UNIVERSITAT DE BARCELONA

New strategies for improving the biotechnological production of pharmaceuticals and biopharmaceuticals in plant cell and organ cultures

Diego Alberto Hidalgo Martínez

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FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

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2017

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FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

DOCTORAT EN BIOTECNOLOGÍA

New strategies for improving the biotechnological production of pharmaceuticals and biopharmaceuticals in plant cell and organ cultures

Memòria presentada per Diego Alberto Hidalgo Martínez per optar al títol de doctor per la Universitat de Barcelona

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Doctorando

A mis padres por su gran apoyo, amor y confianza

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Outline of the PhD thesis

The thesis is presented as a compendium of publications and includes six different sections: General Introduction, Objectives, Results, General Discussion, Conclusions and References.

The General Introduction summarizes the state of the art of Plant Cell and Organ Cultures (Plant Biofactories) and their applications for the pipeline production of Cosme-, Pharma- and Biopharmaceuticals.

Objectives describes the overall aim of the PhD thesis, as well as the specific goals of each chapter of the Results section.

The five chapters of the Results section are based on published scientific articles in ISI journals, where the PhD candidate is always the first author and in each article and when possible according with the editorial rules, it is mentioned that it forms part of his PhD thesis. Each chapter clearly refers to a particular goal of Plant Biofactories employed for the production of biologically active high added-value compounds.

Briefly:

a) **Chapter 1** aims to demonstrate the effectiveness of plant in vitro cultures of *Centella asiatica* for the production of centellosides in optimized conditions by adding elicitors and potential precursors. Centellosides are widely utilized for their therapeutical properties and *C. asiatica* cell lines are also currently applied as cosmetic ingredients.

b) **Chapter 2** focuses on the biotechnological production of *t*-resveratrol, a bioactive compound found in wine and other foods, in engineered hairy roots of tobacco. The study aims to demonstrate the power of metabolic engineering tools for designing new organisms with modified metabolic capacities and their utility for the production of scarcely distributed plant compounds.

c) **Chapter 3** provides a paradigmatic example of how a metabolic pathway can be extended in a host organism by means of metabolic engineering tools. *Silybum marianum* cell cultures, which have the natural ability to produce polyphenol silymarins, are shown

to have a previously unexplored capacity for the heterologous production of *t*-resveratrol, a new polyphenol in this plant species.

d) **Chapter 4** aims to demonstrate the broad capacity of plant cells for bioconverting exogenous substrates to target products, showing that tobacco hairy roots and their derived cell cultures can biotransform *t*-resveratrol into the more active metabolites *t*-piceatannol and *t*-pterostilbene, both high added-value compounds scarcely distributed in nature.

e) **Chapter 5** describes the production of the truncated human tissue plasminogen activator (K2S) in tobacco cell cultures derived from transplastomic plants, aiming to avoid the disadvantages of biopharmaceutical production in field transgenic crops.

The Discussion section includes a general discussion of the main results obtained and described in the five chapters of the PhD thesis in the context of recent studies and the objectives of the thesis.

In the Conclusions section, the main conclusions derived from the results reported in the five chapters are briefly summarized.

The References include the complete references of all the articles cited in the general sections of the PhD thesis.

Introduction

Plant Biofactories Based on Cell and Hairy Root Cultures: Current Status and Future Challenges

Plant biotechnology has advanced enormously in recent decades and today has a considerable social impact. Two key sets of tools, developed in the last century, are responsible for this progress: *in vitro* plant cultures and genetic engineering techniques. Besides allowing a rapid and efficient clonal micropropagation of many plant species, *in vitro* plant culture has led to the development of plant cell factories, in which cell suspensions are established from undifferentiated cells of plant calli. Cells are grown individually, as if they were micro-organisms, and can be cultivated in bioreactors on a large scale for the production of valuable compounds for the chemical-pharmaceutical industry (Georgiev et al., 2009). Currently, these cells are also being applied as cosmetic ingredients, the so-called “plant stem cells”, whose commercial value has increased dramatically in recent years (see **Chapter 1**).

Many natural compounds with pharmaceutical, nutraceutical or cosmeceutical properties are plant secondary metabolites. If their biosynthesis in the plant is restricted to a specific organ and structure-formation, they cannot always be biosynthesized in unorganized systems like plant cell cultures, and their production relies on the establishment of organ cultures such as roots or shoots (Hussain et al., 2012). In this context, hairy root cultures obtained by genetic transformation of the plant material with a pathogenic strain of *Agrobacterium rhizogenes* provide fast-growing plant material that normally produces the same spectrum of secondary metabolites as the roots of the original plant (Pistelli et al., 2010). Like plant cell cultures, hairy roots have been exploited for the production of plant bioactive compounds scarcely distributed in nature and whose complex chemical structures make their chemical synthesis unviable. In this scenario, plant biofactories based on plant cell and hairy root cultures represent a promising alternative to plant crop cultures (**Fig. 1**). In the last decades, huge efforts have been made to develop plant biofactories at an industrial level for the production of high added-value bioactive compounds, including shikonin, taxol and berberine, among other phytochemicals (Sharma et al., 2014).

More recently, metabolic engineering tools have been applied to modify plant genomes and design new plants and cell lines with an improved capacity for the production of specific plant secondary compounds (**Fig. 1**). This strategy depends on the identification of metabolic pathway bottlenecks, lateral branches competing for carbon flux and transcription factors that activate plant secondary metabolite routes (Capell & Christou, 2004). Furthermore, considering the widespread

social rejection of transgenic plants, biotechnological platforms based on transgenic cell or root cultures growing in bioreactors avoid any risk of transgene dissemination. They therefore constitute a safer and more socially acceptable system than transgenic crop cultures for the production of valuable plant compounds.

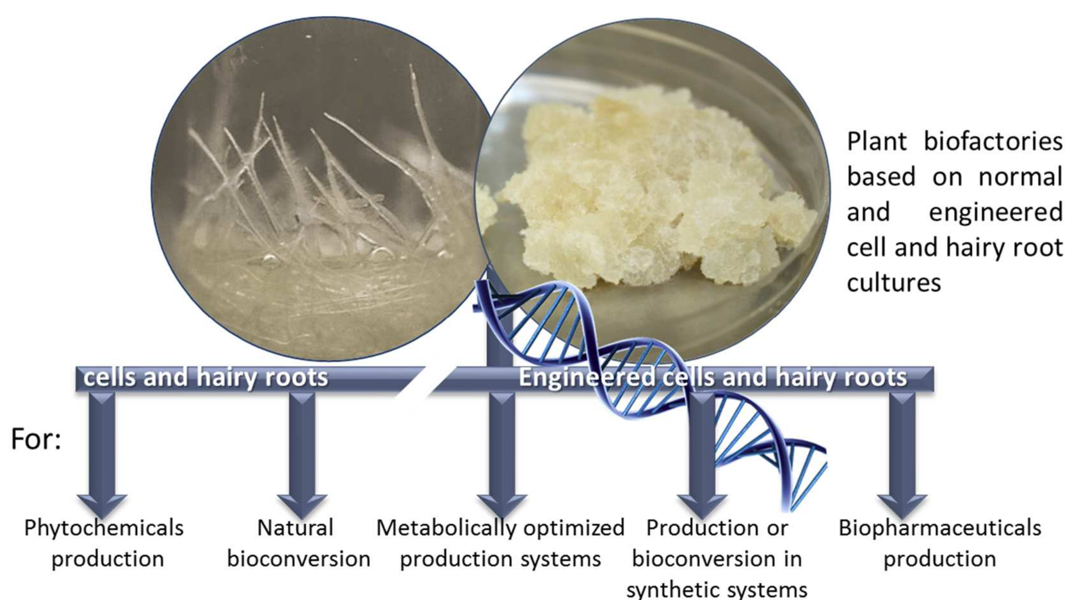

Fig. 1. Principles and main applications of Plant Biofactories based on plant cell and hairy root cultures.

Based on the vast metabolic capacity of plants and their richness in often non-specific enzymes such as hydroxylases, glucosidases, and methyltransferases, plant cell and hairy root cultures have also been used for the bioconversion of exogenous substrates into more active or bioavailable products with improved or new pharmacological activities (Giri et al., 2001; Ishihara et al., 2003) (**Fig. 1**). With the help of metabolic engineering tools, the heterologous expression of foreign genes in *in vitro* cultures is also possible. Equipped with new metabolic capacities, the resulting culture system can convert particular substrates to other bioactive compounds scarcely produced in nature. Also, a biosynthetic plant pathway can be transferred totally or partially from a plant species to a plant cell culture of another plant species (Miralpeix et al., 2013) (**Fig. 1**). This is the case of the pioneering conversion of hyoscyamine into scopolamine utilizing tobacco hairy root and cell cultures as expression hosts for the *Hyoscyamus niger* hyoscyamine-6β- hydroxylase gene (Häkkinen et al., 2005), or more recently piceatannol production in *Vitis vinifera* cell cultures by the heterologous expression of the human cytochrome P450 hydroxylase 1B1 (Martínez-Márquez et al., 2016).

The biopharmaceutical market is currently growing faster than that of pharmaceuticals, and is predicted to reach $US 278.2 billion by 2020 (PMR, 2015). To meet this demand, genetic engineering techniques have been applied to engineer bacterial, yeast and mammalian cells for the production of recombinant proteins with therapeutic properties. In this context, compared with mammalian cells, plant cell cultures offer several advantages for the production of biopharmaceuticals. They are cost effective due to the rapid cell growth in inexpensive chemically defined media, and as eukaryotic systems they have the capacity to correctly fold and assemble multimeric proteins, unlike yeast and bacteria (Santos et al., 2016). Plant cell cultures are also safer than other production systems, being free of mammalian-derived components and bacterial toxins in the production process, and thereby constitute a promising approach for the production of therapeutic proteins (Doran, 2000).

The plethora of potential applications of biotechnological platforms based on plant cell and organ cultures justify the considerable scientific research focused on demonstrating their efficiency for the production of pharma-, biopharma-, nutra- and cosmeceuticals. Work in this field is currently so extensive that the number of related publications is too high to be included or even summarized in a single review. Our aim here is therefore to offer a global vision of the state of the art of plant biofactories in their multiple industrial applications as well as future challenges. Some of the references included are excellent reviews dealing specifically with each of these different applications in the pursuit of bioactive compound production.

Secondary metabolite production in plant biofactories

Secondary metabolism provides plants with a vast array of chemical signals through which they can interact with their environment. Many of them form part of defense systems, helping to protect plants from attack by pathogens and predators, and are synthesized in specific tissues and organs (Freeman and Beattie, 2008). In addition, many secondary metabolites have important biological activities and are responsible for the therapeutic properties of medicinal plants. Overexploitation of wild plants to obtain these compounds has left many species endangered (Georgiev et al., 2009). Since the production of secondary metabolites in plants is influenced by geographical and climatic factors, their cultivation in the field can be difficult (Mishra, 2016). In addition, secondary metabolites often accumulate in plants in tiny amounts and are consequently very scarce in nature. Moreover, their highly complex chemical structures, with numerous chiral centers, make their chemical synthesis unfeasible (Smetanska, 2008).

Their scarcity in nature and high added-value has conditioned the search for new biosustainable sources for these compounds. In this scenario, *in vitro* plant cultures have been developed with the aim of achieving bioactive secondary metabolite production similar to or greater than in plant crops, giving rise to the so-called plant biofactories (Oksman-Caldentey and Inzé, 2004). These new biotechnological systems are based on plant cell and organ cultures and are currently being implemented at an industrial level for the production of several phytochemicals. Examples include arbutin and berberine manufactured by Mitsui Chemicals, Inc.; gingseng by Nitto Denko Corporation; paclitaxel by Phyton Biotech Inc. and Samyang Genex, or shikonin by Mitsui Chemicals Inc. (Sharma et al., 2014). The production systems most frequently used in plant biofactories are cell suspension and immobilized cell cultures. However, when the production of the bioactive compound is not achieved in undifferentiated tissues, hairy root and shoot cultures offer an alternative (Hussain et al. 2012) (**Fig. 2**).

Plant *in vitro* techniques are time-consuming because general rules cannot be defined for the development of callus cultures. Normally, several nutrient media supplemented with different combinations of plant growth regulators (PGRs) have to be tested to obtain an optimum system for the formation of friable calli, which are subsequently disintegrated in liquid medium to obtain the cell suspension cultures. To achieve a high production of secondary metabolites, it is always necessary to optimize the biotechnological process using both empirical and rational approaches (Onrubia et al., 2013). Several steps and parameters need to be considered in order to produce a

high quantity of biomass together with a high accumulation of the target secondary metabolites. These include **a)** the selection of a highly productive cell line; **b)** optimization of the culture parameters (T^a, medium nutrient composition, type and combination of PGRs, light or dark conditions, etc.); **c)** use of elicitors, precursors and permeabilizing agents, which can also play an important role in the bioprocess by improving the productivity and facilitating downstream processes; and d) scaling up the process to bioreactor level (**BOX 1**) (Rao and Ravishankar, 2002).

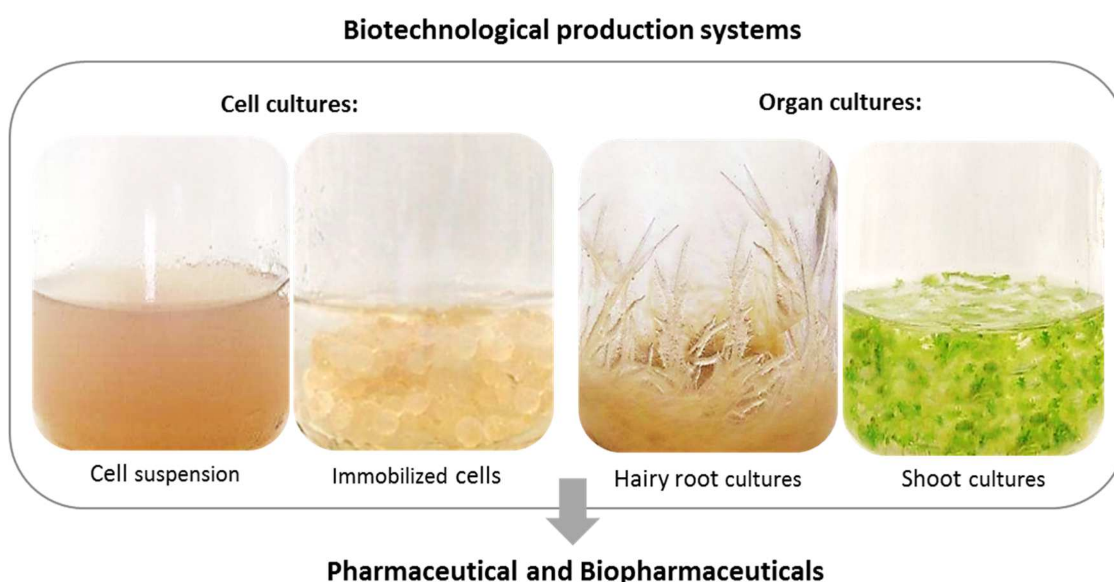

Fig. 2. Most frequently used plant cell and organ systems for the production of pharmaceuticals and biopharmaceuticals

BOX 1

a) Highly productive cell line selection.

b) Optimization of culture parameters.

c) Treatment with elicitors, adding precursors and permeabilizing agents.

d) Scale up to bioreactor level.

In this context, following optimization processes, the production of the anticancer compound taxol in *Taxus baccata* cell lines increased more than 298-fold (Cusido et al., 2014); the antioxidant rosmarinic acid raised more than 3.2-fold in cell cultures of *Satureja kuzistanica*, reaching a final concentration of 244 mg/g dry weight (DW) (Khojasteh et al., 2016), one of the highest achieved to date; and the yield of centellosides, the triterpene saponins of *Centella asiatica,* was improved more than 8.8-fold in cell suspensions (see **Chapter 1**). Table 1 includes some examples of the effectiveness of optimization processes for improving the biotechnological production of plant bioactive compounds.

Plant cells are sensitive to shear forces and the culture medium is difficult to change in cell suspensions, especially when working at bioreactor level. To address these difficulties, cell immobilization protocols have been developed (**Fig. 2**) (Brodelius, 1985). Plant cells can be easily immobilized by microencapsulation in sugar polymers such as alginate, which forms beads in the presence of ion calcium, entrapping the plant cells inside. An alternative method is by adhesion to inert matrices, most typically polyurethane foam or hollow glass fiber (Tatli, 2012).

Table 1. Some examples of improved production of phytochemicals in plant biofactories in optimized processes.

Species	Compound	Culture conditions	Content	Improvement
Taxus baccata	Total taxanes	Initial Optimized	3.5 mg/L 1045 mg/L	298.5
Satureja khuzistanica	Rosmarinic acid	Initial Optimized	76 mg/g DW 244 mg/g DW	3.2
Centella asiatica	Total centellosides	Initial Optimized	190 mg/g DW 1669 mg/g DW	8.8

T. baccata. Optimized system (elicitors, permeabilizing agent): CORO + CDs (Cusido et al. 2014)
S. khuzistanica. Optimized system (elicitors, permeabilizing agent): MeJA (Khojasteh et al. 2016)
C. asiatica. Optimized system (elicitors, precursors): CORO + MER (see **Chapter 1**)
MeJA: methyl jasmonate; CORO: coronatine; CDs: cyclodextrin; MER: Manila elemi resin.

Compared to cell suspensions, immobilized cells have the following advantages: **a)** the process can run continuously, while the product is released from the cells and separated from the culture medium; **b)** the culture medium can be changed more easily, allowing the quick addition or removal of several compounds**; c)** the biomass is rejuvenated *in situ* by perfusion of the growth

medium to the cells at different time intervals; **d)** relatively low amounts of biomass are used efficiently (**BOX 2**) (Brodelius, 1985).

BOX 2

a) Run in continuous mode if the product is released to culture medium.

b) Culture medium easy to change.

c) Rejuvenation of cell biomass by perfusion of growth culture medium.

d) Efficient use of low amount of plant biomass.

The production of target compounds in cell cultures can be very low compared to the whole plant, reflecting the tendency for secondary products to be synthesized by cooperating groups of cells that have reached a particular state of differentiation (Kolewe et al., 2008). A solution to this constraint is to develop plant biofactories based on organized *in vitro* cultures like roots and shoots, consisting of differentiated cells or tissues of multiple interacting cell types involved in the same plant secondary metabolite pathway (**Fig. 2**). In this context, approaches based on hairy root cultures have long been applied for the biotechnological production of pharmaceuticals.

Hairy root cultures are obtained by infection of the plant material with the soil pathogenic bacteria *Agrobacterium rhizogenes*. During this process, *Agrobacterium* transfers a section of DNA from its plasmid, the T-DNA, into the genome of host plant cells. Specifically, T-DNA contains the so-called rol genes responsible for the development of the characteristic phenotype displayed by hairy roots (Georgiev et al., 2012). The role of the T-DNA genes in the activation of secondary metabolism has been discussed (Matveeva et al. 2015). In *in vitro* conditions, roots appear in the plant material at points of bacterial infection, and can be excised and cultured separately, without requiring the addition of plant growth regulators. In a first stage, the roots are cultivated in solid medium, supplemented with antibiotics to eliminate the agrobacteria, and are later transferred to a liquid medium to generate a high quantity of biomass (**Fig. 3**).

In most cases, before scaling up the hairy root cultures to bioreactor level, it is necessary to optimize the culture conditions in shake flasks to maximize the biomass and target compound production. In these circumstances, several factors are essential to reach good product yields, including the selection of a suitable culture medium, elicitors and permeabilizing agents (Ono and Tian, 2011). A plethora of hairy root cultures have been established for the biotechnological production of plant secondary compounds with biological activities (**Table 2**), and currently,

several companies such as Gree2chem from Belgium and Naturex from France are involved in the production of plant bioactive compounds in hairy root cultures (http://www.green2chem.com, accessed on 25/11/2016).

Fig. 3. Sequential steps to develop a plant biofactory based on hairy root culture platforms. a) root induction; b) root line isolation; c) root culture; d) bioreactor culture.

As plant cells have different traits compared to microorganisms, scaling up plant cell cultures to bioreactors can generate various problems stemming from their larger size and shear sensitivity, together with a low doubling time, which conditions a longer fermentation period. However, only slight modifications of classical bioreactors designed for microbial cells can be enough to resolve these issues, and stainless steel stirred tank, bubble column and air-lift reactors have been successfully used to scale up plant cell cultures (Georgiev et al., 2013 and the literature cited therein). Single-use bioreactors, originally developed for highly sensitive mammalian cell culture, are increasingly being used for plant cells that do not exhibit Newtonian fluid behavior: for example, wave-mixed bioreactors for *S. khuzistanica* cell cultures (Khojasteh et al., 2016) and an orbitally shaken CellBag bioreactor for *C. asiatica* cell suspensions (see **Chapter 1**, **BOX 3**).

Table 2. Some examples of the production of phytochemicals in optimized biotechnological systems based on hairy root cultures.

Plant species	Active ingredient	Biological activity	Reference
Cinchona ledgeriana	Quinine	Antimalarial	Hamill et al. 1989
Ajuga reptans	Phytoecdysteroids	Hormones	Matsumoto & Tanaka, 1991
Nicotiana tabacum	Nicotine	Insecticide	Palazon et al. 1998
Datura stamonium	Hyoscyamine	Anticholinergic	Piñol et al. 1999
Artemisia annua	Artemisin	Antimalarial	Rao et al. 1998
Duboisia hybrid *Datura metel* *Hyoscyamus muticus*	Scopolamine and Hyoscyamine	Anticholinergics	Palazon et al. 2003b Moyano et al. 2003 Moyano et al. 2003
Panax ginseng	Ginsenosides	Adaptogens	Palazon et al. 2003a
Scutellaria baicalensis	Baicalin	Antiinflamatory	Hwang 2006
Agastache rugosa	Rosmarinic acid	Antioxidant	Lee et al. 2007
Centella asiatica	Asiaticoside	Healing	Kim et al. 2007
Silybum marianum	Silymarin	Hepatoprotective	Rahnama et al. 2008
Vitania coagulans	Vithanolide A and vithaferin A	Alzheimer Anticancer	Mirjalili et al. 2009
Arnebia hispidissima	Sikonin	Red pigment	Chaudhury et al. 2010
Linum album	Podophyllotoxin	Antimitotic	Chashmi et al. 2013
Dracocephalum kotschyi	Rosmarinic acid	Antioxidant	Fattahi et al. 2013
Coleus forskohlii	Forskolin	Antiobesity	Pandey et al. 2014
V. xanthophoeniceum	Verbascoside	Antioxidant	Alipieva et al. 2014
Taxus x media	Taxol	Anticancerigen	Sykłowska-Baranek et al. 2015
Astragalus membranaceus	Isoflavonoids	Antioxidant	Jiao et al. 2015
Hyperycum spp.	Xanthones	Antifungal	Zubricka et al. 2015
Ajuga bracteosa	Phytoecdysteroids	Hormones	Kayani et al. 2016
Salvia miltiorrhiza	Salvianolic acids	Antiinflamatory	Wang et al. 2016
Stevia rebaudiana	Stevioside	Sweetener	Pandey et al. 2016

It is more difficult to accommodate the specific requirements of plant organ cultures such as hairy roots in bioreactors, which should provide a low-shear environment and ensure reduced mass transfer limitations (Georgiev, 2012). Stirred-tanks can be adapted by using a steel mesh, and pneumatically driven systems such as bubble-column and airlift bioreactors have also been utilized (Mishra et al., 2008 and references therein). Mist and wave-induced bioreactors and several temporary immersion systems are able to deal with the specific requirements of hairy root cultures (Georgiev et al., 2013). Advances in the design of bioreactors have facilitated the industrial implementation of root cultures and currently the South Korean CBN Biotech Company is producing *Panax ginseng* roots in a bioreactor of 10 m^3 (Baque et al., 2012).

BOX 3

The biological activities of the main active components of *C. asiatica* extracts, such as antipyretic, diuretic, and antibacterial, and the use of *C. asiatica* plant stem cells as cosmetic ingredients have generated considerable interest in developing cell cultures of this plant species with improved centelloside accumulation at the cellular level. Recently, our research group treated a *C. asiatica* cell line developed in our laboratory with the new elicitor coronatine (CORO), an analogue of methyl jasmonate (MeJA). CORO was found to have powerful elicitor activity and increased centelloside production (more than five-fold) without altering the centelloside pattern and growth capacity of the cell cultures, even at a concentration 100-fold lower than MeJA (see **Chapter 1**). Taken as a whole, our results, together with those previously described in this section, have fulfilled one of the main aims of this PhD thesis, that is, to demonstrate that Plant Cell Factories constitute a sustainable alternative to plant crops in the field for the production of important pharmaceuticals.

Recommended reviews to gain further insight into plant cell factories for pharmaceutical production:

- Rao SR, Ravishankar GA. Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnol Adv.* **2002**, 20:101-153.
- Oksman-Caldentey KM1, Inzé D. Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. *Trends Plant Sci.* **2004**, 9: 433-440.
- Georgiev MI, Weber J, Maciuk A. Bioprocessing of plant cell cultures for mass production of targeted compounds. *Appl Microbiol Biotechnol.* **2009**, 83: 809-823.
- Karappusamy, S.A. A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. *J Med Plants Res.* **2009**, 3: 1222-1239.

Biotransformation reactions in plant biofactories

Most of the steps involved in plant secondary pathways are catalyzed by enzymes and although the majority have not yet been elucidated, thousands of enzymatic proteins are known to be involved in plant secondary metabolism. Some of them have high specificity for a substrate, whereas others are unspecific and can produce multiple products from different substrates (Miralpeix et al., 2013). Specifically, the core structure of a plant secondary metabolite is frequently conducted by specific enzymes but further modifications including hydroxylations, oxidation-reductions or glycosyl conjugations could be performed by more unspecific enzymes. This fact leads to the use of plant biofactories for biotransformation of exogenous substrates (Ishihara et al., 2003).

Biotransformation processes based on the metabolic capacity of cell suspension cultures as well as immobilized cells and hairy root cultures can be utilized to convert cheap and plentiful substrates into rare and expensive products with improved or new biological activities (Rao and Ravishankar, 2002). Plant cells and hairy root cultures have advantages over enzymatic preparations in that they are able to catalyze stereospecific reactions leading to chirally pure products. They can also perform regiospecific modifications difficult to achieve by chemical synthesis or in biotransformation by microorganisms (Miralpeix et al., 2013).

The use of plant cell cultures for biocatalysis has various benefits (Ishihara et al., 2003): **a)** the cultured cells can accumulate high amounts of the bioconverted products; **b)** the growth cycle for bioconversion are only 1-2 weeks; **c)** the cells can be cultured up to an almost unlimited quantity of biological material. Plant cell cultures also have some constraints: **i)** the necessity to supply enzymes; **ii)** the substrate and product cannot be toxic for the cells; **iii)** the substrate must reach the cellular compartment of the enzyme (**BOX 4**) (Rao and Ravishankar, 2002).

Regio- and stereoselective hydroxylations, glucosylations as well as oxidation-reductions between alcohols and ketones, epoxidations, reductions of C-C double bonds, among other reactions, performed by plant cells from a variety of substrates have been reviewed and summarized previously (Guiri et al., 2001; Ishihara et al., 2003). Some of them are included in **Table 3**, together with more recent examples, illustrating the feasibility of using plant cell cultures to execute these enzymatic reactions.

BOX 4

Advantages:

a) Plant cells accumulate high amounts of target products.

b) Bioconversion cycles of 1-2 weeks.

c) Unlimited plant biomass production.

Weakness:

a) Presence of enzymes required.

b) Substrates and products toxic for plants excluded.

c) Enzymatic subcellular compartmentation.

In this context, Zhua et al. (2010) demonstrated the capacity of *Catharanthus roseus* and *Panax quinquefolium* cell cultures for regio- and stereo-selective hydroxylation of dehydroartemisic acid and artemisic acid., Shimoda et al. (2010) reported the bioconversion of naringin and naringenin to several β-D-glucopyranoside derivatives in *Eucalyptus periniana* cell cultures, and more recently, Häkkinen et al. (2015) described raspberry ketone production by feeding cell cultures of *Plumbago auriculata*, *Hordeum vulgare*, *Rubus articus*, *R. chamaemorus*, *R. idacus* and *Nicotiana tabacum* with *p*-hydroxybenzalacetone or betuligenol.

The potential bioconversion capacity of plant cell cultures also has been harnessed in feeding experiments with putative precursors of high added-value products. In *Centella asiatica* cell suspensions, when α-amyrin was added to the culture together with the permeabilizing agent DMSO, nearly 50% penetrated the cells and over 80% of this was converted into centellosides (Mangas et al., 2008). Although α-amyrin has a high price on the market, some natural resins are rich in amyrin, including Bursera, Protium, Copal and Manila elemi resin (Hernandez-Vazquez et al., 2012). Thus, we recently used Copal extract and Manila elemi resin to increase the centelloside production in coronatine-elicited *C. asiatica* cell cultures (see **Chapter 1**, **BOX 5**).

The use of hairy root cultures as a suitable matrix for bioconversions is gaining ground over plant cell cultures because of their numerous advantages, including biochemical and genetic stability, lower sensitivity to variations in culture conditions, an enzymatic potential very similar to that of the parent plants, and low cost (Banerjee et al., 2012, Srivastava et al., 2016). Some of the myriad studies on the bioconversion of exogenous subtrates in hairy root cultures are also included in **Table 3**.

One of the characteristics of biotransformations mediated by hairy root cultures is their regio-specificity, as demonstrated by the glucosylation of betuligenol into betuloside in *Atropa belladona* (Srivastava et al., 2013). Glycosyl conjugation has also been achieved in *Datura tatula* hairy root cultures, in which 4-hydroxyybenzyl alcohol was glucosidated to gastrodin, an active component of tall gastrodia tuber (Peng et al., 2010). Other types of bioconversions besides glucosylations have been carried out. Recently, Häkkinen et al. (2015) reported the capacity of hairy root cultures of *C. roseus* for reducing 4-hydroxybenzalacetone into raspberry ketone, achieving a higher rate conversion than cell cultures of *Rubus chamaemorys* or *R. idaeus*. Also, we demonstrated that unspecific hydroxylases and methoxylases of tobacco can carry out the bioconversion of *t*-resveratrol, added exogenously to the hairy root cultures, into the more active derivatives piceatannol and pterostilbene (see **Chapter 4**, **BOX 5**).

BOX 5

Another example of the extensive metabolic capacities of plant cell and organ cultures to biotransform exogenous substrates has been provided. The capacity of C. asiatica cell cultures to biotransform precursor amyrins into bioactive centellosides has been previously demonstrated, but the high price of these precursors impedes the industrial application of the system. For this reason, we utilized Copal and Manila elemi resins as rich and sustainable amyrin sources to feed C. asiatica plant cell cultures optimally elicited with coronatine (CORO). Our results showed a high bioconversion rate and the cultures achieved a production 70-times higher than the untreated cultures (see Chapter 1). Similarly, tobacco hairy cultures treated with the exogenous substrate t-resveratrol were able to bioconvert this compound into its derivatives piceatannol and pterostilbene, which have superior pharmacological activities (see Chapter 4). Thus, another of the aims of this PhD thesis, to show the immense metabolic capacity of Plant Cell Biofactories to bioconvert exogenous substrates, was fulfilled.

Table 3. Examples of biotransformations carried out in plant cell and hairy root cultures (for complete review, see Guiri et al., 2001; Ishihara et al., 2003; Banerjee et al., 2012; Srivastava et al. 2016).

Plant cell cultures				
Plant species	Substrate	Product	Reaction type	Reference
N. tabacum	Linalool	Dihydrolinalool	Hydroxylation	Hirata et al. 1981
N. tabacum	α-terpinol	Hydroxy-terpinol		Suga et al. 1983
Catharanthus roseus	Warferin	Warferin alcohol		Hamada et al. 1993
Catharanthus Roseus	Geraniol, nerol, carvone	Hydroxyneo-dihydrocarveol		Hamada et al. 1997
Astasia longa	Carvone	Dihydrocarvone, Isodihydrocarveol		Shimoda & Hirata 2000
Glycyrrhiza Glabra	Papaverine	Papaverinol		Dorisse et al. 1988
Pinus radiata	6-n-pentyl-2H-pyran-2-one	5-(2-Pyron-6-yl)pentan-5-ol		Cooney et al. 2000
Spidorela punctata	3-Alkyl substituted derivative of citronellol and citronellic acid	Derivatives with a hydroxyl group on C-6 or C-7 and the doubled bond at C-7, C-8 and C-5		Pawlowicz et al. 1992
Centella asiática	Thiocolchicine	2-O- and 3-O. onoglucosyl derivatives	Glucosidation	Solet et al. 1993
Crocus sativus	Crocetin	Crocetin di-neapolifanosyl		Dufresne et al. 1999
Papaver somniferum	Silybin	Silybin-7-O-β-D glucopyranoside		Kren et al. 1998
Linum flavum	Deoxypodophy-llotoxin	5-Methoxy-podophyllotoxin β-D-glucoside		Van Uden et al. 1997
Nicotiana plumbaginifolia	Butyric acid	6-O-Butyryl-D-glucose		Kamel et al. 1992
Mytillocatus geometricus	D_2-Carene	Carenols	Red-Ox	Gil et al. 1995
Papaver somniferum	Codeinone	Codeine		Corchete & Yeoman 1989
Daucus carota	Codeinone	Codeine		Lucero et al. 1999

Hairy root cultures				Continuation of table 3
Plant species	Substrate	Product	Reaction type	Reference
Panax ginseng	Digitoxigenine	Digitoxigenin stearate	Esterification	Kawaguchi et al. 1990
Lobelia sessilifolia	Gallic acid	β-Glucogallin	Glucosylation	Ishimaru et al. 1996
Pharbatis nil	Umbelliferone	Skimmin		Kanho et al. 2004
Polygonium multiflorum	1,4-benzenediol	Arbutin		Yan et al. 2008b
Physalis ixocarpa	Hydroquinone	Arbutin		Bergier et al. 2008
Polygonum multiflorum	Esculetin	Esculetin glycoside		Zhou et al. 2012
Datura tatula	Hydroxybenzyl alcohol	Gastrodin		Peng et al. 2010.
Cyanotis arachnoidea	Artemisin	Deoxyartemisin	Red-Ox	Zhou et al. 1998
Levisticum officinale	Geraniol	Linalool		Nunes et al. 2009
Levisticum officinale	Geraniol	Citronellol		Nunes et al. 2009
Anethum graveolens	Geraniol	α-Terpineol		Faria et al. 2009
Atropa belladonna	Trimethoxy acetophenone	Trimethoxy phenyl ethanol		Srivastava et al. 2012

Recommended reviews to gain further insight into plant biotransformations:

- Giri A, Dhingra V, Giri CC, Singh A, Ward OP, Narasu ML. Biotransformations using plant cells, organ cultures and enzyme systems: current trends and future prospects. *Biotechnol Adv*. **2001**, 19:175-99.
- Ishihara K, Hamada H, Hirata, Nakajima N. Biotransformation using plant cultured cells. *J. Mol. Catal. B-Enzym.* **2003**, 23: 145–170.
- Banerjee S, Singh S, Ur Rahman L. Biotransformation studies using hairy root cultures - A review. *Biotechnol Adv*. **2012**, 30: 461-468.
- Srivastava V, Mehrotra S, Mishra S. Biotransformation Through Hairy Roots: Perspectives, Outcomes, and Major Challenges. In: Transgenesis and Secondary Metabolism. Jha S. (Ed). **2016**, Reference Series in Phytochemistry. Springer International Publishing. Pp. 1-24.

