Universidade de Aveiro
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Departamento de Química

Carolina Nogueira Carvalho Gomes

Produção e análise de péptidos com relevância farmacêutica em *Lactuca sativa* e *Medicago truncatula*

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Production and analysis of pharmaceutically relevant peptides in *Lactuca sativa* and *Medicago truncatula*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo de Biotecnologia Alimentar, realizada sob a orientação científica da Prof.ª Sandra Vieira, Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro, e co-orientação da Dra. Sofia Duque, bolseira de pós doutoramento do Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa.

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” Si un peu de rêve est dangereux, ce qui en guérit, ce n’est pas moins de rêve, mais plus de rêve, mais tout le rêve. “

Marcel Proust

o júri

presidente

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palavras-chave

Molecular pharming, leguminosa modelo, alface, hipertensão, enzima conversora da angiotensina I, péptidos inibidores de ACE, transformação de plantas, organogénese, embriogénese somática, alimentos funcionais.

resumo

O molecular pharming permite a produção de proteinas terapêuticas recombinantes a larga escala, de forma segura e a baixo custo. No presente trabalho, é proposta a produção heteróloga de quatro péptidos inibidores da ACE em dois emergentes sistemas de expressão vegetal, *Lactuca sativa* (alface) e *Medicago truncatula* (luz cortada). A utilização da alface, uma planta comestível, pode proporcionar um meio para a administração oral de péptidos anti-hipertensivos, criando um novo alimento funcional. Por outro lado, a utilização de M. truncatula, uma leguminosa modelo, garante não só a facilidade de transformação mas também a extrapolação processual para outras leguminosas. No contexto actual de demanda por terapias alternativas para a hipertensão e de processos mais eficientes de produção de péptidos inibidores da ACE, este trabalho assume particular importância.

keywords

Molecular pharming, model legume, lettuce, hypertension, angiotensin-I converting enzyme, ACE inhibitory peptides, plant transformation, organogenesis, somatic embryogenesis, functional foods.

abstract

Molecular pharming is a cost-effective, scalable and safe system to produce high-quality and biologically active recombinant therapeutic proteins. In the present work the heterologous production of four ACE inhibitory peptides in two emerging plant expression hosts, *Lactuca sativa* (lettuce) and *Medicago truncatula* is proposed. The use of lettuce, an edible plant, can provide a means for oral delivery of antihypertensive peptides, thus creating a novel functional food. On another hand, the use of *M. truncatula*, a model legume, ensures not only the simple transformation process but also the procedural extrapolation to other legume species. In the current scenario of global demand for alternative hypertension therapies and easier ACE inhibitory peptide manufacturing processes, this work assumes particular importance.

List of abbreviations

2,4-D - 2,4-dichlorophenoxyacetic acid

5'-UTR - 5' untranslated region

6X His tag® - Amino acid motif in proteins that consists of six histidine residues

ACE - Angiotensin-I converting enzyme

ACEI - ACE inhibitory

ACEI_CHL - Synthetic gene containing the *M. truncatula* codon optimized coding sequence for ACEI peptide derived from *Chlorella vulgaris* digest

ACEI_CHLLET – Synthetic gene containing the *L. sativa* codon optimized coding sequence for ACEI peptide derived from *Chlorella vulgaris* digest

ACEI_CHLTP - Synthetic gene containing the *M. truncatula* codon optimized coding sequence for ACEI peptide derived from *Chlorella vulgaris* digest and chloroplast transit peptide

ACEI_CHLTPLET - Synthetic gene containing the *L. sativa* codon optimized coding sequence for ACEI peptide derived from *Chlorella vulgaris* digest and chloroplast transit peptide

ACEI_FMK - Synthetic gene containing the *M. truncatula* codon optimized coding sequence for ACEI peptide released during milk fermentation by *Enterococcus faecalis*

ACEI_FMKLET - Synthetic gene containing the *L. sativa* codon optimized coding sequence for ACEI peptide released during milk fermentation by *Enterococcus faecalis*

ACEI_SEA - Synthetic gene containing the *M. truncatula* codon optimized coding sequence for ACEI peptide derived from sea cucumber hydrolysate

ACEI_SEALET - Synthetic gene containing the *L. sativa* codon optimized coding sequence for ACEI peptide from sea cucumber hydrolysate

ACEI_SPI - Synthetic gene containing the *M. truncatula* and *L. sativa* codon optimized coding sequence for ACEI peptide isolated from the pepsin-pancreatin digest of the large subunit of spinach RuBisCO

Act - Actin gene

ADH - Alcohol dehydrogenase

AHP - Antihypertensive peptide

AHPM - Antihypertensive peptide multimer

AMC3 - Multimeric protein containing ACEI peptides (4xVY and IPP) sequences incorporated into the amarantin acidic subunit

BA - Benzyl adenine

BP - Blood pressure

CaMV 35S - Cauliflower mosaic virus 35S RNA promoter

Carb – Carbenicillin

cDNA - Complementary DNA

CEI12 - ACEI peptide with sequence FFVAPFPEVFGK

cGMP - Compliant with good manufacturing practices

CHO - Chinese hamster ovary

CMPV-HT - Hyper-translatable cowpea mosaic virus

ColE1 ori - *E. coli* replication origin

CPMV - Cowpea mosaic virus

CVD - Cardiovascular disease

CVPs - Chimeric virus particles

DH5α™ - *E. coli* strain used

DHFR - Dihydrofolate reductase

DNA - Deoxyribonucleic acid

DNAse I - Deoxyribonuclease I

ECM - Embryo conversion medium

EDTA - Ethylenediaminetetraacetic acid

EHA105 - *A. tumefaciens* strain used

EIM - Embryo induction medium

EPM - Embryo proliferation medium

ER - Endoplasmic reticulum

F1-V - *Yersinia pestis* f1-v fusion gene

FDA - Food and Drug Administration

FDE - Denaturing loading buffer

GFP - Green fluorescent protein

GMOs - Genetically modified organisms

GMP - Good manufacturing practices

GST - Glutathione-S-transferase

Gus - β -glucuronidase gene

GUS - β -glucuronidase protein

HA - Hemagglutinin

hFIX - Human coagulation Factor IX

HIV - Human immunodeficiency virus

HPLC - High Performance Liquid Chromatography

HSP terminator – Heat shock protein terminator

IBA - Indole butyric acid

IC50 - Concentration required to inhibit ACE activity by 50%

IgG - Immunoglobulin G

IPTG - Isopropyl β-D-1-thiogalactopyranoside

Kan - Kanamycin

KanR - Kanamycin resistant

KanS - Kanamycin sensitive

KBP - Kentucky BioProcessing

KDEL - Endoplasmic reticulum-retention signal

LA - LB solidified with 15 g.l-1 microagar

LAB - Lactic Acid Bacteria

LB - Luria Broth rich medium

LB - T-DNA left border

M9-10a - *Medicago truncatula* cv Jemalong embryogenic line

MALDI-TOF - Matrix-assisted laser desorption/ionization time-of-flight

MCS - Multiple cloning site

mRNA - Messenger RNA

MS - Murashige and Skoog culture medium

MS010A - MS basal salts and vitamins, 1% (w/v) sucrose, 0.8% (w/v) agar

MS030A - MS basal salts and vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar

NAA - Naphthalene acetic acid

NisA - Nisin A gene

NOS terminator - Nopaline synthase terminator

NPT II - Selectable marker gene in plant

NPT III - Selectable marker gene in *E. coli* or *Agrobacterium*

OD600nm - Optic density at 600 nm

PCR - Polymerase chain reaction

PEG - Polyethylene glycol

pMP2482 - Plasmid construct that contains the glucuronidase (gus)A::intr/green fluorescent protein (gfp) genes

pRI 201-AN - Binary vector for plant transformation

pRI-ACE - pRI 201-AN constructs containing ACEI peptides coding sequences

pUC19 - Standard *E. coli* vector with a multiple cloning site (MCS) for DNA cloning.

pUC57 - Cloning vector derivative of pUC19

PVX - Potato virus X

RAS - Renin-Angiotensin System

RB - T-DNA right border

Ri ori - Mutant-type replication origin from the *Agrobacterium rhizogenes* Ri plasmid

Ri plasmid - Root-inducing plasmid

RNA - Ribonucleic acid

RNase - Ribonuclease

RT - Reverse transcription

RT-PCR - Reverse transcription-PCR

RuBisCO - Ribulose-1,5-bisphosphate carboxylase/oxygenase

scFV - Single-chain Fv fragment

SHRs - Spontaneously hypertensive rats

T-DNA - Transferred DNA region of Ti plasmid

TBE - Tris borate EDTA buffer

Ti plasmid - Tumor-inducing plasmid

TMV - Tobacco mosaic virus

ubi-1 - Ubiquitin-1 promoter

UH40 - HHL 40 tandem repeat ligated to ubiquitin as a fusion gene

VLPs - Virus-like particles

XGLcA - 5-bromo-4-chloro-3-indolyl-β -D -glucuronide acid

YEB - Yeast Extract Broth

YG-1 - ACEI peptide that consists of ten amino acids derived from yeast glyceraldehyde-3-phosphate dehydrogenase

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1 Bibliographic revision

1.1 Introduction

Humans started using wild plants for food and healing purposes because of their unique nutritional and medicinal properties in pre-historic times (Raskin et al., 2002). In fact, wild plants used to constitute an important part of the diet of ancient human societies and were widely used for illness treatment. Though, along with the transition from the nomad to the sedentary lifestyle and the creation of agriculture, humans started domesticating some target wild plants. The first signs of domestication date back to ten thousand years ago (Doebley et al., 2006) and since then humans have been genetically manipulating plants to meet their needs.

Initially, ancient farmers used to select spontaneous mutations that occurred in wild plant populations in order to achieve desired traits (Gepts, 2002), e.g. larger fruit size, color change, plant habit (Zeder et al., 2006). The plants with the desired characteristics were then used as progenitors for subsequent plant generations, resulting in an accumulation of selected traits over time, the so-called domestication syndrome (Hammer, 1984).

The continuous process of selection, breeding and re-selection, known as classical plant breeding, was a precursor to the modern plant improvement techniques and originated the present-day plant crop varieties, which are completely different from the ones of ten thousand years ago (Gepts, 2002; Doebley et al., 2006).

Whilst traditional plant breeding and selection methods are still widely applied and of foremost importance in food industry, the advent of recombinant gene technology revolutionized plant improvement by enabling plants to be genetically modified with novel traits beyond what was possible through classical breeding and selection techniques. In recombinant gene technology, foreign genes responsible for genetic traits (e.g. insect resistance) are transferred from a source organism (microorganism, animal, plant) to a target plant. Because only the desired gene is inserted into the plant genome, this technology allows a more accurate and less time-consuming plant improvement in

comparison to traditional improvement techniques (Rehbinder, 2008). Plant transformation can be performed using a broad range of gene delivery systems such as *Agrobacterium* Ti plasmid vectors, biolistics, microinjection and physical (electroporation) or chemical (PEG) treatment of protoplasts.

The first genetically engineered plant to be commercially grown was the FlavrSavr™ tomato (Kramer and Redenbaugh, 1994), a tomato engineered to have delayed ripening and subsequently longer shelf life. Although the FlavrSavr™ tomato soon left the market owing to high production costs and consumer concerns, over the last two decades the number of transgenic plants has steadily increased with various important traits related to insect (Barton et al., 1987) and herbicide (Stalker et al., 1988) resistance; abiotic stress tolerance (Wang et al., 2003); nutritional quality, the notable case of golden rice (Ye et al., 2000); virus resistance (Niu et al., 2006); and biofuel production (Chen and Dixon, 2007) being successfully engineered into crop plants.

While most of plant transformations have been focused on conferring agronomic advantages, since the late 1980s (Barta et al., 1986; Hiatt et al., 1989) plants begun to be used as production systems for recombinant pharmaceutical and industrial proteins.

The limitations of microbial fermentation and mammalian cell cultures as therapeutic protein production systems, including cost, scalability, safety and protein authenticity and the growing demands for complex therapeutic proteins have stimulated the advent of molecular pharming. Molecular pharming comprises the use of either whole-plants or *in vitro* cultured plant cell/tissues for the synthesis of recombinant therapeutic proteins in plants (Fischer and Emans, 2000).

Molecular pharming is a cost-effective, scalable and safe system to produce high-quality and biologically active recombinant therapeutic proteins. Plants have the ability to perform most of the posttranslational modifications required for protein bioactivity and pharmacokinetics (Gomord and Faye, 2004). Moreover, some plant tissues provide a means for stable long-term storing of recombinant proteins, minimizing processing or purification steps, costs and labor required to the delivery of injectable therapeutics. Selected tissues are suitable for oral administration, thus reducing the costs, inconvenience and hazards of delivery that are associated with injected therapeutics. Plants are also a safe platform for therapeutic protein production since they do not harbor human or animal pathogens (Streatfield, 2006). Thus, plants are an emerging alternative platform for the

production of pharmaceutically relevant proteins such as vaccines, antibodies and antibody derivatives, and some serum-derived proteins, namely cytokines, growth hormones, interleukins, and interferon (Xu et al., 2011).

Additionally, plants have also been used to produce bioactive and immunogenic peptides. The efficacy, selectivity, specificity, and low toxicity of peptides make them particularly well suited as therapeutic agents for various indications, namely allergy, cardiovascular disease, infectious diseases, immunological disorders, gastrointestinal dysfunction and cancer (Lico et al., 2012).

In the broad range of known bioactive peptides, angiotensin I–converting enzyme inhibitory (ACEI) peptides derived from food proteins have attracted particular attention and have been studied the most comprehensively for their ability to prevent hypertension (Norris and FitzGerald, 2013).

So far, several ACEI peptides have been identified in food proteins, mainly in milk, eggs and plants. These peptides are inactive within the sequence of parent proteins, but they can be released by enzymatic proteolysis *in vivo* or *in vitro*, for example during gastrointestinal digestion or during food processing. Thus, the consumption of these foods is a means for delivery and constitutes a potential benefice for human health.

The industrial production of ACEI peptides is based on enzymatic proteolysis of whole food proteins, which leads to the release of small bioactive peptides with ACE inhibitory activity (Pihlanto and Mäkinen, 2013). The problems associated to such procedures, namely cost and loss of functional properties, have demonstrated the need to develop more straightforward methods to produce ACEI peptides. One viable hypothesis is to genetically engineer plant crops to produce and deliver antihypertensive peptides, therefore creating novel functional foods. Some investigation has been focused on the development of expression methods for ACEI peptide production in plant crops (Matoba et al., 2001). Thus far, two main strategies have been adopted, the over-expression of ACEI peptides precursor proteins and the production of particular peptides as heterologous components (Rosales-Mendoza et al., 2013).

The aim of the present work is the production of ACEI peptides in two emergent plant hosts for recombinant pharmaceutical protein production, namely *Lactuca sativa* L. (lettuce) and the model legume *Medicago truncatula* Gaertn. (barrel medic). The plant expression platform that will be used for ACEI peptide production the stable nuclear

expression of whole plants of lettuce and *M. truncatula*. The benefits of whole plant expression platforms, include the possibility of oral delivery and simplicity of scalability. Furthermore, the availability of straightforward transformation procedures for lettuce and *M.truncatula* and the edibility of lettuce, which ensures the simple oral delivery, are also important advantages of the chosen expression platforms. Moreover, the genetic, genomic, and molecular tools available in model plants such as *M. truncatula*, allow not only the investigation of basic processes important to legumes, but also the extrapolation of that information to other important crop species, including bean, pea and alfalfa.

In addition, and to test for the functionality of the expression vectors constructed transient expression in lettuce and *M. truncatula* will be used.

1.2 Molecular pharming

The term “molecular pharming”, blend of pharmaceutical and farming, surfaced in the literature in the 1980s to refer to the production of high-value compounds in transgenic animals. Nowadays, the expression is mainly employed to refer to the production of recombinant pharmaceutically relevant proteins or secondary products in plants (Lossl and Clarke, 2013; Ma et al., 2013; Paul et al., 2013).

The roots of molecular pharming can be traced back to the mid-1980s when plants started to be genetically engineered to act as bioreactors for production of pharmaceutically relevant proteins. Figure 1 summarizes the milestones in the commercial development of molecular pharming.

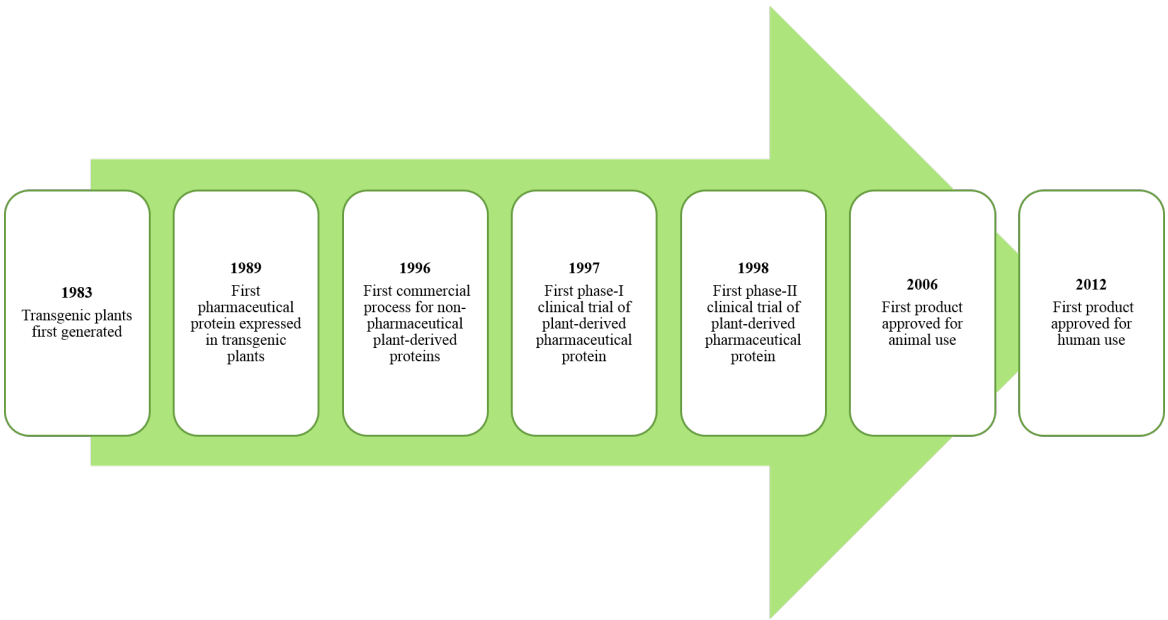


Figure 1 - Milestones in the commercial development of molecular pharming (Sabalza et al., 2014).

Barta et al. (1986) demonstrated that tobacco and sunflower callus tissue were capable of producing transcripts of a human growth hormone fusion gene, even if no protein was detected this was the first report of plants expressing human genes and established plants as a potential recombinant therapeutic protein production system. Later on, the expression of a full-sized IgG in tobacco (Hiatt et al., 1989) was a major breakthrough because it revealed the ability of plants to produce complex functional mammalian proteins with pharmaceutical relevance. In 1990, the structural authenticity of

plant derived recombinant proteins was proved even further with the production of the first native human protein, human serum albumin, in tobacco and potato (Sijmons et al., 1990).

After several studies that demonstrated the capacity of several plant species and systems to produce recombinant pharmaceutical proteins and peptides, during the 1990s the field of molecular pharming gained support and interest from the plant biotechnology community. This scientific attention was followed by commercial interest, with many startup companies being created to materialize the advantages of plants in relation to the established platforms for therapeutic protein and peptide production. Plants provided an inexpensive, highly scalable and safe means of producing pharmaceutically relevant proteins and peptides. In opposition to the fermentation-based traditional platforms that required a massive investment in bioreactors, plants producing pharmaceutical proteins could be established with minimal investment and offered a myriad of different hosts and platforms (Fischer et al., 2012). However, the expectation that plants could compete for the market share of some well-established biopharmaceutical platforms, namely Chinese hamster ovary (CHO) cells, and motivate the mainstream pharmaceutical industry to switch platforms was overinflated. The technical limitations of plants, especially their lower yields compared to mammalian cell lines, allied with the colossal existing investment in fermentation infrastructure, unfavorable public opinion on OGMs and regulatory uncertainty, conduced to caution by the mainstream pharmaceutical industry and consequently to a stagnation in the field of molecular pharming in the 2000s (Stoger et al., 2014). This situation induced a change of paradigm concerning molecular pharming, the initial vision of a highly scalable and low cost production system, while still valid, was replaced by the idea of a production system for certain products that are not easily manufactured by the conventional systems (Fischer et al., 2013).

Conversely to conventional biopharmaceutical production systems that are based on few selected platforms especially the bacterium *Escherichia coli*, yeasts such as *Pichia pastoris*, and well-established mammalian cell lines such as Chinese hamster ovary (CHO) cells (Paul et al., 2013), molecular pharming embraces several technologies with different advantages and limitations, related by their use of plant tissues. The platforms range from plant cells or simple plants growing in fermenters to whole plants growing in soil or hydroponic environments, and the technologies include stable integration of DNA into the nuclear genome or plastid genome and transient expression by infiltrating leaves with

expression vectors based on *Agrobacterium tumefaciens*, plant viruses or hybrids (Paul et al., 2013; Stoger et al., 2014). This great diversity of molecular pharming confers adaptability and flexibility, permitting the selection of the most suitable platform for each product but also conduces to fragmentation. In the early days of molecular pharming, this fragmentation meant that there was no driving force to establish molecular pharming as a single, competitive platform, and consequently no actions were made to match the industry requirements for high yields, standardized procedures and good manufacturing practices (Fischer et al., 2013). As a result, recently, efforts have been made to mimic the mainstream biopharmaceutical industry and focus only on a small number of platforms, especially plant cell cultures, nuclear transgenic plants and transient expression in leafy plants (Paul et al., 2013; Fischer et al., 2015).

Since 2010, the biopharmaceutical industry has given a renewed attention to molecular pharming as a result of its consolidation on a small number of platforms and some target products that meet industry demands (Fischer et al., 2013; Stoger et al., 2014). In 2012, the FDA approval of the first recombinant plant-derived therapeutic for human use, Protalix Biotherapeutics' taliglucerase alfa, commercialized under the name (Elelyso™), was an important breakthrough for molecular pharming. The enzyme taliglucerase alfa is a carrot cell–expressed human recombinant β-glucocerebrosidase and is prescribed for the treatment of Gaucher's disease, a lysosomal storage disorder (Zimran et al., 2011). A recombinant form of glucocerebrosidase under the commercial designation of imiglucerase (Cerezyme®) was already produced in Chinese hamster ovary cells. In this production platform, the enzyme required subsequent *in vitro* exposure to mannose residues in order to have biological activity, resulting in a time-consuming and expensive manufacturing process. Besides, this platform also had some safety problems, namely the risk of viral contamination, allergies and other adverse reactions. In comparison, the plant-based platform is safe, less time-consuming and has reduced production costs, since the desired mannose structure is achieved *in vivo* (Zimran et al., 2011).

Glucocerebrosidase provides a clear example of a target product, which safety, cost and downstream processing issues were solved by switching from a traditional platform to molecular pharming. Following this example of success there has been a continuous increase in clinical trial applications and manufacturing capacity, correlated with the conception of more tangible regulations concerning plant-derived pharmaceuticals.

Even if plants are unlikely to substitute the established platforms given the massive existing investment in fermentation infrastructures and the high product quality, process robustness, and regulatory certainty that have been achieved by decades of incremental improvements (Stoger et al., 2014), the recent promising developments in the field of molecular pharming demonstrate that glucocerebrosidase was not a lone case of success and that plant-based platforms could provide countless opportunities for the biopharmaceutical market. Plants combine the advantage of a full posttranslational modification potential with simple growth requirements and theoretically unlimited scalability in the case of field-grown whole plants (Table 1). Plant-based platforms are versatile, allowing the targeting of recombinant proteins and peptides produced to different organs or subcellular compartments, therefore providing an additional protection against proteolysis. And finally, plants are a safe host for recombinant therapeutic protein and peptide production because they do not harbor human or animal pathogens (Karg and Kallio, 2009).

Hence, instead of facing the red ocean (Kim and Mauborgne, 2005) of established pharmaceutical industries, molecular pharming is now evolving as a disruptive technology that creates its own marketplace by offering rapid drug development and production, unparalleled scalability, unique quality attributes such as tailored glycan structures, individualized therapies, and oral or topical applications of minimally-processed plant tissues thus reducing downstream costs (Sabalza et al., 2014).

