Filipe Silva Nunes de Oliveira

Heterologous production of oligopeptides with antihypertensive effects in *Lactuca sativa* and *Medicago truncatula*

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Sofia Duque, PhD, ITQB-UNL

Co-orientadores: Sandra Vieira, PhD, ibimed-UA

Júri:

Presidente: Professora Doutora Suasana Filipe Barreiros

Arguente: Doutora Dulce Maria Metelo Fernandes dos Santos

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Ao meu irmão,

Que no sul da alma encontres tudo aquilo
que sempre procuraste

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Resumo

No cenário atual onde as doenças cardiovasculares associadas à hipertensão representam um problema à escala global, a necessidade de terapias alternativas tem vindo a aumentar. A consequente pressão por terapias com menos efeitos secundários, mas com a mesma eficácia e menos dispendiosas, deu a oportunidade ao novo paradigma biotecnológico trazido pelo *molecular pharming*, de ganhar a sua quota de mercado na indústria farmacêutica. O *molecular pharming* permite a produção de proteínas recombinantes com efeito terapêutico em larga escala, de forma segura e a custo reduzido. A *Medicago truncatula e a Lactuca sativa* (alface) são dois sistemas emergentes de produção de proteínas recombinantes. A *M. truncatula* é uma planta leguminosa modelo e, por isso, a sua transformação e manipulação *in vitro* são acessíveis, com a vantagem de uma possível extrapolação para outras leguminosas. A *L. sativa* é uma planta consumida crua um pouco por todo o mundo, o que permite a administração via oral de produtos recombinantes com atividade terapêutica. Neste trabalho, foi analisada a expressão heteróloga de quatro péptidos com atividade anti-hipertensiva comprovada nos sistemas de expressão vegetal *M. truncatula* e alface. A produção dos péptidos inibidores da enzima conversora da angiotensina-I - enzima chave na via metabólica que controla a pressão sanguínea - foi verificada ao nível das folhas nas duas plataformas de produção. De forma a permitir a progressão do trabalho, foi mantido um stock de plantas *in vitro*, e foi criado um banco de sementes que permitirá estudar a sua descendência. A transformação estável das plantas foi confirmada pela presença dos transcritos das sequências codificantes dos péptidos inibidores da enzima conversora da angiotensina I na Medicago. Na alface, foi estabelecido um esquema de seleção *in vitro* baseado no antibiótico Canamicina, para a seleção de sementes putativamente transformadas, que foram posteriormente analisadas ao nível genómico. Posteriormente, foi iniciada a otimização do protocolo de extração de proteínas, de forma a possibilitar a sua futura deteção e quantificação através das técnicas ELISA, SDS-PAGE, w*estern immunoblotting* e HPLC.

Palavras-chave: *Molecular pharming*, *Medicago truncatula*, *Lactuca sativa,* hipertensão, enzima conversora da angiotensina I, péptidos inibidores de ACE.

Abstract

In the current scenario of a worldwide problematic situation related to cardiovascular diseases associated with hypertension, the necessity for alternative therapies has become a great challenge. The need for therapies with little side effects and with the same efficacy, but less expensive, has given the opportunity to the new biotechnological paradigm brought by molecular pharming to win its market share from the established pharmaceutical industry. Molecular pharming is a cost-effective, scalable and safe system to produce high-quality and biologically active recombinant therapeutic proteins. *Medicago truncatula* and *Lactuca sativa* (lettuce) are two emerging plant hosts for molecular pharming. *M. truncatula* is a model legume plant amenable for transformation and *in vitro* manipulation, along with the procedural extrapolation to other legume species. *L. sativa* is a worldwide raw edible plant which allows the oral delivery of recombinant therapeutic products. In the present work, the heterologous production of four ACEI peptides - that have proven antihypertensive effect – in Medicago and lettuce hosts was analyzed. The ACEI peptides production was verified at leaf level in the two plant production hosts. An *in vitro* plant stock was kept to allow the progression of the work, and a seed bank was established to allow further studies in the progeny. The presence of the ACEI coding sequences transcripts in Medicago RNA samples confirmed the stable transformation of this host. In the lettuce host, an *in vitro* seed selection scheme based on kanamycin was established to screen ACEI transformants which were further analyzed at genomic DNA level confirming the stable plant transformation with ACEI coding sequences. At protein production level, protocols for the peptide extraction, along with ELISA, SDS-PAGE, western immunoblotting, and HPLC were established for further detection and quantification of ACEI peptide production.

Keywords: Molecular pharming, *Medicago truncatula*, *Lactuca sativa,* hypertension, angiotensin-I converting enzyme, ACE inhibitory peptides.

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List of abbreviations

ACE	Angiotensin-I converting enzyme
ACEI	Angiotensin-I converting enzyme inhibitor
ACEI_CHLTP	Synthetic coding sequence for ACEI peptide derived from *Chlorella vulgaris* digest and chloroplast transit peptide
ACEI_FMK	Synthetic coding sequence for ACEI peptide released during milk fermentation by *Enterococcus faecalis*
ACEI_SEA	Synthetic coding sequence for ACEI peptide derived from sea cucumber hydrolysate
ACEI_SPI	Synthetic coding sequence for ACEI peptide isolated from the pepsin-pancreatin digest of the large subunit of spinach RuBisCO
Act	Actin gene
AP	Alkaline phosphatase
APS	Ammonium persulfate
B_0	Maximum binding solution
CaMV 35S	Cauliflower mosaic virus 35S RNA promoter
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CVD	Cardiovascular diseases
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FDE	Formamide denaturing buffer
GI	Gastrointestinal
GMO	Genetically modified organism
GMP	Good manufactoring practices
HBsAg	Hepatitis B surface antigen
HCl	Hydrogen chloride
hGH	Human growth hormone
His-tag	Poly-histidine tag
HPLC	High performance liquid chromatography

IC_{50}	Half maximal inhibitory concentration
IgA	Immunoglubin A
Kan	Kanamycin
Kan^R	Kanamycin resistant
Kan^S	Kanamycin sensitive
KDEL	Endoplasmic reticulum-retention signal
KNOS	Kinin-nitric oxide system
M910a	Medicago truncatula cv ‘Jemalong’ embryogenic line
mRNA	Messenger RNA
MS	Murashige and Skoog
NaAC	Sodium acetate
NaCl	Sodium chloride
NSB	Non-specific binding solution
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PHB	Polyhydroxybutyrate
RAS	Renin-angiotensin system
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHR	Spontaneously hypertensive rats
sigA	Secretory immunoglubin A
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline and Tween
T-DNA	Transferred DNA region of Ti plamid
TE	Tris-EDTA buffer
TEMED	Tetramethylethylenediamine

TFA Trifluoroacetic acid

Tracer 6x-His linked to alkaline phosphatase

Tris Tris buffer

Ubi-1 Ubiquitin-1 promoter

VLP Virus-like particle

$\chi 2$ Chi-square test

1

Bibliographic Revision

1.1. Modern world & Cardiovascular diseases

At the beginning of the XX century, cardiovascular diseases (CVD) were responsible for less than 10% of deaths worldwide. Nowadays, impressively, it corresponds to nearly one-third of all deaths per year and has been recognized as the leading cause of death worldwide (World Health Organization, 2011). However, death rates are unevenly distributed across the population, with 80% of the burden occurring in low and middle-income countries since these are least able to afford the social and economic consequences of health matters. Among these consequences there are disability, loss of income, premature death and all the related health expenditure that can represent a major problem for families, communities and consequently national finances. In this way, CVD are not only a public health problem but a social and economic problem too. Over the past decades, the early detection and treatment of CVD risk factors like hypertension, have contributed to a decline in mortality in high-income countries (World Health Organization, 2013).

1.1.1. Hypertension: a CVD risk factor

Blood flows through the body in blood vessels pumped by the heart. Each time the heart beats, more blood is pumped, and pressure is created by the force of blood pushing against blood vessels walls, as it flows through them. Hypertension or high blood pressure is a condition in which the pressure of blood in the vessels is persistently elevated, meaning that diastolic pressure and systolic pressure are 140/90 mmHg or above, respectively. This medical condition represents a major cardiovascular disease risk factor but it is also considered as a controllable risk factor (Phelan and Kerins, 2011) since in most of the cases it can be controlled by lifestyle choices like alimentation, although always depending on numerous factors (Roseanne and FitzGerald, 2013) as represented in Figure 1.1.

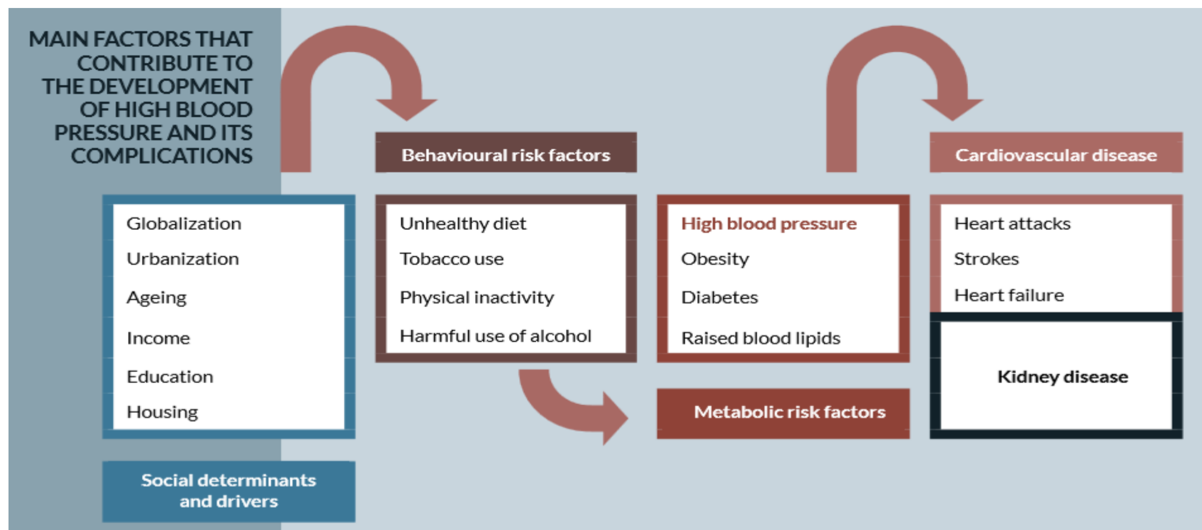

Figure 1.1. Main factors that contribute to the development of high blood pressure and its complications (World Health Organization, 2013)

1.1.2. Current solution and the need for a new paradigm

Although in some cases it is possible to control hypertension by lifestyle choices, in others there is the need for other strategies to cope with the problem. A range of synthetic drugs are available, including diuretics, adrenergic inhibitors such as α and β blockers, direct vasodilators, calcium channel blockers, angiotensin-II receptor blockers and angiotensin-I converting enzyme (ACE) inhibitors (Roseanne and FitzGerald, 2013). Among the synthetic ACE inhibitors there is captopril, enalaprilat and lisonopril; however, their consumption is associated with several side effects including cough, skin rashes, hypotension, loss of taste, angioedema, reduced renal function and fetal abnormalities (Roseanne and FitzGerald, 2013). Also, the cost-effectiveness of these treatments is variable since it is relative to age, sex, level of blood pressure and the presence of other risk factors (Jönsson, 1994). Despite hypertension can be controlled by synthetic drugs, the associated side effects and the high prevalence of the problem worldwide result in a significant and increasing expenditure (Gaziano *et al.*, 2009), therefore new strategies to prevent and deal with CVD and its risk factors have been considered widely.

1.1.3. Bioactive peptides

Besides numerous preventive and therapeutic drug regimens, there has been an increased focus on food-derived bioactive peptides that may contribute to cardiovascular health (Erdmann *et al.*, 2008). Food-derived biopeptides or peptides with biological activity have been described as food components that exert a physiological effect in the body beyond their nutritional value. These biopeptides can be found in diverse candidate proteins that contain these latent biological activities from sources as milk, eggs, meat and fish, as well as different plants such as soy and wheat. Their

biological activity can occur in a direct manner, just by the presence of the food components, or by the release of the bioactive peptide by hydrolysis of its original parent proteins (e.g. during gastrointestinal (GI) digestion or during food processing) (Hartmann and Meisel, 2007; Phelan and Kerins, 2011). Once the bioactive peptides are liberated, they may act as physiological modulators with hormone-like activity. Different health effects have been attributed to food-derived peptides, including antihypertensive activity by the inhibition of the ACE - one of the key enzymes in the blood pressure control metabolic pathway. Thus, the biological activity of these peptides has been recognized and used as a functional food to cope with the problem of hypertension (Roseanne and FitzGerald, 2013). Bioactive peptides with anti-hypertensive activity can be produced by biotechnological means with great advantages.

1.2. Biotechnology – New technological paradigm

The interaction between biology and technology has been promoted by humans since long ago. However, it's since 1919 when Karl Ereky used for the first time the term Biotechnology (Kennedy, 1992) that it became one of the technologies that attracted a greatest attention. It results from the combination of natural and engineering sciences using biological systems, cells or derivates in order to modify products or processes for a specific use (United Nations, 1992; European Federation of Biotechnology, 1989). But it is when compared to more conventional processes as chemical synthesis, that Biotechnology presents its major advantages, by the lower or none generation of residues, lower CO_2 emission, lower resources consumption like H_2O or energy, providing systems with higher overall efficiency (Figure 1.2.). Consequently, from substantially affecting healthcare, production of materials and chemicals, agriculture and forestry, environmental protection, and production and processing of food, Biotechnology has proved capable of generating enormous wealth with influence in every significant sector of the economy (Gavrilescu and Chisti, 2005).

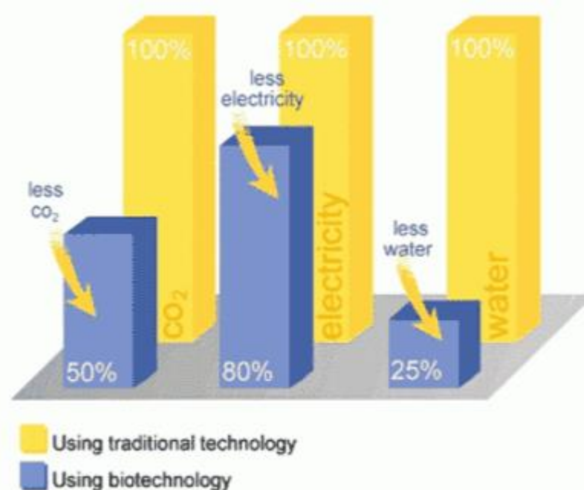

Figure 1.2. Conventional processes versus biotechnological processes when referring to antibiotics intermediate processing (in SusChem - European Technology Platform for Sustainable Chemistry).

1.2.1. Introduction to plant biotechnology

Since the dawn of human race, plants have been used by humans for food, fibers, wood and therapeutic purposes (Boehm, 2007). Ten thousand years ago humans started the transition from nomad to a sedentary lifestyle, changing from hunting and gathering to agriculture. In the early beginning of agriculture, humans started to domesticate certain wild plants, and 4000 years ago ancient civilizations had completed the domestication of all major crop species, including rice, maize and wheat (Doebley *et al.*, 2006). Domestication is a complex evolutionary process where ancient farmers used to select individuals with specific desirable traits, like larger fruit size, desired color or plant behavior (Purugganan and Fuller, 2009; Zeder *et al.*, 2006) and used them as progenitors for subsequent plant generations where the desired traits accumulate over time, leading to what is known has the domestication syndrome (Hammer, 1984). Since farmers used only a limited number of individuals of the progeny, much of the generation genetic diversity is left behind causing a genetic bottleneck that reduces genetic diversity over time. This process, known as classical plant breeding, has originated through time plants completely different from its wild relatives (Figure 1.3) (Wright *et al.*, 2006). Thus, humans have been genetically manipulating plants to meet their needs for long ago.

Figure 1.3. Teosinte the wild ancestor of maize as an example of the effect of genetic bottleneck promoted by domestication adapted from (Doebley et al., 2006).

Although traditional plant breeding and selection methods are still widely applied, the fundamental discoveries of Darwin by the hybridization and selection principles, and of Mendel for defining the fundamental association between genotype and phenotype, provided us the scientific basis for modern plant breeding. Alongside with biotechnology, advances in synthetic biology such as the recombinant gene technology allowed a paradigm shift in plant improvement. By enabling the genetic modification of crops, with the introduction or disruption of a specific gene, or a more specific selection of a desirable trait, it was granted a more accurate and less time-consuming plant improvement (Moose and Mumm, 2008). The new plant breeding paradigm and the commercialization of transgenic crops have been gaining popularity worldwide (James, 2007) for contributing to numerous benefits for

sustainable supplies. The first genetically engineered plant to be commercialized was FlavrSavr™ tomato that was developed using an antisense RNA that regulates the expression of the enzyme polygalacturonase, responsible for softening in ripe tomatoes fruits, what subsequently granted longer shelf life to these genetically engineered tomatoes (Kramer and Redenbaugh, 1994). The famous BT-maize is another example of a genetically engineered crop modified to expressed insecticidal proteins from bacterium *Bacillus thuringiensis,* that confer resistance against certain insect pests like *Ostrinia nubilalis*, the European corn borer, allowing benefits of billions of dollars (Hutchison *et al.*, 2010). One of the most remarkable examples of a modified crop is the notable case of golden rice, engineered with the biosynthetic pathway of provitamin A (β-Carotene) compensating the lack of this essential nutrient in at least 26 countries (Ye *et al.*, 2000). Over the last decades, the number of transgenic crops cultivated worldwide have been steadily increasing as a reflection of the fact that biotech crops have consistently been beneficial for the economy, environment and health to small and large farmers in low, medium and high-income countries (James, 2007).

1.2.2. Molecular pharming

In the 1950s, biomedical research started to unveil the molecular mechanisms of both health and disease, by pinpointing a range of proteins produced naturally in the cells that could have potential therapeutic applications. This, alongside with the discovery that DNA is the molecule responsible for encoding these proteins, which in turn control relevant cellular processes in the organism, provided the impetus for the Biotechnology era. Some examples of these proteins include interferons and interleukins for immune response regulation, growth factors such as erythropoietin responsible for red blood cell production, and neurotrophic factors that regulate the development and maintenance of neural tissue. The pharmaceutical potential of these proteins was widely recognized, but their medical application was in most cases impractical, due to several unworkable reasons such as the small quantities in which they are naturally produced in the body, their complexity, and difficulty in extracting and purifying from living tissue (Crommelin and Sidelar, 2002; Wafelman, 1999). Several difficulties were overcome by the emergence of genetic engineering with the recombinant DNA technology (Ma *et al.*, 2003). This technology has had several positive effects in the production of pharmaceutically relevant proteins, by overcoming crucial problems within its production, including i) source availability, since many proteins with therapeutic potential are naturally produced in the cells in modest quantities, hindering their extraction to meet the clinical demand; ii) product safety, since in the past the direct extraction of products from its native source occasionally led to the transmission of diseases or they come from a downright dangerous source (as for instance the pharmaceutical production of Ancrod, composed of a protein that displays anti-coagulant activity and naturally produced by the Malaysian pit viper; in cases like this the recombinant production in less dangerous organisms like *Escherichia coli* is quite preferable); iii) last but not least, the designing of engineered therapeutic proteins displaying advantages over the native protein becomes a reality with the help of techniques such as site-directed mutagenesis, which allows the introduction of alterations in a protein's amino acid sequence by the

insertion, deletion or alteration of the correspondent DNA coding sequence enabling, for instance, the creation of a novel hybrid protein or the deletion of an entire protein domain (Wafelman, 1999). Morover, the recent CRISPR/Cas9 target genome editing technology, using engineered nucleases, allows a facile, rapid and efficient DNA modification in a wide variety of biomedically important cell types and novel organisms (Sander and Joung, 2014)

The heterologous production of these high-value proteins from several hosts such as bacteria, yeast, plants, animals, and many others is designated by molecular pharming. This term arose in the literature in the 1980s as a portmanteau of farming and pharmaceutical, and nowadays is mainly employed to refer the recombinant production of pharmaceutically relevant proteins and other secondary metabolites in plants (Bhatia *et al.*, 2015; Obembe *et al.*, 2011; Paul *et al.*, 2013). Nowadays, molecular pharming represents an extraordinary opportunity to produce cost-effective modern medicines and make them available worldwide (Paul *et al.*, 2014).

1.2.2.1. Heterologous production systems

Nowadays there is a wide selection of expression systems for the heterologous production of recombinant high-value proteins. These transgenic production platforms include: bacteria such as *Escherichia coli* (Fischer *et al.*, 1999); yeast, particularly *Saccharomyces cerevisiae* but also *Pichia Pastoris*; Fungus, mainly from *Aspergillus* genus; baculovirus-mediated insect cell expression systems; a collection of well-characterized mammalian cells such as Chinese hamster ovary cells (CHO) (Abdullah *et al.*, 2008; Paul *et al.*, 2013; Wafelman, 1999); and plant-based systems (Sharma *et al.*, 2004). Each system has its own advantages in relation to cost, ease of use, and post-translational modification profile, and each of its own characteristics should be considered when choosing the expression system.

Bacteria have been generally used as an expression system for the production of recombinant proteins since its straightforward molecular biology and ease of handling results in low production costs. However, the lack of the eukaryotic biochemical apparatus to perform the post-translational modifications required to produce a fully folded functional protein with optimal biological activity, compromises the use of this production platform to produce eukaryotic proteins hence becoming a less attractive solution in the production, for example, of monoclonal antibodies (Fischer *et al.*, 1999). Fungus and yeast, are robust systems with accessible molecular biology with several molecular tools available. However, despite being eukaryotes and post-translational processing is possible, these systems share some of the difficulties faced when using a prokaryotic system, and differences in metabolic pathways, protein processing, codon usage, and the formation of inclusion bodies, make them less ideal systems to produce mammalian proteins (Giddings *et al.*, 2000; Merlin *et al.*, 2014; Smith, 1996). Several recombinant therapeutic proteins are produced using insect and mammalian heterologous expression systems, and their major advantage is the capability to correctly synthesize and process fully folded mammalian proteins (Giddings *et al.*, 2000; Khan, 2013). On the other hand, these expression systems present certain disadvantages, as product yields are generally low and the

requirement for fetal bovine serum in the growth medium renders the production expensive (Ganz *et al.*, 1996). Moreover, mammalian culture cells are quite sensitive to variations on temperature, pH, dissolved oxygen, certain metabolites and shear forces, making its use as producing host a challenge, especially at industrial-scale since variation in cell growth can negatively affect the product's production and purity (Giddings *et al.*, 2000).

Although some differences exist between plants and mammals, namely in post-translation processing and in codon usage, these are a fewer in comparison with differences between mammals and microorganisms. By having the eukaryotic apparatus for post-translational modifications, safety advantages due to the fact that plant cells are not sensitive to mammalian pathogens (Twyman *et al.*, 2003), potentially high protein production levels and low production costs when compared with mammalian production platforms (Fischer and Emans, 2000), plant-based production platforms offer several interesting advantages as an alternative production system to the well-established traditional expression systems (Table 1.1).

Table 1.1. Comparison of features of recombinant protein production in plants, yeast and conventional systems (Fischer et al., 1999).

Abbreviation: sigA, secretory IgA.