Metabolic engineering tools applied in Plant Biofactories

In the last decade, industrial interest in plant secondary metabolites with biological activities has increased, yet some of these compounds accumulate in plant tissues in very low quantities. Although plant cell biofactories could provide an alternative source of target compounds, the production achieved by these systems is often far from the requirements of commercial exploitation, even under optimized conditions, and examples of successful industrial biotechnological production of plant secondary metabolites are scarce (Wilson and Roberts, 2012).

Plant metabolic engineering considers the cell as if it were a factory, and focuses in optimizing metabolite production or engineering the formation of new compounds. Thus, metabolic engineering has targeted pathways previously elucidated in plants or microorganisms, with the aim of gaining new insights and modifying plant end-products. A metabolic pathway can be altered by the introduction of genes encoding enzymes, transporter proteins or regulators (Miralpeix et al., 2013). In general, metabolic engineering can be defined as the redirection of enzymatic reactions to improve the yield of high added-value compounds, to produce new compounds or to transfer the production of target metabolites to new organisms (Kirasoyan et al., 2009). Strategies frequently utilized in metabolic engineering are summarized in **BOX 6** (Farré et al., 2014).

BOX 6

a) Increase in enzyme activity.

b) Upstream precursor overexpression.

c) Blocking by gene silencing competitive pathways.

d) Generation of a metabolic sink.

Single-gene metabolic engineering approaches aim to bypass rate-limiting steps in plant secondary metabolism by the overexpression of single genes under the control of strong promoters, and by blocking competitive branching pathways, as well as the catabolism of end-products (Capell and Christou, 2004).

There are several examples of overexpression of rate-limiting genes in plant cell and organ cultures to boost plant secondary metabolite production. In this context, Whitmer et al. (1998)

obtained an increase of terpene indole alkaloids in *C. roseus* cell cultures by overexpression of the strictosidine synthase gene. There have been several attempts to increase the biotechnological production of the anticholinergic alkaloid scopolamine in Solanaceae hairy root cultures. Overexpression of putrescine methyltransferase or hyosyamine 6β-hydroxylase, two rate-limiting enzymes involved in this metabolic pathway, gave variable results depending on the plant species, but in general caused only a small rise in scopolamine levels (Palazon et al., 2008). However, when the enzymes were simultaneously overexpressed in *Hyoscyamus niger* hairy root cultures, scopolamine production increased by more than nine-fold (Zhang et al., 2004).

The overexpression of a single gene sometimes fails due to a lack of precursors, which if added or if their production is enhanced, i.e, by means of elicitation, can effectively improve the biotechnological production of a target compound. In this context, overexpression of the putrescine methyltransferase gene in *Hyoscyamus niger* resulted only in a higher amount of methylputrescine, but when the cell cultures were also elicited with methyl jasmonate, the scopolamine production was enhanced (Mehrotra et al., 2010). Similarly, the overexpression of the taxadiene gene in *Taxus x media* cell cultures increased taxane production, but the highest taxane levels were achieved after elicitation with methyl jasmonate (Exposito et al., 2010).

Other metabolic strategies involving inhibition of competitive pathways have also successfully improved the biotechnological production of target compounds. For instance, Kim et al. (2009) reported a 20-fold increase in the production of pinoresinol and its glucoside by down-regulation of the pinoresinol/lariciresinol reductase gene in *Forsythia* cell cultures. On the other hand, taxoid 14β-hydroxylase, an enzyme that catalyzes C-14 oxygenated taxanes, which are formed by a side-route and may compete with taxol for the same precursors, was blocked by the antisense-induced suppression in *T.media* transgenic cell lines (Li et al., 2011).

Alternatively, the overexpression of transcription factors can be used to redirect the plant metabolism by increasing the expression of several genes involved in the same biosynthetic pathway (Gantet and Memelink, 2002; Grotewold, 2008). This could be a more effective strategy, as it may improve the production of target compounds whose biosynthesis has not yet been completely elucidated (Broun, 2004; Grotewold, 2008; Iwase et al., 2009).

In *C. roseus* cell suspensions, overexpression of the ORCA3 transcription factor increased monoterpene indol alkaloids (van der Fits and Memelink, 2000). The same transcription factor

enhanced the catharanthine contents in hairy root cultures of *C. roseus* but only in combination with the gene encoding geraniol 10-hydroxylase, a cytochrome P450 monooxygenase involved in the indole alkaloid pathway (Wang et al., 2010). The ORCA3 transcription factor has also been used to increase camptothecin production in *Camptotheca acuminata* hairy root cultures (Ni et al. 2011). MYB14 and MYB15, cloned from *Vitis vinifera,* belong to the MYB (V-myb myeloblastosis viral oncogene homolog) family of transcription factors, which act as key regulators of the phenylpropanoid pathway (Lu et al., 2016). Höll et al. (2013) demonstrated that the ectopic expression of MYB15 in grapevine hairy roots increased stilbene synthase gene expression and resveratrol accumulation.

As mentioned, plant metabolic engineering is a multidisciplinary field that combines techniques of biochemistry, genetics, molecular biology and plant physiology. Its two fundamental pillars, however, are genetic engineering techniques and *in vitro* plant cultures, and one of its main stumbling blocks is the genetic transformation of non-model plant material. Transient and stable transformation systems can be used in metabolic engineering experiments aimed at exploring the effect of the up- or down-regulation of genes or transcription factors in a biosynthetic pathway. Several transient transformation techniques such as particle bombardment, tissue electroporation, DNA injection, agro-infiltration and viral expression systems have been successfully applied to enhance gene expression levels in plant species, but they only allow for short-term expression (Lessard et al., 2002; Komarova et al., 2010). In contrast, stable transformation techniques, such as *Agrobacterium*-mediated transformation, incorporate genes into the plant genome and expression is maintained over time; however, a wide range of species are recalcitrant to this transformation system (Newell, 2000; Gelvin, 2003).

To avoid transformation problems, one option is to use genetically more amenable model plants such as *Arabidopsis thaliana* or tobacco. Alternatively, microorganisms are also easier to manipulate genetically and have simpler metabolisms, although these systems require a complete knowledge of the biosynthetic pathway that will be introduced in the heterologous host (Miralpeix et al., 2013). Engineered microbial species are ideal systems for the production of high added-value plant compounds because of their faster doubling time in comparison with plant cell cultures, a simpler culture medium composition, easy genetic transformation and well established scale-up systems at bioreactor level (Wilson and Roberts, 2012).

Table 4. Some examples of metabolic engineering applied to plant cell and hairy root cultures.

Plant cell cultures			
Plant species	**Metabolic approach**	**Target compound**	**Reference**
C. roseus *H. niger* *T. media*	Overexpression rate-limitig steps	Terpene indol alkaloids Methylputrescine Taxanes	Whitmer et al. 1998 Mehrotra et al. 2010 Exposito et al. 2010
Forsythia koreana *T. media*	Inhibition of competitive pathways	Sesamin Taxol	Kim et al. 2009 Li et al. 2011
C. roseus *Zea mays*	Overexpression transcription factors	Terpene indol alkaloids Anthocyanins	Van der Fits & Memelink 2000 Grotewold et al. 1998
Hairy root cultures			
Plant species	**Metabolic approach**	**Target compound**	**Reference**
H. niger *Glycyrrhiza uralensis* *Centella asiatica* *Salvia miltiorrhiza*	Overexpression rate-limitig steps	Scopolamine Flavonoids Centellosides Rosmarinic acid	Zhang et al. 2004 Zhang et al. 2009 Kim et al. 2010 Xiao et al. 2011
C. roseus *Salvia miltiorrhiza*	Inhibition of competitive pathways	Terpene indol alkaloids Rosmarinic acid	Runguphan et al. 2009. Xiao et al. 2011
C. roseus *V. vinifera* *Camptotheca accuminata*	Overexpression transcription factors	Catharanthine Resveratrol Camptothecin	Wang et al. 2010 Höll et al. 2013 Ni et al. 2011

In this scenario, several plant secondary pathways have been reconstituted in a heterologous microbial host. Amorphadiene, a precursor of the powerful antimalarial sesquiterpene artemisin, has been produced in *E. coli* (Martin et al., 2003). The same precursor has also been produced by engineering *Saccharomyces cerevisiae* with a mitochondrial-targeted amorpha-4,11-diene synthase (Farhi et al., 2013). In a complex case study, production of the biosynthetic precursor artemisic acid, which can be chemically converted into artemisin, has also been achieved in yeast (Ro et al., 2006). After further improvement, the process yielded a level of artemisic acid of nearly 25 g/L, which is sufficient for industrial production (Paddon et al., 2013). Similarly, taxadiene, an upstream precursor of the anticancer agent taxol, has been formed in transgenic *E.*

coli strains by ectopic expression of genes involved in the methylerythritol-phosphate pathway and the taxadiene gene, opening the way for taxol production in microorganisms (Ajikumar et al., 2010). As summarized by Lu et al. (2016), several approaches to resveratrol production in microorganisms have been reported, but in all cases the cultures need to be fed with specific resveratrol-precursors.

The transfer of a metabolic pathway from a plant species to a microorganism as the heterologous host requires the introduction of a complex set of genes and codon optimization to improve gene expression. The process can be extraordinarly simplified by gene transfer from one plant to another, since more of the primary precursors of a given secondary metabolic pathway may already be present in the host plant and thus only a small set of genes are required. Accordingly, Vasilev et al. (2014) reported the production of geraniol by heterologous expression of only one gene, the *Valeriana officinalis* geraniol synthase, in several hosts, including *N. tabacum* hairy root and cell cultures, the most productive biotechnological platform being tobacco cell cultures. Vainillin, a phenolic compound widely utilized in food, cosmetics and flavoring, has been biosynthesized in hairy root cultures of *Beta vulgaris* by heterologous expression of the *p*-hydroxycinnamoyl CoA hydratase/lyase of *Pseudomonas fluorescens* from the inherently available precursor ferulic acid (Singh et al., 2015).

Other examples of heterologous gene expression for producing active terpenoids in plant cell cultures, including the taxol precursor taxadiene, are summarized by Ikram et al. (2015). Here, we report the production of resveratrol by heterologous expression of the *Vitis vinifera* stilbene synthase gene in an optimized tobacco hairy root system. Also introduced was the transcription factor *At*MYB12 from *Arabidopsis thaliana*, which has been reported to increase the phenylpropanoid pathway in tobacco and tomato (Misra et al., 2010, Pandey et al., 2015). Additionally, the artificial micro RNA for chalcone synthase (amiRNA CHS) was expressed in order to block flavonoid biosynthesis, which competes with the stilbenoid pathway for the same precursors (see Chapter 2, **BOX 7**).

Although there are still very few examples, metabolic engineering techniques can also be applied to enrich the therapeutic properties of plant extracts, achieving the biosynthesis of new and more active compounds. This is the case for scopolamine production in a low scopolamine-producing *Hyoscyamus niger* genotype. As previously mentioned, scopolamine is a tropane alkaloid extensively used as an anticholinergic agent, while its precursor hyoscyamine has a much more

limited use but is more widely distributed in Solanaceae plants. Zhang et al. (2004) reported the heterologous expression of *Nicotiana tabacum* putrescine N-methyltransferase gene together with the overexpression of the hyoscyamine 6β-hydroxylase (H6H) gene under the control of the promotor 35S for the production of scopolamine in transgenic *H. niger* hairy root cultures. In the same context, heterologous expression of the *Hs*CYP1B1 gene led to the production of piceatannol, a resveratrol derivative with more interesting biological activities (Martínez-Márquez et al., 2016). As part of this PhD thesis, we report the heterologous production of resveratrol in stilbene synthase transformed cell cultures of *Silybum marianum*, a plant species which naturally produce the bioactive flavonolignan silymarin (see **Chapter 3**, **BOX 8**).

Another outstanding possibility offered by metabolic engineering is the biotransformation of exogenous compounds into high added-value products by heterologous expression in host systems based on plant cell and hairy root cultures. In this context, Häkkinen et al. (2005) demonstrated the bioconversion of hyoscyamine into scopolamine by heterologous hyoscyamine-6β-hydroxylase in engineered tobacco hairy root cultures. Moyano et al. (2007) corroborated this biotransformation in tobacco cell lines derived from transgenic hairy roots, although at a lower rate of conversion. As mentioned, Martínez-Márquez et al. (2016) employed human cytochrome P450 hydroxylase 1B1 to produce piceatannol in *Vitis vinifera* cell cultures from endogenous t-resveratrol, previously generated by elicitation of cultures. The advantage here was that it was not necessary to add a reaction substrate to obtain the target product, although its production was very low. In contrast, higher levels were subsequently reached by the bioconversion of exogenous t-resveratrol into piceatannol and pterostilbene in transgenic tobacco hairy root and cell suspension cultures (see **Chapter 4**, **BOX 9**).

BOX 7

t-Resveratrol has a wide-ranging therapeutic potential, yet a scarce distribution in nature. Thus, in this PhD thesis, we aimed to develop a biotechnological platform for the production of t-resveratrol based on genetically engineered hairy roots (HR) of tobacco, carrying stilbene synthase (STS)-encoding genes from Vitis vinifera and/or the transcription factor (TF) AtMYB12 from Arabidopsis thaliana and/or artificial micro RNA for blocking chalcone synthase (amiRNA CHS). This comprehensive study model is based on the over-expression of a TF in order to generate a holistic response in the phenylpropanoid pathway and to coordinate the up-regulation of multiple steps; amiRNA CHS would limit the normal flux through the endogenous CHS enzyme, which competes for the precursors of the imported STS enzyme used for the flux deviation (see Chapter 2). In this system, we have demonstrated the capacity of engineered hairy root cultures to produce t-resveratrol, as well as the natural capacity of tobacco roots to bioconvert t-R in other derivatives, such as piceid and piceatannol. It has been shown that the capacity of the cultures to bioproduce t-resveratrol is highly correlated with the expression level of the STS gene in the different hairy root clones. We have also demonstrated that the expression of the transcription factor AtMYB12 is strongly correlated with the increase of flavonoid levels in the culture and a high metabolome perturbation of the system, affecting both primary and secondary metabolism. In contrast, the root lines carrying amiRNA CHS showed the lowest flavonoid levels, due to the blocking of this branching point of the stilbene biosynthetic pathway, and the consequent generation of a greater flow of precursors for the production of resveratrol and its glucoside piceid.

BOX 8

The transfer of a complex plant secondary metabolic pathway to a microorganism normally requires the incorporation of a complex set of genes. Silybum marianum is the main source of the bioactive flavonolignan silymarin. Phenylpropanoid synthesis in cell cultures of this species was demonstrated, with products of the pathway (coniferyl alcohol and some isomers of the silymarin complex) being released to the extracellular medium. Given that stilbene synthase shares the same key precursors involved in flavonoid and/or monolignol biosynthesis, we investigated the potential of metabolically engineered S. marianum cultures for t-resveratrol production. Cell suspensions were stably transformed with Vitis vinifera stilbene synthase and the expression of the transgene led to extracellular t-resveratrol accumulation at the level of milligrams L-1 under elicitation conditions (see Chapter 3). Resveratrol synthesis occurred at the expense of the monolignol coniferyl alcohol. The overall production of the flavonolignan silymarin was less affected in the transgenic cultures, since the flavonoid pathway is already limiting for its synthesis, due to the preferred supply of precursors for the monolignol branch. The new expressed STS gene used excessively produced precursors of non-bioactive compounds (above all, coniferyl alcohol), while maintaining unaltered the metabolic flow for target secondary compounds (i.e. silymarin). This opens a way to extend the applications of plant cell cultures for the simultaneous production of valuable metabolites, both constitutive and foreign, which is another important aim of Plant Cell Biofactories.

BOX 9

Trans-resveratrol (t-R) (3,4',5-trihydroxystilbene) is a phytoalexin available from a wide range of dietary sources, including mulberries, peanuts, grapes and wine, but it has limited bioavailability. In contrast, its derivatives t-piceatannol and t-pterostilbene have superior biological in vivo activities. As mentioned previously, metabolic engineering tools offer us the possibility to transfer a biosynthetic pathway from a plant to another organism. Using this approach, the metabolic richness of certain plant species has been transferred to other plants, or even to more simple metabolic organisms, for the production of high added-value pharmaceuticals. The system can also be applied to bioconvert substrates into scarcer or biologically more interesting compounds. This is the case of the t-R derivatives, t-piceatannol and t-pterostilbene, which are found in plants only in small amounts. In this PhD thesis, by transferring the human cytochrome P450 hydroxylase 1B1 (HsCYP1B1) gene to tobacco hairy roots and cell cultures, we developed a system able to bioconvert exogenous t-resveratrol into piceatannol in quantities near to mg L-1. Similarly, after the heterologous expression of resveratrol O-methyltransferase from Vitis vinifera (VvROMT) in tobacco hairy roots, the exogenous t-resveratrol was bioconverted into pterostilbene. This confirmed the importance of metabolic engineering for the bioconversion of substrates into target products in heterologous host plant systems (see Chapter 4).

Recommended reviews to gain further insight into plant metabolic engineering:

- Capell T, Christou P. Progress in plant metabolic engineering. *Curr Opin Biotechnol.* **2004**, 15: 148-154.
- Miralpeix B, Rischer H, Häkkinen ST, Ritala A, Seppänen-Laakso T, Oksman-Caldentey KM, Capell T, Christou P. Metabolic engineering of plant secondary products: which way forward?. *Curr Pharm Des.* **2013**, 19: 5622-39.
- Farré G, Blancquaert D, Capell T, Van Der Straeten D, Christou P, Zhu C. Engineering complex metabolic pathways in plants. *Annu Rev Plant Biol.* **2014**, 65:187-223.
- O'Connor S. Engineering of secondary metabolism. *Annu. Rev. Genet.* **2015**, 49: 71-94.

Production of biopharmaceuticals in plant biofactories

Biopharmaceuticals are proteins, antibodies or nucleic-acid based products used in the treatment of disease. Their market demand is increasing, as they are selective, effective and also relatively safe and well tolerated. For instance, as reviewed by Fosgerau and Hoffman (2015), the global sales for the peptide-based prostate medicine Lupron™ were worth more than US$ 2.3 billion in 2011 and sales for Lantus™ from Sanofi reached US$ 7.9 billion. Currently over 60 peptides have been approved by the US FDA and more than 140 are in the clinical pipeline. It is expected that by 2020 the global sales of biopharmaceuticals will be worth over $US 278.2 billion (Santos et al., 2016).

Hitherto, the main biotechnological platforms for the industrial production of biopharmaceuticals have been based on microorganisms and mammalian cells growing in bioreactors. *Escherichia coli* was the first expression host used to produce recombinant proteins, and the first biopharmaceutical to be approved was human insulin for diabetes treatment in 1982. Currently, *E. coli* is one of the most commonly used hosts to produce small recombinant proteins, owing to its short production time, easy genetic transformation and feasibility for scaling up to bioreactor level for a high yield of biopharmaceuticals (Baeshen et al., 2015). Biotechnological platforms based on transgenic *E. coli* cultures are simple and inexpensive, but have some limiting drawbacks, such as endotoxin contamination and a frequent failure to produce large and complex proteins. Also, as prokaryotic cells, they do not allow post-translational modifications such as protein folding and glycosylation, which occur in human cells. On the other hand, bacterial systems can produce the target proteins associated with inclusion bodies, which are insoluble, inactive and require refolding (Santos et al., 2016).

Yeast cell biopharmaceutical factories, being based on single-celled organisms, share the advantages of bacteria, namely a fast doubling time (minutes) and facile genetic manipulation. Yet as eukaryotic cells, they also present a secretory pathway leading in most cases to the correct protein, as well as allowing post-translational modifications (Mattanovich et al., 2012). *Saccharomyces cerevisiae* and the methylotrophic yeast *Pichia pastoris* are widely utilized for biopharmaceutical production. Yeasts can produce high yields of proteins larger than 50 kD at a low cost, with the added convenience that signal sequences can be removed, and glycosylation carried out. Nevertheless, sometimes glycosylation by *S. cerevisae* generates only O-linked

oligosacccharides containing manose and not sialylated O-linked chains, which are produced by mammalian cells (Demain and Vaishnav, 2009).

Other eukaryotic systems such as insect and mammalian cells, and even transgenic animals, have been successfully used for the heterologous production of large proteins and mammalian glycosylate proteins, but their major disadvantages are a high production cost, complex protein purification and risk of contamination with human pathogens. In addition, the use of transgenic animals may entail ethical problems (Chu and Robinson, 2001).

In recent decades, a new concept for the production of recombinant proteins has emerged, namely molecular farming, in which the plant crop is harvested for its content of heterologous proteins. Recombinant protein production in plants is much safer and cost-effective than in mammalian and transgenic animals because it requires less time, and contamination with endotoxins, pathogens or oncogenic DNA is not an issue. It is the system of choice for the production of proteins that would be harmful or toxic for mammalian host cells (Demain and Vaishanav, 2009).

Molecular farming, as a large-scale agricultural production system, enjoys the advantages of inexpensive growth media (requiring only water, minerals and sunlight), low capital equipment costs and easy scale-up. However, according to the US Agricultural Department, transgene dissemination and contamination of the human food chain are major concerns in the use of field-grown transgenic crops, especially when edible plant species are used as the host (Wilson and Roberts, 2012). Moreover, the production of heterologous proteins in transgenic plants has to contend with possible contamination with agrochemicals and fertilizers, as well as fluctuations in yield due to variable culture conditions and the impact of bacterial and fungal infections (Hellwig et al., 2004).

Like production systems based on transgenic plants, plant cell cultures have the capacity for proper protein folding and can assemble complex recombinant proteins. Moreover, they combine these advantages with those of the original bioreactor systems, that is, transgene containment, controlled and sterile growth conditions, chemically defined culture media and straightforward compliance with pharmaceutical good manufacturing practices (GMP), which enhances biosafety and productivity (Demain and Vaishnav, 2009; Santos et al., 2016).

Plant cell cultures also offer other intrinsic advantages compared to plant crops, especially for the production of high added-value biopharmaceuticals required in small quantities, or high-purity

proteins for medical applications. The production time is significantly shorter than the growth cycle of a whole plant, allowing proteins to be manufactured in days or weeks, a time-scale compatible with clinical demands, rather than months or years (Doran, 2000). Additionally, *in vitro* plant cell cultures are unaffected by field cultivation factors, such as climate, soil quality, season, day length and weather. Also, downstream processing and protein purification are simplified, especially when the target protein is secreted into the culture medium (Pham et al., 2012).

The plant cell cultures most commonly used as the host for biopharmaceutical production are tobacco suspension cultures, although carrot and rice cells have also been tested, among others (Doran, 2000). Tobacco cell suspensions can be derived from transgenic plants, as for the production of human serum albumin and scFv antibody fragment (Sijmons et al., 1990; Firek et al., 1993), although in most cases heterologous proteins are produced in cell lines genetically transformed with the target gene. For example, BY-2 suspension cultures have been employed for production of human erythropoietin, biscFv antibody fragment or mAb against HBsAg, among other biopharmaceuticals (Matsumoto et al., 1993, 1995; Fischer et al., 1999; Yano et al., 2004). The tobacco cell line NT-1 has also been widely used as a production system of heterologous proteins, as reviewed by Hellwig et al. (2004), including mouse monoclonal heavy-chain γ, heavy chain mAb, and HBsAg.

Cell suspensions of tobacco, a model plant, are easy to transform and have a high growth capacity, although they have the inconvenience of producing toxic compounds, such as the alkaloid nicotine. As a result, research in biopharmaceutical production is moving to food crops with regulatory compliance, for example, hGM-CSF in tomato (Kwon et al., 2003) and human lysozyme and human α1-antrypsin in rice (Huang et al., 2001, 2002). Considering that downstream processes represent a high percentage of the biopharmaceutical production cost, Huang et al. (2015) recently demonstrated that 76-92% of the mGM-CSF produced in rice cell suspension is released to the medium when the signal peptide 33KDsp is added. Similarly, Reuter et al. (2014) reported that in tobacco BY-2 cell suspensions the fusion of fungal hydrofobin-green fluorescent protein ((GFP-HFBI) induced the formation of protein bodies that are selectively captured by a surfactant-based aqueous two-phase separation (ATPS) system (Reuter et al., 2014).

Another amenable plant species for genetic transformation and *in vitro* culture is *Daucus carota*, which has also been used as a host expression-system for biopharmaceutical production.

Hemagglutinin and the polyepitope $[L4T4]_2$ (measles vaccine), proteins ESAT6 and CFP10 (tuberculosis vaccine) or the viral protein PreS2-S (hepatitis B vaccine) have been successfully produced in carrot cell cultures, among others (Rosales-Mendoza and Tello-Olea, 2015). In fact, Taliglucerase alfa, a modified glucocerebrosidase (GCD) enzyme for Gaucher's disease treatment, produced by Protalix Biotherapeutics in the ProCellEx ® platform, based on carrot cell cultures, was the first human recombinant protein to be approved in the US and other countries (Tekoah et al., 2015).

The same ProCellEx® platform based on tobacco BY2 cells has efficiently produced PRX-106, an anti-TNF (tumor necrosis factor) cytokine involved in inflammatory diseases, PRX-102, an α-galactosidase protein used to treat Fabry disease, and PXR-110, a human deoxyribonuclease I proposed for the treatment of cystic fibrosis. All of them are currently in preclinical or clinical studies (Rosales-Mendoza and Tello-Olea, 2015). PRX-112, a human β-glucocerebrosidase enzyme, is naturally encapsulated within carrot cells, taking advantage of the resistance of plant cell walls to degradation in the human digestive tract. It has been proposed for the oral treatment of Gaucher disease, constituting yet another example of the advantages of the plant cell expression system for biopharmaceutical production (Shaaltiel et al., 2015).

Since the first plastid transformation was achieved in *Nicotiana tabacum* 20 years ago, transplastomic plants have been targeted by metabolic engineering approaches to biopharmaceutical production. The high number of chloroplasts per plant cell, the high copy number of the plastid genome, as well as the partial maternal mode inheritance that reduces transgene dissemination by pollen are some of the advantages of this system (Bock, 2014). Transplastomic technology has been widely used to enhance plant resistance to herbicides and plagues and for the production of recombinant proteins, but only scarcely for the production of biopharmaceuticals in cell cultures, despite the problem of gene containment in field-cultivated transplastomic plants (Bock, 2007). Examples include the development of transplastomic tobacco plant cell cultures for the production of phage-derived endolysins, which can be used as an antibiotic against pneumonia (Oey et al., 2009), camelid antibodies (Lentz et al., 2012), and the transforming growth factor (TGFβ3), a cytokine-type protein (Gisby et al., 2011).

Michoux et al. (2011) obtained transplastomic plants for the production of the fragment C of tetanus toxin (TetC) and the derived cell line accumulated up to 7 mg/L of this protein. However, when the cell suspension was inoculated in a temporary immersion bioreactor (TIB), the

regenerated shoots achieved a TetC production of 95 mg/L. Recently, our group obtained tobacco transplastomic plants for the production of the truncated human tissue plasminogen activator (K2S), which is widely used to break down blood clots (Abdoli-Nasab et al., 2013), and the system was optimized for its purification (Abdoli Nasab et al., 2016). As a part of this PhD thesis, we report the production of a truncated form of the tissue plasminogen activator (K2S) in cell suspensions derived from homoplasmic tobacco transplastomic plants (see **Chapter 5**, **BOX10**).

BOX 10

Thrombolysis is the first choice of therapy for Acute Myocardial Infarction. Tissue Plasminogen Activator (tPA) is a potent thrombolytic agent that activates plasminogen to form plasmin. Native tPA is a 70 kDa serine protease with five structural domains and 17 disulfide bonds and the production in Escherichia coli of the active recombinant protein is not possible. For this reason, several deletion-mutant variants including Kringle 2 plus serine protease (K2S) have been considered. Contrary to tPA, K2S (reteplase) is non-glycosylated and is produced in E. coli but for obtaining the active protein it is required a cumbersome refolding processes. This is that currently reteplase is produced by Chinese ovary hamster cells in an expensive process and new sources of reteplase are required. Transplastomic plants are a system of choice for the mass production of several biopharmaceuticals because have the capacity for proper protein folding and can assemble complex recombinant proteins, the polyploidy of the plastid genome could lead a high yield of biopharmaceuticals and the low risk of pollen-mediated outcrossing because of maternal inheritance. However, as field-grown plants, they can suffer contamination by agrochemicals and fertilizers, as well as fluctuations in yield due to climatic changes and infections. Recently, we obtained transplastomic tobacco plants carrying the K2S gene encoding truncated human tPA (reteplase), and confirmed the presence of the target protein in the transgenic plant leaves. Considering the advantages of plant cell cultures for biopharmaceutical production, the last aim of this PhD thesis was to establish a cell line derived from the K2S plants. The transgenic cell line was cultured under light and dark conditions and the obtained results showed the capacity of the cell line to produce the active reteplase form and the positive effect of the light increasing the production of the recombinant protein in more than 3-fold when compared with the production in dark conditions. See Chapter 5.

Recommended reviews to gain further insight into plant cell factories for biopharmaceutical production:

- Doran PM. Foreign protein production in plant tissue cultures. *Curr Opin Biotechnol*. **2000**, 11:199-204.
- Hellwig S1, Drossard J, Twyman RM, Fischer R. Plant cell cultures for the production of recombinant proteins. *Nat Biotechnol.* **2004**, 22:1415-1422.
- Wilson SA, Roberts Sc. Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. *Plant Biotech J.* **2012**, 10: 249-268.
- Tekoah Y, Shulman A, Kizhner T, Ruderfer I, Fux L, Nataf Y, Bartfeld D, Ariel T, Gingis-Velitski S, Hanania U, Shaaltiel Y. Large-scale production of pharmaceutical proteins in plant cell culture-the Protalix experience. *Plant Biotechnol J.* **2015**, 13:1199-1208.

Objectives

As mentioned, plant biofactories are biotechnological platforms based on plant cell and organ cultures used for the production of pharmaceuticals and biopharmaceuticals, although to date only a few of these systems have been successfully implemented at an industrial level. Metabolic engineering is possibly the most straightforward strategy to boost pharmaceutical production in plant biofactories, although some medicinal plants are recalcitrant to genetic transformation. An alternative approach is to use more genetically amenable species such as tobacco or other model plants, extending their metabolic capacities by importing partial or entire biosynthetic pathways.

In addition to technological difficulties, the use of metabolic engineering is also hampered by social opposition to the use of GMOs. Therefore, to circumvent the drawbacks related with genetic transformation, empirical approaches are still being used to improve the biotechnological production of plant cell biofactories.

Plant secondary metabolism involves thousands of different enzymes, some of which catalyze specific reactions, giving one product from a substrate, whereas others can yield multiple products from the same substrate. This trait open plant cell biofactories to new applications, in which the natural metabolic machinery of plants can be harnessed for the bioconversion of phytochemicals or even the production of new bioactive compounds. Synthetic biological pipelines involving the bioconversion of natural substrates into products with a high market value could be established by the heterologous expression of target metabolic genes in model plants.

With this background, the main aim of the work described in this PhD thesis was to demonstrate the high potential of plant biofactories for multiple applications in the biotechnological production of pharmaceuticals and biopharmaceuticals. Accordingly, the PhD thesis has been divided in five chapters, which cover the five most important aspects of Plant Cell Factories.

The specific aims of the study are summarized below:

1) To empirically set up an optimized and scalable biotechnological system based on *Centella asiatica* cell cultures for the efficient production of the bioactive compounds, centellosides, for use in cosmetic and pharmaceutical applications, and to demonstrate the capacity of the biotechnological platform to bioconvert amyrins precursors into the target compounds (**Chapter 1**).

2) To design transgenic hairy root cultures carrying an optimized synthetic pathway for resveratrol production using tobacco as the expression host, and to study the induced metabolic perturbations with cutting edge technologies such as metabolomics, , in response to the promising biological activities of resveratrol and its scarcity derivatives (**Chapter 2**).

3) To transfer the resveratrol production system from the model plant tobacco to *Silybum marianum* cell cultures, which have a natural capacity to produce silymarin with hepatoprotective activity, and to boost the spectrum of biological applications of the *S. marianum* cell extracts (**Chapter 3**).

4) To achieve the bioconversion of resveratrol into its more bioactive derivatives piceatannol and pterostilbene by expression of the human gene CYP1B1 and the *Vitis vinifera* ROMT gene, respectively, in a heterologous system based on tobacco hairy roots and their derived transgenic cell lines (**Chapter 4**).

5) As biopharmaceutical-producing plant biofactories are currently back in the limelight, a further aim is to produce the truncated human tissue plasminogen activator (K2S) in tobacco cell cultures derived from transplastomic plants (**Chapter 5**).