Table 1 - Comparison between the various systems used for pharmaceutical protein production (Sabalza et al., 2014)

Platform	Intrinsic yield	Scalability	Overall productivity	Timescale	Cost upstream	Cost downstream	Main safety risks	PTMs
Bacteria	High*	Low	Moderate	Weeks	Low	High*	Endotoxins	None
Mammalian Cells	High	Low	Moderate	Months	High	High	Viruses, prions	Human-Like
Plant cells	Moderate	Low	Moderate	Weeks to months	Moderate	High	Metabolites	Flexible
Transient expression	High	Moderate	High	Days to weeks	Low	High	Metabolites, endotoxins**	Flexible
Transgenic Plants	Moderate	High	High	Months to years	Low	High (low***)	Metabolites (none***)	Flexible

*The intrinsic yield of bacterial cells is high but many complex proteins are produced as inclusion bodies that need to be resolubilized, thus increasing downstream production costs

** Endotoxins are bacterial contaminants; in the transient expression system they may be present if the system is based on infiltration with *Agrobacterium tumefaciens* but not in platforms based on plant viruses

*** The species is important—if tobacco is used, metabolites such as nicotine must be removed, which increases the processing costs, but if cereal seeds or other edible tissues are used these tissues can generally be regarded as safe and processing costs may be reduced or eliminated completely if the product is orally administered as an unprocessed or partly-processed tissue such as flour paste or fruit juice

1.2.1 Plants as platforms for the production of therapeutic proteins

The continuous development of plant genetic engineering technologies has resulted in an expansion of well-established plant-based platforms (Paul and Ma, 2011). Molecular pharming encompasses platforms based on stably transformed plants obtained by transgene insertion in the nuclear or plastid genome, transient expression using agroinfiltration, viral and hybrid vectors, microalgae, aquatic plants (e.g. duckweed), and *in vitro* culture systems (e.g. cell suspensions, hairy roots and moss protonema) (Xu et al., 2012). Each platform has particular advantages and limitations, therefore its selection is done on a case-by-case basis, depending on economic considerations as well as the product characteristics and intended use (Abranches et al., 2005).

1.2.1.1 Platforms based on transgenic plants

Transgenic plants have been the most widely used platforms for recombinant protein production. To obtain stable transgenic lines the gene encoding the desired protein is cloned into an expression construct, which generally includes a promoter, regulatory elements that ensure efficient RNA processing and protein synthesis, and a polyadenylation signal (Commandeur et al., 2003). This expression construct is then stably integrated into the plant nuclear genome, resulting in the stable inheritance of the transgene and expression of stable pharmaceutical proteins over generations (Chen, 2008). Two major transformation strategies have been employed to insert expression constructs into the nuclear genome: *Agrobacterium*-mediated transformation in dicot species and particle bombardment of DNA coated gold or tungsten beads in monocot species (Paul et al., 2013). Transgenic plant lines offer several advantages as platforms for molecular pharming; they are suitable for long-term production of recombinant pharmaceutical proteins, and also highly scalable, as each line can be used to produce seeds, which increase the number of plants in every generation. Ultimately, the production capacity of recombinant pharmaceutical proteins in transgenic plants is practically unlimited, as it only depends on the number of hectares available for the plant culture. The major drawbacks of transgenic plants are the long development and scale-up timescales, the unreliable production yields, and the potential spread of pharmaceutical crops in the environment and into the food chain by outcrossing and seed dispersal (Paul et al., 2013).

The development of simple transformation technologies has expanded the number of host plants available for molecular pharming. Currently, the major molecular pharming transgenic platforms are based on leafy crops, seeds, fruits and vegetables. Leafy crops are benefic in terms of biomass yield and high soluble protein levels. Additionally, leaf harvesting does not need flowering and thus considerably reduces contamination through pollen or seed dispersal (Makhzoum et al., 2014). One disadvantage of leafy crops is that proteins are synthesized in an aqueous environment, which is more prone to protein degradation, resulting in lower production yields (Ma et al., 2003). In fact, the mature leaves possess very large extra cytoplasmic vacuolar compartments containing various active proteolytic enzymes that are involved in the degradation of native and foreign proteins This is particularly problematic in the case of therapeutic peptide production

because short heterologous peptides have an inherent instability in plant cells (Lico et al., 2012). In addition to the protein instability, the harvested material has limited shelf life and needs to be processed immediately after harvest.

Tobacco has been the most widely used leafy crop for molecular pharming. The major advantages of using tobacco to express pharmaceutical proteins are its high biomass yield, well-established technology for gene transfer and expression, year-round growth and harvesting, and the existence of large-scale infrastructure for processing (Makhzoum et al., 2014). However, the natural production of nicotine and other alkaloids in tobacco poses some safety issues in its use as a host system for heterologous protein production. Therefore, tobacco varieties with low nicotine and alkaloid levels have been produced to diminish the toxicity and overcome those safety issues. Other leafy crops used in molecular pharming include alfalfa and clover (Xu et al., 2012).

As an alternative to leafy crops, plant seeds have been proven to be versatile hosts for recombinant proteins of all types, including peptides or short and long polypeptides as well as complex, noncontiguous proteins like antibodies and other immunoglobulins (Boothe et al., 2010). The expression of proteins in seeds can overcome the shortcomings of leafy crops in terms of protein stability and storage. Seeds possess specialized storage compartments, such as protein bodies and vacuoles, which provide the appropriate biochemical environment for protein accumulation, thus protecting the proteins expressed in seeds from proteolytic degradation (Twyman et al., 2003). Reports have demonstrated that antibodies expressed in seeds remain stable for at least three years at room temperature without detectable loss of activity (Stoger et al., 2000). Furthermore, the small size of most seeds permits to achieve a high recombinant protein concentration in a small volume, which facilitates extraction and downstream processing and reduces the costs of the overall manufacturing process (Stoger et al., 2005). One essential property of seeds is its dormancy, which not only permits the stability of recombinant proteins but also allows a complete decoupling of the cycle of cultivation from the processing and purification of the protein (Boothe et al., 2010). Finally, proteins expressed in the seed do not normally interfere with vegetative plant growth, and this strategy also reduces exposure to herbivores and other non-target organisms such as microbes in the biosphere (Commandeur et al., 2003). Several crops have been studied for seed-based production, including cereals such as maize, rice, barley and wheat, legumes such as pea and soybean,

and oilseeds such as safflower and rapeseed. Maize has several advantages for seed-based expression of proteins; it has the highest biomass yield among food crops, ease of transformation and *in vitro* manipulation, and ease of scale-up (Ma et al., 2003). These potentialities were explored by Prodigene Inc. for the production of the first commercially available plant-made protein, avidin. Other maize-derived protein products developed by this company include β-glucuronidase, aprotinin, laccase, and trypsin (Lau and Sun, 2009). Prodigene was the first company to demonstrate the commercial benefits of plant-based platforms and was also a forerunner in the study of the economic impact of downstream processing in molecular pharming, having developed several successful approaches to recover intact and functional recombinant seeds from maize (Paul et al., 2013). However, Prodigene Inc. was forced to cease its activity following a case of contamination of maize crops by their transgenic counterparts. This incident exposed the risk of using cross-pollinating plants such as maize for the production of heterologous proteins. Maize as also been used to produce recombinant pharmaceutical proteins, including enzymes, vaccines and antibodies (Lau and Sun, 2009). One of the most notable therapeutic proteins produced in maize is Meristem Therapeutics' gastric lipase; an enzyme used in the treatment of cystic fibrosis that has completed phase II clinical trial. In addition to this enzyme, Meristem Therapeutics has developed two other maize-derived products, human lactoferrin (phase I clinical trial), whose intellectual property was later acquired by Ventria Bioscience, and collagen (pre-clinical stage).

Rice is another leading platform for recombinant protein production. Similarly to maize, rice is easy to transform and scale-up but unlike maize, rice is self-pollinating, which reduces the risk of horizontal gene flow. Ventria Bioscience, in its ExpressTec platform, has used rice to produce recombinant pharmaceutical proteins, including human albumin, transferrin, lactoferrin and lysozyme, and vaccines against human rabies and Lyme disease. Its lead therapeutic candidate VEN100, whose active ingredient is lactoferrin, has been shown to reduce significantly antibiotic-associated diarrhea in high-risk patients and recently completed phase II clinical trial (Laffan et al., 2011). Rice has also been widely used as host for peptide expression, especially for the production of allergen peptides (Lico et al., 2012). Recent studies report that rice has the potential to offer an oral delivery system for vaccine antigens and therapeutic proteins and peptides (Takagi et al., 2005; Takagi et al., 2010).

Barley seeds have also been developed as commercial platforms. In comparison to other cereal crops, barley is less widely grown. Though, this fact added to the self-pollinating nature of barley can be viewed as an advantage since the risk of contamination and outcrossing with non-transgenic crops is minimized. Considering this benefit, an Iceland based company, ORF Genetics, has targeted barley grain as the expression site for a number of human cytokines and growth factors (Xu et al., 2012). Other molecular pharming companies, such as Ventria Bioscience and Maltagen have also been developing barley-based production platforms. While barley is still recognized for its recalcitrance to transformation, over the last decade some progress has been made in the development of reliable transformation procedures (Mrízová et al., 2014).

The use of legume seeds, such as soybean and pea, to the production of recombinant pharmaceutical proteins has been less explored than cereal-based platforms, with platforms based on legume seeds having yet to achieve commercial success. However, the fact that legume seeds have exceptionally high protein content (20-40%) can be exploited to produce high yields of recombinant protein (Vianna et al., 2011). Soybean seeds have been used to express recombinant growth factors (Ding et al., 2006; Cunha et al., 2011a), coagulation factors (Cunha et al., 2011b) and vaccine peptides (Maruyama et al., 2014). Transgenic pea seeds have been previously used to produce a single-chain Fv fragment (scFV) antibody (Perrin et al., 2000). In another study pea seeds were used to produce a vaccine that showed high immunogenicity and protection against rabbit haemorrhagic disease virus (Mikschofsky et al., 2009).

Safflower and rapeseed seeds are rich in oil and are, thus, referred as oilseeds. Oilseeds can provide useful recombinant pharmaceutical protein production systems. SemBioSys, with its oleosin-fusion platform, has been a pioneer in that field. Oleosins are the principal membrane proteins of oil bodies; oleosins confer peculiar structural properties to the oil bodies that offer simple extraction and purification procedures (Bhatla et al., 2010). In the oleosin-fusion platform, the recombinant protein is fused with oleosin and consequently targeted to the oil bodies. The fusion protein is then recovered through simple purification of the oil bodies and separated from oleosin by endoprotease digestion. The commercial production of hirudin in safflower by SemBioSys constituted the first report of an oilseed-derived protein (Parmenter et al., 1995). The company has been

focusing on safflower as its primary host ever since, with safflower-derived insulin in phase I clinical trial (Lau and Sun, 2009).

Finally, fruit and vegetable crops can also be employed for molecular pharming. A major advantage of protein expression in fruit and vegetable crops is that edible organs can be consumed uncooked, unprocessed or partially-processed, making them particularly suitable for the production of recombinant subunit vaccines, nutraceuticals and antibodies designed for topical application (Twyman et al., 2003). The oral delivery of recombinant therapeutics is one of the differentiating factors of molecular pharming in comparison to mainstream biopharmaceutical production systems, with several pharmaceutical products being produced in tomato fruits, potato tubers and lettuce leaves for this purpose (Paul et al., 2013). Tomato fruits are particularly useful for protein expression because the fruits are palatable as raw tissue but can also be lyophilized and stored for a long time (Lico et al., 2012). Recently, human coagulation Factor IX was expressed specifically in tomato fruits, constituting the first report on the expression of hFIX in plants. Another study described the expression in tomato fruits of a thymosin α1 concatemer (Chen et al., 2009), an immune booster that plays an important role in the maturation, differentiation and function of T-cells. The chymosin α1 concatemer derived from transgenic tomatoes exhibited biological activity and was proven to stimulate the proliferation of mice splenic lymphocytes *in vitro*. Moreover, the specific activity of the tomato-made protein was higher than that produced in *Escherichia coli*, demonstrating the authenticity of the plant-made product. Other examples of tomato fruit expression include F1-V (Alvarez et al., 2006), a candidate subunit vaccine against plague, and β-secretase (Kim et al., 2012) to serve as a vaccine antigen against Alzheimer's disease.

In conclusion, platforms based on transgenic plants are a promising alternative to the conventional biopharmaceutical production platforms because they provide a stable source of pharmaceutical proteins and are also the most scalable of all molecular pharming platforms. This scalability of transgenic plants ensures the production of recombinant pharmaceutical proteins at levels previously inaccessible, namely the commodity bulk production of monoclonal antibodies. In the current scenario of growing pharmaceutical demand, especially in developing countries, the use of transgenic plants can be game changing since they provide a highly scalable and low cost means of producing medicines.

1.2.1.2 Platforms based on transplastomic plants

Transplastomic plants are a valuable alternative to transgenic plants for the production of recombinant pharmaceutical proteins. Transplastomic plants are obtained by the insertion of expression constructs into the plastid genome, this is done by particle bombardment, as the *Agrobacterium* T-DNA complex is targeted to the nucleus and is therefore unsuitable for gene transfer to chloroplasts (Ma et al., 2003). Following the transformation procedure, the bombarded leaf explants are regenerated in each generation and transplastomic plants with homoplastomic transformation (in which every chloroplast carries the transgene) are finally selected, recurring to a selection medium containing spectinomycin or in combination with streptomycin (Obembe et al., 2011).

Plastid transformation can result in high yields of heterologous proteins because multiple copies of the genome are present in each plastid and photosynthetic cells may contain hundreds or thousands of plastids (Daniell et al., 2009). An example of these high yields was the expression of a proteinaceous antibiotic in tobacco chloroplasts that achieved up to 70% of the total soluble proteins, which is the highest recombinant protein accumulation accomplished so far in plants (Oey et al., 2009). Furthermore, chloroplasts provide a natural biocontainment of transgene flow since genes in chloroplast genomes are maternally inherited and consequently not transmitted through pollen, thereby avoiding unwanted escape into the environment. Other advantages of chloroplast engineering include the ability to express several genes as operons and the accumulation of recombinant proteins in the chloroplast, thus reducing toxicity to the host plant (Ma et al., 2003). Finally, transplastomic production platforms offer the possibility of oral delivery. In fact, it has been demonstrated that chloroplast-derived therapeutic proteins, delivered orally via plant cells, are protected from degradation in the stomach, probably because of bioencapsulation of the therapeutic protein by the plant cell wall.

A shortcoming of expressing proteins via the chloroplast genome is that routine plastid engineering is still limited to tobacco, a crop that is not edible and thus unsuitable for oral delivery of therapeutic proteins. In addition, the synthesis of glycoproteins is not possible in the chloroplast system, as plastids do not carry out glycosylation. Nevertheless, the expression of human somatotropin (Staub et al., 2000) in tobacco established that chloroplasts are capable of proper folding of human proteins with disulphide bonds. In

another study the production of native cholera toxin B (Daniell et al., 2001) demonstrated the capacity of chloroplasts to fold and assemble oligomeric proteins correctly. Other therapeutic proteins expressed in tobacco chloroplasts include interferons alpha-2a and alpha-2b (Arlen et al., 2007; Nurjis and Khan, 2011) and anti-cancer therapeutic agents such as human soluble tumor necrosis factor (Wang et al., 2011) and azurin (Roh et al., 2014). Recently chloroplast transformation of lettuce has also been developed (Lelivelt et al., 2005; Kanamoto et al., 2006) to provide oral delivery systems (Ruhlman et al., 2007; Boyhan and Daniell, 2011). With several therapeutic proteins being expressed in lettuce chloroplast, namely proinsulin (Ruhlman et al., 2007; Boyhan and Daniell, 2011), tuberculosis vaccine antigens (Lakshmi et al., 2013), human thioredoxin 1 protein (Lim et al., 2011a). The chloroplast production platform has yet to achieve commercial success, though the referred developments in this field augur a promising future for therapeutic protein production in chloroplasts.

1.2.1.3 Transient expression platforms

Transient expression is a phenomenon that occurs when genes are introduced into plant tissues and are expressed for a short period without stable DNA integration into the genome (Paul et al., 2013). Traditionally, transient expression was used to verify expression construct activity and to test recombinant protein stability. This strategy allowed the identification and elimination of initial transformation problems and thus the prospect of regenerating the desired transgenic lines was significantly improved. Recently, there has been an emergence of transient expression for the commercial production of recombinant pharmaceutical proteins. The advantages of transient expression platforms include the ease of manipulation, speed, low cost and high yield of proteins. In comparison to transgenic plants, transient expression permits to achieve higher recombinant protein yields because there are no position effects (suppression of transgene expression by the surrounding genomic DNA following integration) (Komarova et al., 2010).

Transient expression systems utilize the beneficial properties of plant pathogens to infect plants, spread systemically, and express transgenes at high levels, causing the rapid accumulation of recombinant proteins (Stoger et al., 2014). Currently, the major transient

expression platforms are based on *Agrobacterium tumefaciens*, plant viruses, or hybrid vectors that utilize components of both (MagnICON®technology).

The agroinfiltration method (Kapila et al., 1997) involves the vacuum infiltration of a suspension of recombinant *Agrobacterium tumefaciens* into the plant leaf tissue, the transgenes are then expressed from the uninterrupted T-DNA. Using this method, milligram amounts of recombinant protein are produced within a few weeks without the need to select transgenic plants, a process that takes months to years to be completed. This system has been commercially developed in tobacco (Whaley et al., 2011) and alfalfa (D'Aoust et al., 2010) but is also applicable in other crops such as lettuce (Negrouk et al., 2005), potato (Bhaskar et al., 2009) and *Arabidopsis* (Kim et al., 2009). An advantage of *Agrobacterium*-mediated transient expression is the fact that it allows the production in plants of complex proteins assembled from subunits (Komarova et al., 2010).

Another transient expression technology is based on the use of plant viruses. In this technology, the gene of interest is inserted among viral replicating elements, episomically amplified, and subsequently translated in the plant cell cytosol (Lico et al., 2008). To date, the most efficient and high-yielding platforms have been developed using RNA viruses (Sainsbury and Lomonossoff, 2008). These plant viruses include *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX), and *Cowpea mosaic virus* (CPMV) (Stoger et al., 2014). The advantages of virus based production include the rapid recombinant protein expression, the systemic spread of the virus, and the fact that multimeric proteins such as antibodies can also be produced by co-infecting plants with non-competing vectors derived from different viruses (Verch et al., 1998; Giritch et al., 2006). Transient expression vectors based on virus have been used to express peptides and long polypeptides (at least 140 amino acids long) as fusions to the coat protein, resulting in the assembly of chimeric virus particles (CVPs) displaying multiple copies of the peptide or polypeptide on its surface (Gleba et al., 2007; Lico et al., 2008). Transient expression based in plant viruses has been commercially adopted by the now-defunct Large Scale Biology Corporation (Vacaville, USA) that used a TMV-based vector for the production of patient-specific idiotype vaccines for the treatment of B-cell non-Hodgkin's lymphoma, which had successfully passed the phase I clinical trials (McCormick et al., 2008).

Finally, the third transient expression strategy is based on systems that incorporate components of the T-DNA transfer system and virus replication functions (Paul et al.,

2013). These hybrid systems use deconstructed viruses obtained by removing the coat protein (responsible for systemic movement) of the noncompeting virus strains and *Agrobacterium* as the vehicle for the systemic delivery of the resulting viral vectors to the entire plant. These systems effectively address most of the major shortcomings of earlier plant-based technologies by providing the overall best combination of the following features: high expression level, high relative yield, low up- and downstream costs, very fast and low cost R&D; and low biosafety concerns (Gleba et al., 2005). Consequently, there has been a commercial development based on several hybrid systems. One of most notable examples is the magnICON® system developed by Icon Genetics (formerly owned by Bayer Innovation, Dusseldorf, Germany; now a subsidiary of Nomad Bioscience, Halle, Germany), which features a deconstructed TMV genome and *A. tumefaciens* as a delivery vehicle (Gleba et al., 2005). Another example is the iBioLaunch platform developed by the Fraunhofer Center for Molecular Biotechnology, which also features a deconstructed TMV genome (Paul et al., 2013). Finally, the CPMV-HT platform is based on a deleted version of *Cowpea mosaic virus* RNA-2 and also allows the "hypertranslation" of recombinant proteins without virus spreading (Stoger et al., 2014). Examples of therapeutic recombinant proteins produced in these platforms have been reviewed in Paul et al. (2013)

Recombinant protein production using transient expression is now being mobilized to large scale with several companies developing scalable, automated plant-based GMP biomanufacturing facilities to efficiently produce large amounts of pharmaceuticals within weeks. Such facilities include the ones of Fraunhofer Center for Molecular Biotechnology (Newark, DE) (http://www. fraunhofer.org), Medicago Inc. (Quebec, Canada) (http://www.medicago.com), Icon Genetics (Bayer; Halle, Germany) (http://www. icongenetics.com), Texas A&M (College Station, TX) and Kentucky BioProcessing LLC (Owensboro, KY) (http://www.kbpllc.com/) (Xu et al., 2012).

In conclusion, the ability transient plant expression systems to produce large quantities of recombinant protein, coupled with use of current technology to increase yields and many technical promising solutions seem to favorably compare with mammalian or insect cell-based systems in quality, cost, and scale (Xu et al., 2012). In case of emerging threats, transient platforms are advantageous because they produce large amounts of recombinant protein rapidly (milligram quantities per plant within a few days) and can be scaled up quickly, currently providing the only reliable platform for rapid

response situations (Fischer et al., 2013). During the H1N1 pandemic, Medicago found that the first batches of H1N1 virus- like particles (VLPs) could be produced three weeks after the Centers for Disease Control and Prevention released the new influenza hemagglutinin (HA) sequence (D'Aoust et al., 2010). Similar lead times were reported for the H5N1 VLP vaccine (Landry et al., 2010). Recently, the application of tobacco plant-based transient production systems, at Kentucky BioProcessing (KBP), to produce antibody lots against Ebola was shown to significantly decrease the amount of time required for production over traditional methods, increase the quantity of antibody produced, and reduce the cost of manufacturing. Finally, at the other end of the market scale transient expression platforms are economical for the production of pharmaceuticals for very small markets, such as orphan diseases and individualized therapies.

1.2.1.4 Plant cell suspension cultures

Plant cell suspension cultures grow as individual cells or small aggregates and are usually derived from *callus* tissue by the disaggregation of friable callus pieces in shake bottles, and are later scaled up for bioreactor-based production. Recombinant pharmaceutical protein production is achieved using transgenic explants to derive the cultures, or by transforming the cells after disaggregation, usually by co-cultivation with *Agrobacterium tumefaciens*. The co-cultivation of plant cell suspensions and recombinant *A. tumefaciens* has also been used for the transient expression of proteins (Schillberg et al., 2013). Since these plant cell suspension cultures are grown in sterilized contained environments they provide a cGMP-compatible production environment, which is more acceptable to the established pharmaceutical industry and regulatory authorities (Spok et al., 2008; Paul et al., 2013). These systems have added benefits of complex protein processing compared to bacteria and yeasts, and increase safety compared to mammalian cell systems, which can harbor human pathogens. Another advantage of plant suspension cultures is the very low maintenance cost in comparison to other fermentor-based eukaryotic systems such as mammalian or insect cells. Moreover, the secretetion of the target protein into the culture medium simplifies downstream processing and purification procedures (Pires et al., 2008). Nevertheless, plant cell cultures also have some limitations such as poor growth rates, somaclonal variation (chromosomal rearrangements are an

important source of these variations; common in plant cell cultures generated by *calli*) and gene silencing, together with inhibition of product formation at high cell densities, formation of aggregates and cell wall growth as well as shear-sensitivity for some species (Hellwig et al., 2004). However, recently it has been shown that it is possible to achieve high levels of a functional recombinant protein in plant cell suspension cultures (Pires et al., 2008).

Tobacco has been the most popular source of suspension cells for recombinant protein production, since these proliferate rapidly and are easy to transform but other plant species have also been used to generate suspension cells, including rice and *Arabidopsis thaliana,* alfalfa, soybean, tomato, *M. truncatula* and carrot (Pires et al., 2012; Schillberg et al., 2013). Carrot suspension cells have been used by the aforementioned Protalix' Biotherapeutics to produce a recombinant glucocerebrosidase. This recent case of commercial success shows that suspension cell cultures have a great potential as a viable system for large-scale protein production.

1.2.2 Optimization of plant expression level

The lower expression levels in comparison to the established biopharmaceutical platforms were one of the major obstacles for the commercialization of molecular pharming (Fischer et al., 2013). Therefore, numerous techniques have been developed to enhance protein expression, including codon optimization of protein sequences to match the preferences of the host plant, targeting to subcellular compartments that allow proteins to accumulate in a stable form, the use of strong, tissue-specific promoters, and the testing of different plant species and systems (Lico et al., 2012).

Protein synthesis can be increased by optimizing the components of the expression construct to maximize transcription, mRNA stability and translation, or by diminishing the impact of epigenetic phenomena that inhibit gene expression (Twyman et al., 2013). In this field, the general strategy is to use strong and constitutive promoters, such as the cauliflower mosaic virus 35S RNA promoter (CaMV 35S) and maize ubiquitin-1 promoter (ubi-1), for dicots and monocot, respectively. However, organ- and tissue-specific promoters are also being used to drive expression of the transgenes in the tissue or organ such as the tuber, the seed and the fruit. Additionally, inducible promoters, whose activities

are regulated by either chemical or external stimulus, may equally be used to prevent the lethality problem. Furthermore, transcription factors can also be used as boosters for the promoters to further enhance the expression level of the transgenes (Obembe et al., 2011).

