeat: a ubiquitous motif involved in macromolecular recognition. *Trends Cell Biol* 1992, 2:127-129.
- Minden JS, Dowd SR, Meyer HE, Stühler K: Difference gel electrophoresis. *Electrophoresis* 2009, 30:S156-161.
- Morse S, Wratten SD, Edwards PJ, Niemeyer HM: Changes in the hydroxamic acid content of maize leaves with time and after artificial damage; implications for insect attack. *Ann Appl Biol* 1991, 119:239-249.
- Muñoz-Mayor A, Pineda B, Garcia-Abellán JO, Garcia-Sogo B, Moyano E, Atares A, Vicente-Agulló F, Serrano R, Moreno V, Bolarin MC: The HAL1 function on Na⁺ homeostasis is maintained over time in salt-treated transgenic tomato plants, but the high reduction of Na⁺ in leaf is not associated with salt tolerance. *Physiol Plant* 2008, 133:288-297.
- Nielsen KM: Biosafety data as confidential business information. *PLoS Biol* 2013, 11:e1001499.
- Park YD, Papp I, Moscone EA, Iglesias VA, Vaucheret H, Matzke AJ, Matzke MA: Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations silence of genes in transgenic plants. *Plant J Bot* 1996, 79:3-12.
- Peters DJ, Constabel CP: Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). *Plant J* 2002, 32:701-712.
- Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001, 29:e45.
- Pineda B: Análisis funcional de diversos genes relacionados con la tolerancia a la salinidad y el estrés hídrico en plantas transgénicas de tomate (*Lycopersicon esculentum* Mill). *PhD thesis*. Universidad Politécnica de Valencia; 2005.
- Prescott VE, Campbell PM, Moore A, Mattes J, Rothenberg ME, Foster PS, Higgins TJ, Hogan SP: Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity. *J Agric Food Chem* 2005, 53:9023-9030.
- Ramirez-Romero R, Desneux N, Decourtye A, Chaffiol A, Pham-Delègue MH: Does Cry1Ab protein affect learning performances of the honeybee *Apis mellifera* L. (Hymenoptera, Apidae)? *Ecotoxicol Environ Saf* 2008, 70:327-333.

- Ricroch AE, Bergé JB, and Kuntz M: Evaluation of Genetically Engineered Crops Using Transcriptomic, Proteomic, and Metabolomic Profiling Techniques. *Plant Physiol* 2011, 155:1752-1761.
- Romero C, Bellés JM, Vayá JL, Serrano R, Culiáñez-Macià FA: Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. *Planta* 1997, 201:293-297.
- Ruebelt MC, Lipp M, Reynolds TL, Schmuke JJ, Astwood JD, DellaPenna D, Engel KH, Jany KD: Application of two-dimensional gel electrophoresis to interrogate alterations in the proteome of genetically modified crops. 3. Assessing unintended effects. *J Agric Food Chem* 2006, 54:2169-2177.
- Rus AM, Rus AM, Estañ MT, Gisbert C, Garcia-Sogo B, Serrano R, Caro M, Moreno V, Bolarín MC: Expressing the yeast HAL1 gene in tomato increases fruit yield and enhances K^+/Na^+ selectivity under salt stress. *Plant Cell Environ* 2001, 24:875–880.
- Schuppener M, Mühlhause J, Müller AK, Rauschen S: Environmental risk assessment for the small tortoiseshell *Aglais urticae* and a stacked Bt-maize with combined resistances against Lepidoptera and Chrysomelidae in central European agrarian landscapes. *Mol Ecol* 2012, 21:4646-4662.
- Shevchenko A, Wilm M, Vorm O, Mann M: Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 1996, 68:850-858.
- Silva FASE, Azevedo CAV: Versão do programa computacional Assistat para o sistema operacional Windows. *Revista Brasileira de Produtos Agroindustriais*, 2002, 4:71-78.
- Spök A, Eckerstorfer M, Heissenberger A, Gaugitsch H: Risk Assessment of “stacked events”. [http://bmg.gv.at/cms/home/attachments/7/7/0/CH1052/CMS1180523433481/cms1200659635294_internetversion_2_07stacked_events.pdf]
- Stam M, Mol JNM, Kooter JM: The Silence Of Genes In Transgenic Plants. *Ann Bot* 1997, 79:3–12.
- Taverniers I, Papazova N, Bertheau Y, De Loose M, Holst-Jensen A: Gene stacking in transgenic plants: towards compliance between

- definitions, terminology, and detection within the EU regulatory framework. *Environ Biosafety Res* 2008, 7:197-218.
- Weiss W, Gorg A: Sample solubilization buffers for two-dimensional electrophoresis. In *Methods in Molecular Biology-2D PAGE: Sample Preparation and Fractionation*. Edited by Posh A. New Jersey: A Humana Press; 2008:35-42.
- Weld R, Heinemann J, Eady C: Transient GFP expression in *Nicotiana glumbaginifolia* suspension cells: the role of gene silencing, cell death and T-DNA loss. *Plant Mol Biol* 2001, 45:377-385.
- Yan J, Wang J, Zhang H: An ankyrin repeat-containing protein plays a role in both disease resistance and antioxidation metabolism. *Plant J* 2002, 29:193-202.
- Wang KL, Li H, Ecker JR: Ethylene biosynthesis and signaling networks. *Plant Cell* 2002, 14:S131-S151.

6. CAPÍTULO II

A new methodological model to address potential adverse effects of genetically modified plants by the use of RNA interference tools

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RESUMO

A segurança e confiabilidade em organismos geneticamente modificados depende significativamente da sua integridade genética e da detecção de potenciais efeitos adversos. A abordagem atualmente utilizada para a avaliação dos potenciais efeitos não intencionais de plantas transgênicas é baseada na suposição de que um organismo geneticamente modificado é composto por duas partes que funcionam de forma linear e aditiva, sendo uma destas o organismo hospedeiro e a outra a proteína transgênica produzida por ele. Este trabalho propõe um novo modelo metodológico para abordar os potenciais efeitos adversos de plantas geneticamente modificadas através da utilização de ferramentas de interferência por RNA em conjunto com a análise proteômica comparativa diferencial. Para tanto, sementes de milho transgênico contendo evento MON810 e sua correspondente não-transgênica isogênica foram submetidas ao tratamento com RNA de fita dupla com sequência homóloga ao do transcrito transgênico expresso. O melhor método de entrega da molécula foi através de solução aquosa alimentando o mesocótilo da plântula. Após 72 h, amostras foliares foram coletadas para confirmar o silenciamento. A solução foi mantida por nove dias e após este período, novas coletas foram realizadas para análise proteômica diferencial e quantificação de proteína transgênica. Foi observada uma redução de aproximadamente 60% nos níveis de

² Este capítulo trata do manuscrito a ser submetido à publicação em revista científica.

proteína transgênica em plântulas de milho sob tratamento. A comparação entre plântulas de milho transgênico sob tratamento e controles evidenciou 22 proteínas que foram diferencialmente expressas ($P < 0.05$). Destas, apenas nove estão relacionadas com a transgênia e foram categorizadas dentro do grupo de metabolismo energético. Portanto, os resultados do presente estudo demonstraram que a expressão de níveis elevados de proteínas transgênicas não pode ser considerado um fator aditivo para a equivalência dos proteomas de plantas transgênicas e convencionais, pois foram detectadas alterações na expressão de proteínas endógenas mesmo na presença de menores níveis de proteína transgênica. Esta abordagem metodológica pode ser de especial interesse para os reguladores e pesquisadores da área de biossegurança. Embora os métodos descritos são específicos para plantas geneticamente modificadas, este trabalho apresenta procedimentos que poderiam ser úteis no desenvolvimento de métodos para avaliar a segurança de outros produtos similares.

6.1. ABSTRACT

The safety and reliability of genetically modified organisms depends significantly on their genetic integrity and the detection of potential adverse effects. The current approach for assessing unintended effects of transgenic plants is based on the assumption that a genetically modified organism is composed of two parts that work in a linear additive matter, being that the host organism and the transgenic protein produced. In this paper we propose a new methodological framework to address the potential adverse effects of genetically modified through the use of RNA interference tools together with the differential comparative proteomics analysis. Therefore, seeds of transgenic maize containing MON810 event and its corresponding near-isogenic non-transgenic plants were subjected to double-stranded RNA treatment with homology to the expressed transgenic transcript sequence. The most efficient method for the delivery of dsRNA was feeding the seedling mesocotyl with an aqueous solution. After 72 h, leaf samples were collected to confirm silencing. Seedlings were kept under dsRNA solution for nine days and after this period, new sampling were performed for quantification of transgenic protein and proteomic analysis. A reduction of about 60% was observed in protein levels in transgenic maize seedlings under treatment. The comparison between transgenic maize

seedlings under treatment and controls showed that 22 proteins were differentially expressed at $p < 0.05$. Out of these, nine are associated with genetic modification and were mainly categorized into the group of energy metabolism. However, these protein categories showed similar expression pattern to those detected in conventional treated and untreated samples. Therefore, the results of this study demonstrated that transgenic proteins couldn't be regarded as a simply additive matter to the proteome equivalence of transgenic and conventional plants because of the detection of differential modulation of endogenous proteins. But also indicate that the reduction of CRY1Ab transgenic protein production consequently reduces the differential modulation of endogenous proteins, thus suggesting that CRY1Ab protein, rather than insertional effects, may be the major source of pleiotropic effects already observed in other studies. This finding could be of special interest for regulators and researchers in the biosafety field. Although the method described is specific for GM plants, this work provides procedures that could be useful in developing methods to assess the safety of other similar products.

6.2. BACKGROUND

The official approval for the introduction of genetically modified (GM) crops in Europe, the United States, Brazil, and many other countries has invoked the concept of 'substantial equivalence'. In other words, if a genetically modified organism (GMO) can be characterized as substantially equivalent to its 'natural' antecedent, the non-transgenic near isogenic counterpart, it can be assumed to pose no new environment and health risks and hence to be acceptable for commercial production and use.

The concept of substantial equivalence has never been properly defined; the degree of difference between a natural food and its GM alternative before its 'substance' ceases to be acceptably 'equivalent' is not defined anywhere, nor has an exact definition been agreed by legislators. It is exactly this vagueness that makes the concept useful to industry but unacceptable to the consumer. Moreover, the reliance by policymakers on the concept of substantial equivalence acts as a barrier to further research into the possible risks of eating GM foods (Millstone et al., 1999).

The current approach for the assessment of the effects on non-

target organisms is a good example on how applicants based on the assumption that a GMO consists of two parts that function in a linear additive fashion: the crop and the novel GM transgene product (Hilbeck et al., 2011). Thus, the transgenic crop plant is declared as safe as its conventional counterpart when no statistically relevant compositional changes are detected and, consequently, only the added transgene product is subject to testing in the environmental risk assessment (Dolezel et al., 2011).

Particularly relevant for herbicide-tolerant varieties, Bohn et al. (2014) argued that compositional studies that have not measured pesticide residues contain serious shortcomings, because if present (i) they are clearly a part of a plant's composition, and (ii) they may add toxic properties to the final plant product either by itself or by affecting the plant metabolism. The authors of this study demonstrated that Roundup Ready GM-soy may have high residue levels of glyphosate and AMPA, and also that different agricultural practices may result in a markedly different nutritional composition of soybeans.

Confidence in the safety and reliability of GM crop species depend significantly on their genetic integrity and the detection of potential adverse effects; however, the frequency of transformation-induced mutations and their importance as potential biohazards are poorly understood (Yin et al., 2004). In fact, the transgene insertion site cannot be predetermined and for this reason transgenes may be inserted in functional genomic regions, thus disrupting the structure and/or altering the regulation patterns of genes from the plant host genome (Rosati et al., 2005). Other secondary unintended effects of genetic modification can also arise during conventional breeding as the result of mutagenesis and hybridization, processes that are integral to breeding programs (Baudo et al., 2006; Barros et al., 2010).

Profiling technologies allow the simultaneous measurement and comparison of thousands of plant components without prior knowledge of their identity. The combination of these non-targeted methods facilitates a more comprehensive approach than targeted methods and thus provides additional opportunities to identify unintended effects of genetic modification (Ruebelt et al., 2006; Deng et al., 2008). Although the use of profiling tools has been seen by many as useful in risk assessment, no consensus has formed on the need or value of these techniques for assessing the risks of all genetically modified organisms (Heinemann et al., 2011).

A number of molecular profiling studies have already investigated possible unintended effects of genetic modification (Zolla et al., 2008; Coll et al., 2010; Agapito-Tenfen et al., 2013). However, these studies do not report consistent or coherent results, which may be explained by their use of a variety of genetic backgrounds and/or different growth conditions, as well as variations in the technologies applied (Ricroch et al., 2011). These inconsistencies highlight the importance of building a “database” of knowledge around natural variability in food crop species (Batista and Oliveira, 2010) and can be addressed through continuous multi-laboratory tasks.

In the present study, we proposed a new methodological model to address potential adverse effects of genetically modified plants by the use of RNA inference tools prior to the comparative proteomic analysis. We have chosen MON810 maize due to available information on its molecular characterization. Therefore, maize seeds were grown under highly controlled conditions and RNAi treatment was performed based on several pilot experiments to test the best time-dose efficiency. After the time course of the RNAi experiment, leaf samples were extracted for their total protein contents. Protein profiles were generated and compared between the plant genotypes (GM and conventional counterpart) and treatments (dsRNA and control-water) to assess differences in protein expression. Differentially expressed proteins were successfully identified and their molecular function and cellular components were analyzed. To the best of our knowledge this study represents the first effort in isolating the potential effect of transgenic protein in endogenous protein variation between transgenic versus non-transgenic plants that are based on highly similar genetic backgrounds.

6.3. MATERIAL AND METHODS

6.3.1. Plant material and growing conditions

The cultivation of MON810 transgenic maize (Monsanto do Brasil Ltda.) was approved in Brazil in 2008. MON810 contains a genomic insert of a modified version of the native *cry1Ab* gene from *Bacillus thuringiensis*. The expression product of this gene is the insecticide protein (Bt toxin) CRY1Ab. Transgenic single cross hybrid seeds AG5011YG (Agrocere Sementes) widely used for whole-plant silage and grain were kindly provided by the company. The near-

isogenic, non-transgenic hybrid AG5011 (Agrocere Sementes) was purchased from local markets. Seeds were tested for the presence of MON810- derived *cry1Ab* insert and its expression product by PCR and immune strip test (Envirologix Inc., Portland, USA), respectively (data not shown). After the confirmation of MON810 event in GM seeds and the absence in its non-GM counterpart, these were used in the experiment. Single cross hybrid seeds are the progeny derived from the cross of a maternal endogamous line “A” with the paternal endogamous line “B”. This seed population is, therefore, supposed to have a high genetic similarity (all individuals are genotype AB). The experimental approach currently applied to the comparative assessment requires the use of control samples that is a non-transgenic, also called “conventional counterpart”, with genetic background as close as possible to the genetically modified plant under investigation (Codex, 2003; AHTEG, 2013; EFSA 2007).

Seedlings were germinated and grown on Plantmax HT substrate (Buschle & Lepper S.A.) and watered daily until dsRNA delivery started. No pesticide or herbicide was either applied.

6.3.2. dsRNA design and off-target effects prediction

dsRNA against *cry1Ab* transgene in genetically modified maize (unique identifier MON-ØØ81Ø-6) was designed to target mid portion of the full coding sequence (CDS) of the protein. The *cry1Ab* transgene sequence does not contain intron sequences (Rosati et al., 2005). Previous work by Mekawi (2009) showed mass fingerprinting and sequences results of tryptic peptides of 69 kDa CRY1Ab fragment isolated from MON810. The expected molecular weight of CRY1Ab is 92 kDa, since MON-ØØ81Ø-6 contains a truncated *cry1Ab* coding sequence that introduce the N-terminal fragment of the full length CRY1Ab protein (130 kDa) of *B. thuringiensis* ssp. *kurstaki* strain HD1 (CTNBio, 2008). However, this author suggests that transgenic CRY1Ab (92 kDa) exposed to plant proteases (such as serine proteases) have their N-terminal and C-terminal peptides removed. Nucleotide sequence was based on the previous work of Hernandez et al. (2003) (GenBank: AY326434.1).

Table 1: Primer characteristics for each analyzed gene transcript by end point PCR and RT-qPCR.

Primer name	Gene product	Genbank Accession	Primer sequence
MEP	Membrane protein PB1A10.07c	GRMZM2G018103 T01	F - GTACTCGGCAATGCTCTTGA P - AACTTCGGTTGGTGAGAGCGGAAA R - CAATCCTGACCCAGACAGATG
LUG	Leunig	GRMZM2G425377 T01	F - GGGACATAAGGGAGAAGAACAC P - TTCCCTGTAGCACTGGATGATGCC R - TCATGGCTTACTGAGGCAAC
CUL	Cullin	GRMZM2G166694 T04	F - CGACAAGGACAACGCCAATA P - ACCTTGCCTGATTGGTGGTTAGTGA R - TCCCAGTGGTATCGCATAGT F - CTTTCCAGGTGCTGGTTACT
FPGS	Folylpolylglutamate synthase	GRMZM2G393334 T01	P - TCAAGAAGTGATACGCCGCTCGAA R - TCATAGTCCAGTTCAGTTTGG
UBPC	Ubiquitin carrier protein	GRMZM2G102471 T01	F - ACAGTGGAGTCCCTGCTTTAAC P - TCAATCTGCTCACTGCTCACGGAC R - GAGCAATCTCAGGGACAAGAG F - GCCCAGAAAGGCTGTCAAT
cry1Ab	cry1Ab protein	AY326434.1	P - AGCTCTTCACGTCCAGCAATCAGA R - GACTTGGTTCGATGTGGTAGTC
ds-cry1ab	Cry1Ab protein	AY326434.1	F - TAATACGACTCACTATAGGGAGA TACTGGTCCGGCCACCAATCAT R - TAATACGACTCACTATAGGGA GAATGTTGACCGGAGGGTGTCTG

After selection of about 700 bp sequence (a shorter fragment of 603 bp was further selected based on the prediction of PCR primers, see next section and Table 1), sequence-based off-target effects were limited by excluding potential siRNAs derived from the dsRNA full sequence with a high likelihood of targeting non-target transcripts by using bioinformatics approaches (Pei and Tuschl, 2006; Birmingham et al., 2007; Grimson et al., 2007; Jackson and Linsley, 2010; Fellmann and Lowe, 2014). In short, The Basic Local Alignment Search Tool (BLAST) was used in order to search for short, nearly exact matches within the Reference RNA Sequences database (REFSEQ_RNA) for *Zea mays* from the National Library of Medicine (NCBI, 2014). Because sequence-based off-target effects can be caused by both guide and passenger strand mediated cross-hybridization with unintended transcripts, both RNA strands were used in the BLAST.

6.3.3. dsRNA synthesis

Genomic DNA template sequences were obtained by PCR amplification of primers targeting the *cryIab* transgene region of previously selected and containing T7 promoter sequence tag (Table 1). DNA was extracted from AG5011YG transgenic maize leaves containing MON-ØØ81Ø-6 insert using NucleoSpin® Plant II (Macherey-Nagel GmbH & Co., Düren, Germany). DNA was quantified using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). Following quantification, DNA amplification was carried out in 40 µl of total reaction volume. Eighty ng of gDNA was mixed with 32 µl of the amplification mixture (10 mM of each dNTP, 10 µM each primer, 1 mM MgCl₂, 1 U Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc.)) The PCR cycling was as follows: initial denaturation for 90 s at 98°C; 30 cycles of denaturation for 10 s at 98°C, annealing for 30 s at 71°C and extension for 30 s at 72°C, and final extension for 10 min at 72°C. PCR products were examined on an agarose gel prior to *in vitro* transcription to estimate concentration and to verify that the products were unique and of the expected size. PCR products were precipitated overnight in ethanol at -20°C, centrifuged for 1 h at 13000 rpm and further re-suspended in nuclease-free water. Re-suspended pellets were quantified using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc.).

Two different protocols for *in vitro* transcription were tested. The

first protocol was mainly based on the MEGAscript® RNAi Kit (Invitrogen™). Following manufactures instructions, this kit is a system for the preparation of dsRNA free of protein and other contaminating molecules and it is designed for the preparation of dsRNAs larger than 200 bp. The procedure begins with a high yield transcription reaction to synthesize two complementary RNA transcripts from template, on this case, PCR products tagged with T7 promoter sequences. The RNA strands are hybridized either during or after the transcription reaction to form dsRNA. Next, DNA and any single-stranded RNA (ssRNA) are removed with a nuclease digestion. Finally, the dsRNA is purified with a solid-phase adsorption system to remove protein as well as mono and oligonucleotides. The other protocol followed similar reagents and reaction conditions but did not use a commercial kit. All enzymes and reagents were bought separately. In this case, one microgram of PCR product (603 bp for *cryIAb* mRNA) was used as template for 20 ul of dsRNA synthesis reaction using the 1 U of T7 RNA Polymerase and Transcription Optimized 5X Buffer (Promega Corporation, Madison, USA), 10 mM rNTP mix (Invitrogen™, Carlsbad, USA) and 100 mM DTT for 2h at 37°C. Afterwards, dsRNA was digested using dnase I (Invitrogen™) and rnase (Invitrogen™) for 1 h at 37°C. The dsRNA was ethanol precipitated overnight, re-suspended in rnase-free water, and quantitated at 260 nm using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc.). The quality and integrity of dsRNA produced by both protocols were determined by agarose gel electrophoresis. dsRNA sequence is available on Table 2. Each of these protocols was tested at least three times and a week time lapse between them.

dsRNA solution molarity was calculated using Oligo Calc: Oligonucleotide Properties Calculator (Kibbe, 2007) in which the oligonucleotide sequence is analyzed for its physical and chemical characteristics using algorithms and assumptions available at <http://www.basic.northwestern.edu/biotools/oligocalc.html>. The obtained formula weight was used to calculate molarity of the solution using the Mass Molarity Calculator from Sigma-Aldrich Co. LLC. available at <http://www.sigmaaldrich.com/chemistry/stockroom-reagents/learning-center/technical-library/mass-molarity-calculator.html>.

Table 2: *Cry1Ab*-derived double-stranded RNA sequence (Genbank accession: AY326434.1) used in this study. Double-stranded RNA sequence is given in a single stranded format.