By developing these five case studies, the thesis aims to reach its ultimate goal of demonstrating the suitability of plant biofactories for the biotechnological production of cosme-, pharma-, and biopharmaceuticals.

Supervisor's report

Javier Palazon, Professor of Plant Physiology of the University of Barcelona and Purificación Corchete, Professor of Plant Physiology of the University of Salamanca as Directors of the PhD thesis entitled "**New strategies for improving the biotechnological production of pharmaceuticals and biopharmaceuticals in plant cell and organ cultures**" certify that the thesis presented here is the result of the work carried out by Diego Alberto Hidalgo Martínez under our guidance and supervision. The own contribution of the PhD candidate to each one of the manuscripts included in the thesis is detailed below.

Chapter 1. An optimized biotechnological system for the production of centellosides based on elicitation and bioconversion of *Centella asiatica* cell cultures.

Hidalgo D[a†], Steinmetz V[a], Brossat M, Tournier-Couturier L, Cusido RM, Corchete P, Palazon J.

Engineering in Life Sciences (2017) 17: 413–419.

Impact Factor (2015): 2.168; Position 76, Q2 in Biotechnology and Applied Microbiology of JCR.

[a] Equal contribution

[†] This article is a part of Diego Hidalgo's PhD thesis

DH: performed the cultures of *C. asiatica* callus and cell lines from small- to bioreactor- scale including elicitors and feeding experiments; centelloside extractions and HPLC determinations; contributed to results interpretation and manuscript writing.

Chapter 2. **Tailoring tobacco hairy root metabolism for the production of stilbenes.**

Hidalgo D[†], Georgiev M, Marche A, Bru-Martínez R, Cusido RM, Purificación Corchete P, Palazon J.

Metabolic Engineering (2017). Under review (Ref: MBE_2017_111)

Impac Factor (2015): 8.201; Position 8, Q 1 in Biotechnology and Applied Microbiology of JCR

† This article is part of the PhD thesis of Diego Hidalgo

DH: performed the genetic transformation, selection and culture of the transgenic hairy root lines of tobacco, PCR and qPCR to analyze the presence and expression of the studied genes and the determination of t-resveratrol and its derivatives and contributes to metabolomics study in the Lab. of Dr. Georgiev. Also, he contributed to results interpretation and manuscript writing.

Chapter 3. ***Silybum marianum*** **cell cultures stably transformed with** ***Vitis vinifera*** **stilbene synthase accumulate** ***t*****-resveratrol in the extracellular medium after elicitation with methyl jasmonate or methylated β-cyclodextrins.**

Hidalgo D[†], Martínez-Márquez A, Cusidó R, Bru-Martínez R, Palazon J, Purificación Corchete

Engineering in Life Sciences (2017) 10.1002/elsc.201600241

Impact Factor (2015): 2.168; Position 76, Q2 in Biotechnology and Applied Microbiology of JCR.

[†] This article is a part of Diego Hidalgo's PhD thesis

DH: performed the genetic transformation, selection and culture of the transgenic cell lines of *S. marianum,* PCR and qPCR to analyze the presence and expression of the *Vv*STS3 gene and determination of silymarins and *t*-resveratrol in the transgenic cell line.

Chapter 4. **Bioconversion of stilbenes in genetically engineered root and cell cultures of tobacco.**

Hidalgo D, Martínez-Márquez A, Moyano E, Bru-Martínez R, Corchete P, Palazon J.

Nature Scientific Reports (2017) 7: 45331.

Impact Factor (2015): 5.225; Position 7, Q1 in Multidisciplinary Sciences of JCR.

DH: Diego Hidalgo performed the genetic transformation of the material to obtain the hairy roots and derived cell lines; *in vitro* cultures, stilbene determinations and contributed to results interpretation and manuscript writing.

Chapter 5. **Biotechnological production of truncate tissue plasminogen activator protein (reteplase) from transplastomic tobacco cell cultures.**

Hidalgo D†, Abdoli Nasab M, Jalali Javaran M, Roque Bru-Martínez R, Cusido RM, Corchete P, Palazon J.

Plant Physiology and Biochemistry (2017). Under review (Ref: PLAPHY-D-17-00494).

Impact Factor (2015): 2.928. Position 41, Q1 in Plant Sciences.

DH: Diego Hidalgo obtained the derived cell lines from transplastomic K2S plants; developed the *in vitro* cultures, characterized and quantified the recombinant protein, and determine the reteplase activity, also he contributed to results interpretation and manuscript writing.

† This article is part of the PhD thesis of Diego Hidalgo

From all the coauthors of the five chapter, VS has not obtained the PhD degree (Chapter 1). We hereafter guarantee that none of the information contained in the chapter co-authored by him will be used to elaborate any other part of someone else's PhD thesis.

For all the above, we consider that the work developed by the PhD candidate grants him the right to defend his thesis in front of a Scientific Committee.

En Barcelona a 22 de Mayo de 2017

Fdo: Javier Palazón Purificación Corchete

Chapter 1. An optimized biotechnological system for the production of centellosides based on elicitation and bioconversion of *Centella asiatica* cell cultures.*

Spanish Summary

Centella asiatica es una planta herbácea de la medicina tradicional Ayurveda desde la antiguedad. Además de en la cicatrización de heridas, esta planta se recomienda para el tratamiento y cuidado de diversas afecciones de la piel como piel seca, la lepra, úlceras varicosas, eczema y/o psoriasis. Un grupo de saponinas triterpénicas, conocidas como centellósidos, son los principales metabolitos asociados con estos efectos beneficiosos. Teniendo en cuenta el interés en estos compuestos activos y su alto valor añadido, existe actualmente un gran interés en desarrollar procesos biológicamente y económicamente viables para producirlos. Trabajos anteriores utilizando la tecnología de cultivo de células vegetales de *C. asiatica* han demostrado la bioconversión eficiente de amirinas en sus derivados, los centellósidos, abriendo una nueva vía de obtención de estas biomoléculas. El presente estudio ha tenido como objetivo aumentar la producción de centellósidos en cultivos celulares de *C. asiatica*. En este artículo se describe la aplicación de un nuevo elicitor, la coronatina, combinado con la adición de resinas enriquecidas en amirinas que son potenciales precursores de los centellosidos, para desarrollar una nueva plataforma biosostenible para la producción de estos compuestos, mediante la generación de un efecto sinérgico de ambos tratamientos. Nuestros resultados han demostrado que la coronatina es un potente elicitor capaz de aumentar la producción de centellósidos, y que los tratamientos con fuentes naturales sostenibles de amirinas aumentan los rendimientos de su producción. Este proceso se puede escalar hasta nivel de bioreactor tipo CellBag agitado orbitalmente, aumentando así la capacidad del sistema para producir biomasa y centellósidos.

***Hidalgo D**, Steinmetz V, Brossat M, Tournier-Couturier L, Cusido RM, Corchete P, Palazon J. Engineering in Life Sciences. **2017**, 17: 413–419.*

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Research Article

An optimized biotechnological system for the production of centellosides based on elicitation and bioconversion of *Centella asiatica* cell cultures

Centella asiatica is a herbaceous plant of Asian traditional medicine. Besides wound healing, this plant is recommended for the treatment or care of various skin conditions such as dry skin, leprosy, varicose ulcers, eczema, and/or psoriasis. Triterpene saponins, known as centellosides, are the main metabolites associated with these beneficial effects. Considering the interest in these high value active compounds, there is a need to develop biosustainable and economically viable processes to produce them. Previous work using *C. asiatica* plant cell culture technology demonstrated the efficient conversion of amyrin derivatives into centellosides, opening a new way to access these biomolecules. The current study was aimed at increasing the production of centellosides in *C. asiatica* plant cell cultures. Herein, we report the application of a new elicitor, coronatine, combined with the addition of amyrin-enriched resins as potential sustainable precursors in the centelloside pathway, for a positive synergistic effect on centelloside production. Our results show that coronatine is a powerful elicitor for increasing centelloside production and that treatments with sustainable natural sources of amyrins enhance centelloside yields. This process can be scaled up to an orbitally shaken CellBag, thereby increasing the capacity of the system for producing biomass and centellosides.

Keywords: *Centella asiatica* / Copal resin / Coronatine / Manila elemi resin / Plant cell cultures

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1 Introduction

Advances in biotechnology, particularly plant cell culture technology, should provide innovative alternatives for the production of renewable sources of biomolecules. The major benefits of cell cultures include: (i) synthesis of bioactive secondary metabolites in a controlled environment, independently of climatic and soil conditions, (ii) elimination of negative biological influences that affect secondary metabolite production in nature (microorganisms and insects), (iii) possibility of improving the production of desired metabolites, and (iv) automatization of cell growth control and metabolic process regulation, which increases production and reduces the cost [1].

Thus, plant cell factories have emerged as a realistic technology for the production of bioactive components of medicines and cosmetics, including high added value products such as shikonin, arbutine, and taxol [2]. In recent years, a number of cosmetic companies have also begun to use plant cell cultures, referred to as "plant stem cells," directly as cosmetic ingredients, thus expanding the role of this technology. Plant stem cell-based products used for this purpose include PCT Malus Domestica and PCT Solar Vitis from Mibelle AG Biochemistry or Centella Stems GX and Echinacea Stems GX from Sederma [3]. Invariably, a biotechnological system based on plant cell cultures requires a previous optimization step, involving the selection of the most productive cell lines, and finding the optimum culture medium and conditions (e.g., temperature, mineral nutrients, carbon source, plant growth regulators, etc.). Moreover, in most cases, the highest yields of secondary metabolites in plant cell

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Abbreviations: CFW, cell fresh weight; **CORO**, coronatine; **GI**, growth index; **MeJA**, methyl jasmonate; **MER**, Manila elemi resin; **SA**, salicylic acid; **YE**, yeast extract

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†This article is a part of Diego Hidalgo's PhD thesis.

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cultures are achieved by harnessing the effect of biotic or abiotic elicitors [4].

Centella asiatica (L) Urban, traditionally named Gotu Kola, is a herbaceous plant belonging to the Apiacea family, widely used in health, food, and cosmetic industries. In traditional medicine, it is recommended as an antipyretic, diuretic, and antibacterial treatment [5]. The active metabolites of *C. asiatica*, triterpene saponins and their sapogenins, are known as centellosides, principally asiaticoside, madecassoside, asiatic acid, and madecassic acid, and several clinical studies have confirmed that they are responsible for the beneficial effects of *C. asiatica* extracts on various disorders [6]. Centellosides, like all triperpenes, are synthesized from the dimerization of farnesyl diphosphate supplied by the cytoplasmic mevalonate pathway, and although their biosynthesis is not well described, structural similarities with amyrins suggest the latter could potentially act as centelloside precursors [7].

Despite considerable research on the development of *C. asiatica* plant cell cultures, centelloside yields have been generally low (the in vitro production of centellosides has been reviewed in detail [7]). In most cases, the highest production was achieved in cell cultures treated with elicitors, principally methyl jasmonate (MeJA) [8, 9] and salicylic acid (SA) [10, 11], which were found to increase the expression of some of the known genes involved in centelloside biosynthesis. Additionally, the capacity of *C. asiatica* cell cultures to biotransform precursors such as amyrins and amyrin-rich resin extracts into centellosides has been reported [12, 13].

In previous work, we established the optimal conditions for *C. asiatica* callus induction [14] and cell suspension growth, and demonstrated the positive effect of 100 μM of MeJA on centelloside yield and on the expression of some centelloside biosynthetic genes [15]. The aim of the current study was to improve the biotechnological production of centellosides in *C. asiatica* cell cultures by testing a new elicitor, coronatine (CORO), which is a bacterial toxin and a jasmonate mimic produced by *Pseudomonas syringae* [16], together with resins rich in amyrins (monohydroxylated pentacyclic triterpene derivatives), potential centelloside precursors [12], before finally scaling up the optimized process to an orbitally shaken CellBag.

2 Material and methods

2.1 Plant cell cultures

Centella asiatica cell line UBCA17 was obtained as described by Mangas et al. [14] and cultured in Murashige and Skoog medium + 30 g sucrose/L + 2 mg of 2,4-dichlorophenoxiacetic acid (2,4-D)/L + 0.1 of benzil aminopurine mg/L, shaken at 100 rpm at 25°C in the dark. The *C. asiatica* cell line was routinely subcultured every 3 wk in fresh medium: 10 g of cell fresh weight (CFW) was inoculated in 250 mL flasks with 100 mL of culture medium. This cell line is characterized by a good growth capacity with an average doubling time of around 7 d and a stable centelloside production. In preliminary experiments, the growth curve (measured as CFW) showed an exponential growth phase until day 12 of culture and the onset of the stationary phase. At day 18, the growth of the cell line decreased significantly, when the growth index (GI: CFW harvested/CFW inoculum) of the system was approximately 3.8–4 (data not shown).

2.2 Elicitor treatments

We have previously reported that MeJA increased centelloside production in the *C. asiatica* cell line UBCA17 more than sevenfold compared to the control (untreated) cells [15], and recently Loc et al. [10, 11] reported that SA and yeast extract (YE) treatment of *C. asiatica* cell cultures enhanced asiaticoside production. In order to confirm these results, in 250 mL shake flasks, we tested the elicitors MeJA, SA, YE, and the new elicitor, CORO, which participates in the jasmonate signaling pathway and was recently found by our group to be more effective than MeJA in increasing taxane production in *Taxus* spp. cell cultures [16]. Thus, UBCA17 cell cultures were supplemented with MeJA (100 and 200 μM), SA (100 μM), YE (4 g/L), and CORO (1 and 2 μM) at day 12 (at the end of the exponential growth phase), and samples were taken in triplicate at days 3 and 6 after elicitation.

2.3 Bioconversion experiments

In the same shake flasks, in order to increase the centelloside production of the system, we tested the centelloside precursor α-amyrin at the concentration of 50 mg/L (120 μmol/L), as well as natural sources of amyrins: 250 mg/L copal resin (containing 20% of amyrins), 125 mg/L copal extract (Ext Copal) (containing 40% of amyrins) [12] and 25 and 50 mg/L Manila elemi resin (MER) (containing 39% of amyrins; quantified by GC analysis). In all the systems, 1% DMSO was added as a permeabilizing agent to enhance cell uptake of precursors [12], and as resins were dissolved in ethanol, 1% of ethanol was also added in mock conditions. Precursors were added after 12 days of growth, and samples were taken in triplicate after 3 and 6 days.

2.4 Combined treatment of elicitors and addition of potential precursors

Once the best elicitation conditions and the most efficient precursors were established, we carried out a combined treatment in the shake flask system in which 1 μM CORO was added to the culture medium at day 12 of growth, together with Ext Copal (125 mg/L) or MER (50 and 125 mg/L). In all the treatments, 1% of DMSO was added and samples were taken as in previous experiments at days 3 and 6 of the treatment. Results were compared with those of mock conditions in which only 1% of DMSO and 1% of ethanol were added to the cell cultures.

2.5 CellBag cultures

In order to scale up the process, a 2 L CellBag (GE Healthcare BioSciences AB, Uppsala, Sweden) with a working volume of 1 L, shaken in a Khuner orbital shaker in the dark at 25°C and 35–38 rpm, with a shaking diameter of 50 mm, was used. We initiated the culture at 35 rpm and gradually the shaking was increased up to 38 rpm to obtain a good distribution of cell biomass

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and oxygen transference without foaming. The sterile airflow was 0.2 L/min and the inoculum size was 10% w/v. In previous experiments, these conditions were, determined as optimal for the growth of the cell line and to avoid out-of-phase phenomena (data not shown). After 10 days of culture (end of the exponential phase in these conditions), MER (125 mg/L), 1 μM CORO, and 1% of DMSO were added. Samples were taken daily in triplicate from day 10 to day 16, in order to measure the biomass and centelloside production. The experiment was run in triplicate.

2.6 Growth measurement

In order to measure the growth capacity of the systems, samples were filtered through 35 mm porous nylon filters, and cell biomass was weighed before (CFW) and after freeze-drying (cell dry weight, CDW).

2.7 Centelloside extraction and quantification

To quantify the centellosides (madecassoside, asiaticoside, madecassic acid, and asiatic acid), freeze-dried samples (1 g) and culture media were extracted as previously reported [17]. HPLC-UV analyses were carried out following the method of Inamdar et al. [18] with some modifications [19]. The analyses were performed at room temperature with a Spherisorb 51 ODS2 (250 × 4 mm) column (Waters Milford, MA, USA) using a mobile phase of ACN and water with 10 mM ammonium dihydrogen phosphate $NH_4H_2PO_4$ (pH 2.5 with orthophosphoric acid) in gradient conditions (ACN: 0–35 min, 20–70%); the flow rate was 1 mL/min and the detector was set at 214 nm. Standard purity was determined by MALDI-TOF MS. Analyses were carried out in triplicate.

2.8 Statistics

Statistical analysis was performed with Excel software. All data are the average of three determinations ± SD. A multifactorial ANOVA analysis followed by Tukey's multiple comparison tests were used for statistical comparisons. A p-value of <0.05 was assumed for significant differences.

3 Results

3.1 Elicitation experiments

In previous growth kinetic studies of the *C. asiatica* cell line UBCA17, we demonstrated that the end of the exponential growth phase and the beginning of the stationary phase is the best moment to perform the elicitor treatment [15]. Thus, the elicitors (see Section 2) were added to the culture at this point, and samples were taken 3 and 6 days later. Figure 1A shows the effect of the different elicitor treatments on the growth capacity measured as the GI (CFW harvested/CFW inoculum). In control conditions and after a culture period of 18 days, the cell biomass of the system increased more than fourfold, which represents an average doubling time of 9 days. Elicitor treatments

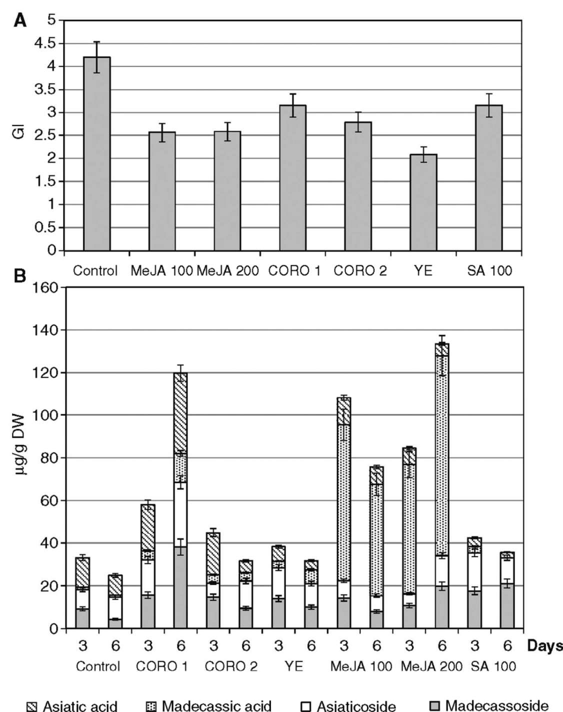

Figure 1. (A) GI (CFW sample/CFW inoculum) of the cell cultures after 18 days of culture. (B) Centelloside production expressed as microgram per gram DW measured 3 or 6 days after the elicitation treatment. CORO 1: coronatine 1 μM; CORO 2: coronatine 2 μM; MeJA 100: methyl jasmonate 100 μM; MeJA 200: methyl jasmonate 200 μM. All the values are the average of three replicates ± SD.

decreased the growth capacity of the cell line, especially MeJA and YE (Fig. 1A).

Measuring the total centellosides as the sum of madecassoside + asiaticoside + madecassic acid + asiatic acid, two treatments dramatically increased the production capacity of the cultures (Fig. 1B). The addition of 200 μM MeJA gave a centelloside production of 134 μg/g DW, which was 5.8-fold higher ($p < 0.05$) than in control conditions on the same day (day 18), although this treatment significantly changed the centelloside pattern, producing a strong increase in madecassic acid. In contrast, treatment with 1 μM CORO increased the centelloside content 5.2-fold ($p < 0.05$) compared with the control but without changing the centelloside profile of the cell line. For this reason, this elicitor was selected for further experiments.

3.2 Bioconversion experiments

The capacity of the *C. asiatica* cell cultures to bioconvert the potential precursor α-amyrin into centellosides, thereby significantly increasing the yield of these bioactive compounds, has been demonstrated [13]. The high cost of pure amyrins impedes their use in industrial cell culture processes, so we decided

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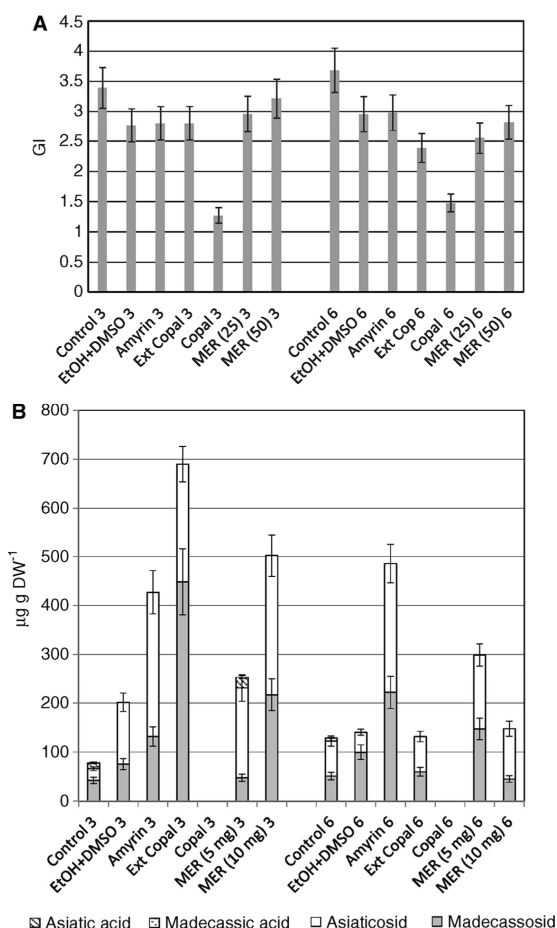

Figure 2. (A) GI (CFW sample/CFW inoculum) of the cell cultures after 18 days of culture. (B) Centelloside production expressed as μg/g DW measured 3 or 6 days after the treatment. CE: copal extract; CR: copal resin; MER: MER at the concentration of 25 or 50 mg/L. All the values are the average of three replicates ± SD.

to test copal and MER, natural and sustainable sources rich in these precursors [12]. Thus, based on our previous results, we supplemented the *C. asiatica* cell cultures at day 12 (end of the exponential growth phase) with 50 mg/L α-amyrin, 250 mg/L copal resin, 125 mg/L Ext Copal, and 25 and 50 mg/L MER. Amyrin and resins were dissolved in ethanol. In order to increase cell permeability and facilitate amyrin uptake by the cells, 1% DMSO was also added.

As shown in Fig. 2A, the precursors had very different effects on the GI of the cell cultures. Whereas cell growth was not affected by α-amyrin and MER, with the biomass at the end of the culture being similar to that in mock conditions (supplemented with 1% DMSO and 1% ethanol), it was largely inhibited ($p < 0.05$) by copal resin. In further experiments, increasing the MER concentration up to 125 mg/L did not negatively affect the growth capacity of the cell line (data not shown). The organic solvents ethanol and DMSO had negative effects on the cell growth and GI, which were lower in mock conditions than in control (untreated cells).

Centelloside production (Fig. 2B) was significantly enhanced ($p < 0.05$) by both α-amyrin and Ext Copal, in some cases more than 70-fold. In the case of Ext Copal, the centelloside content was higher after 3 days of treatment than at day 6, whereas the effects of α-amyrin were most prominent at day 6. In contrast, copal resin was found to cause cell death, probably due to components that are eliminated in the copal extract by washing with petroleum ether [12]. MER treatment also increased the total centelloside content of the culture, being more effective when added at 50 mg/L, and achieving a yield at day 3 comparable to α-amyrin and lower than Ext Copal (Fig. 2B).

3.3 Combined effect of elicitation and addition of potential precursors

In a further approach, we tested the effects of a treatment combining elicitors and precursors. CORO (1 μM) was selected as the best elicitor (Fig. 1) and Ext Copal (125 mg/L) and MER (50 and 125 mg/L) were used as potential precursors, since they enhanced centelloside production without significantly affecting cell growth ($p < 0.05$) (Fig. 2). Neither the CORO treatment nor the addition of resins affected the growth capacity of the cell line (Fig. 3A). The highest centelloside yields were obtained at day 3 by treating the cells with Ext Copal (125 mg/L) and MER (125 mg/L), and were 10.9- and 8.7-fold higher ($p < 0.05$), respectively, compared to control conditions (untreated cells). In both cases, the centelloside production peaked at day 3 of the treatment, decreasing thereafter until day 6 (Fig. 3B).

3.4 Scaling up the process

To scale up the process, a 2 L CellBag with a working volume of 1 L was used, shaken at 35 to 38 rpm in an orbital shaker. The system was working in batch mode. After 10 days (at the end of the stationary growth phase in these conditions), the cell culture was treated with 1 μM CORO, 125 mg/L MER and 1% DMSO. Samples were taken daily. After 10 days of culture, the GI peaked at 4.8, then the cell density decreased at day 11 (after the treatment) but remained stable until the end of the experiment (Fig. 4A). Despite the negative effects of the treatment on cell growth, the combined treatment of elicitor and resin greatly improved the production of total centellosides, with a progressive increase until a maximum yield (7.3 mg g DW^{-1}) at day 15 (Fig. 4B). As in the shake-flask system, the predominant centelloside produced in the bioreactor was madecassoside. The high biomass and centelloside production of the system shows the suitability of the disposable orbitally shaken bioreactor for the culture of *C. asiatica* cell suspensions.

4 Discussion

Centella asiatica has been used in Ayurvedic (Indian) medicine to treat conditions such as skin illnesses, nervous disorders, and

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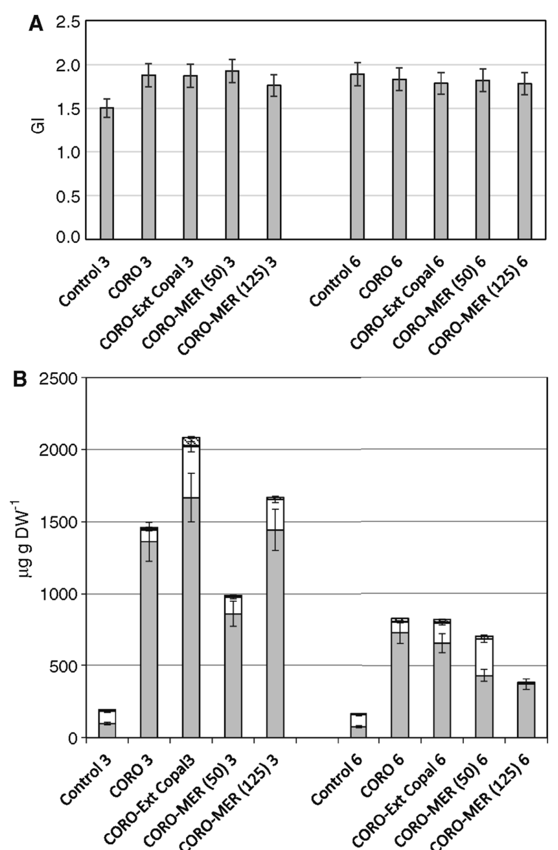

Figure 3. (A) GI (CFW sample/CFW inoculum) of the cell cultures after 18 days of culture. (B) Centelloside production expressed as microgram per gram DW measured 3 or 6 days after the treatment. CE: copal extract; MER: Manila elemi resin at the concentration of 50 or 125 mg/L. All the values are the average of three replicates ± SD.

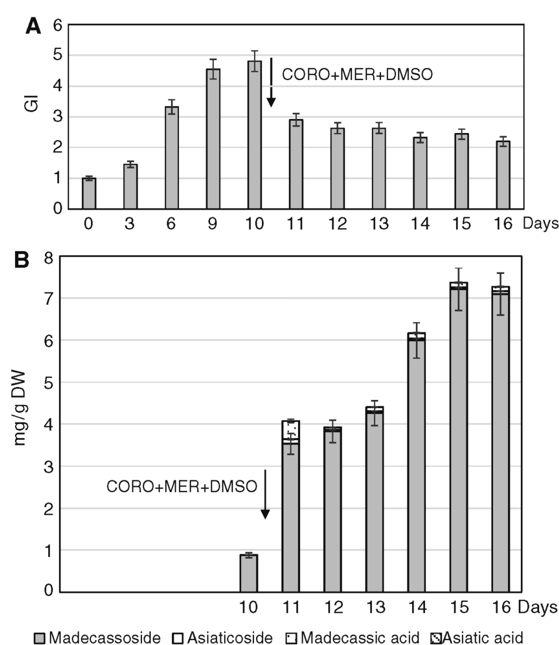

Figure 4. (A) Changes in the GI (CFW sample/CFW inoculum) during the culture period studied. (B) Time course of the centelloside production from the moment of elicitation to the end of the culture period. All the values are the average of three replicates ±SD. Black arrows indicate the time point of the combined elicitor/precursor treatment.

venous insufficiency, and its potent antioxidant, antimicrobial and antifungal, anti-inflammatory, and antirheumatoid activities, among others, are supported by scientific evidence [20–23]. These properties, together with recent applications of *C. asiatica* stem cells as cosmetic ingredients [3], have generated growing interest in *C. asiatica* cell cultures as a new source of centellosides, the triterpenoid saponins responsible for the biological activity of the plant and cell extracts. *Centella asiatica* cell line UBCA17 is characterized by a high capacity for biomass and centelloside production under elicitation [15].

The positive effects of treatments with elicitors such as MeJA, SA, copper ions, and YE on centelloside production have been previously demonstrated in both *C. asiatica* plant cell and hairy root cultures [7]. In this work, we corroborated the positive effect of MeJA, SA, and YE on centelloside biosynthesis, and also for the first time demonstrated the effectiveness of the new elicitor CORO in increasing centelloside production (more than five-fold), even at concentrations 100-fold lower than MeJA, without affecting the growth capacity of the cell cultures (Fig. 1). Additionally, unlike MeJA, which principally enhanced yields of madecassic acid, CORO-treated cultures produced the same centelloside pattern as the control (untreated cells), with madecassoside and asiaticoside predominating (Fig. 1). CORO has been previously used as an elicitor to increase the biotechnological production of other bioactive compounds, such as taxanes in *Taxus* spp. plant cell cultures, achieving a higher activation of taxane biosynthetic gene expression than MeJA and consequently an increased production of taxanes [14].

Like microorganisms, plant cell cultures are able to carry out regio- and stereoselective hydroxylations, hydrogenation, glycosidation, etc. of exogenous substrates, biotransforming them into other compounds with improved pharmacological actions [24]. Thus, biosynthetic precursors have been frequently used to improve the biotechnological production of bioactive compounds in plant cell cultures [25], although this strategy is only suitable for industrial purposes when the precursors accumulate in natural sources in high amounts or can be chemically synthesized. The capacity of *C. asiatica* cell cultures to bioconvert the potential precursor α-amyrin into centellosides with an efficacy of 84% has been reported [13], but the high market price of this compound impedes the scale up of the process

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to an industrial level. However, resins such as Mexican copal or Manila elemi are known to be rich natural and sustainable sources of amyrins [13], so they were selected to feed the *C. asiatica* cell cultures in the present work. Copal resin was found to be highly toxic, unlike Ext Copal, which suggests that cleaning copal resins with petroleum ether removes toxic compounds. Both Ext Copal and MER significantly enhanced the centelloside accumulation in the cell cultures, demonstrating that the system effectively transformed the exogenous amyrins into centellosides.

Empirical approaches to improving secondary metabolite production in plant cell cultures can involve the combination of several strategies. In this study, we tested the effect of resin addition to CORO-elicited *C. asiatica* cells after corroborating the effectiveness of this elicitor. This type of combined treatment has previously achieved good results in, for example, improving taxane production in *Taxus* spp. cell cultures [26]. We found that the two treatments had a synergistic effect, as centelloside production in cells treated with both Ext Copal and CORO increased more than 16-fold compared with the 2.8-fold improvement achieved with the Ext Copal alone.

Rather than in reusable bioreactors, plant cell cultures are increasingly being grown in disposable bioreactors with volumes of up to 200 L for the production of high added value compounds such as proteins and secondary metabolites [27]. These bioreactors have several advantages, as they require less sterilization work, are time saving and cost saving, and produce less waste and environmental contamination [28, 29], but until now they have not been tested for the production of centellosides. In this work, we used a 2 L CellBag and tested one of the treatments producing the best results in the shake-flasks, i.e. elicitation with CORO (1 μM) and feeding with MER (125 mg/L). MER was chosen as the precursor instead of Ext Copal, as it does not require any preparation before adding to the plant cell culture, which is an advantage for industrial application. Under these conditions, the *C. asiatica* cell line grew very well, achieving a GI of 4.8 after 10 days of culture with a final centelloside production of 7.3 mg g DW^{-1}, which was 4.2-fold higher than in shake flasks under the same conditions, thus demonstrating the suitability of this system for scaling up centelloside production.

In summary, our results demonstrate that *C. asiatica* plant cell cultures are a potential sustainable technology for the production of centellosides. In this context, we demonstrated that a combined treatment in which cell cultures are supplemented with the new elicitor CORO and fed with natural amyrin-rich products, such as Ext Copal or MER, significantly enhanced the centelloside production of the system. Moreover, scaling up to a 2 L CellBag was easy and improved the growth and centelloside production capacity of the *C. asiatica* cell suspension. Although this system is limited to 10 L bags, the results obtained open the possibility of scaling up the process further to obtain these valuable compounds in commercial biosustainable plant cell biofactories. New orbital shaken bioreactors with disposable bags with a working volume of up to 50–200 L are available on the market to perform this type of approach.