Protein stability can be increased by targeting proteins to cell compartments that reduce degradation. Protein targeting also affects the glycan structures added to proteins and the type of extraction and purification steps required to isolate the protein from the plant matrix. Proteins can be targeted to the secretory pathway by an N-terminal signal peptide, which is cleaved off for the release of the protein into the endoplasmic reticulum (ER). Proteins that do not require post-translational modifications, e.g. glycosylation, for their activity can be targeted to the chloroplast using N-terminal transit peptides (Kmiec et al., 2014). In addition, the target gene can be used to transform chloroplast directly, with highly enhanced protein accumulation. Moreover, posttranslational modifications of the ER lumen can also be avoided by expressing the protein as translational fusion with oleosin protein, which target the expression of the foreign protein to oil bodies of the seeds (Boothe et al., 2010). Other subcellular compartments like the protein-storing vacuoles are also now being explored for accumulating recombinant protein, as it has been observed in rice seed endosperm (Ou et al., 2014).

1.2.3 Posttranslational modifications and glycoengineering

Glycosylation is the covalent linkage of sugar moieties to proteins, in order to enhance their folding, biological activity, solubility and bioavailability (Lienard et al., 2007). In plant, glycosylation occurs in the secretory pathway in the ER and the Golgi apparatus. However, there are differences in the glycosylation patterns between the plants and animals, with respect to N-glycan composition. Plants add α(1,3) fucose and β(1,2) xylose residues to the N-glycan of their glycoproteins, whereas mammals add α(1,6) fucose moieties, glucose and sialic acid residues to the N-glycan (Figure 2). Glycosylation affects the quality of recombinant proteins because different glycan structures can potentially influence glycoprotein stability, subcellular targeting, immunogenicity, pharmacokinetic behavior, and biological activity (Gomord et al., 2010). Two major strategies have been developed to control the glycosylation of recombinant proteins in plants: subcellular targeting to prevent the addition of undesirable sugar residues and

glycoengineering to prevent the addition of plant glycans and even replace them with human-like counterparts (Obembe et al., 2011).

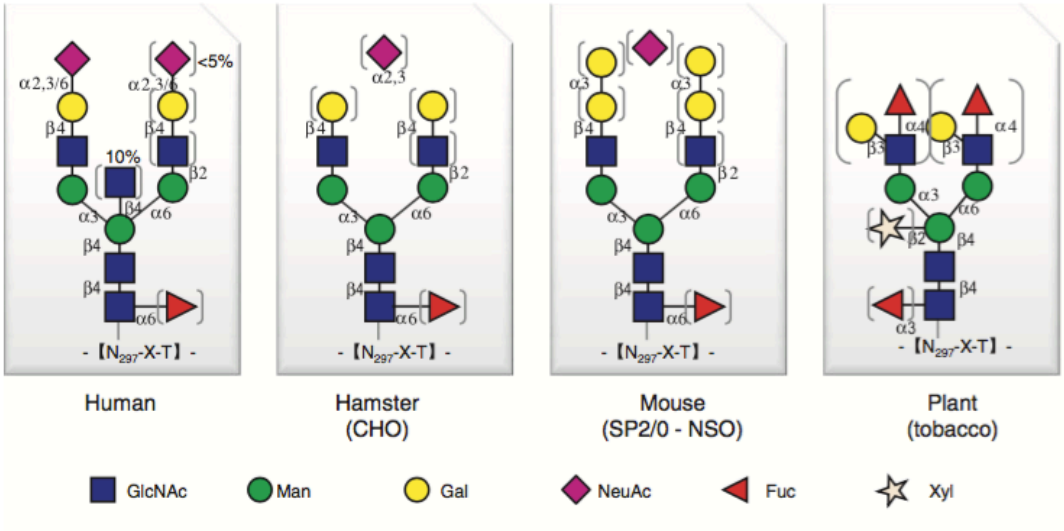


Figure 2 - N-glycans of human native antibodies and recombinant antibodies (Gomord et al., 2010).
A - N-glycans of human native antibodies B - N-glycans of recombinant antibodies produced in hamster CHO cells C - N-glycans of recombinant antibodies produced in murine cells (SP2/0 or NSO cell lines) D - N-glycans of recombinant antibodies produced in tobacco plants.

Targeting is the most straightforward approach because it can be coordinated at construct level by adding specific protein tags. Targeting recombinant proteins to the endoplasmic reticulum using the tetrapeptide tag KDEL and similar derivatives prevents the addition of complex-type glycan structures by avoiding the Golgi apparatus. Another key example of glycan control through targeting is directing proteins to the vacuole, a strategy that Protalix Biotherapeutics uses for the production of taliglucerase alfa (Zimran et al., 2011).

Glycoengineering has been achieved through a variety of approaches, including conventional mutagenesis or homologous recombination to knock out genes encoding unwanted glycosyltransferases, RNA interference to suppress the same enzymes, and transgene expression to introduce heterologous glycosyltransferases that form human-like glycans (Gomord et al., 2010). Engineering the N-glycosylation pathway in different plant species has thus achieved the complete reconstruction of mammalian glycosylation pathways in plants. The versatility of this approach has allowed the development of product-specific designer glycoforms in plants.

Plant glycan structures do not always affect the function of a recombinant protein; for example, the HIV-neutralizing antibody 2G12 produced in plants was just as potent as counterparts produced in mammalian cells, and plant-derived glycoproteins do not appear to induce adverse or allergenic responses in humans even when administered on multiple occasions.

One often disregarded factor is that even native proteins tend to occur as multiple posttranslational variants, and even the industry gold standard, Chinese hamster ovary (CHO) cells, carry out posttranslational modifications that are subtly different from those occurring in humans. Therefore, it should be sufficient for plant-derived pharmaceutical proteins to demonstrate the same or better safety, efficacy, and homogeneity profiles compared with current versions of the same drugs produced in mammalian cells (Stoger et al., 2014).

1.2.4 Downstream processing

In the early years of molecular pharming, scientific studies were focused on demonstrating that plants could produce adequate quantities of recombinant pharmaceutical proteins and confer an orally deliver means. This led to downstream processing and the costs associated to it being basically overlooked. Downstream processing is now known to be an economically critical part of biomanufacturing processes (it can account for up to 80% of the total cost in a therapeutic protein production line) and also to be a key component of the regulatory process for evaluating the safety of pharmaceutical products (Fischer et al., 2012). The goal and the general steps for downstream processing are similar between plant and other expression systems. The goal is to recover the maximal amount of highly purified target protein with the minimal number of steps and at the lowest cost. The basic steps for downstream processes include tissue harvesting, protein extraction, purification, and formulation (Chen, 2008). However, since in molecular pharming the costs of downstream processing are product specific rather than platform specific the evaluation of downstream processing strategies and costs associated to it has to be done on a case-by-case basis. Nevertheless, even if unit operations have to be developed based on the properties of the product, others have to be developed based on the properties of the expression host. Plants produce process-related

contaminants that require specific processing steps to ensure removal of fibers, oils, superabundant plant proteins such as RuBisCO, and potentially toxic metabolites such as the alkaloid nicotine in tobacco (Stoger et al., 2014). These secondary metabolites can be recovered from plant cells or tissues using methods such as adsorption, precipitation and chromatography, often requiring phase portioning and the use of mixtures of organic solvents.

Several approaches have been used to facilitate downstream processing, including secretion of recombinant proteins, eliminating the plant cell disruption step, targeting of proteins into the protein bodies, oil-bodies or plastoglobules, and the use of affinity tags such as poly-histidine tags with the target protein, allowing protein purification by affinity chromatography (Lico et al., 2012). In addition, oral delivery of whole plants or crude extracts containing the pharmaceutical relevant proteins can also be a way to simplify downstream processing and to easily distribute medicines to those in need. Furthermore, the optimization of plant expression level can also ease downstream processing, higher protein concentrations conduct to higher protein volumes (Fischer et al., 2012).

Finally, several purification strategies have been investigated to separate target transgenic protein from host plant proteins, which are tailored for each individual protein based on its solubility, size, pI, charge, hydrophobicity, and affinity to specific ligands and the parallel characteristics of plant host proteins. Chromatographic methods, such as affinity chromatography have been the most extensively used. However, recently increasing attention is being paid to non-chromatographic methods to provide alternatives for large-scale production (Chen, 2008).

1.2.5 *L. sativa* and *M. truncatula* as emerging hosts for pharmaceutical protein production

*Lactuca sativ*a L. (lettuce) is a commercially important crop belonging to the *Asteraceae* family. It is a diploid (2n = 18), autogamous species with a genome size of 2.7 Gb (Truco et al., 2013). This crop is particularly suitable for oral delivery of therapeutics as its raw leaves are consumed by humans, and the time to obtain an edible product is only weeks, compared to the months needed for crops such as tomato or potato. Therefore, recently lettuce has been investigated as a production host for edible recombinant therapeutics (Ruhlman et al., 2007; Boyhan and Daniell, 2011; Martinez-Gonzalez et al.,

2011). Furthermore, the fact that stable transformation procedures for both nuclear (Liu et al., 2012) and plastid genomes (Lelivelt et al., 2005) and transient expression (Negrouk et al., 2005) are widely available is also an advantage. Lettuce has been used as production host for several recombinant therapeutics, virus-like particles (VLPs) and monoclonal antibodies (Lai et al., 2012), antigens (Liu et al., 2012), and human therapeutic proteins (Ruhlman et al., 2007; Lim et al., 2011a).

M. truncatula Gaertn. (barrel medic) is model legume from the *Fabaceae* family. It is a diploid (2n=16), autogamous species with a relatively small genome ($1.8x10^9$ bp for the Jemalong cultivar) and short life cycle of 3-5 months. These characteristics enable this species to be used in molecular genetic studies like analysis of gene expression, promoter functional analysis, T-DNA mutagenesis and expression of foreign genes (Araújo et al., 2004). The phylogenetic distance to economically important crops is crucial in the choice of this plant by many researchers and funding agencies, since it allows comparative genetic studies within the legume family. Furthermore, the potential of *M. truncatula* as expression host has been established for the production of feed additives (Abranches et al., 2005; Pires et al., 2008), human hormones (Pires et al., 2012) and human enzymes (Pires et al., 2014).

1.3 Angiotensin-I converting enzyme inhibitory (ACEI) peptides

Cardiovascular disease (CVD) has been recognized as the leading cause of death in developed countries. Hypertension or high blood pressure is one of the major independent risk factors for CVD (Erdmann et al., 2008), concomitant with cardiovascular disease (CVD) states such as coronary heart disease, peripheral artery disease and stroke. Hypertension is a condition defined by a blood pressure (BP) measurement of 140/90 mmHg or above and is thought to affect up to 30% of the worldwide adult population (Norris and FitzGerald, 2013). The renin-angiotensin system (RAS), Figure 3, plays a crucial role in the control of hypertension. Within the enzymatic cascade of the RAS, Angiotensin I-converting enzyme (ACE) is a key enzyme as it catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II and also inactivates the vasodilator bradykinin (Li et al., 2004).

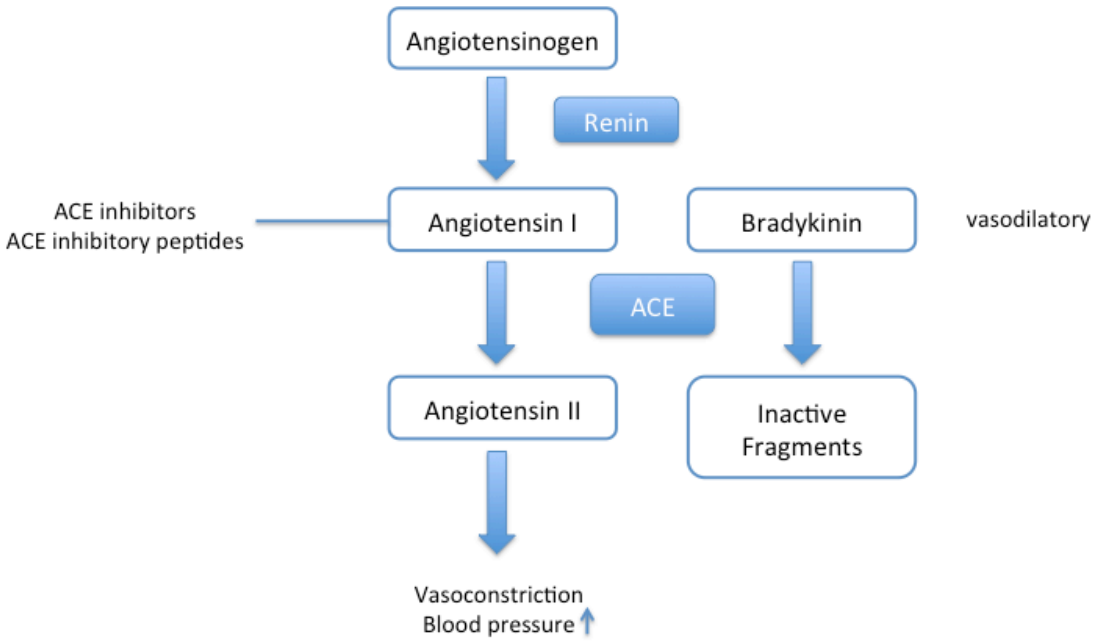


Figure 3 - The renin-angiotensin system (Erdmann et al., 2008).

Several synthetic ACE inhibitors such as captopril, enalaprilat, and lisonopril have been prescribed for treatment of hypertension, congestive heart failure, and diabetic neuropathy (Jimsheena and Gowda, 2010). However, their consumption is associated with various side effects including cough, skin rashes, hypotension, loss of taste, angioedema, reduced renal function and fetal abnormalities (Norris and FitzGerald, 2013). The side effects associated to synthetic ACE inhibitors and the high prevalence of hypertension has led scientists to search for natural and safer therapies for hypertension.

The first exogenous ACE inhibitor was discovered in snake venom (Ondetti et al., 1971), and since then a great number of ACEI peptides have been identified in numerous food proteins, such as milk, eggs, and plants (Table 2 and Table 3) currently constituting the most well known class of bioactive peptides (Iwaniak et al., 2014).

ACEI peptides are generally short chain peptides, i.e., 2-12 amino acids in length, often carrying polar amino acid residues like proline. However, some larger inhibitory sequences have been identified in milk fermented with *E. faecalis* (Quiros et al., 2007), and *L. casei* Shirota (Rojas-Ronquillo et al., 2012), in koumiss (Chen et al., 2010), tuna(Lee et al., 2010), bonito(Hasan et al., 2006) and rotifer (Lee et al., 2009). Studies have also indicated that binding to ACE is strongly influenced by the substrate's C-terminal tripeptide sequence. Hydrophobic amino acid residues with aromatic or branched

side chains at each of the C-terminal tripeptide positions are common features among potent inhibitors. The presence of hydrophobic Pro residues at one or more positions in the C- terminal tripeptide region seems to positively influence a peptide's ACE inhibitory activity. In general, the peptides showing higher activity against ACE have Tyr, Phe, Trp or Pro at their C-terminus (Norris and FitzGerald, 2013). The peptides TQVY from rice (Li et al., 2007), MRW from spinach (Yang et al., 2003), and YKYY from wakame (Suetsuna and Nakano, 2000) are some examples of this principle.

Table 2 - ACEI peptides from plant sources

Source	Sequence	ACE inhibitory activity (IC_{50}; µM)	Antihypertensive activity (mm Hg)	Dose (mg/kg)	Reference
Chlorella vulgaris	VECYGPNRPQF	29.6	Not determined	-	(Sheih et al., 2009)
soybean	YLAGNQ	14	-17.5	2	(Chen et al., 2002)
soybean	VMDKPQG	39	-17.5	2	(Chen et al., 2002)
soybean	NWGPLV	21	Not determined	-	(Kodera and Nio, 2006)
soybean	VLIVP	1.69	Not determined	-	(Gouda et al., 2006)
rice	TQVY	18.2	-40	30	(Li et al., 2007)
spinach	MRWRD	2.1	-13.5	30	(Yang et al., 2003)
spinach	MRW	0.6	-20	20	(Yang et al., 2003)
spinach	IAYKPAG	4.2	-15.0	100	(Yang et al., 2003)
rapeseed	RIY	28	-11.3	7.5	(Marczak et al., 2003)
Spirulina platensis	IAPG	11.40	Not determined	-	(Suetsuna and Chen, 2001)
mung bean	KLPAGTLF	13.4	Not determined	-	(Li et al., 2006)
wakame	YNKL	21	-50	50	(Suetsuna and Nakano, 2000)
wakame	KFYG	90.5	-50	50	(Suetsuna and Nakano, 2000)
wakame	YKYY	64.2	-50	50	(Suetsuna and Nakano, 2000)
wakame	AIYK	213	-50	50	(Suetsuna and Nakano, 2000)
peanut	IETWNPNNQ	72	Not determined	-	(Jimsheena and Gowda, 2011)

Table 3 - ACEI peptides from animal sources

Source	Sequence	ACE inhibitory activity (IC_{50}; µM)	Antihypertensive activity (mm Hg)	Dose (mg/kg)	Reference
sea cucumber	MEGAQEAQGD	15.9	-19	3.12	(Zhao et al., 2009)
fermented milk	LVYPFPGPIPNSLPQNIP P	5.2	-10	6	(Quiros et al., 2007)
milk	LIWKL	0.47	-25.3	10	(Ruiz-Gimenez et al., 2012)
whey	KGYGGVSLPEW	0.70	Not determined	-	(Tavares et al., 2011)
milk	FALPQY	4.3	Not determined	-	(Tauzin et al., 2002)
koumiss	YQDPRLGPTGELDPAT QPIVAVHNPVIV	14.53	Not determined	-	(Chen et al., 2010)
tuna	GDLGKTTTVSNWSPPK YKDTP	11.28	Not determined	-	(Lee et al., 2010)
bonito	TKTGRSAHVLSRYRPRA	2.8	Not determined	-	(Hasan et al., 2006)
bonito	LKPNM	2.4	Not determined	-	(Fujita and Kazunori, 2006)
cuttlefish	VELYP	5.22	-27.6	10	(Balti et al., 2015)
rotifer	DDTGHDFEDTGEAM	9.64	Not determined	-	(Lee et al., 2009)
egg	YAEERYPIL	4.7	-31.6	2	(Miguel et al., 2005)
egg	RADHPFL	6.2	-34	2	(Miguel et al., 2005)
porcine	LGFPTTKTYFPHF	4.92	Not determined	-	(Yu et al., 2006)

ACEI peptides can be classed as inhibitor-type, substrate-type or prodrug-type based on changes in ACE inhibitory activity after hydrolysis of peptides by ACE. Inhibitor-type peptides are ACEI peptides whose activity is not significantly altered as the peptides are resistant to cleavage by ACE. Substrate-type ACE inhibitors show a decrease in ACE activity due to cleavage by ACE. Prodrug type refers to the conversion to potent ACE inhibitors following hydrolysis of larger peptide fragments by ACE itself. The resulting peptides tend to produce long-lasting hypotensive effects *in vivo* (Erdmann et al., 2008). Interestingly, the study of ACEI peptides has revealed that they do not have significant effects on blood pressure in normotensive subjects, suggesting a convenient mechanism that avoids acute hypotensive effects. Based on this finding, it is hypothesized that ACEI peptides could be used in initial treatment of mildly hypertensive individuals or even as supplemental treatments (Rosales-Mendoza et al., 2013).

The activity of ACE inhibitory peptides is generally measured in terms of peptide concentration required to inhibit ACE activity by 50% (IC_{50}). Since *in vitro* ACE inhibitory activity of peptides do not always conduce to antihypertensive activity, *in vivo* activity of these peptides has been generally assessed through tests in spontaneously hypertensive rats (SHRs).

The most common method to produce and identify ACEI peptides is through enzymatic hydrolysis of food proteins with gastrointestinal enzymes such as pepsin and trypsin or with commercial proteases such as Alcalase^{TM} (Pihlanto and Mäkinen, 2013). ACEI peptides have also been produced with *Lactobacillus, Lactococcus lactis* and *Enterococcus faecalis* strains during milk fermentation (Quiros et al., 2007). These methods lead to a complex mixture of compounds, from which the isolation of the peptide of interest is truly difficult. The high cost, the low recovery and the low bioavailability of ACEI peptides that can be produced by *in vitro* enzymatic hydrolysis of their precursor proteins denote the need to develop new and alternative approaches for their production. The application of recombinant DNA technologies for the production of ACEI peptides appears to be a practical alternative to the conventional methods of ACEI peptides production.

1.3.1 Heterologous production of ACEI peptides

The heterologous production of ACEI peptides is a worthwhile approach for the production of these peptides in convenient formulations, at large-scale and low-cost. The implementation of heterologous production systems not only allows the production of ACEI peptides in convenient formulations but also the wide exploitation of beneficial therapeutic effects of these peptides (Rosales-Mendoza et al., 2013).

In recent years, the application of recombinant DNA technologies for the production of ACEI peptides has gathered attention in the biotechnology community. The main approach that has been studied is the generation of multimer proteins containing tandem repeats of ACEI peptides, flanked by protease recognition sequences that allow the peptide release during gastrointestinal digestion. This methodology has been widely applied in *Escherichia coli.*

Park et al. (1998) explored the high-level expression of ACE inhibitor YG-1, which consists of ten amino acids derived from yeast glyceraldehyde-3-phosphate dehydrogenase, with 9, 18, or 27 tandem repeats of the YG-1 gene, a DNA linker encoding Pro-Gly-Arg was inserted between each repeating unit for the cleavage of multimers by clostripain. The multimers have been successfully expressed in *E. coli* at appropriate yields, ranging 15–67 % of total proteins, and active monomers have been rescued following digestion with clostripain. The recombinant YG-1 was identical to the natural YG-1 in molecular mass, amino acid sequence and ACE-inhibiting activity.

An ACEI peptide from a milk casein hydrolysate, KVLPVP, was expressed in *E.coli* as a six tandem dotetracontapeptide linked with the specific cleavage site (Arg-X) of clostripain, and fused to glutathione-S-transferase (GST) tag. The recombinant ACEI peptide fused with glutathione-S-transferase tag reached 24.6% of total intracellular protein. Following digestion with clostripain and carboxypeptidase B, the product was separated with ultrafiltration and reversed-phase HPLC, with an yield of 170 $mg.l^{-1}$. The IC_{50} of the purified recombinant ACEI peptide was 4.6 μM. Remarkably, the oral administration of the recombinant ACEI peptide dramatically decreased the systolic blood pressure of spontaneously hypertensive rats in a dose-dependent manner (Liu et al., 2007).

Rao et al. (2009) described the design and production of an antihypertensive peptide multimer (AHPM), as a precursor of 11 antihypertensive peptides (AHPs) joined by linkers corresponding to gastrointestinal proteases cleavage sites. The recombinant polypeptide AHPM fused with a glutathione S-transferase (GST) tag was expressed in *E. coli* mostly as inclusion body and reached the maximal production, 35% of total intracellular protein. After refolding and purification, simulated gastrointestinal digestion confirmed the release of high active fragments from the AHPM.

The fragmented peptide B (PTHIKWGD), an ACEI peptide retrieved from thermal hydrolysates of tuna meat, has also been produced in *E. coli* in the form of a six tandem repeats. The multimer was produced in the form of inclusion bodies and subsequent acid hydrolysis released single-unit peptides through cleavage of the aspartyl-prolyl bond with yields of 105–115 $mg.l^{-1}$ following reversed phase-HPLC analysis. Interestingly, the *in vitro*-derived biological activity of the recombinant multimer was proven to be indistinguishable to the natural peptide (Fida et al., 2009). Further studies have improved the expression level of the fragmented peptide B in *E. coli* and studied the antihypertensive

activity of this peptide in SHRs. The improved expression level was achieved through the assembly of multimer genes of the peptide and expression as fusion proteins by constructing the vectors with the coding sequences of the single peptide and 2, 4 and 8 tandem repeats. The results showed that the multimer genes were successfully expressed in the constructed systems, and their expression level improved significantly with increasing repeat degree. The peptide monomer was released from the fusion protein with a final yield of 218.9 $mg.l^{-1}$. This peptide resulted in a significant decrease of systolic blood pressure by 36.5 mmHg in SHRs at 4 h (Li et al., 2015).

Furthermore, a DNA fragment encoding an ACEI peptide isolated from sake and sake lees, IYPR, was synthesized and expressed in *E.coli* as seven-copy tandem repeats, linked by the trypsin specific cleavage site. The recombinant protein was produced in the form of inclusion bodies and reached the maximal production of 31% of cellular protein. After purification by affinity chromatography, the recombinant protein was recovered with a high purity of about 90%. The ACEI peptide was released by cleavage of the multimer protein with trypsin and the IC_{50} value was 61 $mg.l^{-1}$. The antihypertensive activity in spontaneously hypertensive rats (SHRs) was also investigated. Single oral administration of this peptide in 10-week old SHRs resulted in a significant reduction of systolic blood pressure to 50 mm Hg at 4 h (Huang et al., 2012).