Cry1Ab-derived dsRNA
UUGUUAUAGUAAGGCAGGAGGGUUUAGUGGGUUUAGGGGGAGUG GUUCAGGUGGUUGGACCCGUCGCGUGGAGGCACCACUUCCCGGG UCCGAAGUGCCCGCCGUCGUAGGACGCGUCCUGGAGGGGCCCCGGU CUAGUCGUGGGAGGCGCAGUUGUAGUGGCGAGGGGACAGGGUCUC CAUGGCGCAGUCCUAAGCGAUGC GAUCGUGGUGGUUGGACGUUAA GGUGUGGAGGUAGCUGCCGUCGCGUAGUUAGUCCAUUGAAGAG GCGGUGGUACAGGUCGCGUCGUUGGAGGUUAGGCCGUCGAAGGC GUGGCACCCAAAGUGGUGGGGGAAGUUGAAGAGGUUGCCGAGGUC GCAAAAGUGGGACUCGCGGGUGCACAAGUUAAGGCCGUUACUCCA CAUGU AACUGGCGUAACUCAAGCACGGUCGGCUCCAGUGGAAGCU UCGGCUCAUGCUGGACCUCUCUCGGGUCUCCGACAGUUACUCGA GAAGUGCAGGUCGUUAGUCUAGCCGGACUUCUGGCUGCAGUGACU GAUGGUGUAGCUGGUUCAGAGGUUGGAGCACCUCACGGAGAGGCU ACUCAAGACGGAGCUGCUCUUCUCCUCGACAGGCUCUCCACUU

6.3.4. Knock down pilot experiments

Pilot experiments were performed to optimize mRNA knockdown and, consequently, reduction of protein concentration. Before primary screening, pilot experiments were done in which RNAi doses, incubation times and other assay variables were ‘titrated’ to optimize assay responses to negative and positive control siRNA molecules; these control values determine baseline, signal and standard deviation values for the system (Sharma and Rao, 2009).

Chemistry of the dsRNA effector molecules. Long dsRNA molecules are taken up and cleaved intracellularly by the cellular type III endoRNase Dicer to yield a pool of overlapping siRNA molecules with high specificity for the target gene (Brodersen and Voinnet, 2006). This approach has been very useful in model organisms (*C. elegans*, *Drosophila* and many plant species) that lack the defensive type I interferon response of the mammals (Bridge et al., 2003). Therefore, long dsRNA molecules are potent inducers of gene silencing.

Delivery systems. Three different delivery systems were tested in time-course pilot experiments using naked dsRNA molecules diluted in nuclease-free water. The delivery of naked dsRNA molecules comprises the direct provision of dsRNA molecules without the need/presence of

any biological vector, such as plasmids or viral vectors. The first pilot experiment consisted of six treatments: dsRNA targeting *cry1Ab* transgene was delivered into maize seedlings at four different concentrations and transgenic and conventional maize seedlings were also fed with water as positive and negative controls, respectively. Intact maize leaves (V3 stage plants, 15 days after sowing; two biological replicates per treatment) were fed through the mesocotyl (Sun et al., 2005). The first experiment was performed in a matrix-like design in order to evaluate reduction in mRNA levels under two factors: time (hours after feeding dsRNA) and dsRNA concentration. Titration of dsRNA solution followed: 5, 10, 50 and 150 ug (approximately 30 nM up to 1000 nM) in 500 ul final volume. Leaf tissue of each plant was collected at 24, 48, 72 and 96 h after feeding dsRNA or water. Leaf samples were placed in cryogenic tubes at -80°C until processed for total RNA extraction, RT-qPCR and lateral flow strip testing.

A second pilot experiment followed similar experimental design. Before spraying, one leaf of each seedling was first wounded with carborundum as an abrasive (silicon carbide, 600 grit; VWR BDH Prolabo, Radnor, USA) (Lu et al., 2003; Tenllado et al., 2003, Andrie et al., 2012). Maize seedlings were sprayed with dsRNA solution or water (Gan et al., 2010). Wounded and unwounded leaves were sampled only at 72h after spraying. The best time-course was defined by the results of the first pilot experiment. Control samples spray with water would isolate the effects deriving from wounding leaves.

The third pilot experiment consisted of feeding a dsRNA water solution through the petiole of V3 stage maize seedlings. Seedlings received three doses of dsRNA solution every 72 h (150ug/150ul). In between dsRNA doses, seedlings were watered or fed with a MS medium without agar and containing 20 g/l of sucrose (MS-20) (Murashige and Skoog, 1962). After the last dose, leaf tissue of treatment and control samples were collected and stored at -80°C for further analysis. These pilot experiments were repeated twice.

The use of a transient delivery system was based on previous observations of stable RNAi experiments that seem to deliver a more complex biological system than transient experiments (Tenllado et al., 2003). The stable expression of dsRNA molecules requires the insertion of a transgene construct into the host organism genome. Most of the methods applied for stable RNAi expression experiments need vector construction and plant transformation, which then requires molecular

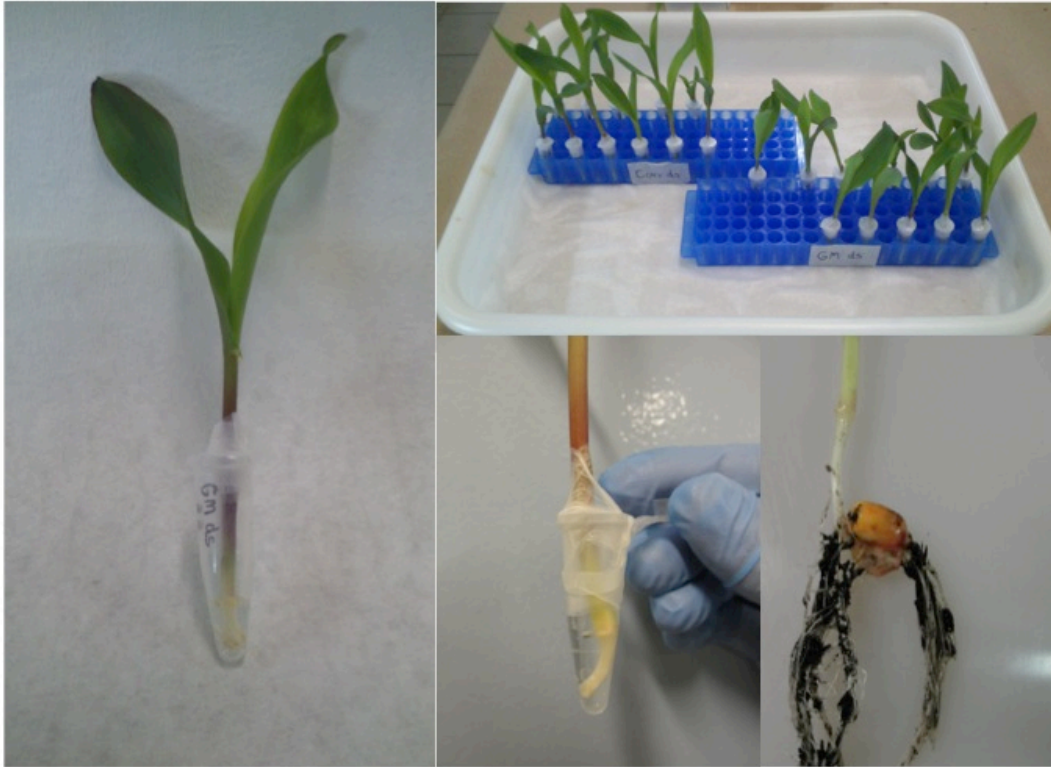
characterization, and a proper investigation of possible pleiotropic effects derived from DNA insertion into host genome (Senthil-Kumar and Mysore, 2011). In addition, for the investigation of possible pleiotropic effects derived from the transformation process of commercial transgenic maize variety for the purpose of addressing biosafety issues, a second transformation in our biological model would mask our observations. It is expected that some transient delivery systems introduce nucleic acid sequences into cells genome that have been targeted (e.g. transfection by bacterial vectors) (for review see Chapter III). Therefore, the use of naked dsRNA molecules without biological vector or chemical carriers was preferred.

6.3.5. Final knock down experiment

Separate time-course pilot experiments were conducted to determine the effect of dsRNA feeding, spraying or mechanically inoculated on silencing *cry1Ab* transgene using the same experimental design, treatments, and parameters as described above with a few exceptions. Results of initial pilot screening tests lead to the design of this optimized knock down experiment. Maize seedlings were grown under controlled conditions as described above. At V3 stage, plants were removed from substrate, radicle/primary roots were cut and the remaining seed was detached from the mesocotyl (Figure 1). Five plants were randomly sampled per treatment to constitute a biological replicate. Around 500 mg of leaf tissue was collected from each one of the three biological replicates for further analysis (ELISA test, RT-qPCR and 2DE). The leaves were cut, weighted and placed in 3.8 ml cryogenic tubes before immersion in liquid nitrogen. The samples were kept at -80°C until extraction.

This experiment consisted of four treatments: (i) GM plants fed with water, (ii) conventional plants fed with water, (iii) conventional plants fed with dsRNA targeting *cry1Ab* transgene, and (iv) GM plants fed with dsRNA targeting *cry1Ab* transgene. Replication was achieved over five independent plants per treatment (considered biological replicates). Plants were placed on 1.5 ml micro tubes and dsRNA solutions of 50ug/50ul were fed overnight. During the day, plants were fed *ad libitum* with MS-20 medium. This was maintained for 12 days. A matrix design graphic is presented in Table 3 showing treatment, controls, dosage and time of sampling.

Figure 1. Plant samples used in the final RNA interference experiment with *cry1Ab* transgene present in MON810 transgenic maize event triggered by *cry1Ab*-derived double-stranded RNA molecules. Transgenic maize seedlings at V3 stage (3 leaves) were initially fed with a water solution containing 30-1000 nM through the mesocotyl.



6.3.6. RNA isolation and RT-qPCR analysis

RNA was extracted from approximately 100 mg of frozen leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. In brief, sample is homogenized with guanidine-isothiocyanate lysis buffer and further purified using silica-membrane. During purification, in-column DNA digestion was performed using RNase-free DNase I supplied by Qiagen to eliminate any remaining DNA prior to reverse transcription and real-time PCR. The extracted RNA was quantified using a NanoDropTM 1000 (Thermo Fisher Scientific, Wilmington, USA).

Reverse-transcription real-time PCR assay was adapted from previously developed assay for the specific detection of MON-00810-6 *cry1Ab* transgene (CRL-GMFF, 2009) to hydrolysis ZEN - Iowa Black® Fluorescent Quencher (ZEN/ IBFQ) probe chemistry (Integrated

DNA Technologies, INC Iowa, USA).

Following quantification, cDNA was synthesized and amplification of each target gene was performed using the QuantiTect Probe RT-PCR Kit (Qiagen) according to manufacturer instructions. RT-qPCR experiment was carried out in triplicates using StepOne™ Real-Time PCR System (Applied Biosystems, Singapore, Singapore). Each 20 µl reaction volume comprised 10 uM of each primer and probe and 50 ng of total RNA from each sample. The amplification efficiency was calculated from relative standard curves provided for each primer using StepOne™ Real-Time PCR System software (Applied Biosystems).

The two most suitable endogenous reference genes out of five candidates (ubiquitin carrier protein, folsylpolyglutamate synthase, leunig, cullin, and membrane protein PB1A10.07c) were selected as internal standard. The candidate genes were chosen based on the previous work of Manoli et al. (2012). The selection of the endogenous reference genes for this study was performed using NormFinder (Molecular Diagnostic Laboratory, Aarhus University Hospital Skejby, Denmark) statistical algorithms (Andersen et al., 2004). Multiple algorithms have been devised to process RT-qPCR quantification cycle (Cq). However, NormFinder algorithm has the capability to estimate both intragroup and intergroup variance, and the identification of the two reference genes as most stable normalizers (Latham et al., 2010). The leunig and membrane protein PB1A10.07c genes were used to normalize *cry1Ab* mRNA data due to their best stability value (SV for best combination of two genes 0.025). Conventional samples were also analyzed in order to check for PCR and/or seed contaminants and to serve as a negative control in the PCR assay. Primer and probe sequences used are provided in Table 1. These have been assessed for their specificity with respect to known splice variants and single-nucleotide polymorphism positions documented in transcript and single-nucleotide polymorphism databases.

The relative expression ratio value (RQ) was calculated for MON-ØØ81Ø-6 transgenic event samples delivered with water relative to samples, which have been submitted to dsRNA treatment, according to the Pfaffl equation (Pfaffl, 2001). Control samples were considered a pool of at least three biological replicates.

Table 3: Time-dose experimental matrix design. Leaf tissue from genetically modified maize event MON810 of three plants were collected at each time-point and dsRNA targeting *cry1Ab* transgene transcript dosage according to the following organization.

dsRNA dosage	Time-point sampling			
	24 h	48 h	72 h	96 h
30 nM	Sample 1.1	Sample 2.1	Sample 3.1	Sample 3.1
100 nM	Sample 1.2	Sample 2.2	Sample 3.2	Sample 3.2
500 nM	Sample 1.3	Sample 2.3	Sample 3.3	Sample 3.3
1000 nM	Sample 1.4	Sample 2.4	Sample 3.4	Sample 3.4

6.3.7. CRY1Ab protein detection and quantification

Confirmation of knock down of the specific target gene is an essential step to ensure the success of any RNAi experiment. The approaches used to validating effective dsRNAs included the demonstration that the expression level of the target mRNA and the gene product is substantially reduced while a negative control dsRNA shows no effect. While mRNA levels were verified using RT-qPCR, transgene product, CRY1Ab protein, was checked using two different methods.

Lateral flow membrane strips. QuickStix™ Kit for Cry1Ab in corn commercial lateral flow test strips (EnviroLogix Inc., Portland, USA) were used to detect CRY1Ab protein present in crude extracts of maize leaves. About 15 mg (1 cm diameter leaf disk) were grinded in 1.5 ml micro tubes with a pestle in the presence of 0.5 ml extraction buffer supplied by the manufacturer. Strips were placed in the tubes and after 10 min they had the bottom section removed for storage purposes. These were then photographed in a MultiDoc-It™ Imaging System (UVP LLC, Upland, USA) under white mode light.

Enzyme-Linked Immunosorbent Assay. Commercial ELISA Bt-Cry1Ab/1Ac kit (Agdia Inc., Elkhart, USA) was used to estimate CRY1Ab concentration in leaf samples of the RNAi experiment. Bacterial CRY1Ab protein (purchased from Dr. Marianne Pusztai-Carey, Case Western Reserve University, USA) was used to perform a standard curve with a serial dilution. Five concentrations (0, 0.1, 0.5, 1, 1.5 ug/ml) of protein standards were used for the calibration. ELISA procedures followed manufacture indications. Results were normalized with leaf sample fresh weight.

6.3.8. Protein extraction

Samples were separately ground with liquid nitrogen in a mortar. Protein extraction was carried out according to Carpentier et al. (2005), i.e. by phenol extraction and ammonium acetate in methanol precipitation. Pellets were resuspended in urea/thiourea buffer (2% v/v Triton X-100 (Sigma-Aldrich Corporation, St. Louis, USA), 2% v/v Pharmalyte (GE Healthcare), 5 mM PMSF, 7 M urea and 2 M thiourea). Protein quantification was performed by means of the copper-based method 2-D Quant Kit (GE Healthcare) and stored at 4°C.

6.3.9. Two-dimensional IEF/SDS–PAGE and protein staining

The extracted proteins were separated by 2-DE as described by Weiss and Görg (2008). In the isoelectric focusing step (IEF), Immobiline™ DryStrip gels with 13 cm and linear pH range 4-7 (GE Healthcare) were used. Strips were previously rehydrated with 750 µg of total protein and rehydration solution (7M urea, 2M thiourea, 2% w/v CHAPS, 0.5% v/v IPG buffer (GE Healthcare), 0.002% w/v bromophenol blue). Strips were then focused on an Ettan IPGPhor IEF system (GE Healthcare) and subsequently equilibrated for 30 min in slow agitation in a Tris-HCl solution (75 mM), pH 8.8, containing 2% w/v SDS, 29.3% v/v glycerol, 6 M urea and 1% w/v dtt or 2.5% w/v iodoacetamide. The strips were then placed on top of SDS-PAGE gels (12%, homogeneous) for the second dimension run using a Hoefer DALT system (GE Healthcare) according to manufacturer's guidance.

6.3.10. Quantitative analysis of maize proteomes

Proteins were visualized by CBB G-250 colloidal stain (MS compatible) as described by Candiano et al. (2004) which increases the staining sensitivity to approximately 1 ng of protein. Each gel was scanned using ImageScanner™ III (GE Healthcare). Cross-comparisons among the different samples were performed using the software Image Master 2D Platinum version 7.0 (GE Healthcare).

After manual verification of spots, gels were matched according to hierarchical condition. Gels from different treatments were first internally matched and only spots that were present on at least three gels within the treatment (with coefficient of variation < 20%) were included

in the analysis.

6.3.11. In-gel digestion and protein identification by MS/MS

Spots were excised and sent to the proteomics platform at the University of Tromsø, Norway for processing and analysis. These were subjected to in-gel reduction, alkylation, and tryptic digestion using 2–10 ng/μl trypsin (V511A; Promega) (Shevchenko et al., 1996). Peptide mixtures containing 0.5% formic acid were loaded onto a nano ACQUITY Ultra Performance LC System (Waters Massachusetts, USA), containing a 5-μm Symmetry C18 Trap column (180 μm × 20 mm; Waters) in front of a 1.7-μm BEH130 C18 analytical column (100 μm × 100 mm; Waters). Peptides were separated with a gradient of 5–95% acetonitrile, 0.1% formic acid, with a flow of 0.4 μl/min eluted to a Q-TOF Ultima mass spectrometer (Micromass; Waters). The samples were run in data dependent tandem MS mode. Peak lists were generated from MS/MS by the Protein Lynx Global server software (version 2.2; Waters). The resulting pkl files were searched against the NCBI nr 20140323 protein sequence databases using Mascot MS/MS ion search (Matrix Sciences; <http://matrixscience.com>). The taxonomy used was Viridiplantae (Green Plants) and ‘all entries’ and ‘contaminants’ for contamination verification. The following parameters were adopted for database searches: complete carbamidomethylation of cysteines and partial oxidation of methionines; peptide mass tolerance ± 100 ppm; fragment mass tolerance ± 0.1 Da; missed cleavages 1; and significance threshold level ($P < 0.05$) for Mascot scores ($-10 \log(P)$). Even though high Mascot scores are obtained with significant values, a combination of automated database searches and manual interpretation of peptide fragmentation spectra were used to validate protein assignments. Molecular functions and cellular components of proteins were compared against ExPASy Bioinformatics Resource Portal (Swiss Institute for Bioinformatics; <http://expasy.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology system database release 69.0 2014 (<http://kegg.jp/kegg/ko.html>).

6.3.12. Statistical Analysis

Real-time relative quantification data were plotted and manually analyzed using Microsoft Excel (Microsoft, Redmond, WA).

Normalized gene expression data was obtained using the Pfaffl method for efficiency correction (Pfaffl, 2001). Cq average from each technical replicate were calculated for each biological replicate and used to make a statistical comparison of the genotypes based on the standard deviation of the difference between means using the estimate of random variation at the level of biological replication. These were calculated at 5% level of significance. Information on real-time data for this study has followed guidelines from the Minimum Information for Publication of Quantitative Real-Time PCR Experiments paper (Bustin et al., 2009). For the 2-DE experiment, one-way ANOVA was used to investigate differences at individual protein levels. The calculations are based on normalized spot volume ratios based on the total intensity of valid spots in a single gel. Differences at the level $P < 0.05$ were considered statistically significant. Statistical analyses were performed using ImageMasterTM 2D Platinum 7.0, version 7.06 (GE Healthcare).

6.4. RESULTS AND DISCUSSION

6.4.1. Performance of *in vitro* transcription of a commercial kit versus *in-house* protocol for dsRNA synthesis

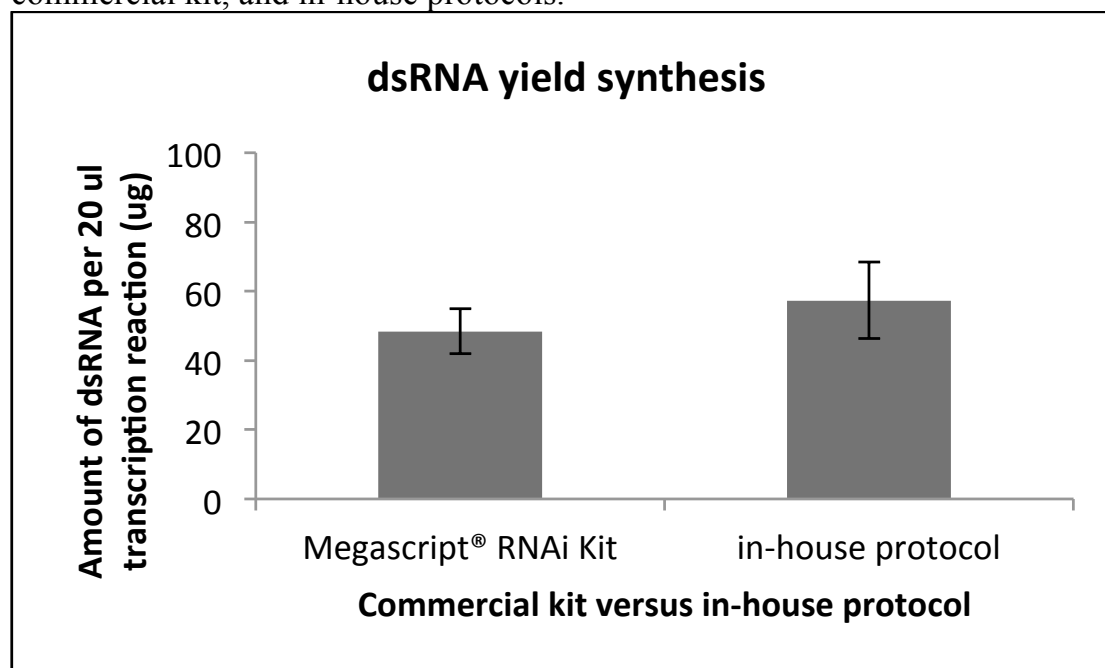
The efficiency of producing large amounts of dsRNA molecules was tested using a commercial kit and an *in-house* protocol. The preliminary results obtained for the parallel test of MEGAscript® RNAi Kit and our *in-house in vitro* transcription shows higher levels of efficiency for the *in-house* protocol at a value of 10 ug more dsRNA for each 20 ul transcription reaction (Figure 2). Nonetheless, taking into consideration that the commercial kit allows only 20 reactions and the *in-house* protocol allows approximately 100 reactions (it will depend on the concentration of each enzyme), and that the kit costs approximately 1.8 times less (it will also depend on values practiced in each country by each manufacturer), the *in-house* protocol yielded an average of 3 times more dsRNA than the commercial kit. It was also taken into account the enzymes used for purification purposes.