Practical application

Centella asiatica extracts are widely used in pharmaceutical and cosmetic industries due to the properties of their bioactive components known as centellosides. Currently, with the utilization of plant stem cells as cosmetic ingredients, there is a need to develop biotechnological systems based on *C. asiatica* plant cell cultures producing high amounts of centellosides. This study shows that a treatment combining elicitors with culture feeding with natural sources of amyrins (centelloside precursors) such as copal and Manila elemi resins can improve centelloside production in *C. asiatica* cell suspensions. The successful scale up of the system to a disposable bench-top bioreactor shows it can be applied for the development of *Centella* plant cell cultures with increased yields of centellosides.

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The authors have declared no conflicts of interest

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Chapter 2. Tailoring tobacco hairy root metabolism for the production of stilbenes.*

Spanish Summary

El amplio espectro de acciones terapéuticas del resveratrol (3,5,4'-trihidroxi-trans-estilbeno) (*t*-R) se ve potenciado en sus derivados *t*-piceatannol (*t*-Pn) y *t*-pterostilbeno (*t*-Pt), pero estos compuestos tienen una distribución muy restringida en la naturaleza. Los cultivos de raíces transformadas de tabaco, que han sido ampliamente utilizados para la producción heteróloga de compuestos diana, tienen la capacidad natural para bioconvertir *t*-R exógeno en *t*-Pn y *t*-Pt. Con el objetivo de desarrollar un sistema biotecnológico capaz de producir *t*-R y realizar su bioconversión en *t*-Pn y *t*-Pt, se establecieron cultivos de raíces transformadas (HR) de tabaco genéticamente ingenierizadas portadoras del gen estilbeno sintasa (STS) de *Vitis vinifera* y / o el factor de transcripción (TF) *At*MYB12 de *Arabidopsis thaliana*, con el fin de generar una respuesta holística en la vía fenilpropanoide y coordinar la regulación de múltiples pasos metabólicos. Además, se utilizó un microARN artificial para la chalcona sintasa (amiRNA CHS) para detener el flujo normal a través de la enzima CHS endógena, que competiría por los mismos precursores que la STS importada para desviar el flujo de fenoles hacia la formación de estilbenos. Los cultivos de raíces transgénicas fueron capaces de biosintetizar los compuestos diana, logrando una producción de 40 µg L^{-1} de *t*-R. Este compuesto fue parcialmente metabolizado en sus derivados *t*-Pn y *t*-Pt, alcanzándose producciones de hasta 2,2 µg L^{-1} y 86,4 µg L^{-1}, respectivamente, así como de su glucósido piceida (hasta 339,7 µg L^{-1}). Sin embargo, el TF *At*MYB12 indujo una fuerte perturbación metabolómica, afectando tanto al metabolismo primario como al secundario, lo que confirma la complejidad de los sistemas biotecnológicos basados en cultivos *in vitro* de plantas para la producción heteróloga de compuestos diana.

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Tailoring tobacco hairy root metabolism for the production of stilbenes

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A R T I C L E I N F O

Keywords:
Nicotiana tabacum
t-resveratrol
t-piceatannol
t-pterostilbene
stilbene synthase
*At*MYB12 transcription factor
amiRNA chalcone syntase

A B S T R A C T

The wide-ranging therapeutic actions of resveratrol (3,5,4′-trihydroxy-trans-stilbene; *t*-R) are evidently more potent in its derivatives t-piceatannol (*t*-Pn) and *t*-pterostilbene (*t*-Pt), but these are scarcely distributed in nature. Tobacco hairy root cultures, which have been widely used for the heterologous production of target compounds, have an innate capacity to bioconvert exogenous *t*-R into *t*-Pn and *t*-Pt. With the aim of developing a biotechnological system, able both to produce *t*-R and perform its bioconversion into *t*-Pn and *t*-Pt, we established genetically engineered tobacco hairy roots (HR), carrying the stilbene synthase (STS)-encoding gene from *Vitis vinifera* and/or the transcription factor (TF) *At*MYB12 from *Arabidopsis thaliana*, in order to generate a holistic response in the phenylpropanoid pathway and coordinate the up-regulation of multiple metabolic steps. Additionally, an artificial microRNA for chalcone synthase (amiRNA CHS) was utilized to arrest the normal flux through the endogenous CHS enzyme, which would compete for precursors with the STS enzyme imported for the flux deviation. The transgenic tobacco HRs were able to biosynthesize the target stilbenes, achieving a production of 40 μg L^{-1} of *t*-R, which was partially metabolized into *t*-Pn and *t*-Pt (up to 2.2 μg L^{-1} and 86.4 μg L^{-1}, respectively), as well as its glucoside piceid (up to 339.7 μg L^{-1}). Major metabolic perturbations were caused by the TF *At*MYB12, affecting both primary and secondary metabolism, which confirms the complexity of biotechnological systems based on seed plant in vitro cultures for the heterologous production of high-value molecules.

1. Introduction

Due to its wide-ranging therapeutic potential, resveratrol (3,5,4′-trihydroxy-trans-stilbene) (*t*-R) is amongst the most studied stilbenes. Its antiviral, antioxidant, anti-inflammatory, and cardioprotective effects have been thoroiughly reported, as well as platelet anti-aggregation and melanoma chemoprevention activities (Baur and Sinclair, 2006). The hydroxylated *t*-R derivative *t*-piceatannol (*t*-Pt) has higher bioavailability and significant anti-

cancer and cancer chemopreventive activity than *t*-R (Szekeres et al., 2011; Piotrowska et al., 2012), while the methoxy derivative *t*-pterostilbene (*t*-Pt) has greater anti-proliferative effects against human colon cancer cells (McCormack et al., 2013). The scarce distribution of these derivatives in nature, however, calls for the development of alternative sources for their sustainable supply. In this scenario, biotechnological factories based on plant cell cultures (so-called "green cell factories" concept) constitute a promising new biosustainable system for plant secondary metabolite (PSM) production. Similarly, hairy root cultures (HR) hold great potential as systems for the bioconversion and bioproduction of PSM (Srivastava et al., 2016), due to their genetic stability (Georgiev et al., 2009; 2012) and facile scale up to bioreactor level (Kim et al., 2002). Additionally, HR do not need exogenous hormones and allow the large-scale harvesting of secondary products (Sevón and Oksman-Caldentey, 2002), in contrast with plant cell cultures, which are frequently unable to produce significant levels of target compounds (Kim et al., 2002).The use of HRC as biofactories has been reviewed by Chandra and Chandra (2011) and Rischer et al. (2013).

Biotechnological studies have shown that *Vitis vinifera* cell cultures need elicitation treatments for an efficient *t*-R production, the best results being achieved by combining methyl jasmonate with the economically expensive cyclodextrins (Lijavetzky et al. 2008). *Silybum marianum* transgenic cell suspensions carrying the *Vv*STS gene, treated only with cyclodextrins have also yielded *t*-R (Hidalgo et al., 2017a), as have HRC of peanut (Halder et al., 2016) and *V. rotundifolia* (Nopo-Olazabal et al., 2014).

Metabolic engineering has been used to enhance biosynthetic pathways in plants (Christou and Klee, 2004; Capell et al., 2004), extending existing routes or introducing new ones to obtain novel compounds (Zhu et al., 2008; Naqvi et al., 2010; Chandra and Chandra, 2011). In approaches based on the modification of single steps, the effects of transgene overexpression are often adsorbed by self-homeostasis. In contrast, holistic strategies, involving the simultaneous overexpression and/or suppression of multiple genes, can be eventually more effective in boosting PSM production (Capell and Christou, 2004). On the other hand, the use of omics technologies has led to significant advances in the elucidation of secondary metabolism and shed light on the competition between primary and secondary pathways (Jacobs et al., 2000; Fukushima et al., 2009).

The heterologous biosynthesis of *t*-R has been reported in both microorganisms and whole plants (reviewed in Delaunois et al., 2009; Kiselev, 2011). Recently, this strategy has been successfully used in metabolically engineered grapevine cell cultures to obtain the highly bioactive stilbenes, *t*-Pn and *t*-Pt (Martínez-Marquez et al. 2016). The capacity of engineered tobacco hairy root cultures to bioconvert *t*-R into the target derivatives has also been demonstrated (Hidalgo et al., 2017b).

The blocking of competitive pathways is another successfully used approach in metabolic engineering to improve PSM production. In amiRNA technology, which involves endogenous primary-miRNAs (pri-miRNAs), the miRNA and miRNA* sequences are replaced with corresponding artificial-miRNA (amiRNA/amiRNA*) sequences for the specific target gene (Tiwari et al., 2014). The application of

amiRNA in plants has led to the elucidation of gene function in the phenylpropanoid pathway (Misra et al., 2010; Pandey et al., 2015), as well as the identification of genes involved in plant growth and development (Dodd et al., 2005; Kim and Somers 2010; Schwartz et al., 2009; Latijnhouwers et al., 2010; Toppino, L. et al., 2011). Recently, Carbonell et al. (2014) developed a relatively straightforward protocol for the fast and effective construction of amiRNAs for plant species, which opens up new possibilities for plant biotechnological research.

The overexpression of transcription factors (TF) is another holistic strategy towards higher PSM yields. In most cases TF are present as gene families and can directly activate all or several of the enzymes involved in metabolite formation in response to biotic and abiotic stress (Wilson and Roberts, 2012; Wang et al., 2016). Moreover, TF allow gaps or undefined stages in metabolic routes to be reduced or eliminated upon transfer to heterologous systems (Christou and Klee, 2004). The MYB TF family is the most useful in enhancing flavonoid biosynthesis (Mehrtens et al., 2005, Stracke et al., 2007); when expressed in tobacco and tomato, TF *At*MYB12 increased the levels of phenolic compounds and insect resistance (Luo et al., 2008; Misra et al., 2010). Recent studies have reported strong modulation in transcriptome behavior by MYB TF, above all affecting genes of the phenylpropanoid pathway (Pandey et al., 2015). In strategies aimed at increasing the production of a specific metabolite, the insertion of a single gene to complete the desired metabolic pathway can be more effective when accompanied by a TF-mediated holistic modification (Misra et al., 2010).

In the present work, we report engineering of tobacco HR carrying the *Vitis vinifera* stilbene synthase gene to tailor the metabolism for the bioproduction of *t*-R and its derivatives *t*-Pn and *t*-Pt. For this, the TF *At*MYB12 was overexpressed to generate a holistic response in the phenylpropanoid pathway and coordinate the up-regulation of multiple steps. In addition, the use of amiRNA-CHS deviated the normal flux through the endogenous CHS enzyme, which competes for precursors with the imported STS enzyme, to the formation of *t*-R (Fig. 1).

2. Materials and methods

2.1. Bacteria and plasmids

Four strains of *Agrobacterium rhizogenes* A4 were used: wild type and three engineered strains carrying the pRiA4. The specific engineered strains and binary plant expression vectors of each one are described in Table 1 and Fig. S1.

Table 1. Transgenic *Agrobacterium rhizogenes* A4 utilized in the experiments.

***A. rhizogenes* ID**	**Expression vector / Gene**
A4-S	pJCV52 / Stilbene synthase 3 (*Vv*STS-3)
A4-TF	pJCV52 / *Arabidopsis thaliana* transcription factor *At*MYB12
A4-Inh	pMDC32B - *At*MIR390a-B/c / Artificial microRNA of CHS (amiRNA-CHS) into *Arabidopsis thaliana* MIR390a precursor

The *Vv*STS-3 gene (Acc. XM_002264953) was cloned as described by Martinez-Marquez et al. (2016). The clone TOPO-U04-A02 that contains the *At*MYB12 gene was purchased from the *Arabidopsis* Biological Resource Center (ABRC). An LR

recombination reaction was performed to generate an expression clone using LR Clonase™ (Invitrogen, Carlsbad, CA). The construction of amiRNA-CHS (see Supp information) was as described by Carbonell et al. (2014) using the following sequences: forward oligo: 5' TGT ATT AAT CAT TGA TTT TTC ACA GAT GAT GAT CAC ATT CGT TAT CTA TTT TTT CTG TGA AAA AGC AAT GAT TAA 3'; reverse oligo: 5' ATG TTA ATC ATT GCT TTT TCA CAG AAA AAA TAG ATA ACG AAT GTG ATC ATC ATC TGT GAA AAA TCA ATG ATT AA 3'. The *Agrobacterium* strains were transformed by electroporation as described by Shaw (1995).

2.2. Stable transformation and hairy root culture induction

Leaf discs of *Nicotiana tabacum* cv Xhanti plantlets grown *in vitro* on Murashige and Skoog (MS) medium were co-infected as described by Gallois and Marinho (1995), using a wild type *A. rhizogenes* A4 or A4-S or A4-S plus A4-TF or A4-S plus A4-TF plus A4-Inh. After 2-4 weeks, HR appeared in leaf discs maintained on MS solid medium with 30 g L^{-1} of sucrose and 500 mg L^{-1} cefotaxime to eliminate the agrobacteria. Further, HR were excised and individually cultured on the same medium plus kanamycin (50 mg L^{-1}) and/or hygromycin (50 mg L^{-1}) for selection. The tobacco HR were kept in these conditions at 25ºC in the dark for several rounds of subculturing until the elimination of agrobacteria and confirmation by PCR analysis.

2.3 PCR analysis

The analysis was performed with DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc). Previously, genomic DNA was isolated as by Dellaporta et al. (1983). Specific primers and amplification reactions used are described in Table S1. PCR products were analyzed by electrophoresis on 1% agarose gels.

2.4 Gene expression by qPCR analysis

Expression of the *Vv*STS-3, *At*MYB12, amiRNA-CHS, phenylalanine ammonia lyase (PAL) and chalcone syntase (CHS) genes was verified by qPCR in selected HR lines as described by Hidalgo et al. (2007b). Briefly, total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (Invitrogen, Carlsbad, CA). Then, cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and finally, qRT-PCR was performed with iTAqTM Universal SYBR Green Supermix (BioRad, Hercules, CA, EEUU) in a 384-well platform system (LightCycler 480 Instrument; Roche). Conditions and primers used are described in Table S1.

2.5 Stilbene extraction and determination

The HR and culture medium were processed as described by Hidalgo et al. (2017b). Briefly, two times one volume of ethyl acetate was added per four volumes of the medium, stirring vigorously, and the non-polar solvent recovered and evaporated. For the HR, 50 mg of freeze-dried powder was extracted twice with 2 volumes of 100 % methanol, sonicated for 30 min, and the supernatant was collected and evaporated. Stilbenes were determined by a Linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer, 4000 Q TRAP of AB Sciex Instruments with MRM scan type in negative mode as described by Hidalgo et al. (2017b).

2.6 Total phenolics content (TPC)

The compounds were extracted as indicated in the previous paragraph and their quantity was estimated by the Folin-Ciocalteu colorimetric method as described by Thiruvengadam et al. (2014) with slight modifications. Briefly, 100 µL of HR extracts were mixed with 3.1 mL of distilled water, followed by addition of 0.2 mL Folin-Ciocalteu reagent. After 5 min, 0.6 mL of 20 % sodium carbonate solution was added, and after 60 min of incubation the solution absorbance was measured at 760 nm. The concentration was calculated as mg of gallic acid equivalents using a calibration curve.

2.7 Total flavonoids content (TFC)

Flavonoids quantity in extracts was estimated using the aluminum chloride colorimetry method described by Thiruvengadam et al. (2014) with slight modifications. Briefly, 200 µL of extract, 100 µL of 10 % (w/v) aluminum chloride solution, 100 µL mL of 1 M potassium acetate solution, and 4.6 mL of distilled water were mixed. After 30 min of incubation the solution absorbance was measured at 415 nm. The concentration was calculated as mg of rutin equivalents using a calibration curve.

2.8 Sample preparation, NMR conditions and data analysis

Six biological replicates of HR were treated as described by Zahmanov et al. (2015). Briefly, in a 2 mL tube 50 mg freeze-dried HR powder, 750 µL of CD_3OD and 750 µL of D_2O (KH_2PO_4, buffer, pH 6.0, containing 0.01% w/v TSPA-d4) were mixed. The mixture was homogenized, sonicated for 20 min, and centrifuged for 20 min at 12,000 rpm. Finally, 800 µL was placed into the NMR tube. The proton spectra (^1H NMR) were recorded at 25°C on an AVII+600 spectrometer (Bruker, Karlsruhe, Germany) at a proton frequency of 600.13 MHz with 4.07 s relaxation time and CD_3OD as the internal lock (Georgiev et al., 2015). The data analysis was processed as described by Marchev et al. (2016). Briefly, the spectra were phased, baseline corrected, set to TSPA at 0.0 ppm and binned to 0.04 ppm using MestReNova software (version 6.2.1, Mestrelab Research, Santiago de Compostela, Spain). The principal component analysis (PCA) was performed with SIMCA-P14.0 (Umetrics, Umea, Sweden), excluding the signals of water and methanol.

2.9 Elicitation assays

The selected HR clones were cultivated in a 200 mL flask with 0.5 g per 25 mL of liquid MS medium at 115rpm, 25ºC in darkness. After 11 days, methyl jasmonate (MeJA) and methylated β-cyclodextrin (MBCD) at final concentrations of 100 µM and 50 mM, respectively, were added. After 3 days of elicitation, samples were taken with their respective control conditions.

2.10 Statistics

The statistical analysis was performed with Microsoft Excel software. All data are the average of three measurements + SE. The multifactorial ANOVA analysis followed by the Tukey multiple comparison test were used for statistical comparisons. A p-value of <0.05 was assumed for significant differences. Correlation studies (Pearson´s correlation) were performed considering the p value <0.1 as significant.

3. Results

3.1. Establishment and genetic characterization of transgenic tobacco root cultures

Transgenic tobacco HR cultures were obtained by co-culture of leaf segments from *in vitro* tobacco plantlets with a wild type *Agrobacterium rhizogenes* A4 (pRiA4) strain (control line) or the engineered bacteria *A. rhizogenes* containing the plasmids [pRiA4 + pJCV52-STS3], [PRiA4 + pJCV52-*At*MYB12] and [pRiA4 + pDMDC32B-*At*MiR390a-B/c amiRNACHS]. The HR appeared at 2-4 weeks, after which they were cultured individually in MS solid medium, supplemented with cefotaxime (500 mg L^{-1}). After successive 2-week subcultures, the antibiotic concentration was reduced. Once the antibiotic was removed, the HR clones generated were tested for the virD gene by PCR to confirm that these are free of agrobacteria infection (Fig. 2). In general, the presence of the transgenes did not affect roots morphology, which were very similar to the wild type HR. Several root clones with a high growth capacity (growth index, GI>3.5) and displaying the typical HR phenotype were selected, and the presence of the respective transgenes in their genome was further confirmed by PCR (Fig. 2). The verified presence of the *Vv*STS3 gene, *At*MYB12 TF and the amiRNA of chalcone synthase in the selected root lines allowed us to establish four tobacco HR populations (Table 2) for further experiments.

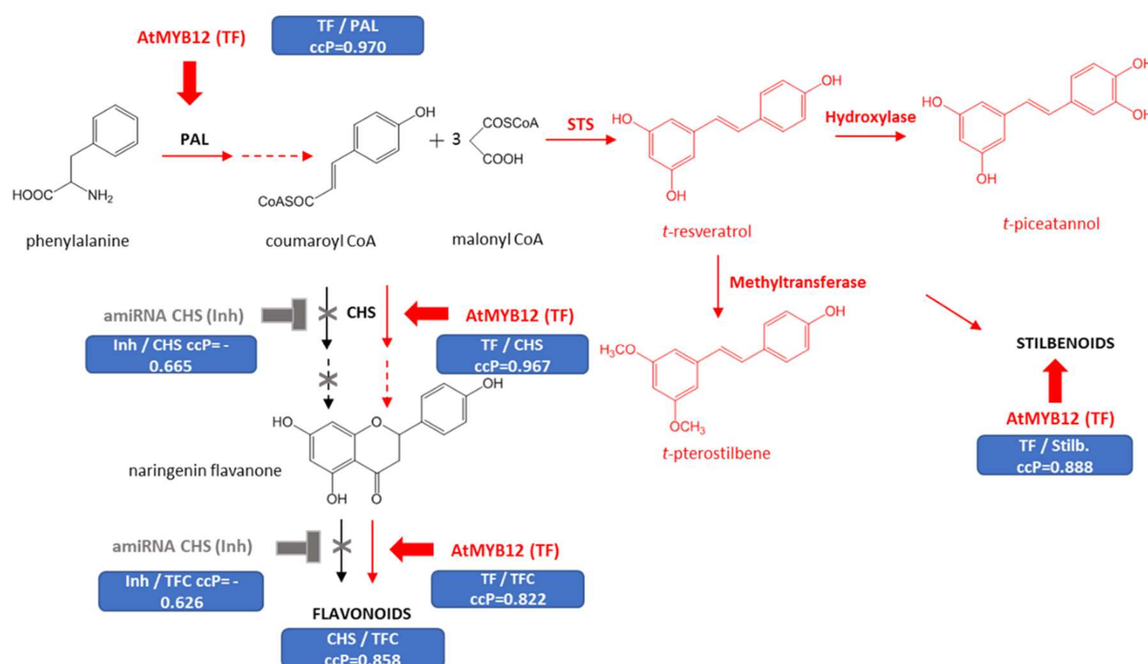

Figure 1. Metabolic perturbations of the phenolic biosynthetic pathway in the transgenic tobacco HR clones caused by heterologous gene expression. PAL, phenylalanine ammonia lyase; STS, stilbene synthase; CHS, chalcone synthase; TFC, total flavonoids content; Stilb, total stilbene contents; Inh, amiRNA expression; TF, *At*MYB12 TF expression; In red, activated pathway; In grey, inhibited pathway; Blue box, value of the mathematical correlation between gene expression and/or plant secondary metabolite accumulation; ccP, Pearson correlation coefficient.

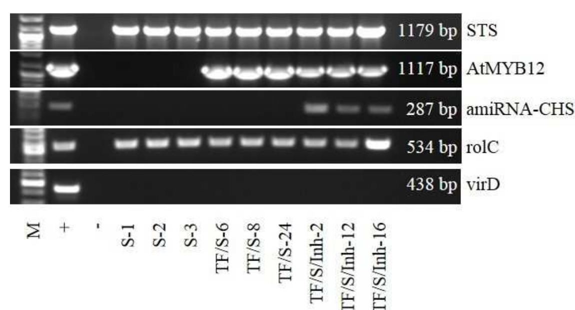

Figure 2. PCR analysis of the genomic DNA of HR lines. (+) positive control (corresponding *A. rhizogenes* used for the infections), (-) negative control (DNA of control HR or DNA of *Nicotiana tabacum* wild type plant for *rolC*). The positions S-1 to TF/S/Inh-16 represent the analyzed HR lines showing the presence of the corresponding transgenes.

Table 2. Description of the different hairy root lines established, indicating the plasmids used for agroinfection and the transgenes integrated in the plant genome and confirmed by PCR.

Line type	Plasmids	Transgenes inserted
C	pRiA4	T-DNA
S	pRiA4 + pJCV52-STS3	T-DNA + *Vv*STS
TF/S	pRiA4 + pJCV52-STS3, pRiA4 + pJCV52-*At*MYB12	T-DNA + *Vv*STS + *At*MB12
TF/S/Inh	pRiA4 + pJCV52-STS3, pRiA4 + pJCV52-*At*MYB12, pRiA4 + pDMDC32B-*At*MiR390a-B/c-amiRNACHS	T-DNA + *Vv*STS + *At*MB12 + amiRNA-CHS

3.2. Transcriptional analysis of the introduced transgenes and their effects on the expression of phenylalanine ammonia lyase and chalcone synthase and the production of stilbenes, total phenolic compounds and total flavonoids

Ten selected HR clones of the different types (denoted as C, S-1, S-4, S-9; TF/S-6, TF/S-8, TF/S-24 and S/TF/Inh-2, TF/S/Inh-12, TF/S/Inh-16) were transferred to liquid medium and subcultured every two weeks. After several rounds of subculture, samples were taken in sextuplicate for each analysis.

The qRT-PCR experiments revealed transgene expression driven by the CaMV 35S promotor in all the selected HR lines, however at variable levels (Fig. 3A). Differences in transgene expression among HR clones can be attributed to the transgene copy number and random transgene insertion into the plant root genome (Hernandez-García et al., 2010).

The expression of the *Vv*STS3 gene was notably low in the S/TF/Inh transgenic roots, even though we took into account the identity between the nucleotide sequence of NtCHS and *Vv*STS-3 (70%) and verified a non-homologous zone to select the amiRNA (see supplementary material). In contrast, as expected, no interactions between *At*MB12 TF and *Vv*STS3 expression were found, because both genes were under the control of the 35S promotor (Fig. 3A).

Among the metabolic changes induced by the heterologous expression of the different transgenes, stilbene biosynthesis was driven by the *Vv*STS-3 gene expression, reaching 57.4 µg L^{-1} of total stilbenes (*t*-R+*t*-Pt+*t*-Pn+Piceid), which were not detected in the wild type HR (C lines; Fig. 3B).

These results demonstrate that the designed biotechnological platform, carrying an active STS enzyme. was able to produce *t*-R, and then metabolize it to other stilbenes by the action of unspecific tobacco enzymes constitutively present in the HR cultures. As shown in Fig. 3B, Table S2, the *Vv*STS gene expression was significantly correlated with the total stilbene content in the HR clones.

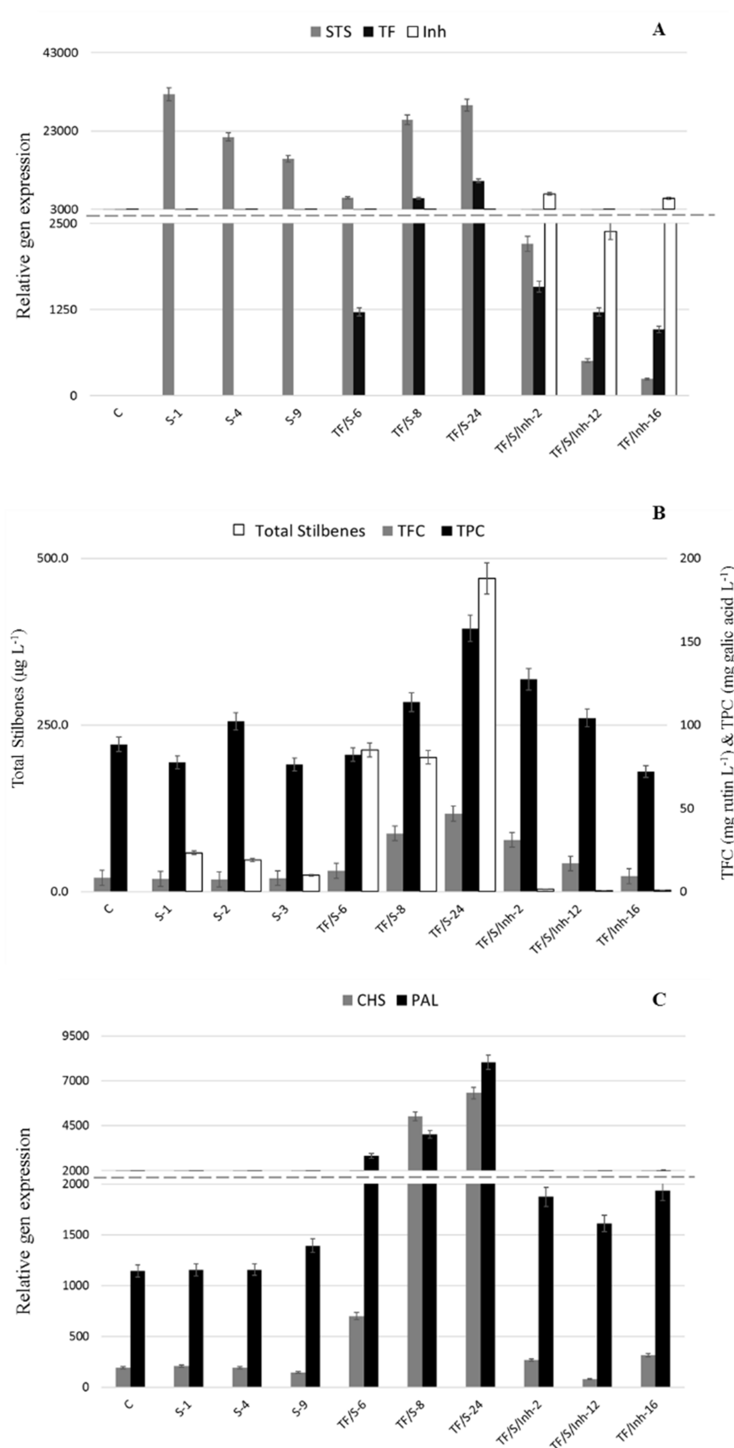

Figure 3. A) Relative gene expression of STS, stilbene synthase; Inh, amiRNACHS and TF, *At*MYB12 TF. B) Total stilbene content measured as the sum of *t*-R, *t*-Pn, *t*-Pt and piceid expressed as µg/L; TFC, total flavonoid content expressed as rutin (mg/L); TPC, total phenolic content expressed as GA, gallic acid (mg/L). C) Relative gene expression of CHS, chalcone synthase, and PAL, phenylalanine ammonia lyase. Each value is the average of 6 biological replicates ± SE.

As expected, the co-expression of the TF *At*MYB12 boosted stilbene biosynthesis, and the total stilbene content in TF/S-type HR lines was more than 6.9-fold higher than in the S-type. A similar increase in total phenolic compounds (TPC) (3.9-fold) and total flavonoids (TFC) (2.3-fold) also occurred in the TF/S-type roots (Fig. 3B). These results show that the TF effectively enhanced phenolic metabolism in the tobacco HR cultures (Fig. 1). Furthermore, a significant positive correlation between the transcript levels of the *At*MYB12 TF and those of the phenylalanine ammonia-lyase (PAL) (ccP=0.970) and CHS (ccP=0.967) genes from tobacco was patent (Fig. 3C and Fig. 1), indicating that the TF could activate early and late genes involved in phenolic metabolism in the tobacco HR cultures.

On the other hand, decreased levels of TFC (Fig. 3B) were observed in the HR lines carrying the amiRNACHS (TF/S/Inh lines), confirming an interference with the natural CHS gene expression in the biotechnological system (Fig. 3C). This was also ratified by the negative correlation between the amiRNA and CHS gene expression (ccP= -0.665) (Fig. 1).

3.3. Study of the metabolomic perturbations induced by the AtMYB12 transcription factor

In order to examined the metabolomic alterations caused by the expression of the *At*MYB12 TF, excluding those induced by the *Vv*STS gene or the amiRNACHS, we obtained transgenic HR clones carrying the T-DNA of *A. rhizogenes* together with the *At*MYB12 TF.

The presence of the transgene was confirmed by PCR (see Fig. S2). ^{1}H-NMR fingerprinting coupled with principal components analysis (PCA) allowed the identification of major perturbations in the metabolome of the transgenic roots harboring *At*MYB12 TF in comparison with wild type HR (control line). To examine in depth the metabolomic changes, the large data set from the ^{1}H-NMR was subjected to PCA to reduce the numerous NMR signals of the extracts (Fig. 4). A total of 79.5% of model variance was described with the principal components PC1 and PC2 (Fig 4A), revealing significant metabolomic differences. Both groups of samples were clearly divided by component two (Fig 4A) and the amino acid (δ 0.5-3.0 ppm), carbohydrate (δ 3.0-5.5 ppm) and aromatic (δ 5.5-9.8 ppm) regions were highly clustered in the root line with *At*MYB12 TF (Fig 4B). Signals of compounds detected by NMR are included in the Table S3.

The TF significantly affected the organic acid profile, both qualitatively and quantitatively, especially that of phenolic acids. The accumulation of two acids from the tricarboxylic acid cycle (TAC), fumaric and malic acid, as well as GABA, was notably enhanced (Fig. 5A-B, Table S3). Also, together with higher levels of cinnamic and *p*-coumaric acid, which are involved in the biosynthesis of stilbenes and flavonoids, the transgenic roots accumulated increased levels of caffeic and sinapic acid, which are eventually involved in lignin/lignan biosynthesis (Fig. 5A-B, Table S3). Similar glutamine, leucine or threonine levels were observed in both transgenic and wild type HR; in contrast, the content of proline and valine were significantly affected by the expression of *At*MYB12 (Fig. 5A-B, Table S3).

