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**Plant bioreactors as a sustainable approach for useful compounds
production: innovation and applied research perspectives.**

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STRUCTURE OF THE THESIS

This thesis is organized into two distinct sections, the first reporting on projects in biotechnology and the second on research studies conducted in plant physiology. Each section presents an introduction and is divided into chapters dealing with a specific project or published paper. The bibliography of each chapter is given at the end of each one.

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SECTION I: PLANT BIOTECHNOLOGY

Abstract

Plants have always been a source of metabolic compounds useful for human life. Today, different genetic engineering techniques allow us to modify both whole plants and *in vitro* cultured material for several purposes. These span from the elicitation of bioactive molecules that are already produced by these organisms to *de novo* biosynthesis of metabolites and recombinant proteins. Several studies have focused on the production of compounds with antioxidant, anti-inflammatory and hypoglycemic activity with the long-term goals of exploiting their protective role in the prevention of neurodegenerative diseases and cancer. Indeed, plant *in vitro* systems appear to be reliable and sustainable platforms for the production of such as molecules.

This section presents three strategies currently being pursued for the production in plants of antioxidant, immunomodulatory compounds and with interesting structural properties for the production of biomaterials. They are based either on stable or transient genetic transformation of plant cells from (Chapter I) *Malus domestica* and (Chapter II, III) *Nicotiana tabacum*. In Chapter I, calli derived from apple pulp have been transformed with the bHLH transcription factor Sn (TF) from *Zea mays*. This TF interacts with MYB and WD40 proteins to upregulate the biosynthesis of anthocyanins and/or proanthocyanidins in different plant species and growing conditions.

In Chapter II, tobacco leaf protoplasts were transiently transformed with the human enzyme IDO1, responsible for immunomodulation response. By promoting the degradation of tryptophan to produce kynurenines, molecules capable to modulate the immune system, this enzyme exerts a powerful immunoregulatory action. In Chapter III a vacuolar storage protein from common bean (*Phaseolus vulgaris*), called phaseolin, was investigated. This protein was engineered adding a Cysteine at C-terminal tail, allowing it to form inter-chain disulfide bridges and complex structures: the production capacity of this biopolymer was investigated both in plant and in *Escherichia coli*, in order to compare distinct bioreactor systems.

Keywords: Bioreactors, Human health, Biopolymer

Abbreviations

CAK	Cyclin-dependent Kinase-Activating Kinase
CHO	Chinese hamster ovary
CPMV	Cowpea mosaic virus
ER	Endoplasmic Reticulum
GMP	Good manufacturing practices
IgE	Immunoglobulin E
LB	Left Border
NLS	Nuclear localization signal
PB	ER-derived protein bodies
pCaMV35S	Cauliflower Mosaic Virus promoter 35S
PHA	Polyhydroxyalkanoate derivatives
PLA	Polylactic acid derivatives
PMF	Plant molecular farming
PSV	Protein storage vacuoles
PVX	Potato virus X
RB	Right Border
RNAi	RNA-interference
SDGs	Seventeen sustainable development goals
SpaA	Surface protein antigen A
T-DNA	Transferred DNA
TF	Transcription factor
Ti plasmid	Tumour inducing plasmid
TMV	Tobacco mosaic virus
TSP	Total soluble proteins
UTR	Untranslated region
VIP1	VirE2-interacting protein 1
<i>VIRs</i>	Virulence genes

1. Introduction

1.1 Plants as bioreactors

The historical progression of medical botany encountered a transformative juncture during the 19th century, propelled by the increasing significance of chemistry. This event shift enabled the isolation, purification, and subsequent synthesis of organic compounds, laying the foundations of the pharmaceutical industry: synthetic molecules exhibit greater specificity in therapeutic actions compared to botanical preparations, instigating a crucial transition. The high demand for bioactive substances across diverse domains, such as chronic disease management, dietary supplementation, and crop pest control, has significantly influenced the evolution of techniques for bioactive molecule production^[1]. *In vitro* cultures under controlled, aseptic conditions have allowed advancements in genetic transformation techniques: thanks to this controlled environment, it is possible to work under selective pressures by antibiotics, inducing mutations, plastid transfer, alterations in ploidy, amplifying the potential for targeted molecular expression^[2]. A defining moment in plant biotechnology unfolded on January 18, 1983, marked by the "Miami Winter Symposium", where independent research groups documented *Agrobacterium tumefaciens*-mediated genetic transformation leading to the development of typical, fertile plants, expressing antibiotic-resistance genes: this event generated a collective awareness within the scientific community regarding the multiple applications of this transformation strategy, spanning agronomy, pharmaceuticals, cosmetics, and nutraceuticals. Subsequent decades presented significant strides in genetic engineering and micropropagation techniques, culminating in the creation of transgenic plants, tissues, and cell cultures capable of yielding elevated quantities of naturally occurring and synthetic compounds, including recombinant proteins. Heterologous protein expression in plant tissues, notably initiated in 1986 in tobacco through the introduction of growth hormone genes by *A. tumefaciens*, has represented a revolutionary innovation. Beyond medical and pharmaceutical applications, the production of marketable bioactive molecules has also involved the cosmetic sector; for example, the synthesis of collagen in tobacco^[3] implies the versatility and potential of plants in biotechnological advancements. Tobacco, renowned for its compatibility with multiple *in vitro* culture techniques and its high biomass and seed yield, continues to serve as a model organism. Tobacco is still an ideal expression host because it has strong transformation protocols and well-defined regulatory components that allow transgene expression to be controlled.

Additionally, because it is a non-food, non-feed crop, there is less chance that transgenic material or recombinant proteins may contaminate feed and human food chains. The high concentration of nicotine and other alkaloids in tobacco, which need to be eliminated entirely during subsequent processing stages, is one of its drawbacks. While tobacco cultivars with less alkaloids are available, the focus of pharmaceutical manufacture has shifted to other leafy crops^[4]. Nonetheless, the techniques pioneered in the late 1990s have extended to various plants, like *Arabidopsis thaliana*, tomato, banana, turnip, cowpea, canola, potato, rice, wheat, and corn. Strategic selection of initial plant materials holds pivotal significance. Notably, preference may be accorded to species prevalent in developing nations for orally administered vaccines, effectively circumventing significant economic and societal barriers^[5]. Plants, acting as biological factories and bioreactors, demonstrate remarkable potential in producing an array of bioactive molecules^[6]: nutraceutical utilization stands prominent among the diverse applications of plant-sourced bioactive molecules. These compounds find their way into dietary supplements, encapsulated within non-food matrices such as pills, capsules, and powders, or naturally integrated into functional foods. In 2008, criteria were defined to characterize functional foods like distinct effects from conventional diets, demonstrable beneficial effects, enhancements in physiological activities, or disease risk reduction^[7].

The exemplary role of anthocyanins, a subset of flavonoids found in fruits and vegetables, is an example of the potential health benefits of plant-derived bioactive molecules. Their consumption has been linked to the alleviation of numerous ailments, including cancer, cardiovascular disorders, and degenerative conditions. Anthocyanins' anti-inflammatory properties, improved visual acuity and potential for preventing diseases such as diabetes and obesity are attributed to their strong antioxidant activity. These compounds counteract free radicals and oxidative molecules, bringing multiple health benefits at the cellular, tissue and systemic levels.

World Health Organisation statistics from 2013 highlight the staggering impact of cancer, with around 9 million deaths out of 14 million diagnosed cases, so new therapeutic techniques are increasingly required to treat these diseases. While conventional chemotherapies and radiotherapies persist, integrating bioactive compounds from plants represents a new frontier. Certain plant-derived compounds exhibit lower toxicity and greater tolerance within healthy human cells, fueling their exploration as potential anticancer agents^[8]. Taxol, an oxygenated diterpenoid, is an effective anticancer agent against gastric, ovarian, lung, and breast cancers.

Its mechanism involves binding to the β -subunits of tumour cell tubulin, preventing mitotic division and inducing cell cycle arrest, particularly at G0/G1 and G2/M transitions. Despite its clinical significance, taxol's extraction from *Taxus brevifolia* bark poses challenges owing to low yield, limited accessibility, and ecological implications due to the plant's diminished populations^[9]. Promising avenues, such as utilizing suspension cultures of *Taxus* cells for sustainable large-scale production, offer potential solutions to mitigate these challenges, ensuring sustainable sourcing while meeting therapeutic demands^[10]. Moreover, the potential of plant systems in the production of recombinant proteins including antibodies, vaccines and hormones represents a convincing alternative to traditional animal cell cultures and bacterial colonies. The modularity of plant-based expression mechanisms makes them adaptable to market dynamics, allowing the scale and purity to be adapted to different requirements. Strategies focusing on the concentration of target compounds within transgenic cereal seeds represent a cost-effective approach that exploits the seeds' innate ability to accumulate high amounts of substances. The temporal trajectory for product acquisition through genetic engineering involves multifaceted processes such as construct preparation, transformation, plant regeneration, validation of successful transformation, and subsequent progeny maintenance. The temporal investment, extending up to two years contingent on plant species, is one of the negative aspects of this technique. Notably, transient expression systems have emerged for rapid, small-scale production of desired proteins/active compounds, facilitating real-time assessment of expression dynamics and product quality.

2. Plant Molecular farming, benefits and critical aspects

In the early 1980s, Fritz Kreuzaler, during a talk on plant viruses at the Max-Planck Institute in Cologne, developed the idea of pathogen-specific antibody expression in plants to achieve antibody-mediated disease resistance. When cDNAs encoding the heavy and light chains of a murine IgM were used for the transformation of *Acetabularia mediterranea*, an algal species with large cells used as a model organism in cell biology, Kreuzaler and his team confirmed the presence of an assembled full-size antibody, despite the doubts of his contemporaries. Later, Avidin and β -glucuronidase, generated in transgenic maize plants, were the first molecular agricultural products sold more than 20 years ago: both proteins are utilized as research

reagents. Since then, a variety of technical and diagnostic proteins have been created by plants. In 2012, the first plant-derived biopharmaceutical for humans was approved for sale: Elelyso (Taliglucerase alfa) is the first recombinant human enzyme (β -glucocerebrosidase) generated by Protalix Biotherapeutics in plant cell suspension cultures of *Daucus carota* for the treatment of the lysosomal storage disorder Gaucher's disease^[11].

In this paragraph, I report an overview of the most frequently mentioned advantages of plant molecular farming, which are safety (for the absence of human pathogens replication, endotoxins formation or animal-derived contamination components), high scalability because of the several strategies disposable for plant culture, and expression system strategies that allow the production of considerable quantities of product in few weeks. Although effective clearing can easily be achieved today, there are some significant disadvantages, such as the need to clarify feed streams with a high particle load and the huge amounts of host cell proteins, the lack of regulatory approval for open field culture, and the absence of a conserved glycosylation process in the different kingdoms.

2.1 Scale-up possibility and cost-effectiveness

Plants are increasingly an attractive and cost-effective emerging alternative as bioreactors for producing compounds for the pharmaceutical and nutraceutical field^[4], such as recombinant proteins, industrial enzymes, vaccines, animal feed, biopolymers and biodegradable plastics. This trend is growing due to the various advantages offered by this technique: plants show significant benefits in the regulation and expression of the desired molecule and, more importantly, significant economic advantages comparing them to mammalian cells and microorganisms, traditionally used to produce foreign molecules^[5]. The expression increasing can significantly help to reduce costs. Furthermore, expression levels for protein products that are not removed and purified before use must be high enough to ensure efficacy. For example, an edible vaccination would come from enough plant tissue to be consumed in one sitting to provide a high enough dose of antigen to ensure protection^[12].

It is estimated that the cost of producing active molecules in plants is only 2-10% compared to traditional microbial fermentation processes and even 0.1% compared to mammalian cell cultures^[5]. Still, in comparison to mammalian systems, the cost benefits may be up to 1000-folds. The expression regulation is crucial in response to the growing demand for recombinant

proteins and natural molecules on a large scale. Plants can become economically viable at yields of 0.1–1.0% of the total soluble proteins (TSP), typical for producing pharmaceutical proteins like recombinant antibodies^[13]. The distinction between scalability, intrinsic and biomass yield should be considered: the intrinsic yield is the quantity of recombinant protein that can be generated expressed as a percentage of total soluble protein or, more conveniently for cross-platform comparisons, as a percentage of wet or dry biomass. Compared to the primary fermenter platforms like bacteria, yeast, and mammalian cells, all plant systems currently have lower inherent yields^[14]. Green bioreactors show low protein yields compared to the common industrial microbial and mammalian production platforms: despite intensive study to enhance protein expression and stability, recombinant protein levels rarely reach 100 µg/kg fresh weight of plant tissue or per liter in suspension cell cultures. In addition to intrinsic factors like protein fusion and targeting strategies, expression strategies, and the co-expression of protease inhibitors, this research has also examined external factors like physical and nutritional cultivation parameters that affect plant growth and fitness. The competitiveness of plants lies in the fact that they produce more biomass than other fermentation systems, mainly when grown in the field; for example, one hectare of tobacco produces on average 1-2 tonnes of leaves per year, on the other hand, a continuous 20-L fermenter may yield 100–150 kg of wet cells. This explains why, even though the intrinsic yields of plants do not match those of CHO cells, plants are likely to be more economically advantageous for large-volume products, especially those with a narrow profit margin, as the cost difference between growing 1, 2, 5, or 10 ha of tobacco is negligible when compared to the cost of commissioning and constructing additional fermentation infrastructure^[14]. On the other hand, open-field cultivation was quickly found to make quality and safety control more challenging. Additionally, downstream processing (which involves removing target proteins from the plant matrix) was found to be a significant cost factor that necessitated process optimization^[11]. Another crucial factor is the production's time scale: producing stable transgenic lines has historically been the primary means of modifying plants to express recombinant proteins, a resource- and time-intensive process. On the other hand, the transient transformation of plant traits proceeds quite quickly, producing recombinant proteins or the byproducts of their action in a few days, and may currently be expanded to production levels that are important to the commercial world^[15]: for example, up to 4 g/kg fresh weight were obtained by transient expression^[11]. The use of plant cells, in particular plant suspension cultures (instead of entire plants), is another commercial attractive area of molecular

farming technology that is currently regaining popularity: even if the term "molecular farming" evokes visions of greenhouses filled to the brim with dense, green leaves expressing pharmaceutical proteins, a large portion of the field's technological and business advancements have been founded on plant cells. These combine the benefits of traditional fermenter systems with those of plants: sterile, controlled, and contained production environments; chemically defined media free of animal components; compliance with the strictest regulatory world guidelines and pharmaceutical good manufacturing practice^[16].

2.2 Expression level optimization: plant selection and genetic strategies

To synthesize recombinant proteins and biomolecules in plants, one of the critical aspects that must be considered is the optimization of expression level. First, one of the main aspects is the species choice: just as the molecules that can be produced are many, so are the plants ideal for producing those specific molecules. Moreover, the host choice is also important because many biological and geographical factors must be considered, as it will be described later. Today, many plants have been used for this purpose, such as tomato, rice, maize, wheat, carrot, soybean, lettuce, alfalfa and potato. Tobacco is one of the main species used in research (as described in the following paragraphs). For transgenic plants and transient expression systems, a good state of health and the growing environment have a significant impact: for instance, even a slight temperature fluctuation of 1–2°C can cause recombinant protein yields to vary by up to 15%. Through a combination of construct design, optimal genetic background, and an evaluated environment, remarkable yields have been attained: up to 10.6% of TSP for human serum albumin expressed in rice seeds, 30% of TSP for industrial enzymes in maize seeds, 36% of TSP for a murine antibody in *Arabidopsis*, and exceeding 70% of TSP for proteins expressed in tobacco chloroplasts.

Plant transformation can be performed in different ways: the production of foreign molecules could be extended to the whole plant (in a constitutive/inducible way) by transforming the nuclear/plastid DNA or by a transient expression system^[17]. To produce recombinant proteins in plants on a large agricultural scale, the most popular technique for genetically modifying plants is stable nuclear expression of transgenes^[18]. Expression of transgenes in chloroplasts often results in a higher yield of recombinant proteins compared to their expression in the cell

nucleus: this is primarily due to the abundant presence of chloroplasts within each cell and the absence of gene silencing^[19]. Numerous benefits arise from chloroplast transformation: a typical photosynthetic cell contains many chloroplasts, which increases the transgene copy number. As mentioned, there is no gene silencing, multiple genes can be expressed in operons, recombinant proteins accumulate within the chloroplast, limiting toxicity to the host plant, and most crops' pollen lacks functional chloroplast DNA, which naturally contains transgenes, so the pollens-transmission is rare. However, chloroplasts cannot perform glycosylation as bacteria^[4].

Although it has been successfully achieved in tobacco and several other Solanaceous species as a routine practice, chloroplast transformation in other plant species has had relatively limited success^[18]. As previously mentioned, transient expression systems are also available in plants, allowing a rapid production of recombinant proteins. While transient expression of foreign genes has been documented in various plants, *Nicotiana benthamiana* is the most used species due to its high protein output and ease of manipulation. As evidenced by the 2014 Ebola hemorrhagic fever outbreak and, more recently, the COVID-19 case, this is particularly helpful when rapidly spreading epidemic or pandemic diseases occur. In these situations, the timely production of recombinant proteins (especially diagnostic reagents, therapeutic antibodies, and vaccine candidates, many of which are complex glycoproteins) is crucial^[20].

Genetic factors must also be considered while transforming plants: one widely utilized strategy for improving recombinant protein synthesis is codon optimization of transgenic coding sequences. Most amino acids are encoded by numerous synonymous codes because of the degenerated nature of the genetic code^[21]. Other significant strategies have been the identification and removal of mRNA destabilizing gene sequences and improving intron splicing by elimination of cryptic splice sites. Moreover, foreign genes that contain erroneous polyadenylation signals that could be misinterpreted by plants have been removed from foreign genes, and 3' mRNA-stabilizing and 5'-UTR (untranslated region) flanking elements have been optimized for a correct expression level of the foreign molecule^[22].

The choice of the expression method, the constitutive or tissue-specific way of transformation, is a vital aspect that must be evaluated case by case. Several factors control the expression of the transgene at transcription, translation and post-translational levels^[23]. The correct evaluation design of the transformation vector is the most crucial aspect after choosing the host plant. In particular, once the transgene of interest has been chosen, it is essential to choose the promoter

that will regulate its expression: generally, plant expression vectors are chimeric constructs made from repurposed plant viruses, like potato virus X (PVX), cowpea mosaic virus (CPMV), alfalfa mosaic virus, and tobacco mosaic virus (TMV)^[24]. Widespread in plant biotechnology is the choice of using constitutive promoters, such as the Cauliflower Mosaic Virus promoter 35S, that allow a continuative expression of the transgene, but also there are tissue- or stage-specific and inducible promoters are widely used. However, the CaMV35S promoter has been used primarily for high levels of expression of target genes in the shoots of dicotyledonous plants. Other promoters, such as ubiquitin promoters, have been extensively used for high-level expression of transgenes in monocotyledonous plants. The transcription level can also be raised by other cis-acting elements, such as plant/synthetic enhancers, which further increase the transcription of the target gene. Because some recombinant proteins are toxic to plant growth and development, especially when produced in large quantities, it is frequently preferable or even necessary to induce the expression of these genes in order to make the products. Numerous plant genes have promoters that allow transgenes to express themselves in response to a range of stimuli, such as hormones, infections, and stressful situations^[18]. There are two reasons for which this approach could be selected: first, compared to constitutive promoters, restricted ones frequently provide higher results in their target tissues. Moreover, since recombinant proteins are more stable and do not affect vegetative growth in selected tissue, it is often advantageous to direct them there^[25]. For example, the endogenous storage protein gene promoters in cereals are typically more active in seeds than the housekeeping gene promoters, which encode actin and ubiquitin: seed-specific and seed-restrictive promoters are frequently employed to determine gene expression for foreign molecules. There are several benefits of using plant seeds for synthesizing recombinant proteins: firstly, seeds have an enormous capacity to naturally store proteins. Second, the processing is separated from the growing and harvesting processes by the seeds' dormancy capacity, allowing the preservation of generated recombinant proteins for long-term storage. Third, the recombinant protein synthesis procedures are less expensive due to the low presence of water and other biomolecules^[26]. For example, rice endosperm was used for various pharmaceutical products, including vaccines, antibodies, cytokines, enzymes, bioactive peptides etc. For many aspects, this tissue represents a valid platform for high-value product synthesis production, scalability, cost-effectiveness, safety, stability at ambient temperature, high-biomass yield, low risk of gene flow and easy production of various recombinant proteins capacity^[27]. External chemical or physical stimuli can control the activity

of a particular class of controlled promoters known as inducible promoters. It can be beneficial to put production under environmental control even though there is frequently an effort to separate recombinant protein creation from environmental impacts so that output is not constrained by light or abiotic stresses^[28]. In which compartments these molecules are mainly preserved? Subcellular localization is another crucial aspect to investigate for expression level optimization and foreign molecule stability.

Protein targeting study can also optimize expression levels: recombinant proteins should not contain amino acids that promote breakdown at N-terminus but should be directed to different organelles. In the absence of a signal peptide, proteins are usually accumulated in the cytoplasm: however, other signal sequences have also been employed that direct recombinant protein to the nucleus, apoplast, plastid, mitochondrion, and vacuolar compartments^[12]. The choice of subcellular compartment is also closely linked to the context, as each of these microenvironments shows chemical differences (e.g. in pH) that affect the stability of the protein itself. More trivially, the space available for protein accumulation must also be evaluated for high expression of the recombinant protein. An example is the dilatation of the membrane network observed with the production of the hepatitis B surface antigen in soybean cell culture, where the recombinant protein was confined to the endoplasmic reticulum, potentially leading to the occurrence of an accumulation limit^[29].

Recently, the accumulation of recombinant proteins in vacuolar sorting determinants has been evaluated: vegetative tissue vacuoles have been studied in tobacco, tomato, and carrot; however, ectopic protein deposition appears to have a significant impact on the hydrolytic nature of vacuoles in vegetative tissues, which is very dynamic. Specific storage proteins, like zeins in maize, accumulate in ER-derived protein bodies after being synthesized on the ER: these are found in large quantities in cereal plants and have also been investigated as potential storage organelles for the manufacture of recombinant proteins in plant cells. Because of their abundance, location within mesophyll cells, neutral pH, and low proteolysis levels, chloroplasts are among the most desirable organelles for the synthesis of recombinant proteins. In order to produce recombinant proteins in chloroplasts, two distinct methods have been employed. As previously indicated, the first strategy is based on the introduction of transgenes into the chloroplast genome by homologous recombination using the biolistic method: chloroplast translation has been studied in the unicellular green alga *Chlamydomonas reinhardtii* and model seed plants such as *Arabidopsis thaliana*, maize (*Zea mays*), and tobacco (*Nicotiana*

tabacum)^[30]. Since the cytosol is where these chloroplast-destined recombinant proteins are synthesized, both the ubiquitin-dependent turnover of their precursors in the cytosol and the chloroplast protein import capacity affect how many of these proteins accumulate in chloroplasts (**Fig.1**)^[31].

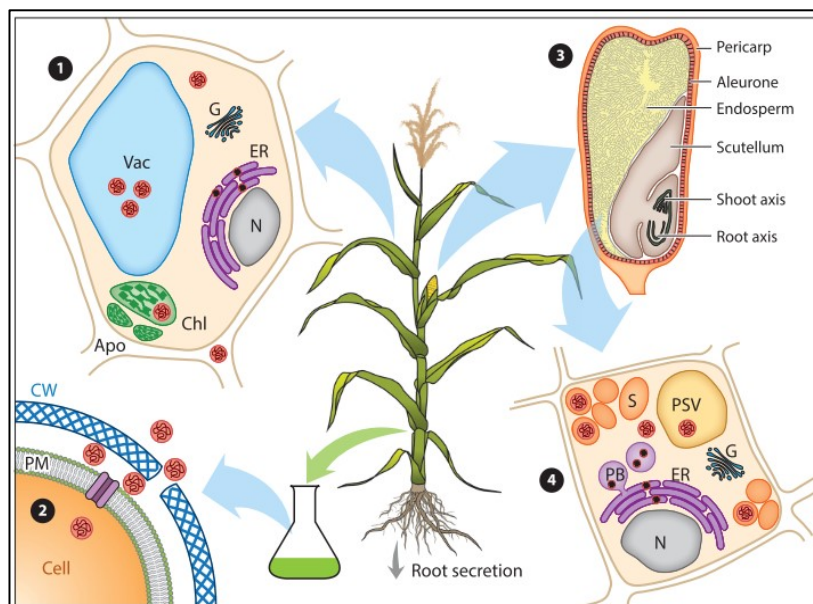


Figure 1. Schematic representation of targeting strategies of recombinant proteins. (1) In vegetative cells, proteins (represented by red spheres), can be retained in the endoplasmic reticulum or sent to the apoplast, vacuole or cytosol. (2) Secretion into the apoplast is widely used in the case of cells in liquid suspension culture, as it greatly simplifies the purification process, as the protein is released into the medium. Another strategy is targeting the foreign molecule to the chloroplast, which is very interesting as it allows high levels of expression. (3) Seeds are natural storage sites for proteins, which are localised for instance in the starch granules. (4) Foreign molecules can finally be sent to natural subcellular storage sites such as protein storage vacuoles (PSV), ER-derived protein bodies (PB)^[38].

2.3 Protein “humanization”

Plants have a significant opportunity to synthesize a wide range of recombinant molecules with suitable post-translational modifications, suggesting a promising future for plant molecular farming. Eukaryotic post-translational modifications such as glycosylation and disulfide bridging are possible, and these are frequently necessary for the biological function of many mammalian proteins. These modifications are critical to the biological efficacy of recombinant therapeutic proteins. Moreover, since there are no known cross-kingdom infections, the risk of pathogen contamination in green bioreactors is not as high as in mammalian cell growth.

Compared to typical cell culture systems, plant growth requirements are low-cost and straightforward, enabling practically infinite scaling at a cheap cost. When plant cell culture is employed, costs are further decreased because it has far simpler growth needs than mammalian or insect cell culture and may use light as its primary energy source. Furthermore, plant systems are resilient and inert, making handling and purification easier, and, in the case of pharmaceutically significant proteins, oral administration needs little processing. There seems to be a wide variety of plant species available for constructing a green bioreactor, and each has advantages and disadvantages that vary based on the intended use^[32]. As mentioned, the process of protein synthesis is widely conserved in both plants and animals: however, significant differences emerge in post-translational modifications, especially regarding the formation of glycans and glycoproteins. While all eukaryotes add glycans during protein secretion, variations in polysaccharide chains are significant among different taxonomic groups because of differences in the enzymes responsible for this modification^[33]. Plant organisms, for example, like insects and yeasts, bind $\beta(1,2)$ -xylose and $\alpha(1,3)$ -fucose to recombinant proteins, unlike animals that bind sialic acid and terminal galactose^[34]. Unlike native proteins, these variations may impact recombinant proteins' distribution, activity, and longevity, resulting in possible immunogenic reactions. To overcome these differences, various strategies have been adopted to "humanize" recombinant glycoproteins: one of the main strategies to avoid unwanted modification of the glycoproteins is to modify the enzymatic complex of the Golgi apparatus by knocking out genes responsible for typical plant glycosylations or adding new glycosyltransferase enzymes to process the newly synthesized glycoproteins further. For example, it is possible to express human enzymes or fusion forms of human galactosyltransferase with a plant N-terminal domain in transgenic plants to produce recombinant galactose-binding antibodies; other strategies considered altering the composition of the growth media to influence glycoprotein composition^[35]. Using RNA-interference (RNAi) to successfully cut down plant transferase activity, several groups have produced simple glycans without IgE action instead of plant-type glycans through protein glycosylation^[36]. Additionally, terminal sialic acid, a crucial component of human glycoproteins, is absent from plant glycoproteins, which could make the proteins inefficient and short-lived: plants that produce biologically active recombinant proteins have effectively manipulated several steps in the sialic acid production process. Among these is the glycosyltransferase known as mammalian α -2,6-sialtransferase, expressed in *Arabidopsis* plants and belongs to the

mammalian trans-Golgi cisternae^[37]. For non-pharmaceutical proteins, glycosylation is less important, but other forms are required for some products to assemble correctly. Proline hydroxylation, for example, is needed for collagen assembly, and plants can be genetically engineered to acquire this ability^[38].

2.4 Risks and potential limits

The use of plants as bioreactors also involves some critical issues: one of the main relates to environmental impact, including transgene spread, gene transfer, and possible toxic effects on nontarget organisms. The dispersal of pollen in cross-pollinated plants is one of the main aspects to be taken into strong consideration in open-field cultivation: to date several strategies against pollen spread are available^[39], such as geographic isolation and the use of male sterile plants. For this reason, self-pollinating species are also considered advantageous for the containment of transgenic material against cross-pollinating crops. Moreover, the use of greenhouses or closed culture systems like callus cultures and cell suspensions, significantly reduce the possibility of pollen contaminations and horizontal gene transfer to bacterial populations^[40].

As mentioned, another aspect that must be considered is the low yields of many proteins currently achieved in plant-bioreactor technology. These are often the result of poor protein stability, challenges with downstream processing yield, inconsistent product quality, and non-authentic glycan structures on recombinant human proteins, which limits the technology^[4]. Another protein-based issue is gluten, which is especially important when employing cereal crops as plant molecular farming hosts. Different proteins, categorized as gliadins or glutenins, are called glutes: these proteins are not harmful in and of themselves, but are strong allergens and exist in far more significant quantities than other potentially dangerous proteins. Due to these concerns, plant-derived biopharmaceuticals have not been routinely approved for clinical studies.

After the expression of the target molecule, the next step to be considered is its purification; downstream processing is the most challenging and cost-intensive aspect of plant recombinant protein production, accounting for up to 80% of the total process costs^[41]. The post-production level processing varies greatly depending on the intended use of the molecules: for example,

pharmaceuticals such as antigen-based vaccines intended for oral administration may not require complex purification processes^[42]. However, achieving a homogeneous final product is essential for drugs intended for intravenous administration and requires more sophisticated purification schemes. In order to extract the product from the plant tissue, a thorough homogenization step is typically required, resulting in the presence of particles in the primary extracts, subcellular impurities of the host cells and secondary metabolites, such as pigments and phenols, which in some cases can bind permanently to the target molecule. Because purification schemes typically include steps that ensure the removal of alkaloids and other bioactive metabolites as well as size-based fractionation steps to remove degradation products and protein aggregates, the risk posed by plant-derived small-molecule impurities is generally low if the product is a recombinant protein^[43]. Using seeds as bioreactors appears to be one of the most promising prospects for producing useful molecules in plants. For example, in seed processing, purification steps could include grain pretreatment, protein extraction and clarification, and protein purification^[44]; however, some starches could induce gel formation during extraction, obstructing the clarification process^[45].

This means that the crucial goal is to develop standards for processing molecules in line with good manufacturing practices (GMP)^[46]: today, different extraction methods are characterised based on centrifugation, flocculation, filter aids utilization, and pre-coat filtration techniques to overcome impurity problems. Moreover, treatments at low pH (~5.5), controlled moderate temperatures (~65°C), and ultrafiltration/diafiltration techniques can reduce 90% of the host cell residues from the target molecule by decreasing the associated cost by more than 75%^[45].

2.5 *In vitro* culture techniques

Micropropagation, also known as rapid clonal multiplication or *in vitro* propagation, is a cutting-edge technology for producing active compounds in plants: it is based on the ability of plant cells to generate entire plants, while preserving the genetic identity of the mother plant. It is a process based on the organogenetic totipotency of plant cells, allowing the formation of complete plants from single or groups of cells taken from plant tissues or organs^[47]. *In vitro* culture consists of taking portions of plant tissue, called explants, which are appropriately sterilized and placed on a sterile culture medium, called substrate, of known chemical composition^[2]. The explants can be isolated by different portions of the main plant: root or

meristematic apex, axillary buds, portions of leaves, and floral meristems are the most common examples. The explants must then be surface sterilized using sodium hypochlorite, ethanol, distilled water, or detergents and placed on the most appropriate substrate^[48]. The growing medium could be liquid or semisolid (depending on the samples) and contains sugars, salts, macro- and micro-nutrients, vitamins, phytohormones, and growth regulators to allow the best cell proliferation and differentiation^[47].

Micropropagation consists of three different principles:

- Proliferation by axillary shoots;
- Organogenesis (direct and indirect);
- Somatic embryogenesis (direct and indirect);

The first mechanism is stimulating buds with appropriate nutritional and hormonal doses to eliminate apical dormancy. Regeneration, also known as morphogenesis, consists of the creation of meristematic centers *ex novo* from already differentiated cells or tissues; morphogenesis can occur through organogenesis if unipolar structures such as roots or shoots originate, or through embryogenesis if bipolar structures (somatic embryos) are produced from a single cell^[48]. Both morphogenetic processes can take place either directly or indirectly: in the latter mode, cells undergo an intense initial proliferative phase that leads to the formation of an amorphous mass of tissue characterised by undifferentiated cells that multiply in a disorganized manner called *callus*^[49]. Plant cells display distinct characteristics depending on their differentiation level and the environment in which they're cultured: fully differentiated plant cells show specific subcellular structures like chloroplasts and larger vacuoles. However, cultured callus cells resemble undifferentiated meristematic cells: they lack features such as chloroplasts and exhibit smaller vacuoles than fully differentiated cells. Remarkably, callus cells can revert to an undifferentiated state, enabling them to regenerate into whole plants under appropriate conditions. Callus cultures may require specific conditions like darkness or day-night cycles for optimal growth. Generally maintained at approximately 25°C, these cultures can take varied forms, ranging from compact masses to friable structures: the latter ones, particularly advantageous, enable the generation of single-cell cultures in gently stirred liquid media (**Fig.2**)^[50]. Nowadays, cell suspension culture systems are employed extensively to culture plant cells on a large scale to extract secondary metabolites. These suspension cultures

are established by transferring the more fragile part of the callus into a liquid medium and maintaining it under optimal conditions of aeration, agitation, light, temperature, and other physical parameters^[16]. Cell cultures enable the production of specific standard phytochemicals in significant quantities and eliminate the presence of unwanted compounds found in plants grown in natural settings. Various types of bioreactors have been utilized for the large-scale cultivation of plant cells, enhancing the efficiency of mass production^[48]. One of the major risks in cell suspension culture on a large scale is cells' natural inclination to stick to bioreactors' surfaces. This event restricts the operational duration of the bioprocess but also affects cellular metabolism by constraining the supply of oxygen and/or nutrients to resulting cell clusters.

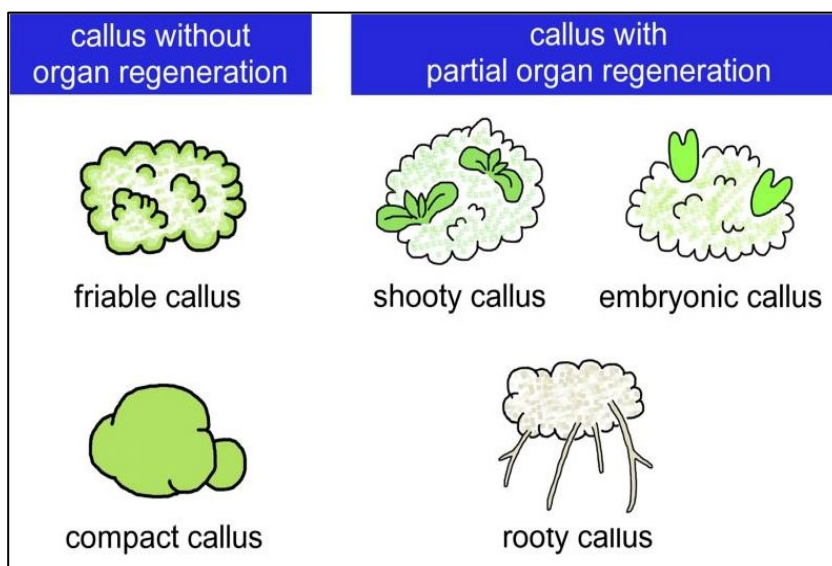


Figure 2. Graphical representation of different plant *callus* types. Depending on the tissue features, calli without any evident organ regeneration are usually referred to as compact callus or friable callus. Depending on the organs they develop, calli with varying degrees of organ regeneration are frequently referred to as rooty, shooty, or embryonic callus^[50].

These unfavorable conditions may induce cell stress, trigger autophagy (such as peroxisome autophagy), and lead to cell death, that could potentially result in (partial) degradation or modification of the product, ultimately causing an increase in product heterogeneity and a reduction in activity^[43].

In vitro culture offers numerous advantages against the previously mentioned risks: it involves culturing plant tissue fragments in a controlled aseptic environment, using sterile culture media,

and providing specific nutrients and growth factors for the accelerated growth of plant cells, tissues, and organs. This method allows the development of engineered genetic material under controlled conditions of humidity, light, and temperature, regardless of environmental or geographical variations^[51]. Compared to obtaining natural plant metabolites, this technique offers significantly reduced production times independent of the plant's developmental stage or stress conditions. Bioactive compound extraction directly from plants is often inefficient, emphasizing the importance of new methodologies such as *in vitro* culture for producing useful molecules like pigments, anti-inflammatory agents, and anticancer compounds^[52]. Generally, secondary metabolites constitute less than 1% of the dry weight of plant cells, necessitating careful research and accurate genotype selection to achieve efficient production. It is important to note how this technique serves as a fundamental starting point for breeding programs, enabling the development of new generations of engineered plants and new varieties while also being essential for conserving endangered species and rare genotypes^[47].

However, in addition to these positive aspects, it is essential to highlight some possible risks associated with utilizing micropropagation: a first aspect that should be taken under consideration is the possibility that the used species and genotypes are recalcitrant to micropropagation, although advancements in research are significantly reducing this obstacle^[53]. In these conditions, plant cells undergo rapid mitotic divisions, which often accumulate unwanted mutations that could potentially compromise the desirable characteristics of the mother plant. This occurrence leads to heterogeneous populations of *in vitro* plants that exhibit a variability known as "somaclonal": however, in some cases, this variability may lead to new varieties of interest^[54]. The final stage of the regenerative process, called "acclimatization", is another challenging aspect to consider at the latter stages of many micropropagation protocols: it consists of seedling transfer from a sterile substrate to the soil. This movement is both an environmental and a metabolic change, because plant tissues move from a predominantly heterotrophic condition to one of complete autotrophy, where the plant undergoes a complex series of morphological, anatomical, and physiological modifications, including the development of new roots and increased photosynthetic activity^[47]. Disadvantages are also linked to production costs: maintaining controlled growth conditions in fresh, dry environments, the production and storage of containers and culture media, the maintenance of the cultures until the target molecule is obtained, and the need for trained operators for this type of production are all aspects that lead to a significant economic increase

compared to field-grown plants. Even today, due to these economic reasons, micropropagation used for molecular farming is more commonly employed in research rather than commercially: advances in understanding biochemical pathways and genes controlling the synthesis of bioactive compounds, coupled with progress in plant synthetic biology and metabolic engineering, could lead to significant improvements in genetic transformation or regeneration techniques^[55].

3. Plant genetic engineering for recombinant molecule production

Molecular farming is therefore based both on the fact that plants are organisms that are naturally capable of producing molecules that are useful to humans, but at the same time it is now possible to produce recombinant heterologous proteins thanks to knowledge of genetic transformation techniques, which exploit the recombinant DNA technique. Different methods are available today: in this thesis I report the biolistic (or gene-gun) method and the more common *Agrobacterium tumefaciens* mediated transformation, which are still the most widely used and efficient, and which have been used in this work. Agrobacterial infection is generally preferred as transgenic plants produced in this way are less susceptible to silencing. On the other hand, the biolistic method is more widely used in the case of transplastomic transformation, where the transgene is inserted into the plastid genome^[12].

3.1 *Agrobacterium tumefaciens* transformation

The phytopathogenic Gram negative *Agrobacterium tumefaciens* is still considered the first “genetic engineer” of plants: in 1983, the first transformed plant was produced, marking a new era in plant biotechnology^[56]. To date, several species of *Agrobacterium* capable of causing tumours has been characterised, including *Agrobacterium rhizogenes*, which is responsible for important root diseases. *Agrobacterium tumefaciens*, however, is the most widely used, so this section will focus more specifically on the infectious process of this latter phytopathogen, the agent of collar or root tumours in numerous plant species.

Within its Ti plasmid, it is capable to transfer DNA (T-DNA) housing virulent genes responsible for triggering tumour or gall formation in the affected plant tissue^[57]. The T- DNA

region is delineated by two 25 bp repeated flanking sequences, called left (LB) and right borders (RB). In nature, this section of the Ti plasmid (which will integrate into the plant genome) contains genes for the production of phytohormones (leading to gall production) and opines. The latter are used by the agrobacterium as a source of carbon and nitrogen providing a selective advantage over competing parasites^[58].

The remaining part of the Ti plasmid is not transferred to the host plant: it contains essential information for T-DNA transfer. It contains seven loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virF* and *virG*) coding for VIR proteins, which literally regulate the transport and integration of the T region^[59]. A lesion on the plant tissue is necessary for infection because it produces phenolic compounds, such acetosyringone, which draw in the pathogen and facilitate its penetration: the bacterial adherence to the host cell's plasma membrane is the initial step in the now well-studied molecular process of *Agrobacterium* ability to operate^[2].