6.4.2. Time and dose-dependent RNAi effectiveness against p35S-cry1Ab transgene expression.

In the present study, it was investigated the silencing potential of a

dsRNA sequence targeting a transgene expressing a insecticidal CRY protein that is regulated by a modified sequence of the Cauliflower Mosaic virus (CaMV) 35S promoter (p35S) present in MON-00810-6 transgenic maize event.

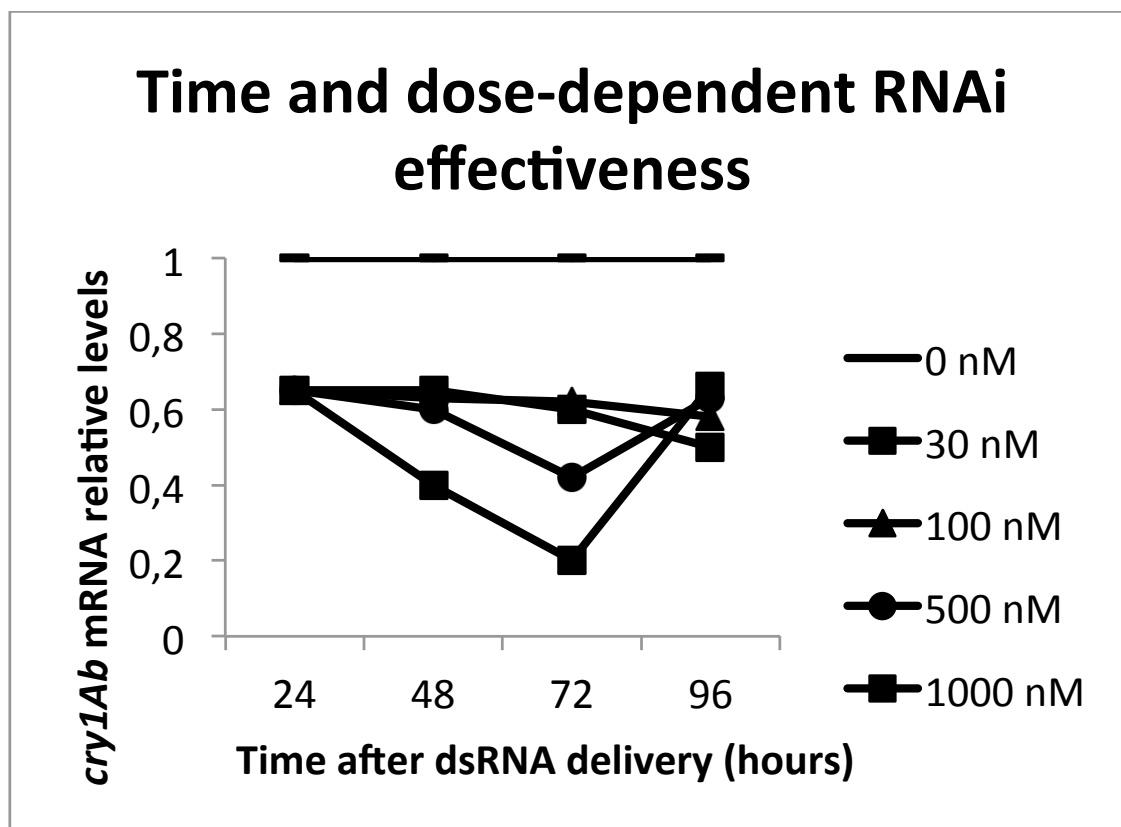
Figure 2: Total amount of dsRNA produced per 20 ul reaction volume according to reagents and protocols from the Megascript® RNAi kit, a commercial kit, and in-house protocols.



The CaMV p35S is a strong promoter used for the constitutive expression of transgenes in nearly all genetically modified crop plants (Vlasak et al., 2003). The RT-qPCR results from our first pilot experiment show that transgenic maize seedlings were silenced at 72 h after 1000 nM dosage of dsRNA delivery through mesocotyl feeding reaching 80% reduction of *cry1Ab* mRNA levels (Figure 3).

Other concentrations of dsRNA solution also showed silencing effects but at lower efficiency. As expected, greater reduction in target mRNA levels were observed at 48-72 h after dsRNA delivery. The third experiment followed the most successful treatment of experiment one, 1000 nM dose after 72 h delivery, but was maintained for 9 days or three dsRNA doses. The results from this assay were equally efficient as the previous one (Figure 4).

Figure 3: Levels of *cry1Ab* transgene transcript detected by RT-qPCR over the course of a time-dose RNAi experiment. Time-point samplings were performed at 24, 48, 72 and 96 h post dsRNA delivery. dsRNA concentrations were 30-1000 nM. $N=3$ for each sampling.



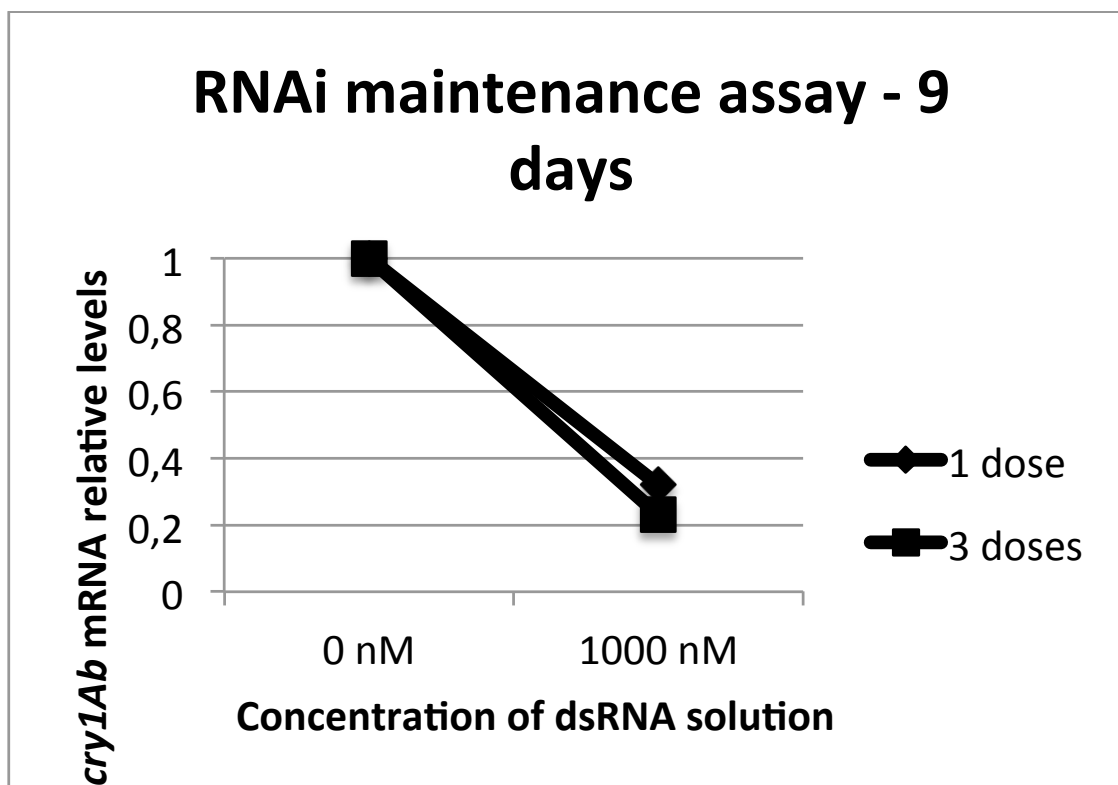
6.4.3. Delivery methods for transient RNAi targeting p35S-cry1Ab transgene

In plant systems, PTGS has been studied by a number of methods of dsRNA or siRNA delivery from transforming plants with dsRNA-forming vectors to introducing a tobacco rattle virus (TRV)-based or by agroinfiltration (Fagard and Vaucheret, 2000; Watson et al., 2005; Eamens et al., 2008). However, because each of these methods consists of a promoter-based expressing transgenic cassette, it could mask the potential adverse effects derived from the original plant transformation event.

6.4.3.1. Mesocotyl feeding

Previous studies suggested that RNA is commonly transported through the phloem (Ruiz-Medrano et al., 1999; Lucas et al., 2001; Tenllado et al., 2003; Tournier et al., 2006; Andrieu et al., 2012) and that the regulation of RNA trafficking plays an important role in plant development in addition to its role in PTGS (reviewed in Vance and Vaucheret, 2001; Haywood et al., 2002; Jorgensen, 2002). These studies were mainly based on the delivery of dsRNA solutions into the vascular system of plant leaves, being spread systemically afterwards (Kalantidis et al., 2008). Sun et al. (2005) proposed a similar approach delivering antisense oligodeoxynucleotide administered to the leaf cells by feeding through the petiole.

Figure 4: Levels of *cry1Ab* transgene transcript over the course of a 9 days long experiment delivering dsRNA at the 1000 nM concentration to V3 stage transgenic maize plants. $N = 3$ plants.



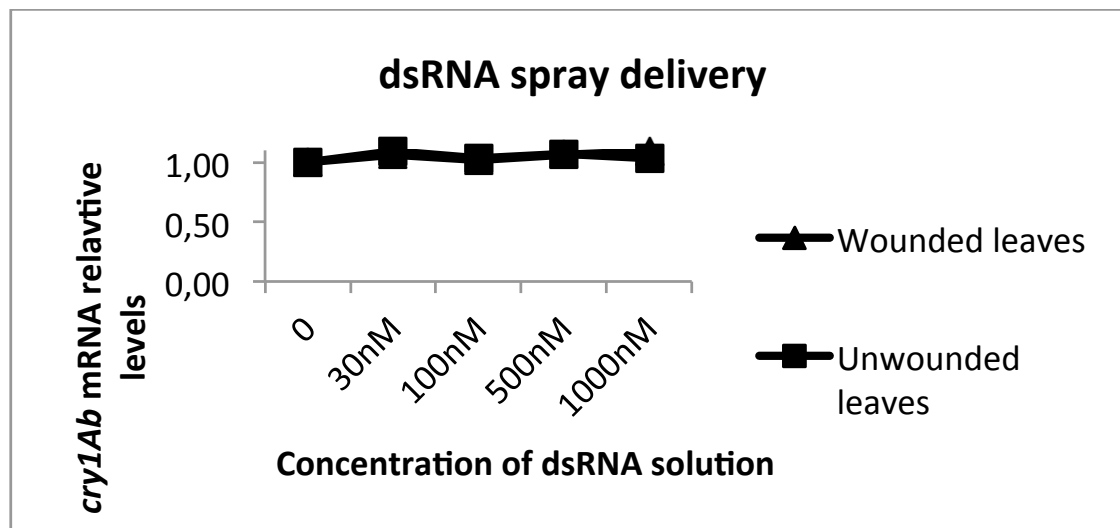
The results from the pilot experiment confirmed systemic silencing from mesocotyl feeding in maize seedlings. Systemic posttranscriptional gene silencing has been reported by many plant studies (for review see Kalantidis et al., 2008). Systemic silencing of transgenes in *Nicotiana benthamiana* was initiated, in localized regions

of the plant, by introduction of transgene- homologous DNA fragments, including those without a promoter, by particle bombardment (Voinnet et al., 1998). This experiment showed a sequence- specific signal of gene silencing spread from cells that had received the ectopic DNA, via a relay mechanism that employs plasmodesmata and phloem channels. Whether dsRNA molecules were transported from mesocotyl to maize leaves by xylem or phloem vascular system is yet unknown.

6.4.3.2. Naked dsRNA spray onto wounded and unwounded leaves

From a practical standpoint, we tested a simple spray technique for the delivery of interfering products onto the surface of plant leaves. In addition, this method provided an alternative to removing seedlings primary roots and aimed at extending their lifetime within the substrate. It was sprayed wounded and unwounded leaves of transgenic maize aiming at silencing transgene expression. Wounds were performed under grafting procedures similar to Tenllado et al. (2003). Neither wounded nor unwounded leaves were silenced at any dsRNA concentration (Figure 5). The success of gene silencing by the application of spray systems to deliver dsRNA into plant varies depending on the biological model. Gan et al. (2010) have demonstrated that crude extracts of *Escherichia coli* HT115 containing large amounts of dsRNA were applied to maize plants (V8 stage) as a spray and the experiment confirmed a preventative efficacy to Sugarcane Mosaic Virus (SCMV) infection. Probably, due to the rnase III-deficient *E. coli* strain that can express dsRNA at high efficiency levels and also the lower amount of viral transcripts present in maize leaves compared to our transgene derived mRNA expression. Rnase III-deficient plasmids are designed to produce large amounts of dsRNA continuously (Timmons et al., 2001; Tenllado et al., 2003; Zhang et al., 2008). Tenllado et al. (2003) were successfully in using purified bacterial dsRNAs spray onto wounded leaves to promote specific interference with the infection in *N. benthamiana* plants by two viruses belonging to the tobamovirus and potyvirus groups. On the other hand, Lu et al. (2003) have tested five different delivery systems in order to investigate RNA-mediated antiviral defense mechanism in plants. But they were not successful when applying direct inoculation of transcripts on *Arabidopsis* infected with tobacco rattle virus (TRV) by applying a naked dsRNA solution onto wounded leaves.

Figure 5: Levels of *cry1Ab* transgene transcript on V3 stage transgenic maize plants, which were sprayed with a solution containing dsRNA at the 1000 nM concentration. Some plants had their leaves wounded with carborundum as an abrasive before dsRNA spray. $N=3$.



6.4.4. CRY1Ab protein turn over

CRY1Ab protein has been detected during the time course of all pilot experiments. Commercially available lateral flow membrane strips were used to monitor protein levels while performing pilot assays. During the time course of the first pilot experiment, CRY1Ab has been detected in all samples, including silenced samples. There was visually no reduction in band intensity, even on silenced plants, thus leading to the conclusion that the time course analyzed was not enough for protein degradation. The second experiment, which had not shown efficient silencing effects showed similar results regarding CRY1Ab detection. The third pilot experiment that maintained seedlings for 9 days under transgene silencing conditions (3 doses of dsRNA every 72 h) showed less CRY1Ab protein, by means of visual band intensity (data not shown).

CRY1Ab present in MON-ØØ81Ø-6 transgenic maize event stability has been also predicted by using Protparam Tool (<http://web.expasy.org/protparam/>) that allows the computation of various physical and chemical parameters for a given protein sequence. The results show that the estimated half-life is 30 h in a supposed mammalian system (using an algorithm predicted from mammalian reticulocytes *in vitro* study) and above 20 h for other biological models

(yeast and *E. coli in vitro*). In addition, the software calculates the instability value, in this case is computed to be 37.77, which classifies this protein as a 'stable' protein. Not surprisingly, other authors have already investigated the crystal nature of these toxins. Douville et al. (2005), for example, showed that corn CRY endotoxin is degraded more rapidly in water than in soils (half-life time of 4 and 9 days respectively), while crystals appeared to be more resilient, as expected. In addition, other studies have found that the degradation of the CRY1Ab protein was significantly prolonged under flooded conditions compared with aerobic conditions, with half-lives extended to 45.9 to 141 days, with results that differ substantially from other assays (Wang et al., 2007).

Monsanto published CRY protein concentration data from MON-ØØ81Ø-6 (Monsanto, 2002). Data was compiled from field trials in Europe and the USA from 1994 to 1996. Means and ranges were given for leaves and other plantorgans. Leaves, for example, showed a CRY1Ab concentration ranging (for all data) from 5.21 to 15.06 µg/g fresh weight. The means of CRY1Ab concentrations in leaves from different trials were reported to range from 8.60 to 12.15 µg/g fresh weight. Across seasons, the average CRY1Ab concentration for MON-ØØ81Ø-6 ranged from 2.4 to 6.4 µg/g fresh weight in top leaves, and, from 3.8 to 5.7 µg/g fresh weight in bottom leaves, during the four development stages tested. Nguyen and Jehle (2007) concluded that their studies corroborate the tendencies of reported CRY1Ab contents of MON-ØØ81Ø-6, a considerable variation in the expression levels of CRY1Ab has been observed. The observed variation exceeds variation levels reported previously and may be due to the large number of analysed samples and different growing years. They suggest a certain plant-to-plant variation in CRY1Ab expression. Then and Lorch (2008) have revised published data on potential factors influencing or correlating with the CRY1Ab content in transgenic plants MON-ØØ81Ø-6.

Care must be also taken when comparing results from different detection methods. Székács et al. (2012) have performed a laboratory ring trial for determination of Cry1Ab toxin in leaf tissue of MON-ØØ81Ø-6 maize using a standardized enzyme-linked immunoassay protocol. Statistical analysis was carried out by the ISO 5725-2 guidelines. They have found that determined concentrations by in-house protocols were statistically not different in one laboratory and different

in two laboratories from the corresponding values by the joint protocol. These authors emphasize the importance of a standardized protocol among laboratories for comparable quantitative Cry1Ab toxin determination. However, even when using a standardized protocol, significant differences still occur among toxin concentrations detected in different laboratories, although with a smaller range of variation.

6.4.5. Analysis of effective transient RNAi assay to silence p35S controlled transgenes

To establish an efficient method for testing substantial equivalence in transgenic crops, we tested different dsRNA delivery systems by using the same dsRNA sequence with different concentrations watering it through the mesocotyl or as a spray solution onto wounded and unwounded leaves; time-course assays were also tested. We aimed at assessing an efficient and less complex delivery of dsRNA molecules into transgenic plants to address potential adverse effects deriving from the insertion of transgenes. The spray method was completely inefficient on maize leaves whatever concentration or wound was performed. Only the method of delivering dsRNA through feeding mesocotyl yielded good results. But because CRY1Ab protein is highly stable in the plant tissue, a longer experiment was required.

As expected, the results from the last experiment showed high silencing effects on *cry1Ab* transgene (Figure 6). The qualitative commercial ELISA kit has been adapted to work quantitatively by performing a standard curve within each run using purified bacterial CRY1Ab protein. ELISA results were interpreted in comparison to the standard curve in order to precisely calculate the concentrations of protein in various samples. The best-fit linear regression equation was $y = -0,6625x + 2,7025$. Unfortunately, due to the lack of more ELISA plants, the samples were tested in pool, each pool representing a treatment. Figure 7 shows relative expression levels of CRY1Ab compared to the control treatment 'GM water', which is the transgenic maize fed with water only. Interestingly, we have found higher amounts of CRY1Ab than those observed in the literature. Our control treatments showed approximately 28 ug of CRY1Ab protein per g of fresh weight. Similarly, our treated samples showed around 22 ug.g⁻¹ fresh weight at 6 days after dsRNA delivery and 17 ug.g⁻¹ fresh weight at 12 days after dsRNA delivery, representing 79 and 63% of expression in relation to

the control samples. This might be explained by inconsistency in measuring concentration of purified protein used in the standard curve.

6.4.6. Proteomic profile of silenced MON810 transgenic maize

The proteomic profiles using 2-DE was determined by the use phenol-based extraction methods, 13 cm gel large and coomassie blue protein staining. Quantitative protein differences between a GM hybrid and its comparable near-isogenic non-transgenic hybrid were investigated by comparison of dsRNA treated versus untreated samples. Experimental variations have been avoided by the exclusion of spots that were not present in at least three gels within each treatment. Each treatment sample consisted of a pool of five biological replicates.

Figure 6: Levels of *cry1Ab* transgene transcript on transgenic maize MON810 leaf tissue under RNAi treatment with a 1000 nM dsRNA solution targeting *cry1Ab* transgene transcript. Two time-point sampling were made for monitoring transgene expression during the entire experiment.

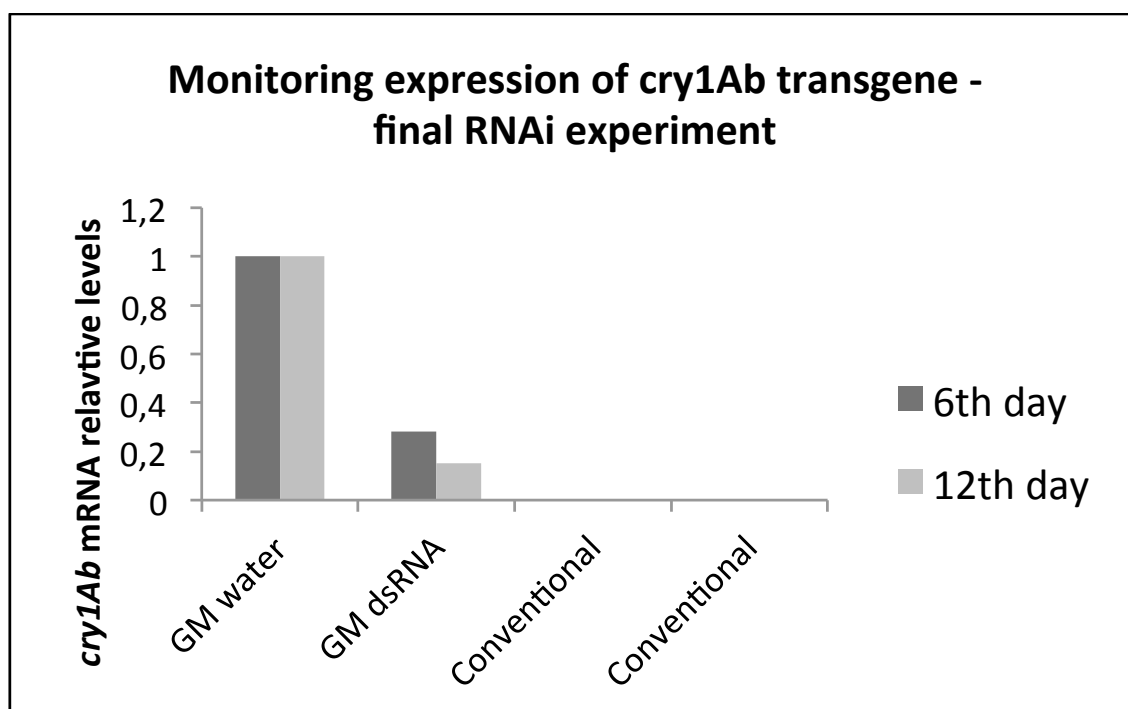
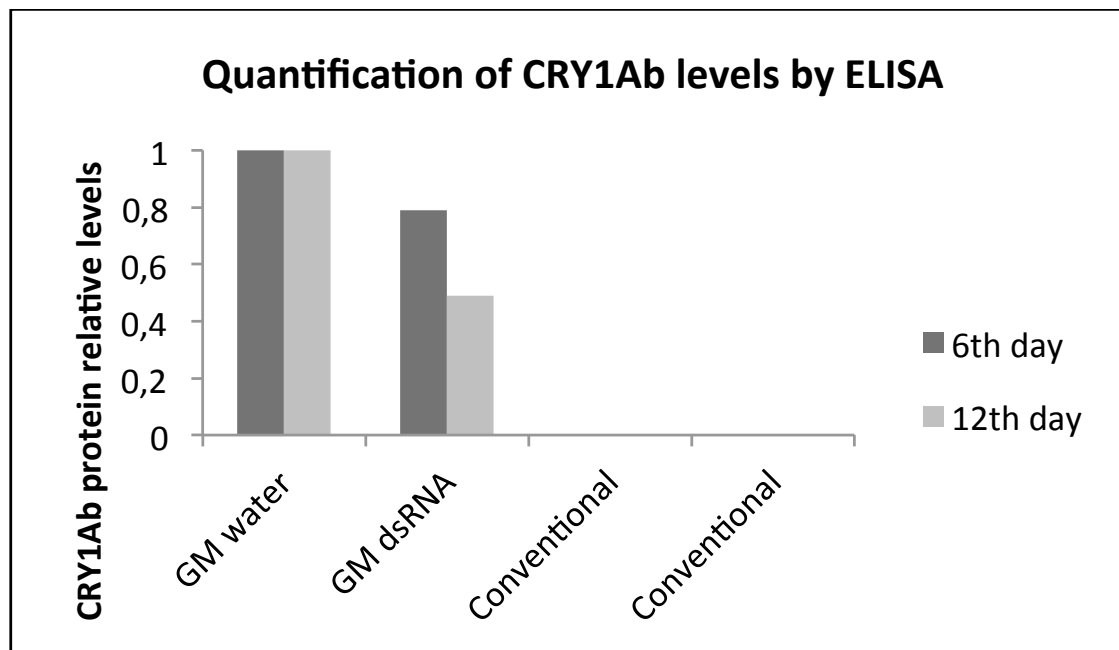


Figure 7: Levels of CRY1Ab transgenic protein on transgenic maize MON810 leaf tissue under RNAi treatment with a 1000 nM dsRNA solution targeting cry1Ab transgene transcript. Two time-point sampling were made for monitoring transgene expression during the entire experiment.