	Transgenic plants	Plant viruses	Yeast	Bateria	Mammalian-cell cultures	Transgenic animals
Risk *	Unknown	Unknown	Unknown	Yes	Yes	Yes
Production cost	Low	Low	Medium	Medium	High	High
Time effort	High	Low	Medium	Low	High	High
Scale-up cost	Low (unlimited biomass)	Low (unlimited biomass)	High (+)	High (+)	High (+)	High
Production	Worldwide	Worldwide	Limited	Limited	Limited	Limited
Product yields	High	Very high	High	Medium	Medium-high	High
Folding accuracy	High?	Medium	Medium	Low	High	Low
Product homogeneity	High?	Medium	Medium	Low	Medium	Low
Multimeric protein assembly (sigA)	Yes	No	No	No	No	Yes
Gene size	Not limited	Limited	Unknown	Unknown	Limited	Limited
Glycosylation	"Correct"?	"Correct"?	Wrong	Missing	"Correct"?	"Correct"
Product vehicle	Yes	Yes	Yes	Yes	Yes	Yes
Delivery vehicle	Possible	Possible	No	No	No	Yes
Storage	Cheap/room temperatura	Cheap/-20ºC	Cheap/-20ºC	Cheap/-20ºC	Expensive/N2	?/N2
Distribution	Easy	Easy	Feasible	Feasible	Difficult	Difficult
Ethical concerns	Medium	Medium	Medium	Medium	Medium	High

*** Residual viral sequences, oncogenes and endotoxins**
(+) In terms of large fermenters, etc.

1.2.2.2. Plants as molecular factories

For its numerous advantages over traditional expression systems, the use of plants for the heterologous production of high-value proteins has been magnetizing the attention of both farmers and scientists for agriculture and molecular pharming purposes (Dong *et al.*, 2014).

The roots of molecular pharming go back to the early 1980s when it was demonstrated that genes could be inserted into the plant genome using a species of soil bacteria called *Agrobacterium tumefaciens*. It was demonstrated not only that the insertion was possible, but also that the plant successfully expressed bacterial genes, and the inserted genes were inherited and expressed by subsequent generations of plants (Bevan *et al.*, 1983; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983).

In 1986, Barta *et al.* demonstrated that tobacco and sunflower callus tissue expressed transcripts of a human growth hormone (hGH) fusion gene and, although no protein was detected since the processing signals of the hGH pre-mRNA were not recognized *in planta,* it was demonstrated for the first time that plants could express human genes. The idea that plants could be used as a recombinant therapeutic protein production system was thus established. Later on, Hiatt *et al. (1*989) reported the first recombinant antibody produced in transgenic plants expressed in the progeny of the cross of two individual transgenic plants. This was crucial since it revealed the ability of plants to produce complex functional mammalian proteins with pharmaceutical relevance. In 1990, the production of the first native human protein, human serum albumin, in tobacco and potato, confirmed once more the structural authenticity of plant-derived recombinant proteins (Sijmons *et al.*, 1990).

Althought plants have proven its potential and have been experimentally used to produce recombinant pharmaceutical proteins since 1989, the first commercial product was not launched until 1997. The first recombinant protein produced for commercial purpose was avidin and, although it has a modest commercial value, it demonstrated that plants could be turned into biofactories for the large-scale production of recombinant proteins (Hood *et al.*, 1997). Over the years it has been proven that plants have the ability to produce complex functional mammalian proteins with therapeutic activity, such as growth regulators, vaccines, cytokines, enzymes and hormones, alongside with a wide range of other recombinant products (Figure 1.4.) (Liénard *et al.*, 2007).

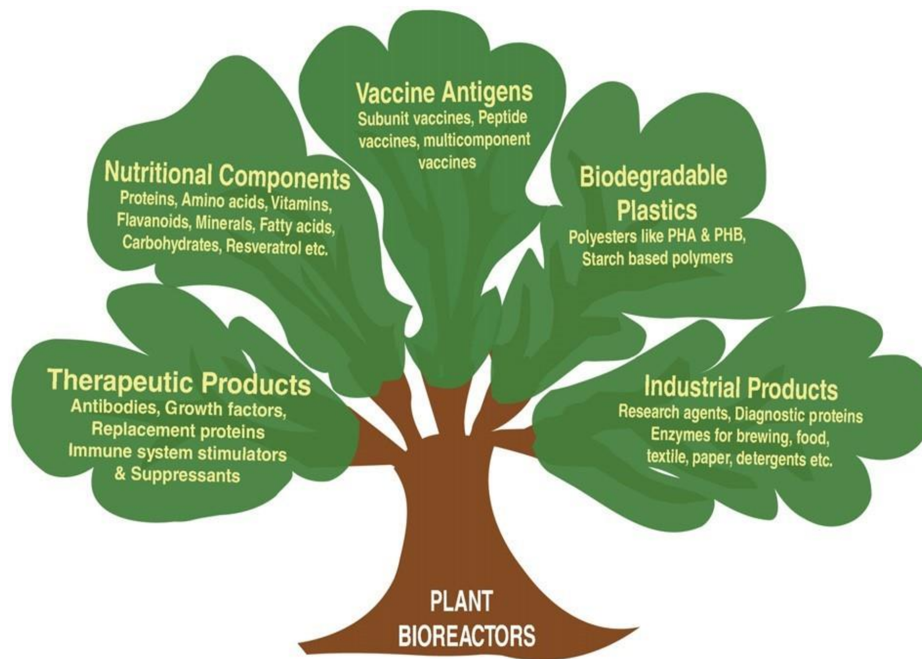

Figure 1.4. Diagram depicting recombinant products using plants as bioreactors (Sharma and Sharma, 2009).

Since the first encouraging results, plants proved to be a promising production platform, and those working in the field of molecular pharming have been laboring to bridge the gap between its scientific principle and its commercial potential (Fischer *et al.*, 2013).

In the 1990s there was a flurry of commercial activity materializing the advantages that plants present in comparison to the established platforms. These advantages included (i) minimal upfront investment to establish and maintain pharmaceutical crops when compared to building and operating an Industrial-scale fermentation reactor; (ii) easy scale-up compared to fermenters; (iii) safety production profile, considering that plants are not susceptible to mammalian pathogens; (iv) immense diversity of hosts and platforms to meet each production specify needs (Fischer *et al.*, 2012; Ma *et al.*, 2003; Twyman *et al.*, 2003).

Despite the promising results, the expectation that plants could compete for a market share over the well-established biopharmaceutical platforms, such as CHO cells, proved to be unrealistic. The technical limitations of plants, especially their lower yields, allied with the great existing investment in fermentation infrastructure, alongside with unfavorable public opinion on genetically modified organisms (GMO), regulatory uncertainty, and concerns from the mainstream pharmaceutical industry, led to the burst of the 1990s molecular pharming bubble, only reevaluated a decade later (Fischer *et al.*, 2013; Spök *et al.*, 2008).

One of the most appealing advantages of molecular pharming was the wide diversity of platforms and techniques available with distinct and overlapping benefits. But this also worked as one of its most significant weaknesses in its commercial development. While biopharmaceutical industry has always focused on a small number of standardized platforms and invested in maximizing their performance, molecular pharming offered diverse platforms and techniques chosen to match the specific

requirements of the recombinant products. The platforms range from plant cells growing in bioreactors or just simple plants growing in synthetic medium, to whole plants growing in soil or hydroponics, and techniques to stable transgene integration into nuclear or plastid genome, to transient expression using bacterial, viral or hybrid vectors. This also meant that it became a fragmented discipline, with no driving force establishing molecular pharming as a competitive platform to meet industry demands for high yields, standardized platforms and regulatory compliance (Fischer *et al.*, 2012, 2013; Twyman *et al.*, 2003)

The commercial implementation obstacles faced by molecular pharming in its early years allowed a change in the early vision of it as replacement of biopharmaceutical current standards. Although still valid, the idea of a platform of highly scalable production at a fraction of the cost of conventional systems shifted to a production system for a niche of products that are not easily produced by the conventional systems. Since this paradigm shift, the focus has been on a small number of platforms, so that they become more competitive and could meet the increasing global demand for biopharmaceutical products (Paul *et al.*, 2013, 2014).

During the last decade the biopharmaceutical industry restored its interest in molecular pharming and, as a result, in 2012 and important breakthrough was achieved when the first recombinant plant-derived therapeutic was approved for human use, the enzyme taliglucerase alfa developed by Protalix Biotherapeutics and commercialized under the name Elelyso® (Zimran *et al.*, 2013). This enzyme is a carrot (*Daucus carota L.*) cell-expressed human recombinant β-glucocerebrosidase, used for the treatment of Gaucher's disease, a lysosomal storage disease (Aviezer *et al.*, 2009). This enzyme represents a clear example of how molecular pharming can overcome the difficulties of the standardized production methods. Initially, the enzyme replacement therapy of glucocerebrosidase was performed using a placenta-extracted enzyme, the alglucerase (Barton *et al.*, 1991), that was subsequently replaced by a recombinant form denominated imiglucerase (Elstein and Zimran, 2009) expressed in CHO cells and that has been used as standard treatment for the past 15 years. However, the production using CHO cells platform is expensive (Beutler, 2006; Futerman *et al.*, 2004) and raises serious security concerns, since a viral contamination already occurred in 2009-2010 at one of its production facilities causing a global shortage (Steinbrook, 2009). Alternatively, the plant-based platform is safer, and unlike imiglucerase, the taliglucerase alfa does not require subsequent exposure of mannose residues to obtain its final structure. Therefore, the downstream process is less time-consuming, and the overall process has reduced production costs (Shaaltiel *et al.*, 2007). Thus, taliglucerase is a great example of how the advantages of a plant-based platform can overcome several issues presented by the traditional platforms. After the successful example of taliglucerase, there has been a continuous increase in clinical trial applications, as for example for insulin produced in safflower approved by the European Union, and also an increase in the manufacturing capacity correlated with a more favorable regulatory reality concerning plant-derived pharmaceuticals (Stoger *et al.*, 2014).

In Figure 1.5 it is chronologically represented the major landmarks in the commercial development of molecular pharming.

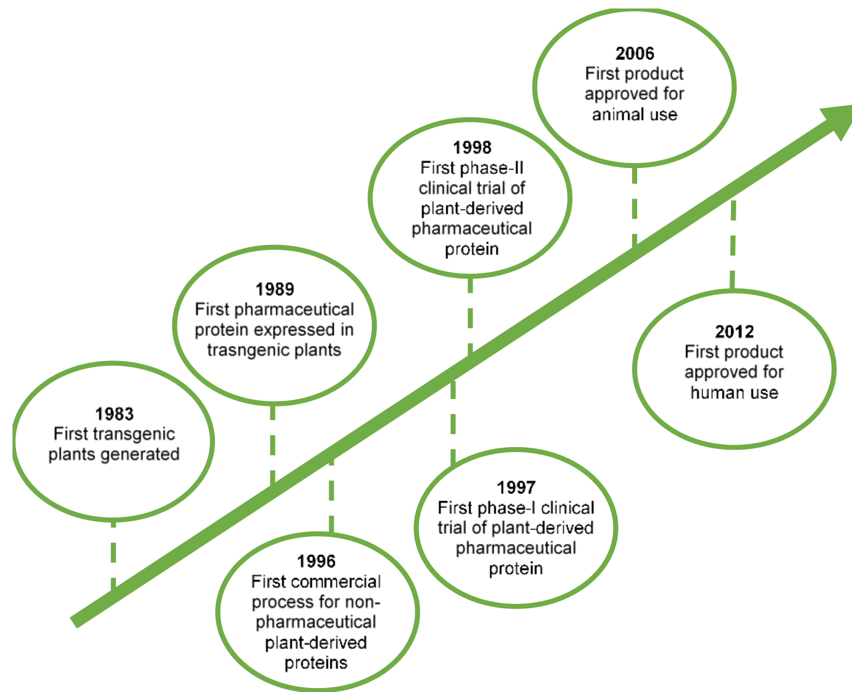

Figure 1.5. Major landmarks in the commercial development of molecular pharming, adapted from Fischer et al., 2013.

Considering the successful and promising results followed by the fast-growing market for recombinant biopharmaceuticals, it is fair to say that molecular pharming provides several unique opportunities within the pharmaceuticals market. However, given the extensive existing investment in industrial fermentation infrastructure, the high product quality, process robustness and regulatory confidence achieved through decades of improvements, it is unlike that plants take over the conventional production platforms, at least in the near future. Thus, acknowledging that plants provide a production platform with full posttranslational modification potential, simple growth demands, theoretically unlimited scalability when considering field-grown plants and accompanied by biosafety guarantee, plant-based production platforms can provide a unique selling point granting the profitable production of biopharmaceuticals that cannot or are difficult to be produced in conventional platforms. Therefore, molecular pharming has been evolving as a disruptive technology seeking to take its own market share not by incremental improvements but by overturning standardized technologies as a new production paradigm (Fischer *et al.*, 2013; Sabalza *et al.*, 2014; Stoger *et al.*, 2014).

1.2.3. Plant-based platforms

Plants can produce a broad variety of products and each one has its own requirements for production. Since molecular pharming embraces several host species, along with several overlapping technologies, with different advantages and limitations, their selection should be carefully considered

depending on product characteristics, intended use, as well as economic considerations (Sharma and Sharma, 2009).

1.2.3.1. Transgenic plants – stable nuclear transformation

Stable nuclear transformation is the most widely used method to produce recombinant proteins (Giddings *et al.*, 2000). It is characterized by the integration of a gene(s) of interest into the plant nuclear genome, leading to its expression alongside with the host genome, thereby conferring the stable inheritance of the trait of interest. To achieve this, the transgene is inserted into an expression construct that commonly includes a promoter, regulatory elements that ensure efficient RNA processing and protein synthesis, including a polyadenylation signal (Commandeur and Al, 2003). The transgene delivery into the host plant is done using either the soil pathogen *Agrobacterium tumefaciens*, commonly to transform dicotyledon species (Horsch *et al.*, 1985) or by biolistic direct transfer of genes, as particle bombardment using DNA coated gold or tungsten beads, more used to monocotyledon species (Christou, 1993).

The stable nuclear transformation encompasses several advantages and disadvantages as a platform for molecular pharming. Once a transgenic plant line has been established it becomes a permanent genetic resource, in contrast to transient expression platforms where each round of production requires a transgene transformation step. Consequently, transgenic plants are the most scalable production platform, since each line can be used to produce seeds increasing the number of plants in each generation and, therefore, the production of recombinant proteins in transgenic plants is theoretically unlimited only dependent on the number of arable hectares. The transgenic seeds can also be used to establish seed banks, working both as a genetic storage unit and/or as recombinant protein long-term storage since the seed desiccation environment enhances protein stability by allowing less conformational modifications. Interestingly, one of its major advantages becomes a considerable drawback since the transgenic plant development and scale-up have long lead times with unreliable production yields. Moreover, the potential spread of transgenic crops in the environment and into the food chain, by outcrossing and seed dispersal, raise ethical and biosafety concerns (Ma *et al.*, 2003; Twyman *et al.*, 2005, 2003).

There is a great variety of plant species that can be used as transgenic platforms, which can be grouped into four major categories: leafy crops, seeds, fruits and vegetables.

Leafy crops are characterized by three major advantages, biomass yields, high soluble protein levels and biosafety convenience since leaf harvesting does not need a flowering stage and therefore the risk of contamination through pollen or seed dispersal is significantly reduced (Conley *et al.*, 2011; Twyman *et al.*, 2003). However, given that in leafy crops proteins are synthesized in an aqueous environment, which is more prone to protein degradation, lower production yields are expected thereby representing a major setback for this platform. In fact, within leaf cells there are large cytoplasmic vacuolar compartments containing numerous active proteolytic enzymes involved in the degradation of native and foreign proteins, even after harvesting or during downstream processing

(Müntz, 2007). This situation is particularly problematic if the products are short peptides that have an inherent instability in plant cells (Lico *et al.*, 2012). To avoid this hurdle, the leaves must be processed immediately after harvested or transported as dried or frozen material, which results in a very limited shelf life (Ma *et al.*, 2011). Furthermore, heterologous protein expression in plant aerial parts can affect the normal growth and development of the host plant (Makhzoum *et al.*, 2014).

Tobacco has been the most widely used leafy crop for molecular pharming for several reasons such as high biomass yield, well-established technology for gene transfer and expression, year-round growth and harvesting, and the already existing large-scale infrastructure for processing. Furthermore, tobacco has a major biosafety advantage since it has little risk of contaminating the food chain because it is a non-edible crop. However, many tobacco cultivars contain high levels of toxic alkaloids, as nicotine, and phenolic substances that have to be removed during the subsequent purification process. Since this downstream process can represent 80% of the product cost, tobacco varieties with low alkaloids and phenolic levels have been produced to overcome this production setback (Conley *et al.*, 2009; Kusnadi *et al.*, 1997). Other examples of leafy crops that are suitable hosts for molecular pharming are alfalfa, lettuce, soybean and (Fischer *et al.*, 2004; Xu *et al.*, 2012).

Although leafy crops are advantageous in terms of biomass yield, plant seeds have the potential to overcome the limitations of protein stability and storage compared to leafy crops platforms (Ma *et al.*, 2003). Seeds offer several advantages, in particular they provide the adequate biochemical environment for protein accumulation, since its desiccation state promotes the decrease in proteases activity thus protecting recombinant proteins against proteolytic degradation (Boothe *et al.*, 2010). It has been demonstrated that seeds are capable to stably storing recombinant proteins, as they are able to maintain their own storage proteins without requiring specialized subcellular targeting. Reports have demonstrated that hirudin, a naturally occurring peptide of leeches that has a blood anticoagulant property, and also antibodies, still remain stable for up to three years at room temperature and for at least three years at refrigerator temperature without detectable loss of activity (Boothe *et al.*, 1997; Larrick and Thomas, 2001; Stöger *et al.*, 2000). This degree of stability means several major advantages for the industrial-scale production of recombinant products such as vaccines, where some cooling requirements could be circumvented (Nochi *et al.*, 2007). Moreover, this stable storage characteristic also allows the complete decoupling of the cultivation cycle from the downstream processing (Boothe *et al.*, 2010). The small size of most seeds allows obtaining a high protein concentration in a small volume, facilitating the downstream process and thereby reducing it significative cost. Therefore, seed-based platforms can be competitive in situations that require large volumes of recombinant proteins per annum (Boothe *et al.*, 2010; Nochi *et al.*, 2007). Furthermore, proteins expressed in seeds do not normally interfere with vegetative plant growth, and have the biosafety advantage of reducing its exposure to herbivores and other non-target organisms (Twyman *et al.*, 2003). Finally, the seed-based system allows the oral delivery of therapeutic products (Nochi *et al.*, 2007).

Several crops have been used as expression hosts for seed-based production. These include cereals such as rice, wheat, barley, soybean and maize, legumes such as pea and soybean, and

oilseeds such as safflower and rapeseed. Maize was the first plant species used for commercial molecular pharming. Since it offers several appealing advantages as the highest biomass yield among food crops but lower overall production costs, ease of transformation and in vitro manipulation, and easiness of scale-up, maize offers the highest potential recombinant yields per hectare and therefore it is generally considered a good production platform for recombinant proteins (Giddings *et al.*, 2000; Hood *et al.*, 1997; Ramessar *et al.*, 2008). Maize has been used for the production of recombinant avidin (Hood *et al.*, 1997), β-glucuronidase (Witcher *et al.*, 1998), recombinant antibodies (Hood *et al.*, 2002), laccase, trypsin and aprotinin (Hood, 2002). The foremost disadvantage of using maize for recombinant protein production is its cross-pollinating nature which represents a considerable risk of contaminating other maize crops (Spök, 2007).

Rice is also a strong candidate as a seed-based production platform. It shares several advantages of the maize platform, well-established transformation technology, high grain yield with agricultural and processing infrastructure, and high protein content. However, unlike maize, rice is a self-pollinated crop, which reduces the risk of gene flow. Furthermore, the endosperm tissue in rice, where proteins accumulate, accounts for more than 90% of the total seed weight (Takaiwa *et al.*, 2007; Yamagata and Tanaka, 1986). A comparison of rice with the successful case of tobacco revealed that despite tobacco has the highest overall yields due to various cropping cycles per year, rice had the higher yields per unit of biomass (Stoger *et al.*, 2002). Rice has been used as a production platform for several recombinant pharmaceutical proteins. Ventria Bioscience, in its ExpressTec platform has been producing human albumin, transferrin, lactoferrin and lysozyme, and vaccines against rabies and Lyme disease. The promising therapeutic candidate VEN100, where Lactoferrin works as an active ingredient that significantly reduces antibiotic-associated diarrhea in high-risk patients, has recently completed phase II clinical trials (Laffan *et al.*, 2011).

Barley is less widely grown when compared to other cereal crops and it is less amenable to transformation. But like rice, barley has the advantage of being self-pollinating and thus the risk of contamination by outcrossing is unlikely. Therefore, despite only marginal interest in this crop, several companies have adopted it as a production platform. ORF Genetics have selected barley as seed-based production platform to produce human-like growth factors, thereby bypassing the use of bacterial or animal cell systems. Matltagen and Ventria Bioscience are other two molecular pharming companies carrying out field trials to produce lactoferrin and lysozyme in transgenic barley (Mäkinen and Nuutila, 2004; Mrízová *et al.*, 2014). Furthermore, over the last decade some progress has been accomplished in the development of reliable gene transfer and expression procedures and there have been some encouraging reports of recombinant yields in transgenic barley (Mrízová *et al.*, 2014; Ramessar *et al.*, 2008).

Legume seeds can also be considered an interesting and promising production system because of their high protein content (20-40%), which can be exploited to produce high yields of recombinant proteins. Soybean share the advantages of a self-pollinating crop combined with a low production cost and, thereby its efficacy in expressing human growth hormone, a humanized antibody against herpes simplex virus, and a functional hypotensive peptide that reduces the systolic blood pressures of a mice

model, has been explored (Russell *et al.*, 2005; Yamada *et al.*, 2008; Zeitlin *et al.*, 1998). Such as soybean, peas present similar advantages and, although there are a few studies reporting the use of pea seeds for commercial molecular pharming, transgenic pea seed are being used for the production of single-chain Fv fragment antibody (Perrin *et al.*, 2000).

The final main category of transgenic crops in molecular pharming corresponds to fruits and vegetables. Considering that recombinant proteins accumulate in edible storage organs and these organs can be consumed uncooked, unprocessed or as partially-processed material, fruits and vegetables consumed raw are the ideal production platform when the product is heat sensitive and therefore cannot be cooked before consumption (Rybicki, 2010; Yusibov and Rabindran, 2008). This strategy has been used in several species such as tomato, banana, potato, and lettuce, aiming the delivery of different therapeutic products such as antibodies and oral vaccines, where these platforms represent a niche advantage over the conventional production platforms (Paul *et al.*, 2013). Potatoes are a widely used system for vaccine production and have been administrated in humans in at least three clinical trials until the date (Daniell *et al.*, 2001). This vegetable has lately been evaluated to produce human serum albumin (Farran *et al.*, 2002), tumor necrosis factor α (Ohya *et al.*, 2002) and antibodies (De Wilde *et al.*, 2002). Other production hosts that have been used to express recombinant products include tomato, banana, carrots, lettuce, among other examples. Fruits, particularly the palatable ones, are also an interesting system since they can be eaten raw and can also be lyophilized for long-term storage (Elena *et al.*, 2007).

Oil and fiber crops can be an economically advantageous platform since the production cost can be balanced by adding revenue derived from their secondary products (Commandeur and Al, 2003). Also, oil crops can be useful hosts because their oil bodies can be exploited to simplify downstream processing by facilitating protein isolation (R. Fischer *et al.*, 2004). Oleosins are the principal membrane proteins of oil bodies (Bhatla *et al.*, 2010) and SemBioSys Genetics developed an oleosin-fusion platform in which the target recombinant protein is fused with oleosin and consequently targeted to the oil bodies. Hereafter, the fusion protein can be recovered by a simple extraction procedure of the oil bodies and separated from oleosin by endoprotease digestion (Moloney *et al.*, 2005). An example of the commercial application of this system is the production of hirudin in safflower, which was the first report of an oilseed-derived protein (Parmenter *et al.*, 1995).