Recently, DNA-coding sequence for the GVYPHK peptide, an ACEI peptide recovered from a partially purified autolysate of bonito bowels, was linked by a trypsin cleavage site to form a ten-copy tandem and expressed in *E. coli*. Optimized expression was achieved with IPTG induction. The produced recombinant protein was purified by affinity chromatography to greater than 95% purity. The release of single ACEI peptides from the multimer protein was also observed after cleavage with trypsin. The *in vivo* results demonstrated that a single oral administration of the recombinant peptide in SHRs resulted in a significant reduction of systolic blood pressure at 2 h (Wang et al., 2015).

In a contrasting approach, a DNA fragment encoding for a single ACEI peptide FFVAPFPEVFGK (known as CEI_{12}) was expressed as a fusion with dihydrofolate reductase (DHFR) in *E. coli* with IPTG induction. However, the expression level of CEI_{12} (fused with DHFR) was relatively low, 500 $\mu g.l^{-1}$ (Lv et al., 2003). More recently, a DNA fragment encoding CEI_{12} has been expressed in *Streptococcus thermophilus* (Renye and Somkuti, 2008). Both *E. coli* and *S. thermophilus* systems require lysis of the host,

followed by expensive purification techniques, such as HPLC, to isolate the peptide for use as a functional food ingredient. In addition, *E. coli* models for expressing ACEI peptides have relied on the use of fusion proteins to protect against degradation by intracellular peptidases, thus enzymatic treatments are necessary to remove the protein tag and used non-food-grade inducer molecules, including IPTG, to optimize peptide production. A recent study (Renye and Somkuti, 2015), has attempted to solve these shortcomings by employing the nisin-induced expression of CEI_{12} in three lactic acid bacteria (LAB) strains *Streptococcus thermophilus* ST128, *Lactococcus lactis* subsp. *lactis* ML3 and *Lactobacillus casei* C2. A synthetic gene encoding for the CEI_{12} peptide was cloned within the pediocin operon, resulting in an in-frame translational fusion with the pediocin leader peptide, which directs the secretion of pediocin from LAB hosts. The recombinant operon was subsequently cloned immediately downstream of the nisA promoter to allow for inducible gene expression. The secretion of the recombinant peptide was observed both in *L. lactis* ML3 and *L. casei* C2. In this system, the use of a nisin as a ‘‘food-grade’’ inducer molecule, and generally-regarded-as-safe LAB species suggests its applicability for the production of functional food ingredients.

1.3.1.1 Examples of engineered ACEI peptides in plants

Plants are a natural source of bioactive peptides, and ACEI peptides are no exception (Table 2). By allowing the insertion of foreign genes into plants and/or the overexpression of target genes, recombinant DNA technology and plant transformation technologies have the potential to expand the functional properties of food crops and/or edible plants.

In plants, two main approaches have been applied for the production of ACEI peptides. The overexpression of proteins that carry these peptides or production of chimeric configurations containing several repeats of desired sequences to promote beneficial health effects of consuming functional foods derived from these genetically engineered crops. Another approach involves the modification of some storage proteins to produce chimeric proteins carrying ACEI peptides (Rosales-Mendoza et al., 2013).

1.3.1.1.1 Rice

Transgenic rice plants that accumulate novokinin (RPLKPW), a potent anti-hypertensive peptide designed according to the structure of ovokinin (2–7) (RADHPF), as a fusion with the rice storage protein glutelin have been generated. The engineered peptide is expressed under the control of endosperm-specific glutelin promoters and specifically accumulates in seeds. Oral administration of either the RPLKPW-glutelin fraction or transgenic rice seeds to SHRs significantly reduced systolic blood pressures, suggesting the possible application of transgenic rice seed as a nutraceutical delivery system and specifically for administration of antihypertensive peptides (Yang et al., 2006).

Recently, (Wakasa et al., 2011) attempted the generation of transgenic rice seeds that would accumulate higher amounts of novokinin peptide by expressing 10 or 18 tandemly repeated novokinin sequences with the KDEL endoplasmic reticulum-retention signal at the C terminus and using the glutelin promoter along with its signal peptide. Although the chimeric protein was unexpectedly located in the nucleolus and the accumulation was low, a significant antihypertensive activity was detected after a single oral dose to SHRs. More importantly, this effect was observed over a relatively longer durations of 5-week intervals at doses as low as 0.0625 g transgenic seeds per kg.

1.3.1.1.2 Soybean

Soybean [*Glycine max* (L.) Merr.] is an attractive option for the production of ACEI peptides given that soybean seeds contain a large amount of total protein. Therefore, there has been an effort to generate soybean lines with improved ACEI properties foreseeing the creation of novel functional foods.

Matoba et al. (2001), introduced Novokinin (RPLKPW) into homologous sequences of a soybean β-conglycinin α’ subunit by site-directed mutagenesis. The sequence encoding this peptide was introduced into three homologous sites in the gene for soybean β-conglycinin α’ subunit. The native α’ subunit as well as the modified, RPLKPW-containing α’ subunit were expressed in *Escherichia coli*, recovered from the soluble fraction and then purified by ion-exchange chromatography. The RPLKPW peptide was released from recombinant RPLKPW-containing α’ subunit after *in vitro* digestion by trypsin and chymotrypsin. Moreover, the undigested RPLKPW-containing α’ subunit

orally administered at a dose of 10 $mg.kg^{-1}$ exerted an antihypertensive effect in SHRs, unlike the native α' subunit. This report introduced for the first time a physiologically active peptide into a food protein by site-directed mutagenesis and demonstrated that this peptide had *in vivo* functionality even at a low dose. Moreover, and founded on this first prospects from an *E. coli* expressed protein, the muted β-conglycinin α' subunit carrying Novokinin repeats was expressed in soybean. This chimeric protein accumulated at levels of up to 0.2 % of extracted protein from transgenic soybean seeds (Nishizawa et al., 2008). Still, the levels of expression were too low and it was not possible to assess the *in vivo* effects of these soybean seeds.

Novokinin has been also expressed in transgenic soybean seeds in a fusion form along with a β-conglycinin α' subunit. Four novokinin sequences were introduced, and the fusion protein with 4 tandem repeat of novokinin ligated to β-conglycinin α' subunit accounted for 0.5 % of total soluble protein and 5 % of the total β-conglycinin α' subunit in transgenic soybean seeds. Interestingly, a reduced systolic blood pressure was observed in SHRs after administering a dose of 0.15 $g.kg^{-1}$ of protein extracts. A similar effect was attained following administration of a 0.25 $g.kg^{-1}$ dose of defatted flour. Thus, it was concluded that this chimeric protein produced in soybean possessed an anti-hypertensive activity (Yamada et al., 2008).

Additionally, a synthetic gene of His-His-Leu (HHL), an ACEI peptide derived from a Korean soybean paste, was tandemly multimerized to a 40-mer and ligated with ubiquitin as a fusion gene (UH40). The recombinant UH40 protein was expressed in *E. coli*, and purified at yields of 17.3 $mg.l^{-1}$. Following digestion with leucine aminopeptidase, the 405-Da HHL monomer was recovered by reverse-phase high-performance liquid chromatography (HPLC) with an average yield of 6.2 $mg.l^{-1}$. MALDI-TOF mass spectrometry, glutamine-TOF mass spectrometry, N-terminal sequencing, and measurement of ACE inhibiting activity confirmed that the resulting peptide was HHL (Jeong et al., 2007). The expression prospect of this chimeric protein in soybean has yet to be assessed.

1.3.1.1.3 Tomato and tobacco

A modified version of amarantin, the main seed storage protein of *Amaranthus hypochondriacus*, carrying four tandem repeats of the ACEI dipeptide Val-Tyr into the

acidic-subunit of amarantin, was expressed in cell suspension cultures of *Nicotiana tabacum* L. NT1. Protein hydrolysate of transgenic *calli* showed high levels of inhibition of the angiotensin converting enzyme, with an IC_{50} value of 3.5 $\mu g.ml^{-1}$ and 10-fold lower than that of protein extracts of wild-type cells, with an IC_{50} of 29.0 $\mu g.ml^{-1}$ (Santos-Ballardo et al., 2013). This was the first time that a chimeric protein comprising an ACEI peptide was produced in plant cell suspension cultures. This modified version of amarantin, was also expressed in the fruit of transgenic tomato plants. The expressed recombinant protein was stably accumulated at levels up to 12.71 % with respect to total protein content of transgenic fruits. A remarkable change in total protein content (5–22 % increase) of transgenic tomato fruits compared to non-transformed samples was stated. Protein hydrolysates from transgenic tomato fruits showed *in vitro* inhibition of ACE, with IC_{50} values that ranged from 0.376 to 3.241 $\mu g.ml^{-1}$; this represented an increase of up to 13-fold in the inhibitory activity compared with the protein hydrolysates of non-transformed fruits. These two results suggest the possible application of tobacco plant cell suspension cultures and transgenic tomato fruits for massive production of this engineered version of amarantin, which could be especially used as an alternative hypertension therapy (Germán-Báez et al., 2014).

1.3.1.1.4 Amaranth

Although amaranth has not been genetically modified to produce ACEI peptides, the feasibility of developing a modified an amarantin acidic subunit has been widely assessed (Yang et al., 2006; Luna-Suárez et al., 2010; Castro-Martínez et al., 2012; Medina-Godoy et al., 2013; Morales-Camacho et al., 2013; Santos-Ballardo et al., 2013; Germán-Báez et al., 2014).

Recently, the *in vivo* effect of an *E.coli* modified amarantin protein, four units of Val–Tyr dipeptides (VY) in tandem and one of Ile–Pro–Pro tripeptide (IPP) incorporated in the amarantin acidic subunit (AMC3), was evaluated in spontaneously hypertensive rats (SHR) by one time oral administration experiments. This study showed that enzymatic hydrolysates of AMC3 containing ACEI peptides (4xVY and IPP) sequences had

significant antihypertensive action by oral administration in spontaneously hypertensive rats (SHR). (Medina-Godoy et al., 2013)

The positive reports of amarantin expression in *E.coli* (Luna-Suárez et al., 2010; Medina-Godoy et al., 2013; Morales-Camacho et al., 2013) along with the sustained expression of amarantin-modified proteins in tomato (Germán-Báez et al., 2014) and tobacco (Santos-Ballardo et al., 2013) prospect the successful production of ACEI peptide fusion proteins in amaranth.

2 Aims

The principal aim of this work is the production of ACEI peptides in two emergent plant hosts as an alternative therapy for hypertension. In order to achieve this global aim the following specific objectives were defined:

- Selection of ACEI peptides for plant transformation purposes
- Construction of plant transformation vectors with the coding sequence for ACEI peptides
- Establishment of a micropropagation scheme for *in vitro* maintenance of lettuce cv. Great Lakes
- Verify expression construct integrity using transient expression in lettuce
- Stably transform *M. truncatula* and lettuce plants with vectors containing the sequence for the ACEI peptides

3 Methods

3.1 Construction of plant expression vectors containing ACEI peptides synthetic genes

3.1.1 ACEI peptide selection for plant transformation purposes

The selection of ACEI peptides was centered in several criteria, the ACE inhibitory activity, the size of the peptide, resistance/sensibility to gastrointestinal enzymes and confirmed antihypertensive activity *in vivo*. Based on those criteria, four ACEI peptides were chosen for plant transformation purposes (Table 4):

1) The first peptide is a nonadecapeptide released during milk fermentation by *Enterococcus faecalis* (Quiros et al., 2007). The 58-76 fragment of β-casein, which was proven to have a low IC_{50} value (5.2 μM) and also demonstrated antihypertensive activity when orally administered to spontaneously hypertensive rats (SHRs);
2) The second peptide is an undecapeptide isolated from the pepsin hydrolysate of algae protein waste, a mass-produced industrial by-product of an algae essence from microalgae, *Chlorella vulgaris* (Sheih et al., 2009). This peptide exhibited a low IC_{50} value (29.6 μM) and was proven to retain the ACE inhibitory activity after *in vitro* gastrointestinal enzyme digestion. Resistance to pH and temperature was also assessed, with the peptide maintaining its ACE inhibitory activity in the pH range of 2-10 and temperatures of 40-100ºC.
3) The third peptide is a decapeptide isolated from *Acaudina molpadioidea* (sea cucumber) hydrolysate, which inhibitory activity was intensified by 3.5 times from IC_{50} 15.9 to IC_{50} 4.5 μM after *in vitro* incubation with gastrointestinal enzymes. This peptide was also proven to have antihypertensive activity in SHRs (Zhao et al., 2009).
4) Lastly, the forth peptide is a tripeptide (MRW) isolated from the pepsin-pancreatin digest of the large subunit of spinach RuBisCO (Yang et al., 2003).

This peptide has been shown to have a low IC_{50} value (0.6 μM) and its antihypertensive activity in SHRs has also been confirmed.

Table 4 - ACEI peptides chosen for plant transformation

Source	Sequence	ACE inhibitory activity (IC_{50}; μM)	Antihypertensive activity (mm Hg)	Dose (mg/kg)	Reference
fermented milk	LVYPFPGPIPNSLPQNIPP	5.2	-10	6	(Quiros et al., 2007)
sea cucumber	MEGAQEAQGD	15.9	-19	3.12	(Zhao et al., 2009)
spinach	MRW	0.6	-20	20	(Yang et al., 2003)
Chlorella vulgaris	VECYGPNRPQF	29.6	Not determined	-	(Sheih et al., 2009)

3.1.2 Design of the ACEI peptides synthetic genes and plant expression vectors construction

Five synthetic genes with optimized codon usage for lettuce and *M. truncatula* and designated ACEI_FMK, ACEI_SEA, ACEI_SPI, ACEI_CHL and ACEI_CHLTP were designed based on the ACEI peptides abovementioned. All genes contain 5' and 3' flanking regions consisting, respectively, of an NdeI restriction site and an incomplete SalI restriction site to facilitate cloning and restriction analysis. The ACEI peptides coding sequences of ACEI_FMK and ACEI_CHL and its 5' flanking regions were joined by a linker of two amino acids corresponding to a cleavage site of trypsin. The gene ACEI_SPI has an eight-tandem repeat of the coding sequence of peptide MRW. The prospect of a tandem construction was evaluated using the peptidecutter tool of ExPASy (http://www.expasy.org/) for peptide cleavage sites prediction. The gene ACEI_CHLTP contains a coding sequence of a 57 amino acid N-terminus transit peptide from the tobacco small subunit RuBisCo for plant chloroplast targeting (Menassa et al., 2004). A 6× His tag® was added to the C-terminus of all synthetic genes for purification and detection of the recombinant peptides. Four synthetic genes (ACEI_FMK, ACEI_SEA, ACEI_SPI and ACEI_CHLTP) were synthesized by NZYTech (Lisboa, Portugal). The synthetic genes

ACEI_FMK and ACEI_CHLTP were cloned by NZYTech in pUC57 by EcoRV and the synthetic genes ACEI_SEA and ACEI_SPI were cloned in pUC57 by EcoRI/HindIII.

The purified PCR-amplified ACEI peptides synthetic genes were cloned into the pRI 201-AN (TAKARA BIO, Japan) (Figure 4) using the *In-Fusion HD Cloning Kit* according to the manufacturer's instructions.

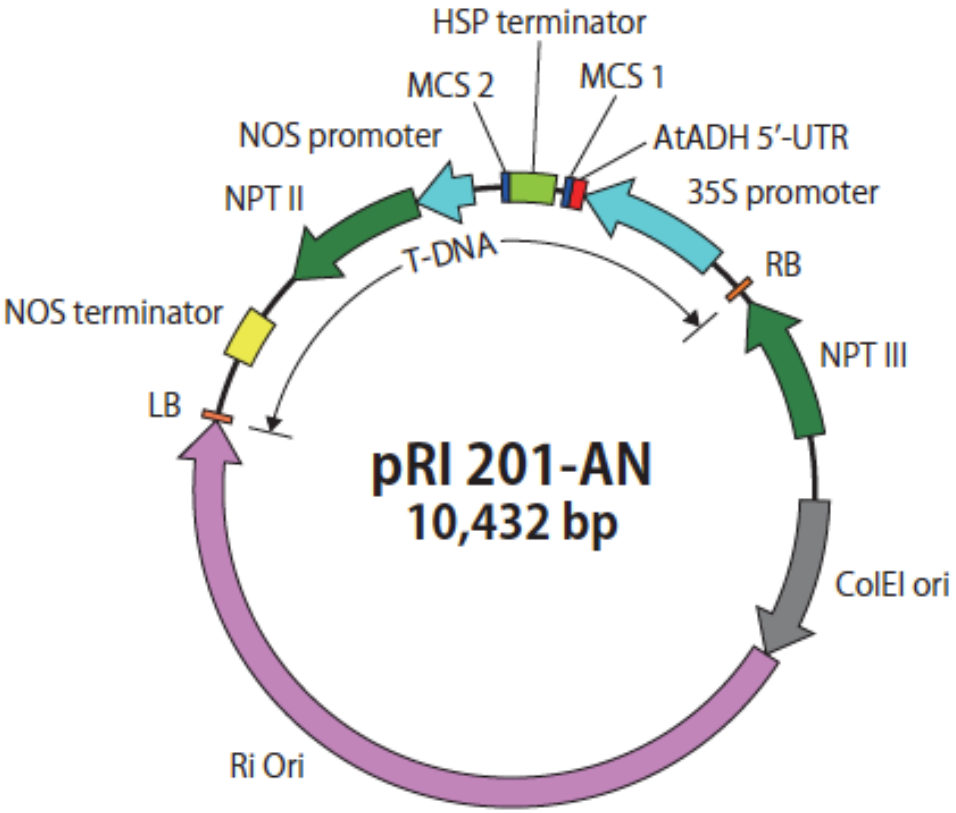


Figure 4 - pRI-201 AN vector map and cloning sites (www.takara-bio.com).

The plasmid pRI 201-AN DNA (TAKARA BIO, Japan) was designed for expressing target genes in transformed plant cells. This plasmid includes an alcohol dehydrogenase (ADH) gene-derived 5' untranslated region (5' -UTR) (translational enhancer region) downstream of the 35S promoter from cauliflower mosaic virus (CaMV) and a NOS terminator. pRI 201-AN DNA is a binary vector for plant transformation and has a mutant-type replication origin (Ri ori) from the *Agrobacterium rhizogenes* Ri plasmid that also allows the replication in *A. tumefaciens*. This vector has both a replication origin (ColE1 ori) derived from pUC plasmids, which allows maintenance at a high-copy-number in *E. coli*, and a multicloning site located near the right border (RB) of

T-DNA relative to the plant selection marker (NPT II), which allows stable integration of the target gene into a plant chromosome.

3.1.2.1 Preparation of DNA fragments for cloning

- Preparation of linearized vector by restriction digestion

For the construction of the plant expression vectors pRI 201- AN was digested with restriction enzymes SalI and NdeI to generate a linearized vector. A double digest was done overnight at 37ºC with 1 U of each restriction enzyme and 1000 ng of vector DNA. The linearized vector was isolated by electrophoresis on an agarose gel and purified from the agarose using the *Zymoclean™ Gel DNA Recovery Kit* (Zymo Research, USA) according to manufacturer's instructions. To test for complete digestion one aliquot (1-2µl) of the digested plasmid DNA was subjected to an agarose gel electrophoresis.

- PCR amplification of ACEI peptides synthetic genes

The ACEI peptides synthetic genes were PCR-amplified from the pUC57 vectors using the CloneAmp HiFi PCR Premix (Clontech Laboratories Inc., USA). The ACEI peptides synthetic genes were PCR-amplified from the pUC57 vectors using specific primers (Table 5). The primers were designed in order to generate PCR products containing 15 bp sequences at 5'- and 3'-ends that overlap with the 3'- and 5'-ends of the linearized vector pRI 201-AN DNA. The 5' primers were specific for each synthetic gene. Since the C-terminus was the same for all genes, the 3' primer was universal (Table 5).

Table 5 - Primers used to amplify the ACEI peptides synthetic genes from pUC57 vectors

Primer Label	Sequence
ACEI_FMK PRIMER	CACTGTTGATACATATGAAATTGGTTTATC
ACEI_SEA PRIMER	CACTGTTGATACATATGGAAGGTGCTC
ACEI_SPI PRIMER	CACTGTTGATACATATGAGATGGATGAG
ACEI_CHLTP PRIMER	CACTGTTGATACATATGGCTTCTTCTGT
UNIV PRIMER	ATTCAGAATTGTCGATCAATGATGATG

PCR conditions were 95ºC/ 2 min for denaturation, followed by 35 cycles of 98ºC/ 10s for denaturation, 55ºC/ 15 s for annealing and 72ºC/ 5 s for extension and 72ºC 5 min for final extension. The target fragments were isolated by agarose gel electrophoresis and purified from the agarose using the *Zymoclean™ Gel DNA Recovery Kit* (Zymo Research, Irvine, USA) according to manufacturer's instructions.

3.1.2.2 In-Fusion® Cloning methodology

The In-Fusion® HD cloning system (scheme represented in Figure 5) allows fast and directional cloning of one or more fragments of DNA into any vector. The foundation of the In-Fusion® HD cloning system is the In-Fusion HD Enzyme, which blends DNA fragments e.g. PCR-generated sequences of a target gene and linearized vectors, efficiently and precisely by recognizing a 15 bp overlap at their ends. In-Fusion® designed primers must contain 15 bases that are homologous to 15 bases at one end of the linearized vector at the 5′ end and a sequence that is specific to the target gene at the 3'-end.

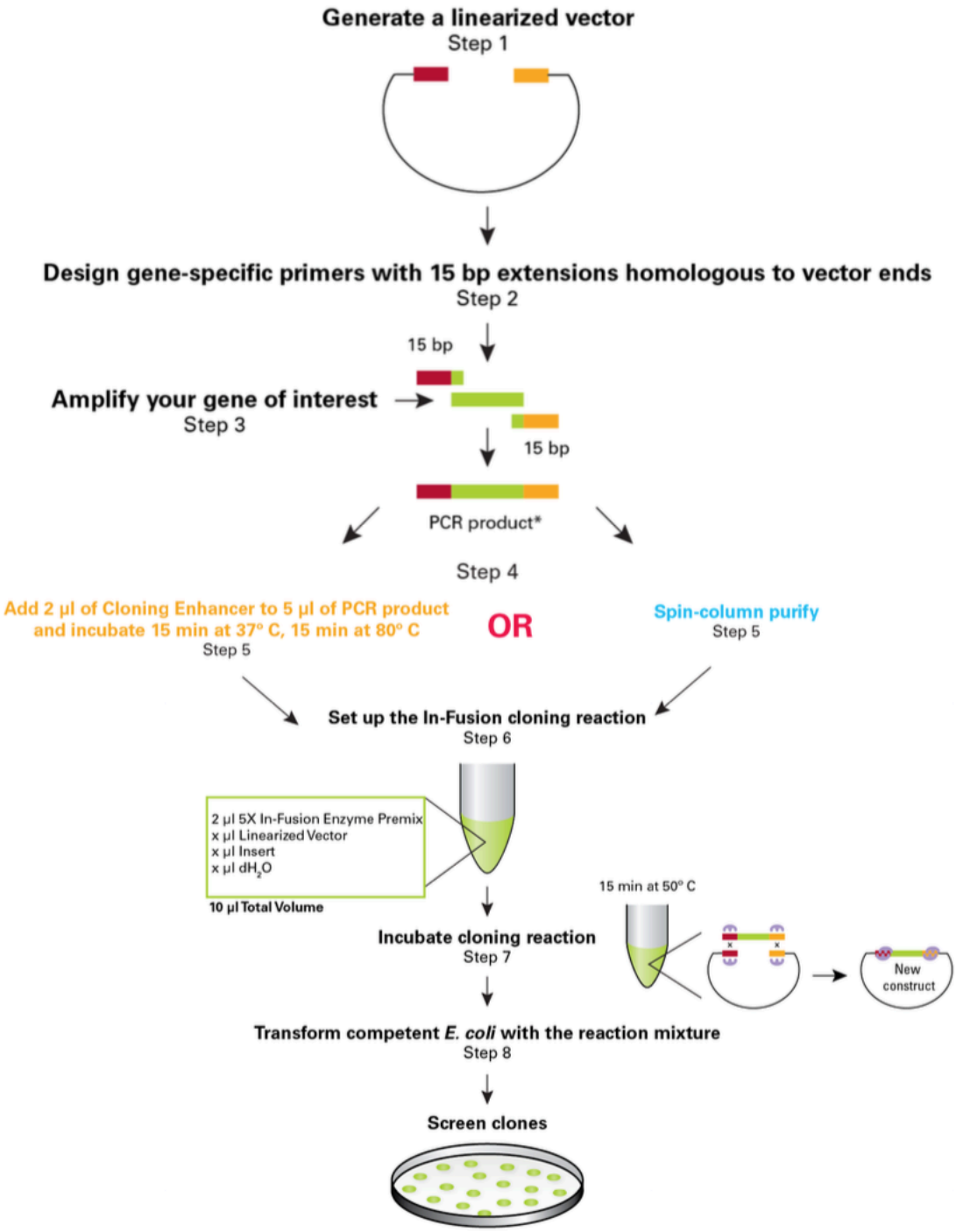


Figure 5 - Schematic representation of the In-Fusion® HD Cloning System (www.clontech.com).