Agrobacterium recognizes the signals released by the host thanks to the transmembrane receptor (VirA): this receptor activates by phosphorylation, in the periplasmic space, the regulatory protein Vir G, leading to the activation of all other *vir* genes. VirD1 and VirD2 genes carry out the cutting near the right-border and left-border regions: in particular, VirD1, a site-specific helicase, splits the two strands of the T-DNA strand, and VirD2 acts as a nuclease by degrading one of the two strands of the helix, giving rise to a linear single-stranded DNA fragment, called a T-strand. The VirD2 protein also binds the 5' cap of the T-strand, while the 3' end obtained by cutting the right-border will act as the primer attachment site for T-DNA replication. At this point, the VirD2-T-DNA strand is ready to be translocated within the plant cell. The transfer takes place by the pilum formed by the Gram- bacteria, obtained by assembling the VirB and VirD4 proteins. Simultaneously with the filament, the hydrophilic VirE2 proteins are transferred into the host cytoplasm via a clathrin mediated endocytosis system^[60]. Once in the cytoplasm, the T-strand associated with the VirD2 protein is coated by VirE proteins, forming the so-called super-T-complex; this protection overcome the risk to be recognized and degraded by host nucleases. Targeting towards the nucleus is enabled by a C-terminal nuclear localization signal (NLS) present in both VirD2 and VirE2 proteins^[61]. Once in the nucleus, the T-complex must be made accessible again to be integrated into the host genome, replicated and finally transcribed. It seems that the super-T-complex breakdown occurs via a ubiquitin-proteasome system, mediated by VirF. The action of the latter virulence protein is enabled by the presence of VirD5, which is supposed to play a stabilizing and

protective role against VirF proteolysis^[62]. The mechanism by which the T-complex reaches chromatin is rather complex. Recent studies have shown that certain TFs, such as the TATA-binding protein, the transcription factor VIP1 and CAK (Cyclin-dependent Kinase-Activating Kinase) appear to mediate the integration of the T-complex into transcriptionally active regions within the genome^[63]. Microhomologies, deletions and duplications have also been identified near the junctions formed by the integration of T-DNA into the plant genome: these rearrangements appear very similar to the typical repairs carried out by eukaryotic cells following double-strand breaks, indicating that DNA integration is mainly carried out by an endogenous host repair system^[64].

Once integration has taken place, the expression of genes carried on the T-DNA strand follows, leading to tumour formation in the plant. Once integration has taken place, the expression of genes carried on the T-DNA strand follows, leading to tumour formation in the plant. *Agrobacterium's* unusual virulence approach caught the interest of plant biotechnologists as soon as it was discovered, and as a result, it was modified to become a never-before-seen tool for plant genetic modification. This adaptation included creating a binary vector system that included a disarmed Ti plasmid that had its T-DNA portion removed and a tiny, controllable plasmid (sometimes up to 20 kb) that was assigned the T-DNA region empty of *Agrobacterium* genes. Since the left and right borders are the sequences that specify the T-DNA area, any type of DNA may be inserted between the borders and used to convert plants (**Fig. 3**)^[65]. The low cost, simple procedure, good repeatability, few integrated transgene copies, and potential for bigger DNA fragment transfer are the benefits of this approach^[66].

The Ti-plasmid, once disarmed, becomes challenging to manipulate *in vitro* due to its large size. Consequently, to address this issue, scientists transferred it to a smaller, independently functioning plasmid, called "helper vector", possessing an *Agrobacterium*-specific replication origin and the *Vir* genes. This step allows the virulence genes to act upon the T-DNA sequences within the same cell in *trans*. Moreover, removing these virulence genes enables the Ti-plasmid to accommodate longer transgenes. To facilitate its replication in various hosts such as *E. coli* and *A. tumefaciens*, a "binary vector" was designed, comprising left and right borders, multiple-host replication origins, selectable marker genes, and the gene(s) of interest. This engineered plasmid is now instrumental in plant genetic transformation processes^[60].

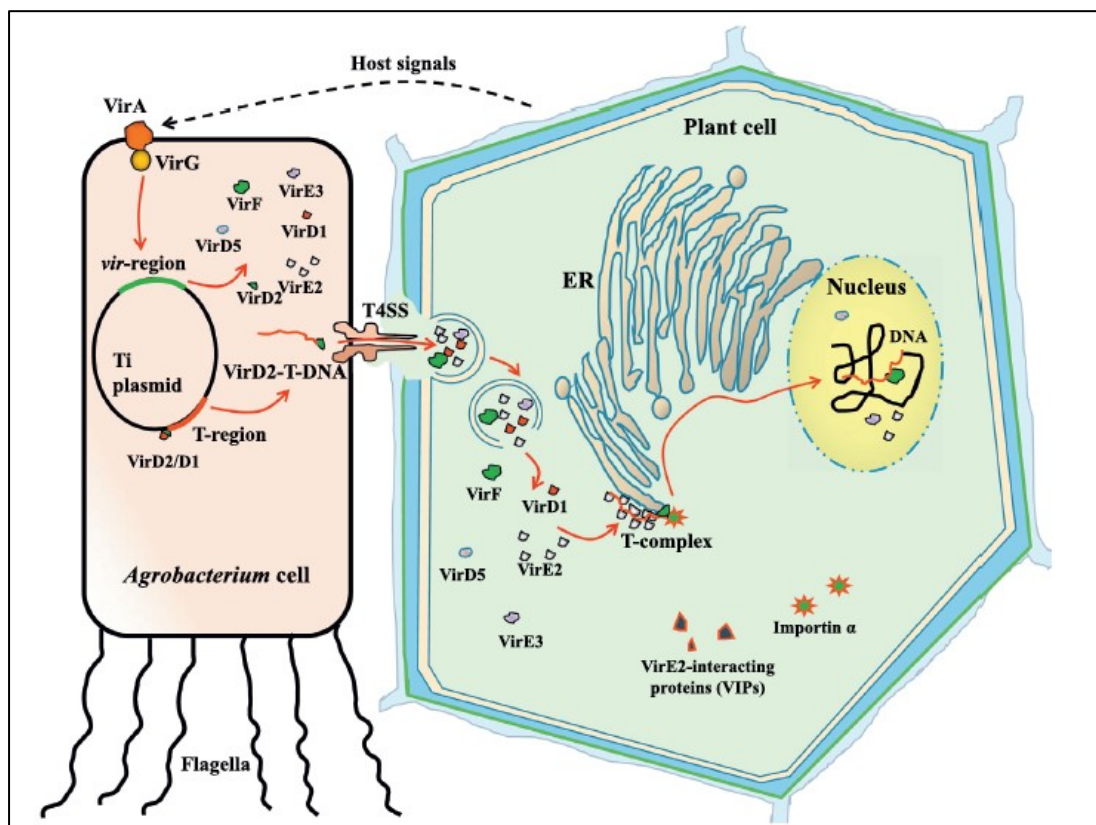


Figure 3. Representation *Agrobacterium tumefaciens* infectious process [101].

3.2 Biolistic method or “Gene-gun”

The biolistic method, also called "Gene-gun" transformation technique, is another pioneering tool for plant genetic engineering. Developed by John Sanford in the late 1980s, this method revolutionized the process of introducing foreign genetic material into plant cells^[67]. All takes place under sterile conditions in a vacuum chamber for a few seconds: the vacuum creates an environment devoid of air, allowing for better particle penetration and minimizing air resistance during particle propulsion. The procedure begins by placing the plant tissue, leaf or stem sections, into the target surface within a vacuum chamber. The principle consists in the production of small particles of heavy metals, called microprojectiles, usually gold or tungsten, coated by DNA fragments containing the transgene(s): these particles act as “Microscopic carriers” of genetic information, which are made to adhere to the lower surface a disk called Macrocarrier. In the upper chamber of the device known as the “Gene gun”, helium (or another high-pressure gas) is compressed until the pressure is able to break a special plastic disk (called

Rupture disk). The burst of gas in the chamber below 'shoots' the plastic disc, called Macrocarrier, against a steel grid (Stopping screen) at a speed of 500m/sec^[2]. The 0.5 to 3 μm gold or tungsten microparticles coated with DNA continue their motion by inertia as they pass between the meshes of the grid and strike the cells to be transformed. The rupture of the wall and membrane allows the DNA to enter the cell. The distance between the gene gun and the plant tissue, as well as the velocity at which the particles are propelled, can be adjusted to optimize transformation efficiency^[68]. Once inside the plant cell, the DNA released from the particles integrates into the plant genome. The main advantage of this method consists in the possibility of transforming intact plant cells such as leaves and seeds: the versatility of this method, which doesn't depend cellular barriers, enable genetic modification in a wide number of plant species, including those with recalcitrant cell walls that might resist other transformation methods^[69]. However, despite its efficacy, the gene gun method shows some limitations: the integration into the plant genome occurs randomly at various sites within the genome. This event can lead to unpredictable outcomes, including gene silencing or disruption of essential genes^[70]. Furthermore, barriers to broad adoption may include the high cost of the Gene-gun equipment and the labor-intensive nature of the procedure. However, the Gene gun method remains an essential tool in plant biotechnology, contributing significantly to crop improvement, biopharmaceutical production in plants, and the exploration of fundamental plant biology.

4. Bioactive compounds for human health produced in this thesis

Pharmaceuticals and agriculture converge in a fascinating and promising field: Plant Molecular Farming (PMF). In this sense, plant cells can be utilized through two possible routes: bioactive molecules typical of plants, such as anthocyanins and vitamins, can be produced by promoting endogenous biosynthetic pathways, or exogenous genes can be introduced. Exogenous genes also include recombinant proteins for pharmaceutical or industrial use, such as antibodies, human proteins and immunoregulatory peptides.

Through this technology it is possible to produce an infinite variety of molecules, including monoclonal natural compounds, antibodies useful in the treatment of various diseases, proteins for human therapies and molecular industry. The following three Chapters report on the three

projects I focused on during this PhD programme. In this paragraph, there is an outline of the three major classes of molecules to which the compounds produced belong.

4.1 Natural bioactive compounds: phenols

Among the compounds naturally produced by plants, two main categories can be distinguished: primary metabolites, which include carbohydrates, lipids, proteins and coenzymes necessary for plant growth and development, and “specialized” metabolites, formerly known as “secondary” metabolites^[71]. Long regarded as metabolic wastes, the latter are now recognized as crucial chemical signals for the interactions between plants and their environment. Their importance for the species’ survival makes them indispensable even though they are not essential for growth. Specialized metabolites, characterised by a highly variable chemical structure and considerable differences within the same species, often possess significant biological activities: many of them show important bioactive properties and are used as drugs^[72]. Once produced, the molecules can be secreted by the plant cell in the apoplast, accumulated in the vacuole (as they are cytotoxic substances), or included in the cell wall^[73].

Among these compounds, phenols constitute a heterogeneous group of about 10,000 molecules characterised by aromatic rings and hydroxyl groups. Phenols are subdivided into simple phenols and polyphenols, which include tannins, flavonoids and lignans, and are often involved in defensive processes against herbivores and pathogens, pollinators attraction and growth inhibition of surrounding plants^[74]. The biosynthesis of phenols occurs via the shikimic acid and malonic acid pathways: depending on the substitutions of the benzopyran ring, flavonoids are classifiable into chalcones, aurones, flavones, flavanols, isoflavonoids, biflavonoids and anthocyanidins. These compounds, as well as having crucial functions in plant reproduction and protection from UV radiation, have antioxidant, anti-inflammatory and anti-tumour properties, making them of increasing interest in the production of drugs^[75]: natural polyphenols are considered to be the most abundant antioxidants in human diets and their activity as radical scavengers is linked to their molecular structure. The phenolic ring can yield a hydrogen atom (of the -OH group) to the radical, thus making it possible to obtain radical (and non-radical) species that are much less reactive, thus interrupting the typical oxidative/radical stress chain reaction^[76]. Certain plant families, such as Solanaceae and Brassicaceae, are particularly rich in phenols, the consumption of which is correlated with a

lower incidence of cardiovascular disease and cancer^[72]. The Mediterranean diet, which includes medicinal herbs such as thyme, mint, ginger and rosemary, is rich in phenols, demonstrating potential therapeutic benefits against cancer, viruses, inflammation, and hypoglycemia. Examples of the beneficial properties of phenols also include antifungal and antibacterial actions, as in the case of oleuropein found in olive oil^[77].

To date, flavonoids have been extensively studied in terms of chemistry, biochemistry, genetics, molecular biology, and dyeing mechanisms among secondary metabolites found in plants. They may be categorized based on their biosynthetic origin and comprise a class of phytochemicals abundant in tea, honey, wine, fruit, vegetables, nuts, olive oil, chocolate, and grains^[78]. Among these, anthocyanins and flavonols are significant pigments that frequently coexist with carotenoids in the same plant organs, leading to a comprehensive combination of colors; however, unlike carotenoids, which are fat-soluble and stored in chromoplasts, anthocyanins are soluble in water and accumulate in vacuoles^[79].

Anthocyanins (formed from anto-, from the Greek άνθος meaning 'flower' and -cyan, from the Greek κύανος meaning 'blue colour') are probably the most important group of visible plant pigments besides chlorophyll^[80], are water-soluble natural pigments responsible for the orange to the blue coloring of fruits and vegetables, which is why they are often used as natural coloring agents.

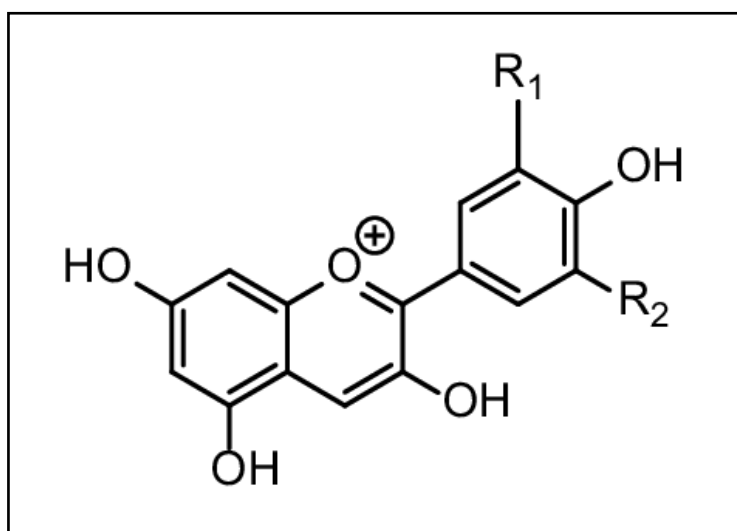


Figure 4. Basic anthocyanin structure ^[102].

Their non-sugar portion (aglycone) is called anthocyanidins, but we generally find them in their glycosylated form. Regarding structure, anthocyanins can be classified on the number and

position of the hydroxyl and methoxyl groups. Six compounds are commonly found in fruit and vegetables: pelargonidin, cyanidin, delphinidin, petunidin, peonidin and malvidin. Among these, cyanidin appears to be the most common and was found in more than 82% of fruits and berries examined^[81]. Due to their potent anti-ageing, anti-tumour, and anti-microbial activities, flavonoids are compounds that have attracted the attention of pharmaceutical companies for multiple applications, in the European Union anthocyanins are designated E163 and permitted for food applications^[82]. In this thesis, as it is discussed above, the production of anthocyanin in callus culture is investigated, mediated by the overexpression of the Sn *Zea mays* transcription factor (Fig.5).

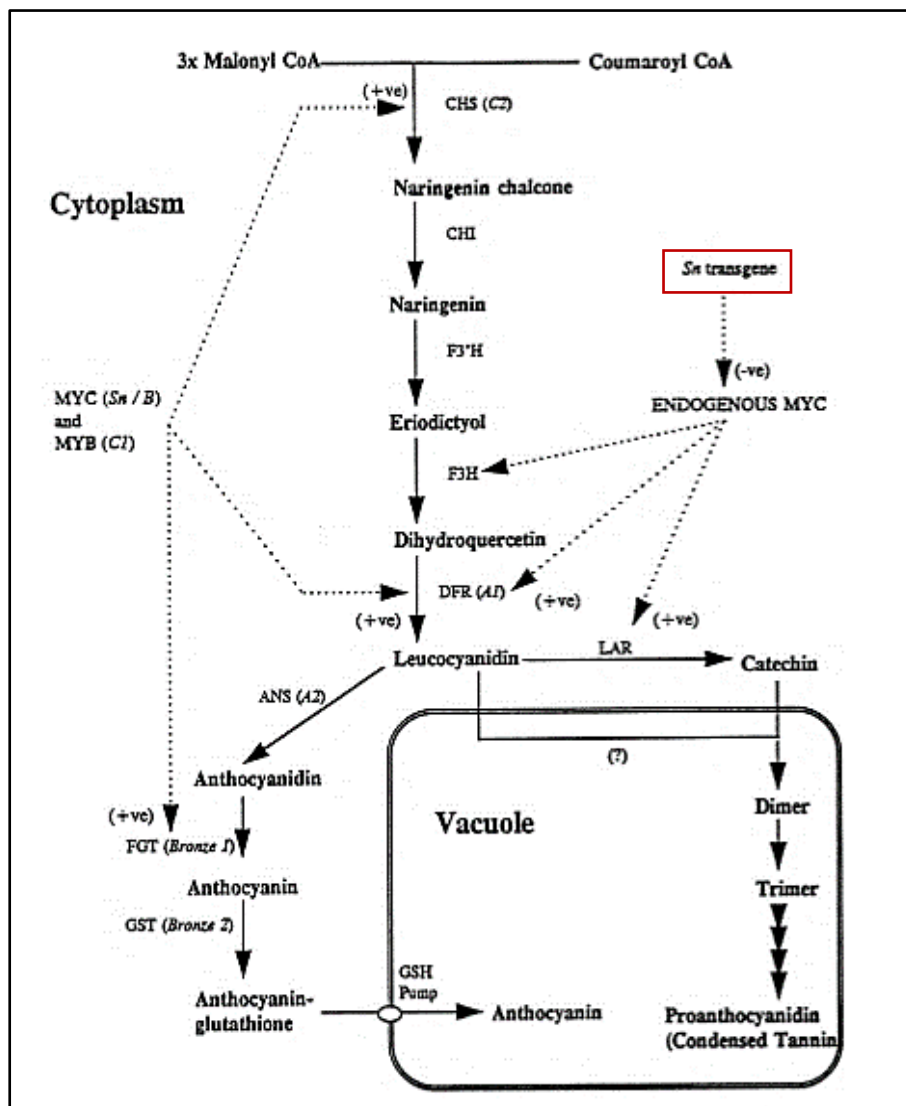


Figure 5. Pro-anthocyanidin and anthocyanin biosynthesis scheme (modified from the original version) ^[103].

4.2 Recombinant proteins for the treatment of human diseases

The growing demand for drugs, linked to the increase in cases of chronic degenerative (insulin resistance, neoplasms, etc.) and autoimmune diseases, has led to a pharmaceutical revolution, which is mainly necessary to satisfy the large number of drugs demanded by the market^[83]. This revolution, which can be defined as “next-generation pharmacotherapy”, has been going on for about thirty years and consists of using organisms modified by genetic engineering techniques, as a source of synthesis of next-generation drugs^[84]. Nowadays, prokaryotic cells (primarily the bacteria *Escherichia coli*) and a select few well-studied mammalian cell lines, such as Chinese hamster ovary (CHO) cells, are the primary producers of recombinant proteins. Although they are less prevalent, other systems such as insect cells, yeast, algae, and cell-free expression platforms are utilized in commercial operations. Nevertheless, using plants as an alternative expression platform has some distinct benefits, especially when the target proteins are complex to produce in traditional systems, require certain qualitative characteristics like specific glycan profiles, or must be produced more widely to meet urgent demand. Plants will often be used to manufacture commercial proteins as expression levels rise and downstream processing becomes more profitable^[85].

Many plant-based products are already on the market, such as certain cancer drugs used in chemotherapy (e.g., taxol, vinblastine, and camptothecin), thanks to which the efficacy of clinical treatments against some of the most aggressive forms of cancer has improved considerably^[86]. Human growth hormone, in particular, was the first recombinant protein to be produced in transgenic plants in 1986, followed in 1989 by the first F1 plant capable of expressing a recombinant antibody derived from two transgenic parents; the actual production of recombinant proteins, however, began in 1997, with the expression of Avidin in transgenic maize plants^[87]. Depending on the plant species, recombinant proteins can be expressed in a variety of plant organs and tissues, such as leaves, seeds, fruits, or edible tubers: alfalfa, carrot, lettuce, tomato, potato, cereal, banana, and tobacco were all tested^[88]. Biopharmaceuticals must be produced, processed, and regulated as pharmaceuticals, keeping them separate from food and feed supplies: this is one of the main concerns regarding using edible plant systems containing biopharmaceuticals. This requires the creation of prevention plans and a robust and safe traceability and labeling system^[89].

The first trial of a vaccine obtained from a plant organism dates back to 1990 when the surface protein antigen A (SpaA) of *Streptococcus mutans* was expressed in transgenic tobacco

plants^[90]. This was followed by who expressed hepatitis B surface antigen in transgenic plants^[91]. Since the creation of a vaccine against the Newcastle virus in 2006, there has been a steady rise in the market for plant-based vaccinations^[92]. In 2005, the World Health Organization met to establish guidelines for developing, evaluating, and using plant-produced vaccines. The outcome of this meeting was that these molecules should be subject to the same precautions and evaluation procedures as conventional vaccines^[88]. Today, several proteins of different origin have been expressed in plants.

4.3 Biopolymers for Bioplastics

The Greek phrases "bio" and "polymer", which combined represent the natural world and living things, are the source of the name "biopolymer": this term can be used to describe two types of molecules, bio-based polymers, which are synthetically made from natural resources, and natural polymers, which are any polymers found naturally in the environment^[93]. Interest in synthesizing new polymers in plants persists due to the increased demand of a growing global population for biomaterials in an era of depleting petroleum supplies. Progress and advances in plant-based polymer manufacturing will be crucial for our understanding of plant metabolism and genetic engineering, expanding the applications of these techniques and, consequently, the economic possibilities for these polymers^[94]. Biopolymers are biocompatible and biodegradable, making them valuable for various uses: in the food business, for instance, they can be utilized as packaging material, emulsions, and edible coatings. They can be employed in the pharmaceutical industry as dressing materials, drug carrier materials, medical prosthetics, and tissue scaffolds^[95]. Biopolymers can be subdivided according to their production method: natural polymers almost unchanged (such as starch derivatives), polymers produced by fermentation (such as polylactic acid derivatives, PLA) and polymers produced by microorganisms (such as polyhydroxyalkanoate derivatives, PHA)^[96].

Bioplastics derived from biopolymers have already entered industrial chains and are beginning to be used in everyday life: these materials are defined as "plastics based on renewable resources or as plastics which are biodegradable and/or compostable" by the European Bioplastics Organization. A polymer is said to be biodegradable if it can be broken down by microorganisms in an enzymatic process to produce carbon dioxide (CO₂), methane, water, inorganic chemicals, or biomass: some of these items can be composted under regulated

environments^[97]. Agriculture could be a crucial resource for sustainable industrial materials by producing new biopolymers directly in plants. Plants naturally generate substances such as starch, cellulose, hemicellulose and lignin, which are used on a large scale in everyday human industry^[98].

Seventeen sustainable development goals (SDGs) were accepted by the United Nations General Assembly to be implemented by 2030, which aim to create a sustainable and healthy society^[99]. The creation of ecologically friendly products is facilitated by the manufacture of bioplastics, making the majority of sustainable development goals achievable since they are widely available and versatile materials. Since monomers for bioplastics are sourced from agricultural waste, the carbon footprint of the end product and its raw materials is reduced. The bioplastics' recyclable nature ensures the product is socially, environmentally, and financially acceptable throughout its sourcing, manufacturing, marketing, consumption, and removal. Adopting sustainable materials in our daily lives may reduce energy, waste disposal, and operating expenses. It seems evident that biopolymers will create new opportunities for a safer world free of dangerous materials^[100].

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CHAPTER I

Genetic transformation of apple pulp calli for antioxidant bioactive compounds production

Abstract

Genetic engineering of biosynthetic pathways is a growing area within botanical research to meet the increasing global demand for compounds with health-promoting properties. The gene transcription regulation of anthocyanin biosynthesis is subject to the control of the MYB-bHLH-WD40 transcriptional complex. Several MYB elements in this complex can activate specific portions of the biosynthetic pathway, responding to signals from cell development and environment^[1]. Studies conducted on maize cell cultures have shown that the expression of a bHLH transcription factor, in association with the MYB gene, both activators of anthocyanin production, induces a significant accumulation of these antioxidant molecules^[2]. A genetic transformation system was proposed to express the *Sn* gene of maize (*Zea mays*), a bHLH transcription factor, in Mela Rosa Marchigiana's pulp calli, a *Malus domestica* variety, to develop a method for anthocyanins production. For this purpose, apple pulp calli were transformed by *A. tumefaciens* with a gene cassette vector to express the *Sn* gene in tandem with a kanamycin resistance marker gene (*NPTII*). Since anthocyanin production is typically associated with stress situations such as attacks by plant pathogens, engineered tissues must be exposed to stimuli in the experimental setting to activate the biosynthetic pathway. In this case, calli were exposed to continuous light in a growth chamber, thus functioning as an initial stress factor to trigger transcriptional activation. Obtained results show the expression of the *Sn* gene is sufficient to significantly favor the synthesis and accumulation of anthocyanins in apple calli exposed to continuous light stress.

Keywords: *Sn*, callus culture, anthocyanins

Abbreviations

bHLH	Basic helix-loop-helix
CaMV35S	Cauliflower mosaic virus 35S promoter
dNTPs	Deoxynucleotide triphosphates
FW	Fresh weight
GC-MS	Gas chromatography-mass spectrometry
GUS	β -glucuronidase
LB	Luria Bertani liquid medium
MRM	Mela Rosa Marchigiana
MS	Murashige & Skoog Basal Medium
MYB	Myeloblastosis transcription factors
NOS	Nopaline synthase
NPTII	Neomycin phosphotransferase
OD	Optical Density
<i>TT2</i>	Transparent testa 2 gene
<i>TT8</i>	Transparent testa 8 gene
<i>TTG1</i>	Transparent testa glabra 1 gene
<i>uidA</i>	β -glucuronidase gene

1. Introduction

Plants produce a wide variety of specialized metabolites, which are thought to be over 200,000 and are essential for symbiotic connections, pathogen defense, survival, and reproduction^[3]. Most of these compounds are pharmacologically significant and several plant secondary metabolites are highly sought after and prized due to their commercial potential; however, the limited quantity of many compounds in plants raises the possibility of overusing native plant species in their natural habitat. Compounds from natural sources are in high demand today due to customer preferences to use such compounds over those of synthetic origin^[4]. Thus, platforms such as chemical synthesis, transgenic plants and/or microorganisms, and plant cell culture have been employed to maintain sustainability while meeting economic demand^[5]. The latter is one of the most effective and promising techniques for producing bioactive compounds in plant cells^[6]: tissue culture methods can produce natural compounds on a big scale in the pharmaceutical industry more affordably than large-scale synthesis.

This work has focused on phenols, a wide range of naturally occurring plant chemicals, showing various biological activities that make them desirable for flavorings, perfumes, medicines, and dyes. Flavonoids and anthocyanin biosynthetic pathways have been the most extensively studied due to the high potential in multiple end-product fields. Two major classes of transcription factors have been identified in maize: the C1/P family, which encodes proteins like the DNA binding domain of the mammalian MYB oncogene products^[7], and the R/B family, which exhibits sequence homology to the basic helix-loop-helix (bHLH) DNA binding proteins found in animal *MYC* protooncogenes^[8]. It has been shown that the expression of both bHLH and MYB anthocyanin trans-activators causes anthocyanin accumulation within anthocyanoplasts in maize cell cultures^[2]. Moreover, three genes have been found in *Arabidopsis*: *TT8* (which encodes for a transcription factor with the bHLH domain), *TT2* (which codes for a protein with the R2R3 MYB domain) and *TTG1*, which affects dihydroflavonol 4-reductase gene expression. These genes influence proanthocyanidin accumulation in developing seed tissues^[9]. Further research in *Lotus corniculatus* demonstrated that the overexpression *Sn* gene (a heterologous bHLH transcription factor involved in the control of the anthocyanin and condensed tannin biosynthesis) can transactivate both anthocyanin and condensed tannin pathways in plant tissues^[10]. In this work, current knowledge concerning the regulation of the anthocyanin biosynthetic pathway was exploited for the stable transformation

of stem cells from Mela Rosa Marchigiana apple pulp, to develop a new bioreactor for producing these antioxidant compounds beneficial for human health.

2. Materials and methods

2.1 Plant material selection and *in vitro* callus culture techniques

The selected plant material consists of the ripe pulp of a variety of *Malus domestica* known as “Mela Rosa Marchigiana” (MRM), that came from the homonymous region and Sibillini mountains, in Italy. This ancient variety is known for its high shelf life and organoleptic properties and is still of interest today for its health benefits^[11].

This variety is rich in polyphenols, flavonoids, catechins, triterpenoids and anthocyanins, but less is known about the secondary metabolites produced by callus cell cultures. Thanks to Verardo and colleagues^[12], it is known that calli obtained from MRM apple pulp contain a significant amount of triterpenic acids, including oleanolic, ursolic, maslinic, pomolic, tormentic, corosolic and annurcoic acids, together with phytosterol, β -sitosterol, which have been screened by GC-MS analysis and are known worldwide for their anti-inflammatory and antioxidant properties. The quantification of these compounds revealed a significant accumulation in calli pulp *in vitro* rather than *in vivo* conditions (**Tab.1**). It is well known that these triterpenic acids are present in many fruits, particularly in the skin and cuticular waxes. For the first time, these compounds are found in disorganized callus cells, probably accumulated in the vacuole or cell wall. The significant properties of this local variety have made MRM the best candidate for genetic engineering for the stimulation of the anthocyanin biosynthetic pathway.

The MRM apples were randomly collected from an orchard growing in Urbino municipality open field (PU, Marche, Italy), global positioning system (GPS) coordinates: latitude 43.713425, longitude 12.626819, altitude 381 m at sea level.

As described by Verardo et al. (2017)^[12], apples' surfaces were sterilized with ethanol and flamed under a laminar flow hood; they were then dissected with a sterile blade. Discs with a diameter of 5 mm and a thickness of 3 mm were isolated from the apple pulp with tweezers about 1 cm from the skin. For obtaining calli from apple pulp, semi-solid Gamborg B5 (3,2 g/L Gamborg B5 salts, 30 g/L sucrose, 8% agar; pH 5,8) was used as a culture medium: the highest

biomass production in the MRM pulp explants was obtained with the addition of 6-benzylaminopurine 2.0 mg/L (BA, Sigma-Aldrich) plus 2,4-dichlorophenoxyacetic acid 0.2 mg/L (2,4-D, Sigma-Aldrich). Callus cultures were grown at 25 °C and in dark conditions. This medium combination was maintained, and subcultures were made in aseptic conditions every 28 days to obtain *in vitro* plant material for further experiments.

Compound	MRM apple pulp Culture (µg)	MRM apple pulp (µg)
β-Sitosterol	123.8 ± 5.7	467.4 ± 11.3
Oleanolic acid	42.1 ± 1.3	1.0 ± 0.1
Phloridzin	n.d.	399.8 ± 12.6
Ursolic acid	170.5 ± 7.8	3.8 ± 0.1
Maslinic acid	948.3 ± 27.6	4.3 ± 0.1
Corosolic acid	681.2 ± 19.8	4.1 ± 0.1
Pomolic acid	16.2 ± 0.6	1.6 ± 0.1
Tormentolic acid	2927.9 ± 50.8	2.0 ± 0.1
Annurcoic acid	351.8 ± 10.6	0.7 ± 0.0
Annurcoic acid isomer	150.8 ± 6.9	n.d.
Unknown	172.7 ± 7.9	n.d.
Total	5585 ± 124.8	824.7 ± 30.0

Table 1. Secondary metabolites content in wild-type MRM apple pulp and callus culture. Powdered apple callus culture (100.0 mg) was extracted twice in MeOH and centrifuged. The supernatants were concentrated *in vacuo* at 40 °C. The residue was suspended in water and extracted with EtOAc and kept at 4 °C until derivatization and GC–MS analysis. The same extraction protocol was followed for 1.0 g of MRM apple pulp. The amount of the various secondary metabolites detected of performed is shown in the table. Data are expressed as the mean value ± standard deviation; n=4 repetitions; n.d., not detectable^[12].

Before the transformation event (which confers to callus culture kanamycin-sulfate resistance), to optimize the transformants-selection system, apple pulp wild-type calli were grown on different kanamycin concentrations to understand which one may cause growth arrest/callus death. The antibiotic stock was prepared by diluting 200 mg of kanamycin powder (Sigma-

Aldrich) in 10 mL of distilled water for a final concentration of 20 mg/mL and sterilized by a 0.22 µm filter (Millex ®- GV Filter Unit, Millipore). The appropriate amount of antibiotic was added to the medium to achieve 100, 200, 300, 500 mg/L kanamycin concentrations.

Growth rates of wild type apple pulp calli grown in Gamborg B5 medium supplemented with increasing doses of antibiotic (kanamycin) after 5, 10 and 15 days were recorded. Apple pulp calli grown on 100-200-300 mg/L of antibiotic show a growth decrease/arrest, while in higher concentrations of kanamycin (500 mg/L), the weight decreases by almost 50%; 100mg/L kanamycin concentration was selected as the optimal one after two independent technical replicates.

2.2 Plasmid construction for genetic engineering

uidA gene sequence from *Escherichia coli* (coding for GUS) controlled by CaMV35S promoter of the pBI121.1 plasmid^[13] was replaced with the *EcoRI* fragment containing the cDNA sequence of *Sn*^[14]. The pBI121.1 plasmid also harbors within the T-DNA left and right border the *NPTII* (neomycin phosphotransferase) gene controlled by the NOS promoter^[10]. To perform *uidA* gene substitution, pBI121.1 plasmid was digested with *SmaI-EcoRI*, the large fragment (about 11 kb) was collected, dephosphorylated and ligation was performed with the 2.5-kbp *EcoRI* fragment containing the *Sn* cDNA: the compatible ends of the pBI121.1 plasmid and the *Sn* fragment allowed the correct ligation. The proper orientation of *Sn* gene was checked through a *PstI* restriction^[15]; the new plasmid containing the transgene was called p121.Sn (Fig.1).

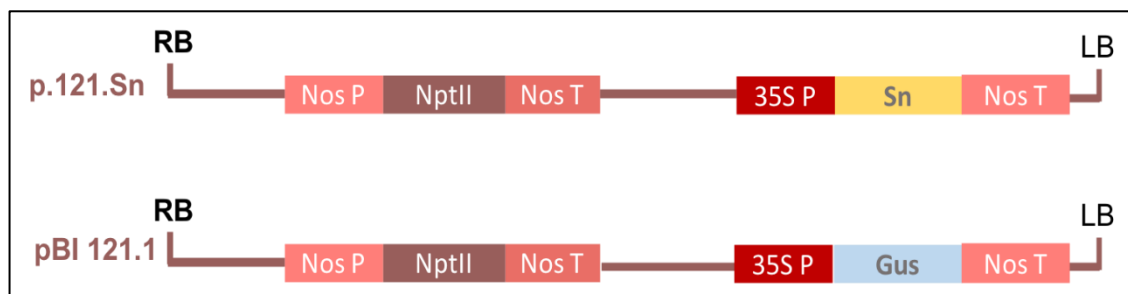


Figure 1. Schematic representation of constructs used for genetic engineering of MRM apple pulp callus culture.

p121.Sn and pBI121.1 plasmids were introduced via triparental mating techniques into two distinct *A. tumefaciens* LBA4404 strain^[16].

A. tumefaciens (p.121.Sn and pBI121.1) strains were grown on two distinct Petri dishes on LB agar medium (Bactopeptone 10 g/L, Yeast Extract 5g/L, NaCl 10 g/L, Agar 15 g/L; pH7.5) plus kanamycin 50 mg/L and rifampicin 100 mg/L (Sigma-Aldrich), and incubated for 2 days at 28 °C. One colony for each construct was transferred to 3 mL of liquid LB medium as pre-inoculum and left to agitate for 8 hours, after which LB medium (plus kanamycin 50 mg/L and rifampicin 100 mg/L) is added to a final total volume of 40 mL. The culture was kept agitating at 28 °C overnight until the culture reached 0.5-1 OD (Optical Density) and used the following day for the agro-infection technique.

2.3 Apple pulp culture agroinfection

The callus culture transformation event was performed by *Agrobacterium tumefaciens*. The agrobacterium's liquid culture (for each construct) is transferred into falcon tubes and centrifuged at 2000xg for 10 minutes. The liquid supernatant is discarded, and the pellet is resuspended in 40 mL of liquid MS0 medium (Murashige and Skoog Salts 4,4 g/L, sucrose 30g/L; pH 5,8) plus acetosyringone, added to a final concentration of 10⁻⁴ M. Under a laminar flow hood, callus clones originating from one wild-type callus of the Mela Rosa Marchigiana variety are fragmented with sterile forceps and scalpels and left to incubate in the previously prepared mix for 30-40 minutes. At the end of the specified time, extra liquid is removed using filter paper, and the presumably transformed fragmented calli are placed on Petri dishes containing Gamborg B5 medium supplemented with phytohormones. The plates thus formed are kept for approximately two days in the dark at 25 °C, until a halo, typical of *Agrobacterium* growth, develops around the calli. The explants are transferred to the appropriately prepared selective medium, Gamborg B5, with kanamycin 100 mg/L supplement. The explants are monitored weekly: after approximately 30-40 days, the transformed calli can be distinguished from the non-transformed ones by their proliferating growths (the others are characterised by tissue darkening and are discarded) (**Fig.2**). Calli expressing the construct pBI 121.1 represent the negative control: just like *Sn* calli, they can grow on selective-kanamycin medium (in contrast, wild types would undergo necrosis), but we expect them to produce the same amount of anthocyanins as a wild-type callus. These portions are isolated and placed on a new culture

medium, identical in composition to the previous one until they reach a large enough size to allow DNA extraction and PCR screening.

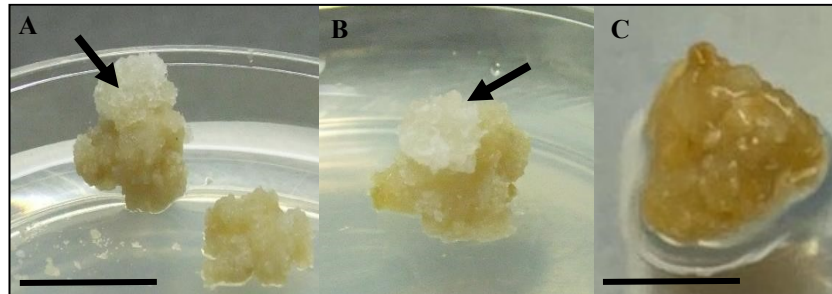


Figure 2. (A,B) MRM calli placed on selective medium (Gamborg B5 + Kanamycin 100 mg/L): after 30 days, the transformed calli budded (black arrows), forming actively proliferating portions. (C) Untransformed calli, on the other hand, do not survive to kanamycin selection and undergo necrosis; Scale bar (A, B) =1cm; Scale bar(C) =5mm.

2.4 PCR screening

DNA extraction from 200 mg of callus culture was performed through Quick-DNA™ Plant/Seed Miniprep Kit (ZymoResearch). PCR was carried out with GRS Taq DNA Polymerase (Grisp Solutions) kit, the mix for 100 ng (4 µL of DNA) samples was made up as follows: MgCl₂ 1.5 µL, dNTPs 1 µL, Primer Forward (5'-AGGTGAACACGCGGAAGATCTCCAA-3') [10 µM] 0.75 µL, Primer Reverse (5'-GCTCAAGCACGCCGCCATAA-3') [10 µM] 0.75 µL, Buffer 2.5 µL, Taq polymerase 0.25 µL, H₂O 14.25 µL. The PCR amplification was performed using the Thermocycler Life touch-865019 (Bioer) according to the following reaction scheme (**Fig.3**).

Amplification products were run on 1% agarose gel (1 g Agarose Duchefa-Biochemie, 100 mL TAE 1X, 5 µL of Ethidium Bromide) by electrophoresis at 100 V for 30 minutes.

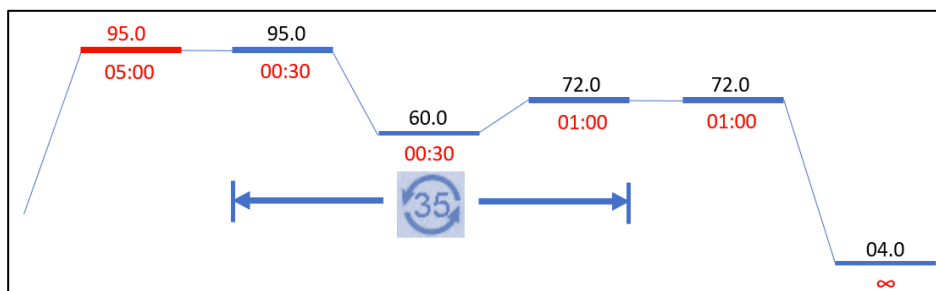


Figure 3. Polymerase chain reaction scheme for *Sn* gene amplification.

2.5 GUS assay for pBI121.1 transformants

While the presence of the *Sn* gene was screened by PCR analysis, the acquisition of the second construct pBI121.1 in apple cell cultures was verified through a Gus assay, using a wt callus as negative control. Apple pulp calli transformed with control vector encoding GUS enzyme were grown in a medium with 100 mg/L of kanamycin for 30 days and then GUS assay was performed, following the method described by Jefferson (1987)^[13]: the tissues were dipped in the X-gluc solution, *vacuum* infiltrated and left overnight at 37°C in the dark.