Finally, the choice of suitable host plants for molecular pharming is crucial for the success of the production system and, given the increasing demand for accessible and good quality pharmaceutical products, a production platform based on transgenic plants can provide a stable source of pharmaceutical recombinant products with the necessary scalability to face the global demand with competitive production costs.

1.2.3.2. Transplastomic plants

Transplastomic plants represent an interesting alternative to transgenic plants for the production of recombinant pharmaceutical proteins. Transplastomic plants are obtained by the stable

transformation of the plastid genome with a foreign gene via homologous recombination (Lelivelt *et al.*, 2005). Plastid transformation is most commonly obtained by biolistic bombardment of leaves but also via polyethylene glycol (PEG) in the case of, for example, tobacco chloroplasts (Daniell *et al.*, 2002; Golds *et al.*, 1993). The resulting tobacco leaf explants are then regenerated and selected on a selection medium containing spectinomycin alone or in combination with streptomycin, to obtain transplastomic plants with homoplastomic chloroplast transformation in which every chloroplast carries the transgene (Obembe *et al.*, 2011).

The stable integration and expression of transgenes in the plastome encompass several advantages for the production of heterologous proteins. Since the plastid genome is highly polyploid, each plastid has multiple copies of the genome and in each photosynthetic cell there are hundreds or thousands of plastids. In combination with the absence of epigenetic control that could lead to gene silencing, the result is a production platform with remarkable levels of protein expression that in some cases can be up to 70% of the total soluble protein for a proteinaceous antibiotic (Ma *et al.*, 2003; Oey *et al.*, 2009). Another advantage of this expression system is its ability to express several genes in prokaryotic-like operons making it possible to express a trait encoded by several transgenes such as an entire biosynthetic pathway as, for example, the metabolic pathway for the synthesis of the bioplastic polyhydroxybutyrate (PHB) (Meyers *et al.*, 2010; Nakashita *et al.*, 2001). Moreover, given that proteins are not known to be exported from plastids to the cytoplasm and/or other organelles, the recombinant proteins will naturally accumulate in the chloroplast where they are less toxic to the host plant (R. Fischer *et al.*, 2004). Furthermore, chloroplasts have the ability to perform disulfide bonds and can fold human proteins which allows the high-level production of biopharmaceuticals using this platform (Staub *et al.*, 2000). Lastly, since plastids are maternally inherited, pollen does not contain plasted genes and, therefore, the unwanted transgene escape to the environment is avoided (Meyers *et al.*, 2010).

Notwithstanding the great potential of this expression platform, transplastomic plants are still limited in its applications. Although it has been achieved in other plant species such tomato, potato and petunia, routine plastid transformation is only possible in tobacco, and a formidable technical challenge outside solanaceous species (Lelivelt *et al.*, 2005). Another disadvantage of this system is that plastids are not able to carry out glycosylation and therefore could not be used to synthesize glycoproteins (Ma *et al.*, 2003).

Nevertheless, more than 100 transgenes have been successfully integrated and expressed using the plastid platform such as industrially valuable enzymes, biomaterials such as bioplastics, biopharmaceutical proteins as proinsulin (Ruhlman *et al.*, 2007b) and somatotropin (Staub *et al.*, 2000), antibodies, antibiotics, vaccine antigens including tuberculosis vaccine antigens (Lakshmi *et al.*, 2013), along with transgenes that confer advantageous agronomic traits as disease or drought resistance (Verma and Daniell, 2007). Although the concept of chloroplast transformation was developed two decades ago (Daniell *et al.*, 2002), the biotechnological potential of this production platform has yet to achieve commercial success as a reliable alternative system for therapeutic protein production.

1.2.3.3. Transient expression

Alternatives to transgenic and transplastomic plants are often desirable when considering biosafety and the long development times that are often required to establish the first generation of transgenic producer lines. The transient expression system is perhaps the fastest production platform, and since transgenes are not stably integrated into the plant genome it is also considered the safest (Rybicki, 2009). This system takes advantage of plant pathogens properties to infect plants and introduce exogenous genes in plant tissues without stable DNA integration (Kapila *et al.*, 1997). Without genomic DNA integration, the episomal DNA is expressed for only a short period of time remaining transcriptionally competent for several days (Komarova *et al.*, 2010). Currently the most promising transient expression approaches are based on *Agrobacterium tumefaciens*, plant virus or hybrid systems that utilize components of both (MagnICON® Technology) (Obembe *et al.*, 2011). Transient expression systems have been mostly used for verification of expression constructs and to attest recombinant protein stability, but now it has been used routinely for the commercial production of recombinant pharmaceutical proteins (Obembe *et al.*, 2011). The transient expression platform encompasses several advantages including ease of manipulation, speed, low cost and high protein yield (milligram quantities per plant within a few days). Since transient expression does not require chromosomal integration it is not susceptible to position effects, where the expression of the transgenes can be suppressed by the surrounding genomic DNA. In this way, this production platform allows to achieve high protein yield when compared to transgenic plants (Komarova *et al.*, 2010). However, scalability within this platform presents certain challenges since these plants are grown in greenhouses and require infiltration infrastructures. Also, the harvested leaf biomass requires immediate processing and, if the recombinant product is destined to human or animal use, an additional purification step is needed to remove endotoxins derived from the infiltrated *Agrobacterium* (Xu *et al.*, 2012). Transient expression is typically carried out in leaves of *N. benthmiana* (Sheludko *et al.*, 2007) but has also been demonstrated to work in other plants such as potato (Bhaskar *et al.*, 2009), green pea (Green *et al.*, 2009), Arabidopsis (Kim *et al.*, 2009) and lettuce (Negrouk et al., 2005).

This production platform is now being mobilized by industry to efficiently meet the worldwide demand for pharmaceuticals. For example, the Fraunhofer Center for Molecular Biotechnology (Newark, DE) (http://www.fraunhofer.org) developed a scalable and automated plant-based factory for the production of large amounts of pharmaceuticals within weeks using plant viral vectors. Another example is the Medicago Inc. (Quebec, Canada) (http://www.medicago.com) that has developed a Good Manufacturing Practices (GMP) Facility to produce vaccines that are under phase II clinical trials. Terrasphere (http://terraspheresystems.com/) developed a high-density vertical hydroponic cultivation system which can be operated year round to produce large amounts of recombinant proteins (Xu *et al.*, 2012). Another remarkable example of the reliability of this platform for rapid response situations was reported by Medicago Inc during the H1N1 pandemic. They found that the first batches of H1N1 virus-like particles (VLPs) could be produced three weeks after the Center for

Disease Control and Prevention released the new influenza hemagglutinin sequence (D'Aoust *et al.*, 2010). A similar situation was reported for the H5N1 VLP vaccine (Landry *et al.*, 2010).

In conclusion, transient expression production platform offers the possibility to produce large quantities of recombinant proteins in an emerging threat case response time, coupled with many technical promising solutions which seems to favorably compare it with the established biopharmaceutical production platforms, as mammalian or insect-based systems, in quality, cost and scale.

1.2.3.4. Plant cell suspension cultures

Plant cell suspension cultures are undifferentiated plant cells that grow as individual cells or small aggregates in a liquid medium under contained, defined and sterile conditions. These plant cells are obtained by the disaggregation of friable *callus* in shake bottles that are later scaled up for bioreactor-based production. Recombinant protein production can be obtained in two ways, using transgenic explants to derive the cultures or by transforming the *calli* cells after disaggregation, commonly by co-cultivation with *Agrobacterium tumefaciens* (An, 1985). Besides stable transformation of explants the co-cultivation has also been used for the transient expression of proteins (O'Neill *et al.*, 2008).

The regulatory burden and concerns about product safety over traditional expression systems, especially for mammalian-based production platforms, have renewed the interest in plant cell cultures as an alternative platform for complex pharmaceutical proteins. Since plant cell suspension cultures grow under a sterile environment, there is a precise control over growth conditions allowing a batch-to-batch product consistency that, together with the associated "containment" of both production and product, aligns the production process with GMP. Hence, this system provides an acceptable production platform to the established pharmaceutical industry with the additional benefits of complex protein processing when compared to bacteria and yeasts, and greater biosafety when compared to mammalian-based production platform (Hellwig *et al.*, 2004; Xu *et al.*, 2012). Furthermore, targeting the protein into the culture medium simplifies the downstream processes reducing the overall production cost (Pires *et al.*, 2008). However, plant cell suspension culture faces certain bottlenecks namely the lower productivity related to poor growth rates, potentially higher capital costs and somaclonal variation such as chromosomal rearrangements, particularly common in plants regenerated from *calli*. Nevertheless, high expression levels of functional recombinant proteins can be obtained using this platform (Pires *et al.*, 2008). The advantages of containment and product consistency, along with reduced regulatory costs, may partly outweigh the lower productivities and high production costs for the production of valuable pharmaceuticals (Commandeur and Al, 2003).

Several different plant species have been used for the preparation of plant cell suspension cultures, namely domestic crops such as tobacco, rice, tomato, soybean and carrot and others as *Arabidopsis thaliana and Medicago truncatula.* Among them, tobacco has been the most popular source of suspension cells for recombinant protein production because transformation and propagation are simple and well-established. Several examples of proteins of medical relevance

produced in plant cell suspension culture include human serum albumin and human erythropoietin produced using *N. tabacum* suspension culture initiated from transgenic plants, hepatitis B surface antigen (HBsAg) using soybean and *N. tabacum* suspension cultures, and glucocerebrosidase using carrot suspension cells (by Protalix biotherapeutics) (Hellwig *et al.*, 2004; Schillberg *et al.*, 2013). The commercial success of these recombinant proteins, such as glucocerebrosidase, shows the great potential of plant cell suspension culture as a viable production platform for large-scale protein production.

1.2.4. Limitations and optimization of plant production systems

Since the last decade, researchers have been struggling to overcome some of the plant's technical limitations that burst the initial molecular pharming bubble. Challenges such as low yield as a result of low-level transcript expression or instability of the recombinant protein, difficulties in the downstream process namely the low recovery during processing and non-mammalian glycosylation and/or the potential impact of plant-specific glycans, represented the major obstacles for the commercialization of molecular pharming products (Obembe *et al.*, 2011).

1.2.4.1. Upstream production – optimization of plant expression

Product yield is a major parameter for the commercial and economic success of any plant-based production platform and it has been addressed by developing technical approaches to increase transgenes expression and increase recombinant proteins stability. Thus, several techniques have been developed to enhance protein expression, namely designing optimal expression constructs to maximize transcription, where both codon optimization of protein sequences to match the preferences of the host plant and improved mRNA stability can be of great advantage. Other strategies include increasing transgene copy number, targeting the recombinant protein to subcellular compartments allowing its accumulation in a stable form, and using strong tissue-specific promoters. Since there is no generalized solution to maximize protein expression, stability and accumulation, the best approach and plant-based platform should be determined empirically in a case-specific manner (Fischer *et al.*, 2012; Twyman *et al.*, 2013). Optimizing transcript expression is generally done using strong and constitutive promoters. For this purpose, promoters such as the cauliflower mosaic virus 35S RNA promoter (CaMV 35S) and the maize ubiquitin-1 promoter (ubi-1) for dicots and monocots, respectively, are commonly used (Fischer *et al.*, 2004). As an alternative, organ- and tissue-specific promoters are used to target the expression of the recombinant products to tissue or organs such as the tuber, seed and fruits, according to the product-specific needs, as for example edible vaccines in edible species or avoid accumulation of the recombinant product in vegetative organs for the cases in which it might negatively affect the plant growth. Moreover, inducible promoters regulated by either chemical or external stimulus are also a resource to prevent the lethality problem (Obembe *et al.*, 2011). Additionally, it has been reported the use of transcription factors to boost promoters and

enhance the transgene expression level (Yang *et al.*, 2001). Additionally, the expression constructs can be designed to improve transcript stability and translational efficiency. For example, by removing native 5' and 3' untranslated regions from the transgene and introducing 5' untranslated leader sequence of rice polyubiquitin gene RUB13 in the expression construct, the transgene expression levels are enhanced (Lu *et al.*, 2008).

Recombinant proteins stability is another major bottleneck of molecular pharming. Proteins stability can be enhanced by targeting the recombinant proteins to cellular compartments where they are less susceptible to degradation (Fischer *et al.*, 2004). Besides influencing protein stability, protein targeting also affects protein structure and activity and can be useful to simplify the downstream processing by including affinity tags or fusions. For example, proteins that require glycosylation for their optimal activity can be targeted to the secretory pathway since these modifications occur in the endoplasmic reticulum (ER) and Golgi apparatus. It was demonstrated that recombinant antibodies fold one hundred times better in tobacco using this strategy than in the cytosol (Schillberg *et al.*, 1999). Targeting to the secretory pathway is achieved by adding an N-terminal signal sequence in the expression construct that targets the ribosome to signal receptors on the ER. By default, this type of targeting in plants will direct the recombinant proteins to the apoplastic space, where they may be retained or secreted depending on their size (Schillberg *et al.*, 2003). For proteins that do not require post-translational modifications such as glycosylation, they can be targeted for plastids using an N-terminal transit peptide (Kmiec *et al.*, 2014). Moreover, unwanted post-translational modifications promoted by the ER can be avoided by expressing the recombinant protein as a translational fusion with the oleosin protein, thereby targeting the recombinant protein to the oil bodies of the seeds (Boothe *et al.*, 2010).

1.2.4.2. Glycoengineering – Protein "humanization"

One of the most promising aspects of molecular pharming is the ability of plants to perform various posttranslational modifications. Therefore, from the beginning there was a great effort to prove that plants can manufacture identical recombinant products to those produced in mammalian cells. For proteins, such as insulin, that are entirely composed by polypeptides, this idea was right from the start. However, for glycoproteins it was a different story since there are some differences between glycosylation patterns in plants and animal cells (Stoger *et al.*, 2014). Glycosylation is the covalent linkage of sugar moieties to proteins. Plant-derived glycoproteins carry the α(1,3)-fucose and β(1,2)-xylose residues at the N-glycan of their glycoproteins, whereas on many native human glycoproteins α(1,6)-fucose, glucose and sialic acid can be found at theirs N-glycan. Glycosylation affects proteins folding, which subsequently influences several characteristics of the recombinant protein, such as its stability, subcellular targeting, immunogenicity, pharmacokinetic behavior and biological activity (Gomord *et al.*, 2010). For these reasons, and to prevent the problem of immunogenicity and allergic reactions that these differences could potentially cause, the "humanization" of the glycans structures produced in plants has been a topic of major concern in the scientific community.

Two leading strategies to control the glycosylation of recombinant proteins have been developed 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 (Stoger *et al.*, 2014). Subcellular targeting is the most straightforward strategy since it can be applied at the transgene construct level by adding a specific protein signal. The tetrapeptide endoplasmic reticulum-retention signal (KDEL) is an example of a tag that can be used in this way, which targets the recombinant protein to the ER. Similar derivate tags prevent the addition of complex-type glycan structures by avoiding the Golgi apparatus (Obembe *et al.*, 2011). The glycoengineering approach has been applied using a range of different techniques, such as *in vitro* modification of plant-derived recombinant proteins by purified human β(1,4)-galactosyltransferase and sialyltransferase enzymes, conventional mutagenesis or homologous recombination to knock out genes encoding unwanted glycosyl-transferases, RNA interference to suppress the same enzymes, and expression of glycosyltransferases (such as β(1,4)-galactosyltransferase) in transgenic plants to produce "humanized" recombinant proteins with galactose-extended glycans (Yusibov and Rabindran, 2008). Furthermore, even when the recombinant protein function is not related to the glycan structure, it is possible to increase product quality favoring its homogeneity by controlling glycosylation. This can be performed, for example, in proteins targeted to the ER are subjected to homogeneous high-mannose glycans (Stoger *et al.*, 2014).

Finally, in several cases plant-based expression platforms offer significant advantages over traditional expression platforms, when plant glycosylation pattern is not an issue, these platforms can surpass bacterial-based expression systems. Plant-based expression platforms can also surpass the industry gold standard, CHO cells, which carry out posttranslational modifications that are subtly different from those occurring in humans, leaving plant-based platforms with the unparalleled advantages of biosafety, efficacy and homogeneity profiles for the production of recombinant therapeutic products.

1.2.4.3. Downstream processing

In the early days of molecular pharming, researchers were mainly focused on demonstrating that plants could produce functional recombinant pharmaceutical proteins. So, it was only by the time of its first commercialization attempts that it was noticed that the costs of product processing and purification had been overlooked. Downstream processing is the production step where the recombinant protein is isolated and purified from the raw biomass, and currently is an economically critical point since it represents up to 80% (depending on the required level of purity which is higher for clinical-grade materials) of the overall production costs. Additionally, it is also a key component of the regulatory process since it is the production step where the product must reach enough purity and homogeneity without contaminants (Fischer *et al.*, 2012). Hence, the development of new approaches to reduce the downstream process is essential for molecular pharming economic efficiency.

The goal of downstream processing in plant-based production systems is similar to other expression systems, and it is basically to recover the greatest amount of highly purified recombinant protein with the minimal number of steps and at the lowest cost. The standardized approach for downstream processing includes tissue harvesting, protein extraction, purification and formulation. Certain products can be processed using standardized approaches, such as affinity chromatography (e.g., when using a poly-histidine tag (His-tag)) to isolate the recombinant product. However, in many cases it is necessary to develop specific processing steps for each recombinant protein, regarding product properties and/or expression host characteristics. Regarding whole-plant expression systems, usually additional steps have to be added for the removal of fibers, oils and other by-products. This matter does not apply when using plant cell suspension cultures where the recombinant proteins are being secreted to the culture medium. Furthermore, plants secondary metabolites such as the toxic alkaloid nicotine in tobacco, and other by-products such as fibers, oils, and superabundant plant proteins as RuBisCO, work as contaminants and have to be removed. This can be done using methods like adsorption, precipitation and chromatography, and often requires phase portioning and the used of mixtures of organic solvents. For recombinant products that are retained within the cell, this step of contaminants filtration is particularly challenging (Hellwig *et al.*, 2004; Q. Chen, 2008; Stoger *et al.*, 2014). Besides affinity chromatography and protein secretion to the culture medium, other strategies have been adopted to simplify the downstream processing. These strategies include eliminating the plant cell disruption step, protein targeting to oil bodies, plastoglobules or protein bodies, and oral delivery of whole plants, fruits or crude extracts containing the recombinant protein (Lico *et al.*, 2012; Obembe *et al.*, 2011). In conclusion, several purification strategies have been investigated in a case-by-case manner, to facilitate downstream processing and purification, offering a greater economy of scale in order to fulfill the initial potential of unlimited scalability of molecular pharming.

1.3. Angiotensin-I converting enzyme inhibitor (ACEI)

1.3.1. Blood pressure regulating mechanism

Blood pressure regulation is a complex process that involves intertwining metabolic pathways. When considering food-derived peptides, the most studied pathways are the renin-angiotensin system (RAS) and the kinin-nitric oxide system (KNOS), where biopeptides can work in the inhibition of the angiotensin-I converting enzyme (ACE). In the RAS system, ACE plays a crucial role in the regulation of blood pressure (Figure 1.6.) (Roseanne and FitzGerald, 2013). Within this enzymatic cascade, ACE catalyzes the conversion of angiotensin I into the potent vasoconstrictor angiotensin II, which promotes the increase in blood pressure. This enzyme is also involved in the release of aldosterone from the adrenal cortex, which also tends to increment blood pressure. Within the KNOS system, ACE inactivates the vasodilator bradykinin, preventing the decrease in blood pressure (Erdos and Skidgel, 1987; Johnston, 1992).

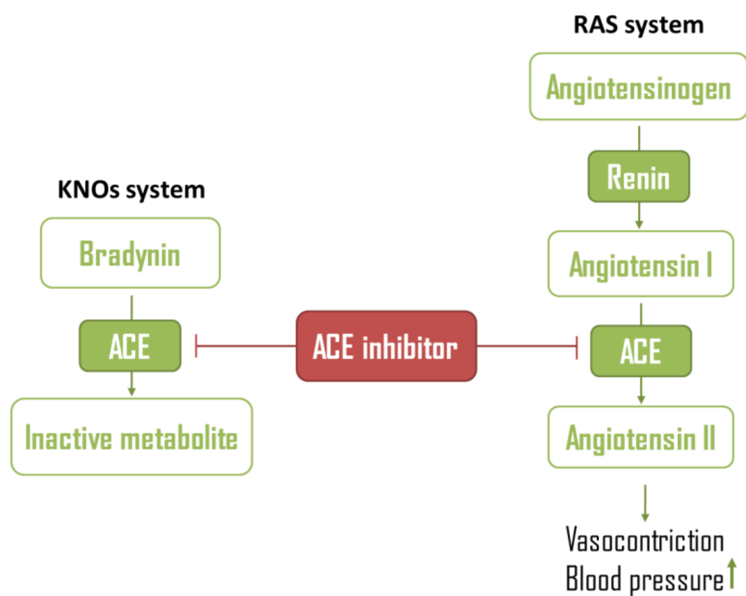

Figure 1.6. The RAS and the KNOS adapted from (Erdmann et al., 2008).

Furthermore, ACE has also been shown to degrade neuropeptides such as enkephalins, neurotensin among others, which may interact with the cardiovascular system (Wyvratt and Patchett, 1985). Considering the important role of this enzyme in blood pressure control, it has been a major target to control hypertension and, therefore, special attention has been given to natural angiotensin I-converting enzyme inhibitory peptides that could be used as a healthier and natural alternative to other type of synthetic ACEI drugs.

1.3.2. ACEI peptides

The first ACE inhibitor to be described was found in snake venom in 1965 (Ondetti *et al.*, 1971). A few years later, Oshima *et al.* (1979) first reported ACEI peptides produced from food protein by digestive proteases. These were the predecessors of many other ACEI peptides found in the most diverse dairy products, from milk and fermented milk products to eggs, plants such as spinach and soybean, and some marine animals as cuttlefish and sea cucumber. Recent examples of ACEI peptides food sources include loach (*Misgurnus anguillicaudatus*) (Li *et al.*, 2012), pork meat (Escudero *et al.*, 2010), lima bean (*Phaseolus lunatus*) (Chel-Guerrero *et al.*, 2012), skate skin (Lee *et al.*, 2011) and boneless chicken leg meat (Terashima *et al.*, 2010).

Most reported ACEI peptides generally contain 2-12 amino acids and have a few shared characteristics, including i) the presence of hydrophobic Pro residues at one or more positions at the C-terminal tripeptide region, which seems to represent a positive influence on peptide's ACE inhibitory activity; ii) among potent inhibitors it is common to find hydrophobic amino acid residues with aromatic or branched side chains at each of the C-terminal tripeptide positions; iii) typically the ACEI peptides with the highest activity have Tyr, Phe, Trp or Pro at their C-terminus (Li *et al.*, 2004; Roseanne and

FitzGerald, 2013). The peptides Thr-Gln-Val-Tyr (Li *et al.*, 2007), Met-Arg-Trp (Yang *et al.*, 2003) and Tyr-Lys-Tyr-Tyr (Suetsuna and Nakano, 2000), from food sources such as rice, spinach and wakame, respectively, are examples of this rule.