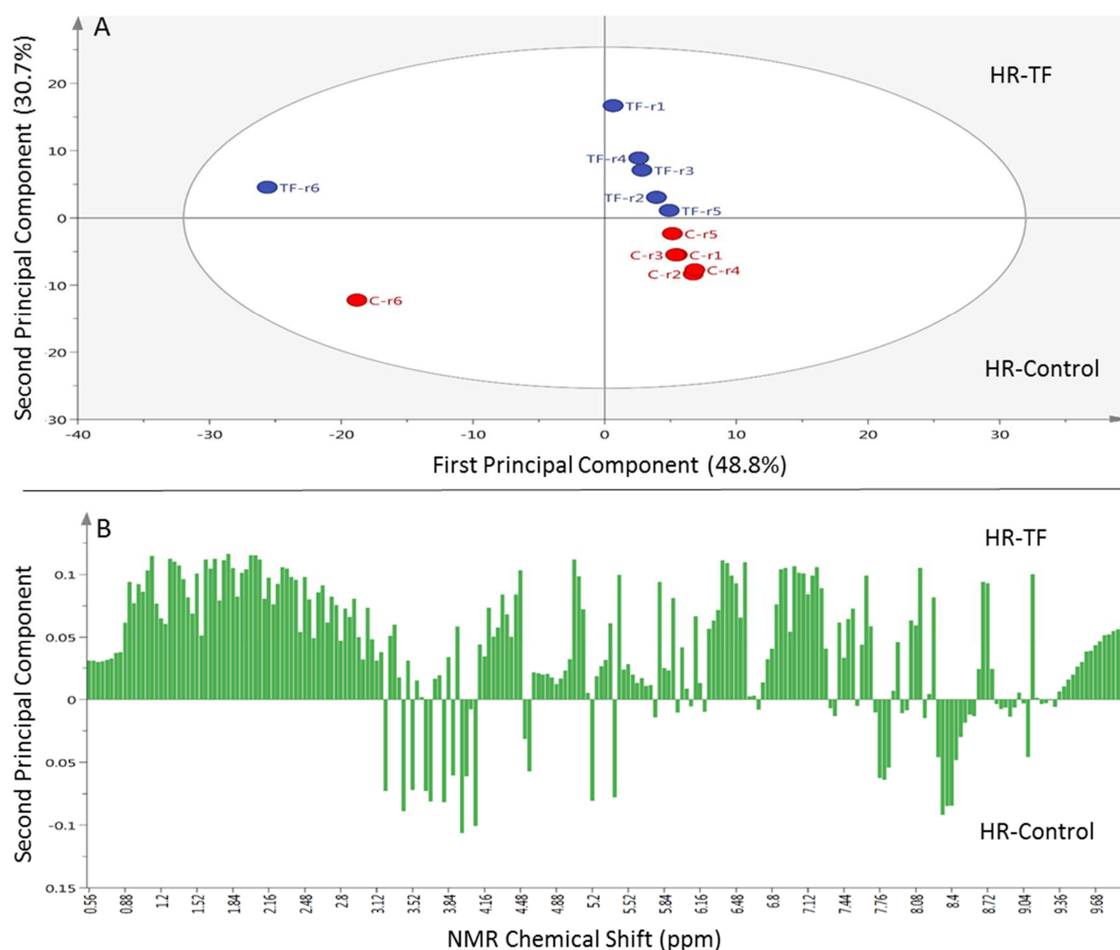

Figure 4. Score plot of principal component analysis (PCA) obtained from all ^{1}H NMR data using PC1 and PC2 (A) and the corresponding loading column plots (B) of control hairy roots (HR) and transgenic HR, harbouring the *At*MYB12 TF. Results are the average of 6 biological replicates

3.4. Bioproduction of stilbenes in elicited hairy roots

In order to investigate the effects of elicitation on the stilbene production in the designed biotechnological system, 100 µM MeJA and 50 mM MBCD were added to the tobacco HR cultures after 11 days of culture and samples were taken 3 days later. This elicitor treatment has been reported as optimum for inducing *t*-R production in *V. vinifera* cell cultures (Lijavetzky et al., 2008). Under the non-elicited control conditions, stilbene accumulation was highest in roots harboring both the *Vv*STS gene and *At*MYB12 TF (TF/S lines). Specifically, the root line TF/S-24, which displayed the highest expression of both transgenes (Fig. 3A), reached a stilbene production of 468.6 µg L^{-1} (Fig. 6A). In most lines, the stilbene accumulation pattern was piceid>*t*-R>*t*-Pt>*t*-Pn. In the TF/S-type lines, the contents were 142.4-339.7 µg L^{-1} for piceid, and 31.3-39.9 µg L^{-1} for *t*-R; the maximum yield of *t*-Pt was 86.4 µg L^{-1}, whereas *t*-Pn levels were significantly lower at 2.2-3.4 µg L^{-1} (Fig. 6A). These results show that unspecific tobacco methyltransferases effectively

bioconverted *t*-R into *t*-Pt when the *At*MYB12 TF was co-expressed with STS in the root cultures. On the other hand, the lowest stilbene production occurred in the TF/S/Inh-type root lines (Fig. 6A), which may be related with the concomitant low expression of the STS and *At*MYB12 genes (Fig. 3A). The combined elicitor treatment (MeJA+MBCD) did not increase stilbene production in the studied transgenic HR (Fig. 6A), but it changed the stilbene pattern to t-Pt>*t*-R≥piceid>*t*-Pn, with a notable increase in the *t*-Pt production. However, as shown in Fig 6B, the elicitor treatment was highly effective in releasing the accumulated stilbenes into the culture medium, with only piceid remaining in the root tissues.

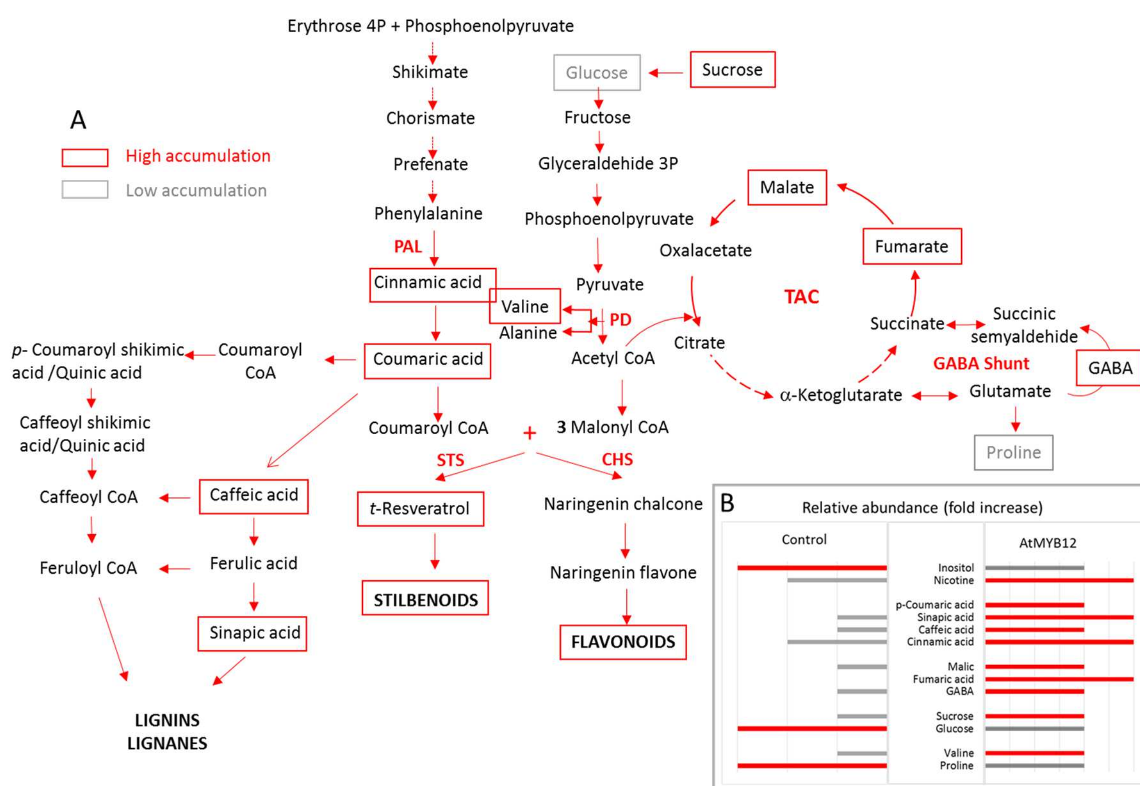

Figure 5. A) Proposed metabolomic alterations in the tobacco HR lines due to the expression of the *At*MYB12 TF. Red boxes, increased pathway/compounds. Grey boxes, decreased pathway/compounds. B) Relative abundance (fold increase) of compounds in the transgenic *At*MYB12 root lines compared with the wild type root lines (in red, increased compounds, in grey, decreased compounds).

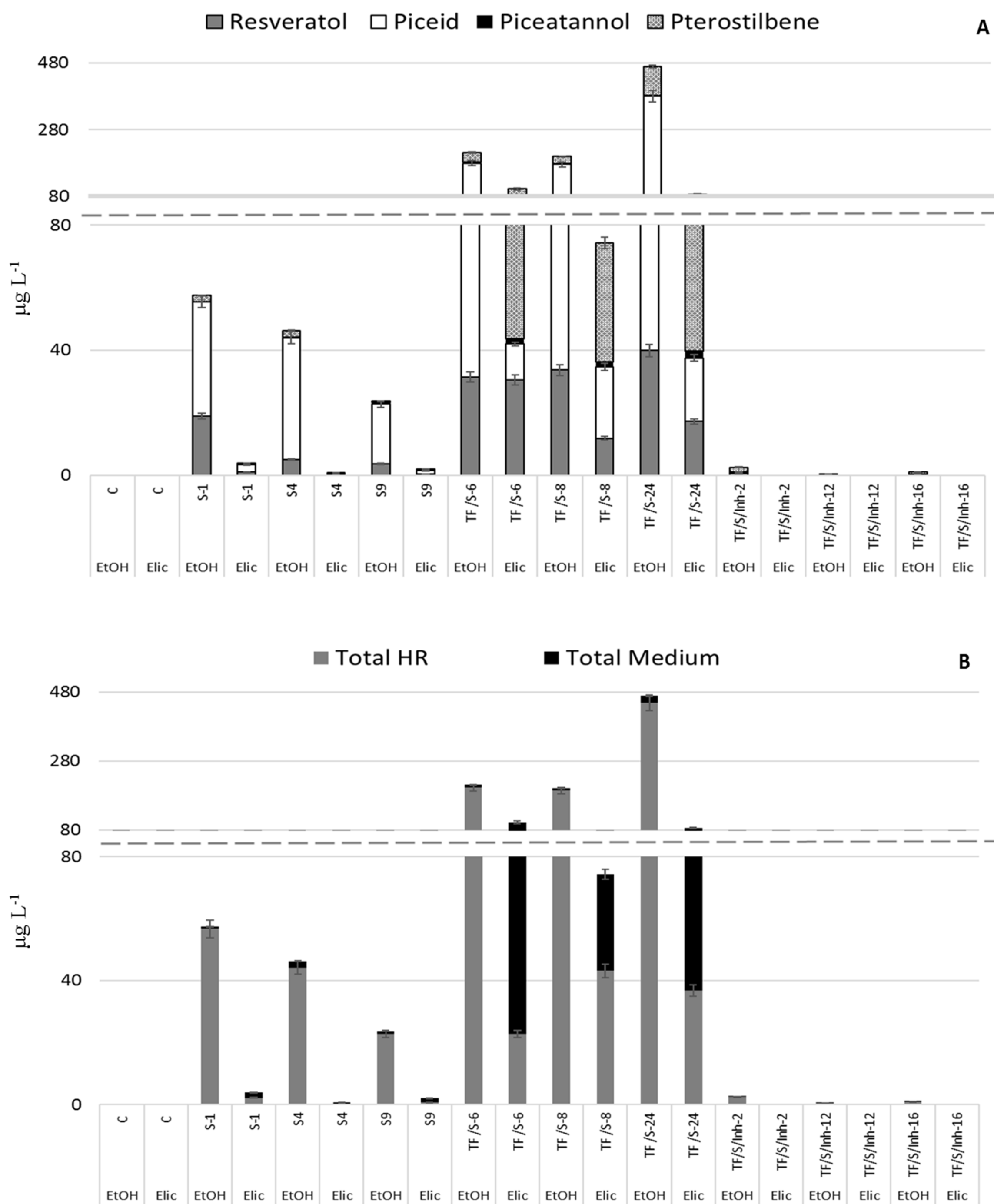

Figure 6. Stilbene production expressed as µg/L in the different HR clones studied after a culture period of 2 weeks. A) Stilbene pattern accumulation. B) Stilbene distribution in the HR or culture medium. Elic, elicited (MBCD + MeJA) conditions; EtOH, control conditions. Each value is the average of 6 biological replicates ± SE.

4. Discussion

As mentioned, tobacco is useful model plant system, which could be easily transformed by *A. rhizogenes* to generate HR cultures and this trait may be harnessed for the heterologous expression of foreign genes harbored in engineered *A. rhizogenes* strains (Vasilev et al., 2014). This fact along with the growing interest in *t*-R and its derivatives *t*-Pn and *t*-Pt (Martinez-Marquez et al., 2016; Hidalgo et al., 2017b), prompted us to set up a production platform based on transgenic tobacco HR. Thus, together with the *A. rhizogenes* T-DNA, the engineered tobacco HR lines harbored the following: the *Vitis vinifera* stilbene synthase (*Vv*STS3) gene for heterologous *t*-R production; the transcription factor (TF) *At*MYB12 from *Arabidopsis thaliana* together with *Vv* STS to generate a holistic response in the phenylpropanoid pathway and coordinate the up-regulation of multiple steps (Pandey et al., 2015); and artificial microRNA for chalcone synthase (amiRNA CHS) together with *Vv*STS3 and *At*MYB12 to limit the phenolic flux through the endogenous CHS enzyme, which competes for precursors with the STS enzyme imported for the flux deviation.

Our results confirmed the potential of engineered HR cultures to produce *t*-R, and the natural capacity of tobacco roots to bioconvert *t*-R into derivatives such as piceid, *t*-Pn and *t*-Pt. The bioproduction of *t*-R was apparently strongly correlated with the expression level of the *Vv*STS3 gene in the different transgenic HR clones obtained. The genetic transformation of plants with STS genes has given interesting results, including plant disease resistance (Fischer and Hain, 1994; Hain and Grimming, 2000), and is still being intensively studied (Delaunois et al., 2009). In this research pipeline, the first experiments were performed by Jeandet et al. (1997), who expressed the STS gene from *Arachis hypogea* in tobacco plants, leading to resveratrol accumulation after treatment with short-wavelength ultraviolet light. Heterologous expression of the STS gene has subsequently been reported in several edible plant species, including tomato, kiwifruit, apple, lettuce, barley and wheat, as a defense against plague attack, based on the phytoalexin activity of resveratrol (Delaunois et al., 2009 and references therein).

Several metabolic engineering strategies have also been developed for *t*-R production in microorganisms, but unlike plant systems, these require the introduction of a complex set of genes. In this context, the entire resveratrol pathway has been induced in modified yeast, *Yarrowia lipolytica* (ATCC 20362 strain), including the genes encoding phenylalanine/tyrosine ammonia lyase (PAL/TAL), cinnamate-4-hydroxylase (4CH), *p*-coumaroyl-CoA ligase (4CL), and STS (Huang et al., 2006). More recently, Trantas et al. (2009) constructed the complete resveratrol biosynthetic pathway in *S. cerevisiae* to produce resveratrol from the precursor phenylalanine (10 mM), obtaining a content of 290 µg L^{-1} *t*-R after 120 h of culture. Higher yields have been achieved in engineered *Escherichia coli* strains; for instance, Katsuyama et al. (2007) reported a production of 171 mg L^{-1} of *t*-R. However, the production of *t*-R in microorganisms always entails the addition of exogenous precursors (Jeandet et al., 2012). The production of *t*-Pn and *t*-Pt was recently reported by Li et al. (2016) in engineered yeast after feeding the culture with phenylalanine,

and Wang et al. (2014) obtained *t*-Pt from *t*-R and *p*-coumaric acid in both engineered yeast and *E. coli.* In seed plant systems, the production of *t*-Pn and *t*-Pt has been made possible by engineering either *V. vinifera* plant cell cultures (Martínez-Marquez et al. (2016) or tobacco transgenic HR cultures (Hidalgo et al., 2017b). In both cases the heterologous genes HsCYP1B1 and *Vv*ROMT were constitutively expressed, whilst *t*-R was supplied by elicitation in the cell cultures, or direct feeding in the HR.

As mentioned in the introduction, TF overexpression can boost a complete metabolic pathway (Capell and Christou, 2004). In this context, the TF of the MYB family have been described as the most useful in enhancing flavonoid biosynthesis (Mehrtens et al., 2005; Stracke et al., 2007). In tobacco plants, *At*MYB12 TF expression causes a strong transcriptome change by up-regulating genes encoding enzymes downstream in the phenolic pathway, which increases flavonoid content as well as the expression of several genes encoding upstream enzymes involved in the biosynthesis of common precursors in the stilbene and flavonoid biosynthetic pathways (Misra et al., 2010).

In our study, in agreement with the results reported by Misra et al. (2010), the *At*MYB12 TF expression was tightly correlated with enhanced flavonoid content in the transgenic HR clones, probably because of an up-regulation of the CHS gene. It has previously been demonstrated that ectopic expression of the *At*MYB12 TF increases the flavonoid content of tomato leaves and fruits (Pandey et al., 2015; Luo et al., 2008). In our tobacco HR, the TF also enhanced the expression of the upstream PAL, resulting in a higher total phenolic accumulation compared with the control roots. Similar results have been reported in *At*MYB12 transgenic tobacco plants, although PAL expression was not regulated by the TF in *Arabidopsis thaliana* (Stracke et al., 2007).

A more detailed view of the metabolism modulation exerted by ectopic expression of TF MYB12 in transgenic hairy roots was provided by NMR-based metabolomics approach. Major qualitative and quantitative metabolomic changes were analyzed in solely TF *At*MYB12-expressing roots and compared with wild type hairy roots (control line). As well as amino acids and sugars, the transgene expression affected organic acids, especially phenolic acids. The accumulation of fumaric and malic acids from the tricarboxylic acid cycle was notably enhanced, and high levels of GABA were found, but contrary to previous reports in transgenic plants, citrate contents were not affected (Misra et al., 2010). These results reflect that the regulation of metabolic pathways will vary according to the plant organs and tissues. Thus, while the levels of several amino acids, including valine, alanine, and phenylalanine, increased in the MYB-12 transgenic tobacco plants, in the HR system we observed the up-regulation only of valine, and the down-regulation of proline biosynthesis.

Among phenolic acids, the transgenic roots accumulated higher levels of cinnamic and *p*-coumaric acid, which are involved in the biosynthesis of stilbenes and flavonoids, as well as caffeic and sinapic acid, which may be involved in lignin biosynthesis. These results are in agreement with those reported in *At*MYB12 TF transgenic

tobacco plants by Misra et al. (2010), who observed an increased expression of genes involved in lignin biosynthesis. Altogether, the results suggest that part of the increased precursor availability for stilbene biosynthesis could be diverted to the lignin pathway, undermining the effectiveness of our biotechnological system for the production of the target compounds, *t*-R and its derivatives *t*-Pn and *t*-Pt (Fig. 5).

The heterologous expression of STS can alter flower morphology, and cause male sterility in tobacco and petunia (Fischer et al., 1997). The sterility may be linked to a competition for substrates between STS and endogenous chalcone synthase (CHS), since fertility can be restored in tobacco by adding exogenous flavonol (Fischer et al., 1997). As mentioned, STS and CHS are both involved in the polyphenol pathway, and it is reasonable to assume that the expression of exogenous STS may lead to a competition for substrates. Effectively, among the analyzed tobacco HR lines, the ones transformed with amiRNA CHS achieved the lowest levels of flavonoids due to inhibition of the CHS gene expression. By silencing the branching point of the flavonoid biosynthetic pathway, a greater flow of precursors was channeled for the production of *t*-R and its derivatives. Unfortunately, the transgenic root lines carrying the amiRNA CHS also showed a low *Vv*STS gene expression and their stilbene contents were lower than in the TF/S root clones.

We have previously discussed that elicitation can improve the heterologous production of a target compound in plant cell cultures (Mehrotra et al., 2010, Exposito et al., 2010). Elicitation of the engineered hairy roots with 50 mM MBCD and 100 µM of MeJA significantly increased the release of *t*-R and its derivatives *t*-Pn and *t*-Pt to the liquid medium, whereas piceid remained mainly inside the roots. In contrast, the positive effects of the combined elicitor treatment (MBCD + MeJA) on the *t*-R production has been previously reported in non-transgenic and transgenic grapevine cell cultures (Jeandet et al., 2014; Martinez-Marquez et al., 2016), and also in transgenic *S. marianum* cell cultures carrying the *Vv*STS gene and supplemented with MBCD (Hidalgo et al., 2017a). The ineffectiveness of the HR culture system in improving the total stilbenoid contents was perhaps due to their fast metabolization in the culture medium, as reported previously (Hidalgo et al., 2017b).

5. Conclusions

Taken as a whole, our results show the suitability of the engineered HR cultures for the heterologous production of *t*-R and its derivatives *t*-Pn and *t*-Pt. The effectiveness of the *At*MYB12 TF in boosting the phenolic pathway and the amiRNA CHS in blocking competitive pathways was also demonstrated. However, these results also show the extreme complexity of metabolically engineering biotechnological systems based on seed plant *in vitro* systems. A large part of the *t*-R biosynthesized by the root cultures was metabolized not only to the target compounds *t*-Pn and *t*-Pt, but also to piceid and probably other non-identified resveratrol-derivatives. Moreover, the boosting of phenolic metabolism, reflected by the high levels of caffeic and sinapic acid in the HR lines harboring the *At*MYB12 TF, could also provide precursors for competitive pathways, such as lignan and lignin

biosynthesis. Therefore new approaches are required in which competitive upstream pathways are silenced by specific amiRNAs and the carbon flux is more effectively channeled to stilbene biosynthesis.

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Conflict of Interest Disclosure

The authors have declared no conflicts of interest

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Chapter 3. *Silybum marianum* cell cultures stably transformed with *Vitis vinifera* stilbene synthase accumulate *t*-resveratrol in the extracellular medium after elicitation with methyl jasmonate or methylated β-cyclodextrins.*

Spanish Summary

La creciente demanda de *t*-resveratrol para usos industriales ha generado un gran interés para su producción biotecnológica. La producción heteróloga de resveratrol en suspensiones celulares, además de requerir la introducción de sólo uno o dos genes, presenta las ventajas de un alto rendimiento de biomasa y un corto tiempo de cultivo, pudiendo constituir una opción para la producción a gran escala. *Silybum marianum* es la fuente natural del flavolignano silimarina. La síntesis de fenilpropanoides en cultivos celulares de esta especie es elicitada por jasmonato de metilo y β-ciclodextrinas metiladas, provocando la liberación al medio de cultivo de alcohol coniferílico e isómeros del complejo silimarina. Dado que la estilbeno sintasa comparte los precursores clave involucrados en la biosíntesis de flavonoides y/o monolignanos, hemos investigado el potencial de los cultivos de *S. marianum* modificados metabólicamente para la producción de resveratrol. Las suspensiones celulares se transformaron con el gen estilbeno sintasa 3 de *Vitis vinifera* y la expresión del transgén condujo a una significativa acumulación extracelular de resveratrol bajo condiciones de elitación, fundamentalmente a expensas del precursor alcohol coniferílico. Por el contrario, la producción de silimarina se vio poco afectada en los cultivos transgénicos, ya que la vía flavonoide es limitante para la síntesis de este producto, debido a que existe un suministro preferente de precursores para la rama de los monolignanos. El hecho de que la expresión del gen STS consumiera precursores de compuestos no bioactivos (ej. alcohol coniferílico) manteniendo inalterado el flujo metabólico para los compuestos secundarios diana (silimarina), abre una nueva vía para ampliar las aplicaciones de cultivos celulares para la producción simultánea de los metabólitos constitutivos de la propia especie como de otros compuestos valiosos producidos heterólogamente.

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Research Article

Silybum marianum cell cultures stably transformed with *Vitis vinifera* stilbene synthase accumulate *t*-resveratrol in the extracellular medium after elicitation with methyl jasmonate or methylated β-cyclodextrins

The growing demand for *t*-resveratrol for industrial uses has generated considerable interest in its production. Heterologous resveratrol production in plant cell suspensions, apart from requiring the introduction of only one or two genes, has the advantage of high biomass yield and a short cultivation time, and thus could be an option for large-scale production. *Silybum marianum* is the source of the flavonolignan silymarin. Phenylpropanoid synthesis in cultures of this species can be activated by elicitation with methyl jasmonate and methylated β-cyclodextrins, with products of the pathway (coniferyl alcohol and some isomers of the silymarin complex) being released into the medium. Given that stilbene synthase shares the same key precursors involved in flavonoid and /or monolignol biosynthesis, we explored the potential of metabolically engineered *S. marianum* cultures for *t*-resveratrol production. Cell suspensions were stably transformed with *Vitis vinifera* stilbene synthase 3 and the expression of the transgene led to extracellular *t*-resveratrol accumulation at the level of milligrams per litre under elicitation. Resveratrol synthesis occurred at the expense of coniferyl alcohol. Production of silymarin was less affected in the transgenic cultures, since the flavonoid pathway is limiting for its synthesis, due to the preferred supply of precursors for the monolignol branch. The fact that the expressed STS gene took excessively produced precursors of non-bioactive compounds (coniferyl alcohol), while keeping the metabolic flow for target secondary compounds (i.e. silymarin) unaltered, opens a way to extend the applications of plant cell cultures for the simultaneous production of both constitutive and foreign valuable metabolites.

Keywords: Flavonolignans / Heterologous gene expression / Plant cell cultures / Resveratrol / *Silybum marianum*

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1 Introduction

The phenylpropanoid pathway, which produces a huge range of secondary metabolites, including lignins, flavonoids, and stilbenes, is ubiquitous in the plant kingdom. These compounds are involved in plant defense, structural support, and survival [1]. Besides their importance to plants, the biosynthesis of flavonoids and stilbenes has attracted increasing attention because of their potential health benefits.

Lignin, stilbene, and flavonoid synthetic pathways share the same precursor molecules (Fig. 1). The first step in phenylpropanoid biosynthesis is the deamination of L-phenylalanine to *t*-cinnamic acid, catalyzed by phenylalanine ammonia lyase

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Abbreviations: CA, coniferyl alcohol; **Cd**, methylated β-cyclodextrins; **CHS**, chalcone synthase; **MJ**, methyl jasmonate; **Sm**, silymarin; **STS**, stilbene synthase; ***t*-R**, *trans*-resveratrol; **Tx**, taxifolin

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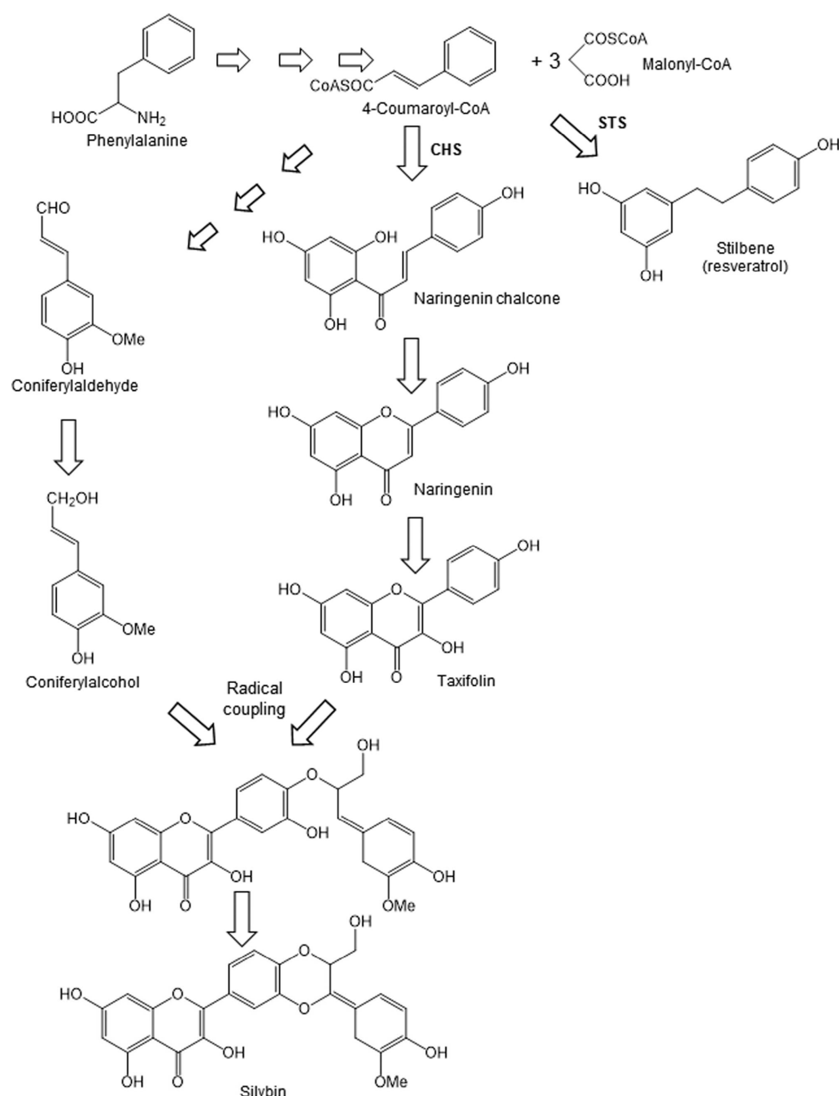

Figure 1. Schematic representation of the phenylpropanoid pathway for monolignol, flavonoid and stilbenoid synthesis. General steps for flavonolignan biosynthesis are also shown.

(EC 4.3.1.5). Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (EC 1.14.13.11) to give 4-coumaric acid, which is then activated by 4-coumaroyl:CoA ligase (EC 6.2.1.12) and the resulting 4-coumaroyl-CoA enters the flavonoid, stilbenoid, and monolignol pathway.

A type III polyketide synthase sequentially adds three acetate extender units derived from malonyl-CoA to a single activated 4-coumaroyl-CoA starter unit. Depending on whether the polyketide synthase is chalcone synthase (CHS, EC 2.3.1.74) or stilbene synthase (STS, EC 2.3.1.95), subsequent folding and cyclization of the generated tetraketide intermediate results in the production of either a chalcone or stilbene ring structure [1].

Hydroxycinnamoyl-coenzyme A shikimate:quinate hydroxycinnamoyl-transferase (EC 2.3.1.133) catalyzes the transfer of the *p*-coumaroyl group to shikimate, thus entering the monolignol pathway [2, 3]. Therefore, carbon allocation in the phenylpropanoid pathway is controlled by the phenylalanine – *p*-coumaric acid pool and by differential induction of genes needed for the described reactions.

The polyphenol *t*-resveratrol (*t*-3,5,4′-trihydroxystilbene) (*t*-R), a member of the stilbene family, has been the focus of a number of studies in medicine and plant physiology. Apart from being a phytoalexin produced in response to stress, such as wounding or pathogen attack [4], this compound is well known for its cardioprotective, antitumor, neuroprotective, and antioxidant activities [5]. Additional work has also demonstrated that *t*-R increases the lifespan in lower and higher organisms through the activation of the sirtuin proteins [6].

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With the growing demand for *t*-R for nutraceutical, cosmetic, and pharmaceutical uses, extensive research work has been devoted to enhancing *t*-R production in plants and plant cell cultures through elicitation or metabolic engineering [6–12].

The first gene transfer experiments were performed with a complete STS gene from *Arachis hypogea* introduced into tobacco [13], leading to *t*-R accumulation after induction with short-wavelength ultraviolet (UV) light, a well-known elicitor of *t*-R synthesis [14]. It has also been shown that the heterologous expression of two grapevine STS genes, *Vst1* and *Vst2*, in tobacco confers a higher resistance to *Botrytis cinerea* infection [15]. Since this pioneering work, STS genes have been transferred to a number of crops, as well as to yeast and bacteria [6].

Due to their capacity for active continuous growth and easy scale-up in large culture volumes, metabolically engineered undifferentiated plant cell suspension cultures could also be a convenient alternative for secondary metabolite production; however, to date, this approach has not been explored in depth for *t*-R production.

Silybum marianum (Asteraceae family) is the source of silymarin (Sm), a flavonolignan prescribed for the treatment of chronic liver disease [16, 17], and more recently for the prevention of recurrent hepatitis C in liver transplant recipients by the European Medicines Agency [18].

Two phenylpropanoid units are needed for Sm biosynthesis: the flavonoid taxifolin (Tx) and the monolignol coniferyl alcohol (CA). The oxidative coupling of the CA moiety to Tx produces the regioisomers of the Sm mixture (silychristin, isosilychristin, silydianin, silybin A and B, isosilybin A and B) [19, 20]. Only the silybin isomer is shown in Fig. 1.

We have previously reported that elicitation is an effective strategy to increase Sm production in cell suspensions [21]. In our studies, it was determined that methyljasmonate (MJ) and random methylated β-cyclodextrins (Cd) induced a massive release of CA, and some of the isomers of the Sm mixture were also detected in the extracellular medium [22, 23]. Expression studies of genes related to the flavonolignan pathway revealed that elicitation with MJ or Cd up-regulated transcription; the change in their expression partially overcame rate-limiting steps for Sm accumulation [24].

Since STS shares the same key precursor metabolites employed for flavonoid and /or monolignol biosynthesis, the heterologous expression of *VvSTS* in *S. marianum* could find a suitable substrate for the purpose of *t*-R production in a non-producing plant species. Based on this rationale, we metabolically engineered *S. marianum* cell cultures with a stilbene synthase from grapes with the aim of assessing whether *t*-R production is achieved and whether its eventual production may have an effect on flavonolignan metabolism in elicited cultures.

2 Materials and methods

2.1 VvSTS3 cloning and construction of the binary vector

An STS coding region (Ref. Seq. XM_002263686.2. PREDICTED: stilbene synthase 3 [*Vitis vinifera*]) was cloned from cDNA of a *V. vinifera* cv. Gamay cell culture. A cell suspension was elicited using 50 mM Cd and 0.1 mM MJ for 24 h as previously described [25] and cells were collected by filtration under gentle vacuum. Total RNA was isolated as described [26] from 1 g fresh elicited cells, and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). First-strand cDNA was synthesized from total RNA using a cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit from ThermoScientific) according to the manufacturer's instructions and used as template to PCR amplify the STS coding region using specific primers (FW 5′-ATGGCTTCAGTTGAGGAATTTAGA-3′; RV 5′-TTAATTTGTAACCGTAGGAACGCT-3′). The amplification reactions consisted of 1 cycle at 94°C for 5 min and 30 cycles at 94°C for 30 s, 54°C for 30 s, 72°C for 1 min, followed by an extension cycle of 10 min at 72°C. Amplified DNA fragments were cloned into pGEM®-T Easy (Promega) and the inserts sequenced.

A binary vector pJCV52-STS3 was built following the protocol described in [27]. The VvSTS3 gene was cloned into the pJCV52 vector (Laboratory of Plant Systems Biology; Ghent University, Belgium) under the control of CaMV35S promoter using the Gateway cloning system (Invitrogen, Life Technologies, NY, USA). The binary vector was transferred into chemically competent *Agrobacterium tumefaciens* strain C58C1 (pGV2260) [28] by standard techniques [29].

2.2 Plant and bacterial culture

Suspension cultures of *S. marianum* were used for transformation. For routine subcultures every two weeks, control cultures and transgenic derivatives were maintained in MS medium containing 3% sucrose, 1 mg/L 2,4-D and 0.5 mg/L BA as described previously (MSS medium) [22]. Cultures were incubated in the dark at 25°C and shaken at 90 rpm.