2.6 Anthocyanins biosynthesis stimulation and extraction

Transformed p121.Sn and pBI121.1 lines and wild-type apple pulp calli were placed under continuous light (Lamp Brand: Osram Fluora T8 G13 L 15 W/77; Luminous Flux (lm): 550) at 25 °C and monitored at 0, 24, 48 and 72 h. The light stress was performed by placing the five plates (one for each genotype: the three transformed strains, the 121.1 and the wild type ones) in a growth chamber at 25 °C in high light conditions. Light stress should trigger the anthocyanin biosynthetic pathway by activating the bHLH, MYB and WD40 transcription factor complex. Over time, we expect the transformed lines to show a reddish color compared to wild-type and pBI121.1 controls, due to the overproduction of anthocyanins after agroinfection. At each time interval, one callus from each genotype was collected and placed at -20 °C: the samples were freeze-dried and the dry weight was recorded. The extraction was performed with a Methanol solution + HCl 2% directly in Eppendorf tubes, keeping them shaking for 1 hour at room temperature. Samples were centrifuged at 16000xg for 10 minutes, then proceeded with spectrophotometric analysis (Jasco V-530 UV/VIS Spectrophotometer; $\lambda=576$ nm). Two independent technical replicates of light-stress induction were conducted for each genotype.

3. Results

3.1 Callus induction of *Mela Rosa Marchigiana* explants

Apple pulp explants were placed on Gamborg B5 medium with the addition of 6-benzylaminopurine 2.0 mg/L (BA, Sigma-Aldrich) plus 2,4-dichlorophenoxyacetic acid 0.2 mg/L (2,4-D, Sigma-Aldrich), as shown by Verardo et al., 2017^[12]. The explants formed whitish friable calli which were maintained for 28 days for further experiments (**Fig.4A,B**).

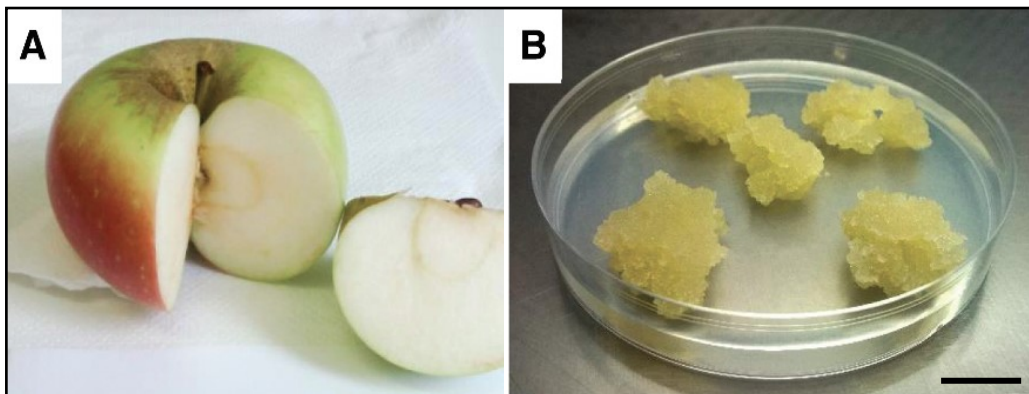


Figure 4. (A) *Malus domestica* Mela Rosa Marchigiana variety used for explants of apple pulp. (B) Callus culture growth on Gamborg B5 medium supplemented with phytohormones after 28 days. Scale bar = 1 cm.

3.2 Culture growth tests

Apple pulp wild-type calli were grown on Gamborg B5 media supplemented by different increasing concentrations of Kanamycin, 100-200-300-500 mg/L, to find the minimum concentration of selective agent that would inhibit the wild-type growth, allowing the normal development of *Sn*-transformed tissues (**Fig.5A, 5B, 5C**). We select 100 mg/L kanamycin concentration as the critical one able to inhibit the growth on wild-type samples for transformants selection.

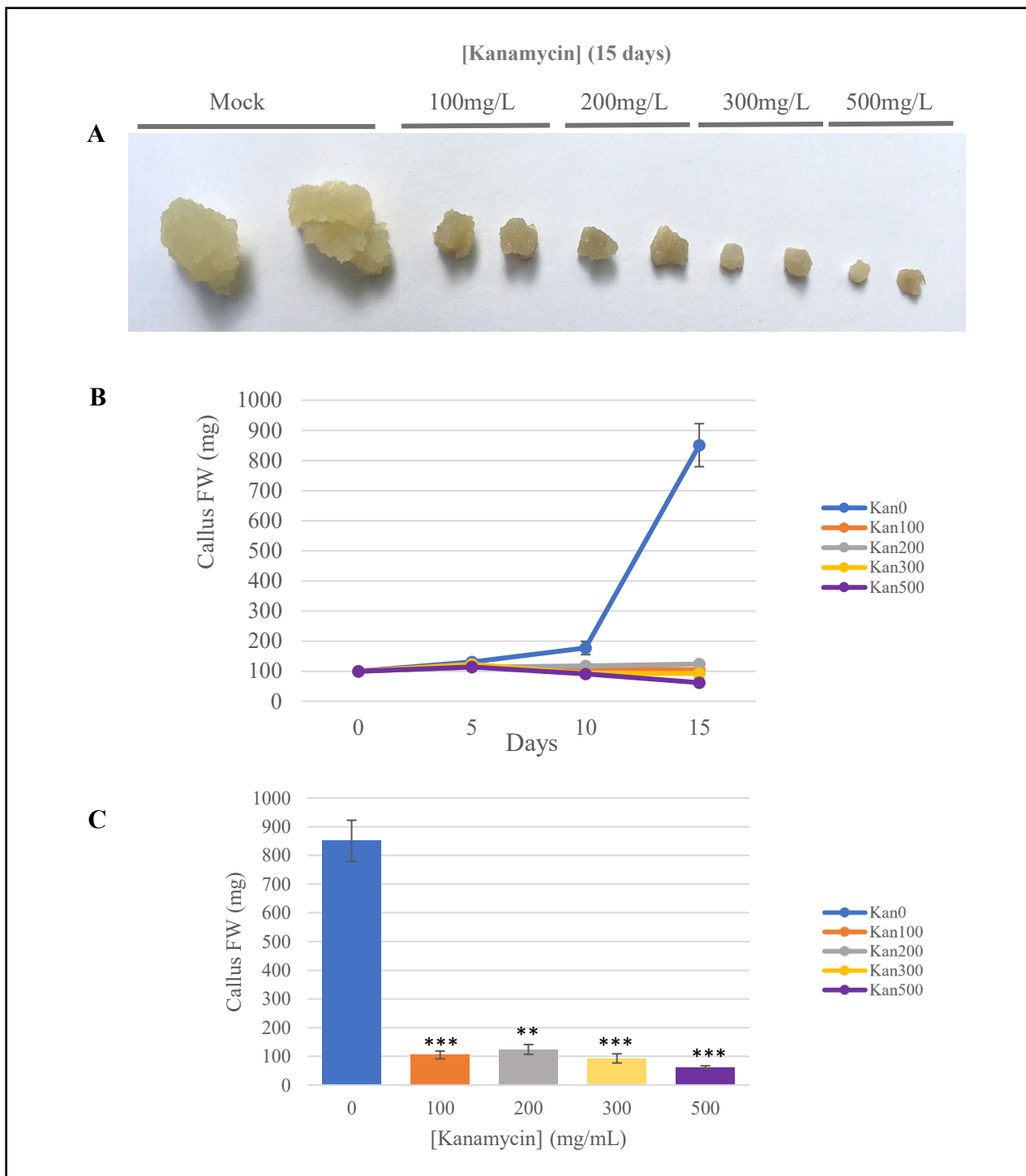


Figure 5. (A) Wild-type calli were grown on Gamborg B5 medium supplemented with phytohormones and increasing concentrations of kanamycin (100, 200, 300, 500 mg/L); Scale Bar = 1 cm. (B) The reported graph shows the growth trend of wild-type MRM calli in presence or absence of kanamycin at different concentrations. Growth monitoring consists of the fresh weight (FW) average recorded at defined time intervals obtained from two technical replicates. Panel (A) and (B-C) data are obtained from distinct technical and biological replicates. (C) The histogram reports the weight after 15 days of wild-type MRM calli placed on a medium containing (or not) different concentrations of kanamycin: a t-student test reveals the significance of the treatment of each kanamycin concentration test against the control (***= t-value < 0,001; **= t-value < 0,01; statistic was performed with PASW Statistic 17, Release Version 17.0.2, SPSS Inc., 2008 software).

3.3 Screening of transformants

The active proliferation areas identified in agroinfiltrated callus culture grown on 100 mg/L kanamycin were isolated and grown for 28 days on the same culture medium. They were screened by PCR and three positive clones, called SN1, SN2 and SN51, were identified and kept growing in the same culture conditions. Wild type sample was used as negative control to confirm the absence of *Sn* gene in untransformed calli (**Fig.6A**).

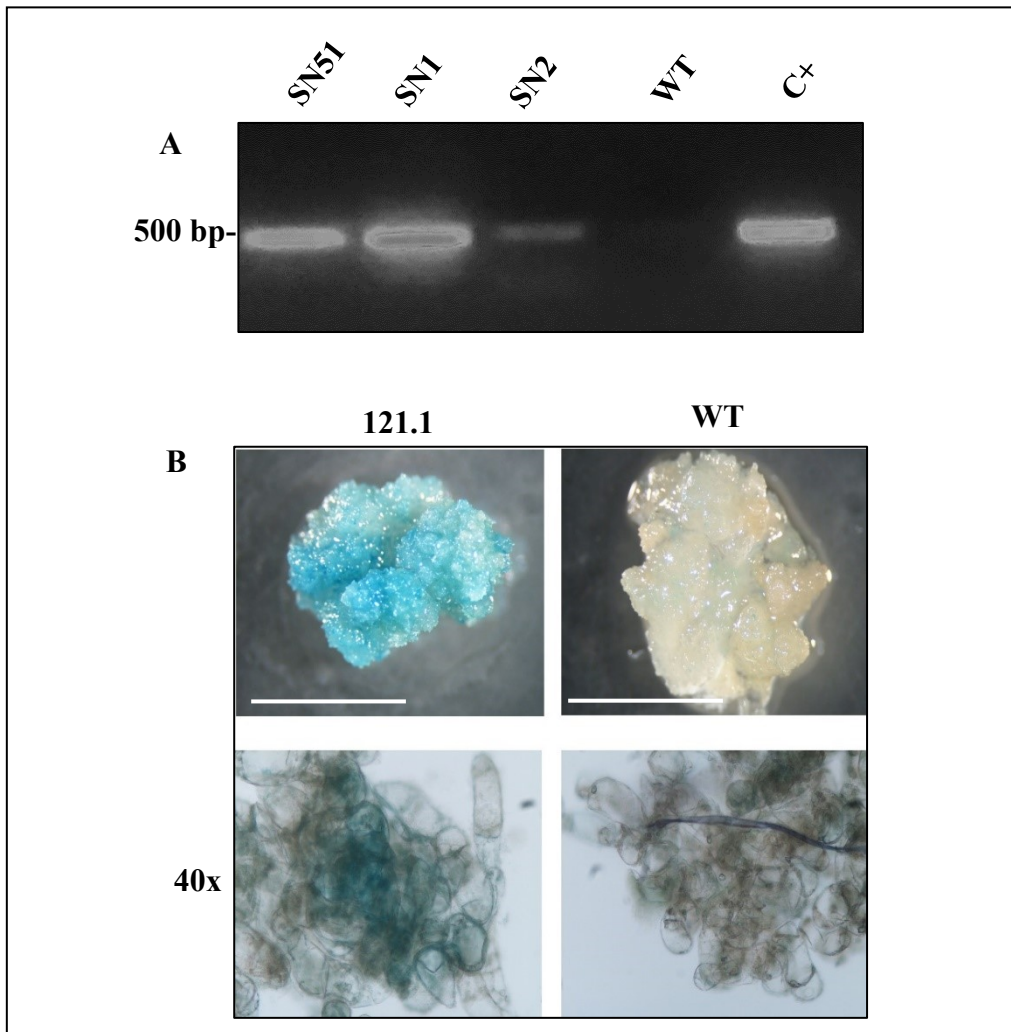


Figure 6. (A) Budd originated on calli surface grown on selective medium were further screened by PCR and compared to wild type sample for *Sn* gene identification (C+: p121.Sn vector). (B) 121.1 and wt calli treated with GUS solution (Scale Bar = 4 mm). A small fragment of each callus was observed under Leitz Dialux 20 Fluorescence Microscope - 40x objective.

The insertion and expression of *uidA* gene sequence in 121.1 sample, coding for GUS, was confirmed by a GUS assay. The technique detects the expression of the GUS gene coding for

the enzyme *Escherichia coli* β -glucuronidase; this enzyme, supplied with specific colourless substrates, can make them colourful and consequently visible. Through GUS analysis, we demonstrate that calli transfected with the control *uidA* gene are correctly transformed (**Fig.6B**).

3.4 Calli light exposure

Wild type calli are usually grown in dark conditions and kept at 25°C: they show yellow-white color, which indicates an absence of anthocyanin synthesis. When subjected to continuous light in a growth chamber at 25°C after 24-48-72 hours, these tissues show they are competent for anthocyanins production, as they gradually begin to show reddish areas, particularly after 72 hours. The same trend is traced from 121.1 samples while they grow in the same selective medium as the *Sn*-transformed calli. This allows us to state that the differences in anthocyanin increase observed in SN51 cannot be attributed to the selective growth medium, which could be responsible for a further stress component, but rather to the light stress induced and the genotype. Anthocyanin accumulation occurs prematurely in SN samples, of which only one is reported in **Fig.7** as an example. After 24 hours, the development of colored areas can be observed in SN51 sample, extending clearly to cover almost the entire surface after 75 hours of exposure.

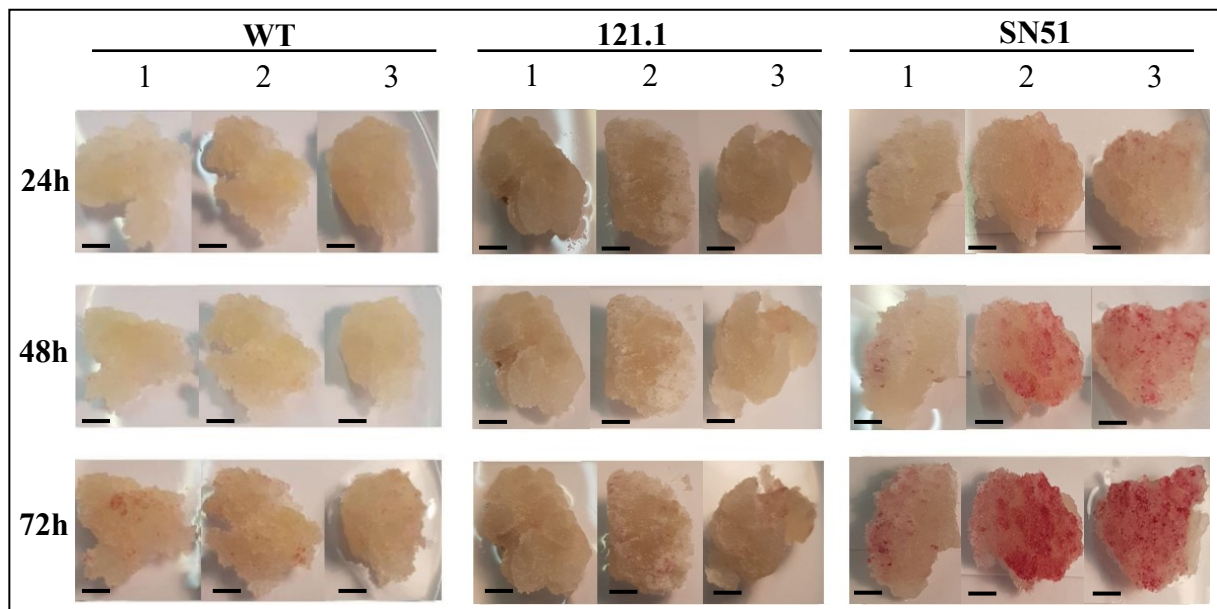


Figure 7. Different callus genotypes exposed to a continuous light treatment: differences are visible at 48 and 72 hours of continuous light stress, were transformed calli (SN51) show widespread reddish areas on callus surface, unlike wild type and 121.1 calli; Scale Bar = 5 mm.

3.5 Anthocyanins content quantification

One callus from each plate (of each genotype and for each stage of light exposure) was collected and freeze-dried. The dry weight of each sample was registered, and the same samples were used for anthocyanins extraction. The total amount of anthocyanins for 100 g of dry weight was calculated. The reported Box Plot (**Fig.8**) shows the difference in anthocyanins content between a stage (24h,48h,72h) and the respective previous one, to illustrate the differential increment in anthocyanins content between phenotypes across the time. At early stages of exposure (0-24 h), no distinctions in antioxidant molecule content are visible between wild-type and transformed calli. This condition changes considerably in prolonged expositions, as it is visible in the two boxes referring to exposure for 24-48 h and 48-72 h. The levels of anthocyanins produced by the transformed calli with the *Sn* gene (SN1 and SN51) are higher than those produced by the wild-type calli and 121.1 samples.

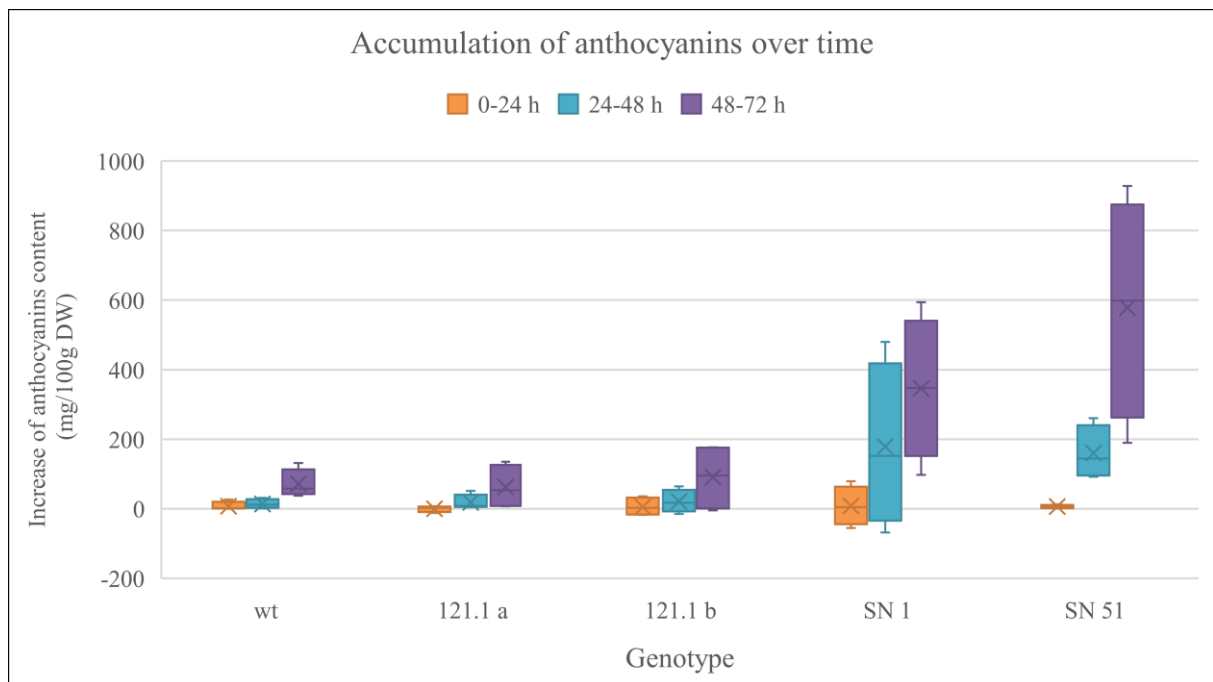


Figure 8. Differential increase of anthocyanins in samples obtained from two independent replicates of the treatment under continuous light. Each box plot represents the difference in anthocyanins amount, between one stage of light exposure and the previous one.

Experiment validity is confirmed by the low level of anthocyanins content in calli transformed with pBI121.1, the sample transformed for the *uidA* gene and which grows on selective medium, as it is comparable to that in the wild-type sample.

The final step of the present work was to statistically demonstrate the relevance of genotype in anthocyanins accumulation after the light treatment. It is globally known that light stress can naturally induce the production of phenols in plant tissues, but in this case, the significant accumulation of the antioxidant compound was due to the introduction of the SN transcription factor, which activates the anthocyanins biosynthetic pathway in several species.

While at the time zero no significant differences were found across the different genotypes, this situation changes in the final 72 h stage as the total accumulation of anthocyanins in *Sn*-genotypes resulted statistically significant (**Fig.9**). Anthocyanins content in wild-type and 121.1 samples is comparable at different stages.

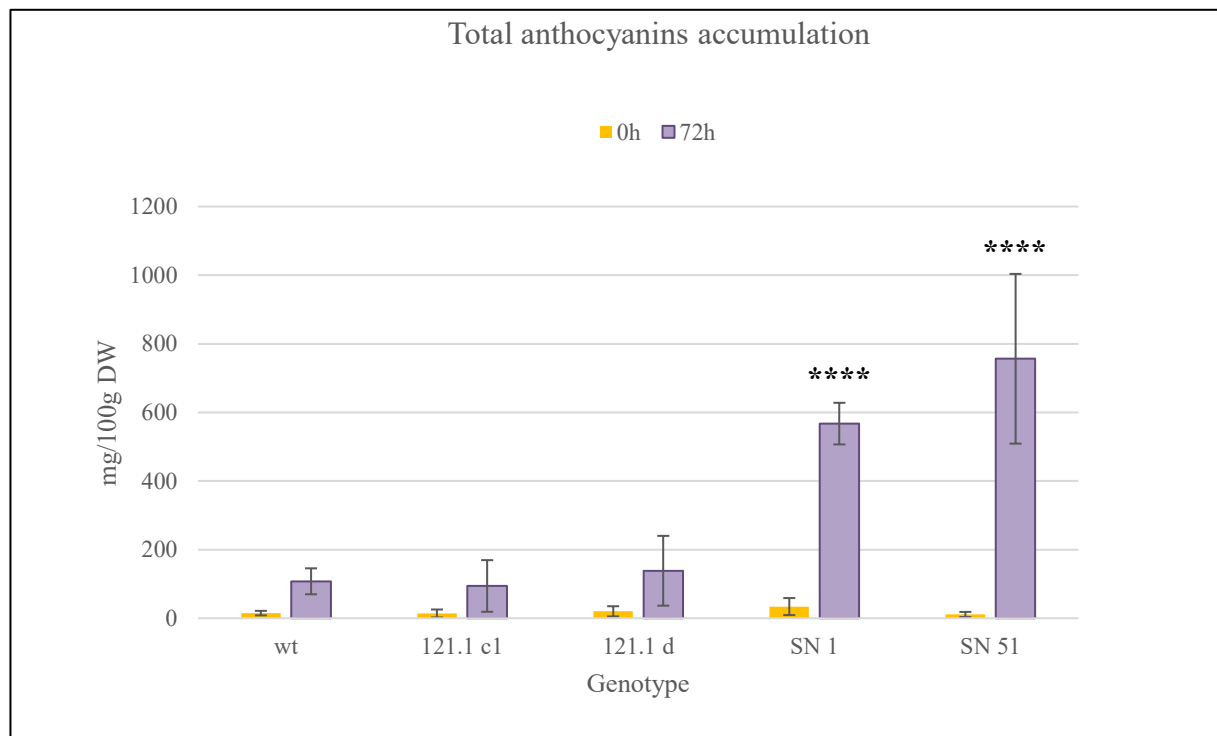


Figure 9. Quantitation at time 0 and at 72 h of anthocyanins content in investigated sample genotypes of two fully independent light treatments (**** = p-value < 0.0001; statistic was performed with PASW Statistic 17, Release Version 17.0.2, SPSS Inc., 2008 software).

4. Discussion

Many studies have attributed to anthocyanins useful and beneficial properties for human health: as already mentioned, they showed antioxidant, anti-inflammatory and anticarcinogenic, as well as protective abilities against cardiovascular diseases, some cancer types, reduction in the onset of diabetes and cognitive function disorders^[17].

In several higher plant systems, bHLH transcription factors have previously been identified as regulators of the anthocyanin pathway. In maize, the *R* gene family controls the quantity, distribution, and timing of anthocyanin colouring. This family consists of a group of regulatory genes located on chromosomes 10 and 2, with *B* on chromosome 2 and the *Lc* and *Sn* genes around two units *R* distant. Each gene controls the tissue-specific colouring of various plant and seed components^[18]. The transcriptional complex of MYB, bHLH, and WD40 has been studied in several plants: in plant models like tobacco and *Arabidopsis*, but also in edible plants like tomato (*Solanum lycopersicum*), one of the most popular vegetables worldwide, have been highlighted the overexpression of this important transcription factor complex in fruit pulp and peel^[19]. This pathway is not just studied for human health promotion but also for ornamental reasons: this biosynthetic pathway was also highly studied in *Petunia*^[20]. In *Lotus corniculatus*, phenotypes obtained from the expression of the bHLH *Sn* reflect the canonical effect of the anthocyanin pathway, although this was circumscribed to the petiole, leaf base and, in some cases, the leaf midrib^[10]. *Sn*-hairy roots generated from a range of dicotyledonous plant species displayed patterns of red pigmentation based on species, genotype, and transformation event^[15]. However, because of their restricted accumulation, the production of secondary metabolites derived from wild populations of plants is not high enough to satisfy commercial demands. Callus, hairy roots, and suspension cultures are a few *in vitro* culture methods that may be used to improve the production of certain metabolites in economically significant plants. Callus culture is thought to be the highest promising method for producing bioactive chemicals in endangered species of most medicinal plants^[21]. These preliminary studies, conducted within the laboratories of plant biology of the Urbino Botanical Garden (University of Urbino Carlo Bo, DISB) and the National Research Council (CNR, IBBR Institute) of Perugia, highlight the possibility of using plant stem cells (calli) as bioreactors.

The biosynthesis of primary and secondary metabolites, as well as metabolic activities, are all significantly influenced by light^[22]. Many studies have demonstrated that light positively impacts secondary metabolite accumulation in callus culture and cell suspensions^[23]: although

calli presented in this work were grown in the dark and only later exposed to light, the ability of calli to catch light stimulus and induce cell differentiation and regeneration is well known. Indeed, calli grown in the dark exhibit characteristic organelles known as proplastids, characterised by a minimal structure and small immature thylakoid membranes^[24]. Moreover, it has been shown in several plant species that exposure of calluses to a light source is able to induce the differentiation of chloroplasts, amyloplasts and leucoplasts, as well as influencing plant growth regulators, leading, in some plant species, to shoots formation^[25,26].

Stem cells derived from apple pulp from Marche region (Italy) were successfully genetically stable-transformed with *Sn*-gene from *Zea mays* for the purpose of stimulating anthocyanin production. Our experiments show that this gene can stimulate anthocyanins overproduction when samples are exposed to a continuous light stress. This research suggests (together with existing literature) that the multigene complex WD40-bHLH-MYB, responsible for the cascade of events which leads to the activation of the enzymatic pathway, is conserved across different plant species. Transformed callus culture would allow us to develop a stable and reproducible biotechnological process that could lead to the accumulation of large amounts of these compounds, as opposed to the whole plant. Indeed, it is important to emphasize the rapidity of growth of these actively proliferating tissues, which under optimize culture conditions can increase in weight almost ten times in about a month. Moreover, the possibility of growing plant stem cells in aseptic semi-solid and liquid culture means a high control of their physical and chemical growth parameters and the possibility to use an external stimulus (in this case, light), to act as a trigger for anthocyanin production, raising to a considerable scale-up of the molecules of interest.

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CHAPTER II

Human indoleamine 2,3-dioxygenase 1 (IDO1) expressed in plant cells induces kynurenine production

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Abstract

Genetic engineering of plants has turned out to be an attractive approach to produce various secondary metabolites. Here, we attempted to produce kynurenine, a health-promoting metabolite, in plants of *Nicotiana tabacum* (tobacco) transformed by *Agrobacterium tumefaciens* with the human IDO1 gene, coding for indoleamine 2,3-dioxygenase 1, an enzyme responsible for the kynurenine production because of tryptophan degradation. The presence of IDO1 in transgenic plants was confirmed by PCR, but the protein failed to be detected. To confer higher stability to the heterologous human IDO1 protein providing a more sensitive method to detect the protein of interest, we cloned a gene construct coding for IDO1-GFP. Analysis of transiently transfected tobacco protoplasts demonstrated that the IDO1-GFP gene led to the expression of a detectable protein and to the production of kynurenine in the protoplast medium. Interestingly, the intracellular localization of human IDO1 in plant cells is similar to that found in mammal cells, mainly in cytosol, but in early endosomes as well. To the best of our knowledge, this is the first report on the expression of human IDO1 enzyme capable of secreting kynurenines in plant cells.

Keywords: protoplasts; kynurenine; genetic transformation

Abbreviations

AhR	Aryl hydrocarbon Receptor
BA	Benzylaminopurine
CaMV35S promoter	Cauliflower mosaic virus 35S promoter
CIAP	Intestinal alkaline phosphatase
DCs	Dendritic cells
GFP	Green Fluorescent protein
HPLC	High-performance liquid chromatography
HRP	Horseradish-peroxidase
IDO1	Indoleamine 2,3-dioxygenase 1
IDO1-GFP	Indoleamine 2,3-dioxygenase 1 fused to Green Fluorescent Protein
ITIMs	Immunoreceptor Tyrosine-based Inhibitory Motifs
Kyn	Kynurenine
LB	Left Border
LPVC	Late prevacuolar compartment
MVB	Multivesicular bodies
NAA	α -Naphthaleneacetic acid
NOS P/T	Nopaline synthase promoter/terminator
OD	Optical density
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
PVC	Prevacuolar compartments
RB	Right Border
RbcL	Rubisco Large subunit
RFP	Red fluorescent protein
Rp	Pearson's correlation
Rs	Spearman correlation
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
TGN	Trans-Golgi network
Th17	T-cells helper 17

Treg	Generation of regulatory T-cells
Trp	Tryptophan
Untr	Untreated

1. Introduction

The production of recombinant proteins in plants dates back to the end of 1980s^[1], and since then many recombinant proteins have been produced in plants for different uses, spanning from biomedical applications to industrial production^[2,3]. Plant platforms employing different expression systems have been developed, with the main distinction between stable transformation, where transgenes can be integrated either in the nuclear DNA or in the plastid DNA^[4,5], and transient expression^[6]. Foreign genes have been transferred into the plants by *Agrobacterium tumefaciens*-mediated transformation methods^[7,8], or other delivery systems including particle bombardment, PEG-mediated DNA uptake in wall-free protoplasts^[9], as well as nanomaterial-based delivery devices^[10]. However, plant expression procedures share some common characteristics such as the production of favorable post-translational modifications and the absence of contaminating endotoxins and animal viruses, which are serious issues in bacterial and eukaryotic expression systems. Several reports have shown the successful expression of bioactive human proteins in plants^[11–13].

Indoleamine 2,3-dioxygenase 1 (IDO1) represents one of the most interesting proteins which links an ancient metabolic pathway with immune regulation. IDO1 is a heme-containing enzyme that catalyzes the oxidative cleavage of the indole ring in L-tryptophan, regulating the catabolism of this essential amino acid at an initial, rate-limiting level in a specific pathway. This activity leads to the kynurenine production, which is the upstream metabolite along the so-called kynurenine pathway^[14]. IDO1 is widely recognized as an authentic immune regulator capable of fine-tuning immune responses not only in pregnancy^[15], but also in several pathological conditions, like autoimmune diseases^[16,17], chronic inflammation^[18], transplantation^[19], and neoplasia^[20]. IDO1 immunoregulatory effects are mainly mediated by Dendritic Cells (DCs), the most potent antigen presenting cells in the mammal immune system that, upon IDO1 upregulation, acquire tolerogenic functions. In fact, through its enzymatic activity IDO1 generates both tryptophan deprivation in local tissue microenvironments and the formation of bioactive metabolites collectively called kynurenines. Particularly, several kynurenines reinforce the immunoregulatory circuit in mammals by acting as a ligand of Aryl hydrocarbon Receptor (AhR) in both T cells and DCs^[21]. As a result, IDO1-expressing DCs mediate multiple effects on T lymphocytes, including inhibition of proliferation, apoptosis, and differentiation towards a regulatory phenotype^[22]. Moreover, apart from being a cytoplasmic enzyme, IDO1 also acts as a signal-transducing molecule by binding tyrosine phosphatases in

its Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) domains^[23] and anchoring to the early endosomes. This localization is a result of the interaction with class IA PI3K subunits which becomes activated^[24]. Thus, the IDO1 ability to restrain inflammation depends on two distinct functions of the IDO1 protein, one is enzymatic function (tryptophan degradation to kynurenine) while the other is free from its enzymatic function.

Potential clinical applications of kynurenine are growing. Kynurenine is required for the production of several metabolites, being the first metabolite in the kynurenine pathway, which leads to the final biosynthesis of nicotinamide adenine dinucleotide (NAD⁺). Among these metabolites, kynurenic acid and xanthurenic acid, as well as their analogues, have neuroprotective activities, suggesting that they may find use in drug development for neurological diseases^[25–28]. Moreover, NAD⁺ is a coenzyme with a pivotal role in a vast number of biochemical processes, in several redox reactions and it is vital to mitochondrial function^[29].

In this study, considering the only options for kynurenine production as chemical synthesis or extraction from human tissues, we expressed human IDO1 in plant cells in order to investigate the possibility to obtain an alternative production platform for both IDO1 protein and biologic kynurenine.

2. Materials and Methods

2.1 Construction of plasmids for plant transformation

To stably express IDO1 in tobacco plants, human IDO1 cDNA was excised from plasmid pEZM02.IDO1 using *EcoRI* and *NotI* restriction enzymes. After excision, both 5' and 3' sticky ends were blunted by DNA Polymerase I large (Klenow) fragment to allow non-compatible ends joining. The result of digestion was separated on a 1% agarose gel and the IDO1 band was isolated and purified using the T-Pro Gel/PCR DNA Purification kit (T-Pro Biotechnology). The plant vector pDHA^[30] was linearized with *BamHI* restriction enzyme, blunted as described above, dephosphorylated using calf intestinal alkaline phosphatase (CIAP) and ligated to the IDO1 fragment to obtain the pDHA.IDO1 intermediate vector, where IDO1 was under the control of the constitutive 35S cauliflower mosaic virus (CMV) promoter and the 35S

terminator suitable for plant expression. For the production of transgenic plants, the expression cassette excised by *EcoRI* digestion from the pDHA.IDO1 plasmid was introduced into the pGreenII.0029 binary vector^[7] opened with the same restriction enzyme, obtaining pGreen.IDO1. This final plasmid was sequenced to verify the correct sequence and orientation of the IDO1 expression cassette.

Strain GV3101 of *A. tumefaciens* was transformed by electroporation with the pGreenII.IDO1 vector and used to produce transgenic tobacco (*N. tabacum* cv. Petit Havana), as described by Pompa and colleagues^[31]. Briefly, tobacco plants were transformed by co-cultivation with *A. tumefaciens* harbouring pGreenII.IDO1 on agar solidified MS medium containing 1 mg/L of 6-Benzylaminopurine (BA) and 0,1 mg/L of α -Naphthaleneacetic acid (NAA) hormones. After co-cultivation, the leaf discs were grown aseptically in Petri dishes at 25 °C under full light on the same medium supplemented with 500 mg/L of cefotaxime and 100 mg/L kanamycin to avoid *Agrobacterium* growth and to select transformed cells, respectively. Shoots emerging from each explant were kept separated to guarantee regeneration of independent transformants. After approximately 5-6 weeks, shoots were plated on MS medium without hormones but still supplemented with antibiotics until new plants developed. Transformed plants (T0) were grown at 25°C in 16 h of light in axenic culture in glass jars without antibiotics and propagated every 5–6 weeks. After PCR results, ten transgenic plants (labelled with numbers from 1 to 10) were chosen and reared to flower to obtain seeds. T1 seeds from self-fertilization were harvested at maturity, seeded onto kanamycin selective medium and scored for resistance. After 21–28 days resistant seeds started to germinate. One T1 plant deriving from each of the ten primary regenerants, T1n (n=1-10), was analysed by PCR to confirm the IDO1 gene presence. To fuse GFP in frame to IDO1 at the C-terminal, IDO1 cDNA was amplified with PCR from pDHA.IDO1 with the forward primer IDO1ClaI (GCAGACTACATCGATGGCACACGCTATGG) and the reverse primer IDO1NheI (GGCCGCTAGCACCTTCCTTCAAAG). The PCR fragment was digested by *ClaI* and *NheI* restriction enzymes and inserted in the vector pJA1, derived from pAW7 after replacing the RFP with GFP^[32]. The resulting vector (pUC.IDO1-GFP) contained the 35S promoter and the IDO1 sequence in frame with a C-terminal GFP, followed by the NOS terminator in a *EcoRI*-*HindIII* cassette. Since IDO1 coding sequence contained another *HindIII* site (**Fig. 2A**), the digestion of pUC.IDO1-GFP by *EcoRI/HindIII* generated two fragments. Both fragments were cloned in the pV binary vector for plant transformation, digested with *EcoRI/HindIII*, to

reconstitute the original IDO1-GFP. This reconstituted cassette was sequenced to confirm the integrity of IDO1 gene and the frame with GFP. The resulting vector pV.IDO1-GFP was used for tobacco protoplast transformation and, after transformation into *A. tumefaciens*, for infiltration experiments.

2.2 Tobacco protoplast isolation

Protoplasts were isolated from transgenic plants as described by De Marchis et al^[33]. Briefly, to isolate mesophyll protoplasts, young fully expanded leaves from tobacco plants grown under aseptic conditions were wounded with a scalpel and placed in a Petri dish with the abaxial side in contact with the 1× enzyme mix solution (in 1% Cellulase Onozuka RS, 0.5% Macerozyme R-10) dissolved in K3 medium (3.78 g Gamborg's B-5 Basal Medium with Minimal Organics, 5.1 mM CaCl₂· 2H₂O, 3.12 mM NH₄NO₃, 0.4 M sucrose, 1.67 mM xylose, BA 1 mg/L, NAA 1 mg/L, pH 5.5) for 16 h at 25°C in the dark. Protoplasts were recovered by resuspending macerated leaves in K3 medium, filtering through a sterile 85 µm nylon mesh and centrifuging for 20 min at 60g with a swinging bucket rotor. The floating green band, representing vital protoplast, was recovered and washed twice with W5 solution (152 mM NaCl, 5 mM KCl, 125 mM CaCl₂· 2H₂O, 5 mM glucose). After testing viability and yield with fluorescein diacetate staining, protoplasts were used for further analysis.

2.3 Tobacco protoplast transformation

After W5 removal by centrifuging for 10 min at 60g, protoplasts were resuspended at a concentration of 1×10^6 mL⁻¹ with MaCa buffer (0.1% MES, 20 mM CaCl₂, 0.5 M mannitol, pH 5.6), heated at 45°C for 5 min and mixed with 60 µg of plasmid DNA mL⁻¹ of protoplast suspension. Protoplasts transformation with polyethylene glycol (PEG) was performed by adding an equal volume of a solution containing 40% (w/v) PEG 4000, 0.1 M Ca(NO₃)₂, 0.4 M mannitol, pH 8. After incubation at room temperature for 30 min transformed protoplasts were slowly diluted to 14 ml with W5 solution. Protoplasts were pelleted at 60g for 10 min at room temperature, resuspended in K3 medium to a final density of 1×10^6 protoplasts mL⁻¹ and

incubated 2 h at 25°C in the dark. Then, tryptophane 100 µM was added to half of the samples, and all the protoplasts were incubated for 24 h at 25°C in the dark before subsequent analysis.

2.4 Plant transformation by Agroinfiltration and microscopy analysis

Soil-grown *N. tabacum* cv Petit Havana^[34] leaves were infiltrated at OD 0.1 with *A. tumefaciens* cultures containing the pV-IDO1-GFP alone or with either organelle marker such as ST-RFP (Golgi marker), RFP-SYP61 (TGN marker), TR2RFP-VSR2 (PVC/MVB marker) or RFP-Rha1 (LPVC marker), as described previously^[35]. The infiltrated areas were analysed 48h post-infiltration by confocal laser scanning microscopy. Confocal imaging was performed using an inverted Zeiss LSM 700 laser scanning microscope. When GFP fusions were imaged with RFP organelle markers, samples were excited with a diode laser at the wavelength of 488 nm for GFP and 555 nm for RFP. Fluorescence was detected with a 552 nm dichroic beam splitter and a 475 to 550 nm bandpass filter for GFP and a 560 to 700 nm bandpass filter for RFP. All dual-color imaging was performed by line switching to obtain adequate live bioimaging data that had not been distorted by organelle motion. Statistical colocalization between IDO1-GFP and TR2RFP-VSR2 was performed as described in Gershlick et al.^[36].