In this work, the vector pRI-201 AN was linearized by double digestion with the restriction enzymes NdeI and SalI. The ACEI peptide synthetic genes were amplified using primers with the following features: the 5' -primer had 15 bases immediately upstream of

the NdeI site on the vector at its 5' -end, and the 3' -primer had 15 bases immediately downstream of the Sal I site on the vector at its 5'– end.

Once the linearized vector and the purified target synthetic genes with 15 bp extensions complementary to the vector ends were obtained, we proceeded to the set up of the In-Fusion® Cloning reaction. Positive and negative control reactions were performed in parallel with the In-Fusion cloning reactions of the target synthetic genes to verify that the system was working properly. The reactions conditions are summarized in Table 6. All cloning reactions were incubated at 50ºC for 15 min. Finally, DH5α™ *E.coli* competent cells were transformed with the reaction mixture.

Table 6 - Recommended In-Fusion® Reactions for Purified Fragments

Reaction Component	**Cloning Reaction**	**Negative Control Reaction**	**Positive Control Reaction**
Purified PCR fragment	100-200 ng*	-	2 µl of 2 kb control insert
Linearized vector	50-200 ng**	1 µl	1 µl of pUC19 control vector
5X In-Fusion HD Enzyme Premix	2 µl	2 µl	2 µl
Deionized Water	to 10 µl	to 10 µl	to 10 µl
*<0.5 kb: 10-50ng,0.5 to 10 kb: 50-100 ng, >10 kb: 50-200 ng **<10 kb: 50-100 ng, >10 kb: 50-200 ng			

3.1.3 Visualization, detection and quantification of Nucleic Acids

Agarose gels were comprised of 0.8-2.0 % agarose (Merck, USA), 1X TBE (0.45M Tris, 10mM EDTA, 0.45M Boric Acid) and 100X SYBR Safe® (Invitrogen, USA). PCR products were also visualized by 12% polyacrylamide gel electrophoresis.

All images from nucleic acid gels were taken using the system/software Gel Doc (BioRad, USA).

When necessary, e.g. after extraction or purification, quantification of Nucleic Acids was performed using the Nanodrop 2000 C spectrophotometer (Thermo Fisher Scientific, USA).

3.1.4 *E. coli* strains and culture media

E. coli DH5α™ competent cells were the host for the plasmids used in the In-Fusion® HD cloning steps. DH5α™ competent cells harboring the constructed plasmids were grown in *Luria Broth* rich medium (*LB*: 10 $g.l^{-1}$ tryptone, $5g.l^{-1}$ NaCl and 5 $g.l^{-1}$ yeast extract) or *LB* solidified with 15 $g.l^{-1}$ microagar (*LA*) (Duchefa, The Netherlands). Kanamycin (50 $mg.l^{-1}$) was added to the media for selection.

3.1.5 Transformation of *E.coli* DH5α™ with plasmid DNA

3.1.5.1 Preparation of DH5α™Competent Cells

Preparation of DH5α™ competent cells was done using the $CaCl_2$ method described in Sambrook et al. (1989) with some modifications (Duque 2010). Briefly, a single colony of DH5α™ from a fresh LA plate was inoculated in 5 ml of LB medium and grown overnight at 37ºC under vigorous shaking (200 rpm). One ml of this culture was inoculated in 100 ml of LB medium and incubated at 37ºC under vigorous shaking until reaching an OD_{600nm} of 0.6 (usually for 3-4 hours). Tubes were then centrifuged for 10 minutes at 5ºC. Supernatant was discarded and the pellet was resuspended in 20 ml of ice-cold 100mM $CaCl_2$. Cell suspensions were maintained on ice for 1 hour and then, cells were centrifuged again. The pellet was resuspended in 2.5 ml of ice-cold 100mM $CaCl_2$ and 0.5 ml of sterile 80% Glycerol was added to each tube. Glycerol stocks of competent cells were flash-frozen in liquid nitrogen and stored at –80ºC, in 200 µl aliquots.

3.1.5.2 Transformation of DH5α™ Competent Cells

Aliquots of 5 µl of In-Fusion reaction mixtures were added to 200 µl of competent cells according to Duque (2010). These bacterial suspensions were incubated on ice for 1h, minimum, to allow the adsorption of plasmids to bacterial cell wall. Subsequently, cells were subjected to 42ºC for 90 sec (heat shock), followed by the addition of 800 µl of SOC medium (5 $g.l^{-1}$ Yeast Extract, 20 $g.l^{-1}$ Tryptone, 0.584 $g.l^{-1}$ NaCl, 0.186 $g.l^{-1}$ KCl, 0.952 $g.l^{-1}$ $MgCl_2$, 1.2 $g.l^{-1}$ $MgSO_4$ and 20 mM Glucose) and incubation for 1h at 37ºC, with

gentle shaking (150 rpm). Cells were pelleted by centrifugation (8000 rpm, 1 min) and 800 µl of supernatant was removed. Cells were resuspended in the remaining medium (200 µl) and spread onto plates containing LA with the appropriate selection antibiotic. Plates were incubated overnight at 37ºC.

3.1.6 Selection of recombinant plasmids

3.1.6.1 PCR Amplification

Colony PCR was performed to attest the presence of the genes of interest in the transformants. Colony replicates were prepared by streak plating in LA plates and colonies were pricked with the help of a sterile toothpick. The PCR screening of transformants containing the constructs pRI-201-ACEI_FMK, pRI-201-ACEI_SEA and pRI-201-ACEI_CHLTP was performed using GoTaq® G2 Flexi DNA Polymerase (Promega, USA) and the synthetic gene specific primers previously enunciated. PCR conditions were 95ºC/ 2 min for denaturation, followed by 35 cycles of 94ºC/ 10s for denaturation, 68ºC/ 1 min for annealing and 72ºC/ 20 s for extension and 72ºC 5 min for final extension. Additionally, PCR screening of transformants containing the construct pRI-201-ACEI_SPI was performed using the CloneAmp HiFi PCR Premix (Clontech Laboratories Inc., USA) and the synthetic gene specific primers previously enunciated. PCR conditions were 95ºC/ 2 min for denaturation, followed by 35 cycles of 98ºC/ 10s for denaturation, 68ºC/ 15 s for annealing and 72ºC/ 5 s for extension and 72ºC 5 min for final extension. PCR products were visualized by 2 % agarose gel electrophoresis and 12% polyacrylamide gel electrophoresis with an Acrylamide/Bisacrylamide (VWR, USA) ratio of 29:1.

3.1.7 Extraction of Plasmid DNA

Bacteria harboring the desired plasmids were inoculated in *LA* (with appropriate antibiotic supplementation) and grown over-night at 37ºC in order to obtain isolated colonies. Single colonies were isolated and inoculated in 2 ml of *LB* with the same antibiotic supplementation in 15 ml tubes. Cultures were grown overnight at 37ºC under shaking (220rpm). The bacterial suspensions were centrifuged at 3500 rpm for 5 min at room temperature and supernatant discarded.

The *QIAprep ® Spin Miniprep Kit* (QIAGEN, Germany) was used for small-scale extraction of plasmid DNA (*miniprep*).

3.2 *Agrobacterium*-mediated transformation of lettuce and *M. truncatula* with plant expression vectors containing ACEI peptides synthetic genes

3.2.1 Plant material and culture media

Lettuce cv. Great Lakes and M9-10a genotype of *M. truncatula* cv. Jemalong were used in this study. The M9-10a genotype was selected due to its high embryogenic potential (Araújo et al., 2004).

Lettuce seeds were surface-sterilized with 50% (v/v) Domestos (commercial bleach with detergent) for 5-10 min, and washed four times in distilled water. The sterilized seeds were germinated on petri dishes containing MS010A - MS (Murashige and Skoog, 1962) basal salts and vitamins, 1% (w/v) sucrose, 0.8% (w/v) agar (Micro-agar, Duchefa, The Netherlands) under light conditions at 24ºC/22ºC (Curtis, 2006). Lettuce and M9-10a plants were maintained in *in vitro* culture conditions and micropropagated in growth-regulator-free medium: MS030A – MS basal salts and vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar (Micro-agar, Duchefa, The Netherlands). To maintain and propagate the plants, 1,5-2 cm explants (with two axillary buds) were subcultured to fresh MS030A every month.

Lettuce shoot organogenesis was induced from *calli*. *Calli* cultures were initiated from 1,5-2 cm explants (with two axillary buds) and leaves of micropropagated lettuce using two different concentrations of growth regulators. MS basal salts and vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar was supplemented with 0.5 mg l^{-1} of benzyl adenine (BA) and 0.1 mg l^{-1} naphthalene acetic acid (NAA) and with 0.5 mg l^{-1} BA and 0.1 mg l^{-1} indole butyric acid (IBA). Shoots regenerated from *calli* were excised when they were 2-3 cm put onto growth-regulator-free medium, MS030A, for rooting. Thereafter, maintenance and multiplication of shoots was achieved in the absence of growth regulators.

Indirect somatic embryogenesis of M9-10 a plants was induced in an embryo induction medium: EIM – MS basal salts and vitamins, 3% (w/v) sucrose, 0.1mg l^{-1} of 2,4-

dichlorophenoxyacetic acid (2,4-D), 0.2 mg l^{-1} of zeatin, 0.2% (w/v) gelrite (Duchefa, The Netherlands) (Araújo et al., 2004). After 21 days in EIM, embryogenic *calli* were transferred to an embryo proliferation medium: EPM – MS basal salts and vitamins, 3% (w/v) sucrose, 0.2% (w/v) gelrite. Embryos ready to be isolated were transferred to an embryo conversion medium (ECM= MS030A). The pH of all media was adjusted to 5.8 before autoclaving (20 min, 121ºC). Growth regulators were filter-sterilized through 0.22-µm filters (Gelman Sciences, Michigan, USA) and added before dispensing the medium in plastic Petri dishes (100 mm ø). All cultures were kept in a growth chamber (PHYTOTRON EDPA 700, ARALAB) with 16-h photoperiod of 100 µmol.m^{-2}.s^{-1} applied as cool white fluorescent light and a day/night temperature of 24 ºC/22 ºC.

3.2.2 *Agrobacterium* strains and culture media

A. tumefaciens disarmed succinamopine strain EHA105 (Hood et al., 1993) was used in all plant transformation experiments. *Agrobacterium* colonies were grown in *LB* solidified with 15 g.l^{-1} microagar (*LA*) (Duchefa, The Netherlands) and liquid cultures were prepared in *Yeast Extract Broth* (YEB) medium (5 g.l^{-1} tryptone, 1 g.l^{-1} yeast extract, 5 g.l^{-1} nutrient broth, 5 g.l^{-1} sucrose, 0.49 g.l^{-1} $MgSO_4 \cdot 7H_2O$, pH 7.2); with the addition of rifampicin (50 mg.l^{-1}).

3.2.3 Transformation of *A. tumefaciens* (strain EHA105)

3.2.3.1 Preparation of EHA105 competent cells

Preparation of freeze/thaw competent EHA105 cells was done based on Wise et al. (2006). Briefly, a single colony of EHA105 from a fresh LA plate was inoculated in 2 ml of YEB medium supplemented with 50 mg.l^{-1} rifampicin and grown overnight at 25ºC under vigorous shaking (200 rpm).

The 2 ml culture was inoculated in 50 ml of fresh YEB antibiotic supplemented medium in a 250 ml flask and left under shaking until cells reached an $O.D_{600nm}$ between 0.5 and 1.0. Cells were pelleted by centrifuging at 4ºC for 8 to 10 min at 10 000 g and the supernatant discarded. The pellet was resuspended in 5 ml of cold 20 mM $CaCl_2$ and the cells were centrifuged again. The supernatant was discarded and cells resupended in 1ml

cold 20 mM $CaCl_2$. Aliquots of 100-150 µl of the cell solution were distributed in chilled 2.0 ml tubes, froze in liquid nitrogen and stored at –80ºC for future use.

3.2.3.2 Transformation of EHA105 competent cells

The plasmids containing the ACEI peptides synthetic genes were mobilized to competent cells of *Agrobacterium* by the freeze-thaw method according to Wise et al. (2006). Briefly, 1 µg of plasmid DNA was added to 100-150 µl of frozen competent cells. The cell/DNA mix was frozen in liquid nitrogen for about 5 min. After, the frozen cell/DNA mixture was thawed for 5-10 min at room temperature and the cells transferred to a culture tube containing 2 ml of YEB medium. *Agrobacterium* cells were incubated at 25 ºC for 2 to 4 h under vigorous shaking (200 rpm). The cells were pelleted by centrifuging at high speed for 2 min and resuspended in 100 µl of YEB medium supplemented with 50 $mg.l^{-1}$ rifampicin and 50 $mg.l^{-1}$ kanamycin. This cell suspension was plated on solid YEB plates with 15 $g.l^{-1}$ agar containing 50 $mg.l^{-1}$ rifampicin and 50 $mg.l^{-1}$ kanamycin for selection of transformants.

3.2.3.3 Screening of EHA105 transformants

Colony PCR was used for screening of transformed EHA105 colonies. Confirmation of the presence of the plasmid constructs was performed by PCR amplification of the target synthetic genes as described in section 3.1.6.1.

3.2.3.4 Preparation of *A. tumefaciens* for transformation

For transient expression of lettuce and M9-10a, *A. tumefaciens* EHA105 was prepared as described in Negrouk et al. (2005) with some modifications. A single colony of *A. tumefaciens* strain EHA105 harboring the appropriate plasmid was inoculated in 2 ml of YEB medium supplemented with 50 $mg.l^{-1}$ rifampicin and 50 $mg.l^{-1}$ kanamycin and grown overnight at 25ºC. For final scale-up, initial overnight cultures were diluted 1:50 in the same YEB medium supplemented with antibiotics and 20 µM of acetosyringone and allowed to grow 18–24 h to an OD_{600} nm of about 2.4. The cell cultures were centrifuged

10 min at 6000 rpm, resuspended to an OD_{600nm} of 0.6 in liquid MS medium with 30 $g.l^{-1}$ sucrose, 100 µM acetosyringone and 100 $µg.ml^{-1}$ 2,4-D and incubated for 1 h at 22ºC to activate the *Agrobacterium* virulence mechanisms.

For stable transformation of lettuce and M9-10a plants, *A. tumefaciens* EHA105 was prepared as described for transient expression with modifications in the final OD_{600nm} and compositions of induction media. The cell cultures were resuspended to an OD_{600nm} of 1.5-1.6 in liquid MS medium with 30 $g.l^{-1}$ sucrose, 100 µM acetosyringone and 0.5 $µg.ml^{-1}$ BA and 0.1 $µg.ml^{-1}$ NAA for lettuce stable transformation and in liquid EIM supplemented with 100 µM acetosyringone for M9-10a stable transformation.

3.2.4 Transient expression studies

Transient expression studies were carried out in lettuce. *A. tumefaciens* EHA105 harboring the plasmid construct pMP2482 (Quaedvlieg et al., 1998) was used to rapidly assess the efficiency of the transient expression protocol in lettuce. The plasmid pMP2482 contains fused coding sequences for GFP and GUS under the control of the same promoter and the detection of the resulting GUS/GFP protein product is strictly related with the gene expression owing to the presence of an intron. The production of the GUS protein product was readily detected with the histochemical GUS staining protocol.

Transient expression studies were also executed with *A. tumefaciens* EHA105 harboring the plasmid constructs pRI-ACEI_FMK, pRI-ACEI_SEA, pRI-ACEI_SPI and pRI-ACEI_CHLTP. Further, a Reverse transcription-PCR assay was done to verify gene integrity within plasmid constructs for plant transformation.

Leaves from 1-month-old lettuce (Figure 6 A) and M9-10a plants grown on MS medium were cut with a scalpel blade (Figure 6 B and C), placed in the *Agrobacterium tumefaciens* strain EHA105 suspensions harboring the pMP2482 and the pRI-ACEI constructs (Figure 6 D) and vacuum infiltrated for 15 min at 0.6 atm pressure (Figure 6 E). The *Agrobacterium* infected leaves were put onto sterile filter paper soaked with MS medium supplemented with 100 µM acetosyringone (Figure 6 F) for a co-cultivation period of 3 days at 24 ºC in the dark (Figure 6 G). As negative control, leaves of lettuce and M9-10a plants were also subjected to the same treatment, without recombinant *A. tumefaciens*.

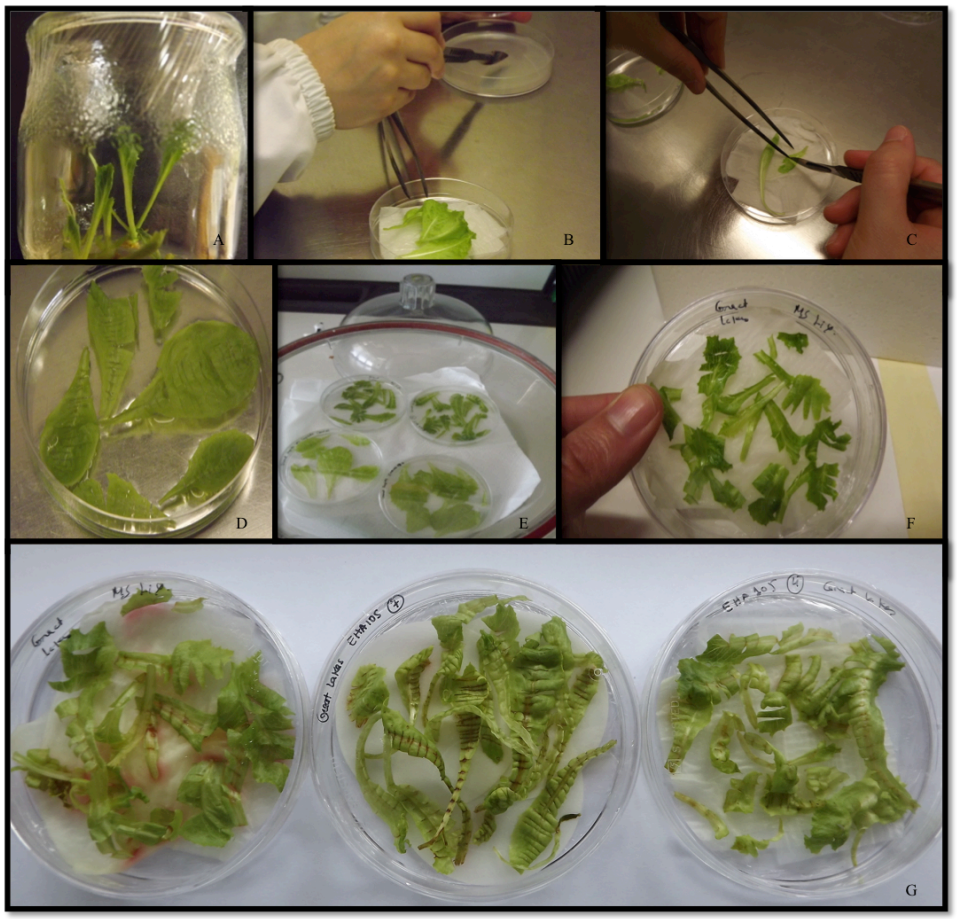


Figure 6 – Transient expression protocol for lettuce.
A - 1-month old leaves from in vitro cultured lettuce; B – Scalpel blade immersion in *Agrobacterium* suspension; C – Wounding of lettuce; D – Placement of explants in *A. tumefaciens* strain EHA105 suspensions; E – Vacuum infiltration with EHA105 suspensions (15 min, 0.6 atm); F - *Agrobacterium* infected leaves placed onto sterile filter paper soaked with MS medium supplemented with 100 µM acetosyringone; G – Co-culture with EHA105 (3 d, 24 ºC, dark conditions).

3.2.4.1 GUS histochemical assay

A histochemical assay was performed using 5-bromo-4-chloro-3-indolyl-β -D - glucuronide acid (XGlcA, Duchefa, The Netherlands) as substrate to detect β – glucuronidase (GUS) activity essentially as described by Jefferson (1987). X-GlcA was dissolved with dimethylformamide (approximately 10 µl per mg of X-GlcA) and directly prepared in 50 mM sodium phosphate buffer pH 7.0. The assay buffer was prepared with 1mM X-GlcA in 50 mM sodium phosphate buffer pH 7.0; 10mM EDTA and 0.1% (v/v) Triton X-100. Leaflets were covered with the assay buffer and the reaction incubated at 37ºC in a wet chamber for 24h. Excess chlorophyll was removed from stained explants with 70% (v/v) ethanol.

3.2.4.2 Total RNA extraction

Total RNA was isolated from leaves infected with recombinant *Agrobacterium* containing the aforementioned plasmids and from non-infected leaves. Frozen leave tissue (100-150 mg) of lettuce and M9-10a plants was homogenized in frozen RNAse free mortals and total RNA extracted with RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. To attest the effectiveness of RNA extraction, a 1.2-1.5 % agarose gel electrophoresis was performed. To allow proper gel migration, RNA samples were denatured in denaturing loading buffer, FDE (10 ml deionized formamide, 200 µl 0.5 M, pH 8.0 EDTA, 1X Green Buffer GoTaq® Buffer).

3.2.4.3 RT-PCR reaction

From the total RNA isolated from the leaves a RT-PCR reaction was performed. The RNA samples were treated with 20 Units of DNase I RNase free (Boehringer, Germany) for 1h at 25ºC, followed by heat inactivation of the DNase I at 65ºC for 5 min. 250 ng of each RNA sample were subjected to reverse transcription–polymerase chain reaction (RT-PCR), using the GoScript™ Reverse Transcription System (Promega, USA), according to manufacturer's instructions (RT-PCR converts RNA into first strand cDNA, which is then used as a template for PCR). Annealing of random primers was carried out at 25ºC, followed by first strand synthesis (RNA-cDNA hybrids) at 42ºC for 60 min on a thermocycler BioRad T100® Thermal Cycler (BioRad, USA), followed by 15 min at 70ºC to denature the reverse transcription (RT) enzyme. The PCR amplification of the ACEI synthetic genes was performed as described in 3.1.6.1. The PCR amplification with specific primers for the Act and Gus genes (Table 7) was performed as follows: 95ºC/ 2 min for denaturation, followed by 35 cycles of 94ºC/ 10s for denaturation, 60ºC/ 1 min for annealing and 72ºC/ 30s for extension and 72ºC 5 min for final extension.

Table 7 - Primers used for PCR amplification of the Act and Gus genes

Primer Label	Sequence
ACTF	TCAATGTGCCTGCCATGTATG
ACTR	ACTCACACCGTCACCAGAATC
GUSF	ACCGTTTGTGTGAACAACGA
GUSR	CATGACGACCAAAGCCAGTA

3.2.5 Stable transformation of *M. truncatula*

Transformation of *M. truncatula* was performed according to Araújo et al. (2004). Leaflets of *in vitro* grown M9-10a plants were used as explants for transformation. Leaflets were put onto a wet sterile filter paper in a Petri dish to prevent excessive desiccation and wounded perpendicularly to the central vein using a scalpel blade previously dipped into the *Agrobacterium* suspension. After a co-culture period of 5 days on solid EIM with 100μM of acetoseryngone, in the dark, at 23ºC, the infected explants were transferred to EIM containing 100 mg l^{-1} of kanamycin as selective agent for transformed tissue and 500 mg.l^{-1} of carbenicillin to eliminate *Agrobacterium*. Embryogenic *calli* cultures were maintained in a growth chamber (PHYTOTRON EDPA 700, ARALAB) with 16-h photoperiod of 100 μmol $m^{-2}s^{-1}$ applied as cool white fluorescent light and a day/night temperature of 24 ºC/22 ºC. To maintain the selective pressure, explants were transferred to fresh selective medium every week. Twenty-one days after infection, embryogenic *calli* were transferred to EPM supplemented with 100 mg.l^{-1} kanamycin and 500 mg l^{-1} carbenicillin. Every 2 weeks, green somatic embryos resistant to kanamycin were transferred to fresh selective ECM until conversion to plantlets. When plantlets developed roots, carbenicillin was eliminated from the medium and kanamycin concentration reduced to 50 mg.l^{-1}.