A. tumefaciens C58C1 (pGV2260) containing the binary plasmid was grown on solid LB medium at 28°C for 48 h. A single colony was inoculated in 50 mL of liquid LB medium that contained 100 mg/L rifampicyn and 75 mg/L spectinomycin, and was incubated overnight at 28°C with shaking (180 rpm). 50 mL of LB medium, supplemented with 75 mg/L spectinomycin, was inoculated with 1 mL of the 1-day-old culture and grown at 28°C and 180 rpm overnight. The bacterial medium was removed by centrifugation at 14 000 rpm for 3–5 min and the resulting bacterial pellet was washed and suspended in MSS medium.

2.3 Stable transformation of suspension cultures

A. tumefaciens C58C1 (pGV2260) carrying the binary plant expression vector pJCV52-STS3 was employed to stably introduce the construct into *S. marianum* cells by a modification of the *Agrobacterium*-mediated transformation method for *V. vinifera* and *Taxus x media* [30].

In brief, exponentially growing cell suspensions (3 days after subculture) were supplemented with 200 mg/L acetosyringone and co-cultured with the bacteria suspended in plant liquid medium (final bacterial OD_{600} in co-culture 0.1). The *Agrobacterium*-infected culture was incubated in darkness at 25°C at 100 rpm for 2 days. The infected cell culture was then

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centrifuged at 3000 rpm for 5 min and the pellet was washed three times with fresh culture medium.

Due to the high proportion of non-transformed escapes to kanamycin when cell suspensions were employed as plant material reported in the literature [30, 31], we previously checked the resistance of *S. marianum* cells to this antibiotic and percentages of viability up to 80% were scored even at 500 mg/L. When testing paromomycin, however, cell viability was lost at 50 mg/L (personal observations). Therefore, for the selection of transformants, the infected cell suspension (2.5 mL) was spread on petri dishes containing solid (10 mg/L agar) MSS medium supplemented with 300 mg/L cefotaxime and 60 mg/L paromomycin. Plates were incubated at 25°C in the dark. Periodical subcultures of the growing callus colonies were carried out at decreasing cefotaxime concentrations. After 3 months, cefotaxime was eliminated from the medium, and the transformed cells were allowed to grow in the MSS selection medium with 60 mg/L paromomycin.

To re-establish liquid cultures, callus tissue was transferred to fresh MSS medium (with 60 mg/L paromomycin.) and incubated under normal cultivation conditions. Subculture was repeated two times at 14-day intervals in the same medium. Subsequent subculturing was carried out into fresh MSS medium (without paromomycin).

2.4 Elicitation and metabolite analyses

For the elicitation experiments, 3 g wet weight 14-day old cells were transferred to 100 mL flasks containing 20 mL medium and incubated for three days prior to the addition of 30 mM final concentration Cd (chemically pure heptakis (2,6-di-O-methyl)-β Cd) purchased from Duchefa (Spain) or 100 μM MJ (Sigma-Aldrich, Spain). The elicitation conditions were selected based on previous results for Sm production in *S. marianum* cultures [22, 32].

Flavonolignans and *t*-R were extracted from the biomass with 80% methanol at 60°C for 4 h. Extracts were filtered, dried in vacuo at below 40°C and resuspended in 1 mL methanol.

The culture medium of cell cultures was separated from the biomass by filtration and flavonolignans and *t*-R were extracted three times with two volumes of ethylacetate. The combined extracts were dried *in vacuo* at below 40°C and resuspended in 1 mL methanol as above.

Flavonolignan and *t*-R analysis was performed by HPLC in a Spherisorb ODS-2 (5 μm) reversed-phase column (4.6×250 mm) at 35°C. The mobile phase was a mixture of 34 volumes of methanol and 66 volumes of acetic acid:water (5:55 v/v) at 1 mL/min [22]. Chromatograms were adquired at 306 nm and, when stated, also at 280 nm. Flavonolignan identification had been previously performed by LC MS (MSD trap XCT and LC 1100 both from Agilent ®), in a Spherisorb S3 ODS2 column (2×100 mm, 3.5 μm) in E.S.I (-) under the same conditions as reported for HPLC analysis of flavonolignans [32]. Identification of *t*-R was carried out by comparison with a commercial standard and also by LC MS as described for flavonolignans. Concentrations of *t*-R and flavonolignan metabolites were estimated using the standard curve generated by pure compounds.

2.5 STS3 transgene analysis

Genomic DNA was isolated from 150–300 mg of *S. marianum* cells using the E.Z.N.A. HP Plant DNA Mini Kit (OMEGA), according to the manufacturer's instructions. The STS3 transgene in transformed suspensions was detected by PCR analysis, where the Fw 5´- CACCATGGCTTCAGTTGAGGAATT-3´ and Rv5´-ATTTGTAACCGTAGGAACGC -3´ primers were used to amplify a 1179-bp coding region of the STS gene. The amplification reactions consisted of 1 cycle at 95°C for 5 min and 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1.30 min, followed by an extension cycle of 10 min at 72°C. DNA from the non-transformed wild-type *S. marianum* cells was used as a negative control. PCR products were analyzed by electrophoresis on 1% agarose gels.

Total RNAs were extracted from *S. marianum* cell cultures using the Nucleospin RNA Plant kit (Macherey-Nagel, Germany). Genomic DNA was eliminated by treating each sample with DNase I (Macherey-Nagel, Germany) according to the manufacturer's instructions. The concentration of total RNA was estimated using a Nanodrop 1000 spectrophotometer. RNA quality was further assessed by agarose gel electrophoresis. RNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen, CA, USA) with oligo dTs as primers.

Expression studies by qRT-PCR were performed in an ABIPRISM 7000 Sequence Detection System with Brilliant SYBRGreen QPCR Master Mix (Stratagene). All PCR reactions were carried out in duplicate in a total volume of 12 μL for 40 cycles under the following conditions: 95°C for 2 min, 40 cycles (95°C, 10 s; 60°C, 20 s; 72°C, 20 s) followed by a melting curve. Threshold cycles (TC) were determined using the 7000 SDS System Software, and TC values were calculated using the actin gene as an endogenous control. The use of actin as an internal reference gene was validated by ensuring that its relative expression remained constant in control cultures and after elicitation. The relative expression levels of target genes were calculated with formula $2^{-\Delta CT}$ [33]. Each sample was run in triplicate, with two biological replicates. Specificity of the primer pairs was evaluated by melting-curve analysis (Mx3000P real-time PCR instrument software, version 2.0) after 40 amplification cycles. The sequences of primers for the expression study were as follows: Actin Fw 5' GCAGGGATCCACGAGACCACC 3'; Actin Rv 5' CCCACCACTGAGCACAATGTTCC 3' and STS Fw 5' AAGGGGAAAAAGCCACCACA3'; STS Rv 5' TTCGATGGTCAAGCCAGGTC 3'

3 Results

Initial transformation of *S. marianum* cell cultures with *A. tumefaciens* C58C1 (pGV2260) harboring pJCV52-STS3 was assessed by antibiotic resistance selection. After 4 weeks on the selection medium, paromomycin-resistant callus colonies were individually transferred to new plates with fresh selection medium. In the selection medium, the non-transformed material did not grow and turned brown, while transformed calli grew vigorously. Within 2–3 months of the initial transformation, sufficient callus material was obtained to check for plant genome T-DNA integration of the STS3 gene by PCR amplification using a primer

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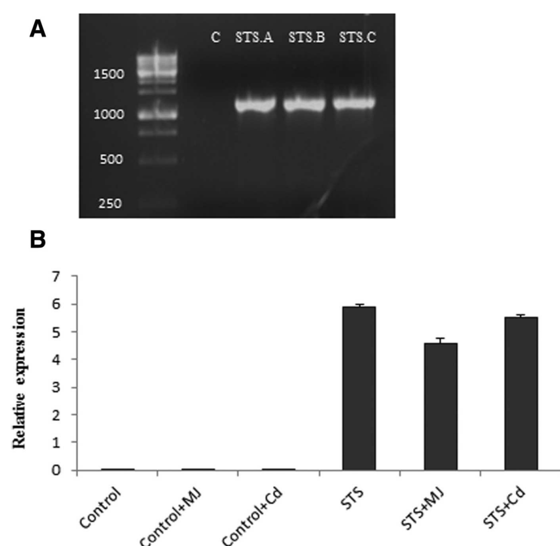

Figure 2. (A) PCR amplification products from genomic DNA of non-transformed and three transgenic cell lines of *Silybum marianum* suspension cultures. Amplification was carried with specific primers for *VvSTS3*. (B) Relative quantitative expression (ratio of gene to actin) of *VvSTS3* in non-transformed and STS transformed S. *marianum* suspension cultures after treatment with MJ or Cd for 24 h. Data represent mean of triplicate cultures ± SD.

pair specific for VvSTS3 under the control of P35S. Three *S. marianum* transgenic calli were selected, and, as shown in Fig. 2A, the STS gene was present in all the transgenic clones, but not in the wild type.

Cell suspension cultures were initiated from one selected transgenic calli maintained under continous paromomycin selection for more than 3 months (cell line STS.A). We had in mind the stimulating effect of the elicitors MJ and Cd on the phenylpropanoid pathway, which provides substrates for flavonolignan synthesis in cell cultures of *S. marianum* [23]. Thus, after two rounds of subculture to stabilize the suspension, for phenotipyc characterization, flavonolignan and *t*-R levels were analyzed in the cell biomass and culture medium of STS3-transformed cultures treated with 30 mM Cd or 100 μM MJ, alone or in combination, for several time periods. The effect of the elicitors on non-transformed control cultures was also determined.

In order to demonstrate the heterologous expression of the STS3 gene in the *S. marianum* cell line, its transcript levels were measured by qRT-PCR using actin as a housekeeping reference gene, under control and elicited conditions. As expected, no transcripts corresponding to STS3 were detected in non-transformed control, while the relative expression of the STS3 gene was between 4- and 6-times higher than the expression of the actin gene in the transgenic cell line (Fig. 2B). Transcript STS3 accumulation was not affected by Cd or MJ treatment, probably because the STS3 gene was under the control of the 35S promoter.

Figure 3 shows the chromatogram profiles, taken at A306, of medium extracts of non- transformed control cultures and of the transformed STS.A line after 48 h in the presence of 30 mM Cd. *t*-R was not detected at any time of the growth cycle under normal culture conditions (i.e. without elicitation), neither in untransformed nor in STS3-transformed cell lines. However, chromatograms of medium extracts of STS3-transformed cultures treated with 30 mM Cd revealed a new peak at a retention time of 21 min. In the same peak mass spectra showed a main representative ion $[M]^-$ at *m/z* 227.2 corresponding to *t*-R. Under the experimental conditions of this work, *t*-R was never detected in biomass extracts. CA and flavonolignans were found both in non- transformed and in STS3-transformed cultures; however, the peak at 21 min corresponding to *t*-R can only be seen in medium extracts from the STS.A cell line.

The total accumulation of *t*-R in STS3-transformed cultures in the presence of elicitors after different times is shown in Fig. 4A. A significant amount of *t*-R was obtained from the extracellular medium of 30 mM Cd -treated cultures, increasing from the beginning of elicitation up to about 12 mg/L at 76 h. A decrease in production was observed at 168 h.

Unlike Cd alone, treatments with MJ alone or MJ + Cd did not improve *t*-R production (Fig. 4B). The promoter used to overexpress the *STS* gene was the strong constitutive CaMV35S promoter. Probably, since enzyme production was constitutive and MJ did not alter its expression, MJ alone or in combination

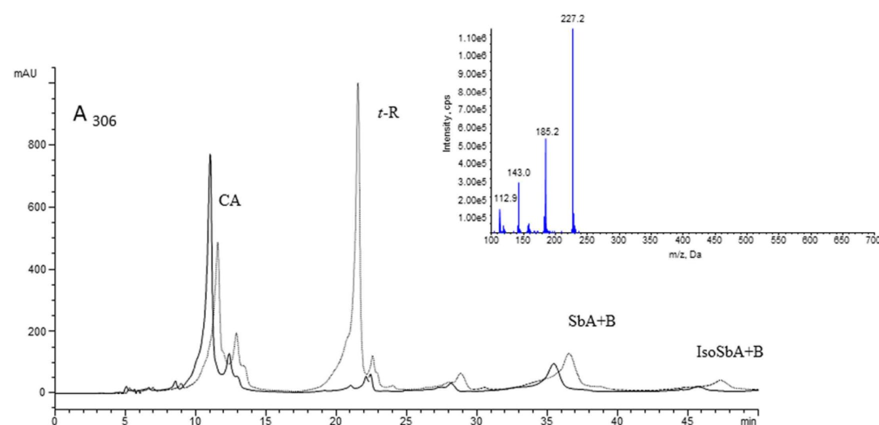

Figure 3. Chromatograms (A306) of medium extracts of non-transformed (solid line) and STS-transformed *S.marian um* cultures (dotted line) treated with 30 mM Cd for 48 h. Inset: mass spectra of the peak (dotted line) at a retention time of 21 min.(Sb: silybin; IsoSb: isosilybin).

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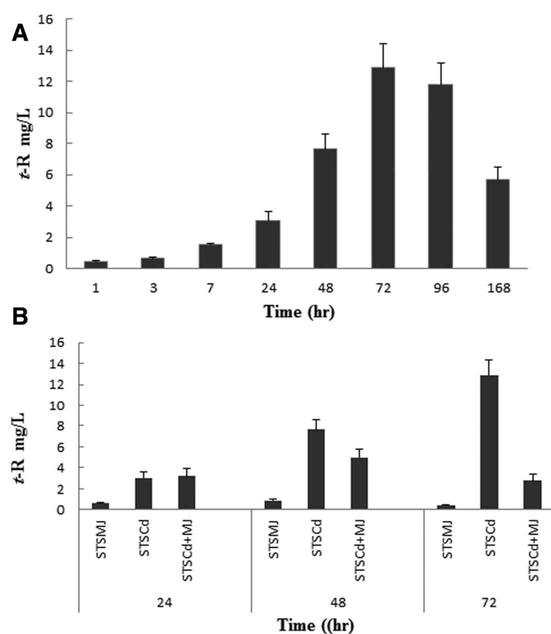

Figure 4. (A) Effect of Cd on *t*-R accumulation in the culture medium of *S.marianum* cultures transformed with *VvSTS3*. Cd (30 mM) were added at day 3 after subculture and samples were extracted at different incubation periods. Data are means ± SD from two independent experiments, each in triplicate. (B) Effect of MJ, alone or in combination with Cd on *t*-R accumulation in the culture medium of *S.marianum* cultures transformed with *VvSTS3*. MJ (100 μM) or 100 μM MJ+ 30 mM Cd were added at day 3 after subculture and samples were extracted at different incubation periods. Data represent mean of triplicate cultures ± SD.

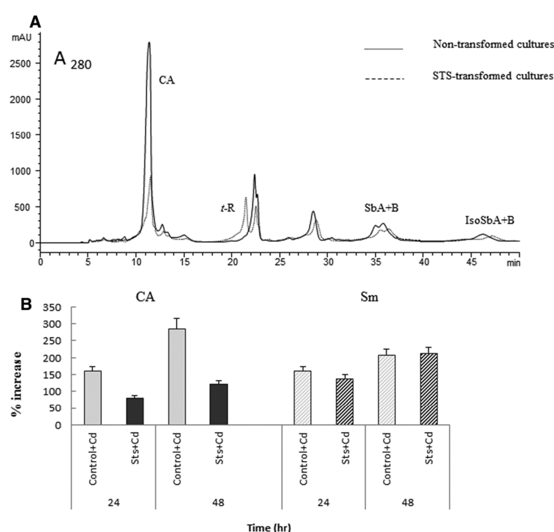

Figure 5. (A) Chromatograms (A280) of medium extracts of untransformed and STS-transformed *S.marianum* cultures treated with 30 mM Cd for 48 h. (B) Percentaje of increase of CA and Sm accumulation in non-transformed and STS-transformed *S. marianum* cultures treated with 30 mM Cd for 24 or 48 h. Data represent mean of triplicate cultures ± SD.

were ineffective. A progressive deceleration after 48 h was also observed, probably due to a toxic effect, since necrosis of cultures was visible after a week of treatment.

Flavonolignan accumulation in STS3-transformed cultures treated with Cd was compared with non-transformed control cultures under the same elicitation conditions. Figure 5A shows the chromatogram profiles, taken at A280, of medium extracts of non- transformed control cultures and of the transformed STS.A line after 48 h in the presence of 30 mM Cd. The relative percentage increases of CA and flavanolignan production both in non-transformed and STS- transformed cultures 24 and 48 h after Cd treatment are shown in Fig. 5B.

From the data shown in Fig. 5A and B, particularly those concerning CA accumulation, it can be recognized that most of the precursors (i.e. p-coumaric acid) for the monolignol pathway have been redirected by the STS protein to the stilbene pathway. Overall production of flavonolignan was less affected in transgenic cultures, since in control cultures the flavonoid pathway was already limiting for Sm synthesis due to the preferred supply of precursors for CA synthesis (more than 20 mg/L was produced in Cd treated cultures [32]; see the peaks for CA in the chromatogram of Fig. 5A).

4 Discussion

Plant cell suspension cultures were originally developed for the production of valuable secondary metabolites with a little commercial success [34–36]. Among several approaches to overcome the low productivity of these systems, metabolic engineering or heterologous expression of genes involved in biosynthesis are receiving considerable attention. At present, microorganisms like bacteria and yeast represent the most frequently used hosts for the production of plant secondary metabolites [34].

Resveratrol, like many plant bioactive metabolites, accumulates in low quantities in plant cell cultures. The wide benefits of this compound and its application potential, has generated considerable interest in the last years, and attempts to implement the heterologous biosynthesis of *t*-R have been reported, both in microorganisms and whole plants [8, 37, 38]. Notably, heterologous production in plants has the advantage of requiring the introduction of only one or two genes, since the other genes from the phenylpropanoid pathway are already present in the plant kingdom [39]. The engineering of *t*-R in plants has led to an increase in antioxidant activities and in disease resistance in the transgenic plants and extended the postharvest shelf life of fruits [39, 40].

Taking into consideration the high cost of introducing genetically modified crops, and public resistance to the acceptance of transgenic food products, heterologous production of *t*-R by plant cell cultures, with a relatively high biomass yield and short cultivation time, could be a viable option for large scale production [41].

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Reports in the literature concerning *STS* gene overexpression in plant cell suspensions are scarce. To our knowledge, heterologous synthesis of *t*-R in plant cell cultures has only been demonstrated in cultured cells of tobacco expressing a STS gene after elicitation with a crude preparation of *Phytophtora megasperma*, with a maximum *t*-R production of 50 ng/g fresh weight [10]. Very recently, Aleynova et al. [42] reported that overexpression of full-length *Vitis amurensis* STS1, *Va*STS2, and *Va*STS7 genes under the control of the double CaMV 35S promoter in grape cell cultures increased stilbene production up to 3.4 times (up to 6.12 mg/L).

In this study, we show that the integration and expression of the VvSTS3 gene in the *S. marianum* genome makes cell suspension cultures competent for *t*-R production and release to the culture medium under elicitation conditions. Although in a number of cases transformation of plants with the *STS* gene has led to the production of *t*-R and derivatives (i.e. piceid and viniferins) [43], no resveratrol-related metabolites were detected in the experiments.

There are many reports on the extracellular *t*-R accumulation in *Vitis* sp. cultures treated with MJ, and/or overall, with Cd (reviewed in [44]). Similarly, in the current study, 10 mg/L *t*-R was released to the culture medium in the presence of 30 mM Cd, although the *t*-R productivity in transgenic *Silybum* cultures was very far from that reported for *V. vinifera* cultures in which more than 3 g/L *t*-R accumulated in the presence of 50 mM Cd + 100 μM MJ [45]. Nevertheless, levels were higher than in elicited transgenic tobacco suspensions or in STS-overexpressing *V. amurensis* cultures, as mentioned above.

We have previously published that elicitation treatments are required for the accumulation of phenylpropanoid–like compounds in *S. marianum*. The monolignol pathway was preferently induced by elicitors, with CA being massively accumulated in the extracellular medium [23]. Thus, monolignols compete with the flavonoid pathway for precursors, which is subsequently the rate-limiting branch for Sm biosynthesis in cell cultures.

In STS-transgenic *S. marianum* cultures, targeted metabolite analysis showed a clear decrease in CA. This suggests that *t*-R synthesis occurred at the expense of monolignol due to the competition with the STS protein for precursors (*p*-coumaroyl CoA, see Fig. 1). On the other hand, the Sm content was less affected since, as mentioned before, in *S. marianum* cultures the flavonoid branch is the rate-limiting step. Although there are no precedents in the literature concerning cell suspensions, competition for common precursors in transgenic plants is not unexpected; for example, a slight decrease in flavonols was seen in transgenic apple modified with STS [46]. In contrast, substantial *t*-R production had no effect on the flavonol concentration in transgenic tomato plants [47,48].

From our results we can conclude that S. *marianum* cell cultures represent a new heterologous host for *t*-R production. The fact that the newly expressed STS3 gene took precursors for unwanted, non-bioactive compound (CA) produced in excess, while keeping unaltered the flow for target secondary compounds (i.e. Sm), opens a way to extend the applications of plant cell cultures for the simultaneous production of valuable metabolites both constitutive and foreign valuable. This potential needs further exploration.

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The authors have declared no conflicts of interest.

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Chapter 4. Bioconversion of stilbenes in genetically engineered root and cell cultures of tobacco.*

Spanish Summary

Actualmente es posible desarrollar total o parcialmente una ruta biosintética vegetal en una planta u otro organismo huésped. Este sistema ha sido explotado para transferir la riqueza metabólica de ciertas especies vegetales a otras plantas o incluso a organismos metabólicamente más simples como son las levaduras o las bacterias, para la producción de compuestos vegetales de alto valor añadido. Otra aplicación de este sistema es la de bioconvertir substratos abundantes en la naturaleza en compuestos más escasos o biológicamente más interesantes, tales como los derivados de resveratrol piceatanol y pteroestilbeno. Estos dos estilbenos, tienen actividades farmacológicas muy prometedoras y se encuentran en plantas en cantidades muy escasas. Mediante la transferencia del gen citocromo P450 hidroxilasa 1B1 humano (*Hs*CYP1B1) a cultivos de raíces transformadas y líneas celulares derivadas de ellas, se ha desarrollado una plataforma biotecnológica capaz de convertir resveratrol exógeno en piceatanol, en cantidades cercanas a los mg L^{-1}. De forma similar, mediante la expresión heteróloga del gen resveratrol O-metiltransferasa de *Vitis vinifera* (*Vv*ROMT), en el mismo sistema biotecnológico, se ha logrado la bioconversión de resveratrol exógeno en pteroestilbeno. Este trabajo también ha demostrado que ambas bioconversiones pueden tener lugar en raíces transformadas de tipo silvestre (pRiA4, sin transgén), lo que demuestra que citocromo P450 hidroxilasas y metiltransferasas inespecíficas de tabaco pueden realizar la bioconversión de resveratrol para formar los compuestos diana, aunque en menor proporción que las raíces transgénicas.

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Bioconversion of stilbenes in genetically engineered root and cell cultures of tobacco

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It is currently possible to transfer a biosynthetic pathway from a plant to another organism. This system has been exploited to transfer the metabolic richness of certain plant species to other plants or even to more simple metabolic organisms such as yeast or bacteria for the production of high added value plant compounds. Another application is to bioconvert substrates into scarcer or biologically more interesting compounds, such as piceatannol and pterostilbene. These two resveratrol-derived stilbenes, which have very promising pharmacological activities, are found in plants only in small amounts. By transferring the human cytochrome P450 hydroxylase 1B1 (*Hs*CYP1B1) gene to tobacco hairy roots and cell cultures, we developed a system able to bioconvert exogenous *t*-resveratrol into piceatannol in quantities near to mg L^{-1}. Similarly, after heterologous expression of resveratrol O-methyltransferase from *Vitis vinifera* (*Vv*ROMT) in tobacco hairy roots, the exogenous t-resveratrol was bioconverted into pterostilbene. We also observed that both bioconversions can take place in tobacco wild type hairy roots (pRiA4, without any transgene), showing that unspecific tobacco P450 hydroxylases and methyltransferases can perform the bioconversion of *t*-resveratrol to give the target compounds, albeit at a lower rate than transgenic roots.

Trans-resveratrol (*t*-R) (3,4′,5-trihydroxystilbene) is a phytoalexin available from a wide range of dietary sources, including mulberries, peanuts, grapes and wine. A plethora of biological activities have been attributed to *t*-R, including inhibiting the progression of cardiovascular, carcinogenic, and neurodegenerative diseases as well as the ageing process, as confirmed by several *in vitro* assays[1]. However, the transfer of the beneficial biological properties of *t*-R from *in vitro* to *in vivo* systems is restricted by its limited oral bioavailability and rapid metabolism[2]. Natural *t*-R analogues found in low abundance, such as the polyhydroxy-derivative trans-piceatannol (*t*-Pn) (Fig. 1), are significantly more bioavailable than *t*-R due to an additional hydroxyl group[3], and they also have higher anti-cancer and cancer chemopreventive activity[4,5]. Indeed, part of the antitumoral effects of *t*-R are attributed to its conversion into *t*-Pn by specific cytochrome P450 hydroxylases, in particular CYP1B1, which over-expresses in a wide range of human tumors[6]. Scarcely distributed in nature, *t*-Pn occurs in plant species such as *Passiflora edulis*, *Cassia marginata* or *Rhodomyrtus tomentosa*, where its accumulation can be more than 1000-fold higher than in grape[1]. Its promising pharmacological activities have prompted a search for new sources of *t*-Pn, including biotechnological approaches.

Another more biologically active derivative of *t*-R is pterostilbene (3′,5′-dimethoxy-resveratrol, *t*-Pt), whose bioavailability is enhanced more than 4-fold by two methoxy groups in its structure (Fig. 1)[7]. This may explain why *t*-Pt has higher antiproliferative effects than *t*-R and a similar activity against human colon cancer cells at half the concentration[8]. Like other stilbenes, the biological activities of *t*-Pt are mainly due to its antioxidant, anti-inflammatory and anticancer properties[7]. Like *t*-Pn, *t*-Pt is scarcely distributed in nature, and is found in *Pterocarpus marsupium*, grapevine and blueberries, where its levels reach 520 ng/g[9,10].

The biotechnological production of plant bioactive compounds based on plant cell and organ cultures constitutes a biosustainable source of scarce and structurally complex plant metabolites[11]. In this context, several

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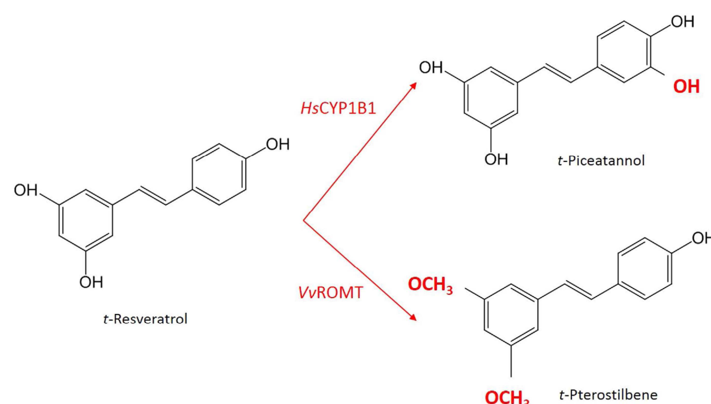

Figure 1. Bioconversion of t-resveratrol into *t*-piceatannol or *t*-pterostilbene by the action of the enzymes encoded by the genes *Hs*CYP1B1 and *Vv*ROMT, respectively.

attempts have been made to develop the biotechnological production of *t*-R in cell factories[12]. In general, *t*-R production in grapevine cell cultures is very low and needs to be enhanced by elicitors. Methyl jasmonate (MeJA) and methylated-β-cyclodextrin (MBCD) have been reported as strong inducers of *t*-R biosynthesis and accumulation, acting synergistically when added together to the plant cell cultures[13,14].

Metabolic engineering contributes a potent set of tools for increasing plant secondary metabolite production in cell cultures. The use of strong promotors to overexpress genes involved in biosynthetic pathway bottlenecks is currently a common strategy for improving the production of target compounds in engineered biological systems[15]. In this scenario, Martinez-Marquez *et al.*[16] recently showed that constitutive expression of resveratrol O-methyltransferase in *Vitis vinifera* led to the production of *t*-Pt, and the heterologous expression of the human cytochrome P450 hydroxylase 1B1 (*Hs*CYP1B1) increased *t*-Pn accumulation in elicited grapevine cell cultures.

Also recently, Li *et al.*[17] described the production of *t*-R and *t*-Pt in engineered yeast after feeding the culture with phenylalanine, and Wang *et al.*[18] reported the production of *t*-Pt from *t*-R and *p*-coumaric acid in two systems, engineered yeast and *Escherichia coli.* Thus, the stilbenoid biosynthetic pathway can be partially reproduced in these microorganisms by means of metabolic engineering tools.

Plant cell cultures have also been used to bioconvert exogenous substrates by exploiting the regioselective and stereospecific properties of plant enzymes as well as the vast potential of plants for biochemical reactions, including oxidation, reduction, hydroxylation, methylation and glycosylation[19]. Hairy root cultures obtained by genetic transformation of plant material with *Agrobacterium rhizogenes* can be an efficient alternative to plant cell suspensions for bioconversions due to their greater genetic/biochemical stability, high growth capacity in hormone-free culture media and relatively low cost. Transgenic cultures have been successfully used for the esterification, glycosylation, hydroxylation, etc. of various substrates, producing known or new compounds, some of them with improved biological activities[20]. Hairy root cultures have also proved useful for the expression of ectopic genes with the aim of bioconverting an abundant natural compound into a scarcely distributed derivative. An example is the efficient bioconversion of hyoscyamine into scopolamine in transgenic tobacco hairy roots carrying the hyoscyamine-6-hydroxylase gene from *Hyoscyamus muticus*[21].

The aim of the present study was therefore to develop a biotechnological platform based on tobacco transgenic hairy roots and cell cultures and explore their capacity to bioconvert exogenous *t*-R into its hydroxylated derivative *t*-Pn and its methylated derivative *t*-Pt by the heterologous expression of the human cytochrome P450 hydroxylase 1B1 (*Hs*CYP1B1) or *Vitis vinifera* resveratrol O-methyltransferase (*Vv*ROMT) genes, respectively. According to the current SIGMA prices, *t*-Pn and *t*-Pt are 25- and 15-fold more expensive, respectively, than *t*-R[22]. Our results show that both types of engineered hairy roots were able to bioconvert *t*-R to produce *t*-Pn or *t*-Pt, and unexpectedly, the target compounds, together with piceid, a glucosylated derivative of t-R, were also generated by the biosynthetic machinery of tobacco wild type hairy roots (pRiA4).

Materials and Methods

Bacteria and plasmids. To infect the plant material, three strains of *Agrobacterium rhizogenes* A4 were used: wild type and two engineered strains carrying together with the pRiA4 the binary plant expression vector pK7WG2_CYP1B1 or pJCV52_ROMT (Fig. S1) for the *Hs*CYP1B1 or *Vv*ROMT genes, respectively. These were preceded by the constitutive Cauliflower mosaic virus 35S promoter, as described in Martinez-Marquez *et al.*[16].

Stable transformation of tobacco and hairy root cultures. Leaf segments of *Nicotiana tabacum* cv Xhanti plantlets grown *in vitro* on Murashige and Skoog (MS) medium[23] were infected by direct inoculation with a needle with a wild type *A. rhizogenes* A4 strain (pRiA4), and the engineered *A. rhizogenes* (pRiA4+pK7WG2_CYP1B1) or *A.rhizogenes* (pRiA4+pJCV52_ROMT). The hairy roots began to appear after 2–4 weeks (Fig. 2). Small roots (1–2 cm) were excised and individually cultured on MS solid medium with 30 g L^{-1} of sucrose and 500 mg L^{-1} cefotaxime to eliminate the agrobacteria. After 6 rounds of subculture in MS medium supplemented with cefotaxime, the antibiotic was removed, and PCR for the virD gene was performed to confirm the

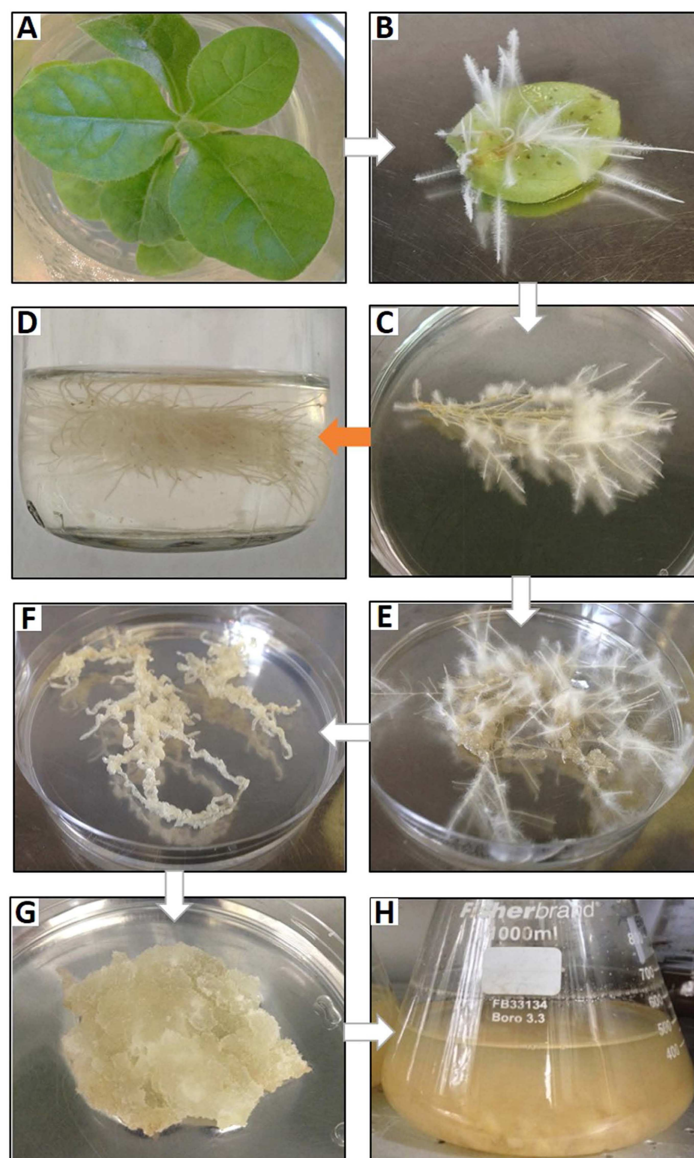

Figure 2. Steps for the establishment of hairy root cultures and the cell suspension derived from them. (**A**) *In vitro* plant of wild type *Nicotiana tabacum*. (**B**) Hairy roots 2–4 weeks after infection. (**C**) Hairy root line in solid MS with antibiotics. (**D**) Hairy roots in liquid MS medium, with or without MBCD. (**E**,**F**) Root dedifferentiation and callus induction in solid MS medium supplemented with NAA and KIN. (**G**) Friable callus. (**H**) Fine cell suspension in the same liquid medium, with or without MBCD.

elimination of *Agrobacterium* (Fig. 3C). In the case of roots obtained after infection with the recombinant *A. rhizogenes*, kanamycin (50 mg L^{-1}) was used for the selection. Hairy root lines were kept in the dark at 25 °C, and after at least 6 rounds of selection by subculturing every 2 weeks in media with antibiotics, they were transferred to MS medium without antibiotics for confirmation by PCR. The growth capacity of the hairy root cultures was measured as the Growth Index (GI, harvested fresh weight/inoculum fresh weight, after 28 days of culture). Only root lines with a high GI were selected for further experiments. *A. rhizogenes* A4 carrying the empty plasmids pK7WG2 or pJCV52 was also used to obtain hairy root cultures, but as the GI and the *t*-R bioconversion of these root lines in preliminary experiments were very similar to those of the wild type *A. rhizogenes*, the latter was used for comparison with the engineered hairy root lines.