2.5 DNA isolation and analysis

Total DNA was isolated from wild type and transgenic tobacco leaves using the GenElute™ Plant Genomic DNA Miniprep kit (Merck KGaA, Darmstadt, Germany). DNA PCR amplification was carried out using the primer pairs forward 35S1 (5'-ACGTTCCAACCACGTCTTCAAAG-3') and reverse IDO1 (5'-GCAAGACCTTACGGACATCT-3') and forward 35S2 (5'-CATGGAGTCAAAGATTCAA-3') and reverse IDO1 for T0 and T1 plants, respectively.

2.6 RNA Extraction and RT-PCR Analysis

Total RNA was extracted from leaf tissues of transformed plants with the NucleoSpin® RNA Plant kit (Macherey–Nagel, Düren, Germany). For the reverse-transcribed PCR (RT-PCR) analysis, RNA was treated with DNase I (Qiagen, Venlo, The Netherlands) and reverse-transcribed (2 g) using oligo(dT)18 and the SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The cDNA preparations were analysed by PCR using specific primers for human IDO1 (forward, 5'-CTCCTGGACAATCAGTAAAGAGTACC-3'; reverse, 5'-ACTTCTCAACTCTTTCTCGAAGCTGG-3') and plant actin (forward, 5'-ATGGCGGATGGGGAGGACATTCA-3'; reverse, 5'-CCTTTTGTTATCCACATCTGTTG-3').

2.7 Protein extraction from protoplasts and western blotting analysis

The protoplasts were washed twice in cold PBS and lysed with Laemmli sample buffer. Proteins were separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to nitrocellulose. Membranes were blocked with 5% (wt/vol) non-fat dried milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 at room temperature for 1 h. After being blocked, membranes were incubated overnight with primary antibodies, namely, anti-IDO1 (Millipore), anti-GFP (ThermoFisher), anti-Rubisco large subunit (rbcL, Agrisera, Sweden), then washed and incubated with appropriate horseradish-peroxidase (HRP)-conjugated secondary antibody (Pierce) for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (BioRad, CA, USA).

2.8 Kynurenine analysis

IDO1 functional activity was measured in protoplast culture supernatants in terms of the ability to metabolize tryptophan to kynurenine, whose concentrations were measured by using a Perkin Elmer, series 200 HPLC instrument (MA, USA). A Kinetex® C18 column (250×4.6 µm, 5 µm, 100 Å; Phenomenex, USA), maintained at the temperature of 37 °C and pressure of 1800 PSI,

was used. Briefly, 1×10^6 transformed protoplasts were resuspended in 1 ml of K3 medium alone or supplemented with exogenous L-tryptophan $100 \mu\text{M}$ (Sigma) was added 2 h after transfection. Then, following an incubation of 24 h at 25°C in the dark, the K3 medium was recovered with a Pasteur pipette and filtered with a syringe containing a cotton filter to eliminate remaining protoplasts. After deproteinization, the samples were eluted by a mobile phase containing 10 mM NaH_2PO_4 (pH = 3.0; 99%) and methanol (1%) (Sigma-Aldrich, MO, USA), with a flow rate of 1.3 ml/min. An UV detector at 360 nm and 220 nm, respectively performed detection of kynurenine and tryptophan. The software TURBOCHROM 4 was used for evaluating the concentrations of kynurenine and tryptophan in the samples by mean of a calibration curve.

2.9 Statistics

Data are representative of at least three independent experiments. All results are shown as mean \pm SD. One-way ANOVA followed by post hoc Bonferroni's test was used when three or more samples were under comparison. All analyses were performed using Graph Pad Prism software 8.0.

3. Results

3.1 Cloning of IDO1 cDNA in a plant expression vector and attempted stably expression in tobacco plants

A comparison of the human indoleamine 2,3-dioxygenase amino acid sequence and the plant protein databases was performed. We found some similarity within plants and green algae only at a low percentage with an identity average of around 30% (data not shown). Since alignment errors can appear when the sequence identity drops below 35%^[37], these sequence homologies seemed to be too low to confirm the presence of IDO1 in the Plantae kingdom. Moreover, most of these proteins were reported to have an unknown function and, in the few cases of being identified as indoleamine 2,3-dioxygenase enzymes, the evidence supporting the existence was inferred only by homology.

Therefore, to express IDO1 in plants, we cloned the human IDO1 cDNA into plant expression vector pGreenII, obtaining pGreenII.IDO1. A schematic diagram of the vector assembly is shown in **Fig.1A**. Then we transformed tobacco (*N. tabacum* cv. Petit Havana) plants with *A. tumefaciens* harboring the pGreenII.IDO1 vector to obtain plants stably expressing human IDO1. Selection with kanamycin allowed the regeneration of transformed plants (T0) as shown in **Fig.1B** (left panel). Regenerated shoots were successively separated from chlorotic leaf discs and placed in fresh selective medium to stimulate further growth and roots formation (**Fig.1B**, central panel). As expected, wild type non-transformed plants failed to regenerate when placed in freshly selective medium (**Fig.1B**, right panel). Total DNA was isolated from leaves of selected regenerated T0 plants and examined by PCR (**Fig.1C**). Only transformed explants showed an amplified fragment of about 500 bp, confirming the presence of the IDO1 gene cassette in their genome. Few T0 events unable to amplify were considered as regeneration escapes. In total, ten PCR positive plants were chosen, transplanted to pots in controlled greenhouse conditions and reared to flower for seed production. Seeds were germinated on kanamycin selective medium and for several plants (T1 events). IDO1 gene presence was confirmed by PCR analysis (**Fig.1D**). However, even if IDO1 mRNA was detected by RT-PCR in leaves (**Fig.1E**), IDO1 protein was undetectable by Western blotting in total proteins extracted from leaves and roots of transformed plants, suggesting a protein stability problem.

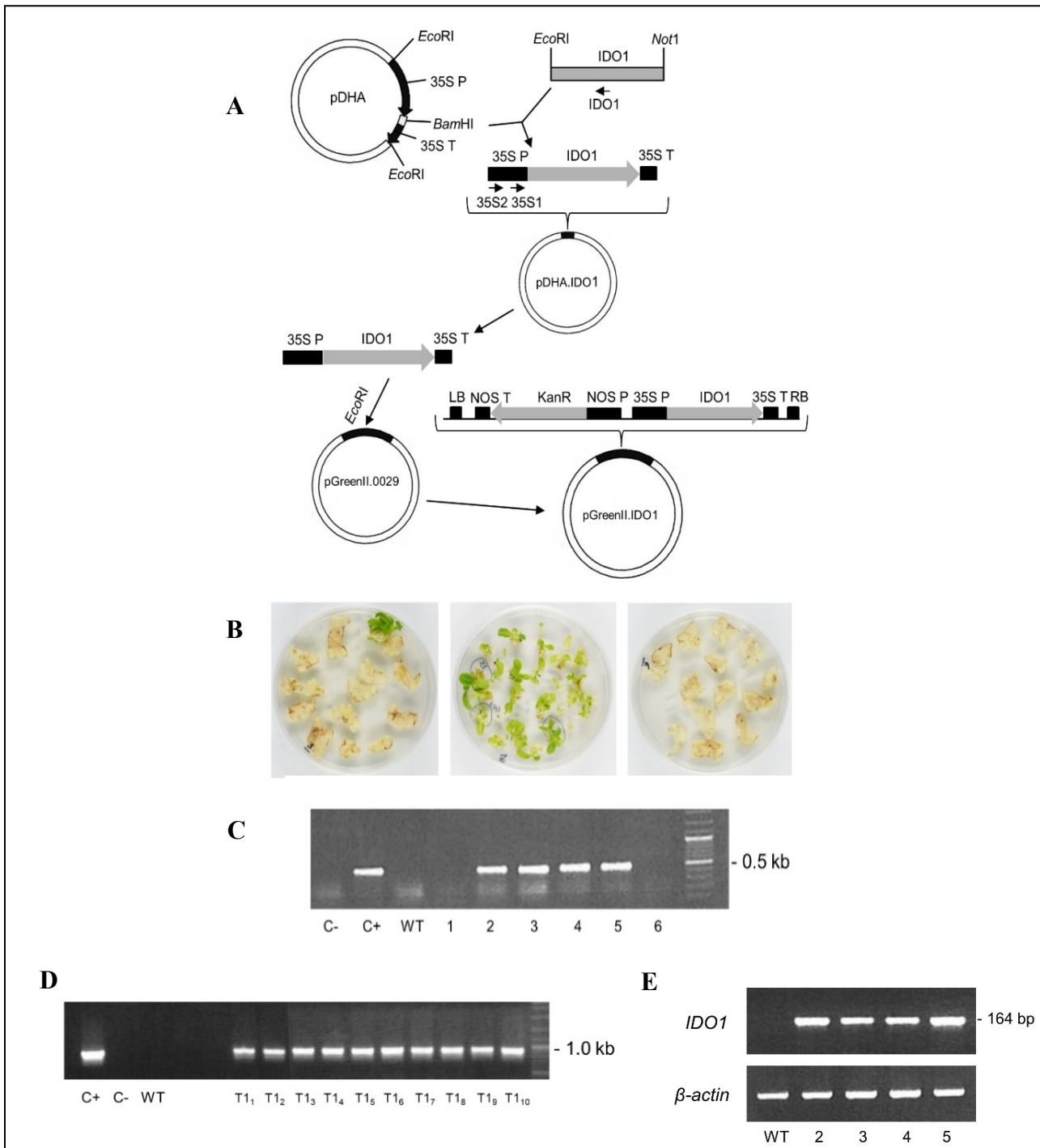


Figure 1. Attempt of human IDO1 protein stable expression in tobacco plants. **(A)** Schematic diagram of pGreen.IDO1 vector cloning. Abbreviations: 35S P, 35S promoter sequence; 35S T, 35S terminator sequence; NOS P and NOS T, *A. tumefaciens* nopaline synthase (*nos*) promoter and terminator sequence, respectively; LB, left border; RB, right border; KanR, gene encoding for kanamycin resistance. The annealing positions of primers 35S1, 35S2, and IDO1 are shown. **(B)** Transgenic tobacco plant selection and regeneration on MS medium with kanamycin after *A. tumefaciens* co-cultivation. Only transformed cells were able to regenerate on selective medium (left panel). Regenerated shoots were separated from chlorotic leaf discs and subcultured to allow growth and rooting (central panel). No regeneration occurred when WT leaf discs were placed on antibiotic selective medium (right panel). **(C)** PCR on some tobacco primary regeneration events (T0) with primers 35S1/IDO1. The amplification signal was detectable only in transformed plants (2–5), whereas plants 1 and 6 were probably regeneration escapes. **(D)** One T1 plant deriving from seeds of each of the 10 primary transformants (T1_n; n = 1–10) were analysed by PCR with primers 35S2/IDO1, confirming the presence of the IDO1 transgene. There were two empty lanes between the lanes WT and T11. **(E)** Different transformed plants (2–5) were analysed for the presence of IDO1 mRNA by RT-PCR. Actin was amplified as a reference gene for the RT-PCR experiment. **(C–E)** WT: wild type plant; **(C,D)** C+: pGreen.IDO1 vector; C-: no DNA.

3.2 Human IDO1-GFP can be transiently expressed in tobacco protoplasts

The lack of human IDO1 expression in plants could possibly be attributed to protein instability. Therefore, a second cloning strategy was carried out by fusing IDO1 sequence to the GFP tag, aiming to get a more stable fusion protein, as frequently observed in fusion technology^[38]. For this purpose and for obtaining a tag useful as both a protein detector and a possible purification

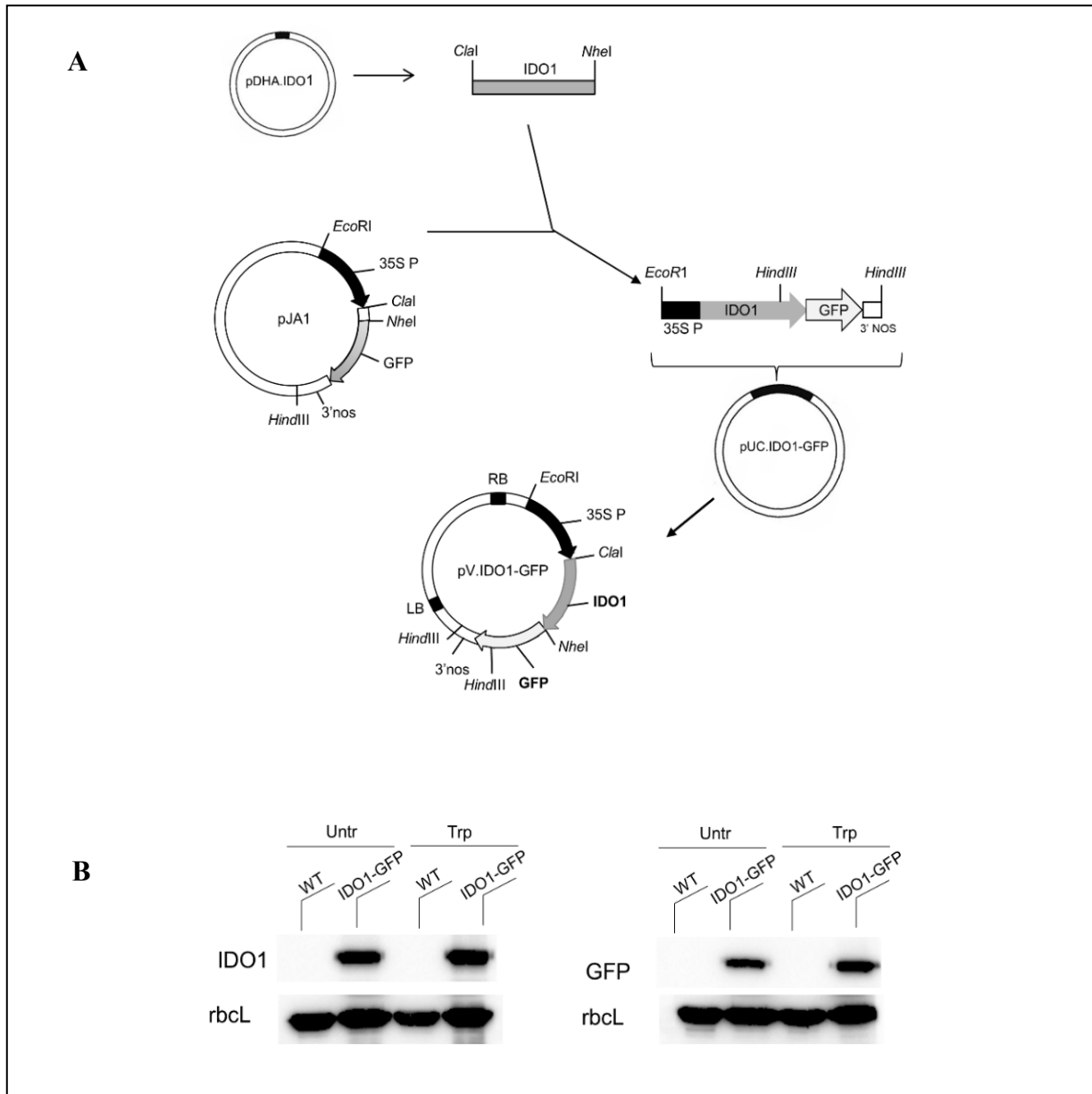


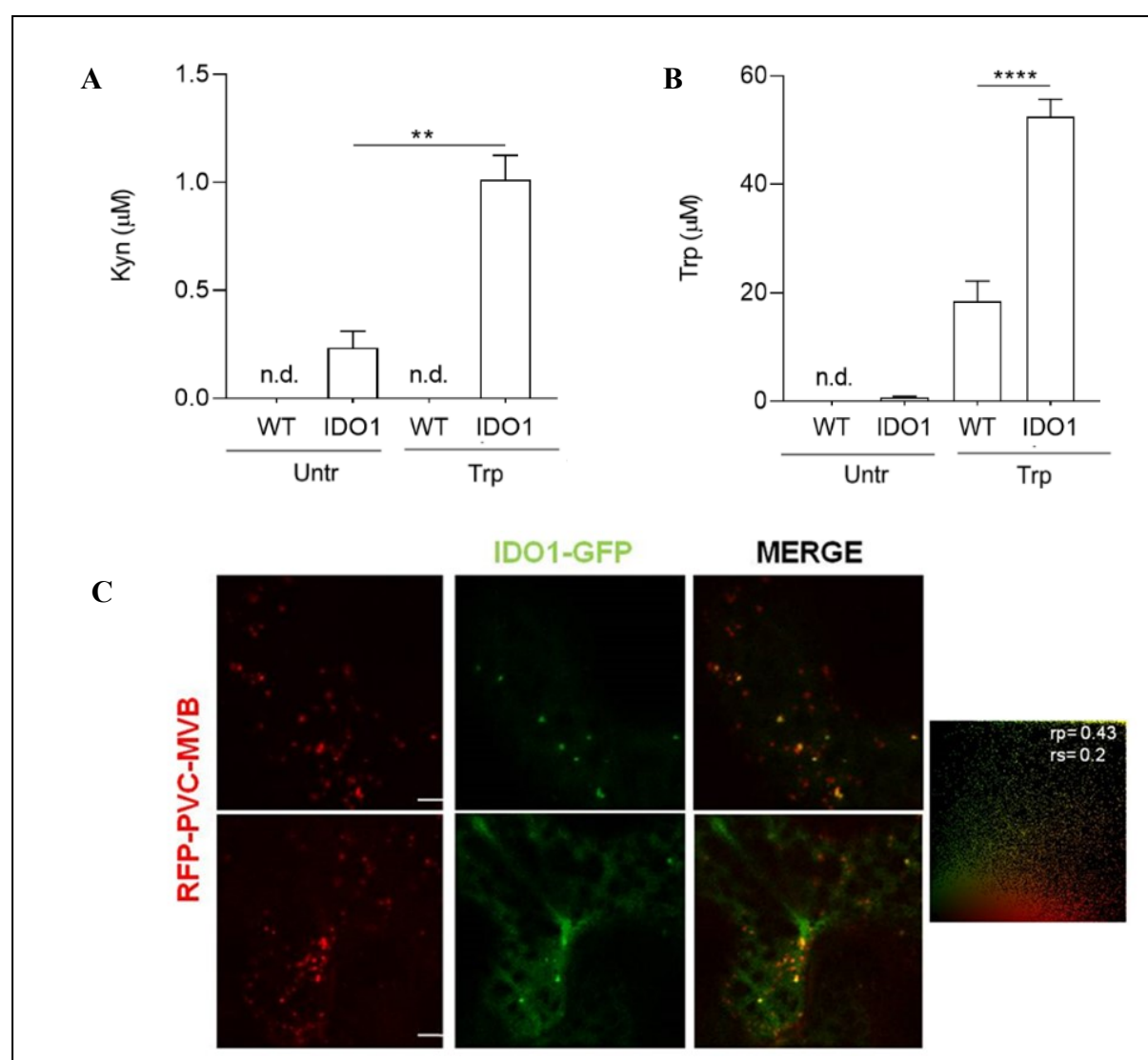
Figure 2. IDO1-GFP expression in protoplasts. **(A)** Schematic diagram of pV.IDO1-GFP construct cloning. Abbreviations are the same of **Fig 1A**. **(B)** Western blot analysis. IDO1 accumulation was analysed in whole cell lysates of protoplasts transfected (IDO1) or not (WT, wild type) with pV.IDO1-GFP vector by using both an IDO1 and a GFP specific antibody. An antibody against rubisco large subunit (rbcL) was used to verify protein normalization. After transformation, half of the protoplasts were incubated with tryptophan 100 μ M (Trp), and the other half remained untreated (Untr). One representative immunoblot analysis of three is shown.

system, we assembled the gene construct coding for IDO1-GFP under the same constitutive promoter previously used in pGreenII.IDO1, the 35S promoter (**Fig.2A**), and we transiently transfected tobacco protoplasts with the new vector pV.IDO1-GFP. By means of western blotting with two different antibodies detecting IDO1 protein and the GFP tag, respectively, we revealed the expression of a 69 kDa protein, matching the size of IDO1-GFP, in plants transfected with pV.IDO1-GFP (**Fig.2B**). In order to avoid possible impact of tryptophan depletion, due to the catalytic activity of IDO1, on cell vitality and protein expression, we added an extra amount of tryptophan to the protoplast medium. The additional tryptophan did not change IDO1 protein expression in protoplasts as shown in **Fig.2B**. As result, IDO1 can be expressed in vitro as a GFP-fused protein in transiently transformed protoplasts.

3.3 Transiently expressed IDO1-GFP has catalytic activity and similar subcellular localization of mammal IDO1

In mammal cells, IDO1 catalyzes the conversion of the essential amino acid tryptophan into kynurenine. Therefore, we investigated if IDO1-GFP transiently expressed in protoplasts had enzymatic activity. For this purpose, we measured kynurenine concentration in K3 medium after a 24 h incubation of protoplasts in medium alone or with extra tryptophan added to enhance and detect the metabolic product (**Fig.3A**). As a control, kynurenine was also measured in non-transformed protoplasts subjected to the same culture conditions. Human IDO1 expressed in protoplasts was found to be capable of producing kynurenine but this effect was strictly related to the enzyme presence. In fact, the WT control protoplasts showed undetectable production of kynurenine in comparison with the IDO1-transfected cells, both in presence and absence of an extra amount of tryptophan in the medium (**Fig.3A**). As an additional control, tobacco protoplasts were also transformed with the empty vector, and the kynurenine concentration measured in their K3 medium resulted undetectable, like what we found in WT protoplasts (data not shown). Moreover, the addition of tryptophan to the medium allowed the production of significantly higher level of kynurenine as compared to untreated IDO1-transfected cells. Unexpectedly, the final (i.e. after the 24 h incubation) tryptophan level measured in the medium with extra-added tryptophan (100 μ M) was significantly higher in the presence of transfected IDO1 cells than in the WT control (**Fig.3B**). As recently demonstrated, in mammal cells IDO1 is localized not only in the cytosol but also in early endosomes^[24]. To

investigate the intracellular localisation of human IDO1 in plant cells, we analyzed leaves of soil-grown tobacco plants transfected in vivo by *A. tumefaciens* cultures with both pV.IDO1-GFP and red fluorescent protein (RFP)-tagged organelle markers. Confocal analysis revealed that IDO1 was primarily found in a diffuse pattern consistent with cytoplasmic localization. However, a partial compartmentalization of IDO1 protein to prevacuolar compartments/multivesicular bodies (PVC/MVB) was also observed when co-expressed with a PVC/MVB marker (TR2RFP-VSR2) (**Fig.3C**). PVC/MVB are intermediate compartments mediating protein transport between trans-Golgi network (TGN) and vacuole, holding overlapping functions with mammalian early endosomes. Since plant organelles are not perinuclear as in mammalian cells, statistical analysis of co-localisation events could be performed to evaluate the relevance of the results.



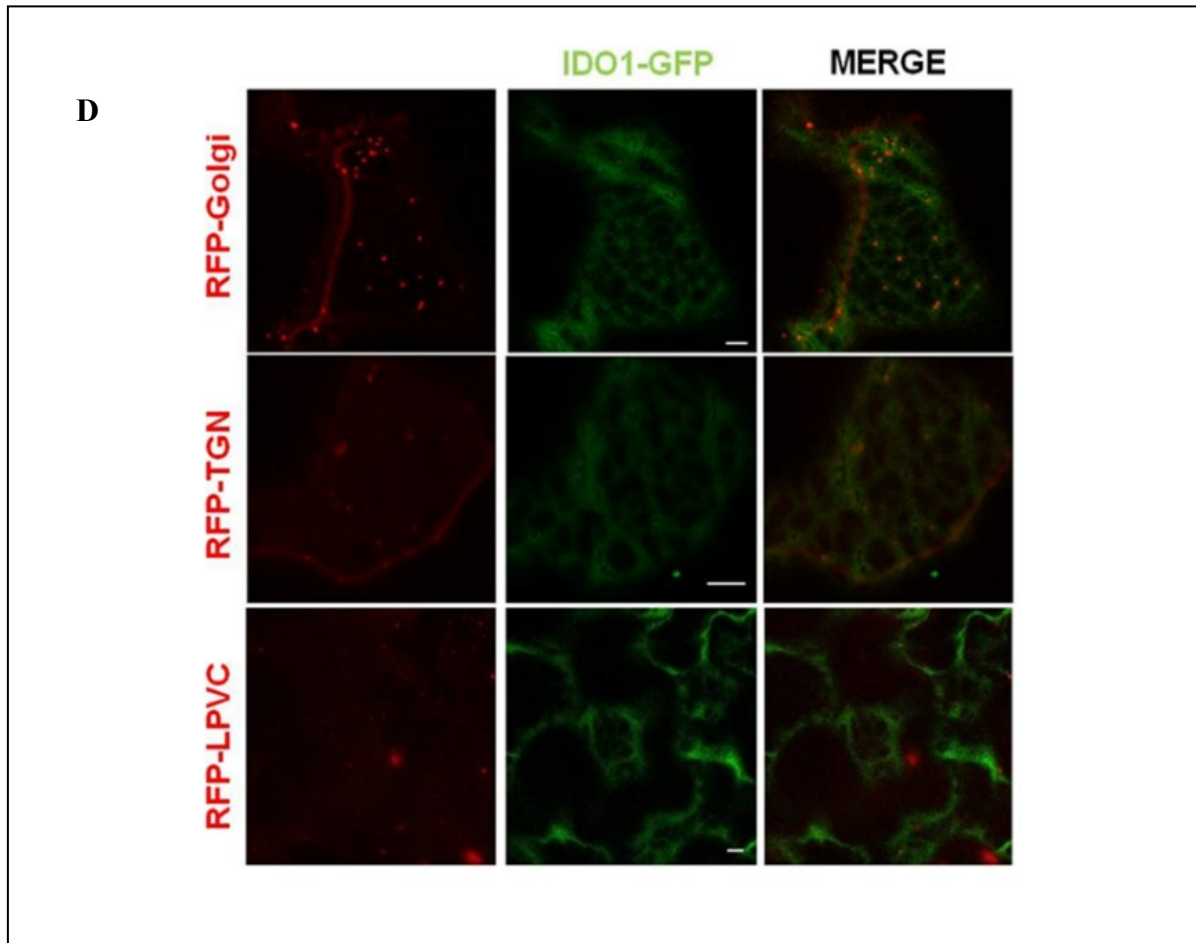


Figure 3. (A) Kynurenine (Kyn) and (B) tryptophan (Trp) concentrations were assessed in the medium of transformed (IDO1) or untransformed (WT, wild type) protoplasts maintained for 24 h in culture in the presence (Trp) or absence (Untr) of tryptophan excess (100 μ M). Concentrations below the detection limit are indicated as not detectable (n.d.). In (A,B), data are means \pm S.D. of three experiments, each performed in triplicate. Data were analysed by one-way ANOVA followed by Bonferroni's post hoc test. ** $p < 0.01$; **** $p < 0.0001$. (C,D) Confocal immunofluorescence microscopy images of *Agrobacterium*-infiltrated tobacco leaf cells coexpressing IDO1-GFP with various organelle markers carrying an RFP tag. The cells are shown in three-channel mode (green, red, and merge). Scale bar, 5 μ m. (C) A partial compartmentalisation of IDO1 protein (green channel) to the prevacuolar compartments/multivesicular bodies (PVC/MVB) marker (red channel) was observed in the merged image. The statistical colocalisation between IDO1 and PVC/MVB marker is shown, with Pearson's correlation coefficient (r_p) of 0.43 and Spearman correlation coefficient (r_s) of 0.2. (D) IDO1 did not seem to colocalise with cell compartments of the secretory pathway such as the Golgi, the trans-Golgi network (TGN), or the late prevacuolar compartment (LPVC).

As seen in **Fig.3C**, Pearson's correlation (r_p) and Spearman correlation (r_s) resulted in coefficients of 0.43 and 0.2, respectively. Since perfect co-localisation would result in statistical coefficients close to 1, our results confirmed the partial localisation of IDO1 at PVC/MVB membrane when co-expressed with TR2RFP-VSR2 (**Fig.3C**). On the contrary, IDO1 could not be found co-localising with other cell compartments of the secretory pathway

when co-expressed with markers of the late prevacuolar compartment (RFP-Rha1, LPVC marker), Golgi apparatus (ST-RFP marker), or trans-Golgi network (RFP-SYP61, TGN marker) (**Fig.3D**). As a whole, based on these data, GFP-tagged IDO1 can be transiently expressed in protoplasts in a catalytically active conformation and has a similar intracellular localization to the one found in mammalian cells.

4. Discussion

The production of biopharmaceuticals in plant has been named as ‘molecular farming’^[39,40]. Particularly, plants can be used to produce pharmaceutical proteins, such as replacement human proteins, antibodies and vaccine candidates^[3]. Several recombinant human proteins with therapeutic activity have been produced in plants, for example, the enzyme alpha-mannosidase is produced in stably transformed plants for the replacement therapy of the lysosomal storage disease alpha-mannosidosis. Another enzyme, named glucocerebrosidase, is produced in plant cell suspension cultures and commercialized^[13] for Gaucher’s disease treatment. Furthermore, plant cell cultures represent interesting options for an easy and scalable production of secondary metabolites, like the anticancer drug paclitaxel^[41]. Thus, in addition to whole plants grown in greenhouse, plant in vitro systems for generating metabolites and recombinant proteins are now considered an attractive alternative to classical technologies^[42,43]. Although the efficiency of plant platforms for biological components production is largely recognized, prokaryotic and mammalian expression systems are still mainly used in biotechnological processes because, provided with a strong regulatory framework, they guarantee robustness and economic sustainability for the industrial platform^[3]. Nevertheless, plant platforms might be attractive for the production of certain biopharmaceuticals with favorable glycan configurations, and for a rapid scale up process in the production of recombinant protein as well. Moreover, plants certainly do not allow the human pathogens growth^[44,45].

In the current report, a cloning strategy was set for the expression of a catalytically active form of human IDO1 in plant cells, with the aim of exploring an alternative platform for the kynurenine production. This molecule is an agonist of AhR, a crucial factor in the immune response regulation that exerts its roles through different immunoregulatory mechanisms. In fact, when activated, AhR is capable of inhibiting the production of several proinflammatory cytokines and of controlling the generation of regulatory T (Treg) cells and/or T helper 17

(Th17) cells in mouse models of auto-immune diseases^[46]. The finding of possible kynurenine participation in a positive-feedback loop in AhR signalling, pave the way to a probable use of kynurenine as an immunoregulatory drug.

Plant organisms naturally produce all amino acids including tryptophan, which not only result to be an essential component of protein synthesis, but also serves as a precursor for a wide range of indole-containing secondary metabolites that are important for plant growth, such as auxin^[47]. Therefore, the catalytic activity of recombinant human IDO1 in transformed plants could be exploited to produce kynurenine in an organism normally producing large quantities of its precursor. Apart from aryl-formamidase, plants do not possess orthologous genes coding for enzymes involved in the kynurenine pathway^[48]. Rice (*Oryza sativa*) seems to be an exception because genomic database searching has revealed the presence of several biosynthetic genes encoding core enzymes in kynurenine pathways, despite a lack of biochemical evidence^[48]. In particular, the sequence of an IDO1 gene (OsIDO) was identified, coding for a protein with a low identity (26%) and low similarity (42%) to *Mus musculus* IDO1. However, the enzymatic activity of OsIDO had not been demonstrated^[49]. Hence, during our study we generated a specific vector for efficient transfection of human IDO1 gene in plants. Although the genetic transformation had been effective and the transgene transcription did occur, transformed plants failed to express IDO1 protein. A possible explanation could be the instability and/or the rapid degradation of human IDO1 protein in a heterologous platform like the plant cell. Therefore, a different construct was cloned encoding for the fusion protein IDO1-GFP with the aim of improving the protein stability. In order to rapidly test the expression of this new gene construct, we transiently transfected protoplasts, plant cells with their cell wall removed and cultivated them in vitro in a liquid medium. Under these conditions, IDO1-GFP fusion protein was expressed, accumulated in the protoplasts and, most importantly, secreted kynurenine in the medium. Heterologous protein expression and kynurenine production in protoplasts were also assessed in the presence of an excess of tryptophan, to compensate the depletion caused by the IDO1 metabolizing activity that could interfere with the plant cell development, as recently demonstrated. In fact, according to He and colleagues^[50] kynurenine is an alternative substrate that can competitively inhibits the key enzymes involved in the conversion of tryptophan into indole-3-pyruvic acid in one of the auxin biosynthesis pathways. They indicated the structural similarity between kynurenine and tryptophan as the cause of kynurenine-mediated inhibition of auxin biosynthesis in roots of *Arabidopsis thaliana*

seedlings, when grown in a medium containing kynurenine. Interestingly, they found that the effect of kynurenine treatment could be suppressed by the presence in the medium of high doses of tryptophan. In our study, we observed that the extra tryptophan supplementation to the protoplast medium, rather than affecting the catalytic activity of IDO1-GFP, increased fourfold the production of kynurenine. These findings suggest that kynurenine production increases according to the availability of the substrate tryptophan in the medium, and IDO1 activity promotes an increased level of tryptophan in the medium by a still unidentified mechanism. The latter aspect is not less important for a nutraceutical application. In fact, tryptophan for humans is an essential amino acid necessary for the *in vivo* biosynthesis of proteins, as well as a precursor of several bioactive compounds. It is widely used as a dietary supplement for several perceived benefits, such as sleep and mood regulation^[51], and it is reported to mitigate the course of several chronic diseases, like Chorn's disease^[52]. Finally, in plants IDO1 keeps the same dual localization recently found in mammalian cells^[53]. In fact, when expressed *in vivo* in tobacco leaves via *Agrobacterium* infiltration and observed by confocal microscopy, the majority of IDO1-GFP are found in the cytoplasm, with partial colocalization with the early endosomes.

In summary, this study demonstrates that in plant cells IDO1-GFP shares the same enzymatic activity and subcellular distribution with native IDO1, paving the way to the possible use of plants as bioreactors to produce kynurenine. To the best of our knowledge, this is the first report on kynurenine production in plant cells by expressing human IDO1 protein. Further studies will be necessary to verify if a negative IDO1-mediated interference with plant metabolism may occur during plant growth.

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CHAPTER III

A biotechnological approach for the production of new protein bioplastics

Published paper*

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Abstract

The future of biomaterial production will leverage biotechnology based on the domestication of cells as biological factories. Plants, algae and bacteria can produce low-environmental impact biopolymers. Here, we have developed two strategies to produce a biopolymer derived from a bioengineered vacuolar storage protein of the common bean (phaseolin; PHSL). The cys-added PHSL* forms linear-structured biopolymers when expressed in the thylakoids of transplastomic tobacco leaves by exploiting the formation of inter-chain disulfide bridges. The same protein without signal peptide (Δ PHSL*) accumulates in *E. coli* inclusion bodies as high-molar-mass species polymers that can subsequently be oxidized to form disulfide crosslinking bridges in order to increase the stiffness of the biomaterial, a valid alternative to the use of chemical crosslinkers. The *E. coli* cells produced 300 times more engineered PHSL, measured as percentage of total soluble proteins, than transplastomic tobacco plants. Moreover, the thiol groups of cysteine allow the site-specific PEGylation of Δ PHSL*, which is a desirable functionality in the design of a protein-based drug carrier. In conclusion, Δ PHSL* expressed in *E. coli* has the potential to become an innovative biopolymer.

Keywords: disulfide bridges, *E. coli*, phaseolin, biopolymer, transplastomic plants

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Abbreviations

AFM	Atomic force microscope
DTT	Dithiothreitol
FFF	Field flow fractionation
FMM	Force modulation microscopy
FW	Fresh weight
HF5	Hollow-fiber flow field-flow fractionation
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
HRP	Horseradish peroxidase
IBs	Inclusion bodies
IPTG	Isopropylthio- β -D-galactopyranoside
MALS	Multi-angle light scattering
MSH	β mercaptoethanol
PBs	Protein bodies
PEG	Polyethylene glycol
PHSL	Phaseolin
PHSL*	Phaseolin (PHSL), with single cysteine residue inserted in the C-terminal tail
R _g	Radius of gyration
RT	Room temperature
TSP	Total soluble proteins
Δ PHSL*	Phaseolin (PHSL), with single cysteine residue inserted in the C-terminal tail lacking its own signal peptide

1. Introduction

The impact of petroleum-based materials on the biosphere is one of the greatest problems to be faced. To this end, the study of biodegradable polymers derived from bioreactors has been constantly increasing. Unfortunately, there are few biodegradable biopolymers produced to date that show processing and mechanical properties of interest for industrial purposes. Due to their high and easy processability, high stability against mechanical and thermal stresses and low cost, petroleum-based plastics have been widely used for a variety of applications, including biomedical uses, drug delivery^[1] systems and cosmetic personal care materials. However, it has been recognized that the widespread use of these synthetic plastics represents a huge environmental problem due to their very slow biodegradability^[2]. The strategy proposed by the research community to address the aforementioned issues concerns the replacing of petroleum-based plastics with environmentally friendly polymers such as polysaccharides, lipids and proteins. This approach has been widely considered for the development of innovative pharmaceutical and cosmetic formulations due to their great availability, high biocompatibility and biodegradability, and great ability to be functionalized^[3,4].

Plant polypeptides like zeins, gluten, phaseolin and soy proteins, are at the center of extensive research, since they can be easily processed into several kinds of materials, such as micro/nanoparticles, hydrogels, films, porous sponges and micro/nanofibers^[5,6]. However, the most well-known issues to overcome in their processing lie in their poor plasmatic half-life and poor mechanical properties, especially in physiological aqueous environments, where they tend to swell until complete dissolution^[4]. Strategies used to address these issues include protein PEGylation to increase the plasmatic half-life, or the use of cross-linking agents to increase stability and mechanical properties. It is well known that the chloroplast compartment could be used to increase production of heterologous proteins of pharmaceutical-industrial interest^[7,8] and biodegradable polymers in transgenic plants^[8]. Furthermore, the plastid compartment accomplishes some of the post-translational modifications of proteins, such as the correct formation of disulfide bonds^[9]. The formation of disulfide bridges within the chloroplast can occur both in the stroma and in the thylakoids, although it seems that the latter have a more suitable environment for carrying out this type of post-translational modification^[10]. Trying to devise a new plant protein to be used as a biodegradable biopolymer, we recently showed that a genetically modified phaseolin (PHSL), a vacuolar seed protein of *Phaseolus vulgaris*, with

a single cysteine residue inserted in the C-terminal tail (PHSL*)^[11], is able to form high-molecular weight species of tens of million Da, when expressed in tobacco chloroplast^[12]. These PHSL polypeptides are linked by inter-chain disulfide bridges, confirming the positive impact of the Cys modification on polymerization.

In this study, we want to compare two different biotechnological platforms for producing a biopolymer based on PHSL* polymerization. The first platform is represented by bacteria expressing a different version of the PHSL* protein, lacking its own signal peptide (Δ PHSL*)^[13,14], which in bean seeds targets PHSL in the endoplasmic reticulum. In fact, it is known that the presence of signal sequences can lead to a decrease in the quality and accumulation of recombinant proteins when expressed in *E. coli*^[15]. A second biotechnological platform consists of transplastomic tobacco plants, those previously obtained^[13] expressing PHSL* and the ones transformed here with the new plastid vector coding for Δ PHSL*. The plastidial Δ PHSL/PHSL*-based polymeric forms were biochemically characterised and the results showed that the absence of the signal peptide dramatically decreased the translation of Δ PHSL* mRNA and the accumulation of the corresponding protein. Conversely, the lack of signal peptide did not produce the same negative effects in bacteria where Δ PHSL* is expressed at higher levels than plastidial PHSL*. Moreover, Δ PHSL* in bacteria accumulated within easily purified protein bodies containing approximately 95% of the desired protein. The data reported here indicated that the bacterial self-crosslinking Δ PHSL* may represent a novel strategy for the production of biopolymers from natural proteins modified by genetic engineering.

2. Materials and Methods

2.1 Gene constructs and plant transformation

The *PHSL* cDNA gene, coding for phaseolin, was amplified from plasmid pDHA.T343F^[16], with 5'NdeI Δ P (5'-tcactttctgectc**acatat**gacttcactccgggag-3') and 3'NotIP* (5'-ccccctccggatc**cgggccg**ctagtacacaaatgcaccctttcttcct-3') oligonucleotides, in order to introduce the NdeI and NotI restriction sites (in bold) at the 5' and 3' ends of the gene, respectively. Furthermore, the 3'NotIP* primer has been designed in order to insert a cysteine residue (underlined) at the C terminus of the protein. After digestion with NdeI/NotI, the PCR product,

where the 72-bp PHSL signal sequence was deleted, was cloned into pCR2.1-5'UTR^[17], to obtain the pCR2.1-5'UTR- Δ PHSL* intermediate plasmid, in which PHSL is under the plastidial psbA promoter/5'UTR control. The psbA/5'UTR- Δ PHSL* cassette was excised from pCR2.1-5'UTR- Δ PHSL* by EcoRV/NotI digestion and subcloned into pLD-CTV^[18], generating the pLD-CTV- Δ PHSL* vector. Homoplasmic Δ PHSL* transplastomic plants were obtained by particle bombardment, as previously described^[13]. T0 transplastomic plants were grown in axenic conditions before being transferred to the greenhouse for seed production. T0 seeds were sown on agar-solidified MS medium with spectinomycin (500 mg/L) in order to obtain T1, and then T2 Δ PHSL* transplastomic plants. Regarding the PHSL* transplastomic tobacco plants used in this study, they were obtained as described^[12].