3.2.6 Stable transformation of lettuce

Leaves from 1-month-old lettuce plants grown on MS medium were cut with a scalpel blade embedded in the suspension of the *A. tumefaciens* harboring the synthetic genes containing the ACEI peptides. Explants were dried with sterile paper towel to

remove excess bacteria, and transferred onto the co-culture *calli* induction medium, MS with 3% (w/v) sucrose, 0.2% (w/v) gelrite, 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA, at 24 ºC in the dark for 3 days. Following co-cultivation, explants were placed onto a selection medium, consisting of MS medium supplemented with 0.1 mg.l^{-1} NAA, 0.5 mg.l^{-1} BA, 100 mg.l^{-1} kanamycin, and 500 mg.l^{-1} carbenicillin. Shoots regenerated from *calli* were excised when they were 2-3 cm in length and put onto growth regulator free medium supplemented with 50 mg.l^{-1} kanamycin and 250 mg.l^{-1} carbenicillin, for rooting.

4 Results and discussion

4.1 Construction of plant expression vectors containing ACEI peptides synthetic genes

4.1.1 Design of the ACEI peptides synthetic genes and plant expression vectors construction

Five synthetic genes were designed for optimal expression in lettuce and *M. truncatula* based on codon usage in these two plant expression hosts (see Annex A and Annex B). In a first approach, 4 synthetic genes (ACEI_FMK, ACEI_SEA, ACEI_SPI and ACEI_CHLTP) with codon optimization for *M. truncatula* were synthesized (Figure 7). The ACEI peptides coding sequences of ACEI_FMK and ACEI_CHL and its 5' flanking regions were joined by a linker of two amino acids (MK) corresponding to a cleavage site of a gastrointestinal protease, trypsin. The gene ACEI_SPI with the eight tandem repeat of the coding sequence of peptide MRW evaluated with the peptidecutter tool of ExPASy showed that the eight repeat tandem construction of this peptide was possible since pepsin cleavage sites are in positions 3,6,9,12,15,18,21 and 24 of the peptide chain. Therefore, it is likely to occur the release of the active peptide after gastrointestinal enzyme digestion of the transformed plants. The gene ACEI_SPI has the same coding sequence in *M. truncatula* and lettuce.

The ACEI peptides synthetic genes cloned into the MCS1 NdeI/SalI restriction sites of the pRI 201-AN vector are represented in Figure 8. The Nde I site of MCS1 was chosen for cloning the target genes because the locations of enhancer and start codon (ATG) might sometimes affect translational activity (Satoh et al., 2004; Sugio et al., 2008; Sugio et al., 2010; Matsui et al., 2012) and the ATG sequence in the NdeI site could be used as a translational start codon. In addition, the SalI site of MCS1 was also used because using two restriction enzymes increases the linearization efficiency and reduces the prospect of plasmid self-ligation. In the In-Fusion Cloning, the SalI restriction site was eliminated to facilitate further restriction analysis. This was accomplished since the synthetic genes contained an incomplete SalI restriction site at the 3' flanking regions.

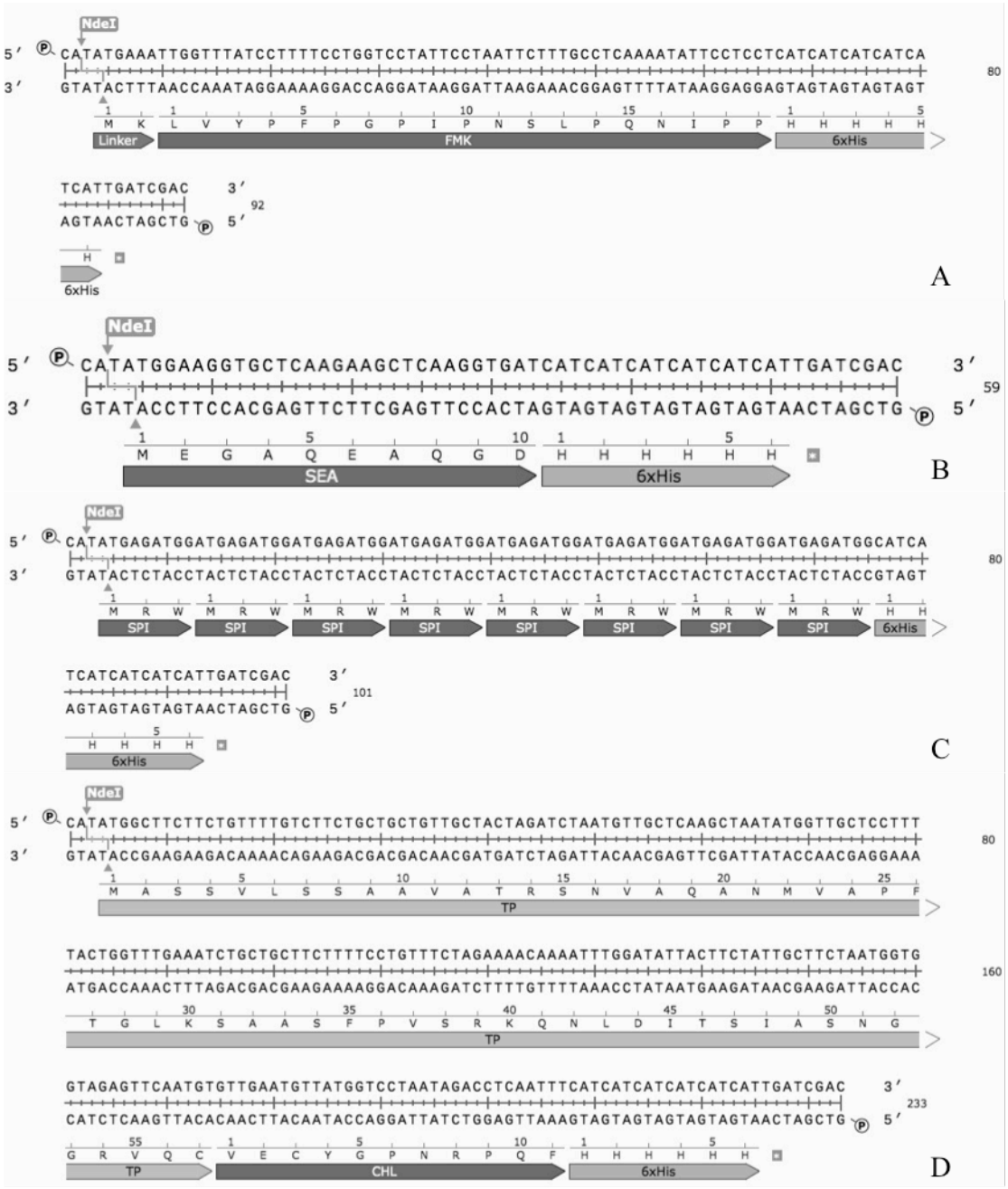


Figure 7 - The ACEI peptides synthetic genes ACEI_FMK (A), ACEI_SEA (B), ACEI_SPI (C) and ACEI_CHLTP (D) with codon optimization for *M. truncatula*.
ACEI_FMK (A) has a 5' flanking region joined by a linker of two amino acids (MK). Gene ACEI_SPIN (C) consists of eight tandem repeat of the coding sequence of peptide MRW. The ACEI_CHLTP (D) construct has a coding sequence of 57 AA transit peptide for plant chloroplast targeting (from tobacco RuBisCo small subunit). All constructs contained a 6x His tag® at 3´ends for purification and detection purposes.

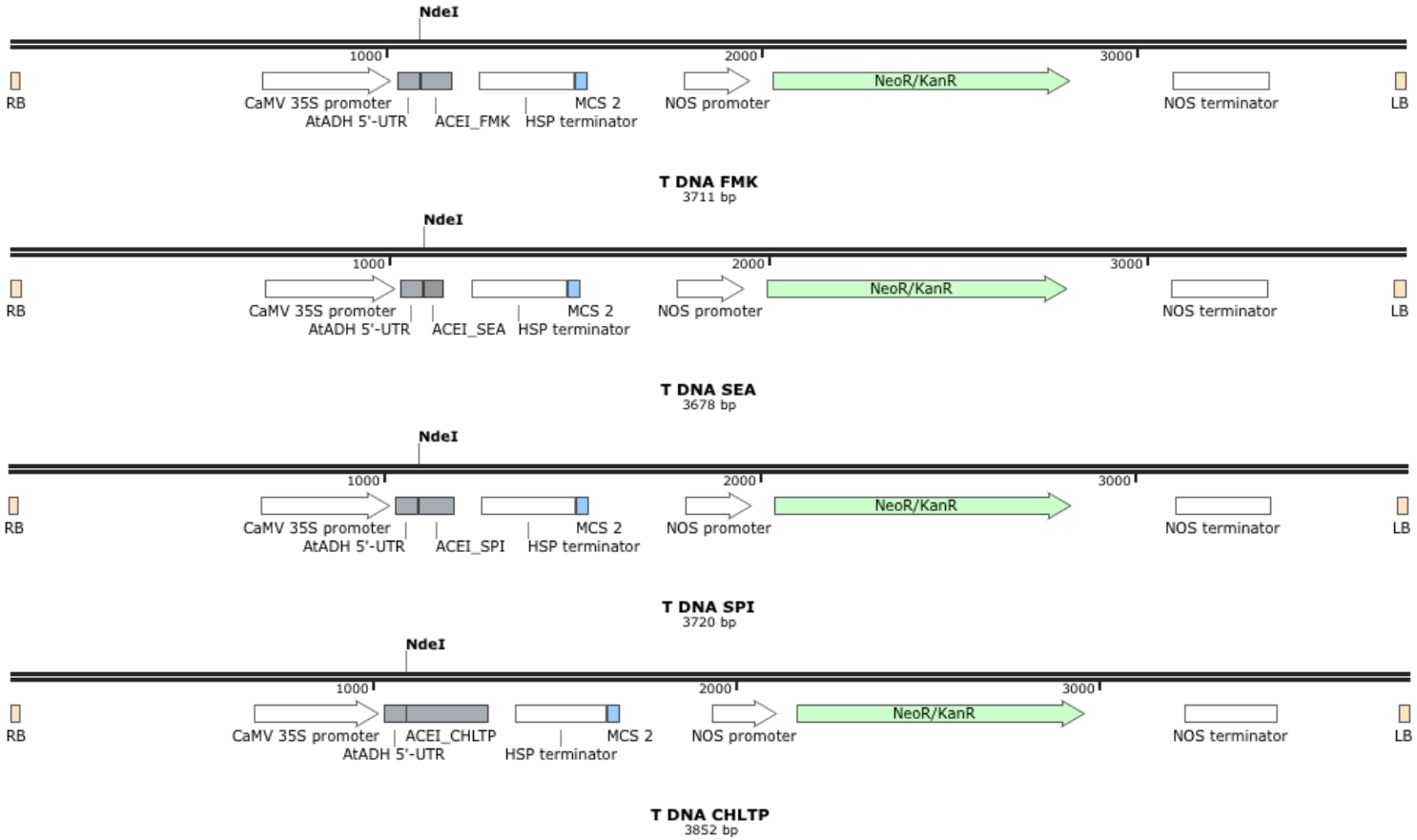


Figure 8 - Schematic representation of the T-DNA region with the ACEI peptides synthetic genes cloned into the MCS1 NdeI/SalI restriction sites of the pRI 201-AN vector.
T-DNA includes an enhancer 5´untranslated region (5´-UTR) downstream of the 35S CaMV promoter and a HSP terminator. The antibiotic resistance gene for selection of Kan resistant transformants is also present under the control of a NOS promoter.

4.1.1.1 Preparation of DNA fragments for cloning

The confirmation of the double digest linearization of pRI-201 AN vector is shown on Figure 9. Previously, the pRI 201-AN vector was successfully linearized by single digests with both enzymes (data not shown).

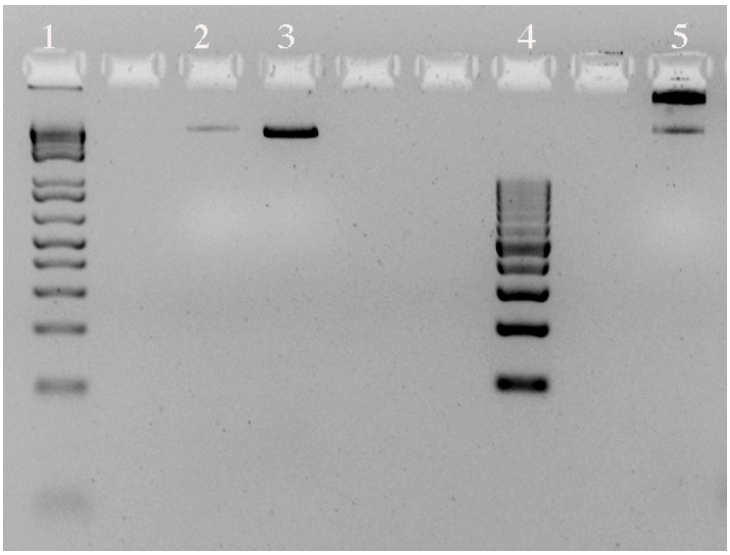


Figure 9 - Agarose gel (0.8%) electrophoresis to monitor NdeI/SalI double digest linearization of pRI-201 AN vector.
1 - 1Kb plus DNA ladder; 2 and 3 - linearized plasmid with expected size (10.4 Kb); 4 - 100bp DNA ladder and 5 - Supercoiled pRI-201 AN vector.

The synthetic genes were amplified directly from the pUC57 vectors and PCR products for subsequent ligation were extracted from agarose gels. Fragments of expected sizes for the 4 synthetic genes were observed: 92 bp for ACEI_FMK; 59 bp for ACEI_SEA, 101 bp ACEI_SPI and 233 bp for ACEI_CHLTP (Figure 10).

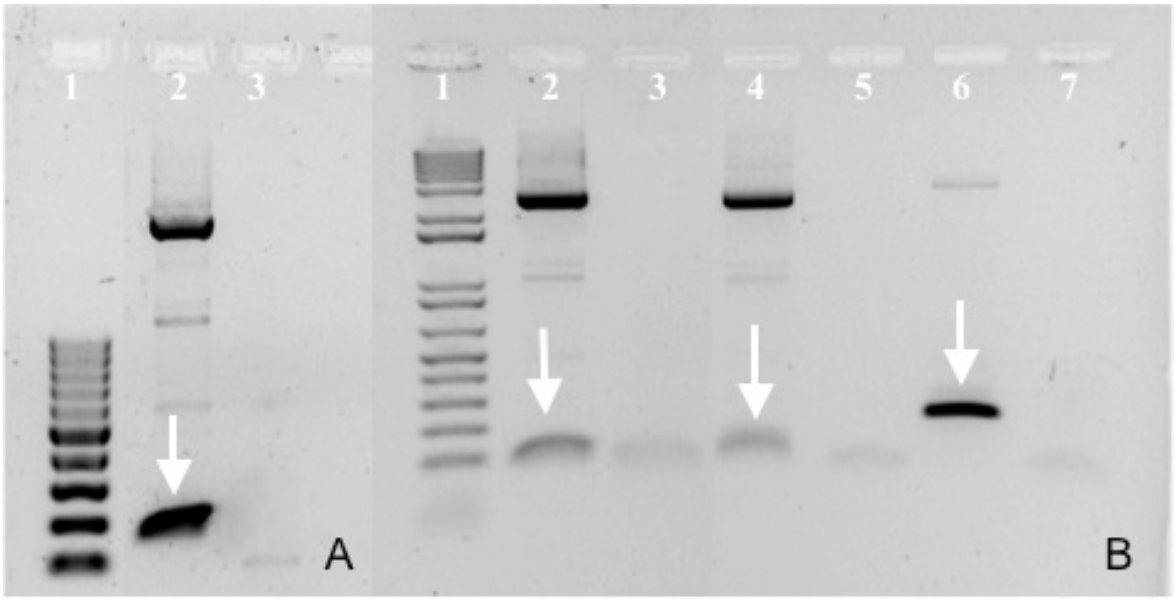


Figure 10 - Agarose gel (0.8 %) electrophoresis to extract the PCR-amplified products with the ACEI peptides synthetic genes.
A - 1 - 100 bp DNA ladder; 2 - PCR-amplified product of ACEI_FMK (92 bp); 3 - control (mix without DNA template); B - 1 - 1Kb plus DNA ladder; 2, 4 and 6 - PCR products of ACEI_SEA, ACEI_SPI, ACEI_CHLTP with expected size (59 bp, 101 bp and 233 bp respectively); 3, 5 and 7 - control (mix without DNA template). Product sizes signalized with arrows.

4.1.2 Selection of recombinant plasmids

After the ligation reaction and DH5α™ transformation > 40 colonies for each construct were obtained. At least 10 colonies were tested for the presence of synthetic genes in the expression vector by colony PCR amplification. Fragments of expected sizes were observed in agarose gels for three synthetic genes (see Figure 11).

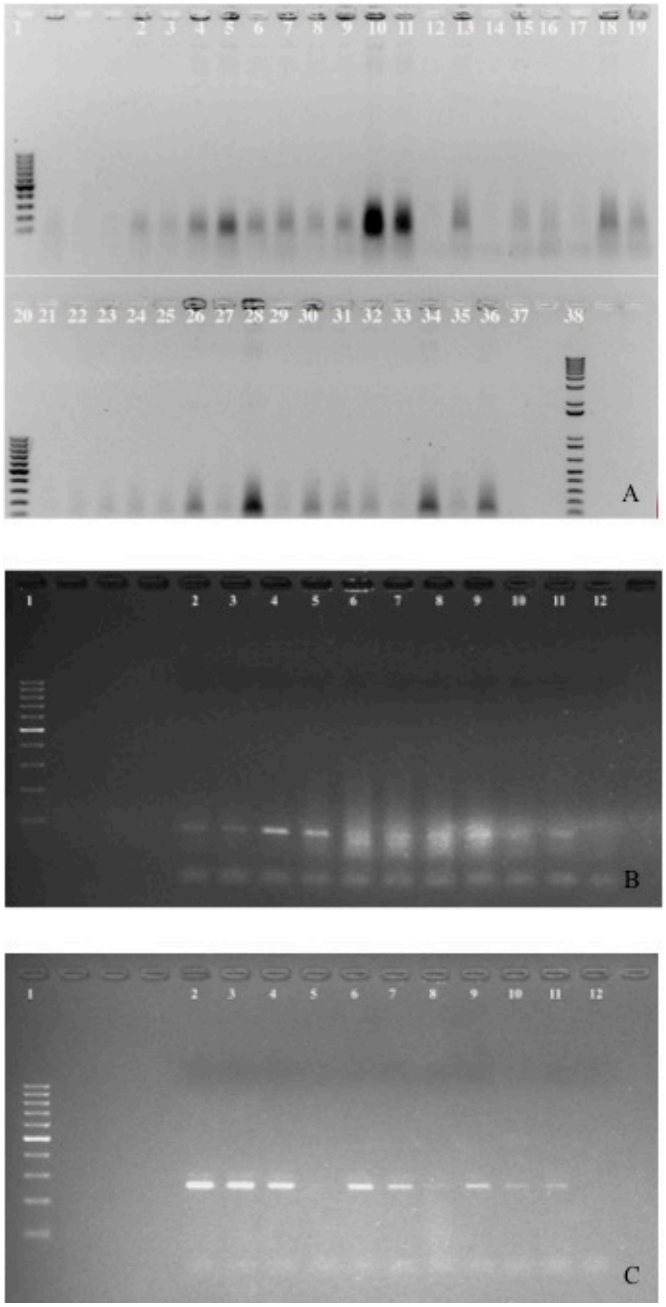
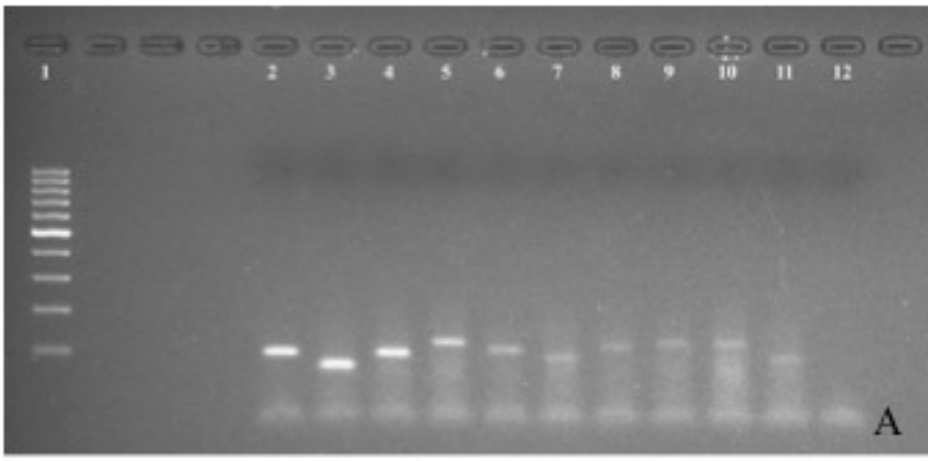
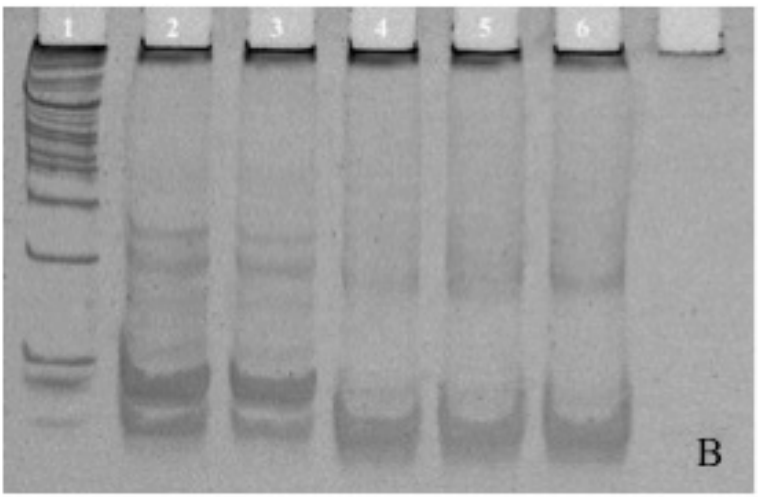


Figure 11 - Agarose gels (1.5 %) electrophoresis of Colony PCR for the ACEI peptides synthetic genes.
A – Colony PCR for gene ACEI_FMK: 1 and 20 - 100 bp DNA ladder; 2-11,13,15-16,18-32 and 34-36 – PCR products of gene ACEI_FMK with expected size (92 bp); 37- control (mix without DNA template); 38- 1 Kb plus DNA ladder; B - Colony PCR for gene ACEI_SEA: 1- 100 bp DNA ladder; 2 to 11- PCR products of gene ACEI_SEA with expected size (59 bp); 12- control (mix without DNA template); C - Colony PCR for gene ACEI_CHLTP: 1 – 100 bp DNA ladder; 2-4, 6-7 and 9-11 - PCR products of gene ACEI_CHLTP with expected size (233 bp); 12 - control (mix without DNA template).

For ACEI_SPI amplification of fragments with different sizes were observed (Figure 12 A). This might be due to the repetitive nature of this synthetic gene sequence. To minimize amplification errors, a high fidelity polymerase was used. Additionally, in this case a higher resolution 12% polyacrylamide gel was used to visualize small

differences in fragment size. However, with this enzyme, fragments of different sizes were also observed (Figure 12 B and C). These results suggest that independently of the fidelity of the used enzyme, non-specific primer hybridization might be occurring as it can be seen on Figure 13, obtained with SnapGene Viewer (www.snapgene.com). The colony corresponding to the fragment of largest size was chosen for the subsequent work.





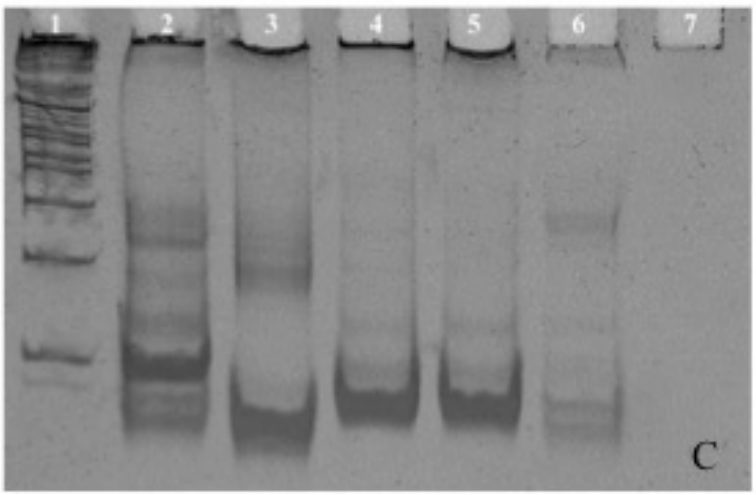


Figure 12 - Agarose gels (1.5 %) electrophoresis and Polyacrylamide gel (12 %) electrophoresis for ACEI_SPI amplified fragments.
A - Agarose gels (1.5 %) electrophoresis for ACEI_SPI amplified fragments: 1 – 100 bp DNA ladder; 2-11 - Different sizes for amplified fragments were observed; 12 - control (mix without DNA template). B and C - Polyacrylamide gel (12 %) electrophoresis for ACEI_SPI amplified fragments to visualize small differences in fragment size: 1 - 1 Kb plus DNA ladder; 2 to 6 - ACEI_SPI amplified fragments with different size were observed; 7- control (mix without DNA template).