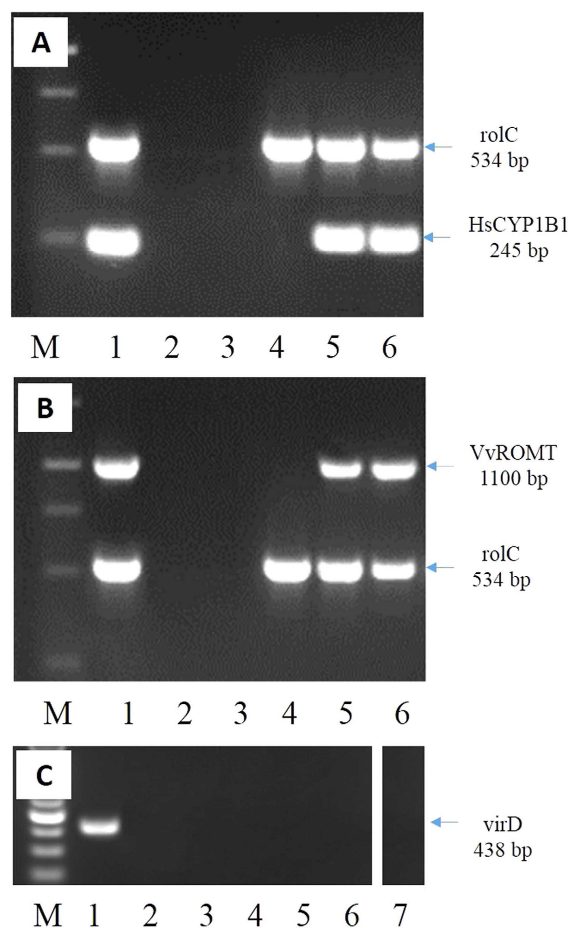

Figure 3. PCR analysis from genomic DNA of transgenic lines of *Nicotiana tabacum*. (**A**) (1) positive control *A. rhizogenes* (pRiA4+pK7WG2_CYP1B1), (2) negative control (DNA of root control) for the *Hs*CYP1B1 gene, (3) negative control (DNA of *Nicotiana tabacum* wild type plant) for the rolC gene, (4) hairy root control, (5–6) CYP1B1L8, CYP1B1L27 lines. (**B**) (1) positive control *A. rhizogenes* (pRiA4+pJCV52_ROMT), (2) negative control (DNA of root control) for the *Vv*ROMT gene, (3) negative control (DNA of *Nicotiana tabacum* wild type plant) for the rolC gene, (4) hairy root control, (5–6) *Vv*ROMT L3, L7 lines. (**C**) (1) positive control *A. rhizogenes*, (2) negative control (DNA of *Nicotiana tabacum* wild type plant) for the VirD gene, (3) hairy root control, (4–5) CYP1B1L8, CYP1B1L27 lines, (6–7) VvROMT L3, L7 lines.

PCR analysis. The hairy root lines were checked by PCR. The analysis was performed using the DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc) with 1 µg DNA. Previously, genomic DNA was isolated from hairy root samples according to Dellaporta *et al.*[24]. Specific primers were used (Table S1) in the amplification of the rolC, *Hs*CYP1B1, *Vv*ROMT and virD genes. The amplification reactions were as follows: 1 cycle at 95 °C for 5 min followed by 35 cycles at 95 °C for 1 min, 57 °C for 40 sec, 72 °C for 1.30 min and an extension cycle of 10 min at 72 °C. PCR products were analyzed by electrophoresis on 1% agarose gels.

qPCR analysis. Expression of the *Hs*CYP1B1 and *Vv*ROMT genes was verified by qPCR in the lines used in the experiments. Total RNA from plant material was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA). For qRT-PCR, cDNA was prepared from 2 µg RNA treated with DNase I (Invitrogen, Carlsbad, CA) and synthesized with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). qRT-PCR was performed using the iTAqTM universal SYBR Green Supermix (BioRad, Hercules, CA, EEUU) in a 384-well platform system (LightCycler_ 480 Instrument; Roche), and each sample was run in triplicate, under the following conditions: 95 °C for 2 min, 40 cycles (95 °C, 10 s; 60 °C, 20 s; 72 °C, 20 s) followed by a melting curve. Gene-specific primers were designed with Primer-BLAST (Table S1). Expression levels were normalized to those of the Elongation factor 1 α (EF-1 α). Stable expression of EF-1α in the different hairy root clones and their derived cell lines was confirmed by the obtained coefficient of variation (CV) of 0.027, which is included within the CV ranking for potential internal reference genes described by Schmidt *et al.*[25].

Step	Total Time (min)	Flow Rate (μL/min)	A (%)	B (%)
0	0	800	100	0
1	1	800	100	0
2	1.5	800	60	40
3	2.5	800	0	100
4	4.5	800	0	100
5	4.6	700	100	0
6	8	700	100	0

Table 1. Gradient solvents used for analysis of stilbenes were A: H_2O + 0.05% acetic acid; B: acetone:acetonitrile (70:30).

Initiation and maintenance of the transgenic cell suspension. Root dedifferentiation and callus induction were performed using solid MS medium supplemented with 2.14 mg/L of naphthalene acetic acid (NAA) in combination with 0.215 mg/L of kinetin (KIN) (Fig. 2). After several subcultures, 30 g of friable callus was placed in 200 mL of liquid MS medium with the same hormones to obtain a fine cell suspension, which was subcultured every 12 days, shaken at 115 rpm and maintained at 25 °C in darkness.

Feeding assays. In a 200 mL flask with 20 mL of liquid MS medium, with or without methylated β-cyclodextrin (MBCD) at a concentration of 5 mM (6.55 g/L), 3.4 g of roots or cells were fed with 200 μL of *t*-R sterile stock solution at 45.64 mg/mL to reach a 2 mM final concentration. Once *t*-R was added to the culture, it was shaken at 115 rpm and maintained at 25 °C in darkness up to the sampling times: 4, 8, 24, 40 and 56 h.

Extraction and determination of stilbenoids. To extract stilbenoids from the culture medium, 1 mL of ethyl acetate was added per 4 ml of the medium, stirring vigorously, and the apolar phase was collected. The extraction was repeated once more, and the apolar phases were combined and evaporated as described in Martinez-Marquez *et al.*[16].

The roots were frozen, freeze-dried and crushed. 50 mg of lyophilized plant material was placed in a tube with two volumes of 100% methanol, sonicated for 30 min to allow the methanol to penetrate the plant tissues, and the supernatant was collected. Again, two volumes of methanol were added and sonicated for 15 min. The methanolic extracts were pooled and evaporated. In order to measure the accuracy of the extraction method, a precisely weighed quantity of *t*-R was added to the culture medium, with or without MBCD, and extracted at different times. At time 0, just after the *t*-R addition, the *t*-R recovery was higher than 95%, demonstrating the efficiency of the extraction method employed.

For stilbenoid extraction from cells, four parts of 100% methanol were added per g of fresh weight, with stirring at 115 rpm for 24 h. The methanol extract was filtered and brought to dryness[16]. All samples were resuspended in 1 mL of 80% methanol and sonicated for 30 min and filtered through a 0.22 μm PVDF filter just before analysis.

Stilbenes were determined by a Linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer, 4000 Q TRAP of AB Sciex Instruments with MRM scan type in negative mode. Standards of t-R, *t*-Pn, *t*-Pt and piceid from LGC STANDARDS, S.L.U. were used to prepare the calibration curves described in Table S2. The gradient used in this system is described in Table 1. The mobile phases were A: H_2O + 0.05% acetic acid and B: acetone:acetonitrile (70:30). The column was a Luna 3 μm C18 (2) 100 A 50 × 2.00 mm s/n: 008–4251-B0 with a temperature of 60 °C and injection volume of 10 uL. The transitions and retention time are described in Table S3 and Fig. S2. Stilbenoid contents are expressed as $\mu g\ L^{-1}$ in both cells and culture medium to facilitate the calculation of the total amount of stilbenoids in the cultures.

Statistical analysis. This was performed with Excel software. All data are the average of three measurements + SE. The multifactorial ANOVA analysis followed by the Tukey multiple comparison test were used for statistical comparisons. A p-value of <0.05 was assumed for significant differences.

Results

Establishment of transgenic root cultures of tobacco. Tobacco hairy root cultures were established by the infection of leaf segments with *Agrobacterium rhizogenes,* harbouring the pRiA4 plasmid alone (wild type), or together with pK7WG2_CYP1B1 or pJCV52_ROMT. All the *A. rhizogenes* were able to induce hairy roots after a period of 2–4 weeks (Fig. 2). Fast-growing root lines (GI > 4, Table S4), wild type or carrying the recombinant plasmid, were selected and their transgenic nature was determined by PCR. Fig. 3 shows a band of 534 bp corresponding to rolC of *A. rhizogenes* in both wild type (pRiA4) and transgenic roots (pRiA4+pK7WG2_CYP1B1), whereas the band of 245 bp corresponding to the *Hs*CYP1B1 gene was observed only in root lines genetically transformed with the binary vector (Fig. 3A). Also, the band of 1100 bp corresponding to the *Vv*ROMT gene was only observed in the hairy root cultures infected with the corresponding agrobacteria (Fig. 3B). All the lines tested negative for the virD gene, indicating the absence of agrobacteria in the hairy root cultures (Fig. 3C). These transgenic lines, as well as some of the wild type lines, were selected for further analysis. All the obtained root lines showed the classical hairy root phenotype, a high growth capacity (Fig. 2, Table S4) and the corresponding gene expression (Fig. 4).

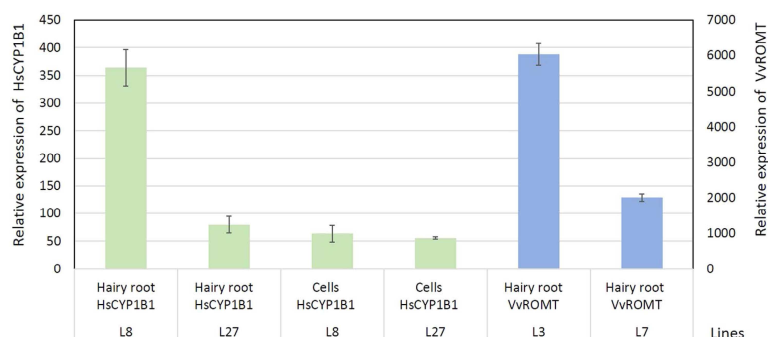

Figure 4. qPCR analysis of the transcript levels of the *Hs*CYP1B1 gene in transgenic hairy root lines CYP1B1L8 and CYP1B1L27 and in their respective derived cell lines. In light grey, the *Vv*ROMT gene transcript levels in transgenic hairy root lines ROMTL3 and ROMTL7. The control lines were used as a negative control to verify non-specific amplification (data not shown). Data are the mean of three independent replicates ± SE.

From these, two selected transgenic root lines carrying the *Hs*CYP1B1 gene (CYP1B1L8 and CYP1B1L27), two lines carrying the *Vv*ROMT gene (ROMTL3 and ROMTL7) and two wild type (pRiA4 alone) lines were fed with 2 mM (456.4 mg L^{-1}) of *t*-R, and samples were taken at different intervals of the culture during a period of 4–56 h.

Bioproduction of *t*-piceatannol in hairy roots and their derived cell lines. The selected transgenic root lines heterologously expressing the *Hs*CYP1B1 gene were able to actively bioconvert the added *t*-R into *t*-Pn, especially when treated with MBCD (Fig. 5A). The highest bioconversion levels were achieved in the MBCD-supplemented CYP1B1L8 line at 8 h, when the *t*-Pn content was higher than 7 ± 0.46 mg L^{-1}. At the same time, the CYP1B1L27 line reached a *t*-Pn content of 4.7 ± 0.29 mg L^{-1}, which increased up to 5.2 ± 0.24 mg L^{-1} at 24 h, after which levels decreased significantly ($p < 0.01$). In the transgenic cultures treated with MBCD, most of the *t*-Pn was released to the culture medium, whereas the significantly lower ($p < 0.01$) levels of *t*-Pn produced by the untreated cultures remained mainly inside the roots (Fig. 5A). Unexpectedly, wild type hairy root cultures (without the *Hs*CYP1B1 gene) were also able to biotransform *t*-R into *t*-Pn, although at a lower rate (0.4%) than the transgenic lines CYP1B1L8 (1.6%) and CYP1B1L27 (1.4%). As before, *t*-Pn levels were also significantly higher ($p < 0.05$) in the MBCD-supplemented wild type cultures and accumulated mainly in the culture medium (Fig. 5A).

Regarding the fate of exogenously added resveratrol in the hairy root cultures (Fig. 5B), *t*-R taken up by the cells was partially metabolized into *t*-Pn and probably other compounds, but this stilbene was also found in the culture medium. In most cases, the remaining *t*-R contents were lower in the MBCD-treated than in the untreated cultures and accumulated mainly outside the cells. For example, the remaining *t*-R in transgenic CYP1B1L27 root cultures was 52 ± 3.26 mg L^{-1} after 24 h of treatment, 77% of which accumulated in the culture medium, whereas when the same line was treated with MBCD, the *t*-R decreased to 40 ± 2.3 mg L^{-1}, 83% being found in the culture medium (Fig. 5B).

The presence of piceid, the glucoside of *t*-R, was detected in both transgenic and wild type hairy root cultures (Fig. 5C). Piceid levels peaked 24 h after the addition of the substrate and then decreased until the end of the culture period (56 h). Glucosylation of *t*-R in the cultures devoid of MBCD was higher than in MBCD-treated cultures, and it was probably a way of detoxifying the excess of exogenously added t-R. Levels of piceid were significantly lower ($p < 0.05$) than *t*-Pn in the transgenic cultures (pRiA4+pK7WG2_CYP1B1), whereas wild type cultures (pRiA4) showed a similar content of both *t*-R derivatives. In contrast with *t*-Pn, piceid accumulated mainly intracellularly, even in the MBCD-treated cultures (Fig. 5C). The presence of *t*-Pn in the wild type tobacco cultures, and piceid and *t*-Pt in both wild type and CYP1B1 root cultures suggests that unspecific hydroxylases and glucosidases from tobacco can transform the exogenous substrate *t*-R into these derivatives. However, the efficiency of these bioconversions was up to 24-fold lower compared with, for example, the capacity of the transgenic root CYP1B1L8 line to biotransform *t*-R into *t*-Pn. Despite the considerable variability among the different control and transgenic root lines, we can infer that the high *t*-Pt production was due to the ectopic expression of the *Hs*CYP1B1 gene, since the average yield of the transgenic CYP1B1 lines (1888 ± 427 μg L^{-1}) was significantly higher ($p < 0.05$) than that of the control (819 ± 138 μg L^{-1}). It was thus demonstrated that the transgene expression effectively increased the bioconversion of *t*-R into *t*-Pt.

The most productive hairy root line (CYP1B1L8) was subjected to a hormonal treatment for dedifferentiation and callus induction. The friable calli were then disintegrated and a cell suspension line obtained (Fig. 2). Transgenic cell suspension cultures grew actively, reaching a growth rate similar to the parental hairy roots (Table S4). The cell line, with or without MBCD, was fed with the same concentration of *t*-R as the hairy root cultures to investigate its capacity to bioconvert this substrate to the hydroxylate derivative *t*-Pn. Like the CYP1B1L8 root line, its derived cell suspension was able to convert *t*-R into *t*-Pn but the production of this system was 8-fold lower than that of the original root line (Fig. 6A). In the MBCD-treated cell cultures, *t*-Pn accumulated in small

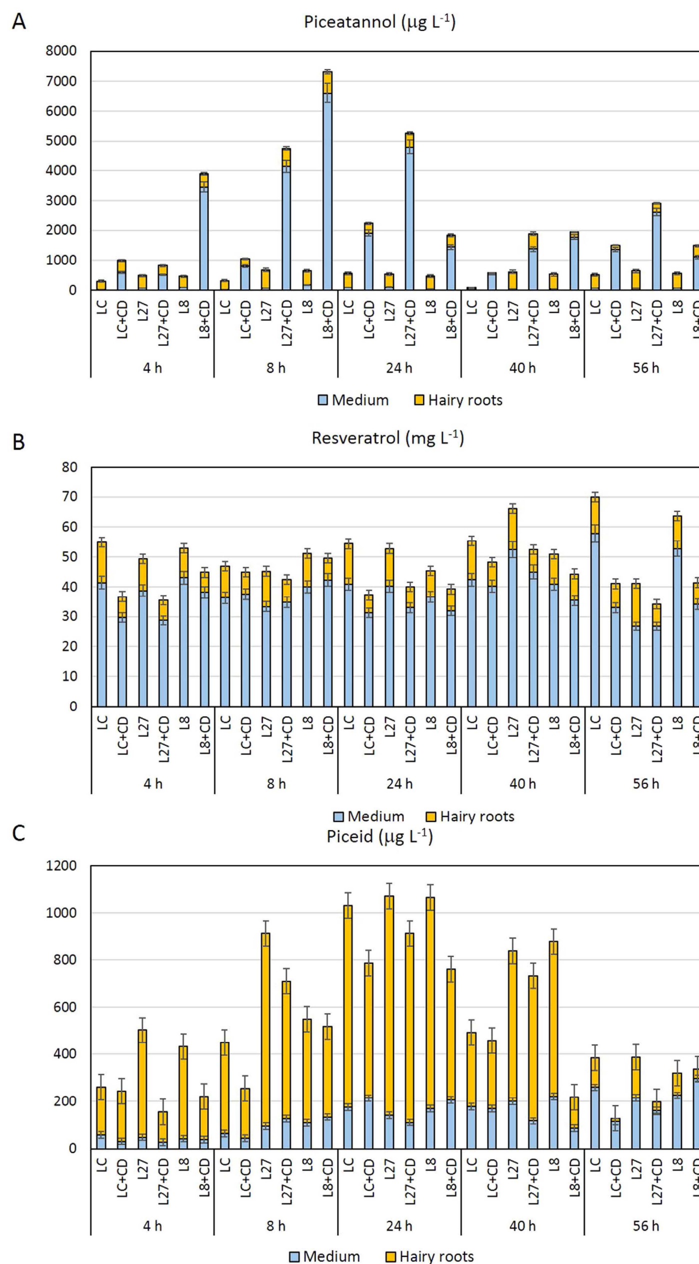

Figure 5. Time course of *t*-piceatannol (**A**), *t*-resveratrol (**B**) and *t*-piceid (**C**) contents in transgenic and wild type tobacco hairy root cultures. LC, A4 wild type root line. L7 and L8, CYP1B1L7 and CYP1B1L8 transgenic root lines. CD, root lines treated with MBCD. Data are the mean of 3 biological replicates ± SE.

quantities in the medium. Its maximum accumulation was at 8 h after feeding the culture with *t*-R, after which it decreased significantly until the end of the culture ($p < 0.01$). In absence of MBCD, only a low amount of *t*-Pn was detected, 24 h after the addition of the precursor (Fig. 6A). Overall, the cell suspension derived from the hairy root line L8 showed only a limited capacity to bioconvert *t*-R into *t*-Pn.

Dedifferentiation of the roots to obtain the cell suspension also affected the exogenous *t*-R accumulation pattern (Fig. 6B). In contrast with the hairy roots, the derived cell suspension culture accumulated *t*-R mainly inside the cells. When treated with MBCD, a small amount of *t*-R remained in the culture medium 4 h after feeding, and only traces were detected inside the cells (Fig. 6B). These results suggest a very low stability of *t*-R outside the cells,

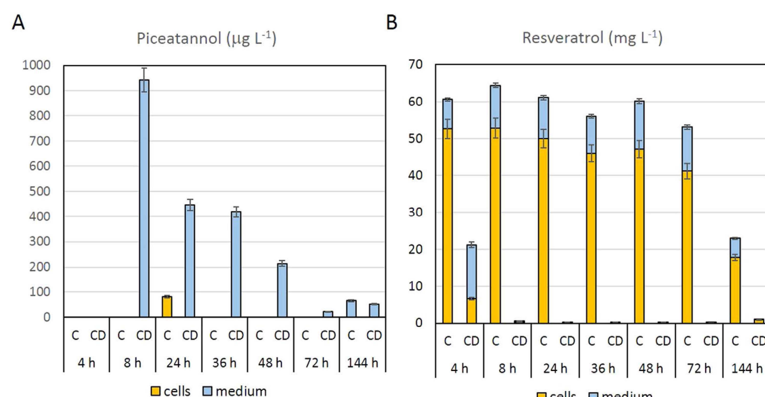

Figure 6. Time course of *t*-piceatannol production (**A**) and t-resveratrol accumulation (**B**) in transgenic tobacco cell cultures. (**C**) control conditions, treatment without MBCD. CD, MBCD treatment. Data are the mean of 3 biological replicates ± SE.

and most of the *t*-R remaining in the culture medium had probably degraded 4 h after the feeding treatment. No piceid was detected in any cell or media samples of the *t*-R-fed cultures, showing that the derived cell suspension lost the capacity to glycosylate the *t*-R, in contrast with the hairy root cultures.

Bioproduction of *t*-pterostilbene in hairy root cultures. Heterologous expression of the *Vv*ROMT gene in hairy root cultures fed with *t*-R led to the bioconversion of this stilbene to its methoxylated derivative *t*-Pt, which was found both inside the roots and in the culture medium, with a maximum production reached by the transgenic root line L3, 24 h after *t*-R feeding (Fig. 7A). In this experiment, the incubation period was not extended because we had previously observed that after 24 h the newly produced stilbene contents in the cultures decreased (data not shown). As well as the control and *Hs*CYP1B1 hairy roots (Fig. 5A), the cultures carrying the transgene *Vv*ROMT were also able to synthesize *t*-Pn and piceid in even greater quantities than *t*-Pt. In particular, root line L3 reached a *t*-Pn content of $2 \pm 0.14\,mg\,L^{-1}$ (Fig.7B), which was very similar to that of the control root line described in the previous experiment (Fig. 5A). The same line *Vv*ROMTL3 also showed the highest *t*-Pt production (Fig. 7A). In this experiment, MBCD significantly increased ($p < 0.01$) the *t*-Pt content in the culture medium, generally without increasing the total yield of the target compounds in the cultures (Fig. 7A). In hairy roots carrying the *Vv*ROMT gene, as in the case of the *Hs*CYP1B1 gene, partial *t*-R degradation occurred, although *t*-R quantities of up to $70 \pm 2.16\,mg\,L^{-1}$ remained in the culture 24 h after feeding (Fig. 7D).

Discussion

Tobacco is a model plant system easily transformed by *A. rhizogenes* to produce hairy root cultures. This trait may be harnessed for the heterologous expression of foreign genes harbored in engineered *A. rhizogenes*[26]. The derived genetically transformed cultures exhibit a high growth capacity and genetic stability for long periods[27,28]. The tobacco hairy root cultures we engineered to ectopically express the human CYP1B1 gene had the capacity to bioconvert *t*-R into *t*-Pn with a yield of up to $7 \pm 0.46\,mg\,L^{-1}$, and others expressing the ROMT gene from *V. vinifera* were able to biosynthesize *t*-Pt, reaching a content of $2.6 \pm .019\,\mu g\,L^{-1}$. Although low, the concentrations of *t*-Pt were more than 25-fold higher than those achieved in the control hairy roots. This biotechnological system thus proved to be suitable for the production of *t*-Pn and, on a lower scale, *t*-Pt, both compounds with promising biological activities and scarcely distributed in nature[1].

Recently, in a similar approach, Martinez-Marquez *et al.*[16] reported a 200-fold enhancement of *t*-Pn production in grapevine cell cultures by heterologous expression of the *Hs*CYP1B1 gene, and the presence of *t*-Pt in transgenic cell lines overexpressing the *Vv*ROMT gene, when both cultures were elicited with MeJA and MBCD. Although the *t*-Pn production achieved in the transgenic *V. vinifera* cell line was higher (up to $20\,mg\,L^{-1}$) than in our study, the greater *t*-Pt production of our transgenic hairy roots, as well as the inherent genetic stability of the system[20,28], confirm that this bioconversion process is also suitable for the production of *t*-R derivatives.

Although examples are few, hairy roots have been previously used for the bioconversion of exogenous substrates. Through the heterologous expression of the hyoscyamyne-6β-hydroxylase gene from *H. muticus*, Häkkinen *et al.*[27] obtained the alkaloid scopolamine after feeding tobacco hairy root cultures with its precursor hyoscyamine. Similarly, hairy root cultures of *Peganum harmala* expressing tryptophan decarboxylase of *C. roseus* produced high levels of serotonin[29], and *Beta vulgaris* hairy roots expressing the *p*-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) gene from *Pseudomonas fluorescens* produced vanillin when the cultures were fed with ferulic acid[30]. Thus, our results further confirm the capacity of engineered hairy root cultures to biotransform exogenous substrates to target products with interesting biological activities.

In this work, the enhancing effects of MBCD on the bioconversion of *t*-R into *t*-Pn in tobacco hairy root cultures were also demonstrated (Fig. 5A). MBCD can act as a precursor solubilizer in biotransformation processes. For example, cell cultures of *Mucuna pruriens* bioconverted 17β-estradiol into 4-hydroxyestradiol when solubilized in β-MBCD, and *Podophyllum hexandrum* cell cultures converted a coniferyl alcohol MBCD complex

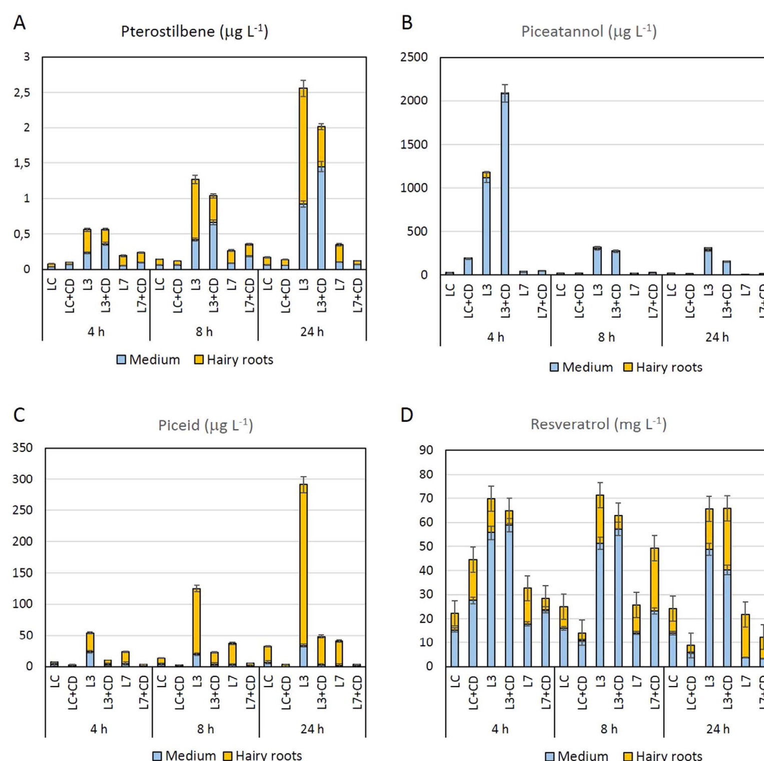

Figure 7. Time course of *t*-pterostilbene (**A**), *t*-piceatannol (**B**), *t*-resveratrol (**C**) and *t*-piceid (**D**) contents in transgenic and wild type tobacco hairy root cultures. LC, A4 wild type root line. L7 and L8, ROMTL7 and ROMTL8 transgenic root lines. CD, root lines treated with MBCD. Data are the mean of 3 biological replicates ± SE.

into podophyllotoxin[31]. In our experiment, considering the poor solubility of *t*-R in water, the solubilizing effects of MBCD may have contributed to the improved efficiency of the hairy root cultures in biotransforming *t*-R to *t*-Pn. Other factors are also likely to have been involved, especially since MBCD did not clearly show any positive effects on *t*-Pt production.

MBCD has also been used as a permeabilizing agent acting on plant cell membranes, thus increasing the release of plant secondary metabolites such as taxol in *Taxus* spp. cell cultures[32]. This effect could be responsible for the higher extracellular *t*-Pn accumulation in the MBCD-treated cultures compared with the untreated control. MBCD may also facilitate the movement of substrates and products through cell membranes during the biotransformation processes and improve the uptake of *t*-R by the hairy roots, thus facilitating the metabolism of this compound inside the cells and its conversion to other stilbenoids like *t*-Pn. However, the positive effect of MBCD on the release of reaction products to the culture medium could negatively affect the production of the system if they are less stable in the medium. This may be the case with *t*-Pt or piceid, which were absent in grapevine cell cultures even after elicitation with MeJA and MBCD[16].

Wild type hairy root cultures have been widely used to biotransform a range of exogenous substrates for the production of pharmaceutical ingredients, including products with enhanced solubility after hydroxylation and glycosydation[20]. In this context, the presence of small quantities of *t*-Pn and *t*-Pt, even in the control cultures, suggests that non-specific tobacco enzymes can also biotransform the supplied *t*-R into its derivatives in the absence of the corresponding transgene. Similarly, the presence of piceid (a *t*-R glucoside) in both transgenic and wild type hairy root cultures confirms the capacity of the tobacco hairy roots to glycosylate *t*-R.

Globally considered, our results show that in tobacco hairy root cultures, *t*-R and its derivatives are not metabolic end-products and may be transformed by unspecific tobacco enzymes into other known or new products. This could explain the lower *t*-Pt contents of our cultures compared with those of other biotechnological platforms based on engineered microorganisms. When the VvROMT gene was expressed in transgenic yeast and *E. coli*[18], concentrations of 170 mg L^{-1} and 150 mg L^{-1} of pterostilbene, respectively, were reached when the cultures were fed with resveratrol[18]. Recently, the *t*-Pt biosynthetic pathway from phenylalanine was transferred to yeast, which required a dozen genetic modifications, and the engineered cultures produced up to 34 mg L^{-1} of pterostilbene[17]. However, only the non-bioactive *t*-R derivative pinostilbene[17] was detected in these cultures and not the high added value *t*-Pt. These results suggest that bioconversion in metabolically engineered microorganisms can

yield a high amount of a target compound, but the metabolic complexity of plant organisms can provide a wider range of compounds, probably including new products.

As mentioned before, plant cell cultures are widely employed for the bioconversion of naturally abundant substrates to scarcer secondary metabolites with important biological activities[19,33]. According to our results, the tobacco transgenic cell cultures carrying the *Hs*CYP1B1 transgene were considerably less able to biotransform *t*-R into *t*-Pn than the parental transgenic roots. Nevertheless, the low levels of *t*-R remaining in the cell cultures, especially when MBCD was added, compared with the root cultures, suggests the transgenic cells have a high capacity to metabolize *t*-R. A greater capacity to bioconvert hyoscyamine to scopolamine in hairy roots compared with the corresponding derived cell lines was also found in tobacco transgenic cultures heterologously expressing the hyoscyamine-β-hydroxylase gene from *Hyoscyamus muticus*[21]. In contrast, when comparing tobacco hairy roots and cell cultures expressing the geraniol synthase gene of *Valeriana officinalis*, Vasilev *et al.*[26] obtained higher levels of geraniol in the cell cultures. However, in this case, the substrate (geranylgeranyl diphosphate) was generated by the plant cells and not added exogenously to the culture.

Conclusions

Taken as a whole, our results show the possibility of developing a *t*-Pn-producing biotechnological platform based on metabolically engineered tobacco hairy roots heterologously expressing the *Hs*CYP1B1 gene, with MBCD playing an important role as a solubilizing/permeabilizing agent. The *t*-Pt production achieved was low, but this is an extremely scarce compound, even in its richest natural sources, such as blueberries, which only accumulate ng/g[7]. Thus, the developed system, based on the heterologous expression of the *Vv*ROMT gene, has potential as a biotechnological source of *t*-Pt after an optimization process. Finally, both untransformed systems were also able to biosynthesize *t*-Pn, *t*-Pt and piceid using the natural genetic capacity of the host plant to perform non-specific hydroxylations, methoxylations and glycosylations, thus demonstrating the immense capacity of plant cells to carry out biotransformations and generate known or even new products.