2.2 Isolation and analysis of nucleic acids

To confirm and verify the homoplasmic state of PHSL* and Δ PHSL* plants, total DNA was isolated from their leaves with the GenElute™ Plant Genomic DNA Miniprep Kit (Merck KGaA, Darmstadt, Germany) and subjected to Southern blotting assay. Total DNA (2 μ g) was digested overnight with BglII restriction enzyme and analyzed as described^[19], using both the *trnI/trnA* tobacco region and the *PHSL* cDNA gene as probes. For expression analysis, total RNA was extracted from PHSL* and Δ PHSL* plants with the Nucleo Spin_RNA Plant Kit (Fisher Scientific, Loughborough, UK), and 2 μ g was electrophoretically fractionated on a 1.4% formaldehyde agarose gel. Hybridization with the cDNA *PHSL* probe was performed as previously described^[19]. Polysomes of PHSL* and Δ PHSL* transplastomic plants were obtained from 300 mg of leaf tissue and analysed as previously reported^[14]. The cDNA of the PHSL gene was used as a probe.

2.3 Protein analysis of transplastomic tobacco plants

Total proteins were extracted from leaves of transplastomic tobacco plants expressing PHSL* or Δ PHSL* homogenized with homogenization buffer (200 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 100 mM Tris-Cl, pH 7.8) supplemented with complete protease inhibitor cocktail

(Roche), and analysed by SDS-PAGE and immunoblotting, as reported previously^[14] using antibodies against phaseolin (1:10000).

Pellet derived from the sucrose gradient of total proteins previously extracted ^[12] from transplastomic tobacco leaves expressing PSHL* was solubilized by homogenation buffer containing SDS. The homogenate was loaded on a linear 5% to 25% (w/v) sucrose gradient made in 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 50 mM Tris-Cl, pH 7.5. After centrifugation at 141,000×g for 24 h at 4°C in a Beckman SW28 rotor, fractions of 900 µL were collected and analysed by SDS-PAGE and protein blotting with antiphaseolin antibodies. To analyse the presence of PSHL* dimeric forms, fractions 5 containing the putative dimers was treated with loading buffer containing 0.1 M DTT. Pulse-chase analyses were performed on protoplasts derived from young tobacco leaves of plants expressing PSHL* and ΔPSHL* as described^[20]. Briefly, protoplasts were subjected to pulse-chase labelling with Pro-Mix (a mixture of [³⁵S]Met and [³⁵S]Cys; GE Healthcare Little Chalfont, Buckinghamshire, United Kingdom) for 1 h and chased for indicated time, and then homogenized using a homogenization buffer (150 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1.5 mM EDTA, 1.5% Triton X-100 and Complete protease inhibitor cocktail [Roche]). Total proteins were immunoselected using antiphaseolin rabbit polyclonal antibodies and analyzed by SDS-PAGE. Gels were treated with AmplifyTM fluorography reagent (GE Healthcare), dried and exposed for fluorography.

2.4 PHSL extraction from beans

For PHSL extraction and purification, *P. vulgaris* seeds (10 g) were stripped of their tegument and placed in a grinder to obtain a flour, then resuspended in PBS (1:8w/v, i.e. 10 g in 80 mL of PBS) stirring for 48 h at 4°C, as described^[12]. Briefly, after filtering through gauze pads and centrifuge at 10000×g for 30 min, pH was adjusted to 4.5 by slowly adding 1M acetic acid to induce protein precipitation. The solution was stirred for 30 min at 4°C and then centrifuged at 10000×g for 30 min. Pellet was collected and resuspended in PBS adjusting to neutral pH with NaOH 1N. Six hundred µl of the prepared sample were loaded on the top of a linear 5% to 25% sucrose velocity gradient made in 150 mM NaCl, 1mM EDTA, 0.1% Triton X-100, and 50 mM Tris-Cl, pH 7.5, and centrifuged at 141,000×g for 24 h at 4°C in a Beckman SW28 rotor. After centrifugation, the gradient was fractionated and three aliquots of the fractions with the major

PHSL content were mixed together and subjected to dialysis for 3 days, during which distilled water was replaced every 12 h, and finally freeze-dried.

2.5 PHSL* purification from tobacco leaves

Leaves from transplastomic tobacco plants, were grinded cold in a mortar with a homogenization buffer (200 mM NaCl, 1 mM EDTA, 100 mM Tris-Cl, pH 7.8) without detergents. The homogenate was filtered with a gauze to eliminate the debris, subsequently was centrifuged at 2500 \times g for 5 min at 4°C to recover the chloroplasts and eliminate most of leaf proteins. The recovered chloroplasts were then solubilized with the same buffer added with 2% of Triton X-100, which solubilizes the plastidial proteins and then loaded on a linear 5% to 25% (w/v) sucrose gradient made in 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 50 mM Tris-Cl, pH 7.5. After centrifugation at 39.000 \times g, for 8 h at 4°C in a Beckman SW40 rotor, the supernatant was discarded and the pellet containing PHSL* aggregates was recovered by a minimal amount of distilled water (1 mL). The resulting PHSL* aggregates were subjected to dialysis overnight to eliminate sugar and other contaminants. The purified sample was used for HF5-UV-FLD-MALS analysis, or lyophilized and analyzed under an optical or electron microscope. The scheme of PHSL* purification is reported in **Fig. S4**.

2.6 Phaseolin expression in *E. coli*

Δ PHSL and Δ PHSL* coding sequences were cloned between PmlI and HindIII sites of the pET-45b(+) vector by GenScript®. This vector allows expression of the proteins with a 6His tag at the N-terminus. Sequences were optimized for bacterial expression. Plasmids were transformed into competent BL21(DE3) *E. coli* cells. Bacteria were grown at 37°C in Luria Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.5) supplemented with ampicillin 100 μ g/ml (A100). Protein expression was induced when the culture reached an OD_{600nm} of 1.0 by adding 0.4 mM isopropylthio- β -D-galactopyranoside (IPTG) (Sigma-Aldrich). Bacterial pellets were resuspended in lysis buffer (Na/K phosphate buffer 50 mM, Triton X-100 0.5% (v/v), pH 7.4), sonicated three times on ice (Ultrasonic Cell Crusher 60 W, 30 sec and 1 min pause) and centrifuged at 4°C for 20 min at 14,000 \times g. Soluble fractions were

transferred into fresh tubes and pellets were solubilized in Na/K phosphate buffer (50 mM, urea 8 M, pH 7.4) when used for analytical purposes.

To perform experiments on recombinant PHSL expression between plants and bacteria, the bacterial pellet was resuspended in Na/K phosphate buffer 50 mM, urea 8 M, pH 7.4. The suspension was sonicated once (Ultrasonic Cell Crusher 60 W, 30 sec) and left rotating 1h at RT. Then, the sample was centrifuged for 20 min at 14,000×g at RT and the supernatant transferred into fresh tubes. For bulk production of Δ PHSL*, the bacterial pellets were disrupted by three repeated passages through the French pressur cell (Avastin, Emulsiflex B15, 10000-13000 Psi) and sonicated three times on ice (Ultrasonic Cell Crusher 120 W, 1 min and 1 min pause). After centrifugation, the insoluble fraction containing the recombinant protein was washed in lysis buffer, then in Na/K phosphate buffer (50 mM, pH 7.4) and finally in MQ water. The resulting pellet was stored at -80°C and then freeze-dried (Edwards freeze-dryer equipped with a vacuum pump Modulyo EF4).

2.7 Analysis of bacteria-produced recombinant PHSL

The presence, identity and integrity of the Δ PHSL and Δ PHSL* proteins were assessed by SDS-PAGE and western immunoblotting. Samples were separated on 10% (w/v) polyacrylamide gels. Proteins were visualized by gel staining with Coomassie brilliant blue R-250 or electroblotted onto nitrocellulose membranes and stained with anti-6×His tag polyclonal antibody (OriGene). Horseradish peroxidase (HRP) conjugated secondary antibody (BioRad) in combination with the enhanced chemiluminescence detection kit WesternBright ECL (Advansta) were used for detection. Images were acquired with a ChemiDoc MP Imaging System (BioRad).

2.8 Bacterial phaseolin PEGylation

For phaseolin PEGylation, the insoluble fraction of the cell lysate was directly suspended in 100 μ l of Tris-HCl 0.1 M pH 6.8, urea 8 M, EDTA 1 mM, supplemented with 5 kDa maleimide PEG (PEGmal₅₀₀₀, Sigma), a cysteine-reactive PEG reagent. The samples were incubated for 2

h at RT in the dark and the reaction was stopped by adding 0.1 M dithiothreitol (DTT). The PEGylation was confirmed by SDS-PAGE and western immunoblotting.

2.9 Preparation of the films by casting

The freeze-dried powders of *E. coli* Δ PHSL and Δ PHSL* were dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) at a concentration of 7% w/v. The dissolution was performed under magnetic stirring overnight. After that, the solution was cast in a mold and the solvent was allowed to evaporate overnight in order to obtain the films.

2.10 Atomic force microscopic analysis of biopolymers derived from bacteria

The XE-100 atomic force microscope (AFM; PARK Systems Inc.) was used in this study. The instrument was equipped with a 50- μ m scanner in the XY plane and a 12.5- μ m scanner in the Z direction, all controlled by the XEP 1.8.1 software. Scanners operated in a closed-loop manner and high voltage mode. The Z scanner resolution was set to 1.8 Å. The nanomechanical properties of the surfaces were acquired by setting the AFM in force modulation microscopy (FMM). For this purpose, a 36A cantilever (Micromasch) with 1 N/m typical force constant and a resonant frequency of 192 KHz was employed. Cantilever free amplitude was calibrated as described by PARK System. Topography, FMM amplitude, FMM phase signal and cantilever deflection were simultaneously acquired during 20 \times 20 μ m imaging at 0.2 Hz. The scanned area was randomly chosen for each film. AFM images were analyzed by XEI software.

2.11 HF5-UV-FLD-MALS analyses

Size separation and molar mass determination of putative biopolymers by HF5-UV-FLD-MALS were performed essentially as described^[12]. The conventional HF5 method is composed of four steps: focus, focus-injection, elution and elution-injection. During the focus step, the mobile phase is split into two different streams entering from the inlet and outlet; and during focus-injection, the flow settings remain unvaried and the sample is introduced into the channel

through the inlet to be focused on a narrow band. In this step, particles smaller than the cutoff are filtered away from the sample^[21]. In the elution step, the mobile phase enters the channel inlet and splits into a radial component out of the fiber's pores (cross-flow, V_x), and a longitudinal component that reaches the detectors (channel flow, V_c); lastly, during elution-injection, the flow is redirected into the injection, allowing for any remaining sample inside the channel and the system to be released. Due to the parabolic flow profile of the carrier flow, smaller particles experience higher average flow velocities than larger ones. In this normal elution mode, the analyte retention time is a function of its apparent diffusion coefficient. Hence, the analyte retention volume can be related to its diffusion coefficient, and consequently, to its hydrodynamic size using Stoke's equation and a calibration can be performed. Multi-angle light scattering (MALS) was used to measure the molar mass of eluted proteins. It allows for the absolute determination of particle radius of gyration (R_g), and, given the dn/dc and absorptivity values of the analysed species, the molar mass value of the eluted species^[22].

To characterize PHSL* plant extract, 30 μ l of a 0.3 mg/ml suspension were injected, while for the *E. coli* extract 1 ml of water was added to 0.1 mg of lyophilized Δ PHSL* protein and the resulting mix was sonicated for three cycles of 5 min. Then, the supernatant was transferred in a vial for injection in FFF.

3. Results

Homoplasmic T2 transplastomic plants expressing Δ PHSL* were obtained in this study using a biolistic transformation protocol^[13]. These plants have the Δ PHSL* gene inserted into the plastome, coding for a modified phaseolin without the signal peptide and contained a cysteine residue just before the vacuolar sorting signal sequence^[23]. Our hypothesis is that the absence/presence of the signal peptide may play a key role in the accumulation of Δ PHSL*/PHSL* in tobacco chloroplasts. Therefore, Δ PHSL* transplastomic plants were characterised and compared with the PHSL* transplastomic plants previously obtained^[12].

3.1 Generation of transplastomic tobacco plants expressing Δ PHSL* protein

Δ PHSL* gene was cloned in a plastid expression cassette based on the same vector, pLD-CtV, already used to clone the PHSL* gene (**Fig. 1A**). These vectors targeted the transgene cassette to the 16S-trnI/trnA-23S region of the chloroplast genome. After particle bombardment of tobacco leaves with the pLD-CtV- Δ PHSL* plasmid and two rounds of regeneration on spectinomycin-containing medium, several T0 transplastomic lines were obtained which were self-fertilized to the T2 progeny. To screen for transgene integration in the plastid DNA and investigate the integrity of the transgene cassette, Δ PHSL* transplastomic lines were analyzed by Southern blot to determine whether they were homoplasmic. The Southern analyses included also the PHSL* transplastomic plants, but these experiments, as all the further analyses in the text on the PHSL* transformants, were new because they were produced only for this study. Total leaf DNA was cut with BglII and probed with a *phaseolin* or a *trnI/trnA* probe, producing the expected fragments for transplastomic DNA (**Fig. 1B**). The *phaseolin* probe produced only a fragment of around 2.0 kb, while the *trnI/trnA* probe identified both the 2.0 kb and the 5.2 kb fragment in the transplastomic plants, and the WT 4.4 kb fragment. The homoplasmic state of the T0 transplastomic plants was demonstrated with a seed germination test on selective antibiotic, showing that all the progenies of these plants were resistant to spectinomycin, as expected for a maternally inherited plastid gene (**Fig. S1**). Northern blotting analyses were carried out in order to verify the correct transcription pattern expected for the transplastomic PHSL* and Δ PHSL* genes (**Fig. 1C-D**). Taken together, these analyses indicate that the insertion of the two exogenous genes within the tobacco plastome occurred correctly.

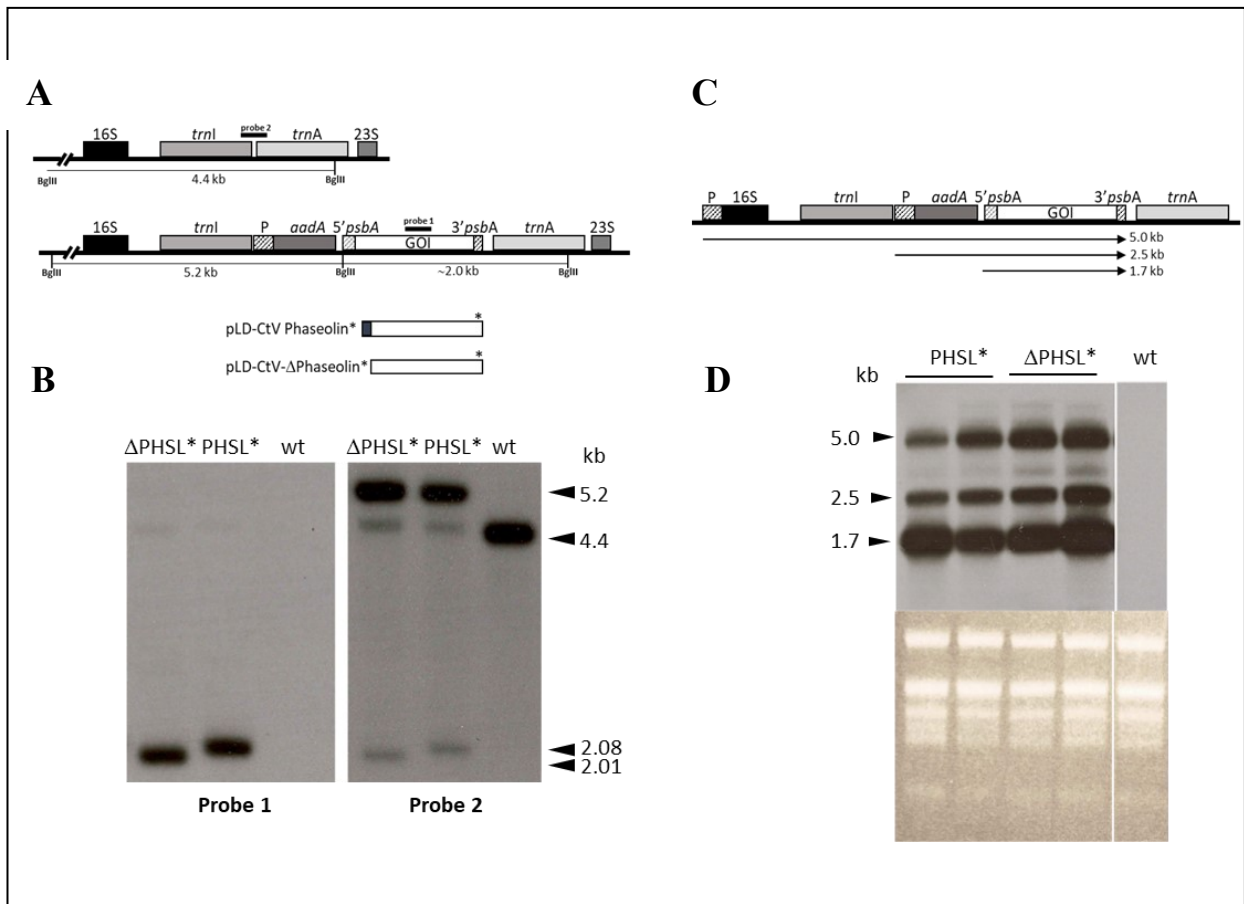


Figure 1. Northern and Southern blot analyses of tobacco transplastomic plants. **(A)** Schematic representation of the *16S/trnI/trnA/23S* region of the tobacco chloroplast genome and of the plasmid transformation vectors, the probes used for Southern blot analysis are indicated as probe1 (*phaseolin* gene) and probe2 (*trnI/trnA* region). The predicted hybridizing fragments, after digestion with BglII restriction enzyme and Southern blot analysis, are indicated in kb. **(B)** Southern blot analysis of PHSL* and ΔPHSL* transformants with probe1, *phaseolin* gene (left panel), and probe 2, *trnI/trnA* region (right panel), showing transgenes plastome integration. The probe 1 produced two slightly different fragments due to the absence of the DNA sequence coding for the signal peptide in the ΔPHSL* plant. The faint fragments around 4.5 kb in the transplastomic plants are likely promiscuous plastid DNA already present in the nucleus, as already reported^[13]. **(C)** Scheme of the transcription pattern expected for the transplastomic PHSL* and ΔPHSL* genes (GOI). Horizontal arrows represent expected transcripts and their sizes. **(D)** Northern blot analysis on total RNA extracted from PHSL*, ΔPHSL* and wild type (wt) plants. As a loading control, rRNA stained by ethidium bromide is shown in the lower panel. The wt sample is shown separately but belongs to the same agarose gel, which contained other four RNA samples close to the wt lane that have been not shown because not inherent to this study. Phaseolin gene was used as a probe. Numbers indicate molecular mass markers in kb. P, Prnn promoter; aadA, aminoglycoside 3'-adenylyl transferase gene; 5'psbA, psbA promoter/5'UTR; 3'psbA, psbA terminator; GOI, gene of interest.

3.2 Targeting to the thylakoid compartment increases the accumulation of PHSL* protein in transplastomic tobacco plants

The intraplastidial localization of heterologous proteins expressed in the tobacco chloroplast appears to have an important effect on their accumulation in the chloroplast. Therefore, we compared the level of accumulation of PHSL* with that of Δ PHSL*, which should have a different localization due to the absence of its own signal peptide^[13].

Total proteins extracted from T2 transplastomic plants expressing the two versions of mutated phaseolins were analyzed using SDS-PAGE and western blot with anti-PHSL antibodies. The signal intensity of the 46-kDa bands was quantified by densitometric analysis (data not shown), indicating that PHSL* accumulated almost 50 times more than Δ PHSL* (**Fig. 2A**). To verify the mechanism that leads to this discrepancy in accumulation rate, pulse-chase experiments were carried out that investigated both the correct synthesis of the exogenous protein and the translation of the foreign gene. Our experimental analyses showed that, while the PHSL* protein was detected at the first chase point and showed a half-life of approximately 4–6 h, the Δ PHSL* protein was undetectable, probably because it was present at levels below the experiment detection limit (**Fig. 2B**). The presence of comparable recombinant mRNA levels between PHSL* and Δ PHSL* plants (**Fig. 1D**) suggests that the lack of accumulation of the Δ PHSL* protein is due to changes in the translation of the mRNA. Thus, polysome analysis was performed in homogenates of transplastomic PHSL* and Δ PHSL* leaves by fractionation on a sucrose gradient, followed by northern blotting experiments with a probe that binds to *phaseolin* mRNA. Our data reveal that, while *PHSL** mRNA was predominantly associated with actively translating polysomes, mRNA from *Δ PHSL** samples was poorly translated, as the mRNA was largely detected in the first fractions of the gradient (**Fig. 2C**). Our experiment indicates a significant reduction in the mRNA translation activity of *Δ PHSL** mRNA compared to *PHSL** mRNA, confirming the existence of a self-regulating mechanism that manages the translation of exogenous mRNAs in the tobacco chloroplast^[14]. To confirm this hypothesis, localization experiments were performed on soluble and membrane fractions of chloroplasts expressing PHSL* and Δ PHSL* separated using SDS-PAGE and analyzed by western blot with anti-phaseolin antibodies (**Fig. S2A**). Our analyses indicate that both recombinant proteins are detected in the membrane fractions containing thylakoid structures. However, experiments to detach the proteins associated with the membranes by treating the thylakoids with salt (NaCl)

or reducing agents (DTT) show that, while PHSL* is integrated into the membrane fraction, Δ PHSL* is only associated with membranes and is effectively removed by both the salt and even more so by the DTT treatment (**Fig. S2B**). We can therefore conclude that a self-regulating mechanism senses the presence of Δ PHSL* protein because it is only associated with thylakoid membranes, whereas PHSL* escapes from this regulatory mechanism due to its integration into the membrane fraction (this study and^[14]).

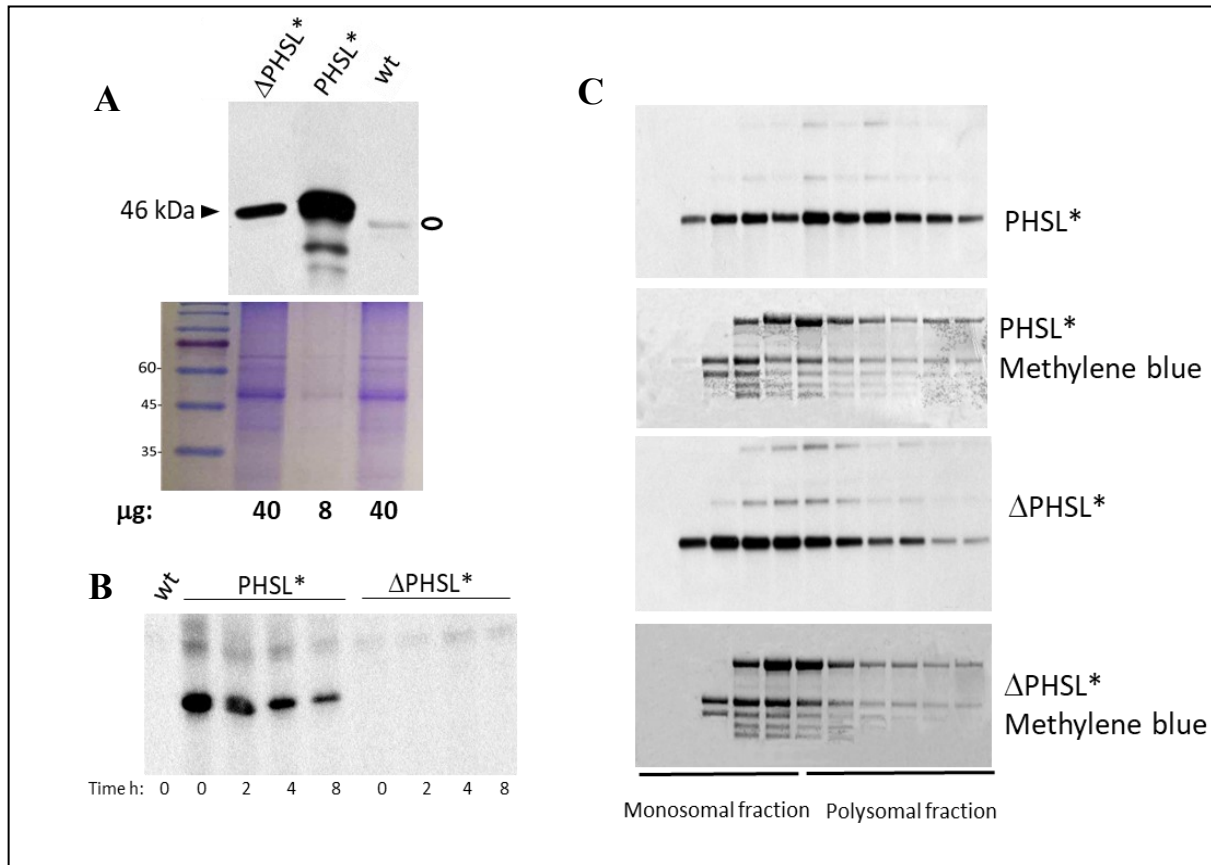


Figure 2. PHSL* and Δ PHSL* accumulation in tobacco chloroplasts. (A) Total proteins extracted from leaves of a WT plant or transplastomic plants expressing PHSL* or Δ PHSL* were separated by SDS PAGE and immunoblotted with antiphaseolin antiserum. The black arrowhead indicates the phaseolin polypeptide. Empty ellipse: contaminant peptide. Numbers on bottom indicate the amount of total proteins loaded in the gel, protein stained by Coomassie is shown as a loading control. (B) Protoplasts of transplastomic tobacco plants were pulse labeled for 1 h and tracked for the indicated times. The homogenized cells were immunoprecipitated with antiphaseolin antiserum and analyzed by SDS PAGE and fluorography. (C) Polysome analysis was performed from PHSL* or Δ PHSL* plant leaves through a 15% - 55% sucrose gradient fractionation. An equal proportion of RNA isolated from each fraction was analyzed by Northern blot using a phaseolin probe. The ribosomal RNA fractionation profile is visualized by methylene blue staining. Lanes 1-5 and lanes 6-1 represent monosomal and polysomal fractions, respectively^[14].

3.3 The plastid environment allows the formation of PHSL* disulfide bonds

Our aim was to produce a protein-based biopolymer and therefore, from here on out, we focused our studies on the transplastomic plants expressing PHSL*, the protein with the highest accumulation rate. The cysteine residue in the C-terminal region of PHSL* was able to trigger the formation of dimers when the protein is expressed at the nuclear level, but not more complex aggregation structures^[11]. These dimers are not present in bean seeds, where PHSL is a homotrimeric glycoprotein, with the three similar polypeptides linked mainly by hydrophobic interactions. Recently, it has been shown that the internal environment of genetically modified tobacco chloroplasts is able to support the formation of supramolecular complexes of PHSL* linked together by inter-chain disulfide bridges^[12]. Here, in order to further investigate the role of the inter-chain disulfide bonds on the aggregation of PHSL* expressed in the chloroplast, the purified supramolecular complexes were homogenated with SDS, which was capable of breaking down PHSL* trimers, but not the covalently-bonded disulfide bridges. The homogenate was analyzed using a velocity sucrose gradient in order to demonstrate the presence of both the PHSL* monomer (46 kDa) and dimers (90 kDa, **Fig. S3A** top). The molecular weights of the two forms are confirmed by the shift of the 90 kDa peak from the 4th to the 5th fraction (**Fig. S3A** bottom). To confirm the presence of disulfide bridges, fraction 5 of the gradient was treated with a strong reducing agent (DTT). The DTT-treated sample was compared with the untreated sample in western blotting experiments. This experiment shows the disappearance of the 90 kDa band in the presence of the reducing agent (DTT), confirming the formation of inter-chain disulfide bridges due to cysteine insertion in the C-terminal tail of the protein (**Fig. S3B**). We then wanted to verify the physical-mechanical structure of the PHSL* supramolecular complexes produced in the chloroplasts of tobacco leaves. The homogenate obtained from the chloroplasts was separated by ultracentrifugation, and the fraction that reaches the bottom of the sucrose gradient containing the PHSL* aggregates was recovered (**Fig. S4**). This material was lyophilized and subjected to macro and microscopic analysis (**Fig. S5**). The images obtained by optical and scanning electron microscope indicated that the structure in which these aggregates are organized was ordered both at macroscopic and microscopic level (**Fig. S5A** and **S5B**).

3.4 Δ PHSL and Δ PHSL* were expressed at a high level in *E. coli* cells and accumulated in inclusion bodies

After having exploited plant cells as an expression system, we next sought to evaluate the expression of mutated PHSL in bacterial cells in order to compare the two biotechnological platforms. Considering that in *E. coli* the signal peptide could negatively influence the folding of the protein itself^[24], we designed two constructs encoding for Δ PHSL* and its related control without *cys*, Δ PHSL. Both constructs were cloned into the pET45b(+) expression vector, which was then transformed in *E. coli* BL21(DE3) cells. These cells were grown at 37°C and expression of the recombinant proteins was induced using IPTG. SDS-PAGE analysis of the soluble and insoluble fractions derived from the lysates obtained from cells induced for different times, showed the appearance of a protein band with a molecular weight consistent with that of Δ PHSL* and Δ PHSL (about 46 kDa) (**Fig. 3A**).

The band was undetectable in non-induced cells (lanes 1 and 6 for Δ PHSL and 10 and 15 for Δ PHSL*). Both recombinant proteins accumulated in the insoluble fraction as inclusion bodies (IBs), where they represented the most abundant protein species (lanes 7–9 and 16–18). The highest level of protein expression was obtained at 2 h post induction (lanes 8 and 17) (**Fig. 3A**). Identity and integrity of the proteins was assessed by immunoblotting analysis using an antibody against the His tag, which selectively stained the 46 kDa protein band observed in the insoluble fraction; only a very faint signal was detected in the soluble fraction, confirming successful expression of Δ PHSL*/ Δ PHSL as IBs (**Fig. 3B**).

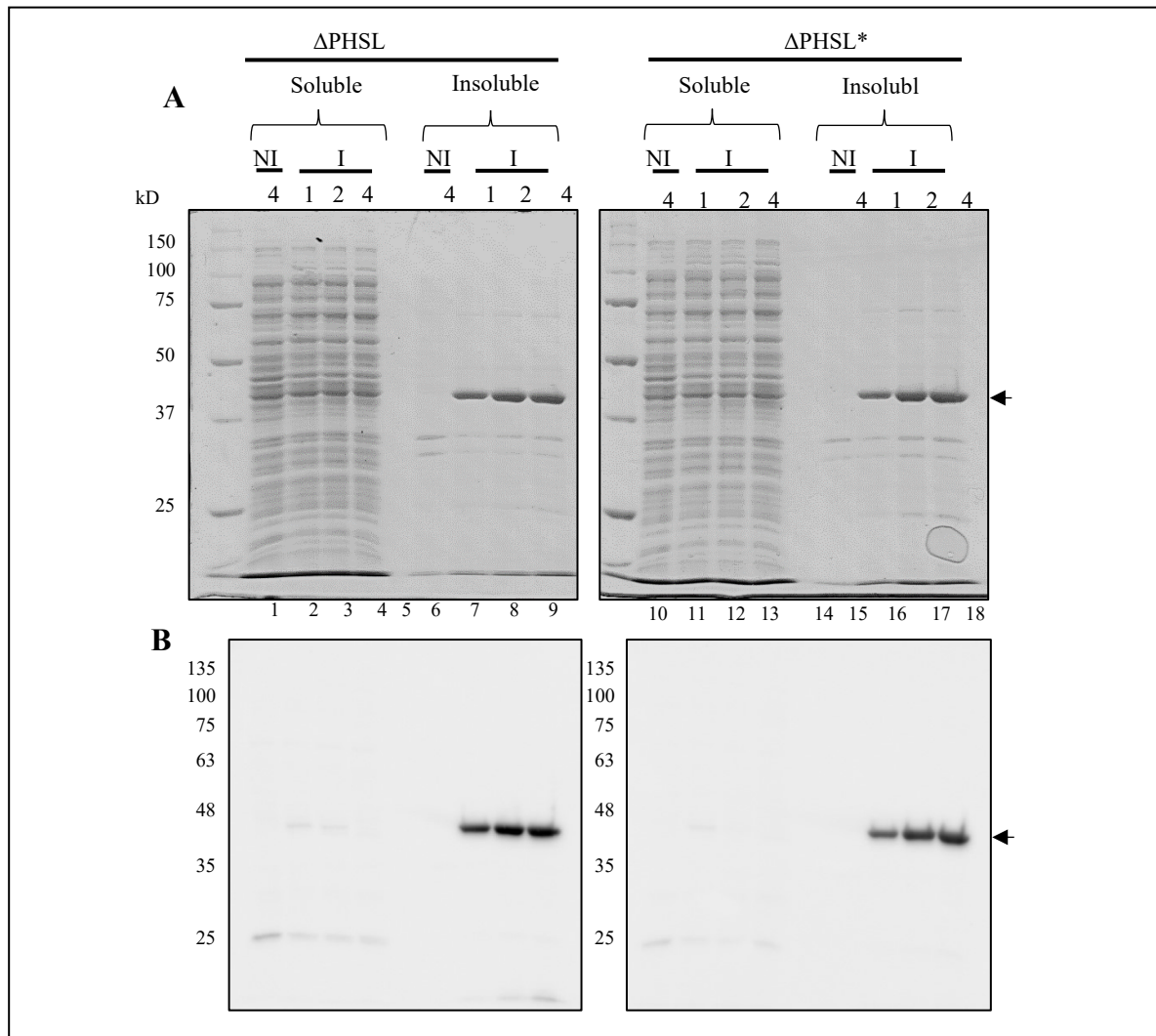


Figure 3. Expression of Δ PHSL and Δ PHSL* phaseolin in *E. coli*. (A) SDS PAGE and (B) western immunoblotting analyses of the soluble (lanes 1-4 and 10-13) and insoluble (lanes 6-9 and 15-18) fractions obtained from lysates deriving from non-induced (lanes 1, 6, 10, 15) and induced (lanes 2-4, 7-9, 11-13, 16-18) bacterial cells for different times (h). Ten μ g of soluble proteins and an equal volume of insoluble proteins were loaded on a 10 % v/v polyacrylamide gel. The gels were stained with Coomassie brilliant blue (A) or electroblotted and stained with an anti-His antibody (B). Molecular weight markers are shown on the left. Arrows at the right indicate the corresponding recombinant phaseolin protein.

3.5 PEGylation and crosslinking of Δ PHSL* in *E. coli*

Based on the above results, bulk protein production for biomaterial preparation involved harvesting the cells at 2 h post induction, complete cell lysis using a French pressure cell, sonication, separation of IBs by centrifugation, and extensive washing of IBs to remove

contaminant proteins. The final material was lyophilized, and an aliquot was analysed by SDS-PAGE under reducing and non-reducing conditions. As shown in **Fig.4A**, both proteins migrated at the expected molecular weight as a single band. Under non-reducing conditions, the electrophoretic mobility of Δ PHSL did not change, while a partial up-shift of the band was observed for Δ PHSL*, demonstrating that it is partially oxidized by forming disulfide-bonded higher molecular weight adducts through its cys residue.

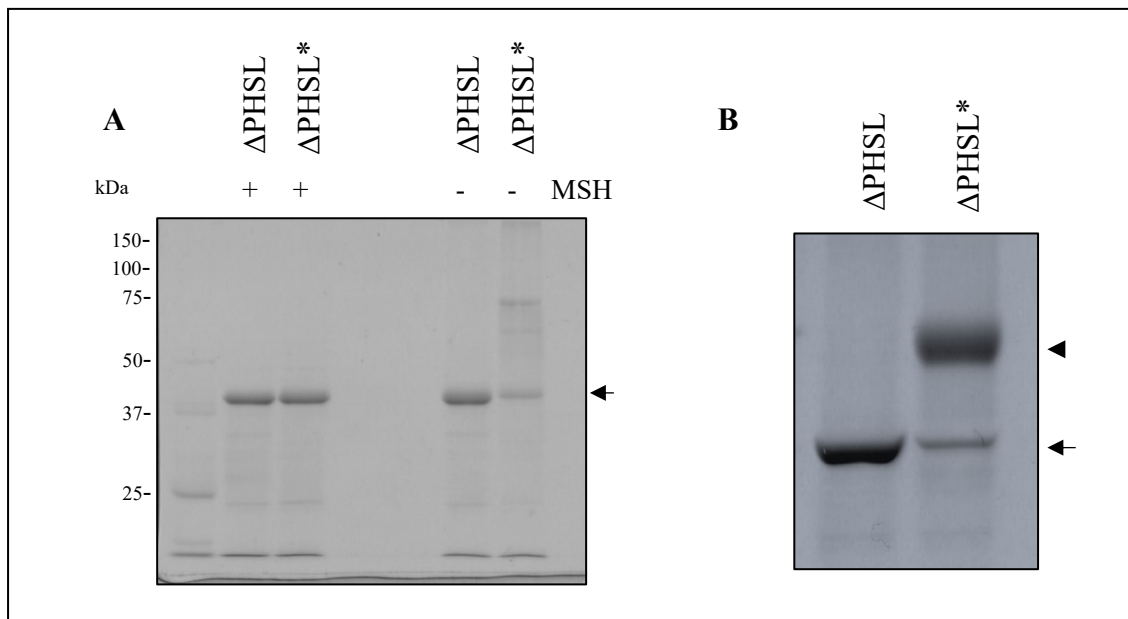


Figure 4. SDS PAGE analysis of Δ PHSL* cysteine oxidation and pegylation. **(A)** SDS PAGE of Δ PHSL and Δ PHSL* under reducing and non-reducing conditions. Five μ g of purified proteins were diluted in sample buffer supplemented or not with β mercaptoethanol (MSH) separated on 10% v/v polyacrylamide gel and stained with Coomassie brilliant blue. **(B)** SDS PAGE of recombinant phaseolin after PEGylation with mPEG 5000. Ten μ g of proteins were loaded on 8% v/v polyacrylamide gel and stained with Coomassie. Arrows at right indicate the corresponding recombinant phaseolin. Arrowhead indicates PEGylated Δ PHSL*.

To assess whether Δ PHSL* partial oxidation occurred inside cells or during IBs processing, the redox state of the cysteine thiol group of Δ PHSL* was evaluated by solubilizing IBs in UREA buffer containing maleimide PEG, a cysteine-reactive PEG reagent. It is known that mal-PEG alkylation of sulfhydryl results in an apparent molecular mass shift when observed by SDS-PAGE. Consistently, SDS-PAGE analysis of PEGylated proteins resulted in an up-shift of the protein band only in the case of Δ PHSL* (**Fig. 4B**), while no differences were observed for

Δ PHSL, which does not contain cys residues, demonstrating specificity of the PEGylation reaction. This evidence highlights that Δ PHSL* is not oxidized within bacterial cells, as expected due to the highly reducing environment of *E. coli*. Therefore, Δ PHSL* probably accumulates in IBs in a reduced form and the oxidation process begins when the protein comes into contact with atmospheric oxygen during the purification process, resulting in partial oxidation as observed in **Fig. 4A**.

In order to study the filmation process, both Δ PHSL and Δ PHSL* extracted from bacteria were dissolved into HFIP at the same concentration of 7% w/v. Polymeric films were obtained by casting, and letting the solvent evaporate. As shown in **Fig. 5**, smooth and transparent films were obtained with both proteins. In order to observe the differences at nano-mechanical level due to the presence of cysteine in Δ PHSL*, an atomic force microscope was used, which receives impulses from a punch that scans the surface of the two films. The mean cantilever amplitude collected in force modulation microscopy analyses (FMM) is sensitive to the local nanomechanical stiffness of the sample; in particular, it increases with increasing sample stiffness. The Δ PHSL surface showed prevalent green-blue regions, indicating cantilever amplitudes between 3.00 and 3.60 nm (**Fig. 5**). On the other hand, the Δ PHSL* surface showed widely diffused green-yellow-red regions, indicating cantilever amplitudes between 7.00 and 10.00 nm. The mean cantilever amplitude of the Δ PHSL* film was about two-fold higher than that of the Δ PHSL film, indicating a significantly higher nanomechanical stiffness of Δ PHSL* compared to the control. This could be attributed to the formation of disulfide crosslinking bridges between the thiol groups of the cys residues present only in Δ PHSL*, which occurred during HFIP evaporation by atmospheric oxidation. An increased stiffness of a biomaterial induced by crosslinking is a very common behavior that has also been observed by several authors for hydrogels present in the extracellular matrix^[25], as well as for polyelectrolyte multilayer films^[26]. Based on these results, we focused our next experiments on Δ PHSL*-expressing *E. coli*.