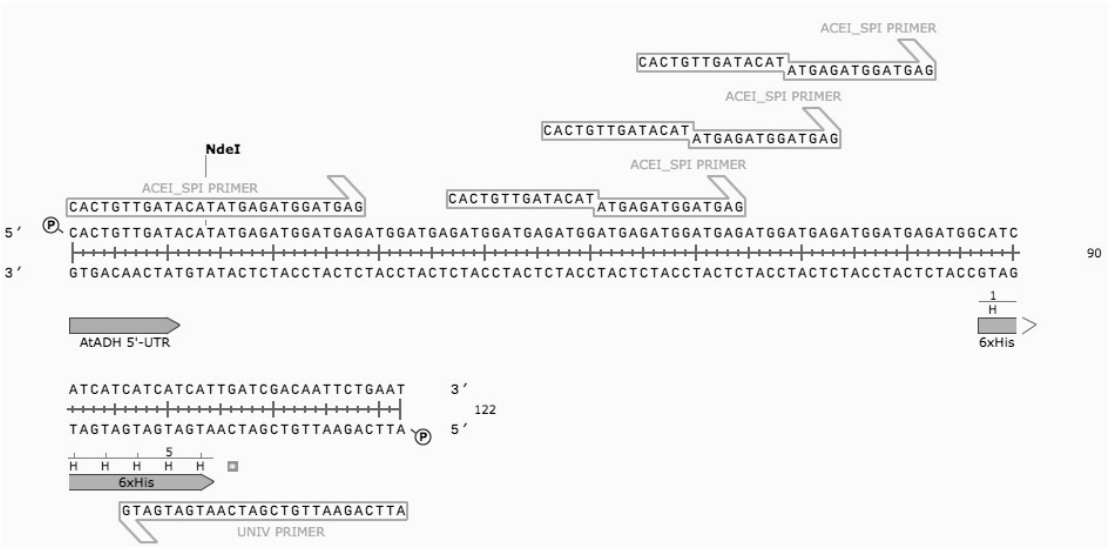


Figure 13 - SnapGene Viewer prevision for non-specific primer hybridization.
Potential amplification of different size fragments due to possible non-specific hybridization of the gene specific primer.

Finally, small-scale extraction of all plasmid constructs was performed for further *Agrobacterium* EHA105 transformation Figure 14. *Agrobacterium*-mediated transformation of lettuce and *M. truncatula* with plant expression vectors containing ACEI peptides synthetic genes

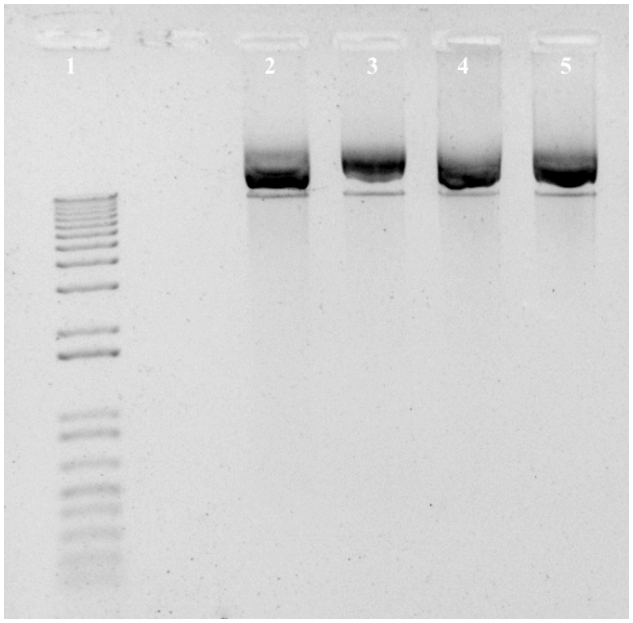


Figure 14 - Agarose gel (0.8 %) electrophoresis after small-scale extraction of all plasmid constructs containing the different ACEI peptides synthetic genes.

1 - 1Kb plus DNA ladder; 2 - pRI-ACEI_FMK; 3 - pRI-ACEI_SEA; 4 - pRI-ACEI_SPI and 5 - pRI-ACEI_CHLTP.

4.2 *Agrobacterium*-mediated transformation of lettuce and *M. truncatula* with plant expression vectors containing ACEI peptides synthetic genes

4.2.1 Screening of *Agrobacterium* EHA105 transformants

Colony PCR of EHA105 colonies confirmed the presence of the plasmid constructs containing the ACEI synthetic genes.

Fragments of expected sizes were observed in colony PCR for three pRI-ACEI plasmids, harboring the genes ACEI_FMK, ACEI_SEA, ACEI_CHLTP. Colony PCR for the construct pRI-ACEI_SPI revealed fragments with different sizes. Agarose gels of the PCR screening of *Agrobacterium* EHA105 transformants are presented on Annex C.

4.2.2 Establishment of a micropropagation scheme for *in vitro* maintenance of lettuce cv. Great Lakes

- Seed decontamination and germination

The percentage of lettuce seed germination (emergence of radicle and cotyledons) was higher using shorter decontamination time, 5 and 7 min (Table 8). With 10 min of decontamination time germination percentage decreased. In all treatments no contamination was observed (Table 8). Seed germination was achieved in light conditions according to Curtis (2006). Dark conditions were also previously tested and resulted in very low seed germination (data not shown).

Table 8 - Percentage of lettuce seed germination and contamination with different decontamination treatments

Treatments	**Germination %**	**Contamination %**
70% etanol + 5 min domestos	99.0 ± 1.0^{a}	0.0 ± 0.0^{c}
70% etanol + 7 min domestos	$97.7 \pm 0.3^{a,b}$	0.0 ± 0.0^{c}
70% etanol + 10 min domestos	95.3 ± 2.3^{b}	0.0 ± 0.0^{c}

Values are mean ±SD; values followed by different letters are significantly different at $p \leq 0.05$ according to ANOVA test n=3.

- Lettuce micropropagation

Radicle emergence was observed 2-3 days after seed plating (Figure 15 A). Seven day-old seedlings of lettuce (Figure 15 B) were transferred to glass flasks containing MS030A and rooted plantlets were developed within 2 weeks (Figure 15 C). Continuous propagation and maintenance of lettuce was accomplished by monthly subculturing explants with two axillary buds to fresh medium (Figure 15 D).

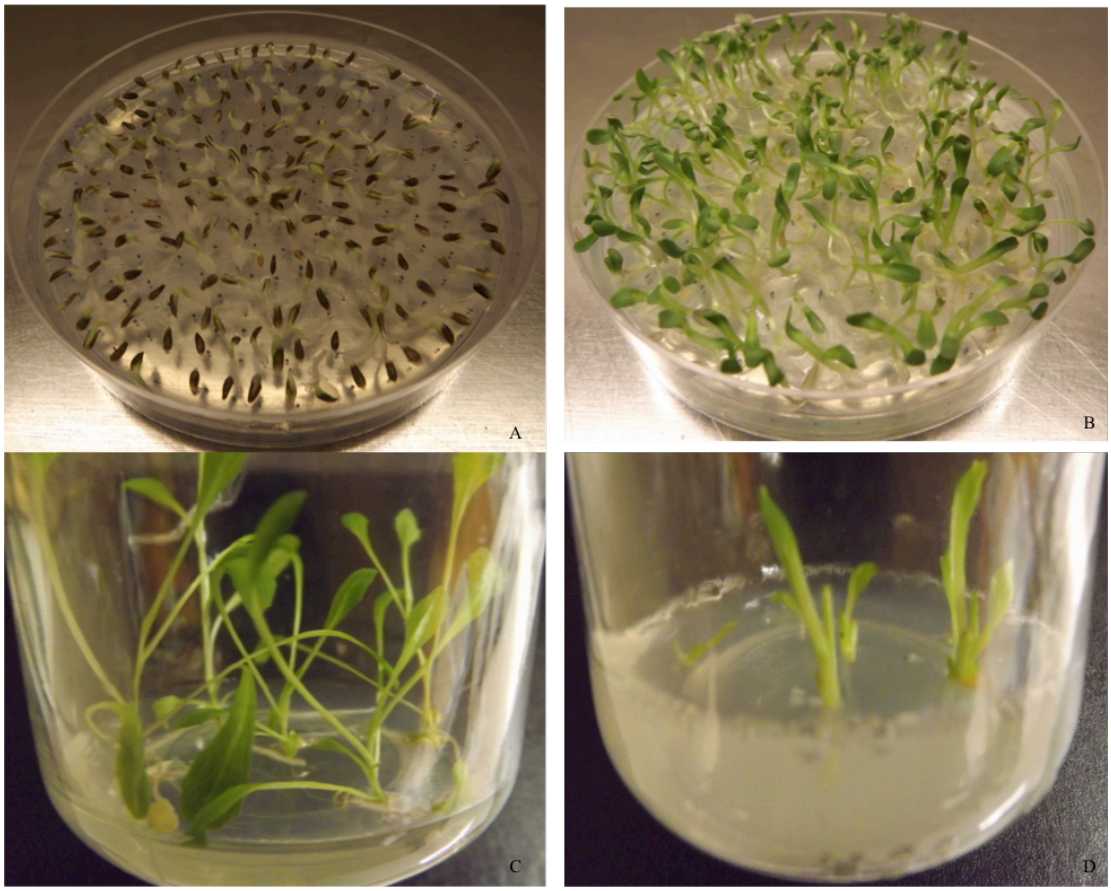


Figure 15 - *In vitro* germination and propagation of lettuce through axillary bud proliferation.
A – Radicle emergence in Great lakes seeds in MS010A (2-3 d); B – Seven day-old seedlings; C – Developed plantlets in MS030A (2 wk); D – Subculture of isolated stem segments with two axillary buds in MS030A (subculture every 4 wk).

- Organogenesis

Calli induction was accomplished after the second week of culture (100% response) in 1,5-2 cm explants (with two axillary buds) and mature leaves of lettuce using both combinations of growth regulators (e.g. Figure 16 A). The combination of benzyl

adenine (BA) and naphthalene acetic acid (NAA) has been extensively used for *calli* induction and shoot regeneration from cotyledonary explants of lettuce (Curtis et al., 1994; Hunter and Burritt, 2002; Hunter and Burritt, 2004; Lim et al., 2011b; Mohebodini et al., 2011; Gómez-Montes et al., 2015) and also from mature leaves (Gómez-Montes et al., 2015). The use of another auxin as indole butyric acid (IBA) could also be favorable for *calli* induction (Olivia Costa personal communication). In this way, 0.5 mg l^{-1} BA and 0.1 mg l^{-1} indole butyric acid (IBA) were also used for *calli* induction. *Calli* hyperhydricity was observed (Figure 16 A), this event has been previously described for lettuce cv Great Lakes and has been proven to be detrimental for shoot regeneration (Teng and Liu, 1993). Some studies have shown that BA levels at low agar concentrations can increase hyperhydricity. This problem can be overcome by reducing the BA concentration or by increasing the agar concentration (Pasqualetto, 1990). A compromise between gelling agent concentration and BA reduction might be a good alternative, since an accentuated reduction of BA concentration will probably cause the lowering of shoot proliferation. Nevertheless, shoot hyperhydricity was reduced upon transference to growth regulator free medium.

Shoots were regenerated in all experiments after 2-3 weeks after transference to MS030A. The highest number of regenerated shoots was obtained with *calli* induced in 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA (Figure 16 B) and subsequently a higher number of plantlets developed roots (Figure 16 C and D). Plants with a well-developed root system were potted in a mixture 2:1 soil–vermiculite and covered with a plastic film for acclimation in a growth chamber according to Araújo et al. (2004) (Figure 16 E).

The maintenance in BA and NAA containing medium allows the perpetuation of organogenic *calli* in cv. Great Lakes. Shoots are always appearing and able to develop upon transference to growth regulator free medium. In this way, a suitable method for regeneration and propagation of lettuce was established. Furthermore, this propagation and regeneration scheme via organogenesis can be coupled to transient expression studies and stable transformation procedures, as a continuous source of explants, and also as regeneration method for transformed lettuce cv. Great Lakes plants.

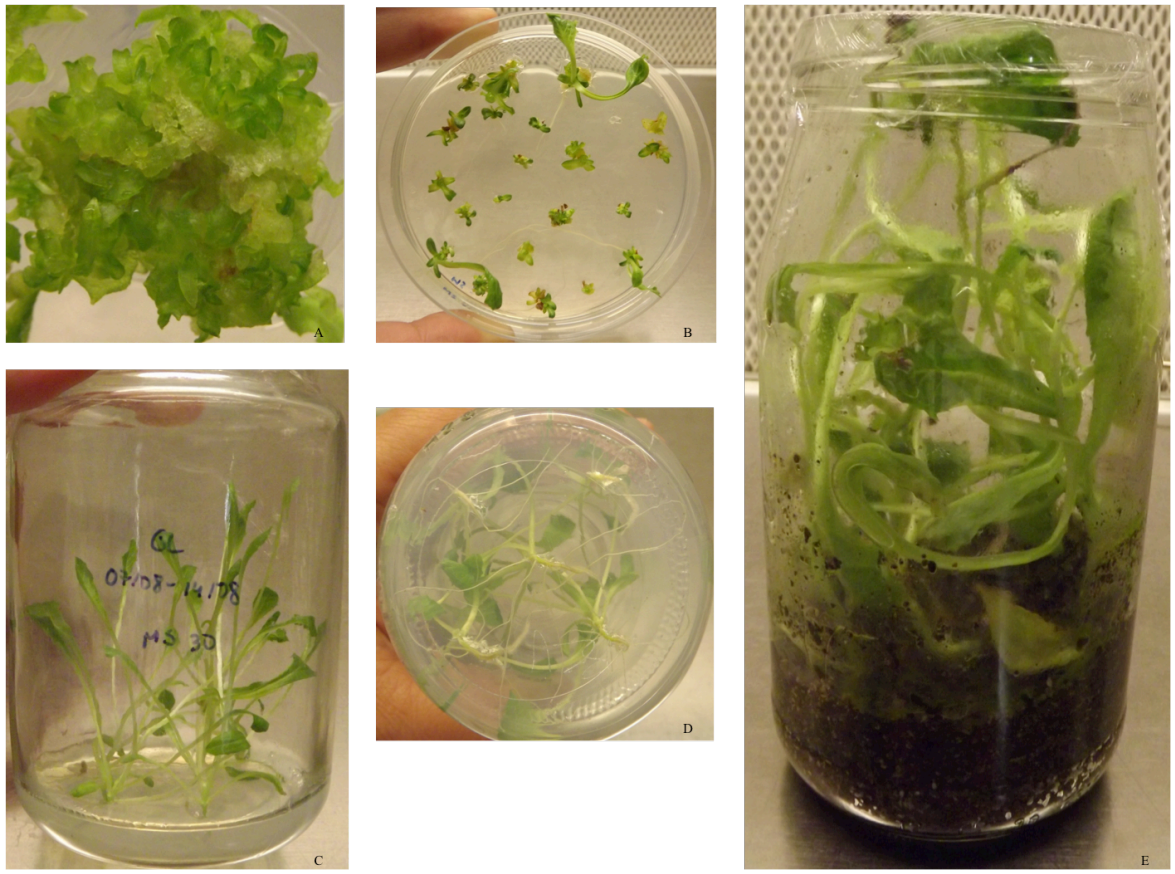


Figure 16 - *In vitro* organogenesis for lettuce.
A – Organogenic *calli* induction after 2 wk in BA and NAA containing medium (*calli* presenting hyperhydricity); B – Shoots regenerated from organogenic *calli* in MS030A; C – Plantlets developed in MS030A; D – Rooting in MS030A; E – Transference to 2:1 soil–vermiculite pots for acclimation.

4.2.3 Transient expression studies

4.2.3.1 GUS histochemical assay

GUS activity was detected in transgenic *M. truncatula* (Figure 17 A) and lettuce leaves infiltrated with *Agrobacterium* suspensions harboring the plasmid pMP2482 (Figure 17 C). GUS activity was not detected in lettuce leaves infiltrated solely with MS030 liquid medium (Figure 17 B). The detection of the GUS protein product validates the effectiveness of the transient expression protocol since its production is strictly related with gene expression owing to the presence of the potato st-lsI intron in the coding region of gusA, confirming that the protein production is occurring exclusively *in planta* (Duque et al., 2007). The GUS histochemical assay also allowed the location of protein production sites, with the majority of protein production occurring in the wounded regions of the leaves. This is an expected result as wounds constitute a portal of entry for *Agrobacterium* and wound repair processes have been proven to facilitate transformation (McCullen and Binns, 2006).

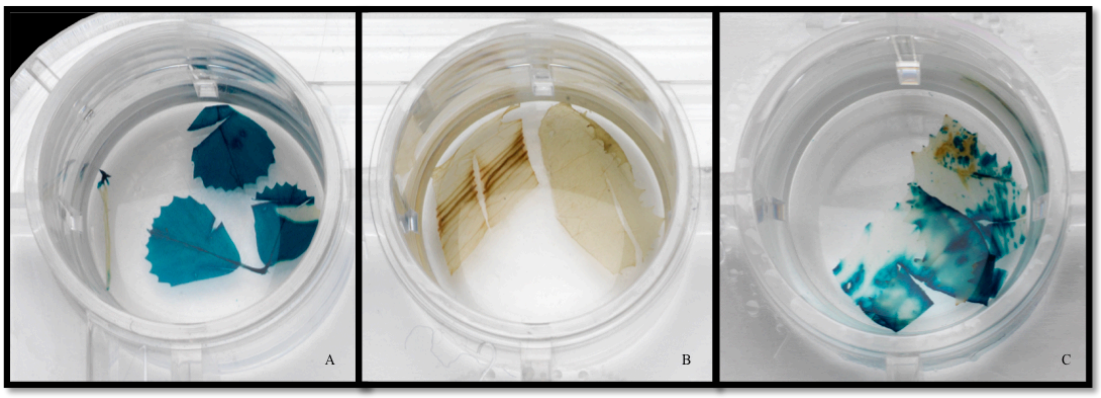


Figure 17 - GUS histochemical assay.
A – *M. truncatula* stably transformed with plasmid pMP2482 (positive control); B – Lettuce leaves infiltrated with MS030 liquid medium (negative control); C – Lettuce leaves infiltrated with *Agrobacterium* suspension harboring the plasmid pMP2482.

4.2.3.2 RT-PCR assay

The results of the RT-PCR assay are presented on Figure 18. The PCR amplification of a 100 bp fragment corresponding to an internal portion of the housekeeping actin (Act) gene confirmed the efficiency of the reverse transcription

reaction. RT-PCR for the Gus gene was also used as positive control for transient expression, and amplification of a 499 bp fragment was observed.

The RT-PCR assay demonstrated the gene integrity of plasmid constructs pRI-ACEI_FMK and pRI-ACEI_CHLTP, with the presence of amplification of fragments with 92 bp and 233 bp, respectively. No PCR amplification was detected in samples agro-infiltrated with genes ACEI_SEA and ACEI_SPI. The absence of amplification can have several explanations, one being the reduced amount of RNA and consequently of cDNA in the samples, suggesting the necessity of RT-PCR protocol optimization. Therefore, it is advisable to repeat the RT-PCR reactions to demonstrate the gene integrity of these plasmid constructs.

These results show that the ACEI_FMK and ACEI_CHLTP synthetic genes are being expressed *in planta* and foresee the prospect of a successful expression in stably transformed plants. Forthcoming testing of the ACEI peptides transient production will be crucial to confirm these encouraging first results.

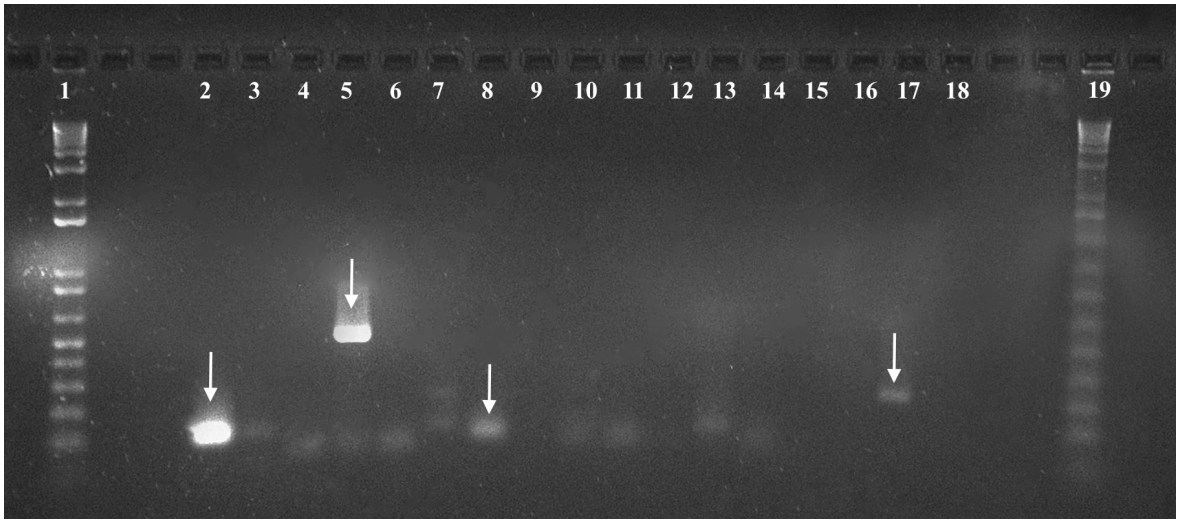


Figure 18 - Agarose gel (2.0%) electrophoresis of RT-PCR products for ACEI synthetic genes.
1 - 1 Kb plus DNA ladder; 2, 4, 7, 10, 13 and 16 – non-infected lettuce leaves amplified with gene specific primers for Act, Gus, ACEI_FMK, ACEI_SEA, ACEI_SPI, ACEI_CHLTP, respectively; 5 – Gus amplified fragment in lettuce infiltrated with pMP2482; 8 – ACEI_FMK amplified fragment in lettuce infiltrated with pRI-ACEI_FMK; 17 – ACEI_CHLTP amplified fragment in lettuce infiltrated with pRI-ACEI_CHLTP; 3, 6, 9, 12, 15 and 18 - control (mix without cDNA template). Product sizes signalized with arrows.

4.2.4 Stable transformation of *M. truncatula*

Agrobacterium-mediated stable transformation of *M. truncatula* executed according to Araújo et al. (2004) is currently underway. These experiments were carried out using *A. tumefaciens* EHA105 harboring the plasmid constructs pRI-ACEI_FMK and pRI-ACEI_SEA.

Initial results from the plant transformation experiments are presented on Figure 19. One-month old leaflets of M9-10a *in vitro* cultured plants were used as explants for *A. tumefaciens* EHA105 transformation (Figure 19 A). In control non-transformed explants, with the presence of antibiotics (100 $mg.l^{-1}$ kanamycin and 500 $mg.l^{-1}$ carbenicillin), the callogenic capacity of the explants was not affected but no embryo formation was observed (Figure 19 B). In control non-transformed explants without antibiotic supplementation, 100% of embryogenic *calli* was observed on EIM containing (Figure 19 C) and extensive proliferation of somatic embryos was observed. The development of somatic embryos in these conditions was faster than in the *Agrobacterium* infected explants in accordance to the described in (Araújo et al., 2004). In the *Agrobacterium* infected explants, Kan^R embryos started to appear five weeks after initiating the selective pressure (Figure 19 D). To maintain the selective pressure, Kan^R somatic embryos were subcultured every 2 weeks to fresh medium with antibiotics where they started to convert into plantlets (Figure 19 E). False Kan^R embryos bleached when they were put in direct contact with the selective medium (Figure 19 F). The first plantlets (Figure 19 G) appeared within 3-4 months after co-cultivation with *Agrobacterium*. At the current stage of the transformation procedure (5 months after co-cultivation*)* three pRI-ACEI_SEA putative plantlets and one pRI-ACE_FMK putative plantlet have been recovered and developed roots in kanamycin selection medium (Figure 19 H and I). These plantlets have been selected as putative transgenic plants, since the rooting in kanamycin medium constitutes a good indication of the stable transgene insertion (Duque, 2010). Since the development of somatic embryos is asynchronous (Araújo et al., 2004), somatic embryos in a late-torpedo/dicotyledonary development stage are still being isolated every week for each transformation event. Molecular analysis of putatively transformed plants will be done to confirm the insertion of the transgene in the plant genome.