ACEI peptides have been generated in several different ways. Commonly, these peptides are produced by enzymatic hydrolysis with GI digestion enzymes including pepsin and trypsin, with enzymatic combinations such as Alcalase™. Likewise, the natural hydrolytic action during GI digestion of additional proteinases, such as chymotrypsin and brush border peptidases, can naturally produce the ACEI peptides (Chabance *et al.*, 1998). These peptides have also been produced by *Lactobacillus* and *Lactococcus lactis* during milk fermentation in cheese production (Okamoto *et al.*, 1995). Furthermore, the production of ACEI peptides was reported in fermented soy products such as soy paste (Shin *et al.*, 2001), sauce (Nakahara *et al.*, 2010), natto and tempeh (Nakahara *et al.*, 2010).

ACEI peptides from various sources can generally be classified into three categories - including inhibitor-type, substrate-type and prodrug-type - accordingly to changes in ACE inhibitory activity after hydrolysis of the peptides by ACE. Inhibitor-type peptides do not have their IC50 values affected by the ACE, therefore peptides in this category such as Ile-Tyr and Ile-Lys-Trp are resistant to cleavage by ACE. Substrate-type peptides are negatively affected by the cleavage of ACE. These peptides are hydrolyzed by ACE, resulting in weaker peptides or with no activity at all. For example, the heptapeptide Phe-Lys-Gly-Arg-Tyr-Tyr-Pro has an IC50 of 0.55 µmol/L and after ACE exposure and consequent hydrolyzation into Phe-Lys-Gly, Arg-Tyr, and Tyr-Pro, the IC50 value increases to 34 µmol/L. Prodrug-type peptides are converted to potent ACE inhibitors after hydrolysis of larger peptide fragments by ACE itself or GI proteases. The result is ACEI peptides with long-lasting hypotensive effect in vivo. For example, a peptide isolated from a thermolysin-digest of Katsuobushi, a Japanese traditional food processed from dried bonito, revealed an 8-fold increase in ACE inhibitory activity when the peptide Leu-Lys-Pro-Asn-Met was ACE-hydrolyzed to produce Leu-Lys-Pro; these results are comparable to the ones of Captopril inhibition (Fujita and Yoshikawa, 1999; Li *et al.*, 2004; Maeno *et al.*, 1996; Roseanne and FitzGerald, 2013). Interestingly, these ACEI peptides do not have significant effects on blood pressure in normotensive subjects, suggesting a convenient therapeutic mechanism that avoids acute hypotensive effects (Rosales-Mendoza *et al.*, 2013).

Besides its numerous advantages, ACEI peptides still need to overcome certain technical obstacles in order to reach their full commercial potential. The high complex mixtures present in ACEI peptides raw samples make its downstream process a real challenge. This, together with the low recovery and the low bioavailability of the peptides produced via *in vitro* enzymatic hydrolysis of their precursor proteins, emphasizes the need for alternative production approaches. Therefore, the use of recombinant DNA technologies for the heterologous production of ACEI peptides appears to be a practical alternative for the large-scale production at a low-cost and in a convenient formulation, for the wide exploitation of the beneficial therapeutic effects of these peptides (Rosales-Mendoza *et al.*, 2013).

The main approach adopted for the heterologous production of ACEI peptides consists in the generation of multimeric proteins containing tandem repeats of these peptides, flanked by protease

recognition sequences that allow their release during protein digestion (Rosales-Mendoza *et al.*, 2013). For example, the ACE inhibitor YG-1 from a yeast glyceraldehyde-3-phosphate dehydrogenase has been expressed with 9, 18 or 27 tandem repeats of itself with a linker between each repeat to allow multimers release by clostripain. This strategy was successfully employed in *Escherichia coli* with convenient yields of 15-67% of total proteins, and with active ACEI peptides been rescued (Park *et al.*, 1998). In another approach, a synthetic coding sequence was assembled to code for the ACE-IP ACEI peptide in the form of 6 tandem repeats in *E. coli*. After acid hydrolysis of inclusion bodies, single peptides were rescued with yields of 105-115 mg peptide/L culture, with the in vitro-derived peptide being equivalent to the natural peptide (Fida *et al.*, 2009).

Besides *E. coli* and other conventional production platforms, plants are an attractive and efficient alternative platform for the heterologous production of ACEI peptides. Rice has been reported as a valuable production platform, where transgenic rice can work as a nutraceutical delivery system. Yang *et al.* (2006) explored the expression of a fusion protein comprising the rice storage protein glutelin and the ovokinin-derived antihypertensive peptide Arg-Pro-Leu-Lys-Pro-Trp, using transgenic rice lines. This recombinant protein was expressed under a selective expression strategy by using an endosperm-specific glutelin promoter. The peptide inhibitory activity was confirmed by significantly reducing the systolic blood pressure of spontaneously hypertensive rats (SHR) after oral administration of the ACEI peptide fraction or transgenic rice seeds. More recently, it was reported an expression construct coding for a protein that comprises 10 or 18 tandem repeats of novokinin combined with the KDEL signal and the glutelin promoter along with its signal peptide in rice. Surprisingly, the recombinant protein was not targeted to the ER but to the nucleolus, and despite the accumulation was low, the transgenic seeds induced a significant antihypertensive activity after a single oral dose, as low as 0,0625 g transgenic seeds/kg (Wakasa *et al.*, 2011).

Soybean is another important example of a plant-platform for heterologous ACEI peptides production. Soybean seeds contain proportionally more essential amino acids than meat, thus being is an important food source worldwide. A strategy adopted for the ACEI peptide expression in soybean was to improve antihypertensive properties by introducing novokinin into homologous sequences of a soybean β-conglycinin α-subunit by site-directed mutagenesis. The recombinant protein was expressed in *E. coli* and showed to exert anti-hypertensive activity following oral administration to SHR. This was the first report on the introduction of a physiologically active peptide into a food protein, by site-directed mutagenesis, which presented *in vivo* antihypertensive effect. Based on this expression evidence, the α-subunit of the seed storage protein β-conglycinin, containing novokinin repeats, was expressed in soybean. However, the recombinant protein accumulated at levels only up to 0.2% of total protein extracts from transgenic soybean seeds; therefore, by being in such low quantities it was not viable observe the *in vivo* effects of these seeds (Matoba *et al.*, 2001; Nishizawa *et al.*, 2008). The novokinin ACEI peptide has been also expressed in transgenic soybeans seeds in a 4-tandem repeat fusion with the β-conglycinin α-subunit. This strategy revealed the 4-novokinin-alpha as 0.5% of total soluble protein and 5 % of the β-conglycinin α-subunit in transgenic soybean seeds. The protein extract led to a reduction in systolic blood pressure in SHR and similar effect was

observed using defatted flour (Yamada *et al.*, 2008). These examples, among others that have already reached commercial success, show the great potential of plant-based systems for the heterologous expression of therapeutic peptides such as the ACEI peptides.

1.4. *Lactuca sativa* and *Medicago truncatula* as hosts for heterologous production of pharmaceutical proteins

Among the plant hosts for pharmaceutical proteins and peptides production, *Lactuca sativa* and *Medicago truncatula* are promising heterologous expression systems (Dong *et al.*, 2014; Twyman *et al.*, 2003).

Lettuce is a worldwide important leafy crop belonging to the Asteraceae family. It is an annual, autogamous, diploid species with nine chromosomes (2n = 18) (Curtis, 2006) and a genome size of 2.7 Gb (Truco *et al.*, 2013). Plus, it is an efficient inbreeding species with robust-growing and it is easy to cultivate. Lettuce is particularly suitable for oral delivery of therapeutics since it is often consumed raw, unprocessed, and is available to be harvested soon, when compared to other crops such as tomato or potato, which is important for protecting the bioactivity of vaccines, antibodies or other pharmaceutical proteins (Joh *et al.*, 2005). These characteristics, alongside with the fact that transformation procedures for both nuclear (Liu *et al.*, 2012) and plastid genomes (Lelivelt *et al.*, 2005), and for transient expression (Negrouk *et al.*, 2005) are widely available, make lettuce a promising production host for several edible pharmaceutical products. These include recombinant therapeutics (Dong *et al.*, 2014; Martínez-González *et al.*, 2011), like hepatitis B virus surface antigen (Kapusta *et al.*, 2001), VLPs, monoclonal antibodies (Lai *et al.*, 2012) and human therapeutic proteins (Lim *et al.*, 2011; Ruhlman *et al.*, 2007a).

M. truncatula is a model legume from the Fabaceae family. It is an autogamous species characterized by its prolific seed production, its diploidy (2n=16), small genome ($1.8x10^9$ bp for the 'Jemalong' cultivar) and a short life cycle that allows several complete growth cycles within a year, of about 3 months each. These characteristics, alongside with the fact that it is easy to manipulate in the laboratory, and that are several molecular tools available and widely used, allow this species to be used in molecular genetic studies like analysis of gene expression, promoter functional analysis, T-DNA mutagenesis, expression of genes for crop improvement and molecular pharming (Araújo *et al.*, 2004; Barker *et al.*, 1990; Cook, 1999). Moreover, this model plant has been suggested as a promising heterologous production system, (Abranches *et al.*, 2005) for the production of food additives (Pires *et al.*, 2008), human hormones (Pires *et al.*, 2012) and human enzymes (Pires *et al.*, 2014).

In previous work from our group (Gomes, 2015), two biological systems were selected for the heterologous expression of ACEI peptides. The *Lactuca sativa* (lettuce) cv. 'Great Lakes' belonging to the Crisphead lettuces type was selected since it is a stress-tolerant and widely adapted cultivar, resistant to the physiological disorder of tip burn (Mikel, 2007; de Vries, 1997). The M910a genotype

of the model plant *M. truncatula* Gaertn cv. 'Jemalong' was also selected as host due to its high regeneration capacity (Barker *et al.*, 1990) and embryogenic potential (Araújo *et al.*, 2004).

The ACEI peptides that were used in the plant transformation experiments were selected according to their ACE inhibitory activity, the size of the peptide, resistance/sensibility to GI enzymes and confirmed antihypertensive activity *in vivo*. Based on those criteria four constructs with peptide coding sequences were design and used for plant transformation (Gomes, 2015).

The first ACEI peptide is a nonadecapeptide released during milk fermentation by *Enterococcus faecalis*. It corresponds to the 58-76 fragment of β-casein, which was proven to have a low IC50 value (5.2 μM) and demonstrated antihypertensive activity when orally administered to SHRs animal models (Quirós *et al.*, 2007). The synthetic codding sequence used for plant transformation was designated by ACEI_FMK. It consists of the ACEI coding sequence and a linker of two amino acids (MK) at its 5' flanking region which gives the ATG starting codon for translation. This linker corresponds to a cleavage site of trypsin for further elimination. At it 3' flanking region the ACEI_FMK has an 6x His-tag (see appendix 6.1.15.; Figure 6.1).

The second ACEI peptide chosen is an undecapeptide isolated from the pepsin hydrolysate of algae protein waste, a mass-produced industrial by-product of an algae essence from the microalgae *Chlorella vulgaris*. This peptide was proven to be resistant to *in vitro* GI enzyme digestion, to pH (range of 2-10) and to temperature (range of 40-100ºC), conditions where it can retain its ACEI inhibitory activity. It exhibited a low IC50 value of 29.6 μM (Sheih *et al.*, 2009). The synthetic coding sequence used for plant transformation was designated by ACEI_CHLTP and is composed by the ACEI coding sequence along with a coding sequence of a 57 amino acids-long transit peptide for plant chloroplast targeting, obtained from the small subunit of the tobacco RuBisCO enzyme. At it 3' flanking region the ACEI_CHLTP has an 6x His-tag (see appendix 6.1.15.; Figure 6.1).

The third peptide is a decapeptide isolated from *Acaudina molpadioidea* (sea cucumber) hydrolysate. It has the particularity of intensified (3.5 times) inhibitory activity after *in vitro* incubation with GI enzymes, from IC50 15.9 to IC50 4.5 μM, and it has demonstrated antihypertensive activity in SHRs (Zhao *et al.*, 2009). The synthetic coding sequence used for plant transformation was designated by ACEI_SEA and consists of the ACEI coding sequence and a His-tag at it 3' flanking region (see appendix 6.1.15.; Figure 6.1).

The forth peptide is a tripeptide isolated from the pepsin-pancreatin digest of the large subunit of spinach RuBisCO. It has an IC50 value of 0.6 μM and its antihypertensive activity in SHRs has also been confirmed (Yang *et al.*, 2003). The synthetic coding sequence used for plant transformation was designated by ACEI_SPI and is composed by an eight-tandem repeat of the coding sequence for MRW peptide, with pepsin cleavage sites in between each MRW coding site. Thus, it is expected the released of the active peptide during GI enzyme digestion of the transformed plants. Also, the ACEI_SPI has a 6x His-tag at it 3' flanking region (see appendix 6.1.15.; Figure 6.1). The His-tag was included in all constructs to facilitate the extraction, purification and detection of the oligopeptides.

To allow the continuous propagation, maintenance, and regeneration of *Lactuca sativa* cv. 'Great Lakes', a micropropagation scheme via axillary bud proliferation and an organogenesis system was

previously established (Gomes, 2015). This regeneration and propagation scheme was used as a regeneration method for transformed lettuce plants and further used as a continuous source of explants in my work.

When I started my research work the following results had already been obtained:

I. Agrobacterium-mediated transient transformation experiments using plasmid constructs demonstrated transient expression of ACEI_FMK and ACEI_CHLTP synthetic genes in lettuce.

II. Agrobacterium-mediated stable transformation with *Lactuca sativa* cv. 'Great Lakes' and *M. truncatula* allowed the selection of plantlets resistant to kanamycin (Kan). This was accomplished for the ACEI_CHLTP in lettuce and ACEI_FMK and ACEI_SEA in *M. truncatula*.

These first encouraging results from (Gomes, 2015) gave me the opportunity to perform the biochemical studies to confirm plant stable transformation, transmission of the traits to the next generations and to try to attest the production of the ACEI synthetic peptides *in planta*.

2

Aims

The principal aim of this work was the analysis of the production of heterologous expressed synthetic ACEI oligopeptides (ACEI transformation) in two emergent host plants, *Lactuca sativa* (lettuce) and *Medicago truncatula* (barrel medic), to be used as an alternative therapy for hypertension. To achieve this main aim several tasks were defined:

- Molecular analysis to confirm stable ACEI transformation of the potentially transformed plant lines.
- Propagation of ACEI transformed plants and obtention of transformed seeds.
- Establishment of a kanamycin selection scheme for *Lactuca sativa* cv. 'Great Lakes'.
- Establishment of conditions for ACEI oligopeptides detection in host plants by enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and high performance liquid chromatography (HPLC) methods.

3

Materials & Methods

3.1. Confirmation of stable ACEI transformed *Medicago truncatula*

3.1.1. Plant material and culture medium

The non-transformed M910a genotype of *M. truncatula* Gaertn cv. 'Jemalong' and putative transgenic *M. truncatula* micropropagated plantlets, obtained from axillary bud proliferation as previously described (see section 1.3.3.;Gomes, 2015)) were used in this study. Plant cuttings were continuously micropropagated and kept in sterile vials sealed with cling film, with 3 or 4 cuttings per vial. MS (Murashige and Skoog, 1962) basal salts and vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar (Micro-agar, Duchefa, The Netherlands) was used as growth medium, and pH was adjusted to 5.8 before autoclaving (20 min, 121ºC). Plantlets were maintained in a plant growth chamber (Phytotron EDPA 700, Aralab) under 12 hours photoperiod at 100 µmol m^{-2} s^{-1} provided by cool white fluorescent light and day/night temperatures of 24ºC/ 22 ºC for 4 weeks. Afterwards, part of the *in vitro* plant stock was transferred to Jiffy® pots within glass flasks covered with cling film, for acclimatization and root development in a growth chamber in the same conditions as described before, for a 3-4 weeks period. Afterwards, plants were potted in a mixture of 4:1 soil-vermiculite in a conventional greenhouse kept cool by ventilation and/or shielding with average daily temperatures from 15 to 35 °C and natural irradiance, to complete the plant life cycle in order to obtain transgenic seeds.

3.1.2. Total RNA extraction

Total plant RNA was isolated from young leaves from control non-transformed M910a and putative transgenic plantlets. For comparison purposes total RNA was also extracted from the roots. Samples were collected in a sterile environment, within a laminar flow cabinet, immediately frozen in liquid nitrogen and stored at -80ºC for further use. Frozen tissues (50-100 mg) were grinded with liquid nitrogen in a frozen RNase-free mortar, and total RNA was extracted with Direct-zol™ RNA MiniPrep (Zymo Research, EUA) according to the manufacturer's instructions. Briefly, plant tissue was

subjected to a TRIzol® based RNA extraction protocol, that simultaneously solubilizes biological material and denatures proteins. Final elution was performed with 35-50 µL DNase/RNase free water provided in the kit. To attest extraction effectiveness, spectrophotometric analysis using NanoDrop™ (Thermo Fisher Scientific, USA) was performed. The ratio of the absorbance at 260 nm to the absorbance at 280 nm was used as nucleic acid purity indicator and samples within the interval [1.8;2.2] were considered with sufficient quality to proceed. To clean contaminated RNA samples, an ethanol precipitation was performed, where 1 mL of absolute ethanol and 4M sodium acetate (NaAC) (0.1x sample volume) was added to the sample and left overnight at -20ºC. Total RNA extracts were then centrifuged for 5-10 min at 15,000 rpm and the supernatant was discarded. After, 400 µL of 70% ethanol was added to clean the pellet, samples were centrifuged for 3 min at 9,500 rpm and supernatant was discarded. Subsequently, 400 µL of absolut ethanol was added, then centrifuged for 3 min at 15,000 rpm and supernatant was discarded. Finally, the residual ethanol in the samples was allowed to evaporate. The pellet was resuspended in 35-50 µl DNase/RNase free water. The RNA samples were treated with TURBO DNA-free™ (ThermoFisher Scientific, EUA) according to the manufacturer's instructions. Briefly, 0.1 sample volumes of 10x TURBO DNase Buffer and 1 µL of TURBO DNase was added to total RNA samples and gently mixed, followed by 37 ºC water bath (Thermo Fisher Scientific, USA) incubation for 30 min. 0.1 sample volumes of DNase Inactivation Reagent was then added and well mixed, followed by 5 min incubation at room temperature. mixing occasionally. Finally, samples were centrifuged at 10,000 g for 1.5 min and supernatant was transferred to a new tube. To confirm RNA integrity, 1.4% agarose gel electrophoresis stained with SYBR® Safe DNA gel stain 1% (v/v) (Invitrogen, EUA) was performed. To allow proper RNA migration in the gel, samples were denatured with formamide denaturing agent (FDE)-including loading buffer (50 µL formamide + 20 µL formaldehyde + 20 µL 10x MPOS) at 65ºC in a thermo block for 10 min, before gel loading.

3.1.3. RT-PCR reaction

After proper confirmation of total RNA integrity, reverse transcription polymerase chain reaction (RT-PCR) was performed. First, from approximately 500 ng of each RNA sample, Reverse Transcription (RT) of RNA into cDNA was performed using the ImProm-II™ Reverse Transcription System (Promega, EUA) according to the manufacturer's instructions. Before the cDNA synthesis reaction, random primers were incubated with the RNA sample at 70ºC for 5 min, followed by immediately chill on ice. RT conditions were: 25ºC for 5 min for annealing of random primers, followed by first strand synthesis (RNA-cDNA hybrids) at 42ºC for 60 min, followed by 70ºC for 15 min to denature the reverse transcriptase enzyme, on a BioRad T100® Thermal Cycler (BioRad, USA). Second, the obtained cDNA was used as a template for PCR. The PCR amplification of an actin gene was used as positive control to confirm cDNA presence, using specific primers (Table 3.1.). The PCR conditions were: 95ºC for 2 min for denaturation, followed by 35 cycles of 95ºC/10s for denaturation, 55ºC/ 1 min for annealing and 72ºC/ 30s for extension, and a final extension 72ºC for 5 min. The PCR

amplification for the first two ACEI synthetic cDNAs (ACEI_FMK and ACEI_SEA) was performed with specific primers presented on Table 3.2. and the conditions were as above described.

Table 3.1. Nucleotide sequence of primers used to amplify the actin cDNA sample from *M. truncatula*

Primer label	Sequence
Actin_Forward	TCAATGTGCCTGCCATGTATG
Actin_Reverse	ACTCACACCGTCACCAGAATC

Table 3.2. Nucleotide sequence of primers used to amplify the ACEI peptides coding sequences from DNA and cDNA plant samples. 'Univ_Reverse' – reverse primer used to amplify the ACEI cDNAs.

Primer label	Sequence
ACEI_FMK_Forward	CAC TGT TGA TAC AT***A TGA AAT TGG TTT ATC**
ACEI_SEA_Forward	CAC TGT TGA TAC AT***A TGG AAG GTG CTC**
ACEI_SPI_Forward	CAC TGT TGA TAC AT***A TGA GAT GGA TGA G**
ACEI_CHLTP_Forward	CAC TGT TGA TAC AT***A TGG CTT CTT CTG T**
Univ_Reverse	***GTA GTA GTA** ACT AGC TGT TAA GAC TTA

* In bold are the areas of complementarity to the ACEI peptides coding sequences

3.2. Confirmation of stable ACEI transformed *Lactuca sativa*

3.2.1. *In vitro* selection scheme for *Lactuca sativa* cv. 'Great Lakes'

3.2.1.1. Plant material and culture media

Seeds were collected from a greenhouse collection of putative transformed lettuce plants previously potted (Gomes, 2015). Wild type and putative transformed seeds were used in this study. Seeds were pre-washed in distilled water, surface-sterilized with 50% (v/v) Domestos (commercial bleach with detergent) for 8 minutes and rinsed four times with sterile distilled water. After the decontamination step, further work was conducted in a vertical laminar flow cabinet. MS (Murashige and Skoog, 1962) basal salts and vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar (Micro-agar, Duchefa, The Netherlands), was used as germination medium and pH was adjusted to 5.8 before autoclaving (20 min, 121ºC).

3.2.1.2. Determination of the optimized kanamycin concentration for seed segregation analysis

To determine the Kan threshold for the inhibition of wild type seed development, seeds were decontaminated as previously described and germinated in increasing Kan concentrations, based on previous published protocols (Pileggi *et al.*, 2001). Seed germination medium was supplemented with five selected increasing Kan concentrations. The following concentrations of Kan were evaluated 0, 150, 300, 600 and finally 750 mg/L (corresponding to 0.00, 0.31, 0.62, 1.24 and 1.55 mM,

respectively). The antibiotic was filter-sterilized through 0.22 µm filters (Gelman Sciences, Michigan, USA). The medium was cooled to approximately 55ºC after sterilization, Kan-supplemented and dispensed in plastic Petri dishes (90 mm diameter). The disinfected wild type seeds were transferred onto germination medium (30 seeds/dish), sealed with cling film, and placed in the growth chamber under 12 hours photoperiod at 100 µmol m^{-2} s^{-1} provided by cool white fluorescent light and day/night temperatures of 24ºC/ for 4 weeks. After four weeks of growth, seedlings were evaluated for their morphology as it was expected to have specific traits related to sensibility to Kan exposure.

3.2.1.3. Kanamycin seed segregation of transformed *Lactuca sativa*

Seeds from 4 randomly chosen putative transformed plants were germinated on germination medium (15 seeds/dish) supplemented with the optimized Kan concentration (300 mg/L). Resistant seedlings (Kan^R) were transferred to new medium without selective pressure to allow root development and general plant growth, in the growth chamber and under the same conditions as previously described (see section 3.1.1.), for 7 weeks. Hereafter, well developed seedlings were transferred to Jiffy® pots within glass flasks, covered with cling film for acclimatization and root growth with the same conditions as previously described, for 2-4 weeks. Afterwards, plants were potted in a mixture 4:1 soil-vermiculite to complete life cycle in a greenhouse in order to obtain transgenic seeds.