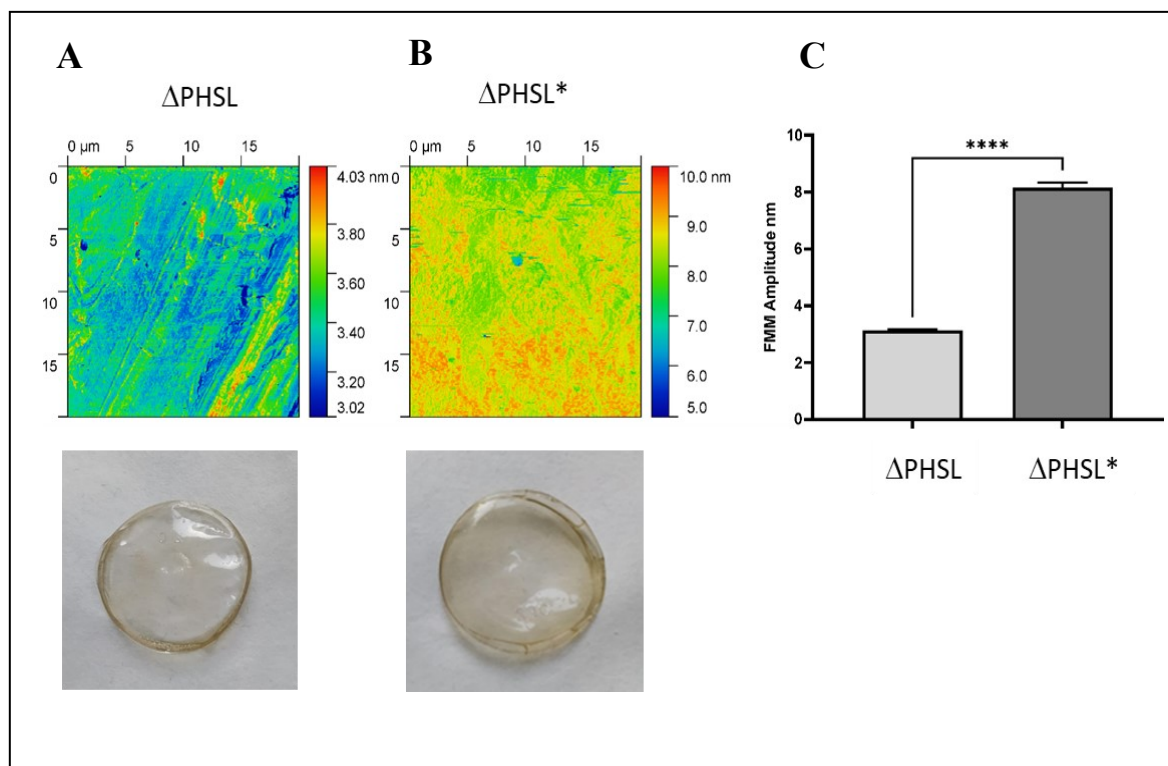


Figure 5. Comparison of the mechanical properties of Δ PHSL (A) and Δ PHSL* (B) expressed in *E. coli*. Representative images of FMM amplitude signal, expressed as nm in the bar graph, of the analyzed samples (above) and films obtained by casting and letting HFIP evaporate (below). FMM signals from all image pixels were collected and plotted as a graph (C). Amplitude signal of Δ PHSL* resulted higher than that of the Δ PHSL sample (Welch's test $p < 0.0001$) indicating an increase of surface stiffness.

3.6 Comparative analysis between the two bioreactors for the production of engineered phaseolin

In order to verify the aggregation states of engineered PHSL extracted from plants or bacteria, we analyzed the protein fraction containing PHSL* purified from transplastomic plants as described in Fig. S4 and the protein fraction containing Δ PHSL* purified from *E. coli* as described in Fig. S6. The analysis was performed via hollow-fiber flow field-flow fractionation (HF5) coupled to UV, fluorescence and multi-angle laser scattering detection. HF5 allows for the selective size-based separation of nano- and micro-sized particles, while smaller species are filtered out in the pre-separation step (see Materials and methods). Online detectors allow the evaluation of protein content and calculate the molar mass values for the eluted species. Moreover, through FFF theory it is possible to calibrate the method to predict the retention time

according to hydrodynamic size. In **Fig. 6**, the fluorescence signal (tuned to protein intrinsic fluorescence) was overlaid with the molar mass calculated by multi-angle light scattering. The method size calibration was also reported (top), and the dependability of the model was confirmed by the retention times of standard injections of proteins (albumin, immunoglobulin, shown as dashed line in **Fig. 6B**) congruent with the predicted ones. The analysis of the plant PHSL*protein fraction showed no evidence of protein species corresponding to PHSL* trimers and oligomers, which should be eluted at earlier retention times, while two protein populations are detected and separated (**Fig. 6A**). The first, peaking at 11 min and consisting of the majority (86%) of the sample, had an average molar mass of 1.2 MDa, while the second eluted at the field release, reached tens of millions of Da. The presence of these high-molar-mass species, together with the lack of PHSL* oligomers, suggested the successful formation of PHSL* polymers. The Δ PHSL* protein fraction from *E. coli* proved to be only partially soluble in water and PBS, suggesting that Δ PHSL* was present at a highly polymerized state. The soluble fraction was injected in FFF and showed to contain a small amount of a first aggregate population (averaging 700 kDa), while the majority of the sample was totally retained, and had a mass distribution reaching 10 MDa (**Fig. 6B**).

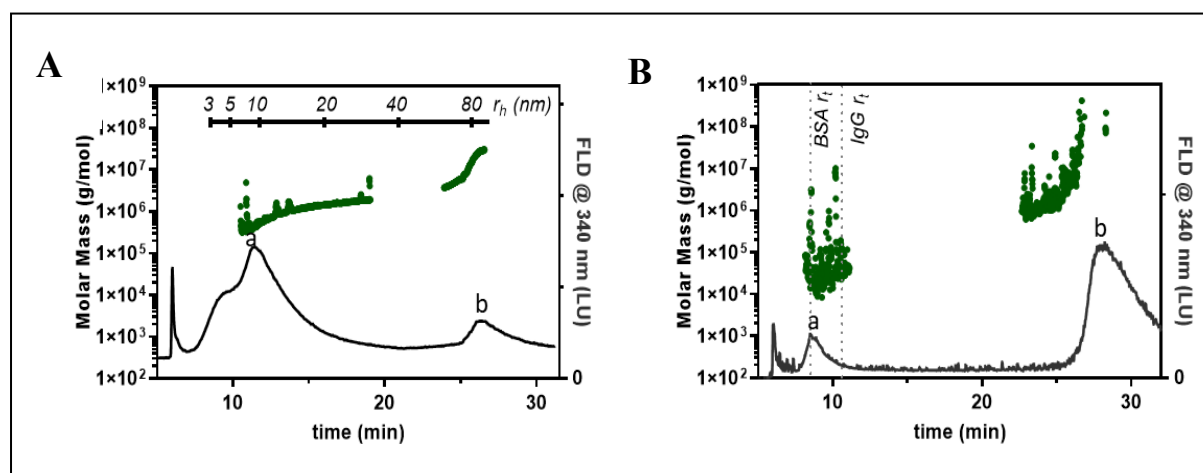


Figure 6. Separation and mass characterisation of PHSL* choroplast extracts (**A**) or Δ PHSL*-expressing *E. coli* extracts (**B**) by hollow fiber flow field flow fractionation with multi angle light scattering, fluorescence and UV detection (HF 5 MALS UV FLD). (**A**) Thylakoid extract. Average molar mass (Da) of the two eluted populations: a, 1.17×10^6 ($\pm 12\%$); b, 1.71×10^7 ($\pm 10\%$). Black line (top): size calibration of the devised method as from FFF theory. (**B**) *E. coli* extract. Average molar mass (Da) of the two eluted populations: a, 6.8×10^5 ($\pm 15\%$); b, 2.3×10^7 ($\pm 18\%$). Dashed lines: experimental retention time values of standard albumin (BSA) and Immunoglobulin G (IgG) injections to verify the reliability of FFF method calibration. Black line: fluorescence signal ($\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 340 \text{ nm}$). Green dots: molar mass values calculated from MALS and UV signals.

To quantify the phaseolin polymers, semi-quantitative western blots using anti-PHSL antibodies were attempted (**Fig. 7**). Western analysis was carried out in triplicate using *E. coli*/plant total soluble proteins, and purified PHSL as a standard, loaded in different amounts. To avoid saturated band signals, the amount of *E. coli* total proteins loaded on the gel was 100 times lower in comparison to the tobacco extracts. The amount of PHSL* polymers in tobacco leaves was first expressed as a percentage of total soluble proteins (TSP) and was roughly 0.03% of TSP, whereas the bacterial Δ PHSL* was expressed at 6.7% of TSP.

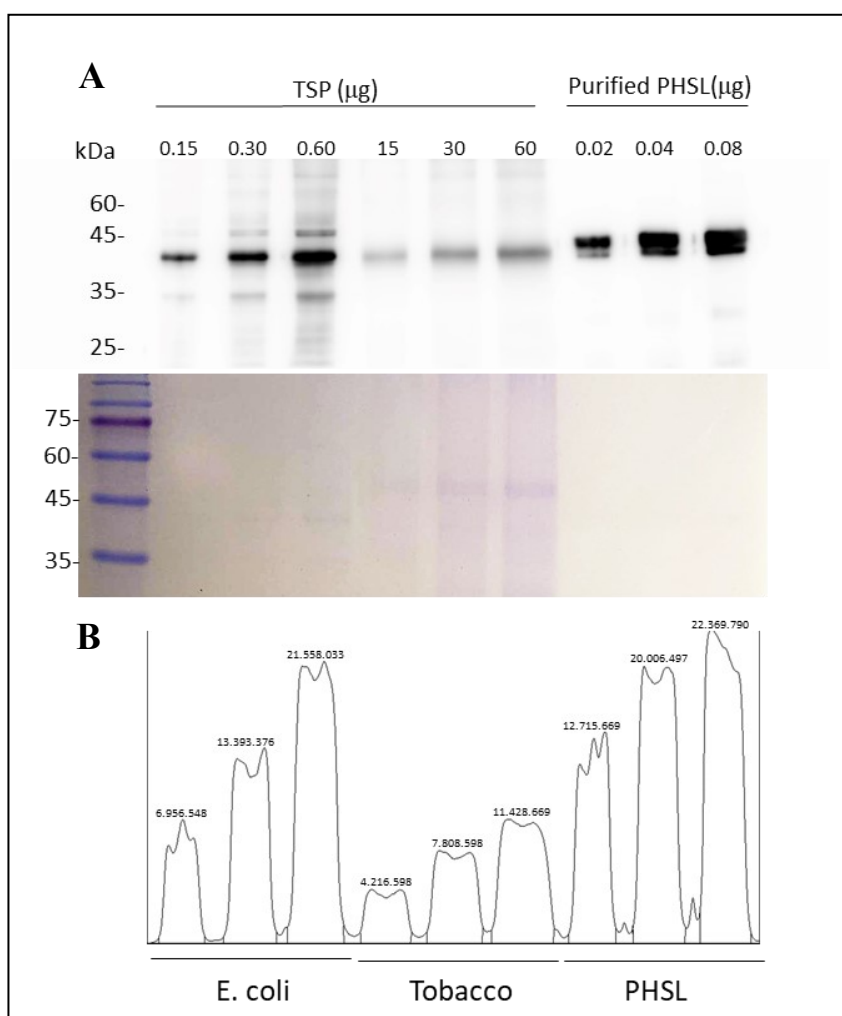


Figure 7. Comparison of engineered phaseolin accumulation in transplastomic tobacco and *E. coli*. (**A**) Total soluble proteins (TSP) extracted from leaves of PHSL* transplastomic plants or from transformed *E. coli* cells expressing Δ PHSL*, were separated by SDS PAGE and immunoblotted with antiphaseolin antiserum. This blot is an example of the three blots performed. Numbers on top indicate the amount of total proteins loaded in the gel and the amount of purified PHSL. Protein stained by Coomassie is shown as a loading control. (**B**) The peaks indicate in arbitrary units the measurement of the phaseolin specific bands in (**A**) by densitometric analysis (Image-J2 image processing software, version 2.9.0/1.53t). Numbers at the left indicate molecular mass in kDa.

There was a 300-fold increase of mutated PHSL polymers in bacterial TSP compared to the plant TSP. As it is not easy to compare the two biotechnological platforms (plants in greenhouse and bacterial liquid culture) for recombinant protein production, it was also determined that in tobacco plants PHSL* yield was 10 µg/g fresh weight (FW), while *E. coli* cells had a ΔPHSL* yield of 1000 µg /L.

4. Discussion

Proteins of high molecular weight (> 25 kDa) are natural heteropolymers, thereby being ideal raw materials for the production of biodegradable plastics alternative to petroleum-derived ones. From the technological point of view, some drawbacks which limit the use of proteins as biodegradable plastics lie in their fragility, low processability, and low mechanical properties^[27]. The blending with other polymers with suitable mechanical properties and/or the use of crosslinking agents are the most common strategies to overcome the aforementioned drawbacks. The aim of this study was to explore a new way to engineer proteins with functionalities targeted to obtain desirable chemical, processing and mechanical properties, so that they can be potential biodegradable alternatives to petroleum-derived polymers. To this end, a genetic modification of the bean storage protein PSHL has been deeply investigated. This protein has been previously modified incorporating a cysteine residue into its C-terminal amino acidic chain (PSHL*) and expressing it in the plant plastids^[12]. Here, we compared two bioplatfroms to produce PSHL* (or its version devoid of the signal peptide, ΔPSHL*), showing that in both plant and bacterial bioreactors engineered PHSL was present as high-molar-mass species polymers. However, if we consider the percentage of TSP, the *E. coli* cells produced 300 times more recombinant PHSL than transplastomic tobacco plants.

Concerning the tobacco plants, an accurate analysis of the intra-plastidial localization and accumulation of PSHL* and ΔPSHL* was performed. Using different molecular biology approaches, it has been shown that the accumulation of PSHL* increased almost 50-fold if the protein was inserted into the membranes of tobacco thylakoids instead that only associated with those membranes (ΔPSHL*). Moreover, it has been shown that the chloroplast environment has the chemical-physical and potential redox characteristics to form the PSHL* disulfide bridges, inducing the accumulation of megadalton-scale biopolymeric PSHL* forms. This

potentially is an excellent result, mainly because this approach could be used in plants of agricultural interest where chloroplast transformation is available, such as tomato^[28] that produce a lot of leaf biomass as waste. The insertion of the PHSL* gene at the level of the chloroplast in these species could increase the economic value of this biomass, from which it would be possible to purify a protein biopolymer usable at a pharmaceutical or industrial level. Unfortunately, the level of expression in PHSL*-expressing transplastomic tobacco plants is very low (0.03% of TSP) in comparison to the accumulation levels reported for other recombinant proteins with the transgenes localized in the plastid genome, that in some cases reached more than 50% of the leaf TSP^[29].

Therefore, we developed an alternative strategy that involves the use of bacterial organisms as bioreactors. Often, heterologous proteins expressed in bacteria (e.g. *E. coli*) are accumulated in PBs, and for this reason, are easy to extract and purify. In this study, we show that *E. coli* expressing Δ PHSL* accumulate this protein in IBs that can be purified by simple centrifugation. Δ PHSL* is present in a reduced form in IBs and is then partially oxidized by atmosphere during the purification process, resulting in a highly polymerized state up to 10 MDa. The presence of cys residues in Δ PHSL* is a functionality that can be exploited in the design of drug delivery systems and/or scaffolds for tissue regeneration. As demonstrated, the thiol groups of cysteine allow the site-specific PEGylation of Δ PHSL*, being a desirable functionality in the design of a protein-based drug carrier. Indeed, the site-specific protein PEGylation, often carried out to increase the protein half-life in blood streamlines, improves the production/purification processes and contributes to retain the pharmacokinetic benefits that accompany PEG attachment^[30]. Moreover, the nanomechanical stiffness of Δ PHSL* revealed that the thiol groups can be also exploited for the protein crosslinking via disulphide covalent bonds. This functionality represents a valid alternative to the use of chemical crosslinkers as glutaraldehyde (with recognized toxic effects), in the design of protein scaffolds for tissue engineering to improve their mechanical properties and to match the degradation rate with the tissue's regeneration one^[31].

In conclusion, our observations suggest that proteins that do not normally possess characteristics suitable for the production of plasticizing materials can be transformed into innovative materials, leading to the production and accumulation of potential useful next-generation products in the materials and pharmaceutical industry.

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SECTION II: PLANT PHYSIOLOGY

CHAPTER I

ERAD-mediated maturation of the regulatory protein of plant meristematic cells CLAVATA 3 emerged during evolution from algae to higher plants

Abstract

The indefinite growth capacity and organogenesis in plants are guaranteed by meristems, tissues highly regulated by intercellular signalling pathways, where cells are in continuous division and differentiation^[1]. In shoots apical meristem of *Arabidopsis thaliana*, a differentiation-promoting peptide obtained by the proteolytic maturation of the protein CLAVATA3 (CLV3) establishes an autoregulatory negative-feedback loop against the expression of cell-promoting transcription factor WUSCHEL^[2]. The shoot apical meristem remains alive for the entire plant life, maintaining a perfect balance between the continuous loss of daughter cells due to organogenesis^[3]. Nowadays, CLV3 is considered a secretory protein, matured by secreted proteases at the extracellular level into the active dodecapeptide form: this matured fragment behaves as a ligand of CLV1/CLV2 receptor complex^[4]. Recent studies in tobacco suggest that the protein's maturation process could not occur in the apoplast but probably through the intracellular ERAD (Endoplasmic Reticulum Associated Degradation) process, which is naturally used by cells to degrade unfolded proteins^[5]. To confirm this hypothesis, we studied the expression of the fusion protein in both *Nicotiana tabacum* plants and callus-cell suspension cultures by analyzing in a steady-state way the localisation of the fusion protein CLV3-GFP. To further investigate the protein maturation process, it has been studied the possible role of ubiquitination in CLV3 maturation process, as it is a key starting point in ERAD. A CLV3-YFP transgenic cell strain of *Chlamydomonas reinhardtii* was produced, to be able to follow the maturation process of this protein in an organism that doesn't show any meristem organization and CLV3 expression. The obtained results suggest a different destiny for CLV3: different genetic and biochemical strategies were carried out to shed light on this maturation process, which follows different ways in different biosystems.

Keywords: CLAVATA3, Plant meristems, ERAD process

Abbreviations

ADP	Adenosine diphosphate
APS	Ammonium persulfate
Arg	Arginine
ARRs	Type-A <i>Arabidopsis thaliana</i> response regulators
ATP	Adenosine triphosphate
BAM1	Barely any meristem 1
BiP	Binding Immunoglobulin Protein chaperone
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CDC48	Cell division control protein 48
CDS	Gene coding sequence
Ch	Chloroplast
CLE	(CLV)/Endosperm surrounding region (ESR)-related
CLV1	Clavata1
CLV2	Clavata2
CLV3	Clavata3
CLV3-GFP	Clavata3 fused to GFP
CLV3-YFP	Clavata3 fused to YFP
CRISPR	Clustered regularly interspaced short palindromic repeats
Cy	Cytoplasm
Cys	Cysteine
CZ	Central Zone
ECM	Extracellular matrix
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
GFP	Green Fluorescent Protein
GUS	β -glucuronidase
HL	High Light
HRP	Horseradish peroxidase
HSP90	Endoplasmic reticulum chaperone protein
Hyp	Hydroxyproline

IgG	Immunoglobulin G
K	Lysine abbreviation
KNAT1	Knotted-like from <i>Arabidopsis thaliana</i>
Leu	Leucine
LL	Low Light
Lys	Lysine
Met	Methionine
MS	Murashige and Skoog salt
OC	Organizing center
PBS	Phosphate-buffered saline solution
pCaMV35S	Cauliflower Mosaic Virus 35S promoter
pCLV3	CLV3 active dodecapeptide form
PM	Plasma membrane
PUB4	Plant U-box4
PZ	Peripheral zone
QZ	Quiescent center
RAM	Root apical meristem
RZ	Rib Zone
SAM	Shoot apical meristem
SDS	Sodium dodecyl sulfate
SP	Signal peptide
STM	Shoot meristemless
TAE	Tris-acetate-EDTA solution
TAP	Tris-acetate-phosphate medium
TCA	Trichloroacetic acid
TSP	Total soluble proteins
Ub	Ubiquitin
UPS	Ubiquitin-proteasome system
V	Vacuole
WOX	Wuschel related homeobox
WUS	Wuschel
YFP	Yellow fluorescent protein

Δ spCLV3

CLV3 protein without signal peptide

[Ara]pCLV3

Arabinose-linked to active dodecapeptide CLV3 form

1. Introduction

1.1 Plant meristems

Plants show a well-organized bipolar structure ensured by the presence of tissues, called meristems, that allow, following respectively phototropism and gravitropism response, the development of the aerial organs and the promotion of root growth. These tissues can be classified by origin, position, and division plane: first, meristems can be distinguished by the source in Pro-meristems, Primary meristems, and Secondary meristems. This work of thesis focuses on Primary meristems, the shoot apical meristem (SAM) and the root apical meristem (RAM), localised at the tip of the seedling: they show a highly organized structure, based on the presence of a niche of undifferentiated totipotent stem cells regulated by a complex network of biomolecular signals^[6]. A “good” meristem satisfies three main conditions: at first, it shows a specific site where indeterminate cells are located, and at the same time, it must self-regulate to not disappear or over-proliferate; from this main niche, cells differentiate and proliferate further away it is from the niche^[7]. Stem cells appear at the early stages of embryogenesis and are continuously produced and kept during the post-embryonic phase of life^[8]. The SAM of Angiosperm plants, located at the growing shoot tip of the plant, is organized into different layers (L1, L2, L3) and zones: the L1 and L2 layers form the *tunica*, while the *corpus*, formed by cells that divide in all planes, consists in the L3 layer. SAM can be also divided into three different domains: Central Zone (CZ), located in the apex, in which cells divide slowly into the next Peripheral Zone (PZ). These low rates of renovation are due to decrease the probability of mutations. In this area, cells divide more rapidly and are engaged by the organ primordia (leaves and flowers) on the flanks of the meristem, acquiring more specified fates and being incorporated into regions of stem between the organs. The upper region of the rib zone (RZ) hosts the organizing center (OC), a niche of cells that keeps the stem cell identity (**Fig.1**)^[9]. The RAM, just proximal to the root cap, shows a similar structure: the “core” of the meristem is represented by the initial cells: in the center of this area, cells slowly mitotically divide and form the Quiescent Centre (QC); this region is fundamental for the root development, behaving like an integrator for many processes and events for meristem establishment and maintenance^[10]. The key point of these tissues is the self-renewing capacity and the ongoing organ formation: a balance between the loss by differentiation and the replacement of stem cells is established, thanks to a complex molecular signalling pathway^[9].

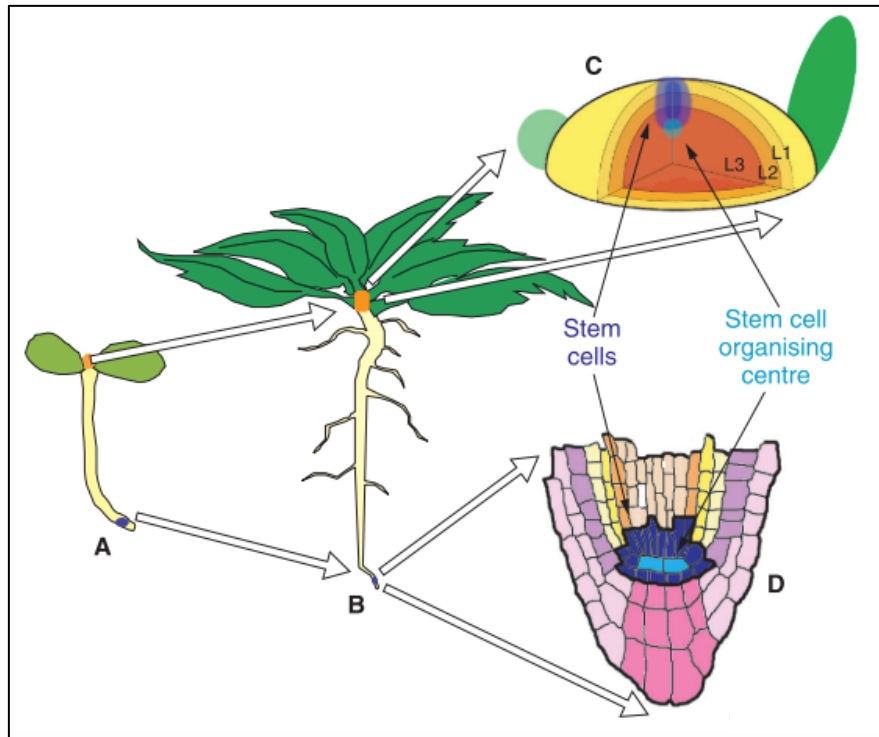


Figure 1. Structure and function of meristems: both are present at post-embryonic level; (A) the SAM generates all aerial tissues, (B) while the RAM is responsible for the primary root growth. (C) SAM meristems are organized into zones and layers already defined during embryogenesis and conserved along plant development. The organ primordia (for flowers and leaf formation) are placed at the flanks of the SAM. OC and (D)QC are niches of totipotent cells responsible for plant growth in both directions [7].

1.2 SAM maintenance signalling pathway

In pluricellular organisms, a strong coordination and differentiation process is required: the interaction between cells acts in “concert” for a correct plant development^[1,3]. Stem cell activity in meristems must be maintained for plant growth and organogenesis over the course of their lives. For hundreds or thousands of years in the case of some trees, the SAM is active for the entire duration of a plant's life. As a result, stem cell division must precisely balance the ongoing loss of daughter cells due to organ creation^[1,3]. The feedback mechanism created by this reciprocal regulation, which involves both positive and negative interactions, keeps the delicate balance needed for stem cell proliferation to occur at the appropriate time and location. In order to maintain appropriate meristematic activity and meristem organisation as well as to coordinate the creation of new organs, both meristems rely on cell-to-cell communication^[2]. In *Arabidopsis thaliana* a crucial regulatory feedback loop was identified in the SAM, between

WUSCHEL (WUS) and CLAVATA3 (CLV3), which is highly conserved across higher plants (Fig. 2)^[6]. WUS is a member of the WOX family of putative homeodomain transcription factors: it acts in a dose-dependent manner in a non-cell-autonomous way^[12], it is expressed in the OC (Layer L3) and moves across these cells or surrounding regions^[13]. WUS has a bi-functional role, repressing and activating the transcription of several proteins^[9].

The accumulation of WUS in the CZ induces the expression of *CLV3*, while lower expression levels of *WUS* repress *CLV3* transcription: this dynamic pathway is allowed by the presence of six cis-elements in *CLV3* locus, one in the 5' and five of them in the 3', that are bound by WUS with different affinities to carry out the regulation^[9]. *CLV1,2,3* genes encode, respectively, a leucine receptor-like protein kinase, a leucine-rich receptor, and a 96-amino acid long protein. *CLV3* induces the expression of type-A ARR genes, which causes a slowdown of cytokinin signalling^[14]. High cytokinin levels normally activate the phosphorylation of type-B ARR proteins and promote activation of type-A ARR genes, that turn off this network and are triggered by this group of hormones. The transcription factor WUS blocks the feedback loop, providing a cytokinin-regulated promotion of meristematic cells^[15].

Loss-of-function mutations on *CLV1* and *CLV3* genes cause the formation of enlarged and abnormal meristems, while *wus* plants show a premature termination of the SAM due to the impossibility of carrying out organogenesis and a consequent impossibility of the plant to develop^[16]. The overexpression of pCaMV35S: *CLV3* leads to plants with a *wus* phenotype and low tracks of *WUS* RNA are detected, suggesting an interdependent control of *WUS* by the protein *CLV3*.

The stem cells feature of meristematic niches in plants is guaranteed also by the expression *SHOOT MERISTEMLESS (STM)*, as shown by recessive *stm* mutants, which do not maintain the SAM and terminate seedling growth. The activity of the putative transcription factor STM is closely related to KNAT1: together, these two proteins help to maintain the indeterminate cellular state in SAM^[7].

clv mutants show an opposite phenotype to that of *stm* plant; *clv* mutants are characterised by the accumulation of stem cells and consequent enlarged meristems formation^[7]: *CLV3* activity consists in the restriction of stem cell fate to the CZ, but it also acts in a non-autonomous way limiting cell division in the Peripheral Zone^[17]. While the expression of *CLV* genes is focused on the CZ, *CLV1* is expressed in the *corpus*; we can mostly find *CLV3* in the *tunica* of this

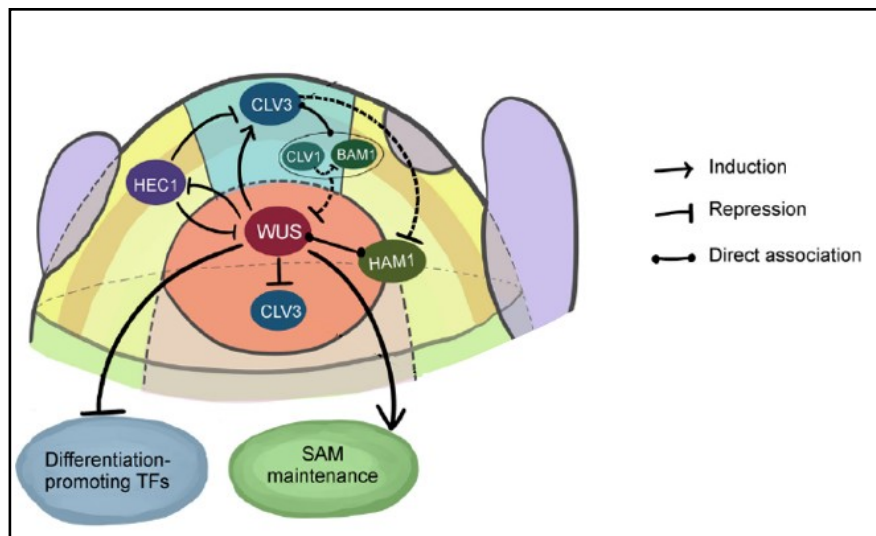


Figure 2. Schematic representation of CLV3-WUS feedback loop. The active form of CLV3 diffuses into the OC layers to link the CLV1 receptors. CLV1 induces a cascade response which leads to *BAM1* expression and *WUS* repression. *WUS* represses the expression of differentiation promoting transcription factors; at the same time, *WUS* moves to the CZ and promotes *CLV3* expression^[9].

area: this means that CLV3 acts as an apoplastic ligand that moves from the *tunica* to the *corpus*, against those cells expressing CLV1^[18].

During embryogenesis, *STM*, *CLV3*, *CLV1*, and *WUS* expressions are not strongly dependent on themselves, but this condition changes at the post-embryonic level. In *clv1wus* and *clv3wus* double mutants, the apex has a normal phenotype, suggesting that the major role of *CLV* genes is to regulate *WUS* expression negatively; on the contrary, the expression of *WUS* induces the expression of *CLV3* in initial cells. The relationship between *STM* and *CLV* is still unclear: the over-proliferation of CZ cells in *clv3* mutants and the premature differentiation of the apex in *stm* plants suggest that these two genes show an antagonistic role, establishing, continuously and dynamically, the balance for the correct development of seedling apex^[18].

1.3 CLE family protein group

For many years it was believed that in plants, the communication event between tissues and organs was entrusted only by phytohormones like auxin, cytokinin, gibberellins, brassinosteroids, jasmonic acid, abscisic acid, and ethylene, which act as a long-distance signal.

More recently, it was discovered that in plants exist, as in animals, peptides with essential roles in cell communication: in the SAM of *Arabidopsis thaliana*, CLAVATA3 (CLV)/ENDOSPERM SURROUNDING REGION (ESR)-related (CLE) showed a fundamental importance in meristem regulation^[11]. CLV3, the main character of this work, is the founding member of this family group: the *CLE* genes encode small polypeptides (< 15 kDa) generated by a proteolytic process of the full inactive form^[19]. *CLE* peptides show multiple functions in plant life cycle: their effect on plant growth and response depends on the different cell-to-cell communication systems^[20]. *CLE* genes show a strongly conserved structure: the *CLE* domain at the C-terminus, the functional part of the protein that will be proteolyzed, a signal peptide, and a variable domain at the N-terminus^[21]: the *CLE* domains were identified in several species like wheat and rice, *Selaginella maellendorfii*, *Medicago truncatula* and some species of nematodes^[20].

This domain consists of 12-14 amino acids: biochemical analyses have shown that the full length of 96-amino acids CLV3 is proteolytically processed to a 12/13-aminoacid peptide^[4], the cleavage occurs between Leu⁶⁹ and Arg⁷⁰ but a recognition domain at the N-terminus of *CLE* domain is required to generate the peptide (**Fig. 3A**)^[9]. Moreover, it was proved that the secreted peptide can assume two forms: the first one consists in 12-aa and it derives from the C-terminus conserved domain of CLV3 (pCLV3) of the *CLE* motif in which the first two prolines are hydroxylated to hydroxyproline^[4]. The second one is a 13-aa and undergoes post-translational modifications; a linear tri-arabinoide chain is linked to the 7th hydroxyproline residue of the peptide by O-glycosylation ([Ara]pCLV3)^[22]; it seems that these modifications are more important to increase the interaction against the receptor rather than the function or activity^[5]. The maturation of this protein leads to secreted signalling molecules in the apoplast: both peptides bind the CLV1/CLV2 receptor complex. *CLV1* encodes a kinase receptor complex with an extracellular domain composed of 21 tandem leucine-rich repeats, a single transmembrane domain, and an intracellular serine/threonine kinase domain^[23] (**Fig. 3B**): this protein form homodimers and is inserted to the plasma membrane (PM). The CLV1 receptor is the first protein that has indirectly demonstrated the homeostatic role of CLV3 in the SAM: it was observed in loss-of-function mutations for this gene, an enlargement vegetative and flower meristem, a phenotype identical to that one of *clv3*^[24]. Moreover, to test if CLV3 signalling requires *CLV1* and *CLV2* expression, mutants of *clv1* and *clv2* but overexpressing *CaMV35S::CLV3* were produced: those plants had shown a typical *clv* mutant phenotype,

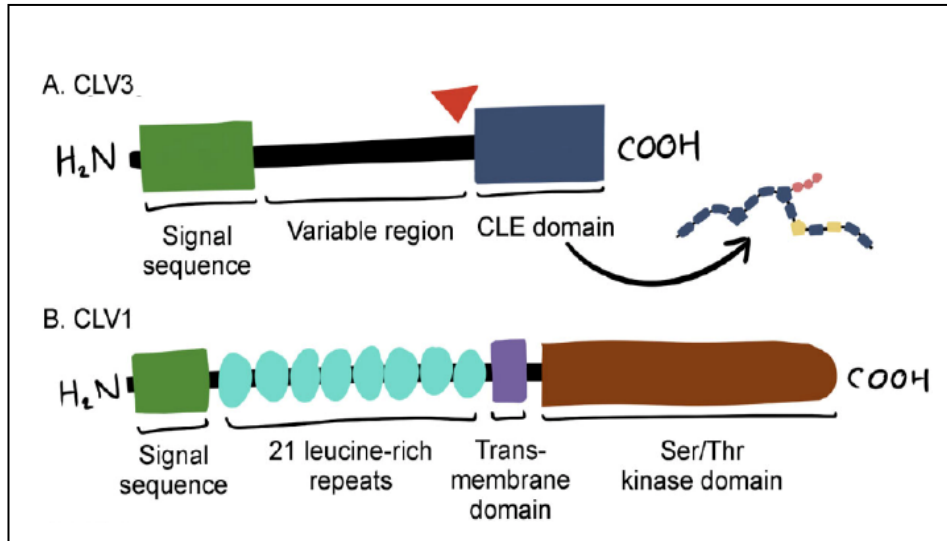


Figure 3. Main functional domains on CLV3 and CLV1 proteins. The pro-peptide CLV3 is cleaved at the N-terminus (red arrow) generating the active ligand ^[9].

demonstrating that CLV3 is not enough by itself to regulate SAM homeostasis^[2]. *clv2* mutants also show several defects in organs development, such as the pedicel (elongated in *clv2*), the anthers, and the valves, which show a reduced number. Genetic analysis has shown that *CLV2* works in the same pathway of meristem development as *CLV3/CLV1*; for this reason, it is believed that *CLV2* works as an additional component of the *CLV* genes signalling pathway and it is considered a heterodimer partner for CLV1 for the detection of extracellular signals^[25].

1.4 CLV3 localisation and maturation process

pCLV3 is one of the most studied plant peptide ligands: the expression of CLV3-GFP fusion protein under the control of its promoter showed a broad distribution rather than its mRNA around the OC by *in situ* hybridization^[26]. Together with the different localisation of the receptor complex CLV1/CLV2, expressed just beneath the *CLV3* expression domain, these evidences have demonstrated this peptide's non-autonomous function. It was proposed that the inactive protein CLV3 is secreted and processed in the apoplast, in the outermost meristem cell layers, and interacts with CLV1/CLV2 complex in deeper cell layers^[2]. In 2002 Rojo and collaborators^[23] showed interesting results about the secretory process of the protein CLV3: it is a soluble secreted protein, with a small hydrophobic region at the N-terminus, which localizes in the apoplast and activates the CLV pathway in the extracellular space. To test this hypothesis,

they produced a CLV3 fusion protein to GFP/GUS and a fusion Δ spCLV3 to GFP/GUS protein, which lacks the signal peptide (SP). It can be observed that, while the GUS/GFP expression is confined to the nucleus (**Fig. 4A**), the fusion protein Δ spCLV3-GUS/GFP is expressed in the cytoplasm (**Fig 4B, 4E**), while CLV3-GUS/GFP localizes in the apoplast between the cells (**Fig 4C, 4D**).

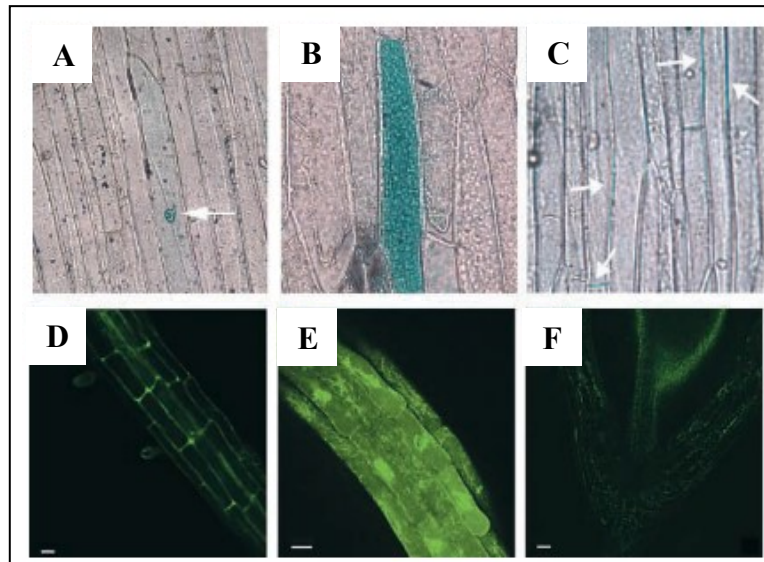


Figure 4. CLV3 is localized in the apoplast: (A-C) Phase-contrast optics used to detect GUS: Cells are transiently transformed expressing (A) GFP/GUS protein alone, (B) Δ spCLV3-GFP/GUS, (C) CLV3-GFP/GUS. (D-F) GFP fluorescence in (D) a root from a 35S::CLV3-G wild-type T2 transgenic plant, (E) a root from a 35S::CLV3 Δ sp-G wild-type T2 transgenic plant; (F) Autofluorescence in the cell wall of an untransformed wild-type hypocotyl. Scale Bars= 20 μ m in (D) and (E) and 50 μ m in (F) [23].

These results indicate that the first 18 amino acids of CLV3 are crucial for the delivery into the secretory pathway, which is blocked when SP is deleted^[23]. For further studies about the crucial role of the signal peptide, the De Marchis and collaborators^[5] transiently transfected tobacco protoplast with the same construct Δ spCLV3- GFP. Protein stability was analyzed by a pulse-chase analysis and fluorescent microscopy, proving a significant stability of the fusion protein inside the cell and demonstrating the requirement of a transient signal peptide for insertion into the ER^[5]. Rojo et al.^[23] demonstrate the activation of the CLV pathway in the apoplast by the protein CLV3, by redirecting the protein to the vacuole: in the conventional protein secretion,

proteins with or without SP are sent to the vacuole or the plasma membrane with an organized sorting mediated by vesicles^[27]. To know more about this process, different lines have been produced of *Arabidopsis thaliana* (cv. Col-0) *clv2-3* mutants expressing a vacuolar-secreted form of CLV3 fusion protein: the obtained results demonstrated that vacuolar-directed forms had reduced activity, not caused by a decrease in transgene expression, shown by mRNA expression levels analysis in transformed plants: this means there's a strong correlation between CLV3 secretion and its activity, crucial for a proper growth and development of the plant^[23]. While many studies discuss the secretory process's involvement in CLV3 maturation, more information is needed about protein processing. As previously mentioned, the CLE domain is proteolytically separated from the 96-aminoacid inactive form, allowing the formation of pCLV3 dodecapeptide. Studies conducted in *Escherichia coli* showed that a serine-secreted protease can process the N-terminus, while C-terminal processing may involve a carboxypeptidase. Moreover, CLV3 protein was overexpressed in *E. coli* and added to the culture media/extract of plant cells to understand if the maturation process occurs in the intra or extracellular compartment and in which way^[28]. However, it is hard to find *in vivo* studies of the maturation process of CLV3: the study of this event is complicated by the absence of antibodies against CLV3 or other CLE polypeptides. To understand more about the maturation process of CLV3, De Marchis and collaborators^[5], in 2018, produced transient lines of tobacco protoplasts: the first population was transfected for CLV3-GFP fusion protein and for GFP secretory protein form, this strategy would help to understand if, after the SP removal in the ER, CLV3 undergoes further processing before reaching the extracellular space. A pulse-chase analysis was performed on transfected protoplasts and revealed a rapid disappearance of the full-length protein at intracellular level. Protoplasts were incubated overnight in their K3 culture medium and pulse-labelled with [³⁵S] Met and [³⁵S] Cys: the chase was performed by adding unlabeled Met and Cys. While the GFP secretory form was found in the cytoplasm and was secreted in the medium, the fusion protein CLV3-GFP was detectable in the cells at very early stages, but no traces of CLV3 were found in the extracellular space (**Fig. 5**).