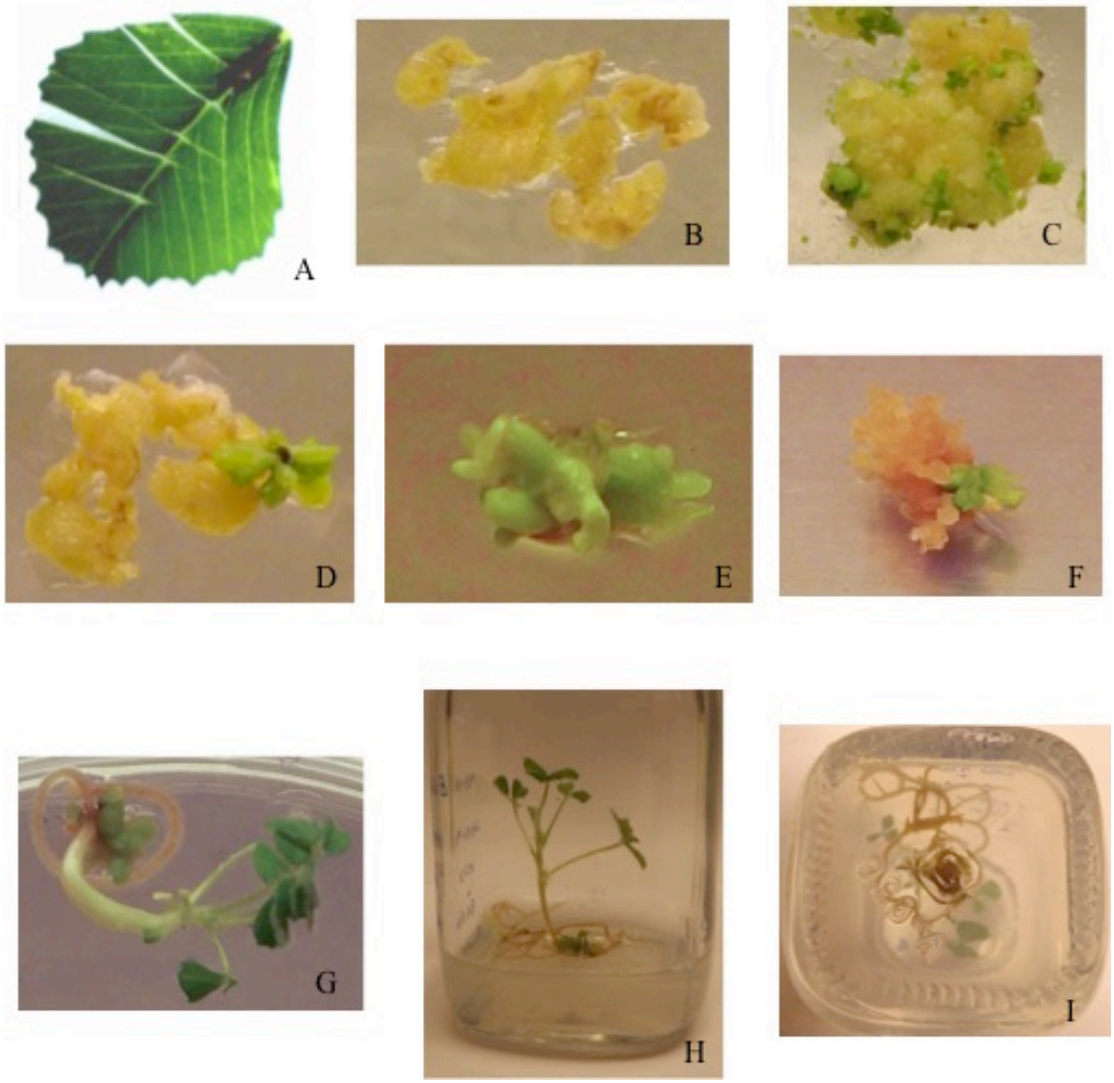


Figure 19 - Transformation-regeneration in *M. truncatula* cv. Jemalong M9–10a genotype.
A – Wounded leaflet of the cv. Jemalong M9–10a genotype; B – Embryogenic *calli* originated from non-infected leaflet in selective conditions (no somatic embryos developed); C – Embryogenic *calli* derived from a non-infected leaflet in EPM; D – First Kan^R embryos (5 wk after initiation of selective pressure) E – Kan^R clump of somatic embryos at different stages of development; F – Kan^S embryos (bleached); G – Kan^R embryo conversion; H – Kan^R plantlets; I – Transgenic T_0 line rooted in 50 mg l^{-1} of kanamycin.

4.2.5 Stable transformation of lettuce

Agrobacterium-mediated stable transformation experiments with are currently underway. *A. tumefaciens* strain EHA105 harboring the plasmid constructs pRI-ACEI_FMK, pRI-ACEI_SEA, pRI-ACEI_SPI and pRI-ACEI_CHLTP were used to infect lettuce. First results from the plant transformation experiments are presented on Figure 20. One-month old leaves from *in vitro* cultured lettuce were used as explants for transformation with EHA105. Previous studies used mature leaves as explants for lettuce

transformation (Lim et al., 2011b), however the majority of *Agrobacterium*-mediated lettuce transformation protocols have used cotyledons as explant source for transformation (Curtis et al., 1994; Sun et al., 2006; Liu et al., 2012). Shoot regeneration and effect of explants age on shoot regeneration has been proven to be genotype-dependent in lettuce (Hunter and Burritt, 2002; Mohebodini et al., 2011). In cv. Great Lakes only shoot regeneration from cotyledons was reported and studies have shown that shoot regeneration was not affected by cotyledon age (Hunter and Burritt, 2002), indicating that the effect of explants age might not be relevant for shoot regeneration on this genotype.

Organogenic *calli* were obtained in *Agrobacterium* infected explants on organogenesis induction medium containing 100 $mg.l^{-1}$ Kan and 500 $mg.l^{-1}$ Carb with the subsequent development of Kan^R shoots (Figure 20 C). In control experiments, the presence of antibiotics (100 $mg.l^{-1}$ Kan and 500 $mg.l^{-1}$ Carb) did not affect the callogenic capacity of the explants, but no shoot formation was observed, as expected for non-transformed tissue. Without antibiotics, lettuce explants underwent *calli* induction and shoot differentiation as described. The development of *calli* and shoots in these conditions (Figure 20 A) was faster than in the *Agrobacterium* transformed explants (Figure 20 B). In the *Agrobacterium* infected explants *calli* formation could be observed 2-3 weeks after the initiation of selective pressure (Figure 20 C) and the first emerging shoots appeared 4-5 weeks after (Figure 20 D). Organogenic *calli* were isolated every week and underwent a vast proliferation (Figure 20 E and F), resulting in a laborious *in vitro* culture procedure. Selective pressure was maintained by subculture every 2 weeks to fresh antibiotic containing medium where they started to convert into plantlets (Figure 20 F). A high number of apparent Kan^R shoots bleached when they were put in direct contact with the selective medium (Figure 20 G), suggesting a large incidence of false positive Kan^R shoots. Moreover, the excessive propagation of organogenic *calli* can also generate numerous false Kan^R clones. This situation also demonstrates the shortcomings of organogenesis as regeneration method in relation to embryogenesis. Embryogenic tissues allow the recovery of numerous transformants that are, in most cases, non-chimeric because of the presumed single cell origin of somatic embryos (Hansen and Wright, 1999).

In cv. Great Lakes the prevalence of apparent Kan^R shoots might be reduced by earlier placement of organogenic *call*i on growth regulator free medium to avoid excessive proliferation and by the increase in kanamycin concentration. For this purpose it will be

important to perform optimization studies for selection of the best kanamycin concentration to be used for cv. Great Lakes transformants selection in future transformation experiments.

The first plantlets appeared within 3-4 months (Figure 20 G) after co-cultivation with *Agrobacterium*. At the current stage of the transformation procedure, 5 months after co-cultivation, plantlets resulting from explants infected with *Agrobacterium* harboring the plant expression construct pRI-ACEI_CHLTP have been isolated and transferred to MS030A medium for rooting (Figure 20 H). Several shoots have developed roots in Kan containing medium (Figure 20 I). These plantlets have been selected as putative transgenic plants. Molecular analysis will be done to confirm the insertion of the transgene in the plant genome. The *Agrobacterium*-mediated transformation of lettuce with the plant expression constructs pRI-ACEI_FMK, pRI-ACEI_SEA and pRI-ACEI_SPI was initiated one month later and the first plantlets are now starting to appear.

To our knowledge, we present here the first transformation/regeneration procedure from leaf explants for the cv. Great Lakes. However, further optimization of this process to overcome the production of non-transformed shoots is required.

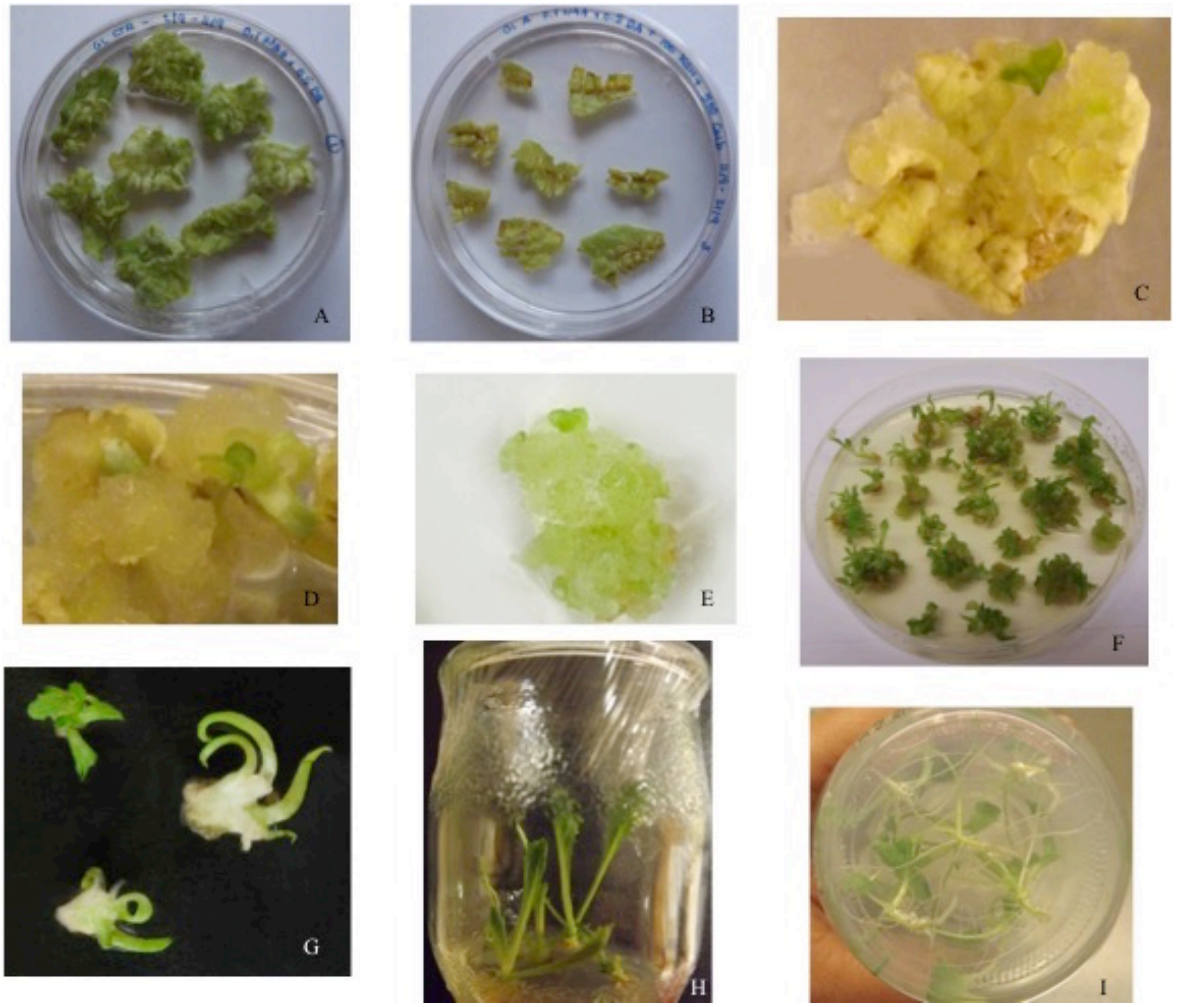


Figure 20 – Transformation- regeneration in lettuce.
A – Organogenic *calli* originated from non-infected leaflet; B – Delay in organogenic *calli* formation for infected leaflets; C – Organogenic *calli* originated from infected leaflet; D - First Kan^R shoot (4-5 wk after initiation of selective pressure); E – Proliferation of organogenic *calli* in supplemented medium; F – Proliferation of organogenic *calli* and development of the first plantlets; G - Kan^S plantlets (bleached) and Kan^R plantlet (green); H – Developed Kan^R plantlet; I – Transgenic T_0 line rooted in 50 mg l^{-1} of kanamycin

5 Conclusion remarks and prospects

As a conclusion, with this work four plant transformation vectors containing genetic information for the heterologous expression of four ACEI peptides, with proven ACE inhibitory activity, and devised from dissimilar organisms (from the sea cucumber to the *Chlorella* microalgae) were obtained.

The establishment of an effective micropropagation scheme via axilary bud proliferation and organogenesis not only allowed the continuous propagation and maintenance of lettuce but also its regeneration. This propagation and regeneration scheme can be coupled to transient expression studies and stable transformation procedures, as a continuous source of explants, and also as regeneration method for transformed lettuce plants.

The GUS histochemical assay demonstrated the efficacy of the established transient expression protocol, and also the convenience of using plasmid constructs harboring the GUS reporter gene for plant transformation experiments. In fact, such plasmids have been used in several lettuce and *M. truncatula* transformation experiments, namely to test lettuce transformation frequency (Torres et al., 1993) and transgene segregation analysis (Torres et al., 1993; Franklin et al., 2011), and for recovery of transformed *M. truncatula* embryos without selective pressure (Duque et al., 2007).

The transient expression of ACEI_FMK and ACEI_CHLTP synthetic genes in lettuce confirmed the gene integrity within the plasmid constructs and foresee the possibility of a successful expression in stably transformed plants. Further plasmid construct sequencing and testing of the ACEI peptides transient production will be crucial to confirm these encouraging first results.

Agrobacterium-mediated stable transformation experiments with lettuce and *M. truncatula* are currently underway. Until now, the initial selection of plantlets resistant to kanamycin has been accomplished for construct pRI-ACEI_CHLTP in lettuce and constructs pRI-ACEI_FMK and pRI-ACEI_SEA in *M. truncatula*. Further molecular and biochemical studies will have to be performed to confirm plant stable transformation and transmission of the traits to the next generations.

Furthermore, the *in vitro* testing of the peptides stability and ACE inhibitory activity will be critical to validate the effectiveness of the two heterologous production systems.

In the case of lettuce, and envisaging the future human administration, besides the molecular and biochemical analysis the active effect of the ACEI peptides will be confirmed by the oral administration to spontaneously hypertensive rats (SHRs), in collaboration with an animal research laboratory.

In the case of *M. truncatula* the induction of *calli* from transformed plants and the establishment of peptide production cell suspension cultures is also a future perspective since this system has already been proven to be an attractive heterologous protein platform production by Abranches et al. (2005). The extraction and purification of the peptides will be facilitated by the presence of the poly-histidine tags.

Finally, in the current scenario of global demand for alternative hypertension therapies and easier antihypertensive peptide manufacturing processes, this work gives an initial contribution to a wider project of ACEI peptides production in edible plants of commercial interest.

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7 Annexes

Annex A– The synthetic gene ACEI_CHL

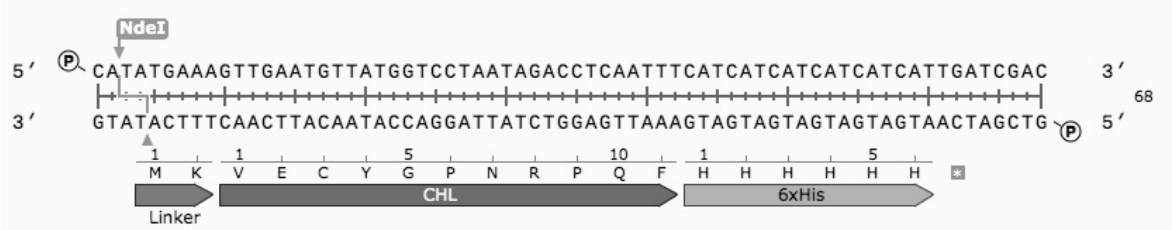

Figure 1 - The ACEI peptide synthetic gene ACEI_CHL with codon optimization for *M. truncatula*. ACEI_CHL has a 5' flanking region joined by a linker of two amino acids (MK).

Annex B – ACEI peptides synthetic genes with codon optimization for lettuce

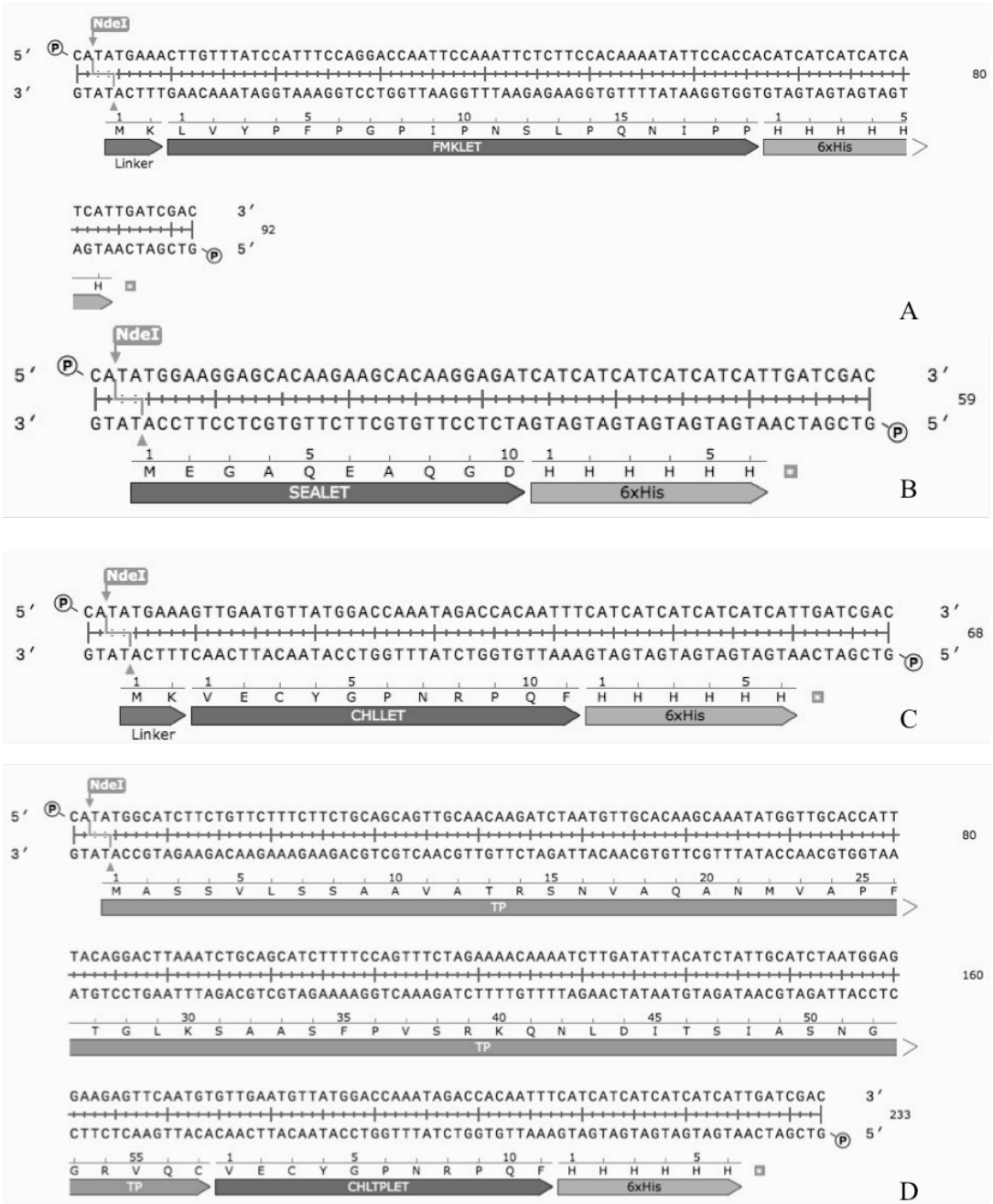

Figure 1 - The ACEI peptides synthetic genes ACEI_FMKLET (A), ACEI_SEALET (B), ACEI_CHLLET (C) and ACEI_CHLTPLET (D) with codon optimization for lettuce.

ACEI_FMKLET (A) and ACEI_CHLLET (C) have a 5' flanking region joined by a linker of two amino acids (MK). ACEI_CHLTPLET (D) construct has a coding sequence of 57 AA transit peptide for plant chloroplast targeting (from tobacco RuBisCo small subunit). All constructs contained a 6x His tag® at 3´ends for purification and detection purposes.

Annex C - Agarose gels of the PCR screening of *Agrobacterium* EHA105 transformants

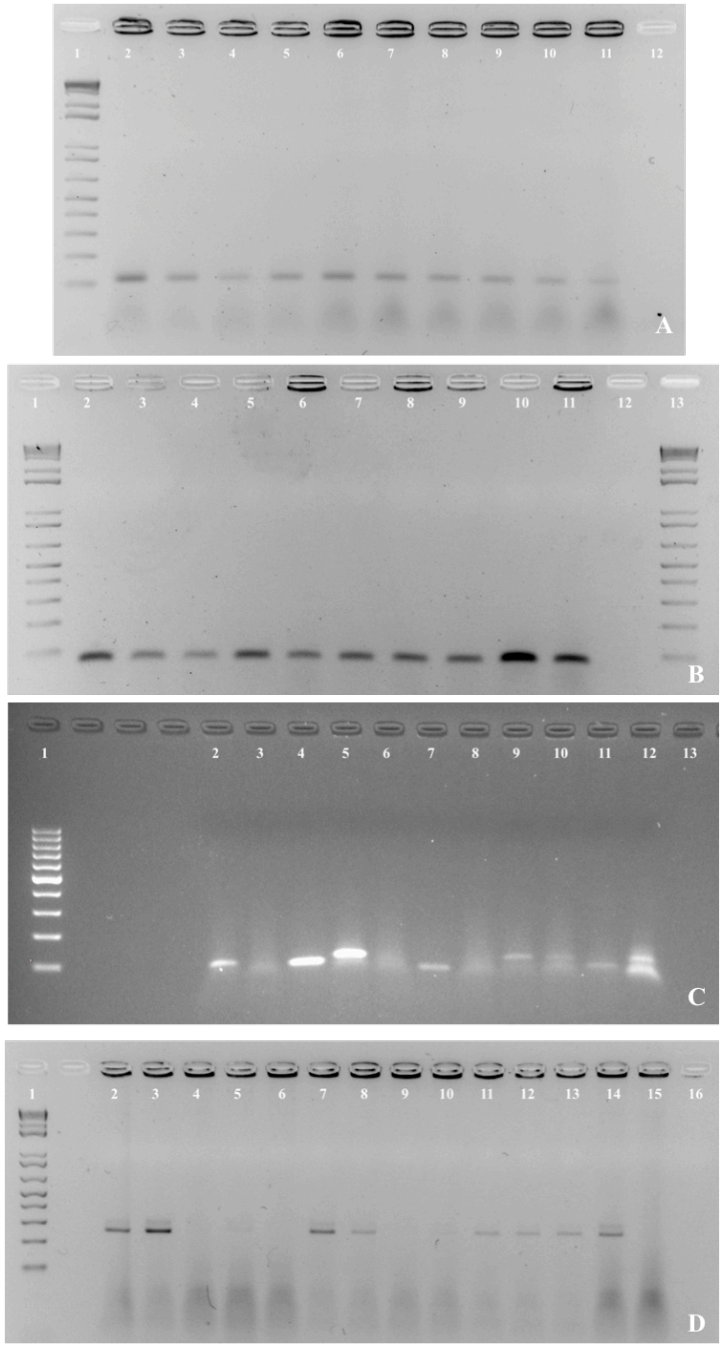

Figure 1 - Agarose gels (1.5 %) electrophoresis of Colony PCR for the ACEI peptides synthetic genes.

A – Colony PCR for gene ACEI_FMK: 1 – 1 kb plus DNA ladder; 2-11 – PCR products of gene ACEI_FMK with expected size (92 bp); 12 - control (mix without DNA template); B - Colony PCR for gene ACEI_SEA: 1- 1Kb plus DNA ladder; 2 to 11- PCR products of gene ACEI_SEA with expected size (59 bp); 12- control (mix without DNA template); 13 – 1 kb plus DNA ladder; C - Colony PCR for gene ACEI_SPIN: 1 – 100 bp DNA ladder; 2-12 - Different sizes for amplified fragments were observed (lane 5 biggest size); 13 – control(mix without DNA template); D – Colony PCR for gene ACEI_CHLTP: 1 – 1 kb plus DNA ladder; 2,3, 7,8 and 11-14 - PCR products of gene ACEI_CHLTP with expected size (233 bp); 16 - control (mix without DNA template).