3.2.1.4. Statistical analysis of kanamycin assay data

An analysis of variance (ANOVA) was performed to test the significance between the means of specific morphologic traits related to Kan exposure. Traits considered were: germination ratio, first emerging green and white leaves, and average root size, obtained in plants subjected to different Kan concentrations, as previously mentioned above. A significant level of $p \leq 0.05$ was considered. The results were expressed as mean values ± standard deviation.

To confirm significance of gene segregation ratios a chi square ($\chi 2$) analysis (Yates, 1934) was performed and observed segregation ratios for Kan^R: kanamycin sensitive (Kan^S) were compared to Mendelian segregation models. The $\chi 2$ with a critical value of 3.841 corresponds to a 95 % level of significance with 1 degrees of freedom (df).

3.2.2. Total DNA extraction

Total plant DNA was isolated from the non-transformed and putative transgenic lettuce seedlings. Plant leaves were collected in a sterile environment within a laminar flow cabinet, immediately frozen in liquid nitrogen and stored at -80ºC for further use. Frozen leaves (50-100 mg) were grinded in a sterile frozen mortar and total DNA was extracted using a DNA extraction protocol adapted from *Arabidopsis thaliana* (Edwards *et al.*, 1991) with one additional extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) step, followed by purification by one Phenol:Chloroform:Isoamyl alcohol (25:24:1) step and one Chloroform:Isoamyl alcohol (24:1) step.

DNA was precipitated with two volumes of absolute ethanol and resuspended in TE (10mM Tris-HCl, pH 7.5, 1 mM EDTA). To attest extraction effectiveness, spectrophotometric analysis using NanoDrop™ (Thermo Fisher Scientific, EUA) was performed. The ratio of the absorbance at 260 nm to the absorbance at 280 nm was used as nucleic acid purity indicator and samples within the interval [1.6;2.2] were considered with sufficient quality to proceed. To attest DNA integrity, 1% agarose gel electrophoresis stained with SYBR® Safe DNA gel stain 1% (v/v) (Invitrogen, EUA), was performed.

3.2.3. PCR reaction

The presence of the ACEI coding sequences in the genome of putative transgenic lettuce seedlings was analyzed by PCR amplification on total DNA plant samples. The PCR was performed using GoTaq® G2 Flexi DNA Polymerase (Promega, USA), according to manufacturer's instructions, and using the specific forward primers and universal reverse primers against the ACEI peptides' coding sequences (Table 3.6). PCR amplification conditions for the transgenes were: 95ºC/ 2 min for denaturation, followed by 35 cycles of 95ºC/ 10s for denaturation, 55ºC/ 1 min for annealing and 72ºC/ 30 s for extension and 72ºC 5 min for final extension. PCR was carried out on a thermocycler BioRad T100® Thermal Cycler (BioRad, USA), with at least 100 ng of template DNA, 0.5 µL of transgene specific primers (10µM), 0.75 mM of MgCl2, 1X Green GoTaq® Reaction Buffer (Promega, EUA) and 0.5 Units of Taq polymerase (Invitrogen, USA), in a final volume of 20 µL adjusted with sterile MiliQ water.

3.3. Oligopeptides analysis

3.3.1. Oligopeptide extraction from *Medicago truncatula* for ELISA

The control non-transformed and transformed *M. truncatula* plants, along with *Populus tremula x alba* plants (kindly provided by Dr. Jorge Paiva), also working as a negative control for the assay, were used. *In vitro* leaf samples were extracted using an ethanol precipitation protocol (Dr. Pedro Fevereiro personal communication). Briefly, frozen young leaves (50-100 mg) were grinded in a sterile frozen mortar. Grinded plant tissue samples were resuspended in 60 µL of Tris buffer (20 mM pH 8) ('Tris') (see section 6.1.1.) and vortexed for homogenization. 9 volumes of absolute ethanol were added to 1 volume of aqueous protein solution and the mixture was cooled to -20ºC for 1 hour. Total protein extract was centrifuged at maximum speed (Eppendorf 5415R Micro-Centrifuge, Germany) and the supernatant was discarded. Pellet was resuspended in 500 µL of Tris and centrifuged at 10,000 rpm for 3 min. The supernatant was transferred to new Eppendorf® tubes, 900 µL of absolute ethanol was added and samples were left overnight. Thereupon, samples were centrifuged at maximum speed for 15 min, supernatants were discarded, and pellets were resuspended in 250 µL of Tris (Dr. Pedro Fevereiro personal communication). To attest extraction effectiveness, spectrophotometric analysis using NanoDrop™ (Thermo Fisher Scientific, EUA) was performed at 280 nm wavelength.

3.3.2. Sample preparation for ELISA

ELISA immunodetection was performed using the His-Tag Detection ELISA Kit (Cayman Chemical, USA) according to manufacturer's instructions. The assay is based on the competition between free ACEI peptides with His-tag, derived from the plant samples, and a 6X-His Tracer [6X-His linked to alkaline phosphatase (AP)] for a limited number of His- specific monoclonal antibody binding sites. Therefore, the presence of ACEI peptides was measured indirectly by an enzyme-linked colorimetric reaction and the intensity of the generated color, determined spectrophotometrically, is proportional to the amount of Tracer bound to the well, which is inversely proportional to the amount of free ACEI peptides present in the well during the assay.

The basic principle of the assay is described in Figure 3.1.

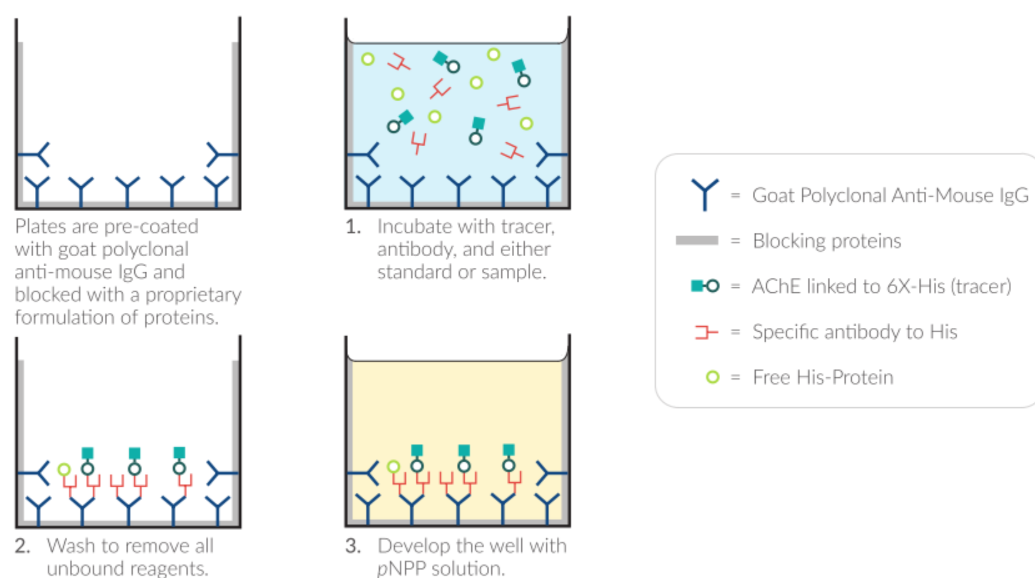

Figure 3.1. Simplified scheme of the basic principle of the ELISA assay (adapted from Cayman Chemical Protocols)

A series of 6 tubes with His-Protein standard was made by serial dilutions from a concentrated standard provided in the kit. The preparation of the Blank (BLK), with just ρNPP reagent, a Non-Specific Binding solution (NSB), a maximum binding solution (B_0), the standards and samples, are summarized in Table 3.3. The samples were diluted with Tris according to nanodrop results to obtain a final concentration approximately to 0.5mg/mL.

Table 3.3. Summary for BLK, NSB, Std and sample preparation for each well of the ELISA plate.

Well	TBS Assay Buffer	Standard	Sample	Ap Tracer	His ELISA Monoclonal Antibody
BLK	-	-	-	-	-
NSB	100 µL	-	-	50 µL	-
B_0	50 µL	-	-	50 µL	50 µL
Standard	-	50 µL	-	50 µL	50 µL
Sample	-	-	50 µL	50 µL	50 µL

The Medicago and populous samples were serially diluted in 1:10, 1:100 and 1:200. The ELISA plate, with all the solutions in the designated wells, was covered with plastic film and incubated for 90 min at room temperature on a standard orbital shaker at 200 rpm. Plate development started by emptying the wells and rinse four times with Wash buffer provided. After the last wash, 200 µL of the ρNPP substrate solution was added to each well of the plate. The plate was covered with plastic film and incubated for 60 min at room temperature. The result was read in a SpectraMax® Plus 384 Microplate Reader (Molecular Devices, USA) at a wavelength between 405 and 420 nm.

3.3.3. Oligopeptide extraction from *Medicago truncatula* and *Lactuca sativa* for SDS-PAGE

The control non-transformed and putative transgenic *M. truncatula*, the control non-transformed and putative transgenic *L. sativa*, and a bacteria cell lysate expressing his-tag protein which worked has positive control, were considered for SDS-PAGE. Young leaf samples were collected and immediately frozen in liquid nitrogen. Samples were grinded using a sterile frozen mortar, transferred to Eppendorf® tubes and weighed in an analytical balance (Sartorius®). Extraction buffer (see section 6.1.2.) was added in 1:1 ratio (w/v) (g of plant tissue: mL of extraction buffer) and samples were vortexed for homogenization. Afterwards, samples were sonicated for 10 seconds (10 pulses with 70% amplitude) on ice and centrifuged at 22,000 rpm for 10 min. Pellet was discarded and supernatant was transferred to new Eppendorf® tubes. Alternatively, two other extraction buffers were tested (see section 6.1.3., and 6.1.4.), alongside with a few differences in the extraction steps. Namely the absence of the sonication step, and the use of a concentration/purification device (Amicon® Ultra-2 Centrifugal Filter Devices, Merck Millipore) used to concentrate proteins with <10 kDa. Considering that the molecular weight of the ACEI oligopeptides is lower than 10 kDA, we used this device to separate the ACEI oligopeptides from the crude total lysate sample. The resulting protein samples were then quantified by BCA assay using Pierce BCA Protein Assay Kit 23225 (Thermo Fisher Scientific, EUA) according to manufacturers instructions.

3.3.4. Oligopeptide separation by SDS-PAGE

The samples were prepared in 1:4 ratio [µL of sample buffer 4x (see section 6.1.6.): µL of protein extraction sample]; span down at top speed (Eppendorf 5417R, Germany) for a few seconds and then heated at 100ºC for 10 minutes in a thermo block with agitation (Eppendorf Thermomixer®). Alternatively, it was also tested another sample buffer (see section 6.1.7.). The final mixtures were loaded in a SDS-PAGE gel.

A 16.5% Mini-PROTEAN® Precast Gel Tris-Tricine 12 wells 20 µL each (Bio-Rad, USA) was used within a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad, USA). After assembling the electrophoresis apparatus, running buffer (see section 6.1.8.) was added until the electrodes were completely covered, and gas bubbles were carefully taken from the buffer surface. Afterwards, samples were loaded using a micropipette with GELoader® tips (Eppendorf, Germany) and 7.5 µL of Rainbow Marker RPN755 (Amersham Life, UK) were also loaded in a separate lane. The electrophoretic apparatus was connected to the power supply and a 90 V (constant) electric field was applied for approximately 45 min. Thereafter, the gel was immersed in Coomassie staining solution (see section 6.1.9.) for 30 min at room temperature with gentle horizontal agitation. The gel was then immersed in a de-staining solution (see section 6.1.10.) to allow the visualization of protein bands. Finally, the gel was digitalized in a Densitometer GS 800 (Bio-Rad, USA).

Alternatively, it was also tested a larger SDS-PAGE gel with a 5-20% gradient resolving gel. The stacking and resolving gels were prepared with 40% 29:1 Acrylamide:Bis acrylamide ratio as described in the tables below (Table 3.4. and Table 3.5.).

Table 3.4. Specifications for preparing the resolving gel (5-20% gradient). Values for a gel with 1.5 mm thickness.

Solution	Resolving Gel (5%)	Resolving gel (20%)
H_2O	18.59 mL	7.34 mL
Lower gel buffer	7.5 mL	7.5 mL
Acrylamide	3.75 mL	15 mL
APS	150 µL	150 µL
TEMED	15 µL	15 µL

Table 3.5. Specifications for preparing the stacking gel. Values for a gel with 1.5 mm thickness.

Solutions	Stacking Gel (3,5%)
H_2O	13.83 mL
Acrylamide	1.75 mL
Upper gel buffer	4 mL
SDS (10%)	200 µL
APS (10%)	200 µL
TEMED	20 µL

The 5-20% gradient resolving gel was prepared on a gradient maker as showed in Figure 3.2. APS and TEMED were added just before adding the gel solutions on the gradient maker since they initiate the gel polymerization.

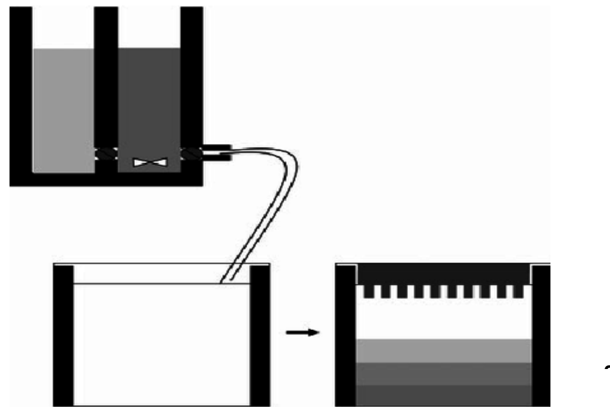

Figure 3.2. Schematic representation of gradient gel procedure. The 5% and 20% acrylamide solutions are poured in each compartment of the gradient maker (1) at the same time. Straight afterwards, the small magneto promotes gradual mixing of the two solutions as they start coming out of the compartments to the gel cassette (2) where polymerization occurs, and gradient gel is formed (3) After, stacking gel solution was added along with the comb. (adapted from Casas-Terradellas et al., 2006).

The resolving gel was left to polymerize for approximately 1 hour at 4ºC (to promote faster polymerization). Soon after, stacking gel solution was added to the gel cassette, along with the comb, and left to polymerize for approximately 30 min at room temperature. After polymerization, the comb was removed.

The electrophoresis apparatus was assembled, and cathode running buffer and anode running buffer (see section 6.1.11. and 6.1.12., respectively) were added until both cathode and anode electrodes were completely covered. Gas bubbles were carefully taken from the buffer surface, and samples were loaded using a micropipette with GELoader® tips (Eppendorf, Germany). 12 µL of Precision Plus Protein™ Dual Color Standards (Bio-Rad, USA) (see section 6: Figure 6.2.) on the first lane and Rainbow Marker RPN755 (Amersham Life, UK) (see section 6; Figure 6.3.) on the last gel lane were also loaded. Afterwards, the electrophoretic apparatus was connected to the power supply and a 45 mA (constant) electric field was applied for approximately 3h40min.

3.3.5. Oligopeptide immunodetection

Oligopeptide transfer from the SDS-PAGE gel to the nitrocellulose membrane was performed using two different transfer methodologies, semi-dry and wet transfer. For both, the filter paper (BIO-RAD, USA), the nitrocellulose membrane (Amersham, UK) and the SDS-PAGE gel were first immersed in transfer buffer (see section 6.1.13.) to equilibrate the components. The semi-dry transfer sandwich was assembled on a Trans-Blot SD (Semi-dry Transfer Cell, Bio-Rad, USA) as shown in Figure 3.3. Electrotransference occurred at 400 mA (constant) for 15 min.

Figure 3.3. Semi-dry transfer assembling scheme.

The wet transfer sandwich was assembled as described before (Figure 3.3.) and placed in a transfer tank filled with transfer buffer. The tank poles were connected to the power supply, which was set to 150 mA (constant) for 16h.

For the immunodetection of the oligopeptides in the nitrocellulose membrane, the membrane was first hydrated in TBS buffer (1x) for 10 min with gentle horizontal agitation (Gerhardt®). TBS was discarded, and the membrane was immersed in blocking solution (see section 6.1.14.) for 2 hours at room temperature with gentle horizontal agitation. Blocking solution was then removed and the membrane was immersed in primary antibody solution. The primary antibody (Anti-6X His tag® antibody [HIS.H8] ab18184, Abcam, UK) was diluted at 1:2000 with TBS-T containing 5% (w/v) of skimmed milk powder (Molico®). The membrane was incubated in this last solution (with the primary antibody) for 30 min at room temperature, and then incubated overnight at 4ºC and 1 hour at room temperature with gentle horizontal shaking on the next day. The membrane was washed three times, 10 min each, with TBS-T (1x) at room temperature with gentle horizontal agitation. The excess of TBS-T from the last wash was carefully removed and secondary antibody solution was added. The secondary antibody (Amersham ECL Mouse IgG, HRP-linked whole Ab, from sheep, GE Healthcare) was diluted at 1:5000 with TBS-T alongside with 5% (w/v) of skimmed milk powder (Molico®). The membrane was incubated in secondary antibody solution for 2 hours at room temperature with gentle horizontal agitation. The membrane was then washed three times, 10 min each, with TBS-T (1x) at room temperature with gentle horizontal agitation. After the last wash, to remove the Tween® (Bio-Rad, USA) (present in the TBS-T), the membrane was washed one last time with TBS (1x) for 10 min at room temperature with gentle horizontal agitation.

To detect the immunolabelled protein bands, the membrane was placed in a plastic sleeve and ECL blotting substrate (Pierce™ ECL Western Blotting Substrate, Thermo Fisher Scientific, USA) was gently spread on the membrane and incubated for 1 min at room temperature. The ECL blotting substrate was prepared by diluting reagent A and reagent B at 100:1, according to manufacturer's instructions (Pierce™ ECL Western Blotting Substrate, Thermo Fisher Scientific, USA). Alternatively, a more sensitive blotting substrate - the Luminata Crescendo Western HRP substrate (Millipore, USA) - was also tested. The substrate was gently spread on the membrane and incubated for 5 min at room

temperature. The membrane was then digitalized in ChemiDoc Gel Imaging System (Bio-Rad, USA). Afterwards, the membrane was washed three times with ddH_2O and left to dry in between bench paper.

3.3.6. Oligopeptide extraction and sample preparation from *Medicago truncatula* and *Lactuca sativa* for HPLC

The control non-transformed and putative transgenic *M. truncatula*, along with the control non-transformed and putative transgenic *L. sativa* previously described (see section 1.3.3.), were considered for High-performance liquid chromatography (HPLC). For the putative transgenic *L. sativa,* a pool of different plant lines for the same ACEI coding sequence was made. The extraction step was performed as described in section (see section 3.3.3.). Three extraction buffers were tested, one consisting predominantly of acetonitrile and a protease inhibitor cocktail (see section 6.1.5.), and two more complex, in an attempt to deal with the possible interfering agents often present in plants extracts (see section 6.1.2. and 6.1.3.) (Laing and Christeller, 2004). After extraction step, samples were diluted in acetonitrile 1:1 (v/v), vortexed for homogenization and centrifuged at 5 min at 13,000 rpm.

Samples were analysed by HPLC equipped with a photodiode array detector (Shimadzu, Japan). ACEI oligopeptides were detected at 215, 220 and 280 nm, with an injection volume of 50 µL at a flow rate of 1 ml min-1. The stationary phase was a Inertsil ODS-P column (particle size of 5 µm; diameter, 4.6 mm; length, 250 mm; GL Sciences, Tokyo, Japan) with solvent A (0.1% TFA) and solvent B (acetonitrile acidified with 0.1% TFA), applying a linear separation gradient up to 70% of solvent B in 40 minutes, to the column previously equilibrated in solvent A. The gradient program was: time 0 min, A-B (100:0); time 40 min A-B (30-70); time 45 min (0:100); time 50 min (0:100); time 50.01 min A-B (100:0); time 55 min A-B (100:0).

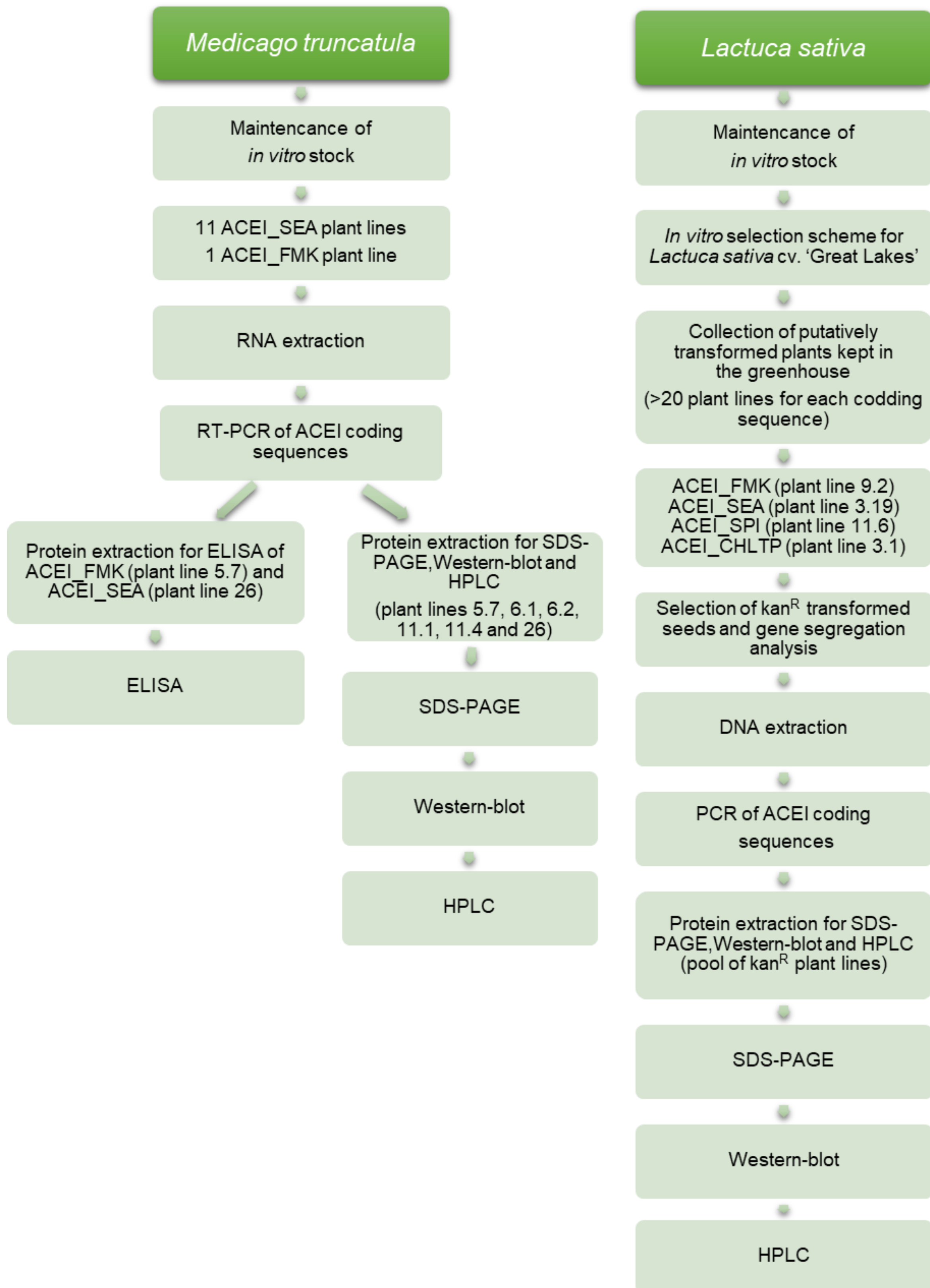

Figure 3.4. Flowchart with the all steps of the flow analysis for *Medicago truncatula* and *Lactuca sativa*.