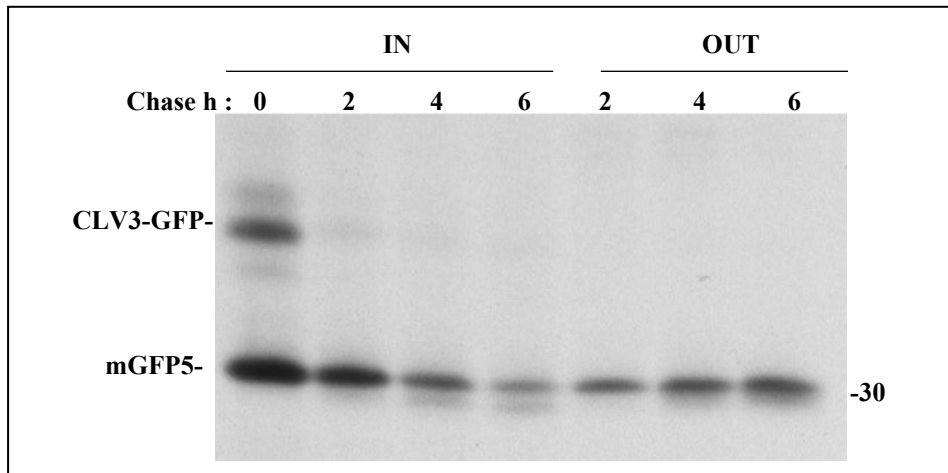


Figure 5. Tobacco leaf protoplast transiently transfected expressing CLV3-GFP and mGFP5 proteins. Protoplast homogenation of intracellular and extracellular protein content, after pulse labelling for 1 h were analyzed by SDS-Page and immunoselected with anti-GFP serum^[5].

Moreover, the same group highlighted that treatments that affect the canonical secretory pathway don't show any effect on the CLV3 maturation process: for example, Wortmannin, which inhibits the receptor-mediated protein sorting from Golgi complex to the vacuoles, didn't affect CLV3 retention. Brefeldin A treatments, responsible for the deregulated fusion of Golgi cisternae with the ER, which means a blocking of secretion and vacuolar sorting, didn't affect the protein maturation process. The pulse-chase analysis conducted on this treated protoplast population showed no change in CLV3 retention or secretion. These results suggest that, after insertion into the endoplasmic reticulum and removal of the signal peptide, CLV3 is further processed before it reaches the apoplast.

1.5 ERAD process is involved in CLV3 maturation

Endoplasmic Reticulum Associated Protein Degradation (ERAD) is a process commonly used by cells to eliminate misfolded or unfolded proteins from the ER. Many chaperones, oxidoreductase, and glycan chain modifying enzymes are in continuous and dynamic work to allow the proper folding of newly synthesized proteins^[29]. A significant fraction of them shows errors in their synthesis or folding processes^[30]: this could be due to several intra or extracellular stresses, like heat, oxidation, or heavy metal ions concentration, or to a perturbation of the homeostatic balance^[29]. To overcome this problem, cells have evolved a quality control system

in the ER that is able to select ERAD targets and send them to the ubiquitin-proteasome system (UPS). Thanks to this mechanism, defective proteins are not released into the secretory transport: the absence of ubiquitin-ligase components in the ER suggests that ERAD targets are retro-translocated to the cytoplasm, a process termed “retrograde transport”^[31]. ERAD process can be summarized in four main steps: first, ERAD targets are recognized by lectins and chaperons and must be sent to the cytosol^[32]. This retrograde transport happens thanks to an ATP-consuming complex that act to send the ERAD target to the proteasome: the AAAtype-ATPase Cdc48 binds the ERAD target and undergoes several conformational changes that allow, together with the hydrolysis of ATP molecules to ADP, the retro-translocation event^[29]. The substrates are then ubiquitinated by several steps; the E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzymes), and E3 (ubiquitin E3 ligases) are essential for the correct ubiquitination event. The ubiquitinated substrate is finally sent to the proteasome 26S, which acts the degradation^[32]. According to previous results, the De Marchis et al.^[5] proposed in 2018 that the ERAD mechanism is possibly responsible for the intracellular maturation process of our protein of interest. According to this model, CLV3 would be proteolyzed by the proteasome at the intracellular level, and the active dodecapeptide would then be secreted in the extracellular space. Bioinformatic predictions showed that the most probable cleavage sites of CLV3-GFP by the 26S Proteasome (Pcleavage: <http://www.imtech.res.in/raghava/pcleavage/>) could form exactly the active dodecapeptide. Furthermore, the same group performed pulse-chase analysis and fluorescence microscopy imaging of transfected protoplasts after MG132 treatment, an inhibitor of proteasome activity, showing an extension of the retention period of the protein at intracellular level (**Fig. 6**)^[5].

CLV3-GFP protoplast lines were transiently transfected with the wild-type form of Cdc48 (CDC48WT) or the mutated form (CDC48QQ), the ATP-dependent complex that allows the retro-translocation event to the cytosol. After 12 hours, protoplasts were pulse-labelled, homogenized again, and analyzed by immunoprecipitation. The amount of CLV3-GFP increased by about 25% in those protoplasts transfected with CDC48QQ, rather than those expressing the wild-type form: the rapid disappearance of the intact precursor, unaffected by inhibitors of secretory traffic but protracted by the inhibitors of the proteasome are interesting features of ER-associated degradation. As described above, CLV3 active peptide is further modified before reaching the extracellular space: the first two-three Proline residues are

hydroxylated and an O-glycosylation with three arabinose residues could further modify the 2nd Hyp.

While the post-translational hydroxylation occurs in the ER lumen, supporting the idea that CLV3 could be an ERAD substrate, the O-glycosylation should assume a previous step in the Golgi apparatus before reaching the proteasome: in this way, the CLV3 substrate would go back into the secretory pathway and it would be then processed by the proteasome^[5]. In fact, in mammalian cells, the N- and O-glycosylation are linked from cis to trans-Golgi cisternae: while N-glycosylation is conserved across animals and plants, little information is known about O-glycosylation conservation across plant species. However, responsible for O-glycosylation was also found in plant cell compartments involved in unconventional protein secretory pathways^[33].

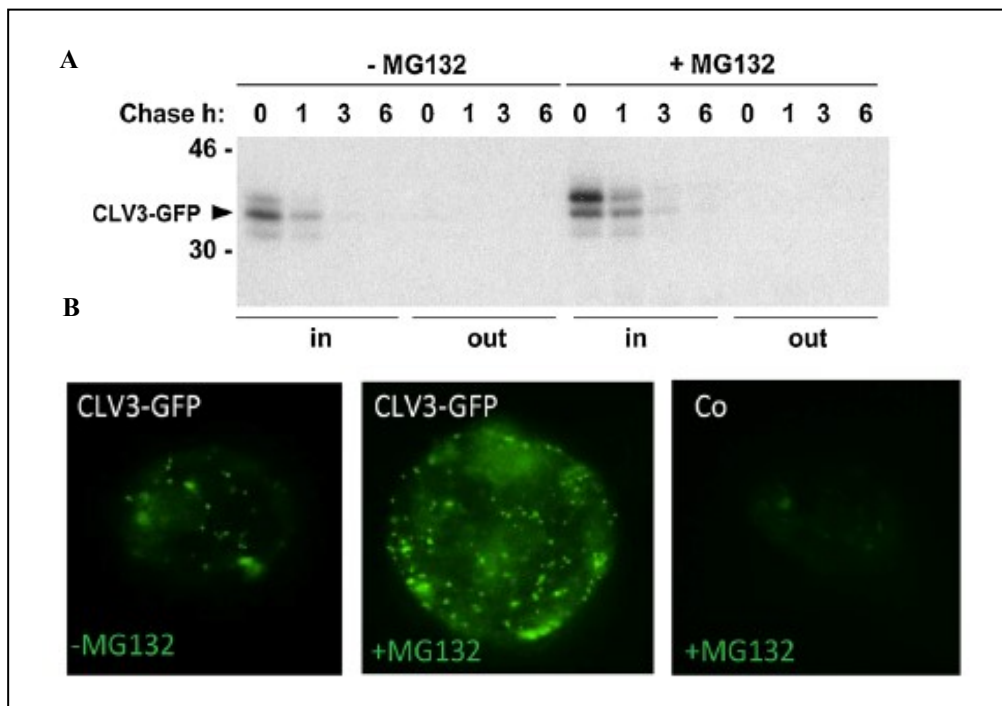


Figure 6. MG132 treatments revealed a possible involvement of ERAD process in CLV3 maturation. **(A)** Transfected protoplasts were pulse-labelled for 1 h and homogenates of intracellular content and precipitates of the culture medium were analyzed by SDS-Page and detected by anti-GFP. **(B)** The same transgenic protoplasts, treated or not with MG132, incubated with anti-GFP antiserum and secondary FITC-conjugated antibody, were analyzed for FITC fluorescence with immunofluorescent microscopy^[5].

The involvement of ERAD in the active CLV3 formation could also explain the role of PLANT U-BOX4 (PUB4) in CLV3 signalling in root meristems: Kinoshita and collaborators^[34], in

2015, describe this study as a “mystery in genetics”. PUB4 is a plant U-box protein with an E3 ubiquitin ligase activity: a first study has shown that loss-of-function mutants showed overproliferated root meristematic cells, suggesting that PUB4 regulates root cell proliferation in both longitudinal and radial axes^[35]. *A. thaliana pub4* mutants (**Fig.7B**) showed a larger SAM than that of wild-type plants (**Fig.7A**), a phenotype comparable to *clv3* mutants (**Fig.7C**): *pub4clv3* plants showed a bigger SAM than each single mutant (**Fig.7D**) and the expression regions of WUS and CLV3 (**Fig. 7F**) were also enlarged^[34].

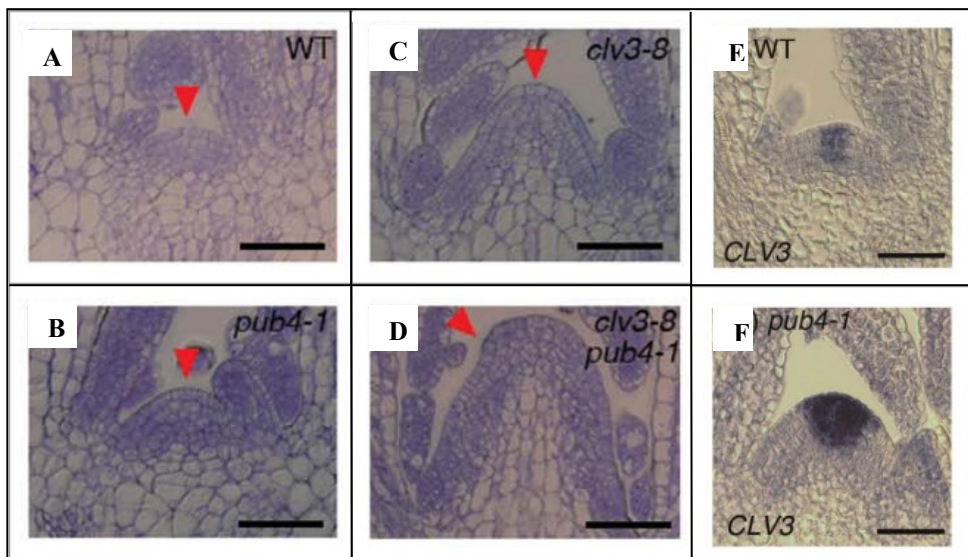


Figure 7. (A-D) SAM of 7-day-old seedlings of each genotype; (E-F) *In situ* hybridization of wt and *pub4-1* SAM using a CLV3 antisense probe^[34].

1.6 Aim of the project

My work of thesis aims to shed light on current knowledge about the maturation mechanism of CLV3 protein, a regulation factor of meristematic centers development in plant organisms. The current maturation model suggests that the pre-protein CLV3 is converted into the active dodecapeptide in the extracellular space: the whole inactive protein follows the canonical secretory pathway, being secreted in the apoplast and cut by extracellular proteases.

However, the cited results suggest a different CLV3 protein behavior: an alternative maturation system was proposed by De Marchis et al. in 2018^[5], which would involve the degradation system of misfolded proteins within the endoplasmic reticulum (ER), called ERAD.

Following previous results, our current approach to investigate more about CLV3 secretion and maturation is to follow this process in time in different biosystems. At first, we confirmed previous results by stable-expressing the CLV3-GFP fusion protein in *N. tabacum* plants and analyzing the stability of the protein at intracellular and extracellular levels in a steady-state way. Moreover, we compare this process in suspension cell cultures by using callus liquid cultures from tobacco CLV3-GFP cells to understand if the expression and maturation process is conserved across different cell culture conditions.

We selected an organism, *Chlamydomonas reinhardtii*, which lacks CLV3 protein and the meristematic organization, to study protein's maturation where it is not recognized. A different behavior of CLV3-GFP in this organism could indicate that the maturation process of the protein of interest has potentially evolved gradually with the conquest of emerged lands by plant kingdom and could significantly help us for further studies by creating multiple-transformants lines and mutants in both tobacco cells and microalgae strains.

Our attention was also focused on the *pub4 Arabidopsis thaliana* mutant, which develops a very close phenotype to those reported for *clv3*: we assumed that the ubiquitin ligase enzyme is involved in CLV3 recognition in the ER and, therefore, in its targeting to the proteasome to produce the active hormonal peptide. Several strategies have been carried out to investigate more about the ubiquitination role in CLV3 maturation process. These studies lay the foundations to highlight the complex biochemical/evolutionary mechanism of the regulation of plant meristematic cells.

2. Materials and methods

2.1 Plant model: *Nicotiana tabacum*

Nicotiana tabacum is an annual perennial plant of Solanaceae family. It has a poorly branched stem, which can reach a height of 1-3 meters, and large, lanceolate, rough leaves. The inflorescence, often red or light pink, is a panicle with a distinct rachis; the capsule fruit contains numerous spherical, brown seeds with a diameter of 0.5 mm^[36]. Plant biotechnology development has significantly affected tobacco studies, which today is one of the most important plant models for several studies, together with *Arabidopsis thaliana*. The rapid life cycle and the total genome-sequencing allow this plant to be used in genetic engineering, protoplast isolation, and biochemical studies^[37]. While the overexpression of *CLV3* in *Arabidopsis* leads to a strong lethality in the majority of plant individuals, the same *35S::CLV3* expression in *N. tabacum* surprisingly didn't show any macroscopic significant phenotypic differences compared to wild-type plants: three main phenotypes were found, but the most representative one was macroscopically indistinguishable from the wt plant. However, as expected, different morphological changes were observed in meristematic cell number of SAMs, which was reduced in those transgenic plants, and in leaf cell size^[38]. The vitality of tobacco plants instead of *Arabidopsis* ones, aspect still considerate unclear, allowed us to carry out biochemical experiments and cell biology analysis for this project.

2.2 Microalgae model: *Chlamydomonas reinhardtii*

The big world of algae can be divided into two main groups: microalgae, a heterogeneous group of unicellular organisms that includes eukaryotic protists, prokaryotic cyanobacteria, and green multicellular macroalgae^[39]. Like higher plants, these organisms possess a photosynthetic apparatus: they are easily cultivated, and they multiply exponentially in a very short time in favorable environmental conditions^[40], some of them can double approximately every 8 hours^[41]. Today, high-value pharmaceutical compounds, recombinant proteins, biofuels, and bioremediation applications have been produced from microalgae.

In the mid-20th century, a unicellular alga called *Chlamydomonas reinhardtii* started to be considered one of the primary model organisms in plant and cell biology world: it presents

oval-shaped cells, 10 μm long and 3 μm wide, with two flagella at the anterior end. The chloroplast allows the photosynthetic event, and it occupies 40% of the cell volume together with several mitochondria, the genome of which was completely sequenced, together with the genomic DNA. The nuclear genome sequence also allowed the construction of a genome-wide library, insertional mutants, and CRISPR-mediated techniques for gene-targeted disruptions. Different nuclear and plastid transformation techniques (such as electroporation, particle bombardment, and *A. tumefaciens*-mediated transformation) are available; however, the plastid transformation strategy generates significantly greater yields^[42]. Many features make *C. reinhardtii* an excellent laboratory species: genetic studies take advantage of the vegetative growth as fast as that of haploids, which allows mutant and transformant strains to be biochemically analyzed immediately^[43]. These cells could be grown under different conditions of light, nutrients, and CO₂ supply (for phototrophic growth). Moreover, the growth capacity in dark conditions on an acetate-containing medium keeps the photosynthetic apparatus functional and allows light-sensitive photosynthetic mutants to be isolated^[43].

2.3 Construct design

The 35S promoter, the sequence encoding CLV3-GFP, and the 35S terminator were excised by *Pst*I from pAVA120, a plasmid in which the cDNA coding for CLV3 had been introduced upstream of the GFP sequence. To create pGreen, this portion was inserted into the *Pst*I site the pGreenII binary vector, obtaining pGreen.CLV3-GFP^[5].

The most probable ubiquitination sites of CLV3 have been verified by RUBI - Rapid UBIquitination prediction tool (<http://old.protein.bio.unipd.it/rubi/>). The system revealed that, in the 96-amino acid sequence of CLV3, Lysine at position 61 has the highest probability of ubiquitination. However, two other Lysines were identified, which could offset the effect of Lysine 61 if the latter is mutated: therefore, a single-mutant construct for Lysine 61 and a second triple-mutant construct for Lysines at 57-59 and 61 positions were produced. Mutagenesis of pGreen.CLV3-GFP construct was performed using QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies), which allows site-specific mutation in virtually any double-stranded plasmid. Primers for mutagenesis were designed with the QuikChange Primer Design tool (<https://www.agilent.com/store/primerDesignProgram.jsp>) and are reported in **Tab.1**.

<i>k61</i> mutant K61	Forward	5'-CATGTAGTCCTAAACCCCTCGTCTTTGCCTTCTCT-3'
	Reverse	5'-AGAGAAGGCAAAGACGAGGGGTTTAGGACTACATG-3'
<i>triple-</i> mutant K57- K59-K61	Forward	5'-GTCCTAAACCCCTCGTCCTTGCCTTCTCTGCTTCTCCATTTGCTC-3'
	Reverse	5'-GAGCAAATGGAGAAGCAGAGAGGGCAAGGACGAGGGGTTTAGGAC-3'

Table1. Primers designed for selective mutagenesis of pGreen.CLV3-GFP through QuikChange XL Site-Directed Mutagenesis Kit. Lysines coding sequence was mutated into Arginine's one.

The obtained amplicon was further amplified by transformed *E. coli* thermo-competent cells, and purification was performed by ZymoPURE™ II Plasmid Midiprep Kit (ZymoResearch). For *Chlamydomonas reinhardtii* transformation, the CLV3 sequence was synthetically redesigned with codon optimization and intron spreading as recently described^[44]. The synthetic optimized gene coding sequence (CDS) was cloned into pOpt2_mVenus_Paro vector^[45] to generate a protein which contains a C-terminal mVenus (YFP) fusion.

2.4 Genetic transformation

2.4.1 *Agrobacterium tumefaciens* transformation of *Arabidopsis* and tobacco plants

GV3101 *Agrobacterium tumefaciens* strain was transformed by electroporation with pGreen.CLV3-GFP and used to produce transgenic *Arabidopsis* (Col-0) and tobacco (*Nicotiana tabacum*) cv. Petit Havana SR1. Young plants of *Arabidopsis thaliana* (Col-0) were stable-transformed by floral dipping. A colony of *Agrobacterium* grew on 100 mg/L kanamycin selective medium was transferred into a flask containing LB culture medium (Bactopectone 10 g/L, Yeast Extract 5g/L, NaCl 10 g/L, Agar 15 g/L; pH7.5), and kept overnight shaking at 28 °C. The bacterial suspension was centrifuged at 6,000xg (4°C) for 10 minutes and the pellet was resuspended in an equal volume of 5% freshly prepared sucrose solution + Silwet L-77 (Lehle Seeds, Texas, USA). Plants were dipped by inversion into the *Agrobacterium tumefaciens* suspension such that all above-ground tissues are submerged and kept in the

solution for 30 seconds. Treated plants have been covered with a plastic bag to maintain humidity and returned to the growth chamber. The dipping procedure was repeated after three days. Plants have been grown until siliques were brown and dry, then they were collected in separated bags. Seeds were sterilized (as described in Paragraph 2.5.1) and placed on a MS0 medium + 100 mg/L kanamycin to select transformants, then the seedlings are screened by PCR. Leaf disc explants from tobacco wild-type plants were transformed by co-cultivation with *A. tumefaciens* harbouring pGreen.CLV3-GFP. Leaf discs were then grown at 25°C under full light on a regeneration medium containing 250 mg/L cefotaxime and 100 mg/L kanamycin. Regenerated shoots of each explant were isolated and grown separately to guarantee the regeneration of independent transformants. After 5 weeks, shoots were plated on half-strength Murashige and Skoog salts, 100 mg/L kanamycin, and 250 mg/L cefotaxime until the new plants developed. Transformed plants were grown at 25°C in 16 h of light in vessels without antibiotics. Plants were then screened by PCR and Western Blot analysis, and some positive plants were reared to flower for seed collection for further experiments.

2.4.2 Protoplast transient transformation

Protoplasts obtained as described (see Paragraph 2.5.2) are centrifuged for 10 minutes at 60xg. Supernatant is removed and precipitated protoplasts are resuspended in approximately 1 mL of MaCa buffer (0.5 M mannitol, 20 mM CaCl₂, 0.1% w/v 2-[N-morpholino] ethanesulfonic acid (MES); pH 5.8) to reach a concentration of 1 million protoplasts/mL. Thermal shock is induced keeping protoplasts for 5 minutes at 45°C. After cooling, transfection is performed, adding 60µg of total in a volume not exceeding 10% of the total transfection volume (e.g., total volume 1 mL, DNA in 100µL). Tobacco protoplasts were transfected with *k61*-pGreen.CLV3-GFP and *triple*-pGreen.CLV3-GFP mutant constructs and pGreen.CLV3-GFP was used for Mock-protoplasts. An equal volume of 40% w/v PEG (for 100 mL: 40g PEG 4000 (Merck) dissolved into 70 mL of 0.1M Ca(NO₃)₂·4H₂O and 0.4M mannitol) is gently added to the tube. The tube is left at room temperature for 30 minutes, shaking gently every 5 minutes. Protoplasts are washed again with W5 buffer, adding 3 ml step by step until the tube is full. The tube is centrifuged 10 minutes at 60xg, and the supernatant is removed. The cells are resuspended in 1mL of K3 buffer and left at 25°C overnight in the dark.

2.4.3 Microalgae transformation

Glass bead agitation protocol was performed to carry out UVM4 strain of *C. reinhardtii* nuclear transformation^[46] using 10 µg of linearized plasmid DNA. Selection of transformants was done on TAP agar plates supplied by paromomycin. Expressing colonies were screened looking at YFP fluorescence and confirmed by Western Blot.

2.5 *In vitro* cultures

2.5.1 *Arabidopsis* and tobacco plants *in vitro* culture

Arabidopsis and Tobacco SR1 wild-type seeds' surface is sterilized keeping them shaking in an Eppendorf tube with 1.0 mL of ethanol 70% for 30 seconds. The liquid is then substituted with the same volume of 10% sodium hypochlorite, keeping them shaking for 5 minutes. Wild-type seeds are washed five times with sterile distilled water and placed on semi-solid MS0 medium (Murashige and Skoog Salts 4,4 g/L, sucrose 30g/L; agar 1.5%; pH 5,8) in Petri dishes. Seeds obtained from transformed plants are sterilized in the same way and placed on the same medium + kanamycin 75 mg/L. The plates are placed under 16 hours of light and 8 hours of dark photoperiod for 2 weeks at 25°C. Tobacco seedlings are transferred into sterile vessels containing respectively the same culture medium and maintained in the same controlled conditions (Fig.8).



Figure 8. Tobacco seedlings and plants *in vitro* cultures.

2.5.2 Tobacco protoplasts isolation

Tobacco protoplast isolation starts with 2-5 weeks old leaves of medium-sized plants. The center rib of the leaves is removed with a sterilized scalpel, and the lower surface of the leaf is incised, forming superficial cuts that should not go entirely through the leaf. The explants are then transferred into a Petri dish containing 9 mL of K3 buffer (For 1L solution: 3.78g Gamborg's B5 basal medium with minimal organics Sigma G5893; 750mg CaCl₂ 2H₂O; 250mg NH₄NO₃; 136.2g Sucrose; 250mg Xylose; 1mg 6-benzylaminopurine; 1mg α -naphthalenacetic acid) and 1mL of the hydrolytic enzyme mix (3% Macerozyme Onozuka R-10; 6% Cellulase Onozuka R-10). The lower page of the leaf must be in contact with the buffer while the upper page must be wetted as little as possible. Petri dishes are then placed in the dark at 25°C overnight. The following day, the protoplasts are isolated: the digestion mix is removed and approximately 10 mL of K3 is added directly on the leaves. The plate is shaken so the leaves are disaggregated and the protoplasts floating in the K3 buffer are released. The protoplasts are recovered and filtered through an 85-100 μ m pore size nylon membrane into a Falcon tube and centrifuged for 20 minutes at 60xg. Vial protoplasts foliate, and the underlying buffer is removed. Four volumes of W5 buffer (9g NaCl; 0.37g KCl; 18.37g CaCl₂ 2H₂O; 0.9g glucose) are then added to the cells, shaken gently, and centrifuged for 10 min at 60xg. Live protoplasts pellet is conserved and the supernatant is removed with a Pasteur pipette. The protoplasts are gently resuspended in an equal volume of W5, and a second wash is performed. After removing the supernatant again, 10 mL of W5 are added (500 μ L cells in 4.5 mL of W5). Cells are diluted 1:10 and counted with a Burker Chamber with a Leitz Dialux 20 Microscope. Approximately 1 million protoplasts are required for each transient transformation (**Fig.9**).

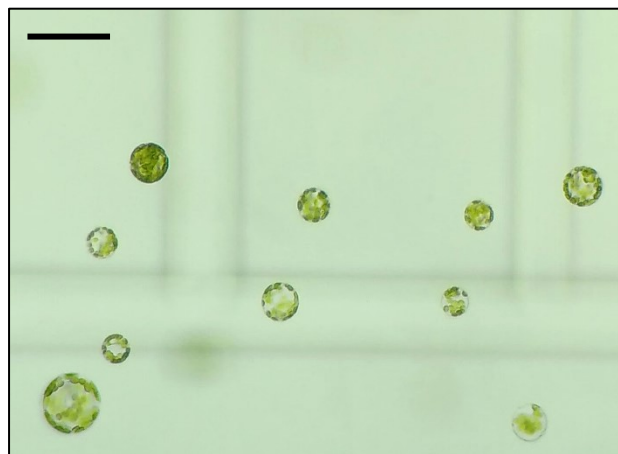


Figure 9. Tobacco protoplasts counting after isolation; Scale Bar = 100 μ m.

2.5.3 Tobacco cell suspension cultures from calli

Callogenesis was induced from *in vitro* cultivated tobacco (of both wild-type and pGreen.CLV3-GFP transformed plants) leaves by cutting leaf explants of approximately 5 mm x 5 mm, and placed on MS medium (4.4 g/L Murashige and Skoog Salts; 30g/L Sucrose; 2,4-D 0.05 mg/L; pH 5.8) with Agar 0.8% supplemented with kinetin 0.1 mg/L. The explants were placed at 25 °C and grown in the dark. Callus masses obtained were isolated and kept in an equally constituted culture medium for 28 days, after which some of them were transferred into a liquid culture medium. Calluses are finely ground using a sterile scalpel and then transferred to a flask containing the same MS + kinetin 0.1 mg/L medium without agar (1 g callus/100 mL of liquid medium). The culture is kept shaking in the dark at 25 °C for approximately 10 days.

2.5.4 *C. reinhardtii* cultivation

All stages of transformation and growth monitoring of the microalga *Chlamydomonas reinhardtii* were carried out at the Sole Lab laboratory of Prof. Matteo Ballottari, Department of Biotechnology, University of Verona. Different culture systems of *Chlamydomonas reinhardtii* were tested, growing the different genotypes under mixotrophic conditions at different light exposures, Low Light (LL, 100 $\mu\text{mol photons/m}^2/\text{s}$) and High Light (HL, 500 $\mu\text{mol photons /m}^2/\text{s}$). Mixotrophic growth of *Chlamydomonas* occurred in Tris-acetate-phosphate (TAP) growth medium, in liquid shake flasks at 25°C. Growth tests were conducted using Multi-Cultivator MC-1000 (Photon Systems Instruments, Drasov, Czech Republic). Parameters used for monitoring growth were cell productivity, cell area and Fv/Fm. Cell densities were measured using Countess II FL Automated Cell Counter (Thermo Fisher Scientific, Waltham, Massachusetts). Dry biomass was evaluated by overnight lyophilization of washed cell pellets and gravimetric determination.

2.6 DNA extraction and PCR screening

DNA extraction from tobacco callus culture and leaves were performed through Quick-DNA™ Plant/Seed Miniprep Kit (ZymoResearch). The filtered DNA is suitable for PCR and other

downstream applications. PCR was carried out for *CLV3-GFP* sequence in tobacco samples. PCR was performed with GRS Taq DNA Polymerase (Grisp Solutions) kit, the mix for 100 ng of DNA samples was made up as follows: MgCl₂ 1.5 µL, dNTPs 1 µL, Primer Forward [10 µM] 0.75 µL, Primer Reverse [10 µM] 0.75 µL, Buffer 2.5 µL, Taq polymerase 0.25 µL, H₂O 14.25 µL. Primers are reported in **Tab.2**. The PCR Amplification was performed using the Thermocycler Life touch-865019 (Bioer). Amplification products were run on 1% agarose gel (1 g Agarose Duchefa-Biochemie, 100 mL TAE 1X, 5 µL of Ethidium Bromide) by electrophoresis at 100 V for 30 minutes.

<i>CLV3-GFP</i> Tobacco plants/calli	Primer forward	5'-GTGATGTATACGTTGTGGGAG-3'
	Primer reverse	5'-ACGTTCCAACCACGTCTTCAAAG-3'

Table2. Primers used for PCR screening of transgenic tobacco plants/calli.

2.7 Protein extraction

2.7.1 Plant material and microalgae homogenization

For total soluble protein extraction (TSP) from leaves and cell cultures, 200 mg of leaf or 500 mg of callus are homogenized. Homogenation takes place in a mortar kept cold in an ice basket. An appropriate amount of homogenation buffer (Tris-HCl 0.1M, NaCl 0.2 M, EDTA 1mM, 0.2% Triton X-100) (2-3 times the weight of the leaves and 50% of the callus' weight) is prepared just before use, in which a tablet of Complete™ (Roche) is added to a final concentration 1x. The homogenate obtained is transferred to an appropriate tube and centrifuged at 1500xg for 10 min at 4 °C. The supernatant contains the total soluble proteins. Microalgae's cells and protoplasts have been homogenate adding an adapted homogenation buffer (150 mM Tris-Cl pH 7.5, 150mM NaCl, 1.5mM EDTA, 1.5% Triton X-100) + Complete™ (Roche) as described and vortexed. To load the extraction product into an SDS-Page blot ¼ volume of Denaturation Buffer 6x (120 mM Tris-Cl pH 8.6, 6% SDS, 2% β - mercaptoethanol, 50% Glycerol) and ¼ volume of Bromophenol blue (0.1% Bromophenol blue, 10% Glycerol) are added to each sample. Samples are boiled for 5 minutes at 90 °C before each use.

2.7.2 Supernatant TCA precipitation

CLV3 possible secretion leads to different localisation of the protein at intra- or extracellular level, for this reason the liquid culture medium of tobacco suspension culture/microalgae, and protoplast suspension buffer were precipitated to investigate the presence of the protein CLV3-GFP (or CLV3-YFP in microalgae). 400 μ L of 100% w/v trichloroacetic acid solution (TCA) are added to 1600 μ L of sample and let incubate for 10 minutes at 4°C, then centrifuged at 18620xg for 5 minutes. The supernatant is removed, and the protein pellet is washed twice with 200 μ L of cold acetone: each wash is followed by a further centrifugation of 10 minutes at 18620xg. Acetone is removed and the pellet is left at 95 °C for 5-10 minutes until dryness. ¼ volume of Denaturation Buffer 6x and ¼ volume of Bromophenol blue are added to each sample, which must be boiled for 5 minutes at 90 °C before each use.

2.8 Protein SDS-Page and Immunoblot

CLV3 protein localisation linked to GFP/YFP was verified by SDS-PAGE and western immunoblotting. Samples were separated on 1 mm 12% w/v polyacrylamide gels (Separating gel: 2.1 mL H₂O, 1.2 mL TRIS buffer 1.5M pH 8.8, 1.46 mL Bis-Acrylamide 40%, 48.75 μ L SDS 10%, 37.5 μ L APS 10%, 4.5 μ L TEMED; Stacking gel: 1.88 mL H₂O, 0.75 mL TRIS buffer 0.5M pH6.8, 0.33 mL Bis-Acrylamide 40%, 30 μ L SDS 10%, 15 μ L APS 10%, 3 μ L TEMED). Proteins were visualized by gel staining with Coomassie brilliant blue or electro- blotted into a PVDF membrane and stained with anti-GFP polyclonal antibody (GFP Polyclonal Antibody, Invitrogen, Thermo Fisher scientific) diluted 1:2000. Horseradish peroxidase (HRP) conjugated to goat anti-rabbit secondary antibody (Invitrogen, Thermo Fisher scientific) 1:10000 in combination with the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher scientific) kit was used for detection. Images were acquired with a ChemiDoc MP Imaging System (BioRad).

2.9 Electron Microscopy

Leaves from wt or CLV3-GFP tobacco plants were analyzed by immuno-electron microscopy. Explants of young leaves were fixed in 1.6% (w/v) paraformaldehyde and 1.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer pH 6.9 for 1 h at room temperature. Samples have been washed with 0.1 M phosphate buffer and dehydrated in ethanol and placed overnight in LR white resin at 60°C. Ultrathin sections (70–80 nm) were cut using a Leica Microsystems Ultracut E (Leica Microsystems Nussloch GmbH, Nussloch, Germany), mounted on 300-mesh nickel grids and immunogold labeled.

Grids were floated on drops of double-distilled water, phosphate-buffered saline (PBS), normal goat serum diluted 1:10 in PBS for 10 min, and 5% bovine serum albumin (BSA) in PBS for 10 min. They were then incubated with antiGFP antiserum (1:2000 dilution) for 1 h at room temperature. Controls were incubated in pre-immune rabbit serum. The sections were washed with 0.1% BSAc in PBS and incubated in the same buffer with goat anti-rabbit secondary antibody (1:25 dilution) conjugated with 15 nm gold particles (BBInternational, Cardiff, UK). The grids were washed in drops of 0.1% BSAc in PBS, PBS and double-distilled water, post-stained in Uranyl acetate, and examined under an electron microscope (EM 400 T; Philips, Eindhoven, The Netherlands)^[47].

2.10 Root growth assay

Seeds of *Arabidopsis thaliana* (Col-0) have been sterilized as described in Paragraph 2.5.1 and placed on MS0 basal salts medium agar plate (4.4 g/L Murashige and Skoog Salts; 30g/L Sucrose; pH 5.8) and 1.5% agar. Basal medium was supplemented with microalgae's liquid culture medium of different genotypes, wild-type and CLV3-YFP (1 mL for each plate). Seedlings were grown in a vertical position at 25 °C and 16-h light/8-h dark photoperiod for 10 days before root length measurement.

3. Results

3.1 *Arabidopsis* shoots do not survive to CLV3-GFP over-expression

Seeds collected from CLV3-GFP *Arabidopsis* transformed plants were sterilized and placed on MS0 medium and left them germinate at 25°C and under a 16h light photoperiod. While wild-type plants normally develop, CLV3-GFP shoots showed hyperpigmentation, a condition probably due to severe stress, and abnormal phenotypes characterised by small and aberrant cotyledons. Gene acquisition was verified by PCR. After 5 days, *Arabidopsis*-transformed sprouts died: in 2018, Kuluev and collaborators^[38] successfully transformed tobacco plants, which didn't show any macroscopic defect in plant development. Although it is still unclear the phenomenon thereby CLV3 overexpression doesn't show deleterious effects in tobacco, in the present work tobacco plants were stable-transformed and used for following experiments.

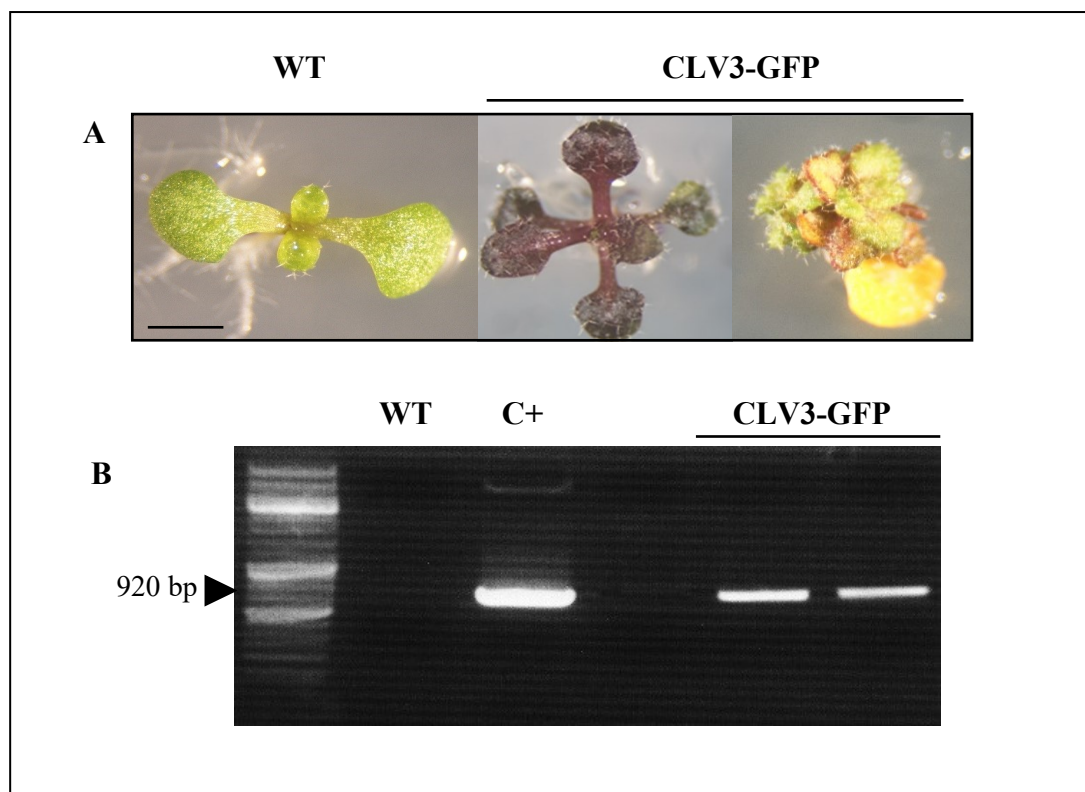


Figure 10. (A) *Arabidopsis* shoots overexpressing CLV3-GFP fusion protein are not able to generate the entire plant; Scale Bar = 1 mm (B) PCR analysis confirms transformation event in those aborted shoots, indicating that overexpression of this gene is probably not compatible with adult plant development.

3.2 Stable Expression of CLV3-GFP fusion protein in tobacco plants

Regenerated shoots originated from tobacco leaf explants co-inoculated with *A. tumefaciens* were isolated and selected by placing explants on MS0 medium + 100 mg/L kanamycin concentration (**Fig.11A**). The gene acquisition was verified by PCR, and an immunoblot revealed the proper synthesis of CLV3-GFP fusion protein in selected plants. These plants were rear to flower for seed production and collection, and further used for callus induction (observed in one week by placing CLV3-GFP leaf explants on the medium supplemented with phytohormones) and suspension cultures obtained from tobacco-calli liquid culture (**Fig.11B**).

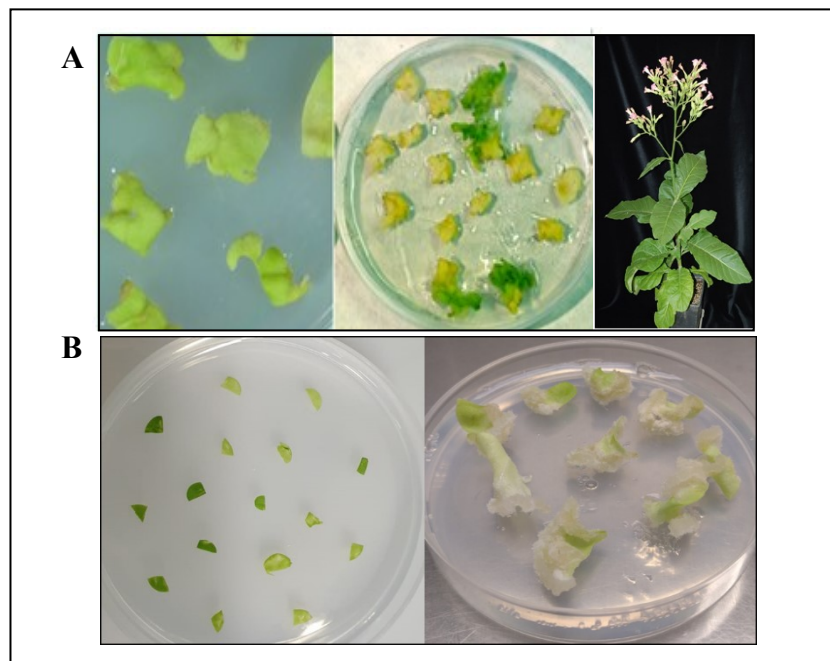


Figure 11. (A) Shoots regeneration on kanamycin selective medium from agro-infiltrated tobacco explants. Transformed tobacco plants showed a wt phenotype as observed by^[38]. (B) Callus induction from CLV3-GFP leaf explants placed on MS0 medium supplemented with phytohormones; callus formation was observed after 1 week.