The total protein content mean was $1.48 \pm 0.04 \text{ mg.g}^{-1}$ of fresh weight. No statistically significant differences were found between treatments. The average number of spots (864) on the 2-DE gel from GM and non-GM plants grown under dsRNA treatment and control samples showed similar patterns and they were considered well resolved for a 13 cm gel stained with coomassie blue. No statistically significant differences were found between and within treatments (data not shown).

Figure 8a, 8b, 8c and 8d show representative gels for dsRNA GM and conventional treated and untreated samples, respectively. We have renumbered spots ID to facilitate reading. Table 4 described all possible proteome comparisons in this study. We have performed only the two last comparisons for the purpose of addressing our biological question on testing substantial equivalence of transgenic versus non-transgenic near isogenic maize plants.

The first comparison between GM treated and untreated samples showed a total of 11 spots that were differentially expressed in both gel images (Spots ID from 1 to 11). By detecting spots changes between GM dsRNA with GM water treated samples, we can detect protein

differences that are related to dsRNA treatment and to the reduction in transgenic protein levels.

Table 4: Schematic design of possible gel-to-gel comparison and expected results.

Gel Comparison		Expected outcomes
Gel 1	Gel 2	Observed effects
GM dsRNA treated	Conventional dsRNA treated	1. Transgenic insertion 2. 0-50% protein
GM dsRNA treated	Conventional water treated	1. Transgenic insertion 2. 0-50% protein 3. RNAi process
GM water treated	Conventional dsRNA treated	1. Transgenic insertion 2. 0-100% protein 3. RNAi process
GM water treated	Conventional water treated	1. Transgenic insertion 2. 0-100% protein
GM dsRNA treated	GM water treated	1. 100-50% protein 2. RNAi process
Conventional dsRNA treated	Conventional water treated	1. RNAi process

The second comparison between treated and untreated Conventional samples showed a total of 9 differentially expressed spots (Spots ID 12 to 20); with this comparison, all detected changes are related to the RNAi process alone because the dsRNA does not have homology to any known maize endogenous protein.

The results from the previous comparative analyses were plotted together and spots present in treatment versus control samples in both genotypes and with identical protein identities were considered to be linked to off-target effects of the exogenous RNAi machinery. Thus, by excluding these from the analysis, we were able to isolate the effects derived from the reduction of CRY1Ab protein only.

Interestingly, when we plot these differentially expressed spots together, we found out that spots 11 (GM water) and 14 (Conventional water) match, as well as spots 13 (Conventional ds) and 3 (GM ds). The first protein is repressed in both GM and Conventional treated with dsRNA solution probably due to some metabolic change deriving from the saturation of the RNAi machinery by treated cells (Dai et al., 2014). On the other hand, the second protein is oppositely regulated by the

induction of *cry1Ab* transgene silencing. For some unknown reason, this protein is only activated when large amounts of dsRNA enter cell, regardless of its efficiency in targeting a homologous mRNA. Thus, the 9 spots left in the GM comparison are proteins that are altered due to potential pleiotropic effects. And the 7 spots left from the Conventional comparison are then related to the RNAi triggers. Therefore, these results show that even in the absence of high levels of CRY1Ab transgenic protein, these plants cannot be considered substantial equivalents and further experiments should be performed in order to address their biological relevance.

6.4.7. Mass spectral identification of differentially expressed proteins

Comparison of GM and the near-isogenic variety treated with *cry1Ab* target dsRNA and water control samples, revealed a total of 20 different proteins that were either present, absent, up- or down-regulated in one of the hybrids, at a statistically significant level ($P < 0.05$) (Table 5). Proteins that were not detected in this study, they were either not present or below the detection limit of approximately 1 ng, and were then considered absent in the sample.

Eighteen spots were identified with Mascot scores value greater than 120 using Quadrupole Time-of-Flight (Q-TOF) tandem mass spectrometry analysis (MS/MS) ($P < 0.05$). The other two spots were not able to be identified due to their low concentration in the gel, thus revealing low Mascot scores. The proteins were all identified in *Zea mays* species with high confidence. Table 5 presents the MS/MS parameters and protein identification characteristics for this experiment.

Figure 8: Representative two-dimensional gel electrophoresis (2-DE) maps of the proteome of genetically modified maize plants (MON810 event) and non-modified maize under dsRNA delivery at a concentration of 1000 nM for 12 days. Linear isoelectric focusing pH 3–10 for the first dimension and 12% SDS–PAGE gels in the second dimension were used. Molecular masses range from 250 to 10 kDa are given on the left side. The red arrows point to differentially expressed protein spots selected for mass spectrometry identification. ID of identified proteins from Table 5 are indicated in red numbers.

Figure 8. Continued.

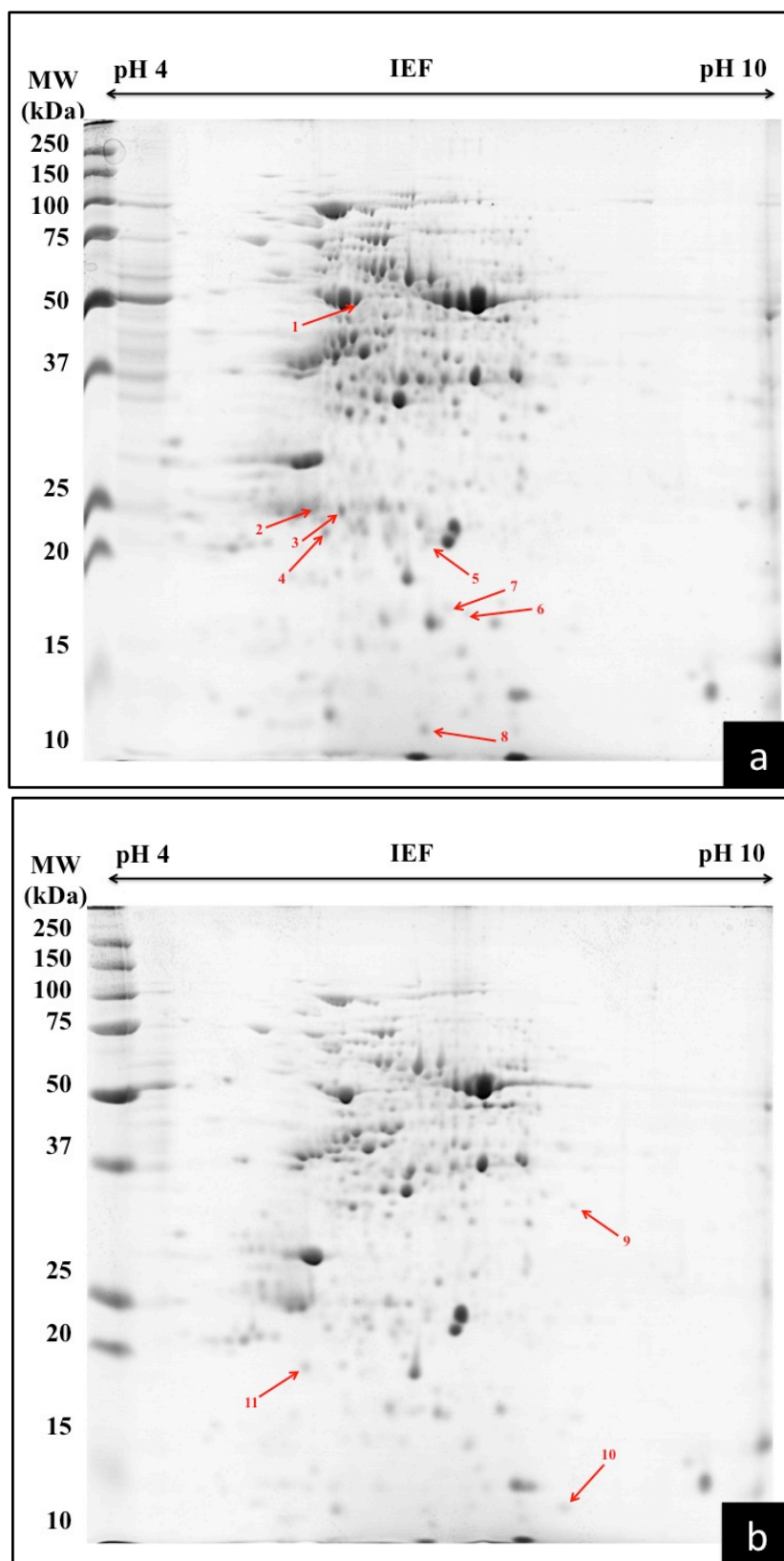
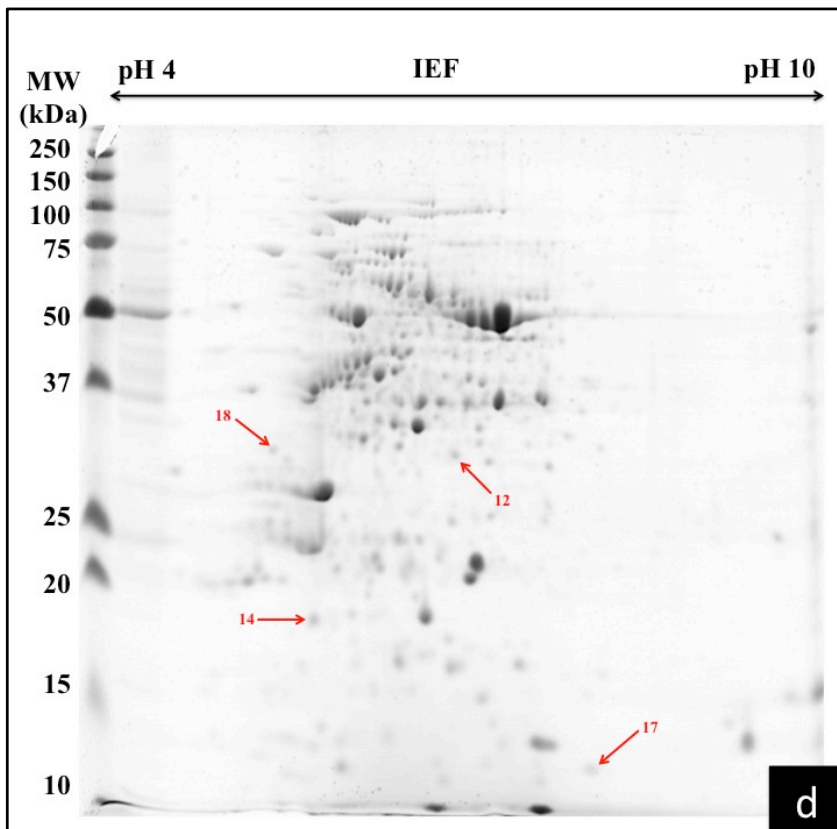
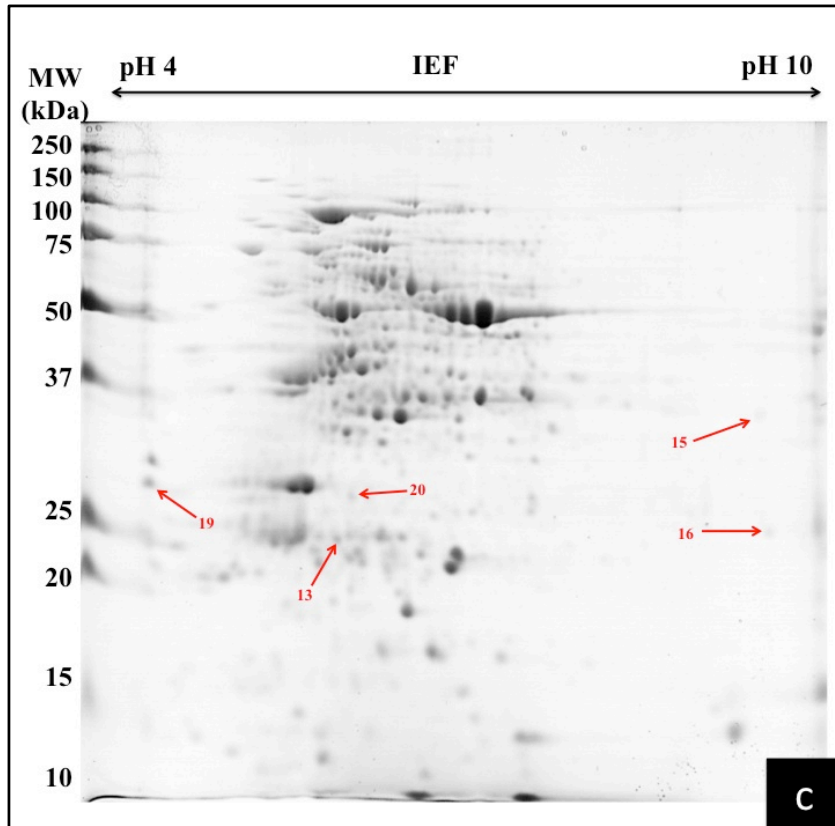


Figure 8. Continued.



Functional classification of the identified proteins, carried out in accordance with the KEGG Orthology system database, showed that they were assigned to one out of these seven ortholog groups: (a) Energy metabolism, (b) Carbohydrate metabolism, (c) Metabolism of co-factors and vitamins, (d) Genetic Information Processing, (e) Signaling and cellular processes, (f) Stress metabolism, (g) Lipid metabolism.

The identification of shared proteins in both genotypes (GM and conventional) that were differentially modulated in dsRNA-treated and water control samples show that these proteins are related to the RNAi triggered by cry1Ab dsRNA sequences. The detection of ferritin 1 protein (Spots 3 and 13) that was identified in both GM and conventional treated samples indicates that the dsRNA treatment affects the expression of this protein irrespective of the presence of its transcript target. Ferritin-like proteins are iron storage proteins that are highly correlated to protection against oxidative stress (Briat et al., 2010). Similarly, the thylakoid lumenal 19 kDa protein (Spots 11 and 14) was found to be present only in water treated samples, both GM and conventional plants. This protein is a calcium binding protein that participates in the photosynthesis. The repression of this protein in both water treatments in plants is not yet conclusive and further studies should be made in order to elucidate this.

We have observed the up regulation of proteins related to the energy metabolism, carbohydrate metabolism and genetic information processing in conventional samples treated with dsRNA compared to untreated control conventional samples. On the other hand, proteins related to both carbohydrate and stress metabolism were up regulated in control samples only. The seven proteins that were found to be differentially modulated in conventional samples are identified as follows: hypothetical protein (Ricin family; Spot 12), NADPH-protochlorophyllide oxidoreductase (Spot 15), hypothetical protein precursor (Spot 16), hypothetical protein ZEAMMB73_690514 (Spot 18), ribonucleoprotein (Spot 19), and electron transporter/ thiol-disulfide exchange intermediate (Spot 20).

Hypothetical proteins without annotations in the database were further analyzed by searching for protein sequence homology to any other conserved domains. Peptides corresponding to Spot 12 were identified to contain domains of ricin-type beta-trefoil which is a carbohydrate-binding domain formed from presumed gene triplication.

The domain is found in a variety of molecules serving diverse functions such as enzymatic activity, inhibitory toxicity and signal transduction. The second hypothetical protein, Spot 16, matched to conserved domains of the ascorbate peroxidase protein family. Ascorbate peroxidases are enzymes that detoxify peroxides using ascorbate as a substrate. The ascorbate-glutathione cycle is one of the major hydrogen peroxide detoxifying system in plant cells. Ascorbate peroxidase enzymes play a key role and are directly involved in the protection of plant cells against adverse environmental conditions (Carvezan et al, 2012). The family of plant proteins induced by water deficit stress, or abscisic acid stress and ripening was observed in identified peptides of Spot 18 and no further annotations were available for this protein domain.

Other proteins were also detected at statistically significant different levels in both conventional samples treated and untreated. These were ribonucleoproteins which present RNA recognition motif and are a highly abundant domain in eukaryotes found in proteins involved in post-transcriptional gene expression processes including mRNA and rRNA processing, RNA export, and RNA stability. NADH dehydrogenase proteins are well-known enzymes that participate in the mitochondrial oxidative phosphorylation system (Hatefi et al., 1985). In addition, the electron transporter thiol-disulfide exchange intermediate belong to the thioredozin-like protein superfamily and function as protein disulfide oxidoreductases, altering the redox state of target proteins via the reversible oxidation of their active site dithiol.

In regards to the GM genotype, other proteins were identified at statistically different levels in GM treated compared to untreated control samples. These nine proteins were assigned to the following protein categories: energy metabolism; carbohydrate metabolism, metabolism of cofactors and vitamins, genetic information processing, and signaling and cellular processes. These proteins were: ATP synthase CF1 beta subunit (Spot 1), TPA: triosephosphate isomerase (Spot 2), 50S ribosomal protein L21 (Spot 5), NADH dehydrogenase I subunit N (Spots 6 and 7), glycine-rich RNA-binding protein 2 (Spot 8), annexin2 (Spot 9), and PL3K2 (Spot 10). ATP synthase is a well-known and important enzyme that provides energy for the cell to use through the synthesis of adenosine triphosphate (ATP). Similarly, triosephosphate isomerase enzymes play an important role in glycolysis and are essential for efficient energy production. These enzymes are directly related to

the energy/carbohydrate metabolism together with NADH deshydrogenases.

Annexins are Ca^{2+} and phospholipid binding proteins and participate in the regulation of membrane organization and membrane traffic and the regulation of ion (Ca^{2+}) currents across membranes or Ca^{2+} concentrations within cells (Gerke et al., 2005). Annexin activities comes from *in vitro* studies, including exocytosis, actin binding, peroxydase activity, callose synthase regulation and ion transport (Baucher et al., 2012). Plant annexins have also been found to be stimulated by abiotic stress including salinity, cold, oxidative and mechanic stress (Vandeputte et al., 2007).

Phosphatidylinositide 3-kinases, PL3K2, are jacalin-like lectins with sugar-binding protein domains (referencia). These proteins may bind mono- or oligosaccharides with high specificity and are important for development and signaling, similar to yeast and animal systems. This includes involvement in endocytosis, reactive oxygen species (ROS) production, and transcriptional activity (Lee et al., 2010).

In addition to these protein categories, glycine-rich RNA-binding protein presents a RNA recognition motif, just as the ribonucleoprotein present in conventional samples. Another protein related to genetic information processing is the 50S ribosomal protein L21 that acts in the synthesis and modification of chloroplastic proteins.

Most interesting, the modulation of proteins groups in both genotypes (GM and conventional) present similar pattern. Different from the results obtained by other authors (see review Table at Agapito-Tenfen et al., 2013). Because most of proteins are assigned to the same biological function, these results indicate that the reduction of CRY1Ab transgenic protein production consequently reduces the differential modulation of endogenous proteins. This suggests that CRY1Ab protein rather than insertional effects may be the major source of pleiotropic effects already observed in other studies (Agapito-Tenfen et al., 2013).

6.5. CONCLUSIONS

This work describes the development of a new method to test substantial equivalence of genetically modified plants expression new transgenic proteins thru RNAi tools. The method provides the description of RNA interference and comparative proteomic protocols detailing the reasons of each protocol choice and the results obtained by

these. Seeds of transgenic maize containing MON810 event and its corresponding near-isogenic non-transgenic plants were subjected to double-stranded RNA treatment with homology to the expressed transgenic transcript sequence. As observed, the most efficient method for the delivery of dsRNA was feeding the seedling mesocotyl with an aqueous solution. After 72 h, leaf samples were collected to confirm silencing. Seedlings were kept under dsRNA solution for nine days and after this period, new sampling were performed for quantification of transgenic protein and proteomic analysis. It was observed a reduction of about 60% in protein levels in transgenic maize seedlings under treatment. The comparison between transgenic maize seedlings under treatment and controls showed that 22 proteins were differentially expressed at $P < 0.05$. Out of these, nine are associated with genetic modification and were mainly categorized into the group of energy metabolism. However, these proteins categories showed similar expression pattern to those detected in conventional treated and untreated samples. On the basis of international quality assessment protocols and previous establishment of the minimum testing criteria for each new GMO event, this method could be used to check proteomic changes while performing the risk assessment prior to environmental release. This application could be of special interest for regulators and researchers in the biosafety field. The method can be also used for research purposes, to study the effect of inserting different DNA sequences and consequently expressing new proteins on a variety of host genomes. Although the method described is specific for GM plants, this work provides procedures that could be useful in developing methods to assess the safety of other similar products.