4

Results & Discussion

4.1. Analysis of ACEI expression at the transcript level in transformed *Medicago truncatula*

4.1.1. RNA extraction

RNA samples extracted with Direct-zol^{TM} RNA MiniPrep (see section 3.1.2.), from control non-transformed M910a and 12 putative transformed Medicago *in vitro* plantlets, were analyzed spectrophotometrically with Nanodrop®. Results revealed general good purity (Abs260/Abs280 >1.8). One sample, corresponding to plants putatively transformed with the ACEI_SEA synthetic coding sequence, had to be subjected to an ethanol purification step to improve its purity, as described in section 3.1.2. RNA concentrations obtained were very variable, from 98.5 ng/µL to 922.7 ng/µL. Total RNA integrity was confirmed in a 1.4% agarose gel electrophoresis as described in section 3.1.2. Overall, migration patterns were consistent with non-degraded total RNA, by the presence of two clear bands corresponding to 28S and 18S rRNA (Figure 4.1).

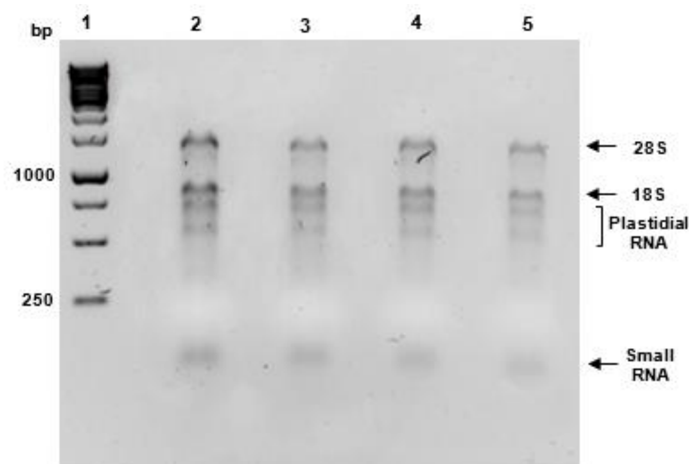

Figure 4.1. Agarose gel (1.4%) electrophoresis to monitor RNA integrity. Lane 1 - 1 Kb DNA ladder; Lanes 2 to 5 - total RNA from Medicago plants putatively transformed with ACEI oligopeptides.

Although most samples presented a migration profile corresponding to non-degraded RNA, some samples from putative Medicago plants transformed with the ACEI_SEA sequence presented a consistent altered migration pattern, possibly corresponding to degradation, even after repetition of the extraction procedure (Figure 4.2.).

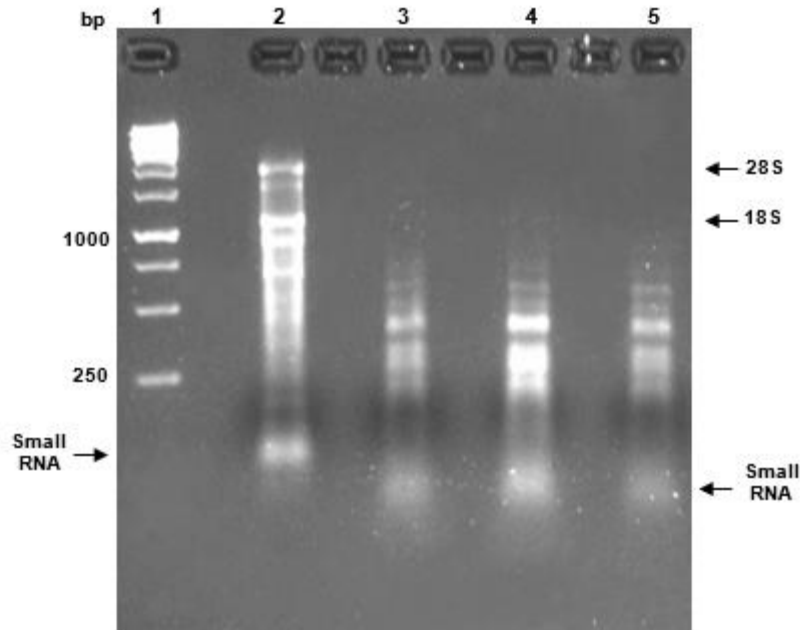

Figure 4.2. Agarose gel (1.4%) electrophoresis to monitor RNA integrity. Lane 1 - 1Kb DNA ladder; lane 2 – control Medicago (M910a); lanes 3 to 5 - total RNA from putative transformed Medicago with ACEI_SEA coding sequence.

We proposed the hypothesis that the different migration pattern occurred because of the unintentional presence of different plant tissues, such as leaves and roots. Plant tissues can be very heterogeneous in terms of problematic compounds (e.g. secondary metabolites such as phenolic compounds that can bind to nucleic acids and interfere in the subsequent reactions), varying from low (e.g. spinach) to high number (e.g. pine needles) (Esra Maltas, 2011). In our case we decided to test the extraction of total RNA, separately, from roots and leaves from random Medicago samples. The results obtained did not corroborate the proposed hypothesis since non-degraded RNA migration pattern was observed in both root and leaf samples. The presence of plastidial RNA on leaf samples was the only significant difference among the two different plant tissues (Figure 4.3.).

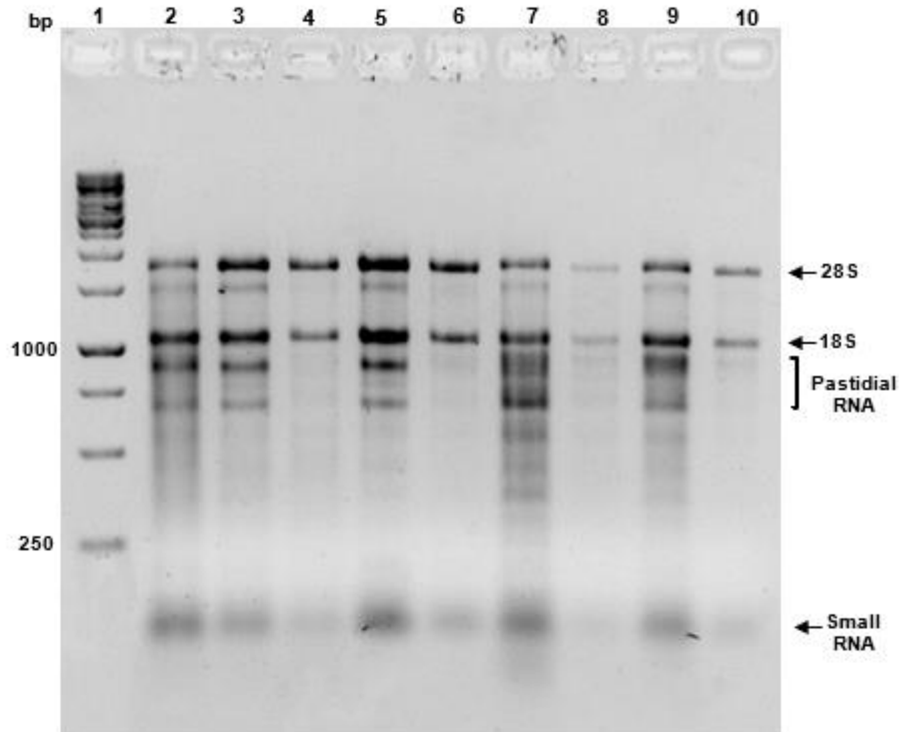

Figure 4.3. Agarose gel (1.4%) electrophoresis to monitor RNA integrity of total RNA samples from Medicago leaves and roots. Lane 1 - 1 Kb DNA ladder; lane 2 - leaves from control Medicago (M910a); lanes 3, 5, 7 and 9 - leaves from putative transformed Medicago.

Considering the results in Figure 4.3. and the pattern observed in Figure 4.2. the subjacent hypotheses was rejected. No altered migration pattern was obtained after extraction repetition with the different tissue samples. We were not able to find an alternative explanation for these results.

Considering the nanodrop results obtained after extraction repetition, and figure 1 results, these ACEI_SEA samples were considered for further ACEI expression analysis.

4.1.2. RT-PCR

The *M. truncatula* transformation system is a well stablished porotocol in our laboratory (Araújo *et al.*, 2004, 2013; Duque *et al.*, 2004) and have been used with success by other groups (Pires *et al.*, 2014, 2008, 2012). Using this optimized protocol, with a tight kanamycine selection, the posibility of obtining non-transgenic plants that escaped selection is extremely low (Araújo *et al.*, 2004). Therefore, considering the transformation protocol efficiency we move direcltely to the analysis of ACEI expression at the transcript level in transformed *Medicago truncatula*. In this way, to confirm ACEI expression at the transcript level, RT-PCR was performed using the RNA mentioned in section 4.1.1. The technique consists of two parts: the synthesis of complementary DNA (cDNA) from RNA by RT using random primers and the amplification of specific cDNA by the polymerase chain reaction (PCR) using specific primers, as described in section 3.1.3. The efficiency of the RT reaction was confirmed

by the amplification of a 101 bp fragment corresponding to an internal portion of the housekeeping actin gene (Actin, 'Act') working as a positive control for the presence of quality cDNA (section 3.1.3; Table 3.1.) (Data not shown). The synthesized cDNA was used in a PCR amplification reaction using the ACEI_FMK_Forward and ACEI_SEA_Forward specific primers (section 3.1.3; Table 3.2.) for the amplification of the synthetic ACEI_FMK and ACEI_SEA coding sequences, respectively, along with the Univ_Reverse primer which is common to all ACEI coding sequences. The results are presented in Figure 4.4 and Figure 4.5. The presence of two amplification fragments of aparenttly 59 and 92 bp in length, sugests the presence of the ACEI_SEA and ACEI_FMK transcripts, respectively. Although some ACEI_SEA total RNA samples presented the RNA altered profile in agarose gels electrophoresis as previously described (Figure 4.2.), PCR amplification was also observed in these samples.

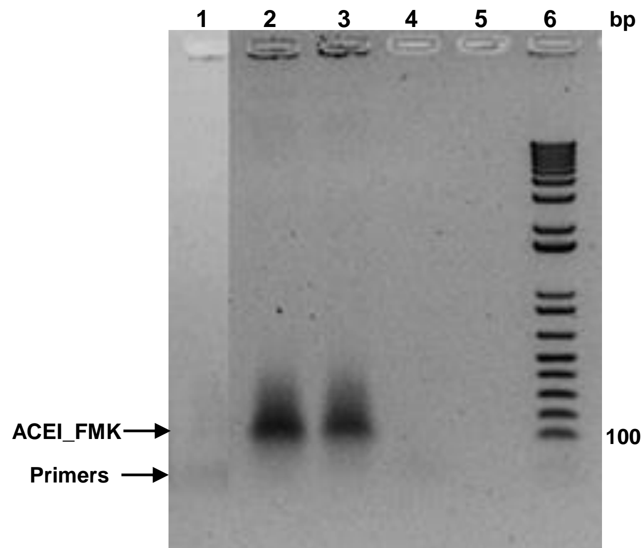

Figure 4.4. Agarose gel (1.7%) electrophoresis of PCR products for ACEI synthetic genes. Lane 1 - control Medicago (M910a); lane 2 and 3 (replicates) - ACEI_FMK (plant line 5.7) 92 bp amplicon; lane 4 – PCR mix without DNA template; lane 6 – 1 Kb plus DNA Ladder. The image was edited to eliminate empty lanes.

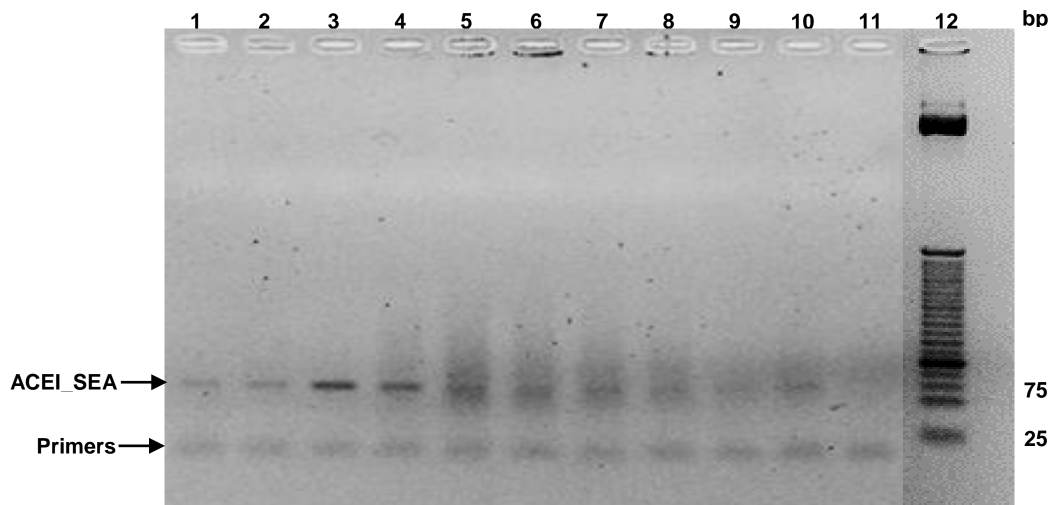

Figure 4.5. Agarose gel (2.2%) electrophoresis of PCR products for ACEI synthetic genes. Lane 1 to 10 - ACEI_SEA 59 bp amplicon (plant lines 6.1, 6.2, 11.1, 11.2, 11.3, 11.4, 11.5, 25, 26 and 27, respectively); lane 11 – PCR mix without DNA template; lane 12 – 25 bp DNA Ladder. The image was edited to eliminate empty lanes.

The results obtained demonstrate that the ACEI_FMK and ACEI_SEA coding sequences are being expressed *in planta* and consequently confirm their presence in the genome, corroborating a successful plant stable transformation for the Medicago host.

4.2. *In vitro* selection scheme for *Lactuca sativa* cv. 'Great Lakes'

4.2.1. The effect of kanamycin on seed germination and seedling development in wild-type *Lactuca sativa*

In order to determine the threshold concentration of the antibiotic Kan beyond which wild type *L. sativa* seed development is inhibited, seeds were germinated in seed germination medium supplemented with increasing Kan concentrations as described in section 3.2.1.2. After four weeks of growth, seedlings were evaluated for morphology as it was expected to have specific traits related to Kan exposure (Duque *et al.*, 2004). The traits evaluated on germinated plantlets were: green and white first emerging leaves, and root development (Table 4.1.).

Table 4.1. Percentage of *L. sativa* cv. 'Great Lakes' seed germination (%), green emerging leaves (Kan^R), white emerging leaves (Kan^S) and average root size, with different Kan concentrations (mg/L).

Kanamycin concentration (mg/L)	Germination (%)	First emerging green leaves (Kan^R)	First emerging white leaves (Kan^S)	Average Root size (mm)
0	85.8 ± 5 ^{a*}	99.1 ± 1.3 ^{a*}	0 ± 0 ^{a*}	8 ± 6.3 ^{a}
150	87.5 ± 3.2 ^{a}	7.5 ± 3.6 ^{b}	92.5 ± 3.6 ^{b}	7.3 ± 1
300	84.2 ± 7.4 ^{a}	0 ± 0 ^{c}	88.1 ± 9 ^{b}	5.5 ± 0.6
600	85.8 ± 4.2 ^{a}	0 ± 0 ^{c}	37 ± 18.5 ^{c}	4.3 ± 0.5
750	88.3 ± 5.8 ^{a}	0 ± 0 ^{c}	7.6 ± 3.1 ^{d}	4.3 ± 0.5

*values followed by the same letter within a column are not significantly different by ANOVA ($p<0.05$)

The resistance of non-transformed seedlings was influenced by culture period and Kan concentration. Seed germination (emergence of radicle) was achieved after 2-3 days and was not affected by the presence of the antibiotic, as germination ratios in all different concentrations were consistent with the control plates (Figure 4.6.; A, C, G, E and I). However, normal seedling formation with radicle, cotyledon and first emerging leaves, suffered great inhibition at higher Kan concentrations (600 mg/L and 750 mg/L) (Figure 4.6.; H and J).

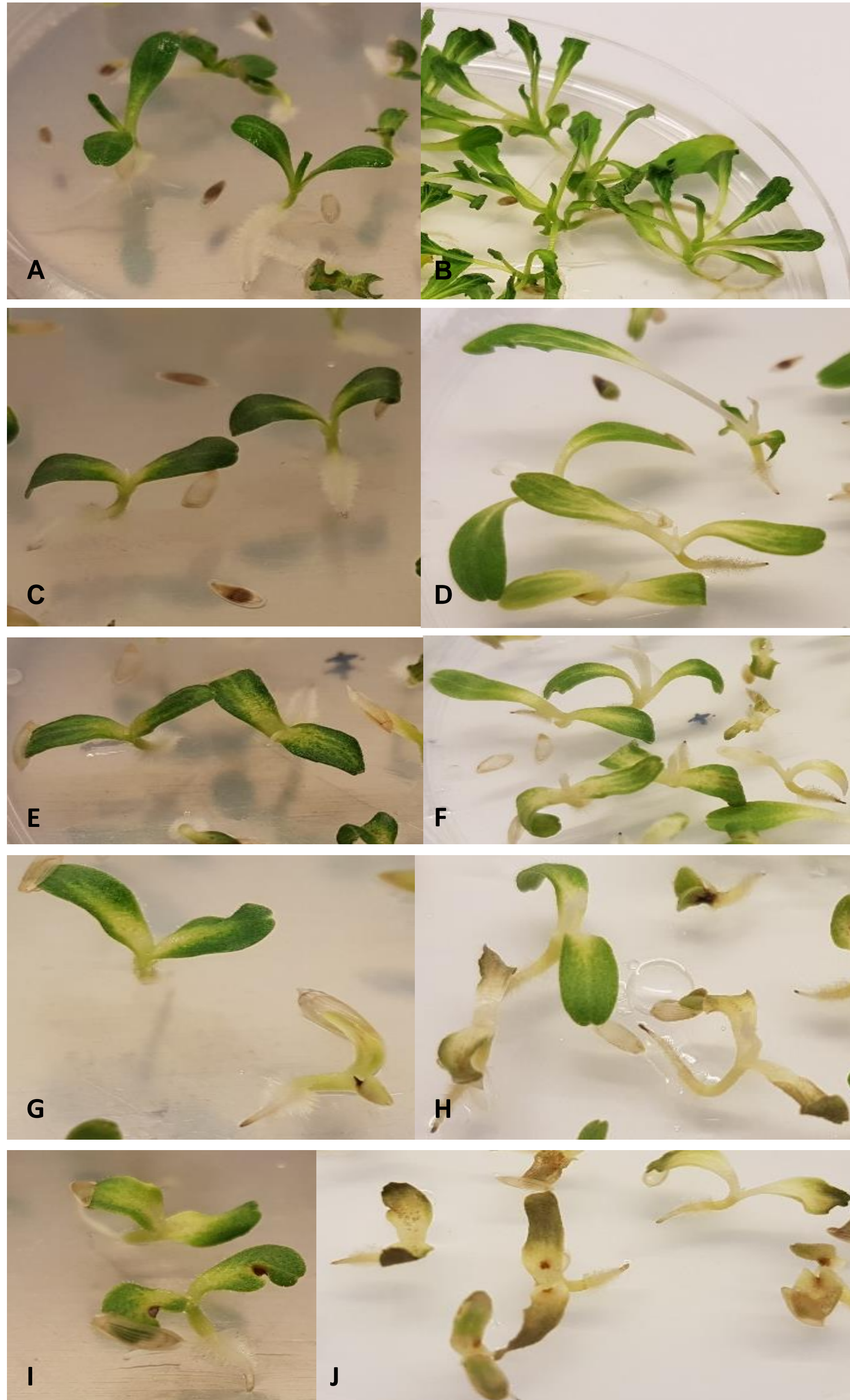

Figure 4.6. Seed germination in wild type Lactuca sativa cv. 'Great Lakes'. A, C, E, G and I, seedlings 10 days after the beginning of the assay with 0, 150, 300, 600 and 750 mg/L of Kan, respectively; B, D, F, H and J, 28 days after the beginning of the assay with 0, 150, 300, 600 and 750 mg/L of Kan, respectively. Seedlings disposed in plastic Petri dishes with 90 mm diameter.

Etiolated (white) first emerging leaves were expected as a specific morphological trait related to Kan exposure (Duque *et al.*, 2004), and this was consistently observed from 300 mg/L Kan concentrations (Figure 4.6. F). At higher Kan concentrations a generalized inhibition of first emerging leaves was observed (Figure 4.6. H and J). Furthermore, as Kan concentration increased the number of roots with root hair and secondary roots decreased drastically until no longer being present at higher antibiotic concentrations (data not shown).

According to these results, the 300 mg/L Kan concentration was the one selected for further seed segregation analysis in transformed lettuce. To our knowledge, we present here the first optimized selection scheme using Kan for the *Lactuca sativa* cv. 'Great Lakes'.

4.2.2. Seed segragation analysis in transformed *Lactuca sativa*

Seeds from 4 randomly putative transformed *L. sativa* lines were germinated on germination medium supplemented with 300 mg/L Kan. Four weeks after germination, Kan^S seedlings had almost completely etiolated cotyledons and first emerging leaves (Figure 4.6) and root development was partially inhibited (Figure 4.7.; B and C; arrow). In most cases, development of first emerging leaves was completely inhibited (Figure 4.7. D; see Table 4.1.).