3.3 CLV3-GFP fusion protein is not found in the apoplast

De Marchis and collaborators^[5] has demonstrated in 2018 the intracellular localisation of CLV3-GFP in tobacco protoplasts, by a pulse chase analysis which revealed a rapid disappearance of the fusion protein at intracellular level and the total absence of the whole protein in the apoplast. To further investigate the protein localisation in plant cells, an

Immunoblot was carried out on tobacco protoplasts (of both genotypes, wild-type and CLV3-GFP) kept incubating after isolation in K3 medium in dark conditions at 25 °C. The steady-state analysis of the intra/extracellular protein content revealed the absence of CLV3-GFP in the culture medium, confirming what previously observed (**Fig. 12A**)^[5].

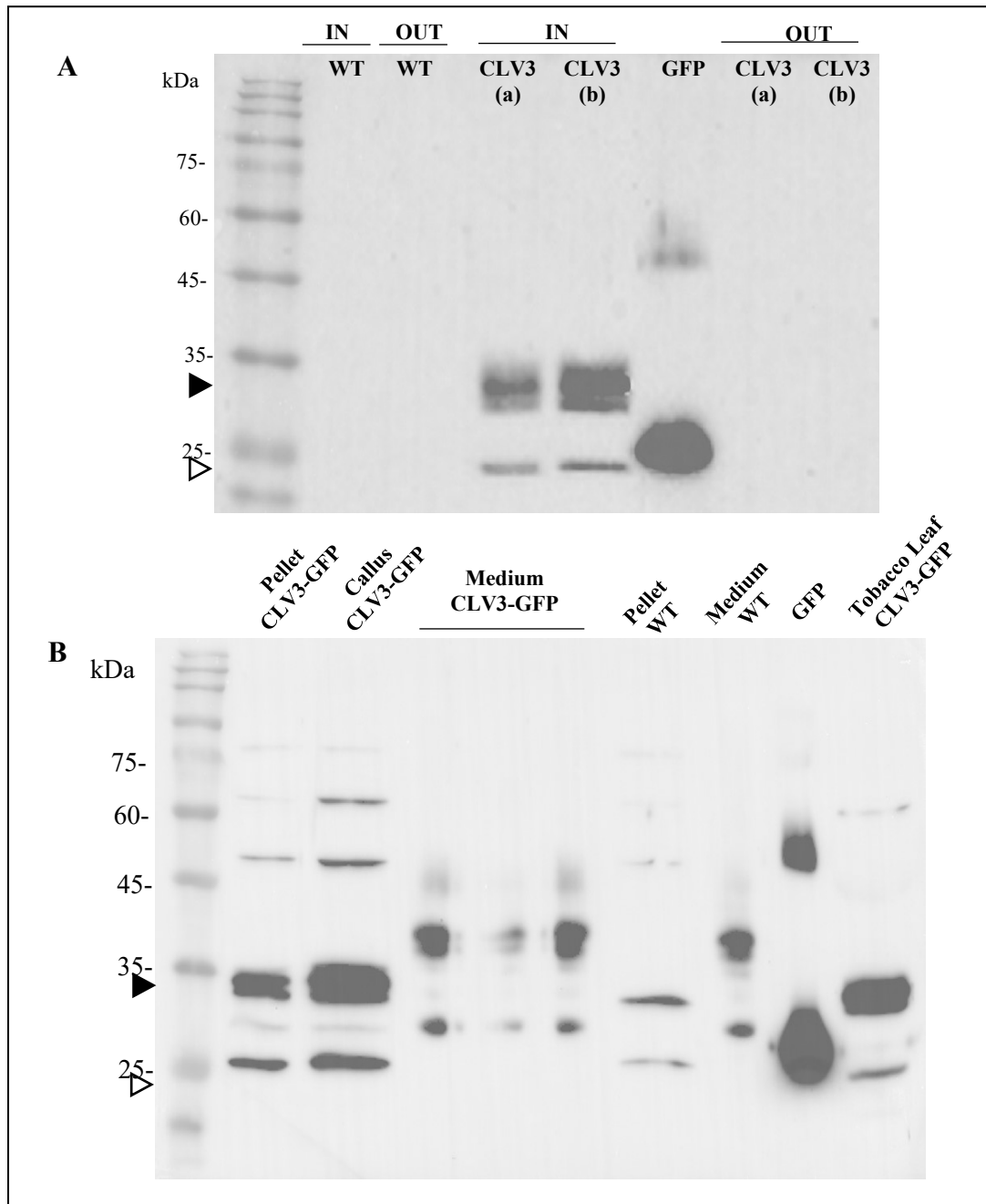


Figure 12. (A) (IN) Total soluble protein extracted in both wt and CLV3-GFP tobacco protoplasts; (OUT) supernatants of each protoplast culture medium was precipitated with TCA and loaded; GFP = 10 µg. (B) Callus culture from CLV3-GFP tobacco plants was induced, and the expression of the protein was verified (Callus CLV3-GFP); the liquid suspension culture obtained from calli of both genotypes (wt and CLV3-GFP) was screened as described in (A) for the intracellular and extracellular protein content (Pellet wt/CLV3-GFP and Medium wt/CVL3-GFP, respectively; GFP and tobacco CLV3-GFP Total soluble protein extract were used as Mocks). Black arrow: CLV3-GFP, White arrow: GFP.

Moreover, to further verify the maturation process is not affected by the culture conditions, the expression of CLV3-GFP was also tested in calli originated by transformed tobacco plants and grown on a semisolid medium. A suspension cell culture was started from these positive calluses and grown for 1 week in dark conditions and continuous shacking at 25°C. The intracellular and extracellular content suggest again the intracellular synthesis of CLV3 but the lack of the secretion of this protein in plant cells (**Fig.12B**).

To further examine CLV3 behavior *in vivo*, ultrathin sections of wt and CLV3-GFP tobacco leaves were incubated with antiGFP antiserum analyzed by immuno-electron microscopy. The detected signal due to the gold-conjugated secondary antibody confirmed the cytoplasmatic localisation of the fusion protein, while no clusters were observed in the extracellular matrix (**Fig.13**).

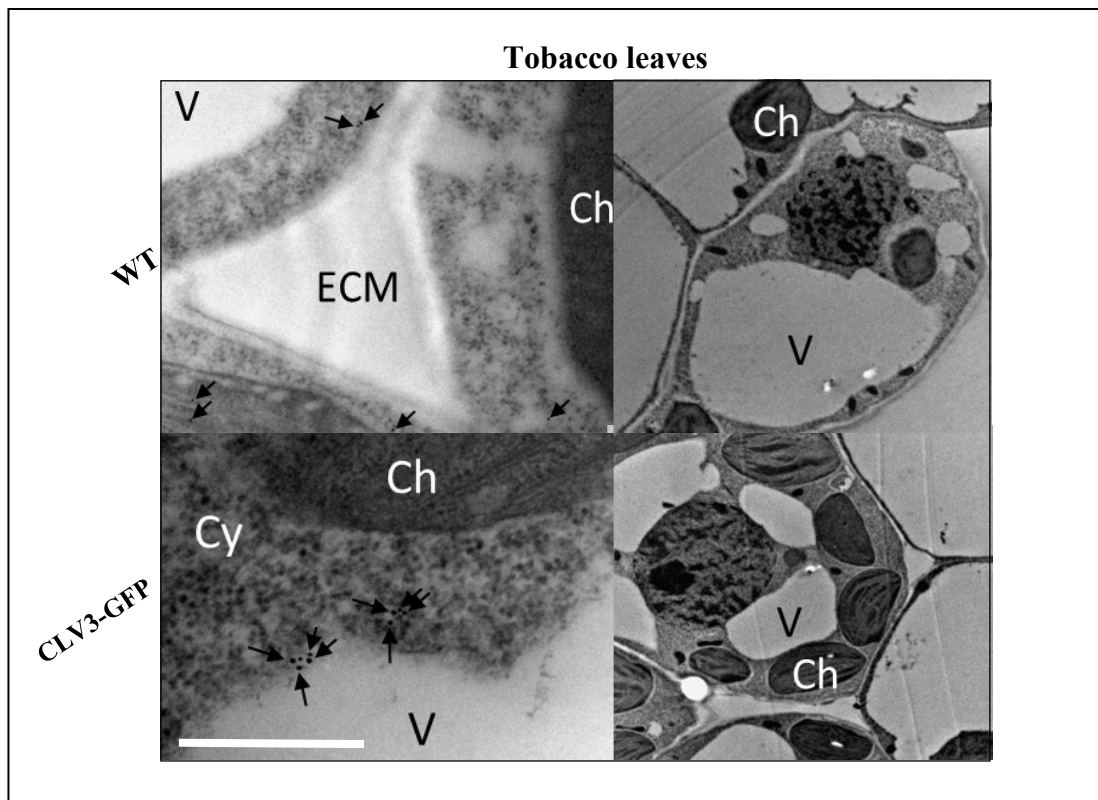


Figure 13. Localisation of CLV3-GFP by immunoelectron microscopy analysis. Arrows indicate the 10-nm gold-conjugated goat anti-rabbit IgG. Clusters of CLV3-GFP proteins within the cytoplasm, but no signal was found in the apoplastic space; Scale Bar = 5000 nm (V, vacuole; Ch, chloroplast; Cy, cytoplasm; ECM, extracellular matrix).

3.4 *CLV3* expression in algae strains

To further study the maturation process of *CLV3*, the first approach was to understand how this protein behaves in an organism that lacks *CLV3* gene or homologous sequences (as confirmed by nucleotide and protein BLAST Alignment Search Tool) and does not show the meristematic organization. Such an organism, in our case *C. reinhardtii*, was successfully transformed with the *CLV3*-YFP construct. Growing test of wild-type and transformed algae in different conditions were performed (**Fig.14**), showing that the transformation event had no effects on recorded growth parameters, as there are no significant differences between the wild-type strain alga and the transformed *CLV3*-YFP trends. This approach aims to retrace an evolutionary gap between unicellular algae and vascular plants, in which modern tissue organization is present, by studying protein behavior over time in phylogenetic terms (**Fig.15A**). The presence of the signal peptide in the amino acid sequence and the absence of an actual secretion mechanism in tobacco suggest a change in the destiny of *CLV3* during evolution, which, therefore, could revert to the canonical pathway followed by secretion proteins in the alga. The liquid culture of wild-type and *CLV3*-YFP *C. reinhardtii* grew in mixotrophic conditions was centrifuged, to separate cells from the liquid fraction. Pellet and medium protein content was analyzed by immunoblot confirming *CLV3*-YFP is correctly synthesized intracellularly and is secreted in the extracellular space. This observation suggests that *CLV3*-GFP is not processed in microalgae which does not recognize the fusion protein, releasing it in the full-length form in the medium (**Fig. 15B**).

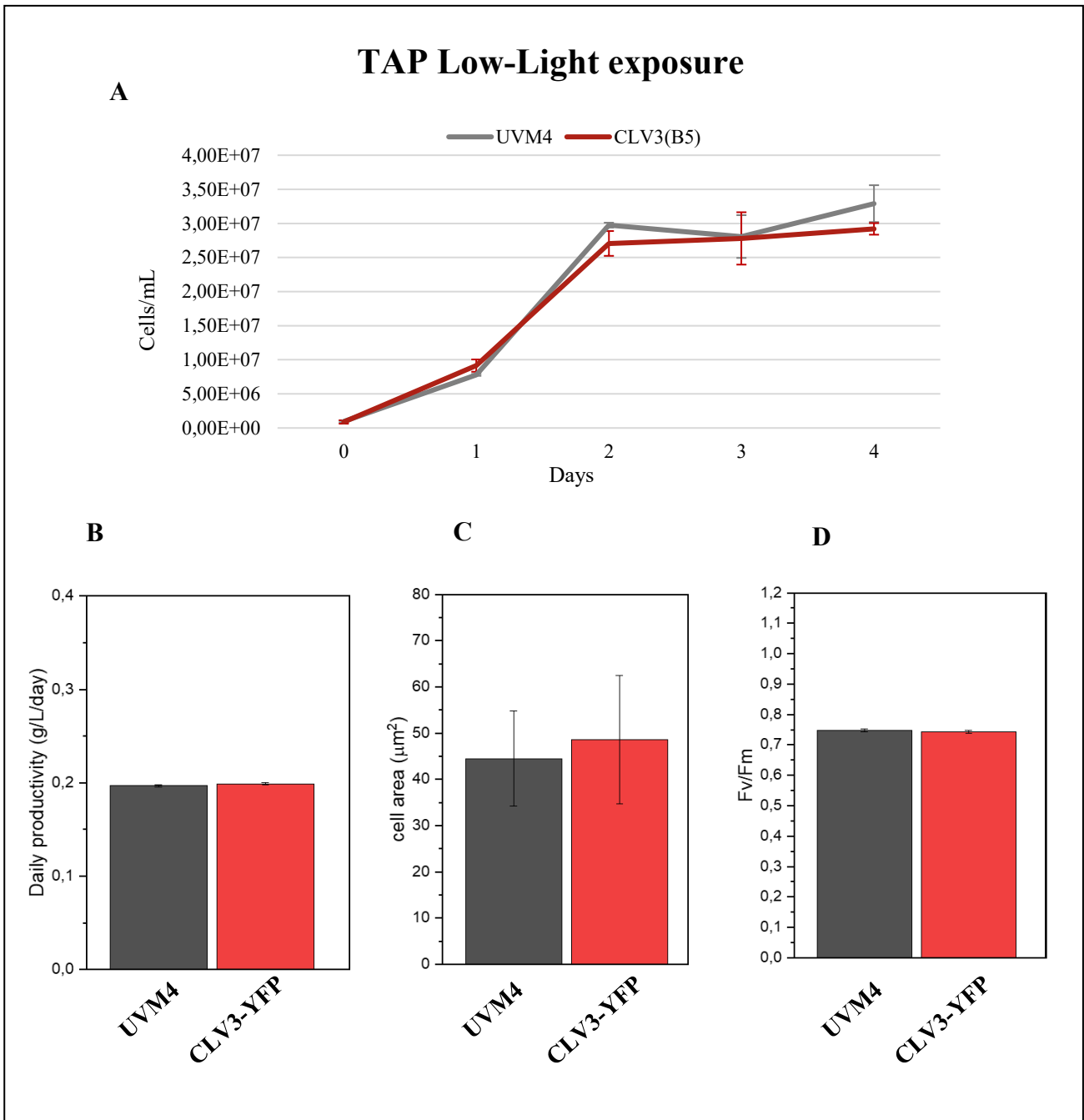


Figure 14. (A) Growth curve of both strains of *Chlamydomonas reinhardtii*, wt and CLV3YFP, grown at a low light exposure for 4 days, carried out in duplicate. Graphs (B, C, D) show the main parameters investigated for the growth quality of wt and transformed algae (Daily productivity, Cell area and Fv/Fm). One-way ANOVA demonstrate there are no significant differences between the growth performance of the two lines, either under low light exposure or high light conditions (Graphs not shown).

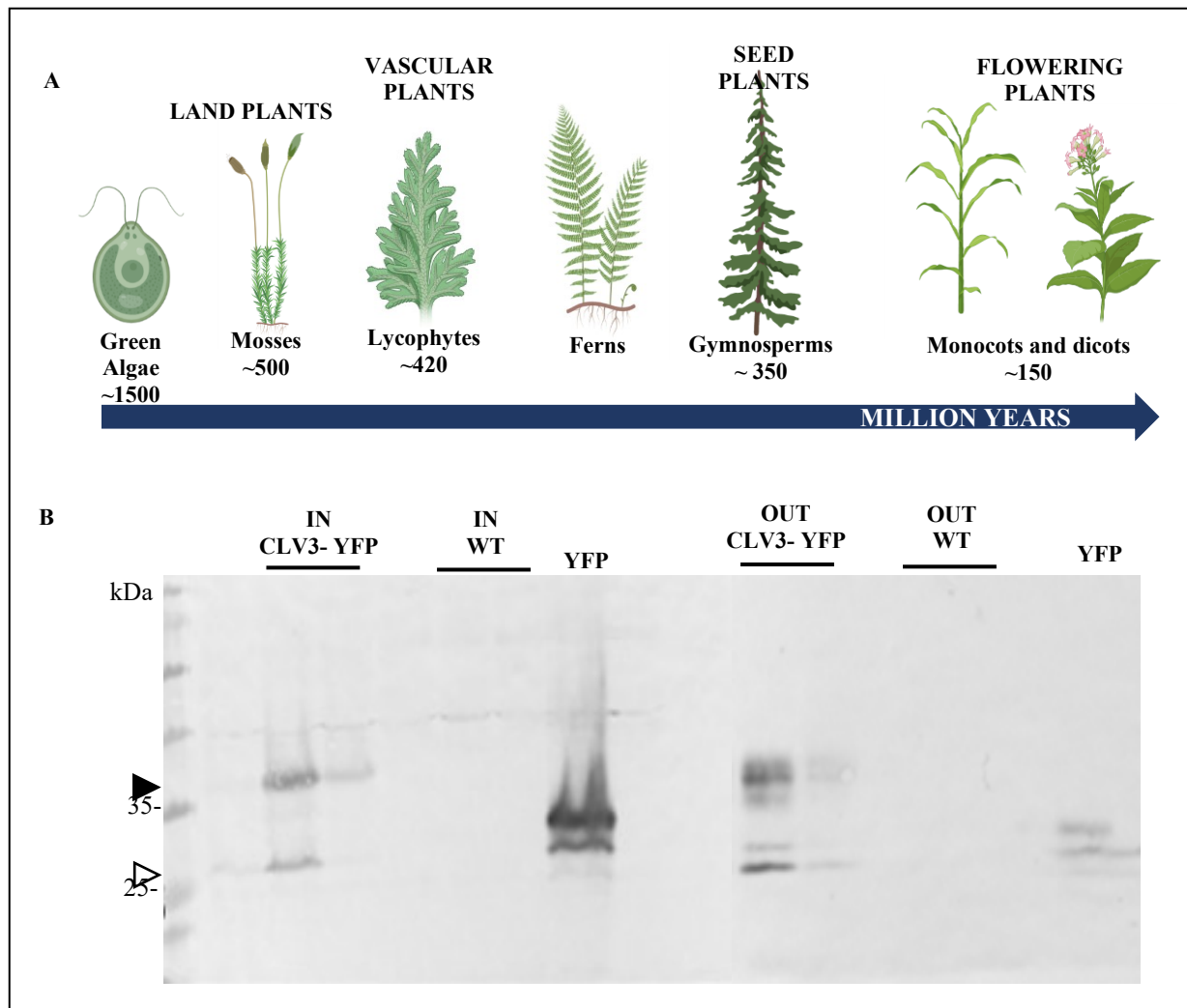


Figure 15. (A) During evolution, plants have adopted mechanisms for niche-meristematic cells regulation. Most unicellular algae lack the *CLV3* gene in contrast to higher plants. The hypothesis is that the maturation process of *CLV3* emerged in the transition from unicellular algae to the first forms of plants that conquered the mainland and was completely fine-tuned in higher plants. (B) *C. reinhardtii* cells were transformed with *CLV3*-YFP. Five million of transgenic and wt microalgae cells and the respective supernatant were homogenized and precipitated. Separated proteins were analyzed by immunodetection using an anti-GFP (Green Fluorescent Protein) antibody (Agrisera, Sweden). This result indicates the presence of the full-length protein both in the cell homogenates and in the culture medium after three days of incubation. Black arrow: *CLV3*-YFP; White arrow: YFP.

3.5 Physiological activity of *CLV3* in microalgae

In order to investigate whether the degradation of *CLV3*-GFP is absent in the microalgae and therefore the active *CLV3* peptide is not detectable in their culture medium, physiological tests were conducted growing *Arabidopsis* seedlings on MS0 supplemented with microalgae supernatant. It has been observed that when the chemically synthesized dodecapeptide corresponding to the functional dodecapeptide of *CLV3* (RTVPSGPDPLHH, p*CLV3*) is added

to the culture medium of *Arabidopsis* seedlings, the root meristems are strongly affected by its presence, showing a strongly shortened and branched phenotype of the roots, indicative of CLV3 activity^[5].

The culture medium of wild-type and transgenic CLV3-YFP microalgae was then added to the MS0 solid growth basal medium, and sterilized *Arabidopsis* seeds were placed equidistant from each other, as previously described in the study of the maturation mechanism of the CLV3 protein in wt and transformed tobacco protoplasts by De Marchis and coworkers (2018)^[5]. After 10 days from seed placing, measurements of roots length were recorded: no effects on root development were observed on those seedlings placed on transformed CLV3-GFP algae medium, compared to the untreated control (Fig.16A, B). This evidence suggests a lack of maturation of CLV3 in *C. reinhardtii*, potentially due to an absence of the proteolytic processing responsible for maturation of CLV3 in microalgae.

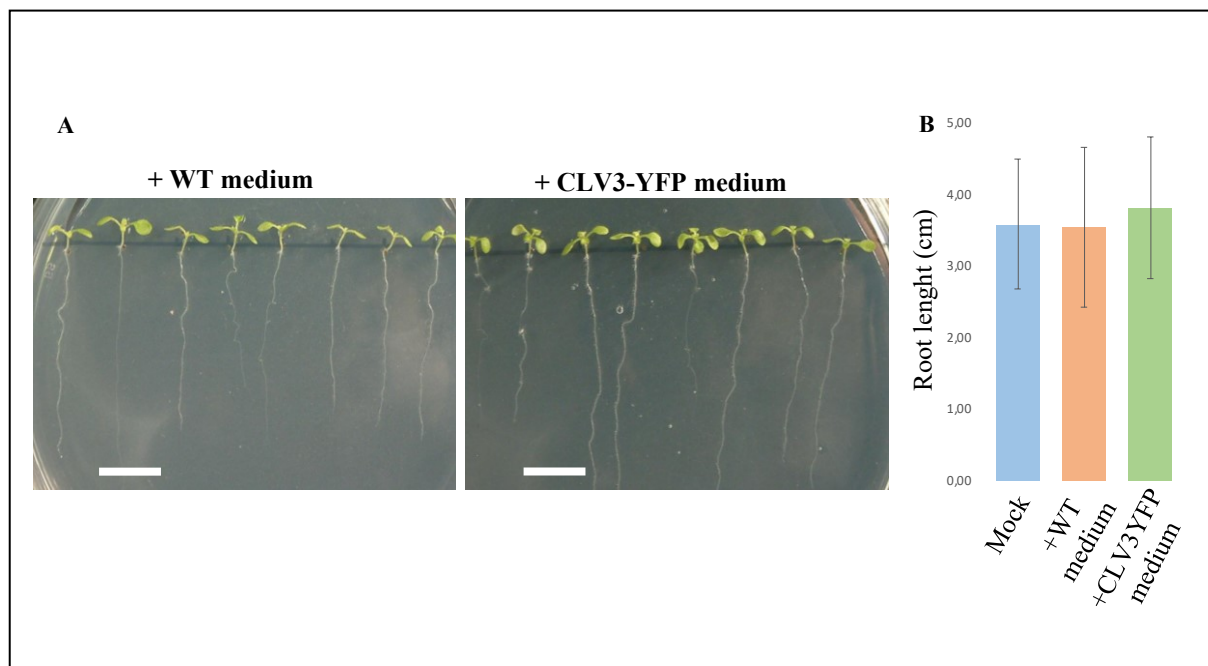


Figure 16. (A) To determine if the medium obtained from transgenic algae influences root growth suggesting the presence of the active dodecapeptide in the culture medium, a physiological assay was carried out. *A. thaliana* seeds were placed on MS0 with the addition of 3-days culture medium from *C. reinhardtii* cells wt or CLV3-YFP. Scale bar = 1 cm (B) One way ANOVA reveal that *Arabidopsis* roots growth is not affected by the presence of the CLV3-YFP protein in the culture medium, suggesting the absence of proteolytic processing in microalgae cultures (Mock: Seedlings growth on standard MS0 medium; +WT/CLV-YFP medium: seedlings growth on standard MS0 medium supplemented by 1 mL of microalgae medium).

3.6 Ubiquitination role in CLV3 maturation

ERAD process assumes the retrograde ATP-consuming transport which allows the retro-translocation event of targeted proteins from the ER to the cytosol. In this way, the substrate can be subjected to several ubiquitination steps and sent to the proteasome for degradation (as described in Paragraph 1.5). Moreover, Kinoshita and collaborators^[35] identified a possible correlation between the action of CLV3 and PUB4, an E3 ubiquitin ligase studied in *Arabidopsis*. To investigate more about the possible role of ubiquitination in CLV3 maturation process, a transient transformation of wild-type tobacco protoplasts was conducted with three different constructs. Ubiquitination generally occurs on Lysine residues of the amino acid sequence: a qualitative analysis was performed by using The RUBI - Rapid UBIquitination tool, which identified the 61-Lys as the most probable one for the ubiquitin ligase recognition. A first single-mutant (*k*) was obtained for the 61-Lys, substituting this residue with arginine. A second mutant construct has been designed were, together with the mutation of Lys-61, the Lys-57 and Lys-59 have been substituted with three arginine residues, to avoid any possibility of restoring the ubiquitination process (Fig.17).

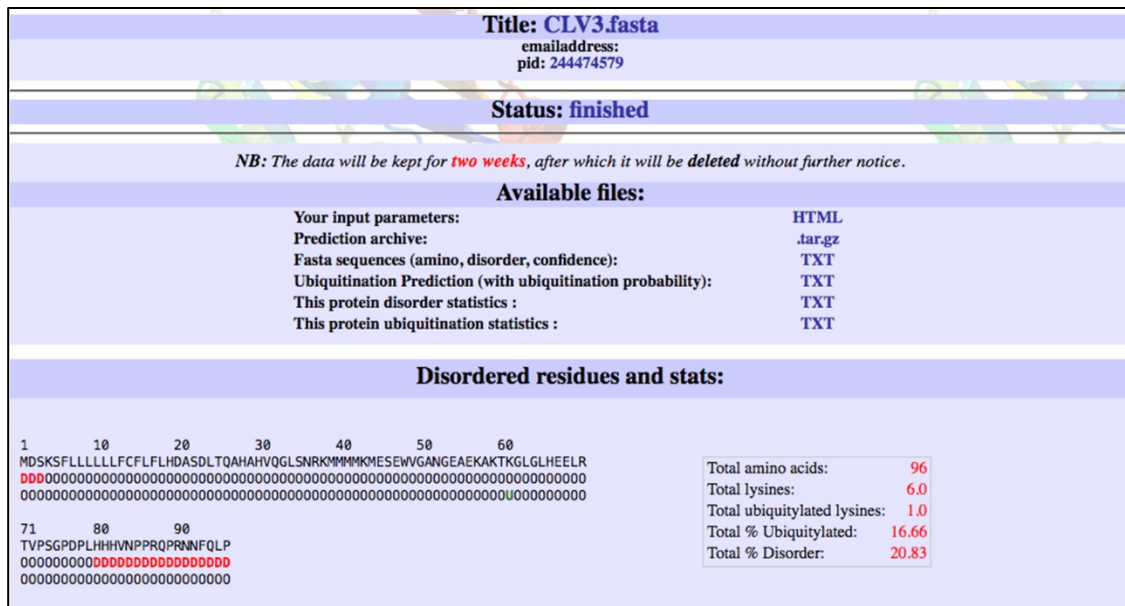


Figure 17. Output of RUBI - Rapid UBIquitination tool for CLV3 ubiquitination sites prediction.

Acquisition of *k*-mutations was confirmed by sequencing. The wild-type tobacco protoplasts were transiently transformed with the three constructs (PGreen.CLV3-GFP, *k61*-PGreen.CLV3-GFP mutant, *triple*-pGreen.CLV3-GFP mutant); 1 million of wild-type

protoplasts were used as a negative control, following the same transfection procedure but replacing the DNA with H₂OmilliQ. Wild-type and transformed protoplasts were left incubate for 1 day in the dark at 25°C. Once the incubation was completed, the pellet and the protoplast culture medium were separated: the first was homogenized, while the latter was purified with a 0.22µm filter to avoid any cellular contamination and precipitated with trichloroacetic acid. The procedure was repeated for each protoplast culture. Immunoblot analysis of these samples confirmed the transfection event: at intracellular level the synthesis of the CLV3 fusion protein was observed in all transformed protoplasts, which shows the same molecular weight of that observed in previous experiments, both in protoplasts transfected with pGreen.CLV3-GFP and those with pGreen.CLV3-GFP mutant vectors. Wild-type pellet showed no protein signal, as expected. Extracellularly, no detectable traces of the CLV3 protein were found in any of the investigated lines. Both absence of CLV3 protein in wild-type and pGreen.CLV3-GFP tobacco protoplast culture medium confirm what was previously observed. A different behavior was expected in those cells transfected with Lys-mutated pGreen.CLV3-GFP vectors: the most obvious condition for a determining role of ubiquitination in the CLV3 maturation mechanism would have been to find the fusion protein in the culture medium of this protoplasts in its full-length form, suggesting a canonical secretion (**Fig.18**).

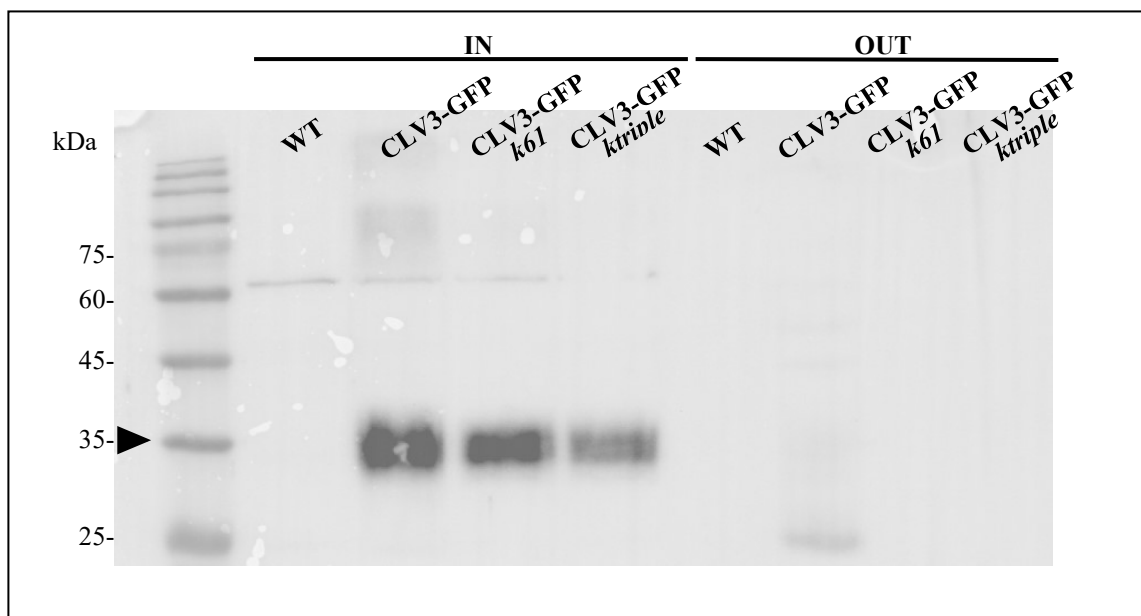


Figure 18. 150-thousand protoplasts of each strain (wild-type and transformed) were homogenized for loading intracellular TSP. The supernatants derived from 1 million protoplasts from the same strains were precipitated with trichloroacetic acid. Western blot analysis shows the successful transformation of the CLV3-GFP protoplast lines as the synthesis of the protein at the intracellular level is confirmed. In the medium, the presence of the protein is not detectable: this aspect could indicate a lack of secretion of CLV3-GFP fusion protein or rapid intracellular/extracellular maturation of CLV3-GFP. Black arrow: CLV3-GFP band.

4. Discussion

CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE) peptides are shoot and root apical meristems developmental regulators: these peptides are part of a negative feedback loop which establishes a fine-tuned balance between stem cells proliferation and differentiation in meristematic niches^[48]. CLV1 and CLV2 create a heterodimer receptor for CLV3 ligand, expressed in cells next to those that produce CLV1^[49]. Many studies have shown that the exogenous addition of the active form of CLV3 significantly affects root growth in *Arabidopsis*^[50], suggesting that CLV3 is secreted and processed in the extracellular space to explain its function, binding the receptor complex on adjacent cells in its active form. However, little is known about the mechanism responsible for the maturation of this protein and *in vivo* tests are necessary to determine whether CLV3 processing occurs: the rapid turnover of generated fragments makes very difficult the experimental detection of this phenomenon^[51]; for this reason, reduced studies are disposable in which the intact precursor of CLV3 is detected biochemically in intra- and extracellular compartments of plant cells. In 2006, Jun and coworkers^[51] described the *in vitro* proteolytic activity of cauliflower plant extracts capable of processing CLV3 and CLE1 at their C-terminal, not ruling out the possibility that the processing is mediated by unspecific proteases in cauliflower extracts, so further studies are necessary to characterize this process more deeply.

To fill the knowledge gap in this sense, De Marchis and coworkers^[5], in 2018, suggested the possible involvement of the Endoplasmic Reticulum-Associated protein degradation in the rapid maturation process of CLV3 protein. According to this study, ERAD could be directly responsible for the active peptide production or involved in the regulation of the CLV3 effective processing. The stabilizing effect of the proteasome inhibitor MG132 and the CDC48 mutation effect suggests that a relevant proportion of CLV3-GFP newly synthesized polypeptide is an ERAD substrate^[5]. The present study confirmed the rapid degradation of the fusion protein CLV3-GFP observed by this group in several tobacco cell culture systems and *in vivo*: CLV3-GFP was detected intracellularly in tobacco protoplasts, tobacco callus-obtained suspension cultures and tobacco leaf explants, but the full-length fusion protein was not detected in the extracellular space.

In this context, a possible relationship between CLV3 stability and chaperone proteins has also been hypothesized, studying the phenotype of *Arabidopsis shepherd* mutant, which shows reduced expression levels of the endoplasmic reticulum chaperone protein HSP90, called

endoplasmin^[5]. The close similarity between the *shepherd* phenotype and loss-of-function mutants of *CLV* genes suggests a direct or indirect interaction between these chaperone proteins and the CLV system. Biochemical assays conducted under ER stress conditions, using tunicamycin, which causes an inhibition of the glycosylation process and secretion protein synthesis, revealed a strong inhibition of CLV3-GFP expression. The finding that CLV3-GFP is a functional and highly unstable protein is in line with the characteristics of HSP90 clients. Moreover, endoplasmin, in ER-stress conditions CLV3-GFP tobacco protoplasts, completely abolished the tunicamycin effect, demonstrating a connection between these two proteins.

In order to delve deeper into the hypothesis that ERAD has a crucial role in CLV3 processing, ubiquitination step was further investigated: mediated by ubiquitin ligase, which binds Ub-molecules to lysine residues, ubiquitination represents a crucial checkpoint for sending the misfolded protein to the proteasome in ERAD degradation. Previous studies^[35] have highlighted a close relationship between CLV3 and PUB4, an E3 ubiquitin ligase. To investigate the potential role of ubiquitination in CLV3 processing, CLV3-GFP mutant lines (*k-61* and *k-triple*) of tobacco protoplast were homogenated in order to follow protein localisation at intracellular and extracellular level, as in previous experiments. The fusion-protein was detected intracellularly, but not in the apoplast. If ubiquitination plays a role in CLV3 maturation, it would be expected to observe a different behavior of the protein in samples transformed with CLV3-GFP and those transfected with *k*-mutant constructs. A clear sign of the involvement of ubiquitination in CLV3 protein processing would have been to find the intact protein also in the apoplast, suggesting that if the un-ubiquitinated protein is not sent to the proteasome, it is fully secreted. However, the absence of the intact protein in the medium of transformed protoplasts expressing *k*-mutant forms of CLV3 suggests two possible scenarios: ubiquitination may not be decisive for the maturation of the protein since, as previously described by De Marchis et al.^[5], ERAD mechanism could represent a regulatory mechanism and not the maturation process itself. However, if the ERAD process is really involved in this maturation, it would be possible that CLV3, recognized as misfolded by chaperone proteins but not being ubiquitinated, is not even secreted but it remains blocked in the endoplasmic reticulum "waiting" to begin its processing. This hypothesis would agree with what was observed by De Marchis et al.^[5], which shows that the stability of the protein under ER-stressed conditions is strongly increased upon co-expression with endoplasmin, suggesting an important link between CLV3 and chaperones. To explore this topic further, the stability of

the protein at the intracellular level should first be verified with techniques that allow us to follow proteins in time. A pulse-chase analysis would reveal whether, in the *k*-mutant CLV3-GFP lines, where presumably ubiquitination does not occur, the protein remains stable over time at the intracellular level rather than being rapidly degraded, as previously observed. Confocal microscopy would also help verify the fusion protein's localisation in both transiently transformed protoplasts and stable-transformed tobacco plants for the *k*-mutant pGreen.CLV3-GFP: this technique could reveal protein's accumulation in specific cellular compartments, presumably in the Endoplasmic Reticulum. This aspect could be further studied by isopycnic gradient, followed by Immunoblot, which would highlight the co-localisation of the protein of interest with marker proteins of specific cellular compartments, such as the endoplasmin/BiP chaperone proteins for the Endoplasmic Reticulum.

To further investigate whether the intact CLV3 protein is matured in a different biosystem than the original one, *C. reinhardtii*, which lacks *CLV3* gene and meristematic tissue organization, was successfully transformed. Intra- and extracellular localisation of the protein confirmed the secretion of the full-length CLV3-YFP fusion protein. Algae culture medium showed no effects on the growth of *Arabidopsis* roots, which develop strongly shortened and branched root phenotype in presence of the active dodecapeptide pCLV3. This evidence suggests that, in microalgae, CLV3 protein is not recognized as misfolded and therefore is not processed but secreted in its whole form in the extracellular space. In this case, the whole protein would not be able to perform its function on *Arabidopsis* roots: however, to confirm these hypothesis, qualitative analyses proving the absence of the active pCLV3 or unspecific degradation products in the algae medium are required.

If all these hypotheses are true, these results suggest that the maturation mechanism of the CLV3 protein has changed over time: however, the proposal of this alternative protein maturation pathway raises pertinent questions regarding both the mechanism of CLV3 peptide secretion and the alignment of the results presented here with the existing literature on CLV3. The hypothesis that the secretory nature of the CLV3 protein, recognizable by its sequence, has changed during evolution is therefore still open: the study of the CLV3 signalling still requires further investigation to shed light on the maturation process of this regulatory protein.

Conclusion

Plant Molecular Farming (PMF) is becoming a viable method for creating recombinant pharmaceuticals and for natural product synthesis, for which consumer demand is constantly increasing. Its capacity to produce safe, high-quality, and physiologically active proteins has been well documented: plant *in vitro* culture is an innovative method that makes it possible to produce added-value compounds regardless of seasonal, regional, or environmental fluctuations, keeping extraction costs relatively low. The majority of the benefits of plant-based expression systems are carried over by plant cell culture, most notably the capacity to create complex proteins that are correctly glycosylated, folded, and assembled without contamination by endotoxins and pathogens^[52].

Plant usage for human health is also expanding due to recent advances in plant genetic engineering, which make it possible to produce plants with desired characteristics and properties: different techniques for genetic transformation have been addressed in this thesis, many of which have been in use for more than forty years ^[53]. Without mentioning the difficulties of a stable, high-frequency transformation, the traditional tissue culture technique involves many stages after gene delivery, including callus induction, proliferation, rooting and somatic-embryos formation: tissue culture could represent a bottleneck for the creation of plants with altered genes and for creating new features to enhance agricultural types ^[54].

Due to significant genotype dependence, the callus proliferation phase after transformation is usually the most time-consuming and rate-limiting step: in this sense, recent research has demonstrated the strong correlation between the expression of many plant-morphogenetic regulators, such as *BABYBOOM* and *WUSCHEL*, and the genetic transformation efficiency of plant material in both dicots and monocots^[55,56]. These studies have demonstrated how combinatorial expression of *WUS*, *MERISTEMLESS*, and *BABYBOOM* can effectively induce meristem induction *de novo*, leading to the expansion of somatic embryos also in recalcitrant species, in addition to agroinfection efficiency improvement^[57]: gene manipulation is promising for developing genotype-independent genetic transformation techniques in a wide range of crops^[58].

The study presented in Section 2 of this thesis about the maturation of the regulatory protein CLV3, the main antagonist of WUS, could, therefore, reveal an interesting regulatory approach for the multiple applications of PMF presented in Section 1: the fine control of this important stem cell regulator in the SAM could represent an essential turning point for the transformation

of plant species, callogenesis and organogenesis induction, leading to a significant reduction of time and higher control of plant material regeneration.

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