6.6. REFERENCES

- Agapito-Tenfen SZ, et al. (2013). Comparative proteomic analysis of genetically modified maize grown under different agroecosystems conditions in Brazil. *Proteome Science* 11(1):46.
- AHTEG (2011) Guidance Document on Risk Assessment of Living Modified Organisms, <http://www.cbd.int/doc/meetings/bs/mop-06/official/mop-06-13-add1-en.pdf>, United Nations Environment Programme Convention for Biodiversity.

- Andersen CL, et al. (2004) Normalization of real-time quantitative reverse transcription-pcr data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 64:5245-5250.
- Andrieu A, et al. (2012) An in planta, Agrobacterium-mediated transient gene expression method for inducing gene silencing in rice (*Oryza sativa* L.) leaves. *Rice* 5:23.
- Barros E, et al. (2010) Comparison of two GM maize varieties with a near-isogenic non-GM variety using transcriptomics, proteomics and metabolomics. *Plant Biotechnol J* 8:436-451.
- Batista R and Oliveira M (2010) Plant natural variability may affect safety assessment data. *Regul Toxicol Pharmacol* 58: S8-S12.
- Baucher M, et al. (2012) Insight into plant annexin function: from shoot to root signaling. *Plant Signal Behav* 7(4):524-8.
- Baudo M, et al. (2006) Transgenesis has less impact on the transcriptome of wheat grain than conventional breeding. *Plant Biotechnol J* 4 (4):369–380.
- Birmingham A, et al. (2006) 3' UTR seed matches, but not overall identity, are associated with RNAi off- targets. *Nat Methods* 3:199–204.
- Bøhn T, et al. (2014) Compositional differences in soybeans on the market: Glyphosate accumulates in Roundup Ready GM soybeans. *Food Chemistry* 153:207–215.
- Brodersen P and Voinnet O (2006) The diversity of RNA silencing pathways in plants. *Trends Genet* 22(5):268-80.
- Bustin SA, et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry* 55(4)611–622.
- Candiano G, et al. (2004) Blue Silver: a very sensitive colloidal coomassie G-250 staining for proteome analysis. *Electrophoresis* 25:1327–33.
- Carpentier SC, et al. (2005) Preparation of protein extracts from recalcitrant plant tissues: an evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics* 5:2497-2507.
- Caverzan A, et al. (2012) Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. *Genetics and Molecular Biology* 35 (4):1011-1019.

- Codex (2003) The Guideline for The Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants - CAC/GL 45-2003, Codex Alimentarius Commission.
- Coll A, et al. (2010) Natural variation explains most transcriptomic changes among maize plants of MON810 and comparable non-GM varieties subjected to two N-fertilization farming practices. *Plant Mol Biol* 73:349-362.
- Dai Z, et al. (2014) Early lethality of shRNA-transgenic pigs due to saturation of microRNA pathways. *J Zhejiang Univ Sci B* 15(5):466-73.
- Deng PJ, et al. (2008) The definition, source, manifestation and assessment of unintended effects in genetically modified plants. *J Sci Food Agr* 88:2401–2413.
- Dolezel D, et al. (2011) Scrutinizing the current practice of The environmental risk assessment of GM Maize applications for cultivation in the EU. *Environmental Sciences Europe*, 23:33.
- Douvillea M, et al. (2005) Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. *Biochemical Systematics and Ecology* 33:219–232.
- Eamens J, et al. (2008) RNA Silencing in Plants: Yesterday, Today, and Tomorrow. *Plant Physiol.* 147(2):456–468.
- EFSA (2011) Scientific Opinion on Guidance on selection of comparators for the risk assessment of genetically modified plants and derived food and feed, *EFSA Journal* 9, 2149.
- Fagard M and Vaucheret H (2000) (Trans)Gene Silencing in Plants: How many mechanisms? *Annu Rev Plant Physiol Plant Mol Biol* 51:167-194.
- Fellmann C and Lowe SW (2014) Stable RNA interference rules for silencing. *Nature Cell Biology* 16:10–18.
- Gan D, et al. (2010) Bacterially expressed dsRNA protects maize against SCMV infection. *Plant Cell Rep* 29(11):1261-8.
- Gerke V and Moss SE (2002) Annexins: From Structure to Function. *Physiological Reviews* 82:331-371.
- Gerke V, et al. (2005) Annexins: linking Ca²⁺ signalling to membrane dynamics. *Nat Rev Mol Cell Biol* 6:449-61.
- Grimson A, et al. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* 27:91-105.
- Hatefi Y, et al. (1985) The enzymes and the enzyme complexes of the mitochondrial oxidative phosphorylation system. In Martonosi A

- (1985) *The Enzymes of Biological Membranes 4* (2nd ed.). New York: Plenum Press. pp. 1–70.
- Haywood V, et al. (2002) Plasmodesmata: Pathways for Protein and Ribonucleoprotein Signaling. *The Plant Cell* S303–S325.
- Heinemann JA, et al. (2011) Molecular profiling: a tool for addressing emerging gaps in comparative risk assessment of GMOs. *Environ Int* 37:1285-1293.
- Hernández M, et al. (2003) A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard based on the 3'-transgene integration sequence. *Transgenic Res* 12:179-189.
- Hilbeck A, Meier M, Römbke J, et al. (2011) Environmental risk assessment of genetically modified plants - concepts and controversies. *Environmental Sciences Europe* 23:13.
- Jackson AL and Linsley PS (2010) Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat Rev Drug Discov* 9:57-67.
- Jorgensen RA (2002) RNA traffics information systemically in plants. *PNAS* 99(18):11561–11563.
- Kalantidis K, et al. (2008) RNA silencing movement in plants. *Biol Cell* 100:13–26.
- Kibbe WA (2007) OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Res* 35: W43-6.
- Laohavisit A and Davies JM (2011) Annexins. *New Phytol* 189:40-53.
- Latham GJ (2010) Normalization of microRNA quantitative RT-PCR data in reduced scale experimental designs. *Methods Mol Biol* 667:19–31.
- Lee Y, et al. (2010) Lipid Signaling in Plants Plant Phosphatidylinositol 3-Kinase. *Plant Cell Monographs* 16: 95-106.
- Lu R, et al. (2003) Virus-induced gene silencing in plants. *Methods*. 30(4):296-303.
- Manoli A, et al. (2012) Evaluation of candidate reference genes for qPCR in maize. *Journal of Plant Physiology* 169(8):807–15.
- Millstone E, et al. (1999) Beyond ‘Substantial equivalence’. *Nature*, 401:525-526.
- Murashige T and Skoog F (1962): A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant* 15:473-497.

- Nguyen HT and Jehle JA (2007) Quantitative analysis of the seasonal and tissue-specific expression of Cry1Ab in transgenic maize MON810. *Journal of Plant Diseases and Protection* 114(2):820-887.
- Novak W, et al. (2000). Substantial Equivalence of Antinutrients and Inherent Plant Toxins in Genetically Modified Novel Foods. *Food Chem Toxicol* 38(6):473-83.
- Pei Y and Tuschl T (2006) On the art of identifying effective and specific siRNAs. *Nat Methods* 3:670-676.
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* 29(9):e45.
- Ricroch AE and Bergé JB (2011) Evaluation of genetically engineered crops using transcriptomic, proteomic, and metabolomic profiling techniques. *Plant Physiol* 155:1752–1761.
- Ruebelt MC, et al. (2006) Application of two dimensional gel electrophoresis to interrogate alterations in the proteome of genetically modified crops. 1. Assessing analytical validation. *J Agr Food Chem* 54:2154-2161.
- Ruiz-Medrano R, et al. (1999) Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. *Development* 126:4405– 4419.
- Senthil-Kumar M and Mysore KS (2011) New dimensions for VIGS in plant functional genomics. *Trends in Plant Science* 16(12):656–665.
- Sharma S and Rao A (2009) RNAi screening: tips and techniques. *Nat Immunol* 10(8):799–804.
- Shevchenko A, et al. (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal Chem* 68:850-858.
- Sun C, et al. (2005) Antisense oligodeoxynucleotide inhibition as a potent strategy in plant biology: identification of SUSIBA2 as a transcriptional activator in plant sugar signaling. *The Plant Journal* 44:128–138
- Székács A, et al. (2012) Inter-laboratory comparison of Cry1Ab toxin quantification in MON 810 maize by enzyme-immunoassay. *Food Agric Immunol* 23:99–121
- Tenllado F, et al. (2003) Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. *BMC Biotechnol* 3: 3.

- Then C and Lorch A (2008) A simple question in a complex environment: How much Bt toxin do genetically engineered MON810 maize plants actually produce? In Breckling B, Reuter H and Verhoeven, R (2008) Implications of GM-Crop Cultivation at Large Spatial Scales. Theorie in der Ökologie 14. Frankfurt, Peter Lang.
- Tournier B, et al. (2006) Tabler M, Kalantidis K. Phloem flow strongly influences the systemic spread of silencing in GFP *Nicotiana benthamiana* plants. *Plant J.* 47(3):383-94.
- Vance V and Vaucheret H (2001) RNA silencing in plants--defense and counterdefense. *Science* 292(5525):2277-80.
- Vandeputte O, et al. (2007) The tobacco Ntann12 gene, encoding an annexin, is induced upon *Rhodococcus fascians* infection and during leafy gall development. *Mol Plant Pathol* 8:185-94.
- Vlasak J, et al. (2003) Comparison of hCMV immediate early and CaMV 35S promoters in both plant and human cells. *Journal of Biotechnology* 103:197-202.
- Voinnet O, et al. (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* 95(2):177-87.
- Wang H, et al. (2007) Biodegradation of Cry1Ab protein from Bt transgenic rice in aerobic and flooded paddy soils. *J Agric Food Chem* 55(5):1900-4.
- Watson JM, et al. (2005) RNA silencing platforms in plants. *FEBS Lett.* 31;579(26):5982-7.
- Weiss W and Gorg A (2008) Sample solubilization buffers for two-dimensional electrophoresis. In *Methods in Molecular Biology-2D PAGE: Sample Preparation and Fractionation*. Edited by Posh A. New Jersey: A Humana Press; 35-42.
- Yin Z, et al. (2004) Transgene inheritance in plants. *J Appl Genet* 45:127-144.
- Zolla L, et al. (2008) Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. *J Proteome Res* 7:1850-1861.

7. CAPÍTULO III

Rational transient RNA interference designs for gene function studies in plants

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RESUMO

Interferência por RNA tornou-se uma ferramenta valiosa para estudos de perda de função gênica em quase todos modelos biológicos eucariotos. Ainda, a transformação de plantas tem sido uma das metodologias mais utilizadas para para geração de genótipos RNAi estáveis. No entanto, os efeitos adversos decorrentes do processo de engenharia genética pode apresentar desafios à interpretação dos resultados de silenciamento. Por outro lado, ao permitir que o silenciamento gênico seja transiente, é possível estudar fenótipos a curto prazo, reverter o silenciamento e analisar as consequências da inibição da transcrição temporária e *in vivo*. A implementação eficiente de experimentos que abordem ensaios de RNAi transientes em sistemas vegetais tem sido dificultada pela informação dispersa na literatura científica. Neste trabalho nós discutimos os potenciais efeitos não-intencionais do silenciamento estável de genes e fornecemos informações sobre experimentos de RNAi transientes.

7.1. ABSTRACT

RNA interference has become a valuable tool for loss-of-function studies across eukaryotes. Stable plant transformation RNAi methodologies seem to be preferred by plant biologists. However, off-target effects deriving from the genetic engineering process might pose extra challenges to the interpretation of silencing results. On the other

³ Este capítulo trata do manuscrito submetido à publicação em revista científica.

hand, by enabling transient gene silencing, it is possible to study short-term phenotypes and to examine the consequences of temporary target inhibition *in vivo*. Efficient implementation of transient approaches in plant systems has been hindered by the scattered information in the scientific literature. Here we discuss the potential unintended effects of stable gene silencing and provide information on transient RNAi experimental designs and protocols that disfavor such effects.

7.2. INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved gene down-regulation mechanism and has been demonstrated to exist in many eukaryotes. It occurs naturally against double-stranded RNA (dsRNA) that interferes with the translation of target mRNA transcript eventually suppressing the gene expression (Fire et al., 1998). The small non-coding RNAs are the cleavage product of dsRNA called microRNA (miRNA) and small interfering RNA (siRNA) that carried out by a ribonuclease called DICER or Dicer-like enzyme (Zamore et al., 2000). The small non-coding RNAs in association with RNA-induced silencing complex (RISC) (Mette et al., 2000), Argonaute (AGO) (Tabara et al., 1999) and other effector proteins lead to the phenomenon called RNAi (reviewed in Brodersen and Voinnet, 2006).

RNAi is arguably one of the most significant discoveries in biology in the last two decades. First recognized in plants (where it was called post-transcriptional gene silencing, PTGS), the starting point of RNAi study can be attributed to the early years of modern plant biotechnology. In the late 1980s and early 1990s, an attempt to increase the pigment content in petunia flowers by genetic transformation lead to the decrease of such pigments (Napoli et al., 1990; van der Krol et al., 1990). Some transgenic plant lines exhibited a coordinate suppression or “gene silencing” to which mechanism was at that time unknown, but the authors referred to as “co-suppression” (Eamens et al., 2008). The biology of endogenous RNAi pathway in plants revealed that plant miRNA genes are independent transcription units; their processing is determined by flanking sequences, rather than the miRNA sequence itself; their central sequences are more important than 3' sequences for targeting (Parizotto et al., 2004).

One of the most important features of RNAi is that the endogenous pathways can be activated by providing exogenous triggers

that enter the pathway. Methods based on RNAi have quickly become popular approaches for loss-of-function experiments due to their efficiency, ease of use, and cost as compared to traditional approaches. It is, for instance, relatively easy to prepare dsRNAs or siRNAs for a gene of interest and introduce them into cells or organisms. In plants, long dsRNA/hairpin RNA (hpRNA) enters the cytoplasm and is processed by the RNase-III enzyme Dicer into 21 nucleotide (nt) duplex-long siRNAs with two nt 3' overhangs (Carthew and Sontheimer, 2009). One of the siRNA strands is incorporated into RISC and acts as a guide for binding to the complementary messenger RNA (mRNA). The cognate mRNA is subsequently cleaved in the middle of the base-pairing region by a "slicer" (Ago2 protein) and rapidly degraded (Ghildiyal and Zamore, 2009). Figure 1 demonstrates the main features of exogenous and endogenous RNAi triggers and main enzymes involved in each step of the RNAi pathway. RNAi can be quickly and easily employed in a wide range of genotypes or even species, whereas identification of gene mutations is limited to certain plant species that have mutant genetic stocks. In addition, RNAi has the ability to silence genes in a sequence-specific manner and the expression of RNAi constructs can be controlled in a tissue-specific development or time-dependent manner (Senthil-Kumar and Mysore, 2011). Moreover, RNAi facilitates the study of essential genes whose complete inactivation would lead to lethality or extremely severe pleiotropic phenotypes.

There seems to be a scientific consensus to separate the main approaches of experimental RNAi into two categories: stable or transient RNAi expression (Bauer, 2010). Stable RNAi expression is defined as continuous persistent gene-knockdown, mediated by dsRNA-generating DNA vectors that are integrated in the host genome by genetic transformation methods. In general terms, stable RNAi expression mimics pre-miRNAs, which is then expected to provide a heritable source of RNAi triggers. Under stable expression, introduction of the nucleic acid of interest and an appropriate selection marker allows the rapid selection of the few cells that stably integrated the nucleic acid into their genome. Once propagated, these cells inherit the nucleic acid and express any dsRNA-encoding gene continuously. Transient RNAi expression is related to the delivery of exogenous nucleic acids that are temporarily expressed in the host cell (i.e. few hours or few days), and can also be integrated into the host cell genome, but do not follow genetic transformation steps. Subsequently, the nucleic acids are usually

expressed for a short period of time within a delimited part of the plant tissue. They may eventually be recognized as foreign genetic material and become degraded or diluted through cell division (Figure 1).

Exogenous dsRNA can be used to silence the expression of target genes in plants. Upon introduction, the dsRNAs enter a cellular pathway that provokes PTGS. The dsRNAs get processed into 21–23 nucleotide duplexes by Dicer, then called siRNAs. The siRNAs assemble into RISC and the siRNA antisense strand subsequently guide the RISCs to complementary mRNA molecules, where they cleave and destroy the cognate mRNA that takes place near the middle of the region bound by the siRNA strand (Senthil-Kumar and Mysore, 2011). These steps are elaborately reviewed elsewhere (Waterhouse and Helliwell, 2003; Agrawal et al., 2003; Meister and Tuschl, 2003). Thus, by exploiting these properties RNAi can, in principle, be used to suppress the expression of any gene.

However, results obtained from *in vitro* studies on gene expression profiling of siRNAs disclosed that RNAi, in general, might be compromised by off-target effects (Jackson et al. 2003; Sledz et al. 2003). Since siRNAs can also function through a naturally occurring miRNA-like pathway, imperfect complementarity to 3'UTRs of other than the target mRNA might lead to translational repression and/or degradation of non-target mRNAs (Carthew and Sontheimer 2009; Birmingham et al. 2006). These are called 'sequence-determined' effects (Heinemann et al., 2013). In addition, the so-called 'non-specific' off-target effects (Jackson and Linsley 2004) or 'sequence-independent' effects, caused by siRNA-induced interferon response or the disturbance of the endogenous miRNA pathway, may lead to global changes in gene expression, unspecific effects, and cellular toxicity, which profoundly compromise the conclusions for any RNAi experiments that are designed to study the functional role of a particular target gene (e.g. safety of RNAi-based therapeutic approaches) (for review see Bauer, 2010).

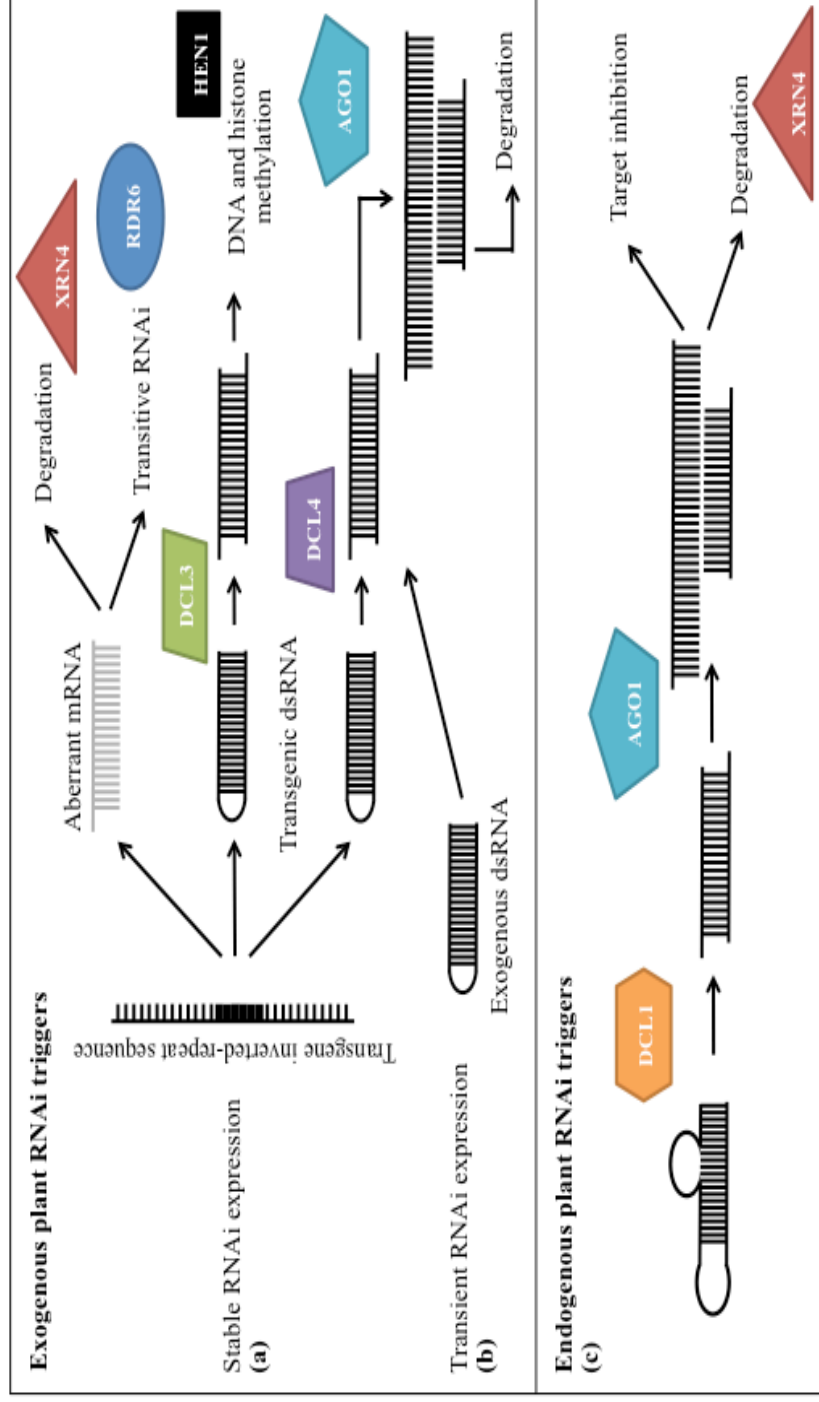
RNAi off-target effects were first described by Jackson and co-workers (2003). Using genome-wide microarray profiling, as a method of detection, the authors identified 1.5- to 3-fold changes in the expression of dozens of genes following transfection of individual siRNAs. The levels of complementarity between the sense or antisense strands of the siRNA and the off-targeted genes varied considerably and

the overall off-target expression profile was unique for each siRNA, suggesting a sequence-specific component to the phenomenon.

Nonetheless, unintended gene silencing is a common outcome of the genetic engineering process. Engineering-associated production of new dsRNA molecules may be created when truncation of inserted DNA are used as template for transcription. The resulting single-stranded RNA may bind to the target mRNA to create regions of linear dsRNA that can be processed into siRNA. Another possibility is when the insert contributes to the formation of a stem-loop, from which the “stem” may be processed into a miRNA-like molecule (Heinemann et al., 2013). In 1994, the first genetically engineered whole food organism, the Flavr Savr tomato, was intentionally developed to increase storage life through suppression of the tomato polygalacturonase gene (PG), resulting from transformation of an antisense expression cassette of the PG cDNA (Sheehy et al., 1988). Although efficient suppression of target transcript, transformation events were found to contain multiple T-DNA insertions (Redenbaugh et al., 1992; Sanders and Hiatt, 2005), thus read-through transcription of multiple PG cassettes resulted in RNAi-mediated PG suppression, rather than antisense-mediated sequestration, of the endogenous PG transcript (Watson et al., 2005). Krieger et al. (2008) were able to verify that polygalacturonase suppression correlated with accumulation of 21-nt small interfering RNAs.