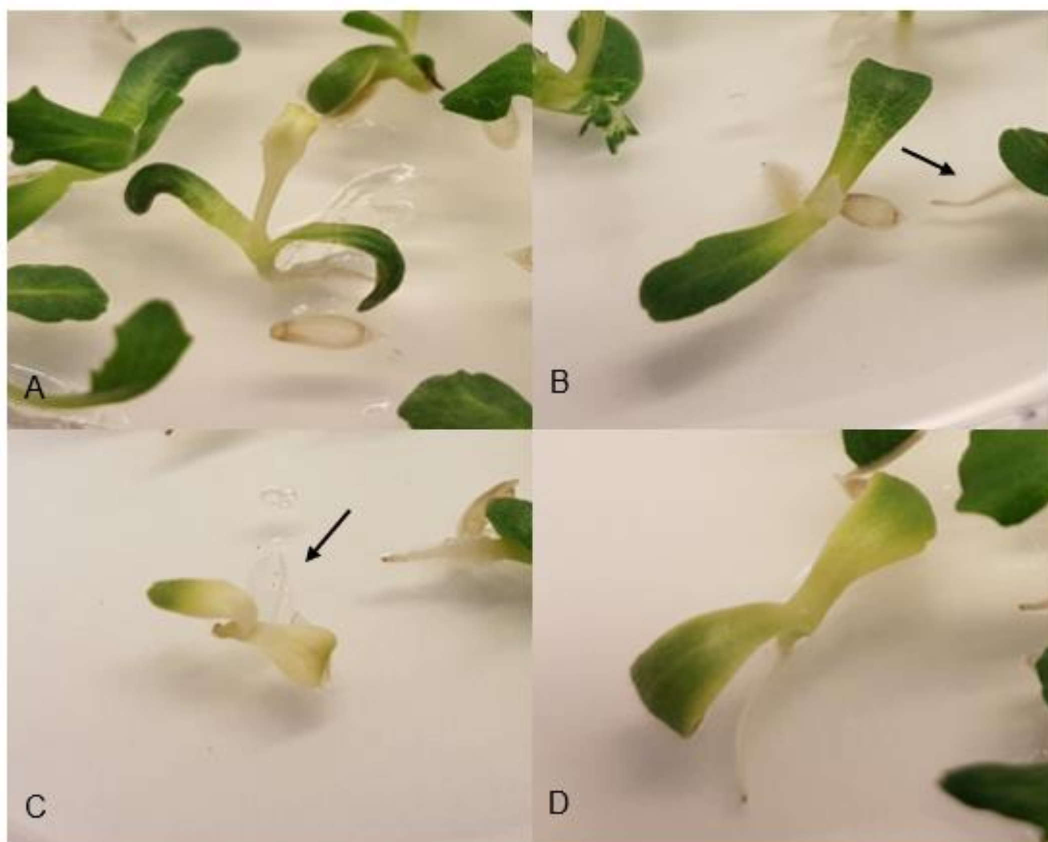

Figure 4.7. Kanamycin sensitive seedlings (KanS) from putative transformed lettuce with ACEI_FMK, ACEI_SEA, ACEI_SPI and ACEI_CHLTP, corresponding to A, B, C and D, respectively.

By contrast, Kan^R seedlings had produced at least one pair of leaves (Figure 4.8.).

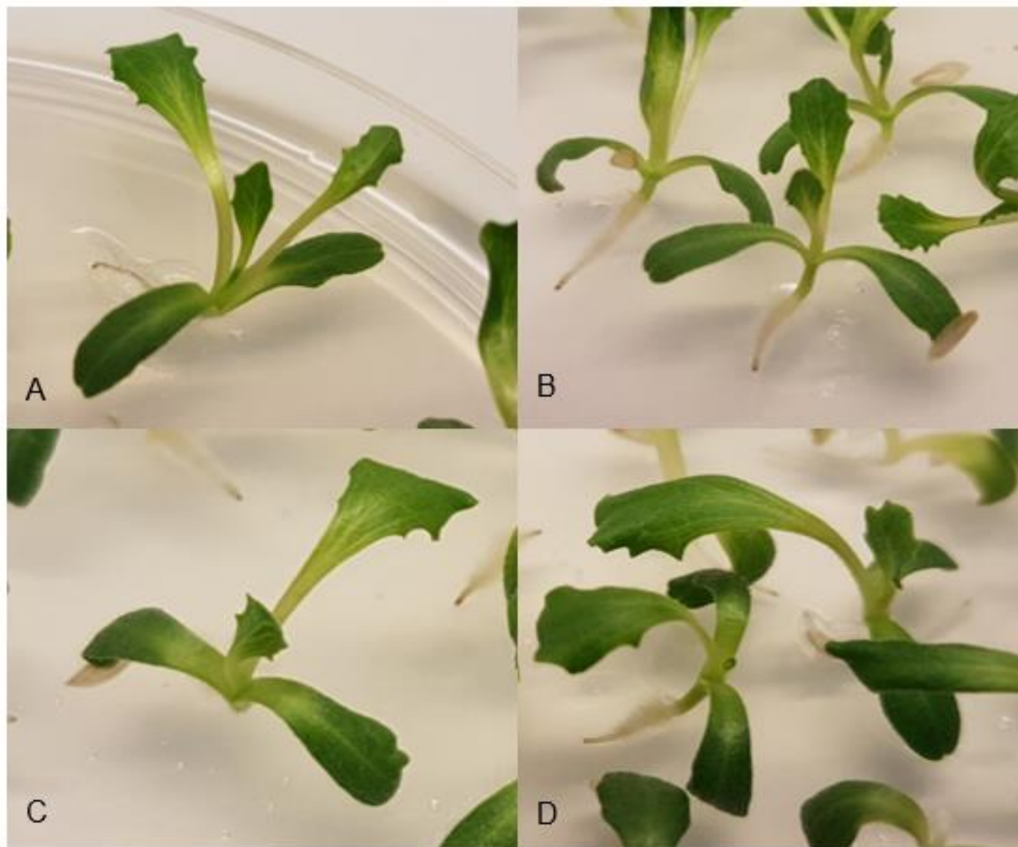

Figure 4.8. Kanamycin resistant seedlings (KanR) from transformed lettuce with ACEI_FMK, ACEI_SEA, ACEI_SPI and ACEI_CHLTP, corresponding to A, B, C and D, respectively.

Seedlings with a different resistance pattern profile were also observed, where some organ tissues presented Kan^S phenotype whilst others presented Kan^R (Figure 4.9.).

Figure 4.9. Different kanamycin resistance profile observer in seedlings from putative transformed lettuce with ACEI_SPI (arrows).

Although Kan^R plants exhibited normal aerial plant development, root system appeared to be partially inhibited. So, in further work, after putatively transformed plants had been selected, Kan was removed to allow normal root development and guarantee seedling viability when transferred to Jiffy® pots for acclimatization. All seed lines tested, with the different ACEI coding sequences (ACEI_FMK, ACEI_SEA, ACEI_SPI and ACEI_CHLTP), showed both Kan^R and Kan^S plants.

4.2.3. Stable inheritance of the transgenes and Mendelian segregation patterns

Starting from T_0 self-pollination lettuce plants, stable inheritance of the neomycin phosphotransferase gene (Npt II) conferring kanamycin resistance, was tested in four independent T1 seed lots (Figure 4.10.).

Figure 4.10. Putative transformed lettuce plants (T0) in the greenhouse.

The analyses of segregation ratios for Kan resistant/sensitive seedlings (T1) are presented in Table 4.2. With 300mg/L Kan, we were able to distinguish Kan resistant seedlings (first emerging green leaves) from Kan sensitive ones (first emerging etiolated leaves) resulting from the progeny of self-fertilized T_0 plants.

Table 4.2. Chi-square (χ^2) analyses of segregation ratios for kanamycin resistant/sensitive seedlings among two T_1 lines obtained from self-pollination of transgenic *L. sativa* primary T_0 independent lines.

Plant lines	Total seedlings	Observed Kan^R	Observed Kan^S	Expected Kan^R	Expected Kan^S	Test ratio (Kan^R: Kan^S)	χ^2 (df=1)
ACEI_FMK	43	39	4	32.25	10.75	3:1	5.65
ACEI_FMK	43	39	4	40.3	2.7	15:1	0.67
ACEI_SEA	41	32	9	30.25	10.25	3:1	0.2
ACEI_SPI	44	28	15	33	11	3:1	2.21
ACEI_CHLTP	45	32	13	33.75	11.25	3:1	1.34

Kan^R = kanamycin-resistant seedlings; Kan^S = kanamycin-sensitive seedlings; Test ratio = Mendelian expected ratio of hemi - to homozygous lines; χ2 = Results from Yates chi-square (χ2) test using the formula [χ2= Σ (Observed value-Expected Value)^2/Expected value]; with 1 degree of freedom (df=1) and 0.05 probability level (*P*= 0.05).

The results are consistent with a Mendelian segregation pattern of 3:1 for the ACEI_SEA, ACEI_SPI and ACEI_CHLTP, and 15:1 for the ACEI_FMK, corresponding to the presence of a single and double functional insert, respectively (χ2 analysis; df = 1; p = 0.05), conferring kanamycin resistance in transformed plants.

4.3. Confirmation of stable ACEI transformed *Lactuca sativa*

4.3.1. DNA extraction

Genomic plant DNA was extracted from control non-transformed (Kan^S), putative transgenic (Kan^R) and seedlings with a different resistance pattern profile, previously used in the χ^2 statistical analyses, using a DNA extraction protocol adapted from *Arabidopsis thaliana* (Edwards, Johnstone, & Thompson, 1991) as described in section 3.2.2. Nanodrop® results revealed general good purity (Abs260/Abs280 >1.8) and DNA concentrations ranged from 62.3 ng/μL to 618.5 ng/μL. Genomic DNA integrity was confirmed by a 1% gel electrophoresis as described in section 3.2.2. Overall migration pattern was consistent with non-degraded genomic DNA since the majority of samples presented a tight band and small smearing. Although in some cases it was verified the presence of putative impurities, these would not be in conformity with Nanodrop purity ratios and may seem to represent DNA that did not entered the gel (Figure 4.11.).

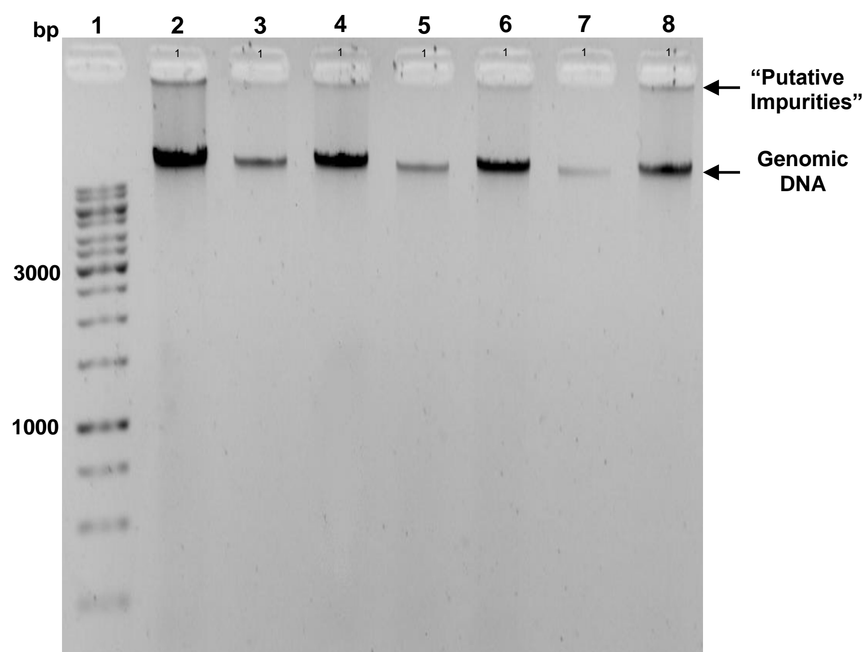

Figure 4.11. Agarose gel (1%) electrophoresis to monitor genomic DNA integrity. Lane 1 - 1Kb DNA ladder; lane 2 – control non-transformed lettuce genomic DNA; lanes 3-8 – putative transformed lettuce genomic DNA.

4.3.2. PCR screening for stable transformed *Lactuca sativa* seedlings

PCR amplification with specific primers for the four ACEI synthetic coding sequences was performed as described in section 3.2.3. PCR products were visualized by 1.7 % agarose gel electrophoresis. The efficiency of the PCR amplification reaction was confirmed by the amplification of an internal portion of the housekeeping actin gene (Act), which functioned as a positive control PCR reaction. The presence of an amplicon with 92 bp confirmed the presence of the ACEI_FMK coding sequence in the lettuce genome (Figure 4.12.). The ACEI_SEA, ACEI_SPI and ACEI_CHLTP coding sequences could not be visualized. To rule out problems in the PCR amplification conditions, these should be optimized. Temperature, primers concentration and magnesium concentration should be optimized for each ACEI coding sequence, especially in the case of the ACEI_SPI since it consists of an eight tandem repeat of the small MRW peptide coding sequence, whose PCR amplification can be problematic. Furthermore, a DNA Polymerase Proofreading enzyme could be used in parallel, as it has higher performance in polymerizing correct sequences with the minimum error.

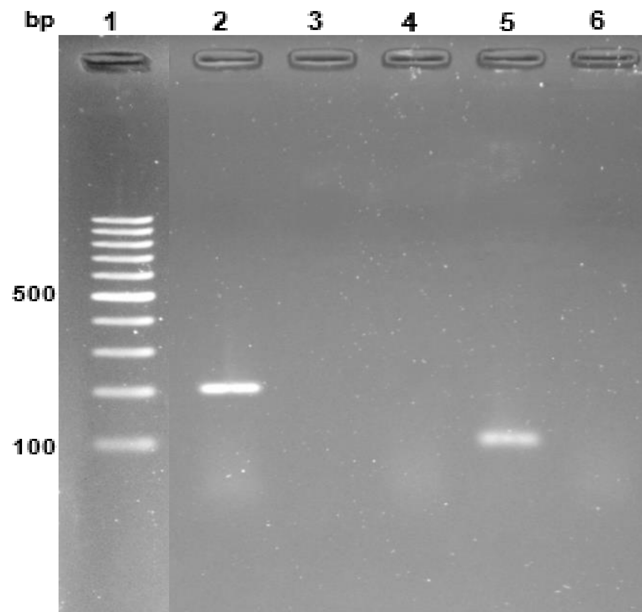

Figure 4.12. Agarose gel (1.7%) electrophoresis of PCR products for ACEI synthetic genes. Lane 1 - 100 bp DNA ladder; lane 2 – Positive control of 101 bp Act amplicon; lane 4 – control non-transformed lettuce; lane 5 - ACEI_FMK (plant line 9.2) 92 bp amplicon; lane 6 –DNA sample from kanamycin sensitive seedlings (Kan^S). The image was edited to eliminate empty lanes.

These results, together with seed segregation analysis (see section 4.2.3.), showed that the T-DNA region containing the ACEI_FMK synthetic coding sequence was stably inserted in the genome of the lettuce T_0 transformed plants and was transferred to the T_1 progeny.

4.4. Analysis of ACEI expression at peptide level in *Medicago truncatula* and *Lactuca sativa*

4.4.1. Oligopeptide extraction analysis employing an ELISA assay

Considering the existing experience in the lab using an ethanolic precipitation protocol for protein extraction, and that the ACEI oligopeptides are not membrane proteins and, therefore, the use of detergent in the extraction buffer was not essential (Mika and Lüthje, 2003; Santos, 2012), total proteins extraction from lettuce, Medicago and Populos, leaves was performed according to section 3.3.1. for ACEI oligopeptides expression analysis. Thereafter, the total protein samples were subjected to a semi-quantitative ELISA to confirm the presence of the His-tagged ACEI oligopeptides in the host plants. The ELISA assay performed was a competitive enzyme immunoassay, where the antigen - the ACEI oligopeptides with the His-tag– in the plant total protein extract sample competed for limited antibody binding sites with a tracer antigen (antigen bound to a reporter enzyme) in a colorimetric assay (see section 3.3.2.). Although the ACEI oligopeptides are low molecular weight targets (< 10 kDa) their specificity for the antibody in the assay, given by the presence of a His-tag,

would allow to extrapolate their relative concentration from a standard curve, from a crude/impure total protein leaf sample. The standard curve plot is represented in Figure 4.13.

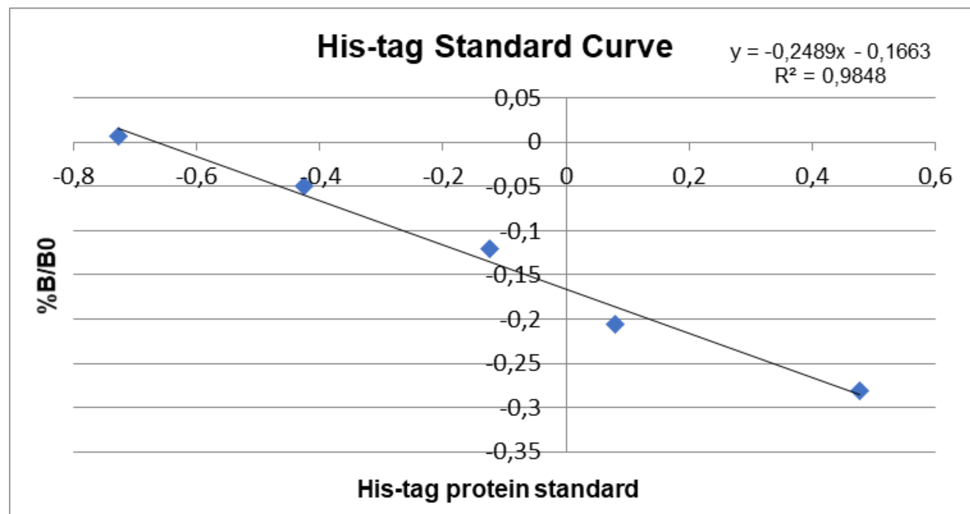

Figure 4.13. His-tag standard curve plot and linearized curve with the R^2 value. The %B/B0 was obtained by dividing the samples or standards abs by the maximum binding solution (B0) abs.

The standard curve plot obtained considered five of the six His-protein standard samples measurements since standard 6 was considered out of scale. The data was linearized using a logarithmic transformation. The linearized standard curve meets the requirements to continue the work since the R^2= 0.98 (Figure 4.13.).

The absorbance results obtained are presented on Table 6.1. on annex 6.1.16. These absorbance results should have been used to determine samples concentrations using the equation obtained from the standard curve plot (Figure 4.13.). However, since the expected results were not achieved these calculations were not performed. Instead, it was observed a similar colorimetric result (similar Abs) among all total lysate samples, from the negative controls, with the Medicago M910a and Populos, to the transformed Medicago. All different protein samples presented a high degree of biding to the specific anti-His antibody in the assay in the less diluted samples, with relative similar increase of colorimetric result in the serially diluted samples. Thus, it was hypothesized that we were in the presence of a nonspecific binding of a certain product present in the total crude protein samples, which produced the false positive results recorded. Although the ELISA kit used is indicated for non-specific crude cell lysates, the total plant protein samples could contain materials that interfere in the assay producing the false positive or simply erroneously high levels of His-tagged peptides. In order to overcome this situation, in further work, the samples should be subjected to a His-tag purification procedure, such as the affinity His-tag purification column commercialized by ABT beads.

4.4.2. Oligopeptide extraction analysis by SDS-PAGE

To attempt to confirm the production of the ACEI peptides at the protein level, total lysates of Medicago and lettuce leaves were subjected to SDS-PAGE gel electrophoresis to separate proteins according to their electrophoretic mobility. The protein extraction and SDS-PAGE gel electrophoresis were performed as described in section 3.3.3. and 3.3.4., respectively. The result is shown in the following Figure 4.14.

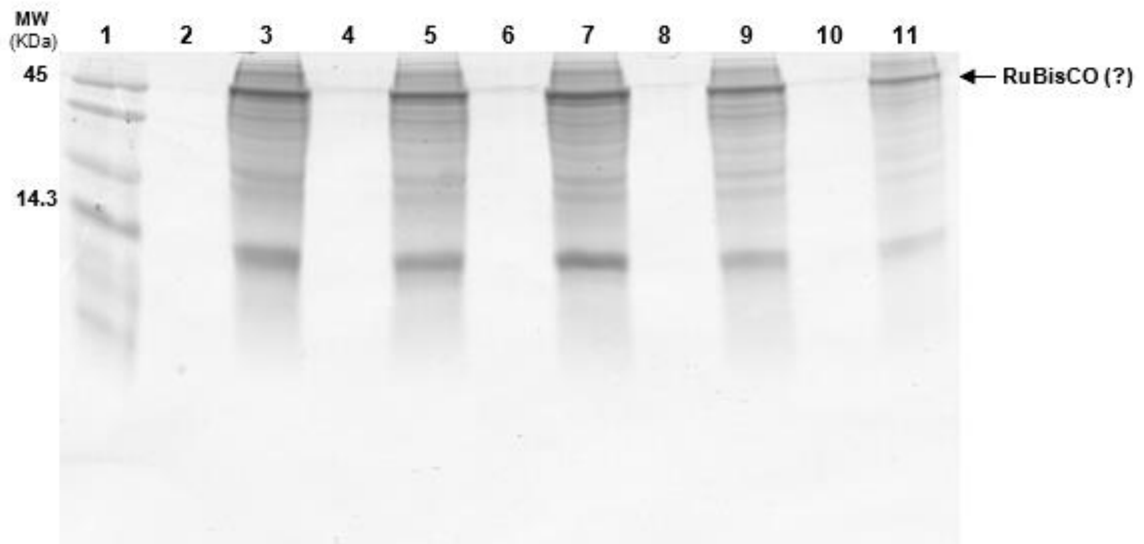

Figure 4.14. SDS-PAGE gel electrophoresis (Mini-PROTEAN® Precast Gel Tris-Tricine) of total lysate samples from Medicago leaves. Lane 1 – Low range protein standard (Rainbow Marker RPN755); lane 2 and 3 – control Medicago M910a samples; lanes 4 and 5 – transformed Medicago samples with ACEI_FMK (plant line 5.7) (expected size 3.14 kDa); lanes 6 to 11 – transformed Medicago samples with ACEI_SEA (plant lines 6.1, 6.2 and 11.1, respectively) (expected size 1.86 kDa).

The total lysate samples represented in Figure 4.14. were subjected to the concentration device mentioned in section 3.3.3. In lanes, 3, 5, 7, 9 and 11 are the samples with proteins with molecular weight higher than 10 kDa, while in lanes 2, 4, 6, 8 are the samples with proteins with molecular weight under 10 kDa. Higher molecular weight samples present a stronger band intensity, in comparison with the lower molecular weight samples, corresponding to a higher protein concentration. Also, it was observed a protein distribution pattern related to leaves with a strong band possibly corresponding to RuBisCO (Armbruster *et al.*, 2009), especially in the samples with higher molecular weights. In all different electrophoretic conditions tested (see section 3.3.4.) it was not possible to visualize the expected low weight bands for none of the ACEI oligopeptides. Similar results were obtained for the total lysate samples from lettuce leaves (data not shown).

In an attempt to identify the ACEI peptides a more sensitive technique for protein detection was employed namely the western immunoblotting.

4.4.3. ACEI oligopeptides analysis by SDS-PAGE and western blot

Total lysate samples from lettuce and Medicago leaves were subjected to SDS-PAGE and subsequent western blotting as described in sections 3.3.4. and 3.3.5., respectively, to attempt to confirm the presence of the ACEI oligopeptides. The results are shown in Figures 4.15. and 4.16.

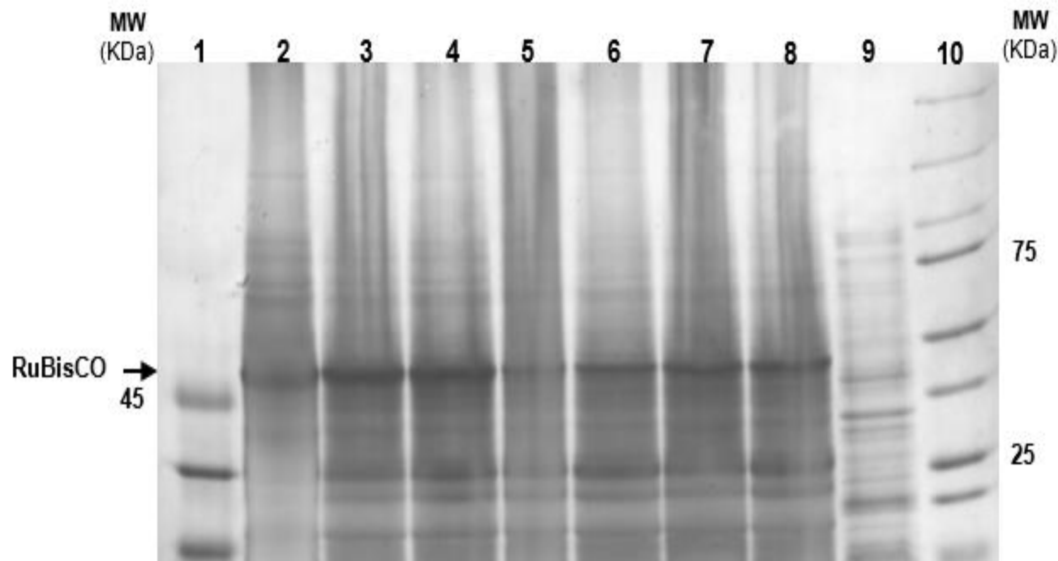

Figure 4.15. SDS-PAGE gel electrophoresis with a 5-20% gradient resolving gel of total lysate samples from Medicago leaves. Lane 1 – Low range protein standard (Rainbow Marker RPN755); lane 2 – negative control (non-transformed Medicago M910a); lane 3 - ACEI_FMK (plant line 5.7) (expected size 3.14 kDa); lanes 4 to 8 - ACEI_SEA (plant lines 6.1, 6.2, 11.1, 11.4 and 26, respectively) (expected size 1.86 kDa); lane 9 – positive control (bacteria cell lysate expressing His-tag protein); lane 10 - High range protein standard (Precision Plus Protein™ Dual Color Standards).