Figure 1. Post-transcriptional RNA silencing pathways in plants. (a) Stable RNAi expression system. An inverted-repeat transgene construct produces dsRNA transcripts with perfectly complementary arms. Two distinct Dicer-like (DCL) enzymes process the dsRNA transcripts. DCL3 probably produces siRNAs of the 24 nt size class and DCL4 is probably the preferred enzyme for production of 21-nt siRNAs from the dsRNA. The first DCL product can direct DNA or histone modification at homologous loci, one of the siRNA strand of the second DCL product incorporates into AGO1-loaded RISC to guide endonucleolytic cleavage of homologous RNA, leading to its degradation. Aberrant transgenic transcript can also be produced from truncated transgene insertion, these undergo degradation or lead to transitive RNAi depending on the sequence complementarity to other non-target mRNAs. XRN4 and RDR6 are the required enzymes for each of the pathways respectively. (b) Transient RNAi expression system. Exogenous delivery of dsRNA molecules undergo similar pathways of siRNA production by DCL3 and DCL4. This leading to mRNA degradation and/or DNA or histone methylation. (c) Endogenous RNAi expression. Pri-miRNA transcripts are transcribed from host genome sequences. The combined nuclear action of DCL1 and other enzymes produces a mature

miRNA. This is then incorporated into AGO1-loaded RISC to promote two possible sets of reactions that are not mutually exclusive: endonucleolytic cleavage of homologous RNA (directed by 21-nt siRNAs) or inhibition of translation, possibly at the initiation level.



Therefore, owing to our incomplete understanding of the mechanisms behind miRNA biogenesis and target inhibition, in addition to off-target effects deriving from transformation process itself; the RNAi process becomes somewhat unpredictable and may not be as efficient and precise as desired. Hence, as global databases on outputs from gene silencing analysis and off-target effects become available, they could provide a highly desirable benchmark for the future of RNAi-based assessments on gene function and its commercial applicability. In this review we discuss the potential unintended effects of stable gene silencing and provide information on transient RNAi experimental designs and protocols that disfavor such effects. We intend to provide a compilation of information on plant systems, which seems to be scattered in the scientific literature, as well as to provide sequential approach for designing and performing transient experiments with plants.

7.3 HOW STABLE IS RNAI “STABLE EXPRESSION”?

The stable expression of exogenous dsRNA molecules requires the insertion of a transgene construct into the host organism genome. Most methods applied for stable RNAi expression experiments need vector construction and plant transformation, which then requires molecular characterization, and a proper investigation of possible pleiotropic effects derived from DNA insertion into host genomes (Senthil-Kumar and Mysore, 2011). Unless there is a real need for a stable expression of RNAi mode (e.g. long-term phenotypes), stable RNAi experiments seems to deliver a more complex biological system than transient experiments (Tenllado et al., 2003).

Usually, the transgene construct is composed of a promoter and terminator between which an inversely- repeated sequence of the target gene is inserted (with a spacer region between the repeats), which then produces an hpRNA structure. The RNA transcribed from a transgene like that hybridizes to itself to form the hairpin structure. This comprises a single-stranded loop region, encoded by the spacer region, and a base-paired stem encoded by the inverted repeats. The whole length of the stem appears to be used as a substrate for the generation of siRNAs, but few or none are generated from the loop. Since a spacer region is needed for the stability of the transgene construct, although is not involved in siRNA production, an intron sequence is often used in this position,

especially because it appears to enhance the efficacy of silencing (Watson et al., 2005).

In the genetically stable RNAi plants, the location(s) of and copy numbers of RNAi constructs integrated into the plants genome are factors influencing the extent of its transcription, thereby influencing the level and the sequence of dsRNA production and the effectiveness of intended RNAi (Kerschen et al., 2004). Truncation of DNA inserted construct might lead to transcription of unintended sequences, transcription in inverted orientation, or when the insert contributes to the formation of a stem-loop which may be processed into an miRNA-like molecule. In addition, the simple duplication of intact DNA constructs may also lead to unintended effects in RNAi plants.

Transgenes can become silent after a short or long phase of expression, and can sometimes silence the expression, at least partially, of homologous elements located at ectopic positions in the genome (Matzke and Matzke, 1998; Kooter et al., 1999). The widespread occurrence of transgene inactivation in plants, and classical cases of silencing of duplicated sequences suggests that all genomes contain defense systems that are capable of monitoring and manipulating intrusive DNA. Such DNA might be recognized by its structure, its sequence composition relative to that of its genomic environment and possibly by its disruption of normal biochemical functions (Kumapatla et al., 1998). Although methylation, especially of repeated sequences, is widely associated with gene inactivation, other attributes, including chromatin modification, may be involved. Elimination of inactivated intrusive DNA (presently best documented for filamentous fungi) may also contribute to genomic defense mechanisms in plants (Kumapatla et al., 1998) Still, it is interesting to note that since early studies of RNAi in plants using transgenic lines, there are only a few reports that provide comprehensive data on genomic context of transgenes in well-characterized transgenic lines.

The most notable example of such effects recalls one of the first reports on PTGS or co-suppression observed by Napoli et al. back in 1990 (Napoli et al., 1990). As an attempt to overexpress chalcone synthase (CHS) in pigmented petunia petals by introducing a chimeric petunia CHS gene, the authors unexpectedly observed a block in anthocyanin biosynthesis. After segregation analysis of several dozens of different transgenic lines, they concluded that, in the altered white flowers, the expression of both genes was coordinately suppressed. The

mechanism responsible for the reversible co-suppression of homologous genes in trans was unknown by that time, but the authors suggested the possible involvement of methylation. Van der Krol and coworkers (van der Krol et al., 1990) had also observed similar patterns with transgenic lines in early studies on the interference of RNA strands through mechanism of suppression by sense genes, which were involve in the transcription process itself.

In animal systems, the use of strong promoters in RNAi vectors to drive hpRNA production could favor a completely different type of off- target effect due to the inhibition of natural miRNA or siRNA regulation through saturation of the pathways with exogenous or transgene siRNAs (Grim et al., 2006; Grimm, 2011). More recently, Dai et al. (2014) have investigate the feasibility of inhibiting classical swine fever virus (CSFV) replication by shRNA in vitro and in vivo. These authors have observed early lethality of shRNA-transgenic pigs due to abnormal expressions of miRNAs and their processing enzymes are also observed in the livers of shRNA- transgenic pigs and other in vitro experiments, indicating saturation of miRNA/shRNA pathways induced by shRNA.

Apparently, the use of strong constitute promoters might also trigger similar off- target effects in plant systems. Martínez de Alba et al. (2011) observed that plants harboring dsRNA transgenes exhibit increased levels of binding ARGONAUTE1 (AGO1) protein and that increased AGO1 levels leads to increased PTGS efficiency.

This indicates that the miRNA pathway dampens the efficiency of PTGS by limiting the availability of AGO1. Regarding this, the authors then propose that during the transgene PTGS initiation phase, transgene siRNAs and endogenous siRNAs compete for binding to AGO1. High dsRNA levels produced in inverted repeated PTGS promote the activities of different Dicers and RISCs, which would normally act in distinct pathways, to mediate silencing redundantly. Recent analyses of combinatorial Dicer knockouts in *Arabidopsis* support this idea (Gascioli et al., 2005; Xie et al., 2005).

Large-scale loss-of-function screens have begun to systematically interrogate entire genomes to identify the genes that contribute to a certain cellular response. However, the complex interplays among cellular building blocks, which, in their concurrence, give rise to the emergent properties observed in cellular behaviors and responses requires a holistic approach (Machado et al., 2011). The holistic

approach not only aims to understand the interactions among components within a system, but also aspires to decipher how a system as a whole responds to perturbations. One cannot forget that this perspective thus provides a contrasting, yet complementary, vision to the classical reductionist paradigm (Neumuller and Perrimon, 2010).

7.4. BIOLOGY OF TRANSIENT SILENCING: THE DELIVERY SYSTEM

Although there is a diversity of RNA silencing pathways in plants, they all share three biochemical features: (i) formation of dsRNA; (ii) processing of dsRNA to small 20–26-nt duplex dsRNAs with staggered ends; and (iii) inhibitory action of a selected siRNA strand within effector complexes acting on partially or fully complementary RNA or DNA (Brodersen and Voinnet, 2006).

In general terms, what differentiates the ‘stable’ from the ‘transient’ expression of RNAi in plants is the delivery system of dsRNA molecules. By using a transient approach, the researcher can deliver the dsRNA molecule to any tissue and at any time during the life cycle of the plant. This sets this approach apart from others based on transgenic plants over-expressing palindromic constructs predicted to encode dsRNA under the control of constitutive promoters (Waterhouse et al., 1998; Chuang and Meyerowitz, 2000). The delivery of ectopic dsRNA molecules facilitates the measurement of dose, the performance of rescue experiments, as well as single-cell analysis. Although widely used as a research tool, transgenic approaches remain one of the least understood plant RNA silencing processes (Brodersen and Voinnet, 2006). Nonetheless, some transient approaches have the advantage that they negate the need to introduce a transgene into the target plant genome. Consequently, they allow RNAi to be carried out in plant species that are recalcitrant to genetic transformation (Small, 2007).

Some transient delivery systems may, however, lead to stable integration of nucleic acid sequences into the target cell genome (e.g. transfection by bacterial vectors). Hence, misconceptions arose in the literature because some authors referred to any transient approach as non-transgenic, regardless of possible nucleic acid introgression. This was more common for articles published prior to 2003, most probably due to the lack of an international guidance on the use of terms such as given for instance by The Cartagena Protocol on Biosafety (CPB)

(<http://bch.cbd.int/protocol/text/>). According to the CPB, *in vitro* nucleic acid techniques and direct injection of nucleic acid into cells or organelles are considered modern biotechnology, and any living organism that possesses a novel combination of genetic material obtained through the use of such techniques is considered a genetically modified organism. Off-target effects derived from that can be compared to those originating from transgenic-based approaches. Table 1 features the main differences between stable and transient RNAi experimental approaches.

7.4.1. Particle bombardment (biolistics or “gene gun”)

A historical overview of RNAi in plants goes back to the late 1980s and early 1990s, when plant biotechnology researchers were using genetic engineering to alter flower color (for review see Eamens et al., 2008; Watson and Wang, 2012). The researchers expected an increase in floral color by expression of numerous copies of transgenes but instead they observed the suppression of both the transgene and the homologous endogenous plant genes (Napoli et al., 1990; van der Krol et al., 1990).

It seems that particle bombardment was used in one of the first transient RNAi methodologies that have been reported in the plant science literature. Most probably due to the need of a efficient and rapid experiment, but also due to the fact that several plant species (i.e. cereals) are difficult to be transformed with *Agrobacterium tumefaciens*, thereby limiting reverse genetic approaches with currently available technologies (Schweizer et al., 1999b; Schweitzer et al., 2000). The idea at that time was that the expression of RNAi pathway still needed host genome integration.

The basics of biolistic for transient expression relies on the use of tungsten or gold particles coated with coiled plasmids/vectors, followed by “gene gun” delivery into tissue (i.e. leaf) segments by a particle inflow gun (Schweizer et al., 2001). In that case, single-cells present in the tissue sampled will have the plasmid sequences integrated into their genomes. Several DNA vectors as well as naked DNA sequences have been used to trigger RNAi in plant cells.

Systemic and posttranscriptional silencing of transgenes in *Nicotiana benthamiana* was initiated by introduction of transgene-homologous DNA fragments, including those without a promoter,

through particle bombardment (Voinnet et al., 1998). This experiment showed a sequence-specific signal of gene silencing spread from cells that had received the ectopic DNA via a relay mechanism that employs plasmodesmata and phloem channels.

Table 1. Similarities and differences between stable and transient RNAi experimental approaches in plants. Only the most common features of RNAi expression methods and modes of gene silencing are shown here.

RNAi experimental properties	Stable RNAi system	Transient RNAi system
Targeting design		
• Target sequence	Same as transient	Same as stable
• Long or short dsRNA	Both possible	Both possible
• siRNA screens	Not possible	Possible
• Expression cassette	Required	Not required
Off-target prediction		
• Bioinformatics	Same as transient	Same as stable
Delivery system		
• Time specific	Not possible	Possible
• Tissue specific	Not possible	Possible
• Dose specific	Not possible	Possible
• Transmission	Systemic, all cells	Not necessarily systemic
• Amplification	More likely	Less likely
• Rescue experiments	Not possible	Possible
• Plant transformation	Required	Not required
• Vectors	Not required	Not required
Knock down confirmation	Only once	Every assay
Potential off-targets		
• Due to saturation of RNAi pathway	Most likely	Less likely
• Due to transformation	Most likely	No
• Due to delivery system	No	Most likely, require proper controls
• Due to concentration of dsRNA	Most likely	Less likely, require proper controls

Shang et al. (2007) have also observed the spread of a systemic silencing effect onto large zones of *Antirrhinum* (eng. “Snapdragon”) petal tissue lacking pigmentation. The transmission of the silenced state had spread both laterally within the affected epidermal cell layer and into lower cell layers. The authors noticed that it is not possible to know the precise extent to which the silencing signal was promulgated from an individual transformed cell.

Since biolistics is a physical and not a biological transfection procedure, it is not limited to compliant cell types as vector-based deliveries are. However, efficiently transfection of small cells and the possibility of significant tissue damage limit its potential usefulness. Novel strategies and adaptations in order to increase efficiency have been developed, such as the use of nanoparticles for biolistic transfection into human embryonic kidney HEK293 cells (O’Brien and Lummis, 2011). But these have not yet been tested in plant cells.

7.4.2. Agrobacterium infiltration and infection (agroinfiltration and agroinfection)

A. tumefaciens and related *Agrobacterium* species have been known as plant pathogens since the beginning of the 20th century. However, only in the past three decades the ability of *Agrobacterium* to transfer DNA to plant cells has been harnessed for the purposes of plant genetic engineering. Since the initial reports in the early 1980s using *Agrobacterium* to generate transgenic plants, scientists have attempted to improve this “natural genetic engineer” for biotechnology purposes (Gelvin, 2003).

The strategy to use *Agrobacterium* for transient expression in plants emerged from different research needs, ranging from a less time-consuming assay to a simple wish of not wanting a transgene or its product to be present after the initial few hours or days following transformation (Gelvin, 2003).

As said, *Agrobacterium* has always been used to transform plants, and more recently, used as a delivery method in transient dsRNA expression protocols. If T-DNA is integrated into cells and those follow selection and regeneration protocols, the end-point product will be a stable transgenic plant. In other cases, *Agrobacterium* might be delivered transiently, with T-DNA introgression into a few cells, and

these are better called transient transformation via agroinfiltration (Liu et al., 1992; Shang et al., 2007; Zottini et al., 2008).

The lack of complete knowledge about the roles of position effects, chromatin effects, and T-DNA integration patterns in transcriptional and post-transcriptional gene silencing demands the development of new strategies to enhance the extent and stability of transgene expression (Gelvin, 2003; Singer et al., 2012).

Two other transient strategies are currently being developed to permit the expression of gene products in plants via *Agrobacterium* without prior host genome disruption or integration. These are the use of “non-integrating” T-DNA systems (Nam et al., 1993) and virus-based vector (agroinoculation) (Vaghchhipawala et al., 2011).

Agroinoculation methodologies have been developed with both DNA and RNA viruses. Tobacco, tomato, and barley vectors have shown extensive silencing with attenuated symptoms, and agroinfiltration with tobacco rattle virus (TRV) and potato virus X (PVX) vectors are becoming the vectors of choice for many investigators (for review see Robertson, 2004). An overview of viral vectors is presented in the following section.

The most popular method of agroinfiltration involves introduction of *Agrobacterium* into plant leaves using a needleless syringe. Simple *Agrobacterium*-mediated gene overexpression protocols (agroinfiltration) have now been optimized for several plant species (Wroblewski et al., 2005). Grafting plant leaves with an *Agrobacterium* suspension was shown highly efficient since the first reports on gene silencing (Voinnet et al., 1998). Agroinfiltration can also be conducted in non-sterile conditions by placing leaf tissues in Petri dishes with leaf tissues and bacterial suspension in a desiccator and subject them to vacuum for a few minutes whereupon vacuum is quickly released to let the bacterial suspension enter the leaf tissues (Bertazzon et al., 2012).

7.4.3. Viral vector-based delivery

The discovery of RNAi in plants lead to the finding that plants can recognize and degrade viral RNA that is invading the cells. The understandings of plant-viral interactions and defense mechanisms have undoubtedly evolved after this paradigm shift.

Virus-induced gene silencing (VIGS) involves three major steps: engineering viral genomes to include fragments of host genes that are

targeted to be silenced, infecting the appropriate plant hosts and silencing the target genes as part of the defense mechanism of the plant against virus infection. Because it allows the targeted down-regulation of a particular gene through the degradation of its transcripts, the potential of VIGS as a tool for the analysis of gene function was quickly recognized (Baulcombe, 1999). VIGS is rapid (3–4 weeks from infection to silencing), does not require development of stable transformants, and allows characterization of phenotypes that might be lethal in stable lines (Burch-Smith et al., 2004). Despite improvements in the protocols used for VIGS in several plant species, several limitations of VIGS remain unaddressed (for review see Senthil-Kumar and Mysore, 2011).

The lack of appropriate VIGS vectors for some plant species might be considered one of the main drawbacks in its applicability. Nonetheless, efforts in developing new vectors are increasing. It has, for instance, been reported that Apple latent spherical virus (ALSV) vectors have been used to induce reliable and effective VIGS in a broad range of plants including tobacco, tomato, Arabidopsis, cucurbits and legumes (Igarashi et al., 2009). Furthermore, Sasaki and co-workers (2011) have recently developed an efficient virus-induced gene silencing method in apple, pear and Japanese pear using ALSV vectors; and Yamagishi et al. (2013) developed an ALSV-based protocol to obtain reduced generation time in apple trees.

The delivery of virus-vectors by *Agrobacterium* is mainly restricted to dicot plants, and an alternative method is sap inoculation. The sap method involves multiplication of the virus in “virus-friendly” *N. benthamiana* (a tobacco plant relative) or another appropriate host plant followed by inoculation of the target species with the sap (Lu et al., 2003). It is also relevant to consider the potential interference from viral symptoms with interpretation of data since silencing of certain plant genes can allow more viral replication resulting in severe developmental phenotypes in which viral titer are required (Burch-Smith et al., 2004). It has been claimed that ALSV is the best vector alternative to avoid such problems (Iragashi et al., 2009). There are several comprehensive reviews, updates on technical applications and methodological papers available for VIGS (Becker and Lange, 2009; Lu et al., 2003; Ramegowda et al., 2013; Burch-Smith et al., 2004; Robertson, 2004; Senthil-Kumar and Mysore, 2011).

7.4.4. Bacteria vector-based delivery

Several commercial and non-commercial plasmid vectors have been widely used for delivery of dsRNA constructs. Some protocols are developed using agroinfiltration and some by the directly delivery of plasmids.

Another strategy for transient systems has been described for maize, barley and wheat, which applies micro particles that were coated with supercoiled plasmid constructs followed by biolistic delivery into leaf segments (Schweizer et al., 1999).

Tournier et al. (2006) have made use of a modified grafting approach using plasmid expression vector for the delivery of dsRNA molecules to elucidate the driving force behind long-distance transport of the silencing signal. The authors have grafted silenced leaves in different position within the plant shoot (up and lower leaves). They have showed that the direction of systemic spread of silencing from inducer to sensor could be manipulated by altering sink/source relations in the plant.

Another elegant approach uses recombinant plasmids that were transformed into *Escherichia coli* HT115, an RNase-III deficient strain. The crude extracts of *E. coli* containing large amounts of dsRNA were applied to plants as a spray and the experiment confirmed a preventative efficacy against virus in *N. benthamiana* and maize respectively (Tenllado et al., 2003; Gan et al., 2010).

7.4.5. The delivery of naked dsRNA molecules

Due to delivery problems such as those described in the preceding paragraphs, much attention has been paid in the recent years to the delivery of naked dsRNA into a target tissue. Accumulating experimental evidence suggests that specific cell types may take up naked oligonucleotide-based drugs where they exert suppression of their targets. Those findings warrant more detailed analyses of this mode of delivery.

Laufer et al. (2010) have reviewed delivery possibilities of naked dsRNA into animal systems. The authors suggested various degrees of enhanced cellular uptake of nucleic acids. Currently, liposomes and cationic polymers are used as standard tools to transfect animal cells in vitro. However, these procedures are characterized by a significant lack

of efficiency, accompanied by a high level of toxicity, rendering mostly inadequate for *in vivo* applications. In this context, cell-penetrating peptides (CPPs) represent an interesting alternative, as they generally are less toxic than liposomes or cationic polymers. In addition, they are commonly better suited to transfer cargo into different cell types such as non-adherent cells and primary cells, which are hard to transfect using commercially available standard protocols. The most advanced approaches in the field are complex carrier systems combining vantages of assorted strategies to generate nanoparticles with better-defined properties, aimed toward enhanced uptake as well as intracellular trafficking in combination with cell-specific functionalities. But so far, most of these efforts have been tested only in animal cells, probably due to its potential therapeutical purpose.

For the generation of large-scale enzymatically prepared dsRNA libraries, researchers have developed a robust and simple protocol that basically consists of a cDNA fragment tagged with T7 promoter sequence by PCR, which is then transcribed to produce dsRNA *in vitro* (for review see Buchholz et al., 2006). Alternatively, *E. coli* RNase-III deficient strains are also been used to produce large amounts of dsRNA molecules that are afterwards purified (Tenllado et al., 2003; Gan et al., 2010).

7.5. SEQUENTIAL APPROACH TO DESIGN A TRANSIENT RNAI EXPERIMENT IN PLANTS

In response to old and new challenges, important knowledge related to assessment of plant gene functions by transient RNAi assays has evolved. At the same time, however, it is also clear that such knowledge is scattered, and not satisfactorily described in the scientific literature.

7.5.1. Targeting design: identifying the right dsRNA

Efforts to identify effective RNAi triggers have led to design rules and algorithms based on empirical and systematic analyses of siRNAs using conventional and machine learning-based approaches. Such studies have advanced our ability to predict efficient siRNAs (Fellmann and Lowe, 2014).

The position of dsRNA within the mRNA sequence is flexible. Often, dsRNAs derived from the coding sequence of the cognate transcript are used. However, the 3' untranslated region (3'UTR) of the transcript can be targeted by dsRNA as well. While the relative position of dsRNA at the cognate transcript does not play a crucial role, dsRNA length may influence efficient silencing. It has been observed that dsRNA sequences shorter than 500 base pair (bp) may not be effectively processed by Dicer. Although it is not clear if there is an upper limit for dsRNA length, for practical reasons, dsRNA length typically ranges from 500 to 800 bp and rarely exceeds 1000 bp (Sharp, 1998). The great advantage of using long dsRNA to induce RNAi is the simplicity, which can be appreciated by the production of high yields of molecules from simple in vitro transcription (IVT) protocols.

However, there are many possibilities for dsRNA silencing failure: (i) the lack of ability of dsRNA molecules to enter the cell, (ii) mutation and/or deletions within the target sequence, (iii) structure of mRNA target, (iv) sense strand is being loaded into RISC, and (v) presence of a pseudo-gene inhibiting the target molecule. Identifying potent sequences through the tiling of entire transcripts or assessment of preselected candidates can minimize these drawbacks. To build such a candidate list, several design criteria must be considered to maximize the number of potent dsRNAs recovery, beginning with NCBI's Reference Sequence (RefSeq) collection. The unique or common part of multiple RefSeq transcripts per gene can be used to design dsRNA/shRNA molecules (Fellmann and Lowe, 2014).

7.5.2. Knock-down confirmation: target mRNA and protein levels

The effectiveness of any siRNA target site is examined by the level of mRNA and protein that is left after RNAi, and is determined by the factors such as the half-life of the protein and protein turnover. Expression of the siRNA target gene is frequently analyzed by Western blotting, Enzyme-Linked Immunosorbent Assay (ELISA) and immunostaining using protein-specific antibodies, Northern blotting with gene specific probes or RT-qPCR assays with gene specific primers. Each assay has its own advantages and disadvantages. Standard protocols for each of these assays can be used. Whereas RNA-based detections are more sensitive and quantitative, immuno-staining enables tracking changes in the protein expression directly at a single cell level. Protein

stability and turn over should be kept in mind to see the effect of siRNA on the level of expressed protein and its loss-of-function phenotype (Wadhwa et al. 2004). Thus, validation of siRNA duplexes is required prior to its use in experimental systems, ideally by western blotting or ELISA to show a reduction in protein levels. However, in many cases good antibodies are not available, and researchers must rely on RT-qPCR to detect knockdown of the mRNA species.

Holmes et al. (2010) reported upon a phenomenon, which affects RT-qPCR quantification of gene knockdown that could result in false negative results and the rejection of valid siRNA duplexes. The authors observed that for certain transcripts, the degradation of the 3' mRNA fragment resulted from siRNA-mediated cleavage is blocked. This leaves an mRNA fragment that can still act as a template for cDNA synthesis, giving rise to false negative results and the rejection of a valid siRNA duplex. Therefore, these authors recommend that, when possible, primers should ideally be designed to flank the siRNA target sequence. A comprehensive review on best practices for RT-qPCR assays can be found elsewhere (Bustin et al., 2009).

7.5.3. Off-targeting investigation: less is more

Initially, gene silencing by RNAi was believed to be highly specific, thus requiring complete sequence homology between siRNA and target mRNA. However, recent reports have showed that non-specific effects, often referred as off-target gene silencing can occur during RNAi. This non-specificity of RNAi can be distinguished into two distinct categories: (i) sequence-determined off-target effects and, (ii) sequence independent off-target effects.

Sequence-determined effects, as named, depend on the sequence of the RNA trigger. One of the most important aspects influencing off-target silencing is the nature of trigger sequence, being that used in an RNAi vector or directly delivered to cells. Off-target silencing is mainly influenced by trigger sequence homology with mRNA (Senthil-Kumar and Mysore, 2011). By computational analysis with genome and/or transcriptome sequences of 25 plant species, Xu et al. have predicted that about 50% to 70% of gene transcripts in plants have potential off-targets when used for PTGS that could obscure experimental results (Xu et al., 2006). Therefore, in order to design siRNAs, two important aspects must be considered: (i) the potency in knocking down target

genes and (ii) avoiding the off-target effect on any non-target genes. Although many studies have produced useful tools to design potent siRNAs, off-targets effect prevention has mostly been delegated to sequence-level alignment tools such as BLAST. Other research groups have dedicated their work on the development of whole-genome thermodynamic analysis that can identify potential off-targets with higher precision (Chen et al., 2013). In addition, the chance of off-targets increases with greater length of the initial dsRNA sequence (Qiu et al., 2005; Warthmann et al., 2008). Xu et al. (2006) showed that 21-nucleotide duplex stretch of 100% identity between the silencing trigger sequence and endogenous target gene sequences is not absolutely required to provoke gene silencing. This is especially true for plant systems, which can tolerate small number of nucleotide base pair mismatches compared to animals (Stephan et al., 2008). Several computational tools have been developed to predict the influence of trigger sequence used in hpRNA or miRNA vector construction or siRNA design off-target gene silencing (Qiu et al., 2005; Xu et al., 2006). The influence of the seed region on off-target silencing is not widely reported in plants but has the potential to occur (Birmingham et al., 2006).

Sequence independent off-target effects have been also reported. Certain position- specific, sequence-independent chemical modifications in siRNAs have been shown to reduce off-target effects in animals (Senthil-Kumar and Mysore et al., 2011). In addition, the concentration of dsRNA molecules might also have a positive correlation to off-target effects, as higher dsRNA concentrations are known to favor off-target effects. In some cases, RNA-dependent RNA polymerase can mediate amplification using small RNA or piece of dsRNA producing “secondary” dsRNA, termed transitive RNAi (Alder et al., 2003; Bleyes et al., 2006). This can considerably influence the dsRNA number in cell (Senthil-Kumar and Mysore et al., 2011). Amplification of secondary dsRNA molecules can produce new dsRNAs with different sequence and unpredictable targets (Baum et al., 2007; Gordon and Waterhouse, 2007; Pak and Fire, 2007; Sijen et al., 2007).

Robertson et al. (2012) have described the results of a quantitative proteomic analysis of tomato fruit lines transformed with the carotenogenic gene phytoene synthase-1 (Psy-1), in the sense and antisense orientations, in comparison with a non-transformed, parental line. Statistical analyses of the number and quantity of proteins upon

transformation and the introduction of the Psy-1 transgene, in either the sense or antisense orientation, show that unintended effects on the proteome did occur. The changes detected in the azygous line show that the transformation process itself does perturb the proteome, presumably because a fragment of the vector became integrated within the genome, thus perturbing the proteome, although the transgene was lost in the second generation of plants. Previous studies have also shown significant alterations to the metabolome and transcriptome of both Psy-1 lines (Fraser et al., 2007), emphasizing the need for a holistic approach.

Khan et al. (2009) have investigated competition among transfected small RNAs and the endogenous pool of miRNAs for the intracellular machinery that processes small RNAs. To test this hypothesis, the authors have analyzed genome-wide transcript responses from 151 published transfection experiments in seven different human cell types. Their results show that targets of endogenous miRNAs are expressed at significantly higher levels after transfection, consistent with impaired effectiveness of endogenous miRNA repression. In addition, this effect exhibited concentration and temporal dependence.

Notably, the green fluorescent protein (GFP)-derived double-stranded RNA (dsRNA- GFP), which is currently commonly used as control in honey bee RNAi experiments, showed undesirable effects on gene expression, pigmentation or developmental timing in *Apis mellifera* (Nunes et al., 2013). The authors have performed three independent experiments using microarrays to examine the effect of dsRNA-GFP treatment (introduced by feeding) on global gene expression patterns in developing worker bees. Their data revealed that the expression of nearly 1,400 genes was altered in response to dsRNA-GFP, representing around 10% of known honeybee genes. Expression changes appear to be the result of both direct off-target effects and indirect downstream secondary effects.

Ideally, off-target effects should be investigated by performing gene expression profiling (e.g., microarray, proteomics, etc.) to assay the expression pattern of non-target genes. While off-target gene modulation is well documented (Lin et al., 2005), little is known about the possible off-target effect on overall cellular physiology.

7.6. CONCLUSIONS

RNAi analysis is a powerful method for gene-function analysis, but it is relevant to keep in mind the limitations of this approach. Unfortunately, a failure to recognize these limitations has led to the publication of many articles that display RNAi data without sufficient information.

Stable and transient RNAi systems have misconceptions regarding the integration of foreign DNA sequences into host genomes. Nonetheless, stable RNAi plants may have adverse effects inherent to genetic transformation. Therefore, the development of transient RNAi experiment in plants seems to accomplish a less complex biological system, with less potential off-target effects, which can lead to an increased confidence in interpretation of results. Off-target effects are derived from the production of intended dsRNA molecules or unintended dsRNA molecules that target unintended mRNA molecules.

We reviewed experimental approaches on the development of a transient RNAi experiment in plants that disfavor these effects. The revision includes the following: (i) conceptual differences between stable and transient RNAi systems; (ii) stable delivery systems; (iii) transient approaches; (iv) bioinformatics to identify the most suitable dsRNA sequence; (v) knock-down confirmation by measurements of mRNA and protein levels; (vi) bioinformatics and molecular profiling techniques to predict off-target effects.

Thus, understanding the limitations of RNAi is critical to enabling researchers to choose the best loss-of-function method for the study of their genes as well as to allowing readers to critically evaluate the results of papers using this technology.

7.7. REFERENCES

- Abbas-Terki T., et al. (2002) Lentiviral-mediated RNA interference. *Human Gene Therapy*, 13, 2197-2201.
- Alder M.N., et al. (2003) Gene silencing in *Caenorhabditis elegans* by transitive RNA interference. *RNA*, 9, 25–32.
- Agrawal N., et al. (2003) RNA interference: biology, mechanism, and applications. *Microbiology and Molecular Biology Reviews*, 67, 657–685.

- Angell S.M. and Baulcombe D.C. (1997) Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *EMBO Journal*, 16, 3675–3684.
- Bauer M. (2010) Strategies to Prevent siRNA-Triggered Cellular Toxicity. In: Erdman V.A. and Barciszewski J. (Eds), *RNA Technologies and their applications*. Springer, Berlin, Germany: 451pp.
- Barton G.M., et al. (2002) Retroviral delivery of small interfering RNA into primary cells. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 14943-5.
- Becker A. and Lange M. (2010) VIGS--genomics goes functional. *Trends in Plant Science*, 15, 1-4.
- Bertazzon N., et al. (2012) Transient silencing of the grapevine gene *VvPGIP1* by agroinfiltration with a construct for RNA interference. *Plant Cell Reports*, 31, 133-43.
- Bleys A., et al. (2006) Down-regulation of endogenes mediated by a transitive silencing signal. *RNA*, 12, 1633-1639.
- Birmingham A., et al. (2006) 3' UTR seed matches, but not overall identity, are associated with RNAi off- targets. *Nature Methods*, 3, 199–204.
- Brodersen P. and Voinnet O. (2006) The diversity of RNA silencing pathways in plants. *Trends in Genetics*, 22, 268-80.
- Buchholz F., et al. (2006) Enzymatically prepared RNAi libraries. *Nature Methods*, 3, 696-700.
- Burch-Smith T.M., et al. (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant Journal*, 39, 734-46.
- Carthew R.W. and Sontheimer E.J. (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell*, 136, 642–655.
- Chen J., et al. (2013) Whole-Genome Thermodynamic Analysis Reduces siRNA Off- Target Effects. *Plos One*, 8, e58326.
- Chuang C.F. and Meyerowitz E.M. (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 4985–4990.
- Eamens J., et al. (2008) RNA Silencing in Plants: Yesterday, Today, and Tomorrow. *Plant Physiology*, 147, 456–468.

- Echeverri C.J., et al. (2006) Minimizing the risk of reporting false positives in large-scale RNAi screens. *Nature Methods*, 3, 777-779.
- Fagard M. and Vaucheret H. (2000) (Trans)Gene Silencing In Plants: How Many Mechanisms? *Annual Review of Plant Physiology and Plant Molecular Biology*, 51, 167-194.
- Fellmann C. and Lowe S.W. (2014) Stable RNA interference rules for silencing. *Nature Cell Biology*, 16, 10-18.
- Fire A., et al. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391, 806-811.
- Fraser P.D., et al. (2007) Manipulation of phytoene levels in tomato fruit: effect on isoprenoids, plastids and intermediary metabolism. *The Plant Cell*, 19, 3194-3211.
- Gan D., et al. (2010) Bacterially expressed dsRNA protects maize against SCMV infection. *Plant Cell Reports*, 29, 1261-8.
- Gascioli V., et al. (2005) Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Current Biology*, 15, 1494-1500.
- Gelvin S.B. (2003) Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiology and Molecular Biology Reviews*, 67,16-37.
- Ghildiyal M. and Zamore P.D. (2009) Small silencing RNAs: an expanding universe. *Nature Reviews Genetics*, 10, 94-108.
- Grim D. (2011) The dose can make the poison: lessons learned from adverse in vivo toxicities caused by RNAi overexpression. *Silence*, 2, 8.
- Hannon G.J. (2002) RNA interference. *Nature*, 418, 244-251. Hamilton A.J. and Baulcombe D.C. (1999) A species of small antisense RNA in post-transcriptional gene silencing in plants. *Science*, 286, 950-952
- Heinemann J., et al. (2013) A comparative evaluation of the regulation of GM crops or products containing dsRNA and suggested improvements to risk assessments. *Environment International*, 55, 43-55.
- Holmes J., et al. (2010) Detection of siRNA induced mRNA silencing by RT-qPCR: considerations for experimental design. *BMC Research Notes*, 3, 53.

- Igarashi J., et al. (2009) Apple latent spherical virus vectors for reliable and effective virus-induced gene silencing among a broad range of plants including tobacco, tomato. *Virology*, 386, 407–416.
- Jackson A.L. and Linsley P.S. (2004) Noise amidst the silence: Off-target effects of siRNAs? *Trends in Genetics*, 20, 521–524.
- Jackson A.L., et al. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnology*, 21, 635–637.
- Kerschen J., et al. (2004) Effectiveness of RNA interference in transgenic plants. *FEBS Letters*, 566, 223–8.
- Kooter J.M., et al. (1999) Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends in Plant Science*, 4, 340–347.
- Krieger E.K., et al. (2008) The Flavr Savr Tomato, an early example of RNAi technology. *HortScience*, 43, 962–964.
- Kumpatla J., et al. (1998) Genome intruder scanning and modulation systems and transgene silencing. *Trends in Plant Science*, 3, 97–104.
- Laufer P., et al. (2010) Selected Strategies for the Delivery of siRNA In Vitro and In Vivo. In: Erdman V.A. and Barciszewski J. (Eds), *RNA Technologies and their applications*. Springer, Berlin, Germany: 451pp.
- Lindbo J.A. (2012) A historical overview of RNAi in plants. *Methods in Molecular Biology*, 894, 1–16.
- Liu C.N., et al. (1992) Multiple copies of *virG* enhance the transient transformation of celery, carrot and rice tissues by *Agrobacterium tumefaciens*. *Plant Molecular Biology*, 20, 1071–87.
- Lu R., et al. (2003) Virus-induced gene silencing in plants. *Methods*, 30, 296–303.
- Machado D., et al. (2011) Modeling formalisms in Systems Biology. *AMB Express*, 1, 45.
- Manche L., et al. (1992) Interactions between double-stranded RNA regulators and the protein kinase DAI. *Molecular and Cellular Biology*, 12, 5238–5248.
- Martínez de Alba P., et al. (2011) The miRNA pathway limits AGO1 availability during siRNA-mediated PTGS defense against exogenous RNA. *Nucleic Acids Research*, 39, 9339–9344.
- Matzke A.J. and Matzke M.A. (1998) Position effects and epigenetic silencing of plant transgenes. *Current Opinion in Plant Biology*, 1, 142–148.

- Meister G. and Tuschl T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature*, 431, 343–349.
- Mette M.F., et al. (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO Journal*, 19, 5194–5201.
- Nam J., et al. (1997) Differences in susceptibility of Arabidopsis ecotypes to crown gall disease may result from a deficiency in T-DNA integration. *Plant Cell*, 9, 317–333.
- Napoli C., et al. (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell*, 2, 279–289.
- Neumüller R.A. and Perrimon N. (2011) Where gene discovery turns into systems biology: genome-scale RNAi screens in *Drosophila*. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, 3, 471–8.
- O'Brien J.A. and Lummis S.C.R. (2011) Nano-biolistics: a method of biolistic transfection of cells and tissues using a gene gun with novel nanometer-sized projectiles. *BMC Biotechnology*, 11, 66.
- Parizotto E.A., et al. (2004) In vivo investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. *Genes and Development*, 18, 2237–2242.
- Qiu S., et al. (2005) A computational study of off-target effects of RNA interference. *Nucleic Acids Research*, 33, 1834–1847.
- Ramegowda V., et al. (2013) A high-throughput virus-induced gene silencing protocol identifies genes involved in multi-stress tolerance. *BMC Plant Biology*, 1, 193.
- Redenbaugh K., et al. (1992) Safety assessment of genetically engineered fruits and vegetables: a case study of the Flavr Savr tomato. CRC Press, Boca Raton, USA: 288pp.
- Robertson D. (2004) VIGS vectors for gene silencing: many targets, many tools. *Annual Review of Plant Biology*, 55, 495–519.
- Robertson F.P., et al. (2012) Proteome changes in tomato lines transformed with phytoene synthase-1 in the sense and antisense orientations. *Journal of Experimental Botany*, 63, 6035–6043.
- Sanders R.A. and Hiatt W. (2005) Tomato transgene structure and silencing. *Nature Biotechnology*, 23, 287–289.

- Sasaki J., et al. (2011) Efficient virus-induced gene silencing in apple, pear and Japanese pear using Apple latent spherical virus vectors. *Plant Methods*, 7, 15.
- Saxena S., et al. (2003) Small RNAs with imperfect match to endogenous mRNA repress translation: implications for off-target activity of siRNA in mammalian cells. *Journal of Chemical Biology*, 278, 44312–44319.
- Scacheri P.C., et al. (2004) Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 1892–1897.
- Schweizer P., et al. (1999) A transient assay system for the functional assessment of defence-related genes in wheat. *Molecular Plant-Microbe Interactions Journal*, 12, 647-654.
- Schweizer P., et al. (2000) Double-stranded RNA interferes with gene function at the single-cell level in cereals. *Plant Journal*, 24, 895–903.
- Senthil-Kumar M. and Mysore K.S. (2011) New dimensions for VIGS in plant functional genomics. *Trends in Plant Science*, 16, 656–665.
- Shang Y., et al. (2007) Methods for transient assay of gene function in floral tissues. *Plant Methods*, 8, 1.
- Singer K., et al. (2012) Formation of complex extrachromosomal T-DNA structures in *Agrobacterium tumefaciens*-infected plants. *Plant Physiology*, 160, 511-22.
- Sledz C.A., et al. (2003) Activation of the interferon system by short-interfering RNAs. *Nature Cell Biology*, 5, 834-9.
- Small I. (2007) RNAi for revealing and engineering plant gene functions. *Current Opinion in Biotechnology*, 18, 148-53.
- Tabara H., et al. (1998) The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell*, 99, 123–132.
- Tenllado F., et al. (2003) Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. *BMC Biotechnology*, 3, 3.
- Tournier B., et al. (2006) Phloem flow strongly influences the systemic spread of silencing in GFP *Nicotiana benthamiana* plants. *Plant Journal*, 47, 383-94.

- Van der Krol A.R., et al. (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene- expression. *Plant Cell*, 2, 291-299.
- Vaghchhipawala Z., et al. (2011) Agroinoculation and agroinfiltration: simple tools for complex gene function analyses. *Methods in Molecular Biology*, 678, 65-76.
- Voinnet O., et al. (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell*, 95, 177-87.
- Wadhwa R., et al. (2004) Know-how of RNA interference and its applications in research and therapy. *Mutation Research*, 567, 71-84.
- Waterhouse P.M. and Helliwell C.A. (2003) Exploring plant genomes by RNA- induced gene silencing. *Nature Reviews Genetics*, 4, 29–38.
- Waterhouse P.M., et al. (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 13959-13964.
- Watson J.M., et al. (2005) RNA silencing platforms in plants. *FEBS Letters*, 579, 5982-7.
- Weld R., et al. (2001) Transient GFP expression in *Nicotiana glauca* suspension cells: the role of gene silencing, cell death and T-DNA loss. *Plant Molecular Biology*, 45, 377-385.
- Wroblewski T., et al. (2005) Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. *Plant Biotechnology Journal*, 3, 259–73.
- Xie Z., et al. (2005) DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 12984–12989.
- Xia H., et al. (2002) siRNA-mediate gene silencing in vitro and in vivo. *Nature Biotechnology*, 20, 1006-1010.
- Yamagishi N., et al. (2013) Reduced generation time of apple seedlings to within a year by means of a plant virus vector: a new plant-breeding technique with no transmission of genetic modification to the next generation. *Plant Biotechnology Journal*, 12, 60-8.

- Zamore P., et al. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, 101, 25–33.
- Zottini M., et al. (2008) Agroinfiltration of grapevine leaves for fast transient assays of gene expression and for long-term production of stable transformed cells. *Plant Cell Reports*, 27, 845-53.
- Watson J.M., et al. (2005) RNA silencing platforms in plants. *FEBS Letters*, 579, 5982–5987.