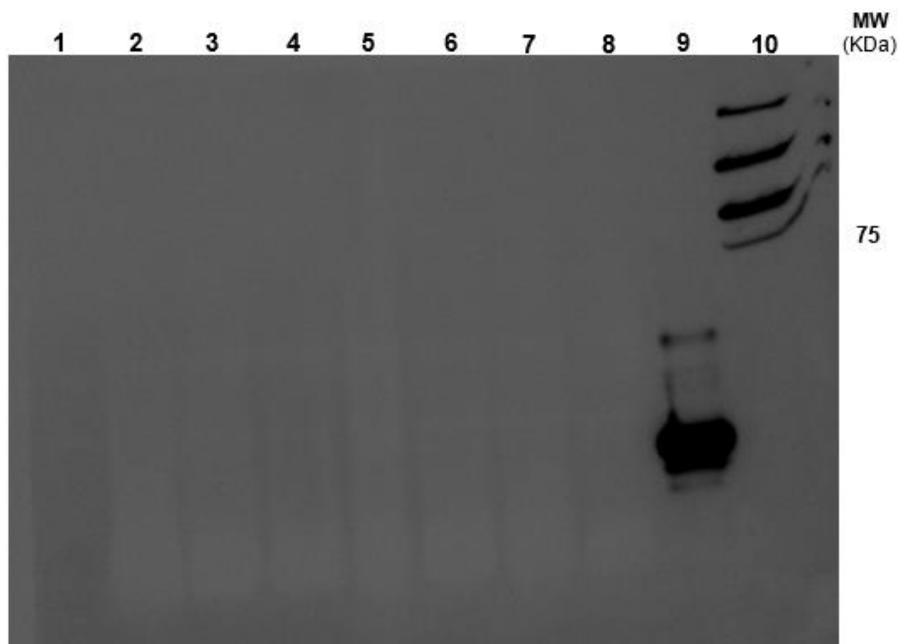

Figure 4.16. Western blot of total lysate samples from Medicago leaves. Lane 1 – Low range protein standard (Rainbow Marker RPN755); lane 2 – negative control (non-transformed Medicago M910a); lane 3 - ACEI_FMK (plant line 5.7) (expected size 3.14 kDa); lanes 4 to 8 - ACEI_SEA (plant lines 6.1, 6.2, 11.1, 11.4 and 26, respectively) (expected size 1.86 kDa); lane 9 – positive control (bacteria cell lysate expressing His-tag protein); lane 10 - High range protein standard (Precision Plus Protein™ Dual Color Standards).

In Figure 4.15., the protein distribution pattern related to leaves with a strong band possibly corresponding to RuBisCO, is once more evident in lanes 2 to 8. In this assay the control Medicago (M910a) was used as a negative control, corresponding to lane 2, and also a positive control, consisting of a bacterial lysate expressing a His-tag 25 kDa peptide (lane 9). The expected band for the ACEI oligopeptides was still absent.

Since the ACEI peptides could be present in low concentrations, making it difficult to visualize them on an electrophoresis gel, a western blot was performed. An antibody against the 6x His-tag was used and, therefore, one band on the positive control and another in each transformed Medicago sample were expected. In Figure 4.16. it is possible to recognize a band on lane 9, corresponding to the His-tag positive control, proving that the antibody used was working with the desired performance. Despite that, it was not possible to visualize any of the expected bands for the ACEI oligopeptides in all the different conditions tested. This was consistent, either using the precast Tris-Tricine gel or the gradient resolving gel, and either using the semi-dry or the wet transfer techniques, as described in section 3.3.5.

Considering the lack of positive results three possible scenarios were hypothesized:

1) The first is regarding the protein extraction procedure. Bering in mind that plants have rigid cellulose cell wall, protein extraction required mechanical lysis by grinding. Along with the cellulose wall, cell organelles are also broken up upon grinding. Among these organelles there is the vacuole that contains secondary plant products (especially phenolics and polyphenolics), organic acids, and proteinases (Müntz, 2007). These released substances can have an influence in peptides by modifying, inactivating or degrading them (Laing and Christeller, 2004). Regarding this aspect, in our protein extraction procedure we used several protective substances and technique to protect proteins. Among the substances used in these procedures, there was: i) proteinase inhibitors such as EDTA and a commercial cocktail of proteinase inhibitors; ii) PVP used to prevent polyphenols from binding and/or inactivating proteins (Spencer *et al.*, 1988); iii) ascorbic acid or β-Mercaptoethanol working as an antioxidant to maintain the reduced state of free sulfhydryl groups in enzymes; iv) Tris to maintain stability of peptides from both a pH and an ionic- strength standpoint. Even with the better extraction buffer tested (buffer 1) and the obtention of 25 μg/μL (average) of total protein concentration per sample, it is still possible that during the extraction procedure the ACEI oligopeptides were lost, considering their low molecular weight and consequent susceptibility to degradation.

2) The second scenario hypothesized was that although ACEI coding sequences are present in host plants genome, and in the case of Medicago there is messenger RNA being expressed, it is possible that the peptides are degraded after protein synthesis. In leafy crops proteins are synthesized in an aqueous environment, which is more prone to protein degradation. This situation is particularly problematic in our case since we are dealing with short peptides that have an inherent instability in plant cells (Lico *et al.*, 2012). To avoid this complication, we immediately froze plant material after harvesting, but peptide degradation can still occur *a priori,* while the plants are alive and growing in the

growth chamber. To avoid this situation one of the ACEI oligopeptides, the ACEI_CHLTP has a transit peptide in its sequence for plant chloroplast targeting (Gomes, 2015).

3) The third scenario hypothesized, was that the ACEI oligopeptides are being produced in a low concentration and, thereby, in order to make its extraction and concentration more efficient, the total lysate samples should be subjected to a His-tag purification procedure, such as the one mentioned on section 4.4.1.

In conclusion, the encouraging results obtained at the transcript level in transformed Medicago, and at genomic level in lettuce, along with the favorable total protein concentrations obtained using extraction buffer 1, we foresee the possibility of using these optimized procedures considering the previous mention suggestions.

4.5. ACEI oligopeptide analysis by HPLC

Total lysate samples from Medicago and lettuce leaves were subjected to HPLC as described in section 3.3.6., in an attempt to find the optimal conditions for the extraction and subsequent chromatography of protein samples in future detection and quantification analyses of the ACEI oligopeptides.

Total protein extraction was performed with three different extraction buffers as described in section 3.3.6. Oligopeptides were detected at 215, 220 and 280 nm, considering that 215 and 220 nm are recommended wave lengths for peptide detection and 280 nm for protein detection. The results are shown on the following chromatograms in Figures 4.17. and 4.18.

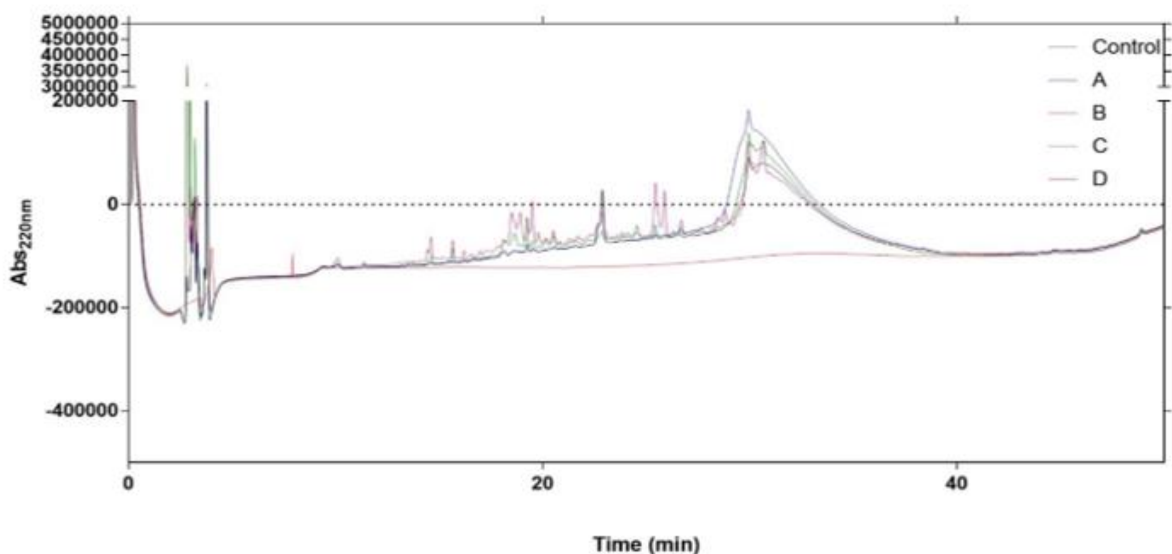

Figure 4.17. Chromatogram of total lysate samples with absorbance at 220 nm, extracted with extraction buffer 4 from control non-transformed and transformed lettuce leaves. Label A – transformed lettuce with ACEI_FMK coding sequence (plant line 9.2); label B – transformed lettuce with ACEI_SEA coding sequence (plant line 3.19); label C - transformed lettuce with ACEI_SPI coding sequence (plant line 11.6); label D – transformed lettuce with ACEI_CHLTP coding sequence (plant line 3.1).

The chromatogram on Figure 4.17. from lettuce protein samples extracted with extraction buffer 4 (see section 6.1.5.) revealed reduced quality. The chromatogram basal line is not well defined, there are negative absorbance values, an absence of defined peaks was verified and sample with label B (transformed lettuce with ACEI coding sequence) reveals a flat pattern consistent with the possibility of an extraction or HPLC preparation error. The negative absorbance values are possibly related to a greater affinity of the solute to the stationary phase than to a given mobile phase moiety (Kowalska, 1990).

In comparison, the chromatogram on Figure 4.18. from Medicago protein samples extracted with extraction buffer 1 (see section 6.1.2.) revealed overall better quality. Since the HPLC preparation was consistently the same in all tested samples, the major source of variation was the protein extraction buffer. Among the extraction buffers tested, the extraction buffer 1 produced the best results and, therefore, should be considered in further procedure optimizations. In further work, the ACEI oligopeptides should be synthetically synthesized to create a positive control to allow the detection and quantification of the ACEI oligopeptides.

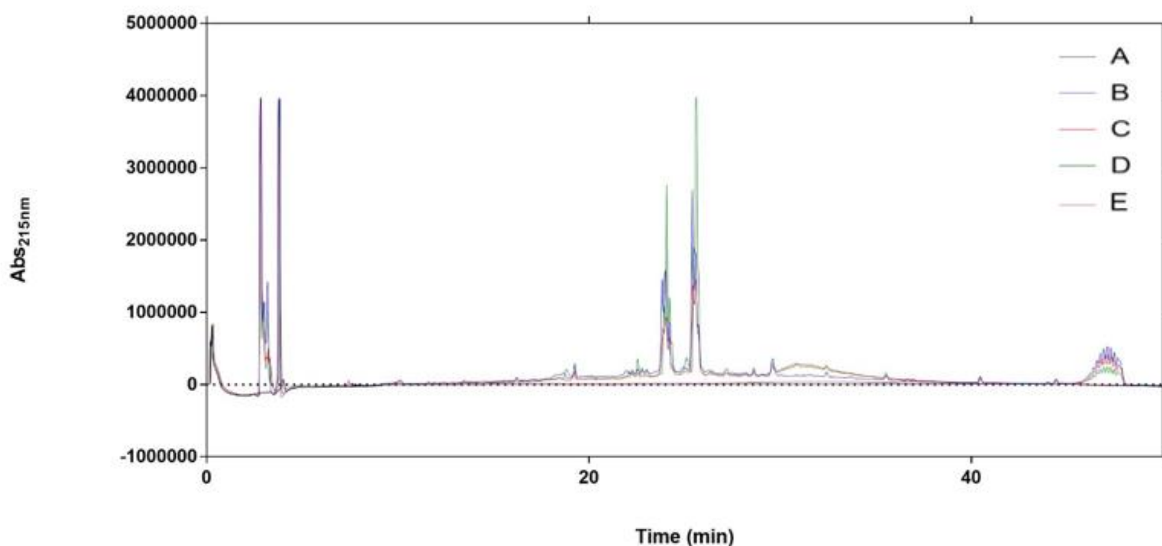

Figure 4.18. Chromatogram of total lysate samples with absorbance at 215 nm, extracted with extraction buffer 1 of transformed Medicago leaves. Label A to E - transformed Medicago with ACEI_SEA coding sequence (plant line 26).

In conclusion, the positive results obtained: i) in the ACEI_FMK and ACEI_SEA expression at the transcript level in transformed Medicago; ii) the encouraging results obtained using the optimized *in vitro* selection scheme for transformed lettuce; iii) the confirmation of stable transformation of lettuce with the ACEI_FMK coding sequence; and finaly, iv) the establishment of optimized condition for protein extraction and detection are promising results for further heterologous production of the ACEI oligopeptides in these plant hosts.

Conclusions and future perspectives

In conclusion, the work developed for my master thesis and here presented proved to be a contribution to the study of the heterologous expression of four ACEI oligopeptides, with proven ACE inhibitory activity, produced in dissimilar plant hosts, *Medicago truncatula* and *Lactuca sativa*.

In this work, the expression of ACEI_FMK and ACEI_SEA coding sequences *in planta* confirmed their presence in the plant genome and validate a successful plant stable transformation for the *M. truncatula* host.

Medicago and lettuce hosts were successfully maintained using an *in vitro* micropropagation scheme as source of plant material. An ongoing stock of non-transformed and transformed medicago and lettuce plants are currently maintained at BCV lab using this procedure. A seed bank of both transformed hosts was created, providing an alternative to the *in vitro* livestock, allowing further molecular characterization studies in the descendance.

An optimized screening strategy for *L. sativa* transformants was established, which proved to be vital for the selection of transformants for further transformed seed selection and molecular analysis. Although there are already optimized transformants selection systems using Kan in the model plant *M. truncatula*, this procedure should always be performed when using a new type of explants or genotype. In this way, it was necessary to optimize the selection efficiency in lettuce to reduce the possibility of selection of resistant but non-transformed seedlings without compromising the whole plant viability and ability to produce offspring. This study could work as a suitable approach for other transformation procedures within this specie.

By seed segregation analysis in lettuce it was possible to confirm a Mendelian segregation pattern of 3:1 and 15:1, corresponding to the presence of a single and double functional insert, respectively. These results, along with further molecular analyzes, that, at lest for the ACEI_FMK synthetic coding sequence, the T-DNA region was stably inserted in the genome of the lettuce and was transferred to the progeny.

Using different protein analysis methodologies such as SDS-PAGE, western immunoblotting, and HPLC, we were not able to obtain positive results for the detection of the ACEI oligopeptide. Nevertheless, the first steps in the establishment of the working procedures were made, providing direction for further optimization strategies.

In the case of *M. truncatula*, we can envisage the establishment of a plant cell suspension culture (e.g. by *calli* induction from the transformed plants leaves) for heterologous production of ACEI peptides, that could represent a promising and very attractive solution for the established biopharmaceutical production platforms. The procedure for establishing the plant cell suspension culture from Medicago has already been established at BCV lab. For the lettuce host it would have to be optimized.

For both production platforms, cell suspension culture and leafy crops, in the lettuce and Medicago hosts, the downstream process should be optimized. In the Medicago, the presence of the His-tags in the ACEI peptides will facilitate the extraction and further purification processes. In the case of lettuce, since the ACEI peptides will be administrated orally, within the plant leaves, the downstream process must be optimized considering, for example, the shelf life of the leaves in order to avoid peptide degradation.

The *in vitro* testing of the ACEI peptides activity will be a crucial proof to validate the effectiveness of both heterologous production systems. If, for example, the presence of the His-tag in the ACEI oligopeptides has a negative influence in their antihypertensive activity, a peptide cleavage site should be added so that the His-tag could be removed upon GI enzyme digestion to occur the release of the active peptide. Furthermore, and considering the future human administration of these peptides, the *in vivo* testing of its active effect should be tested by oral administration to SHRs. Bering in mind that in the case of lettuce the peptides will be administrated within the plant leaves, while in Medicago it will be administrated as a purified product, the different pharmacokinetics should be considered and described.

Lastly, in the current scenario of an ongoing problematic situation related to cardiovascular diseases associated with hypertension along with the worldwide demand for alternative therapies, the demand for therapeutics with less side effects, the same efficacy but less expensive, and more environmentally friendly*,* has given the opportunity to the new biotechnological paradigm brought by molecular pharming to win its market share from the stablished pharmaceutical industry. This thesis represents a small contribution in the way to this brighter future.

6

Appendix

6.1. Reagents and Solutions

6.1.1. Tris Buffer 1 M

6.05 g of Tris base

30 mL of distilled water (dH_2O)

pH 8

The 6.05 g of Tris base was dissolved in 30 mL of dH_2O. pH was then adjusted to 8 with 1 M HCl, and final volume was adjusted to 50 mL with dH_2O.

6.1.2. Extraction buffer 1

Tris-Hcl (pH8) 50 mM

NaCl 100 mM

EDTA 1 mM

Triton x100 1% (v/v)

PVP 2.5% (w/v)

6.1.3. Extraction buffer 2

Ascorbic acid 100 mM

NaCl 500 mM

β-Mercaptoethanol 5 mM

Triton (x100) 1% (v/v)

PVP 2.5 % (w/v)

pH 8 (adjusted with NaOH 1M)

Stored at 4ºC.

6.1.4. Extraction buffer 3

NaCl 500 mM

PVP 2.5% (w/v)

SDS 2% (w/v)

Protease inhibitor cocktail (20µL per 500 µL of sample)

β-Mercaptoetanol

pH 8

6.1.5. Extraction buffer 4

Acetonitrile 1:1 (v/v)

Protease inhibitor cocktail (20µL per 500 µL of sample)

6.1.6. Sample Buffer 1 (4x)

Tris 250 mM (pH6.8)

SDS 8% (w/v)

Glycerol 40% (v/v)

β-Mercaptoethanol 20% (v/v)

Bromophenol blue 0,01% (w/v)

6.1.7. Sample Buffer 2 (4x)

Tris 100 mM (Ph 6.8)

SDS 1% (w/v)

Glycerol 15% (v/v)

B-Mercaptoethanol (2%) (v/v)

Bromophenol blue 0,01% (w/v)

6.1.8. Running Buffer Tris-Tricine-SDS (1x)

Tris base 100 mM

Tricine 100 mM

SDS 0,1% (w/v)

pH 0,3

6.1.9. Comassie Staining Solution

Coomassie Brilliant Blue 0,2% (w/v)

Methanol 50% (v/v)

Acetic acid 10% (v/v)

6.1.10. De-staining Solution

Methanol 25% (v/v)

Acetic acid 5% (v/v)

6.1.11. Cathode Running Buffer (10x)

Tris 1 M

Tricine 1 M

SDS 1%

pH 8,25

6.1.12. Anode Running Buffer (10x)

Tris 1 M

HCl 0,225 M

pH 8,9

6.1.13. Transfer Buffer

Tris 25 mM

Glycine 192 mM

Methanol 20%

pH 8,3 (adjusted with HCl

Methanol was added just before utilization. Before adding it, buffer was cooled to avoid the formation of gas bubbles upon methanol addition.

6.1.14. Blocking solution

Milk powder 5% (w/v)

TBS-T (10x stock) 1% (v/v)

ddH2O (sterile)

Stored at -20ºC and reused.

6.1.15. ACEI peptide synthetic coding sequences

Figure 6.1. ACEI synthetic coding sequences ACEI_FMK (A), ACEI_SEA (B), ACEI_SPI (C) and ACEI_CHLTP (D). ACEI_FMK (A) has a 5' flanking region joined by a linker of two amino acids (MK). ACEI_SPIN (C) consists of an eight-tandem repeat of the coding sequence for the MRW peptide. 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.

6.1.16. ELISA measurements

Table 6.1. Absorbance mesuarments at a wavelength between 405 and 420 nm.

Samples	Absorbance
Standard 1	0,9 ± 0,1
Standard 2	1,2 ± 0,2
Standard 3	1,4 ± 0,2
Standard 4	1,7 ± 0,2
Standard 5	1,8 ± 0,2
Standard 6	1,5 ± 0,2
NSB	0 ± 0
BLK	0 ± 0
B_0	1,9 ± 0,3
M9	0,6 ± 0,1
Populus (0,41 mg/L)	0,5 ± 0,1
Populus (1:10)	1,3 ± 0,2
Populus (1:100)	1,8 ± 0,2
Populus (1:200)	1,5 ± 0,2
M910a (0,41 mg/L)	0,5 ± 0,1
M910a (1:10)	1,5 ± 0,2
M910a (1:100)	1,9 ± 0,3
M910a (1:200)	2,0 ± 0,3
ACEI_FMK (0,41 mg/L)	0,5 ± 0,1
ACEI_FMK (1:10)	1,5 ± 0,2
ACEI_FMK (1:100)	2 ± 0,3
ACEI_FMK (1:200)	2 ± 0,3
ACEI_SEA (0,41 mg/L)	0,4 ± 0,1
ACEI_SEA (1:10)	1,5 ± 0,2
ACEI_SEA (1:100)	2,1 ± 0,3
ACEI_SEA (1:200)	2,1 ± 0,3

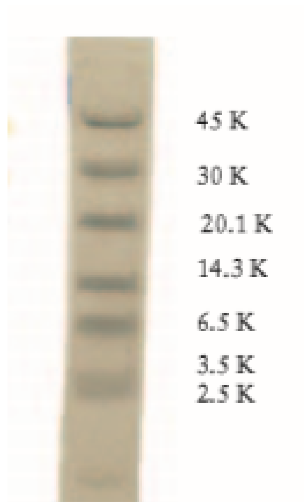

Figure 6.2. Rainbow Marker RPN755 (Amersham Life, UK)

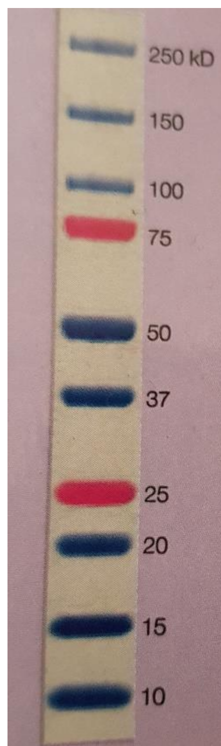

Figure 6.3. Precision Plus Protein™ Dual Color Standards (Bio-Rad, USA)

7

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