8. CONCLUSÕES

Em conclusão, nossos resultados mostraram que as proteínas inseticidas CRY e enzimas EPSPS tolerantes a herbicidas à base de glifosato, expressas tanto em eventos transgênicos simples quanto estaqueados, foram a principal fonte de influência na expressão proteica em milho transgênico. Destaca-se que as amostras contendo o evento transgênico estaqueado foram agrupadas, estando distantes de outros genótipos analisados pela Análise dos Componentes Principais. Além disso, observamos evidências de possíveis interações sinérgicas e antagônicas mediadas pela piramidação de transgenes por melhoramento convencional, uma vez que vinte e duas proteínas foram estatisticamente diferencialmente moduladas. Estas proteínas foram designadas principalmente para o metabolismo de energia / carbono (77 % de todas as proteínas identificadas). Também, os níveis de expressão dos transcritos transgênicos tiveram uma redução significativa de cerca de 50% quando comparados com as variedades parentais de eventos transgênicos simples. Tais observações indicam que as alterações no genoma do milho transgênico estaqueado pode ter um impacto sobre a expressão gênica global e que por sua vez possam ser relevantes para as avaliações de segurança. Algumas destas proteínas não estavam dentro da faixa de variabilidade natural encontrada em uma variedade local de milho crioulo. Baseado no nosso conhecimento, este foi o primeiro estudo sobre a análise proteômica comparativa de eventos transgênicos estaqueados. No entanto, a detecção de alterações no perfil de proteínas não apresenta um problema de segurança *per se*. Portanto, mais estudos devem ser realizados a fim de se abordar a relevância biológica e as implicações de tais alterações. Os resultados obtidos com milho transgênico silenciado demonstraram que a expressão de níveis elevados de proteína transgênica não é um fator aditivo para a equivalência dos proteomas transgênico e convencional. Níveis baixos de expressão CRY1Ab e/ou mudanças oriundas da inserção do transgene *per se* podem ter um efeito adverso sobre a célula de planta hospedeira. Mais estudos precisam ser realizados para compreender os mecanismos bioquímicos e fisiológicos envolvidos na regulação gênica sob controle de promotores fortes constitutivos como p35S. Conclui-se que pouca atenção é dada aos possíveis efeitos adversos oriundos da recombinação de sequências de DNA, mesmo em ensaios que visam apenas investigar a função gênica de um determinado gene endógeno. Ainda, a falha em

reconhecer as limitações da técnica de RNAi levou à publicação de vários artigos que exibem dados sem informações suficientes sobre os ensaios. Compreender tais limitações é fundamental para permitir que investigadores possam escolher o melhor método para o estudo de genes de interesse, bem como permitir aos leitores a avaliação criteriosa dos resultados destes trabalhos.

9. CONSIDERAÇÕES FINAIS

Os resultados obtidos neste trabalho demonstraram que as novas abordagens metodológicas testadas foram capazes de preencher algumas lacunas do conhecimento científico sobre a regulação gênica de transgenes inseridos em genomas vegetais.

A atual abordagem que utiliza o critério de ‘equivalência substancial’, adotada pelas agências regulatórias de grande parte dos países, é baseada na suposição de que um organismo geneticamente modificado é composto por duas partes, a planta e a proteína transgênica, e que funcionam de forma linear e aditiva. Portanto, não são adequadas para a detecção de alterações no proteoma de plantas geneticamente modificadas oriundas de interações sinérgicas e antagonistas da integração e expressão de transgenes.

O presente trabalho obteve sucesso ao desenvolver e testar metodologias e protocolos mais adequados e que puderam detectar alterações de forma mais sensível e robusta. Através dos ensaios realizados foi possível detectar alterações no proteoma de milho geneticamente modificado em dois modelos biológicos: (i) quando dois transgenes estão inseridos convencionalmente em uma única variedade de milho transgênico e, (ii) quando a expressão do transgene é silenciada por RNAi. A combinação de dois ou mais transgenes na mesma planta através de cruzamento convencional também acarreta alteração nos níveis de expressão dos transgenes. Os resultados obtidos para a quantificação dos transgenes via PCR em tempo real sugerem que tal alteração pode ser ocasionada pela maior demanda energética, bem como possíveis rotas metabólicas de silenciamento de transgenes devido à presença de sequências homólogas de promotores. Ainda, essas alterações não foram observadas na mesma intensidade nas amostras de milho silenciadas, confirmando assim a hipótese anteriormente descrita sobre a influência do acúmulo de proteínas transgênicas na célula na expressão de genes endógenos.

A detecção de alterações no proteoma do milho transgênico não representa um risco *per se*. A realização de ensaios similares com alimentação de animais modelos (*feeding-studies*) para estudos toxicológicos e de alergenicidade com as variedades de milho utilizadas, em sua forma estaqueada e silenciada, pode ajudar na elucidação da potencial relevância biológica de tais alterações.

REFERÊNCIAS

- Agapito-Tenfen SZ. et al. 2013. Comparative proteomic analysis of genetically modified maize grown under different agroecosystems conditions in Brazil. *Proteome Sci* 11(1): 46.
- Amman D, et al. 2007. Procedure for the implementation of the precautionary principle in biosafety commissions. *Journal of Risk Research* 10: 487-501.
- Balsamo GM. et al. 2011. Proteomic analysis of four Brazilian MON810 maize varieties and their four non-genetically-modified isogenic varieties. *J Agr Food Chem* 59: 11553-59.
- Barros E. et al. 2010. Comparison of two GM maize varieties with a near-isogenic non-GM variety using transcriptomics, proteomics and metabolomics. *Plant Biotechnol J* 8: 436-451.
- Batista R e Oliveira M. 2010. Plant natural variability may affect safety assessment data. *Regul Toxicol Pharm* 58: S8-S12.
- Bayer M. et al. 2009. Paternal control of embryonic patterning in *Arabidopsis thaliana*. *Science* 323: 1485–1488.
- Bird A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev* 16: 6–21.
- Bohn T. et al. 2010. Demographic responses of *Daphnia magna* fed transgenic Bt-maize. *Ecotoxicology* 19(2): 419–430.
- Borges F. et al. 2011. MicroRNA activity in the *Arabidopsis* male germline. *J Exp Bot* 62(5): 1611-1620.
- Carman JA. et al. 2013. A long-term toxicology study on pigs fed a combined genetically modified (GM) soy and GM maize diet. *Journal of Organic Systems* 8(1).
- Carthew RW and Sontheimer EJ. 2009. Origins and Mechanisms of miRNAs and siRNAs. *Cell* 136 (4): 642-655.
- Chuang JC, Jones PA. 2007. Epigenetics and MicroRNAs. *Pediatr Res* 61: 24R-29R.
- Coll A. et al. 2008. Lack of repeatable differential expression patterns between MON810 and comparable commercial varieties of maize. *Plant Mol Biol* 68: 105-117.
- Ding D. et al. 2009. Differential expression of miRNAs in response to salt stress in maize roots. *Ann Bot* 103(1): 29-38.
- Frank T. et al. 2012. Metabolite profiling of maize kernels--genetic modification versus environmental influence. *J Agric Food Chem* 60(12): 3005-12.

- Garrick D. et al. 1998. Repeat-induced gene silencing in mammals. *Nat Genet* 18: 56-59.
- Gassmann AJ. et al. 2014. Field-evolved resistance by western corn rootworm to multiple *Bacillus thuringiensis* toxins in transgenic maize. *PNAS* 111(14): 5141-6.
- Grimaud C. et al. 2006. RNAi Components Are Required for Nuclear Clustering of Polycomb Group Response Elements. *Cell* 124: 957-971.
- Heller C. et al. 1995. Chromosomal insertion of foreign (adenovirus type 12, plasmid, or bacteriophage lambda) DNA is associated with enhanced methylation of cellular DNA segments. *PNAS* 92 (12): 5515-5519.
- Heinemann JA. et al. 2011. Molecular profiling: a tool for addressing emerging gaps in comparative risk assessment of GMOs. *Environ Int* 37: 1285-1293.
- Hilbeck A, et al. 1998. Toxicity of *Bacillus thuringiensis* Cry1Ab toxin to the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environmental Entomology* 27 (5): 1255-1263.
- Hilbeck A. et al. 2012. A controversy re-visited: Are ladybeetles and lacewings adversely affected by Bt toxins? *Environmental Sciences Europe* 24: 10.
- Holck A. et al. 2002. 5'-Nuclease PCR for quantitative event-specific detection of the genetically modified MON 810 MaisGard maize. *Eur Food Res Techn* 214: 449.
- Khvorova A. et al. 2003. Functional siRNAs and miRNAs exhibit strand bias. *Cell* 115(2): 209-16.
- La Paz JL. et al. 2014. The Use of Massive Sequencing to Detect Differences between Immature Embryos of MON810 and a Comparable Non-GM Maize Variety. *PLoS ONE* 9(6): e100895.
- Leon C. et al. 2009. Metabolomics of transgenic maize combining Fourier transform-ion cyclotron resonance-mass spectrometry, capillary electrophoresis-mass spectrometry and pressurized liquid extraction. *J Chromatogr A* 1216(43): 7314-23.
- Li LM. et al. 2010. Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. *Cancer Res* 70: 9798-9807.
- Manetti C. et al. 2004. NMR-based metabonomic study of transgenic maize. *Phytochemistry* 65(24): 3187-98.
- Matsuoka, T. et al. 2000. A method of detecting recombinant DNAs

- from four lines of genetically modified maize. *Shokuhin Eiseigaku Zasshi* 41: 137-143.
- Matsuoka T. et al. 2002. Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *J Agric Food Chem* 50(7): 2100-2109.
- Meier M. et al. 2013. Possible consequences of an introgressed insect resistance trait in a shared-herbivore crop-wild relative assemblage. *Ecol Evol* 3(2): 416–423.
- Mekawi EMM. 2009. *Biochemical investigations on genetically modified crops*. 83 f. Tese (Doutorado em Ciências Naturais) – Faculdade de Ciências Naturais, Universidade de Hohenheim, Hohenheim, 2009.
- Ng EK. et al. 2009. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut* 58: 1375-1381.
- Poerschmann J, et al. 2005. Molecular composition of leaves and stems of genetically modified Bt and near-isogenic non-Bt maize: characterization of lignin patterns. *J Environ. Qual* 34.
- Remus R. et al. 1999. Insertion of foreign DNA into an established mammalian genome can alter the methylation of cellular DNA sequences. *J Virol* 73(2): 1010-22.
- Reinhart BJ. Et al. 2002. MicroRNAs in plants. *Genes Dev* 16(13): 1616–1626.
- Reinhart B. et al. 2002. Shared Role for Differentially Methylated Domains of Imprinted Genes. *Mol Cell Biol* 22(7): 2089–2098.
- Reyna-López GE. et al. 1997. Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. *Mol Gen Genet* 253: 703-710.
- Rice P. et al. 2000. EMBOSS: The European Molecular Biology Open Software Suite. *Trends in Genetics* 16(6): 276-277.
- Sambrook J. et al. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Sanger F. et al. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA*. 74 (12): 5463–7.
- Saxena D e Stotzky G. 2001. Bt corn has a higher lignin content than non-Bt corn. *Am J Bot* 88 (9): 1704-1706.
- Sawazaki E. et al . 2000. Potencial de linhagens de populações locais de milho pipoca para síntese de híbridos. *Bragantia* 59 (2).

- Seralini GE. et al. 2014. Republished study: long-term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Environmental Sciences Europe* 26: 14.
- Scheir AF. 2007. The maternal-zygotic transition: death and birth of RNAs. *Science* 316: 406–407.
- Schuettengruber B, et al. 2009 Functional Anatomy of Polycomb and Trithorax Chromatin Landscapes in *Drosophila* Embryos. *PLoS Biol* 7(1).
- Schumacher A. et al. 2000. Epigenetic and Genotype-specific Effects on the Stability of the Novo Imposed Methylation Patterns in Transgenic Mice. *J Biol Chem* 275: 37915-37921.
- Schuettengruber B. et al. 2007. Genome Regulation by Polycomb and Trithorax Proteins. *Cell* 128(4): 735–745.
- Steinberg TH. 2009. Protein gel staining methods: an introduction and overview. *Methods Enzymol* 463: 541–563.
- Stitzel ML and Seydoux G. 2007. Regulation of the oocytes-to-zygote transition. *Science* 316: 407–408.
- Traavik TI and Lin LC (Eds.). 2009. Biosafety First: Holistic Approaches to Risk and Uncertainty in Genetic Engineering and Genetically Modified Organisms. Third World Network and GenØk, Norway. 616p.
- Wang W. et al. 2014. A novel 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase transgene for glyphosate resistance stimulates growth and fecundity in weedy rice (*Oryza sativa*) without herbicide. *New Phytol* 202(2): 679-88.
- Zhang B. et al. 2006. Conservation and divergence of plant microRNA genes. *The Plant Journal* 46: 243–259.
- Zhang L. et al. 2011. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Research* 2:107-126.
- Zhang L. et al. 2009. A Genome-Wide Characterization of MicroRNA Genes in Maize. *PLoS Genet* 5(11).
- Zolla L. et al. 2008. Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. *J Proteome Res* 7: 1850-1861.

APÊNDICE

Capítulo I

Additional file 1. Primers sequences and gene indentification used in this study.

Primer name	Gene product	Genbank Accession no.	Primer sequence
MEP	Membrane protein PB1A10.07c	GRMZM2G018103 T01	F - GTACTCGGCAATGCTCTTGA P - AACTTCGGTTGGTGAGAGCGGAAA R - CAATCCTGACCCAGACAGATG
LUG	Leunig	GRMZM2G425377 T01	F - GGGACATAAGGGAGAAGAACAC P - TTCCCTGTAGCACTGGATGATGCC R - TCATGGCTTACTGAGGCAAC F - CGACAAGGACAACGCCAATA
CUL	Cullin	GRMZM2G166694 T04	P - ACCTTGCCTGATTGGTGGTTAGTGA R - TCCCAGTGGTATCGCATAGT F - CTTTCCAGGTGCTGGTTACT
FPGS	Folylpolyglutamate synthase	GRMZM2G393334 T01	P - TCAAGAAGTGATACGCCGCTCGAA R - TCATAGTCCAGTTCAGTTTGG
UBPC	Ubiquitin carrier protein	GRMZM2G102471 T01	F - ACAGTGGAGTCCTGCTTTAAC P - TCAATCTGCTCACTGCTCACGGAC R - GAGCAATCTCAGGGACAAGAG F - TACGATTTGACAGCACCTTC
EPSPS	CP4-epsps protein	Company document only	P - TTGAACCCGCTGCGCGAAATG R - GTCACCGTCTTCCGATTTC F - GACGTGGAGGAACAGAACAA
cry1A.105	cry1A.105 protein	FV532179	P - TTGTGCCTGAGTGGGAAGCTGAA R - CCTCTACCTGGACAGACTCTAA F - GCGACTACCTGAAGAACTACAC
cry2Ab2	cry2Ab2 protein	FV532179	P - CAACACCTACCAGTCGGCCTTCAA R - TGTCGTGAAGCCTCGTATTG

Table 1: Differentially expressed proteins in stacked transgenic maize variety versus controls (single event transgenic maize variety with the same genetic background) and non-genetically modified counterpart and a landrace by 2D-DIGE analysis. Proteins were considered differentially modulated at statistical significant difference in normalized volume in stacked vs. single GM events and control samples at ANOVA $P < 0.05$. The Table reports spot number (Match ID), accession number and protein name, together with Mascot score, sequence coverage, number of matched peptides, theoretical and experimental molecular weight (MW), isoelectric point (pI) and fold change. Abbreviations for each plant variety are provided within 'Material and Methods' section.

Match ID	Genebank ID	Protein Name	Mascot Score	Sequence Coverage (%)	Pep	Theor. Mass (kDa)	Theor. pI (pH)	Exp. Mass (kDa)	Exp. pI (pH)	Fold change (ANOVA $P < 0.05$)
55	gi 11467199	ATP synthase CF1 beta subunit [<i>Zea mays</i>]	2248	72	62	54	5.31	56	5.80	Conv, RR, RRxBt>Bt > Land
155	gi 413948212	hypothetical protein ZEAMMB73_661450 [<i>Zea mays</i>]	723	44	21	46	5.62	44	5.96	Land>Conv, RR, Bt, RRxBt
156	gi 413939324	glutamate-oxaloacetate transaminase2 [<i>Zea mays</i>]	1201	61	43	50	8.43	44	6.12	Land>Bt>Con onv, RR, RRxBt
231	gi 195622374	fructose-bisphosphate aldolase [<i>Zea mays</i>]	798	40	19	40	5.39	37	5.50	Land>Conv, RR, Bt, RRxBt
406	gi 414591286	APx2-cytosolic ascorbate peroxidase [<i>Zea mays</i>]	1036	59	20	31	5.77	27	5.78	Conv, RR, Bt, RRxBt>Land
426	gi 226504576	APx1 - cytosolic ascorbate peroxidase [<i>Zea mays</i>]	772	54	18	27	5.65	26	5.74	Bt>Conv>RRxBt>RR>Land

171	gi 414586172	3-isopropylmalate dehydrogenase [<i>Zea mays</i>]	1042	44	23	43	5.62	42	5.18	Conv>Bt< Land> RR>RRxBt Bt>Conv, RR, RRxBt>La nd Conv, Bt>RR, RRxBt>La nd RR>Conv, Bt, RRxBt>La nd Bt, RRxBt>Co nv, Land, RR
175	gi 195645514	acyl-desaturase [<i>Zea mays</i>]	441	24	8	45	6.61	42	6.15	RR>Bt< RRxBt>RR
177	gi 308081433	coproporphyrinogen III oxidase [<i>Zea mays</i>]	321	24	9	47	7.23	42	6.12	RR>Bt< RRxBt>La nd RR>Conv, Bt, RRxBt>La nd Bt, RRxBt>Co nv, Land, RR
762	gi 226499080	dihydroflavonol-4-reductase [<i>Zea mays</i>]	534	36	14	35	5.43	33	5.83	RR>Bt< RRxBt>RR
64	gi 226492645	vacuolar ATP synthase subunit B [<i>Zea mays</i>]	711	45	17	54	5.07	54	5.19	RR>Bt< RRxBt>RR
105	gi 162458207	enolase 1 [<i>Zea mays</i>]	1604	67	29	48	5.20	49	5.60	RR>Bt< RRxBt>RR
437	gi 413951084	hypothetical protein ZEAMMB73_536198 [<i>Zea mays</i>]	416	32	9	28	5.14	26	5.39	RR>Bt< RRxBt>RR
714	gi 195619804	enolase [<i>Zea mays</i>]	663	40	16	48	5.59	56	6.05	RR>Bt< RRxBt>RR
137	gi 226505740	DIMBOA UDP-glucosyltransferase BX9 [<i>Zea mays</i>]	1197	49	23	50	5.15	45	5.43	RR>Bt< RRxBt>RR
415	gi 41459136-		333	28	7	35	7.71	26	5.08	RR>Bt< RRxBt>RR

	66	phosphogluconolactonase isoform 1 [<i>Zea mays</i>]								Bt, RRxBt>La nd Bt, RRxBt>Co nv, Land, RR
421	gi 1956112 74	14-3-3-like protein [<i>Zea mays</i>]	858	67	23	29	4.82	26	4.93	
572	gi 2265046 88	uncharacterized protein LOC100272933 precursor [<i>Zea mays</i>]	202	13	9	22	6.02	19	6.62	Bt, RRxBt only
345	gi 1956192 62	gibberellin receptor GID1L2 [<i>Zea mays</i>]	244	20	5	33	4.93	31	5.06	Bt, RRxBt only
545	gi 1956265 24	2-cys peroxiredoxin BAS1 [<i>Zea mays</i>]	160	23	4	28	5.81	21	4.56	Bt, RRxBt only
38	gi 2264932 35	LOC100281701 [<i>Zea mays</i>]	1110	41	17	61	5.20	59	5.15	RR only
750	gi 2265301 74	ankyrin repeat domain-containing protein 2 [<i>Zea mays</i>]	619	57	16	38	4.57	36	4.66	RR only

Capítulo II

Table 5: Differentially expressed proteins in MON810 transgenic maize treated with dsRNA targeting cry1Ab transgene versus controls (untreated samples of GM and conventional counterpart plants) by 2-DE analysis. Proteins were considered differentially modulated at statistical significant difference in normalized volume in treated vs. untreated GM events and control samples at ANOVA F test ($P < 0.05$). The Table reports spot number (Spot ID), accession number and protein name, together with Mascot score, sequence coverage, number of matched peptides, theoretical and experimental molecular weight (MW), isoelectric point (pI) and fold change.

Match ID	Spot ID	Protein Name	Mascot Score	Sequence Coverage (%)	Pep	Theor. Mass (kDa)	Theor. pI (pH)	Exp. Mass (kDa)	Exp. pI (pH)	Fold change (ANOVA $P < 0.05$)
1	gi 1146719 9	ATP synthase CF1 beta subunit [Zea mays]	1620	65	50	54	5.31	50	5.4	GM treated
2	gi 1947026 98	TPA: triosephosphate isomerase [Zea mays]	530	32	11	32	6.14	24	4.9	GM treated
3	gi 4139246 31	ferritin1 [Zea mays]	432	31	12	37	6.68	24	5.1	GM treated
5	gi 1956492 11	50S ribosomal protein L21 [Zea mays]	425	30	7	23	9.34	21	6.0	GM treated
6	gi 1946973 74	NADH dehydrogenase I subunit N [Zea mays]	399	47	12	23	9.07	17	6.3	GM treated
7	gi 1946973 74	NADH dehydrogenase I subunit N [Zea mays]	120	21	4	23	9.07	17	6.1	GM treated
8	gi 1956096 54	glycine-rich RNA-binding protein 2 [Zea mays]	273	43	5	15	6.10	10	5.8	GM treated
9	gi 1624596 61	annexin2 [Zea mays]	422	27	10	35	6.82	30	7.5	GM untreated
10	gi 1624637 24	PL3K2 [Zea mays]	225	42	7	17	6.73	11	7.2	GM untreated
11	gi 2264914 84	thylakoid lumenal 19 kDa protein [Zea mays]	422	27	9	27	5.48	19	4.8	GM untreated
13	gi 22276	ferritin [Zea mays]	343	29	9	31	6.12	24	5.1	Conv treated

15	gi 4601998 2	NADPH- protochlorophyllide oxidoreductase [Zea mays]	69	32	11	40	9.19	35	9.1	Conv treated
16	gi 2265315 56	hypothetical protein precursor [Zea mays]	352	24	7	38	8.62	24	9.5	Conv treated
19	gi 2198856 17	ribonucleoprotein [Zea mays]	186	22	6	30	4.60	28	3.5	Conv treated
20	gi 2265015 82	electron transporter/ thiol- disulfide exchange intermediate [Zea mays]	361	26	8	33	6.47	27	5.2	Conv treated
12	gi 2265331 40	hypothetical protein [Zea mays]	257	24	6	33	5.96	30	6.1	Conv untreated
14	gi 2264914 84	thylakoid lumenal 19 kDa protein [Zea mays]	345	32	6	27	5.48	19	4.8	Conv untreated
18	gi 4145872 71	TPA: hypothetical protein ZEAMMB73_690514 [Zea mays]	246	14	3	28	5.07	31	4.3	Conv untreated
1	gi 1146719 9	ATP synthase CF1 beta subunit [Zea mays]	1620	65	50	54	5.31	50	5.4	GM treated
2	gi 1947026 98	TPA: triosephosphate isomerase [Zea mays]	530	32	11	32	6.14	24	4.9	GM treated
3	gi 4139246 31	ferritin I [Zea mays]	432	31	12	37	6.68	24	5.1	GM treated
5	gi 1956492 11	50S ribosomal protein L21 [Zea mays]	425	30	7	23	9.34	21	6.0	GM treated