As previously mentioned, metabolically engineered yeast and *E. coli* cultures have been developed for *t*-R production from simple and abundant precursors such as phenylalanine and *p*-coumaric acid. However, production in these systems requires the introduction of the whole gene set of the metabolic pathway for stilbenoid synthesis. In contrast, since the direct natural *t*-R precursors, malonyl CoA and *p*-coumaryl CoA, are already found in plant tissues, heterologous production in biotechnological platforms of plant origin has the advantage of requiring the introduction of only one or two genes.

Therefore, and according with our results, it is conceivable that in the near future new biotechnological systems based on plant cell or hairy root cultures will be designed to produce *t*-R by heterologous expression of the stilbene synthase gene, as well as the resveratrol derivatives *t*-Pn and *t*-Pt, if they also carry the transgenes VvROMT and/or HsCYP1B1. In support of this hypothesis, Xiao *et al.*[34] dramatically activated rosmarinic acid biosynthesis by the genetic manipulation of only two genes of the metabolic pathway in hairy root cultures of *Salvia miltiorrhiza*.

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Author Contributions

J.P. and P.C. conceived the experiments. D.H. helped by A.M.-M. and E.M. conducted the experiments. J.P., P.C. and R.B.-M. analyzed the results. J.P. and P.C. wrote the manuscript. All authors contributed to and reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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Chapter 5. Biotechnological production of truncate tissue plasminogen activator protein (reteplase) from transplastomic tobacco cell cultures.*

Spanish Summary

Las plantas transplastómicas son un sistema de elección para la producción de proteínas recombinantes de interés biofarmacéutico, debido a la elevada poliploidía del genoma de los plastos y el bajo riesgo de dispersión del transgén a través del polen, ya que la herencia plastídica es vía materna. Sin embargo, al tratarse de un sistema agronómico de producción, puede presentar contaminación por agroquímicos y fertilizantes, así como fluctuaciones en su rendimiento debido a las variaciones climáticas y a posibles infecciones del cultivo. El "Human Tissue Plasminogen Activator" (tPA), es una proteína obtenida por recombinación genética que se utiliza en el tratamiento del infarto cardíaco agudo, ya que convierte el plasminógeno en plasmina, que por digestión de la fibrina induce la disolución de los coágulos de fibrina. Recientemente, nuestro grupo de investigación ha obtenido plantas transplastómicas homoplásmicas de tabaco portadoras del gen K2S que codifica un tPA humano truncado (reteplasa) y que presenta una actividad biológica mejorada en relación con el tPA. Nuestros estudios confirmaron la presencia de la proteína diana en las hojas de las plantas transplastómicas. Teniendo en cuenta las ventajas que presentan los cultivos de células vegetales para la producción de proteínas recombinantes, en este trabajo hemos establecido una línea celular derivada de las plantas K2S. Los resultados de este estudio confirmaron la producción de la forma activa de reteplasa por los cultivos celulares de tabaco. La proteína recombinante se cuantificó en los cultivos crecidos en condiciones de luz y oscuridad, alcanzándose una producción 3 veces superior de reteplase bajo condiciones de la luz blanca que en oscuridad.

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Biotechnological production of recombinant tissue plasminogen activator protein (reteplase) from transplastomic tobacco cell cultures.

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A R T I C L E I N F O

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A B S T R A C T

Transplastomic plants are a system of choice for the mass production of biopharmaceuticals due to the polyploidy of the plastid genome and the low risk of pollen-mediated outcrossing because of maternal inheritance. However, as field-grown plants, they can suffer contamination by agrochemicals and fertilizers, as well as fluctuations in yield due to climatic changes and infections. Tissue-type plasminogen activator (tPA), a protein used to treat heart attacks, converts plasminogen into plasmine, which digests fibrin and induces the dissolution of fibrin clots. Recently, we obtained transplastomic tobacco plants carrying the K2S gene encoding truncated human tPA (reteplase) with improved biological activity, and confirmed the presence of the target protein in the transgenic plant leaves. Considering the advantages of plant cell cultures for biopharmaceutical production, we established a cell line derived from the K2S tobacco plants. The active form of reteplase was quantified in cultures grown in light or darkness, with production 3-fold higher in light.

1. Introduction

Biopharmaceuticals based on proteins, antibodies or nucleic acids are increasingly being used for disease treatment. Although only about 60 peptides have been approved by the US FDA to date, more than 140 are under clinical study and by 2020 the global sales of biopharmaceuticals are expected to be worth over $US 278.2 billion (Santos et al., 2016). Tissue type plasminogen activator (tPA), which induces the dissolution of fibrin clots by converting the zymogen plasminogen into the serine protease plasmin, is a clinically useful thrombolytic agent (Clark, 2001) and a target for biotechnological production. tPA has five domains, N terminal finger, epidermal growth factor, serine protease, Kringle 1 and Kringle 2 (Youchung et al., 2003). The active part of tPA, the

thrombolytic Kringle 2 domain, serine protease domain, two functional regions of proteasa (176-527 amino acid residues), plus the 1 to 3 amino acids of the N-terminal is known as the truncated human tissue plasminogen activator (K2S, reteplase), which has a longer plasma half-life and higher fibrinolytic activity than tPA (Nordt and Bode, 2003).

The main biotechnological systems for the production of recombinant biopharmaceuticals are based on microorganism cultures such as *Escherichia coli* and yeast at bioreactor level, while large proteins are generally produced by mammalian cell platforms (Demain and Vaishnav, 2009). As an agricultural system, molecular farming for biopharmaceutical production has many advantages, including inexpensive growth media (only water, minerals and sunlight are required), low capital equipment costs and easy scale-up. However, a drawback of the system according to the US Agricultural Department is a lack of guaranteed transgene contention and the risk of contamination of the human food chain if edible plant species are used as the host (Wilson and Roberts, 2012). Moreover, transgenic crops used for the production of heterologous proteins are exposed to agrochemicals and fertilizers in the field, while variable culture conditions and the impact of bacterial and fungal infections can lead to fluctuations in yield (Hellwig et al., 2004).

As an alternative production system, plant cell cultures share the capacity of transgenic crops for proper protein folding and can assemble complex recombinant proteins. They also have similar advantages to bioreactor systems based on microorganisms and mammalian cells, as they avoid transgene dissemination and provide controlled and sterile growth conditions, chemically defined culture media, and compliance with pharmaceutical good manufacturing practices (GMP), ensuring the biosafety and productivity of the system (Demain and Vaishnav, 2009; Santos et al., 2016). Furthermore, they allow proteins to be manufactured in days or weeks, rather than the months or years required when depending on the growth cycle of a whole plant (Doran, 2000).

Several factors affecting transgenic crops, such as climate, soil quality, season, day length and weather, are not issues for biotechnological platforms based on plant cell cultures. Additionally, the secretion of heterologous proteins into the culture medium simplifies downstream processing and protein purification (Pham et al., 2012). The first human recombinant protein approved in the US and other countries was Taliglucerase alfa, a modified glucocerebrosidase (GCD) enzyme used to treat Gaucher's disease, produced by Protalix Biotherapeutics in the ProCellEx® platform based on carrot cell cultures (Tekoah et al., 2015).

Transplastomic plants have been targeted for the production of biopharmaceuticals due to the high number (approx. 100) of chloroplasts per plant cell, the high copy number (approx. 10000) of the plastid genome, as well as the maternal mode inheritance, though low-level leakages of transgenes in pollen may occur (Bock, 2014). Transplastomic technology has enhanced field-grown plant resistance to herbicides and plagues and has been used for the production of recombinant proteins. However, the derived cell cultures have been scarcely applied for the biotechnological production of heterologous proteins (Block, 2007). Examples of biopharmaceuticals produced in transplastomic

tobacco plant cell cultures include the phage-derived endolysins, used as an antibiotic against pneumonia (Oey et al., 2009), camelid antibodies (Lentz et al., 2012), the transforming growth factor (TGFβ3), a cytokine-type protein (Gisby et al., 2011), and fragment C of tetanus toxin (TetC). The latter was accumulated up to 7 mg/L, but when the transplastomic cell suspension was cultured in a temporary immersion bioreactor (TIB), regenerated shoots achieved a TetC production of 95 mg/L (Michoux et al., 2011).

Recently, our group obtained tobacco transplastomic plants harboring the K2S gene driven by the promotor Prrn for the production of the truncated human tissue plasminogen activator (K2S, reteplase), which is one of the most important pharmaceutical recombinant proteins, widely used to break down blood clots through the conversion of plasminogen to plasmin (Abdoli-Nasab et al., 2013). The purification system of reteplase has been recently optimized, reaching a production of up to 30.6 μg/100 mg of leaf tissue (Abdoli-Nasab et al., 2016). Taking into account the potential advantages of plant cell cultures for the production of biopharmaceuticals, in this work we demonstrate for the first time the capacity of cell suspension cultures derived from K2S transplastomic tobacco plants to produce the bioactive target peptide.

2. Material and Methods

2. 1. Plant material

In this work, we utilized seeds from homoplastic plants carrying the K2S gene driven by the promotor Prrn obtained as described by Abdoli-Nasab et al. (2013). Sterile seeds were germinated on solidified MS (Murashige and Skoog, 1962) medium supplemented with 500 mg/L spectinomycin, in Magenta vessels (SIGMA). The *in vitro* plantlets were cultivated in a climate chamber at 25ºC under a 16 h photoperiod and an approximate light intensity of 60 μmol m^{-2} s^{-1}.

2.2. Initiation and maintenance of the transgenic cell suspension

Leaf discs of young K2S plants were cultivated in darkness or under light conditions for callus induction in solid MS medium (Murashige and Skoog, 1962) supplemented with 2.14 mg/L of naphthalene acetic acid (NAA) in combination with 0.215 mg/L of kinetin (KIN) (Piñol et al., 1985) and 500 mg/L of spectinomycin (Fig. 1). After several subcultures, 30 g of friable calli were placed in 300 mL of liquid MS medium with the same hormones and antibiotic to obtain a fine cell suspension, which was subcultured every 12 days, shaken at 115 rpm and maintained at 25 °C in darkness or light conditions.

2.3. qPCR analysis

Expression of the *K2S* gene in the cell suspension was verified by qPCR. The elongation factor 1 α (EF-1 α) as the nuclear-encoded reference (Schmidt et al., 2010) and the accD-like plastid-encoded (Lee et al., 2004) were used for gene normalization. Total RNA from the plant material was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA). For the qRT-PCR, cDNA was prepared from RNA treated with DNase I (Invitrogen, Carlsbad, CA) and synthesized with SuperScript IV reverse transcriptase (Invitrogen, Carlsbad, CA). qRT-PCR was performed using the iTAq™ universal SYBR Green Supermix (BioRad, Hercules, CA, EEUU) in a 384-well platform system (LightCycler_480 Instrument;

Roche), and each sample was run in triplicate, under the following conditions: 95 °C for 2 min, 40 cycles (95 °C, 10 s; 60 °C, 20 s; 72 °C, 20 s) followed by a melting curve. Gene-specific primers were designed with Primer-BLAST (Table 1).

Table 1. Sequences of the primers used to amplify the genes by qPCR

Gene	Primer Sequence	Amplicon size bp	Reference
Elongation factor (EF1)	FW: TGGTCAGGAGATTGCGAAAGAGC RV: ACGCAAAACGCTCCAATGGTG	130	Hidalgo et al. (2007)
accD	FW ACAACTGGTGGAGTGACAGC RV ATGCAATGTAGGCGTTGGGT	76	This work
K2S	FW: GCATGACTTTGGTGGGCATC RV: CGGGACATCCTTCTGTCCAC	59	This work

2.4. Protein extraction

Total soluble protein (TSP) was extracted as described by Wang et al. (2005) with slight modifications. Briefly, 4 g of the harvested cell suspension was ground in liquid nitrogen and placed in a 15 mL tube, which was filled with 10% trichloroacetic acid / acetone, and centrifuged at 10000 g for 5 min (4°C). The supernatant was removed, washed with 0.1 M ammonium acetate / 80% methanol and centrifuged. The pellet was washed with 80% acetone, and air-dried at room temperature for 10 min. The tube with the pellet was filled with phenol/SDS buffer (Wang et al., 2005), mixed thoroughly and incubated for 5 min on ice. After centrifugation, the upper phase was recovered in a new tube, which was filled with 0.1 M ammonium acetate / 80% methanol, incubated over night at -20°C and then centrifuged at 10000 g for 10 min (4°C). Finally, the pellet was washed once with 100% methanol and once with 80% acetone and air-dried. The protein was resuspended in 6 M urea and quantified using the RC DC Protein Assay Kit II (Bio-Rad, CA, USA).

Native proteins were extracted using finely ground powder under liquid nitrogen. Per 5 g of cells, 10 mL of cold buffer solution was used (buffer: 250 mM sucrose, 50 mM HEPES, 5% glycerol, 10 mM $Na_2O_5S_2$, 1% PVP, 10 mM ascorbic acid and 100 mM PMSF, pH=7.5). The mixture was thoroughly homogenized and centrifuged at 12000 g for 15 min (4°C). Finally, the supernatant was purified for further analysis.

2.5. Protein purification

For purification of the recombinant protein, a His Gravitrap™ TALON® (GE HealthcareBio-Sciences AB, Uppsala, Sweden) was used following the manufacturer's instruction. The eluted protein was concentrated by Amicon® Ultra-2 (30K) Centrifugal Filter Devices (Millipore Corp., Bedford, MA), and the buffer exchange was carried out with buffer phosphate (50 mM sodium phosphate, 300 mM

NaCl, pH=7.4). Finally, the quantification was done by the RC DC Protein Assay Kit II (Bio-Rad, CA, USA).

2.6. SDS-PAGE and sample preparation for mass spectrometry analysis

The TSP extracted from the cell suspension was placed (100 µg) in 12% acrylamide gel followed by staining with Coomassie brilliant blue (Laemmli, 1970). The gel bands located at 48 kDa were processed as described by Gundry et al. (2010). For digestion, sequencing grade modified trypsin (V5111, Promega, Madison, WI) was used and samples desalted with PepClean™ C-18 Spin Columns (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's recommendations.

2.7. Liquid chromatography-mass spectrometry

For protein identification, MS and MS/MS data were acquired in an Agilent XCT plus ion trap mass spectrometer with a ChipCube interface fed by an Agilent 1100 series nanopump HPLC system (Martínez-Esteso et al., 2011). The four most intense precursor ions in the MS scans were selected for MS/MS and then passed to an active exclusion list released after 1min. Raw data were converted into a peak list with the extraction tool of SpectrumMill Proteomics Workbench (SMPW) (Agilent). The reduced data set was searched against the Swissprot forward and reversed protein database without taxonomical restrictions in the identity mode with the MS/MS search tool of SpectrumMill Proteomics Workbench, using the following parameters: trypsin, up to 1 missed cleavages, fixed modification carbamidomethylation of cystein, the variable modification oxidation of methionine, and a mass tolerance of 2.5 Da for the precursor and 0.7 Da for product ions. Peptide hits were validated first in the peptide mode and then in the protein mode according to the score settings recommended by the manufacturer.

The MRM selector tool of SMPW was used to generate transition lists from validated peptide identifications by selecting the five most intense precursor/product ion pairs for each target peptide above the precursor m/z, and calculating the collision energy for each precursor ion.

The MRM experiments were performed on an Agilent's standard flow LC-MRM-MS platform (Agilent Technologies, Palo Alto, CA) consisting of a 1290 Infinity UHPLC interfaced to a 6490 triple quadrupole mass spectrometer via a JetStream ESI source. Protein sample digests were separated using an Advance Bio Peptide Map 2.1*150 mm 2.7 µm column thermostated at 50ºC at 0.4 mL/min flow rate over a 6-min run along a linear gradient from 0 to 70 % of solvent B consisting of 0.1% FA in 90% ACN. Source parameters were: 3000-3500 V capillary voltage, 15 L/min N_2 gas flow, 150ºC gas temperature. Each transition was acquired at unit resolution in standard mode for 10 ms dwell time at 3 duty cycles per second.

A project was created in the open source application Skyline (MacLean et al., 2010) using the sequence of identified peptides and selecting manually the previously generated transitions, in order to import, visualize, refine the acquisition method and compare the LC-MRM runs.

2.8. Activity assay

The protein assay was performed with a tissue-type plasminogen activator (tPA) Human Chromogenic Activity Assay Kit (ab108905, abcam, Cambridge,

UK). This assay measures the ability of tPA to activate the plasminogen to plasmin. The amount of plasmin produced is quantitated using a specific substrate releasing a yellow p-nitroaniline chromophore. Briefly, human plasminogen, plasmin substrate and tPA standard were prepared following the manufacturer's instructions. Then, a standard curve was prepared in triplicate by serial dilution, considering 7 points (40, 10, 2.5, 0.625, 0.156, 0.039 and 0 IU mL^{-1}). Next, a mixture of specific diluent, plasminogen and plasmid substrate (60, 10 and 10 µL respectively) was distributed in a 96-well microplate. Finally, 20 µL of standard points or samples were added, incubated at 37 °C and the absorbance was periodically read at 405 nm for up to 9 h.

3. Results and Discussion

3.1 Establishment of transgenic cell suspension cultures and K2S gene expression.

After 2 months of the initial callus induction, sufficient material was obtained to establish the cell suspension. A characteristic green color was observed in the callus and cell suspension grown in light (Fig. 1 A, B). After 12 days of culture, samples from the cell suspension growing in light or darkness were taken for the planned analyses. Their growth capacity was measured as a growth index (harvested fresh weight / inoculum fresh weight, GI) and biomass productivity (r_x). Light was found not to affect the biomass production of the system, and in both conditions, the cell cultures reached a GI > 3, which represents a $r_x > 18.5$ g $L^{-1}d^{-1}$ (Fig. 2), a growth capacity very similar to the untransformed tobacco cell lines (data not shown). These results show the high capacity of the system based on tobacco suspension cultures to produce biomass, which was not affected by the K2S gene expression. Similar results for growth capacity were reported previously for tobacco cell cultures genetically designed for the heterologous production of the *t*-resveratrol derivative, *t*-piceatannol (Hidalgo et al., 2017), and for the production of scopolamine in transgenic tobacco cell lines carrying the *Hn*H6H gene (Moyano et al., 2007).

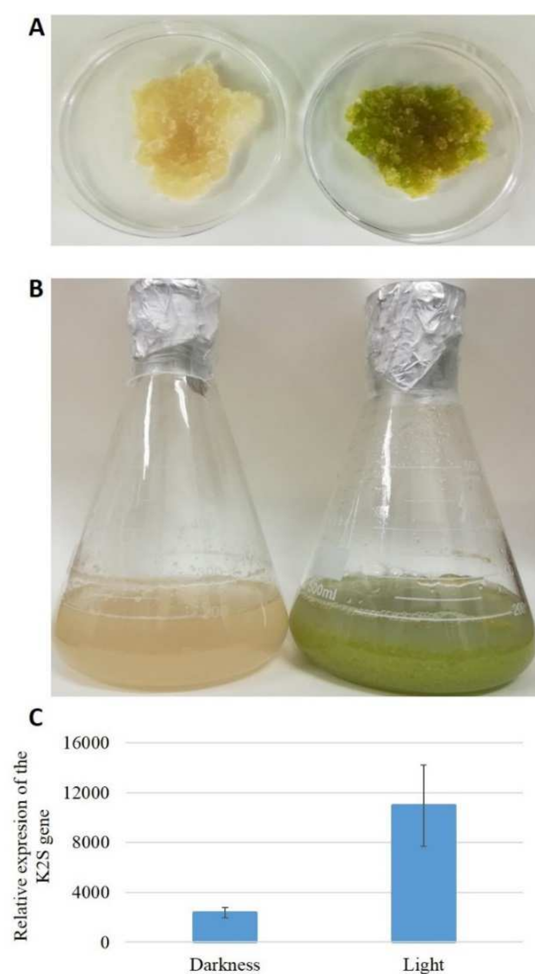

Figure 1. Appearance of the callus cultures (A) and the derived cell suspensions (B) growing under dark and light conditions. (C) Analysis of the relative K2S gene expression normalized to Elongation Factor 1α.

In order to normalize the gene expression data more precisely in the qPCR analyses, cp values of housekeeping genes (EF1 and accD) were used, in combination or separately, to calculate and compare the coefficient of variance (CV) between them. The results, $CV_{EF1} = 0.0247 > CV_{EF1\&accD} = 0.0307 > CV_{accD} = 0.0513$, demonstrated that the accD gene

expression was affected by light and was therefore not useful for this experiment, while EF1 proved to be suitable for use as a housekeeping gene. (Figure 1 C).

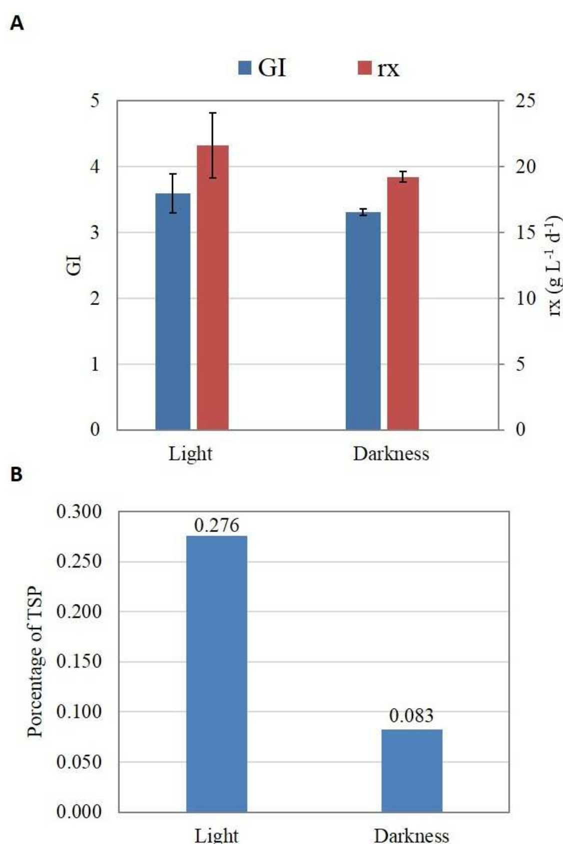

Figure 2. A) Growth capacity of the cell suspension measured as growth index (GI) and biomass productivity (rx) and B) reteplase production of the cell suspensions growing under dark and light conditions. TSP, total soluble protein. Each value is the average of 3 biological replicates ± SE.

In contrast with the growth capacity, the analysis of the K2S gene expression showed significant differences according to the conditions, with 4.6-fold higher gene expression when the cell line was cultured under light. These results could be related to the green color of this cell suspension, which is probably caused by a higher degree of chloroplast organization and/or their number per cell. Recently, Barreto et al. (2017) developed transgenic calli for the heterologous production of the fragment C of tetanus toxin (TetC), and reported a positive correlation between green shoot development and TetC yield when the callus was cultivated in a temporary immersion culture.

3.2 Characterization of rtPA *heterologous protein by Mass Spectrometry*

The acrylamide gel bands corresponding to the predicted molecular weight of the K2S protein were successfully submitted to qualitative analysis. A method was previously established to detect 7 different reteplase peptides, 3 of which were proteotypic (Fig. 3), i.e. they were exclusive to reteplase and with no homology with related species (Fig.4). Each peptide was detected with at least 3 transitions, which gives specificity to the analysis. In all the samples from transgenic material overexpressing the K2S gene, peptides associated with reteplase were found and as expected, the signals were not detected in the wild type cell suspension. In all samples, the most intensive signals were detected for the peptides GGLFADIASHP WQAAIFAK and VYTAQNPSAQALGLGK (Fig. 5 A), at a retention time of 3.4 min and 2.0 min respectively (Fig. 5 B). The transitions of the peptide GGLFADIASHPWQAAIFAK were detected both in samples kept in the dark and in light without significant quantitative differences probably because the transitions for this peptide give rise to many unspecific signals in the tobacco proteome background. Transitions of the peptide VYTAQNPSAQALGLGK were well above noise in light treated samples while only noise could be detected in darkness treated samples. The later is consistent with the higher plastid abundance and the K2S expression levels in light conditions, thus

validating the suitability monitoring VYTAQNPSAQA LGLGK by MRM as a surrogate peptide of K2S. The results confirmed the capacity of the K2S transgenic cell line to biosynthesize the target biopharmaceutical.

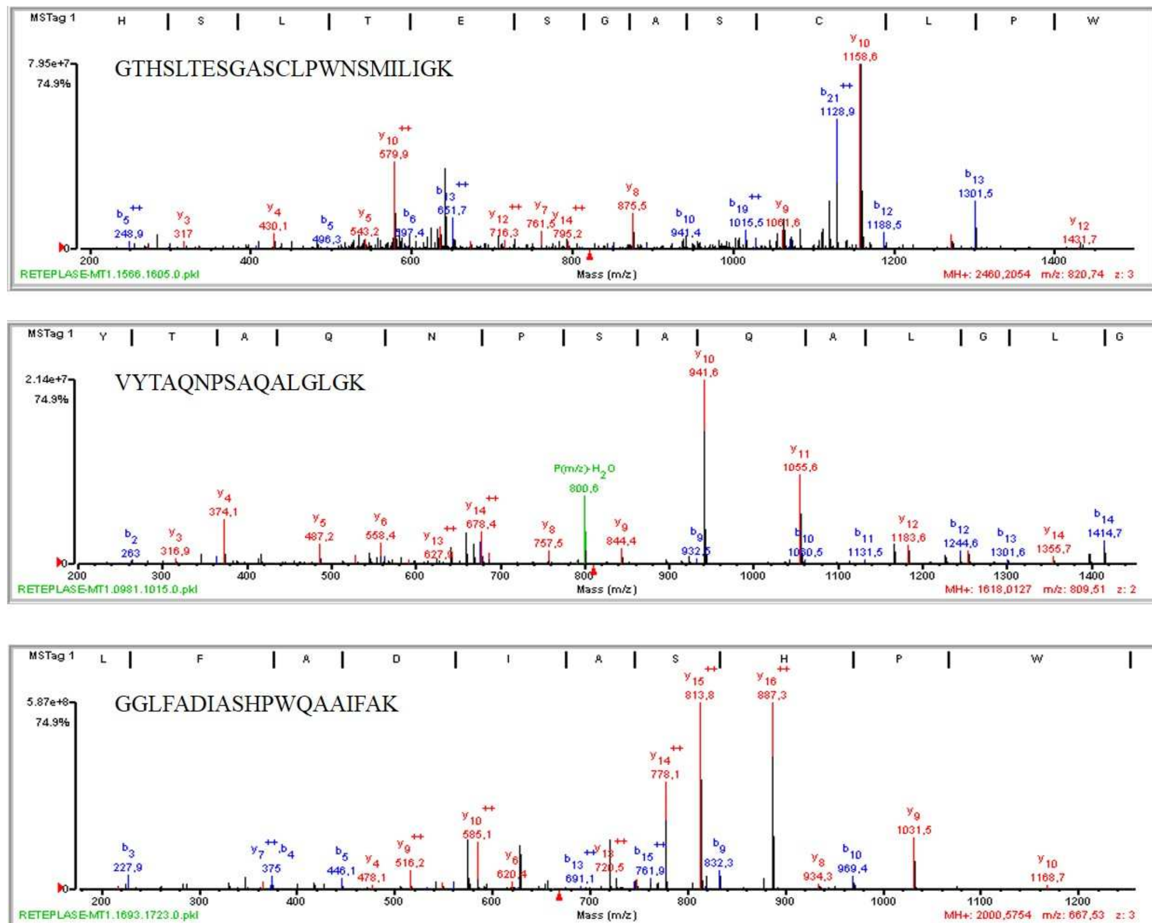

Figure 3. Mass spectrum and fragment ion of the 3 proteotypic peptides for the human tPA protein.

ID	Sub Group #	Length	Identical AA's	%ID	Species	Protein Name
Q28198	1	566	566	100	BOVIN	Tissue-type plasminogen activator
Q8SQ23	1	562	468	83.3	PIG	Tissue-type plasminogen activator
P00750	1	562	460	81.9	HUMAN	Tissue-type plasminogen activator
Q5R8J0	1	562	458	81.5	PONAB	Tissue-type plasminogen activator

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Q28198 (1) MMSAMKTEFLCVLLLCGAVFTSPSQETYRRLRRGARSYKVTCRDGKTQMTYRQHDSWLRPLLRGNQVEHCWCDGGRAQCHSVPVRSCSEPWCFNGGTCRQ
Q8SQ23 (1) -MYALKRELWCVLLLCGAICTSPSQETHRRLRRGVRSYRVTCRDEKTQMIYQQHQSWLRPLLRGNRVEHCWCNDGQTQCHSVPVKSCSEPRCFNGGTCLQ
P00750 (1) -MDAMKRGLCCVLLLCGAVFVSPSQEIHARFRRGARSYQVICRDEKTQMIYQQHQSWLRPVLRSNRVEYCWCNSGRAQCHSVPVKSCSEPRCFNGGTCQQ
Q5R8J0 (1) -MNAMKRGLCCVLLLCGAVFALPSQEIHARVRRGARSYQVICRDEKTQMIYQQHQSWLRPVLRSNRVEYCWCNSGRAQCHSVPVRSCSEPRCFNGGTCQQ

Q28198 (1) ALYSSDFVCQCPEGFMGKLCEIDATATCYKDQGVAYRGTWSTAESGAECANWNSSGLAMKPYSGRRPNAIRLGLGNHNYCRNPDQDSKPWCYVFKAGKYI
Q8SQ23 (1) AIYFSDFVCQCPVGFIGRQCEIDARATCYEDQGITYRGTWSTTESGAECVNWNTSGLASMPYNGRRPDAVKLGLGNHNYCRNPDKDSKPWCYIFKAEKYS
P00750 (1) ALYFSDFVCQCPEGFAGKCCEIDTRATCYEDQGISYRGTWSTAESGAECTNWNSSALAQKPYSGRRPDAIRLGLGNHNYCRNPDRDSKPWCYVFKAGKYS
Q5R8J0 (1) ALYFSDFVCQCPEGFAGKCCEIDTRATCYEDQGISYRGTWSTAESGAECTNWNSSALAQKPYSGRRPDAIRLGLGNHNYCRNPDRDSKPWCYVFKAGKYS

Q28198 (1) SEFCSTPACAKVAEEDGDCYTGNGLAYRGTRSHTKSGASCLPWNSVFLTSKIYTAWKSNAPALGLGKHNHCRNPDGDAQPWCHVWKDRQLTWEYCDVPQC
Q8SQ23 (1) PDFCSTPACTKEKEE---CYTGKGLDYRGTRSLTMSGAFCLPWNSLVLMGKIYTAWNSNAQTLGLGKHNYCRNPDGDTQPWCHVLKDHKLTWEYCDLPQC
P00750 (1) SEFCSTPACSEGNSD---CYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSC
Q5R8J0 (1) SEFCSTPACSEGNSD---CYFGNGLAYRGTHSLTESGASCLLWNSMILIGKVYTAQNPNAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSC

Q28198 (1) VTCGLRQYKRPQFRIKGGLFADITSHPWQAAIFVKNRRSPGERFLCGGILISSCWVLSAAHCFQERYPPHHLKVFLGRTYRLVPGEEEQTFEVEKYIIHK
Q8SQ23 (1) VTCGLRQYKEPQFRIKGGLYADITSHPWQAAIFVKNRRSPGERFLCGGILISSCWVLSAAHCFQERFPPHHVRVVLGRTYRLVPGEEEQAFEVEKYIVHK
P00750 (1) STCGLRQYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTVILGRTYRVVPGEEEQKFEVEKYIVHK
Q5R8J0 (1) STCGLRQYSQPQFRIKGGLFADIASHPWQAAIFARHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTVILGRTYRVVPGEEEQKFEVEKYIVHK

Q28198 (1) EFDDDTYDNDIALLHLKSDSLTCARESASVRTICLPDASLQLPDWTECELSGYGKHESSSPFFSERLKEAHVRLYPSSRCTSQHLFNRTVTNNMLCAGDT
Q8SQ23 (1) EFDDDTYDNDIALLQLKSDSLTCAQESDAVRTVCLPEANLQLPDWTECELSGYGKHEASSPFYSERLKEAHVRLYPSSRCTSKHLFNKTITNNMLCAGDT
P00750 (1) EFDDDTYDNDIALLQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDT
Q5R8J0 (1) EFDDDTYDNDIALLQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVADNMLCAGDT

Q28198 (1) RSGGDHTNLHDACQGDSGGPLVCMKDNHMTLVGIISWGLGCGRKDVPGVYTKVTNYLDWIRDNTRP
Q8SQ23 (1) RSGGDNANLHDACQGDSGGPLVCMKGNHMTLVGVISWGLGCGQKDVPGVYTKVTNYLNWIRDNTRP
P00750 (1) RSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNMRP
Q5R8J0 (1) RSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGEKDVPGVYTKVTNYLDWIHDNMRP
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Figure 4. Multiple sequence alignment of tPA in related species. Those that are highlighted represent the seven peptides for method detections and those that are framed correspond to proteotypic peptides for human tPA (protein ID P00750).

3.3 Determination of the protein content and activity assay.

As the reteplase was polyhistidine-tagged (Abdoli et al. 2013), the recombinant protein expressed in chloroplasts was estimated by measuring the TSP before purification and after concentration of the eluted protein from the column, as described in Material and Methods. The rtPA content reached in the tobacco cell suspension when cultivated in darkness was 0.083 % of the TSP, rising to 0.277% in light conditions, which represents an increase of more than 3-fold. These results are consistent with the MRM signal intensities for the peptide VYTAQNPSAQALGLGK and the production values were lower than those obtained in the transplantomic mother plant (approx. 1% of TSP) (Abdoli Nasab et al., 2016), but in that case the protein was harvested after 8 weeks rather than the 2 weeks of the cell suspension culture period. In accordance with previous reports (Michoux et al., 2011; Hong et al., 2002; Hellwig et al., 2004; Magnuson et al., 1998), this result shows that the production of recombinant protein in plant cell suspensions is lower than in the whole plant.

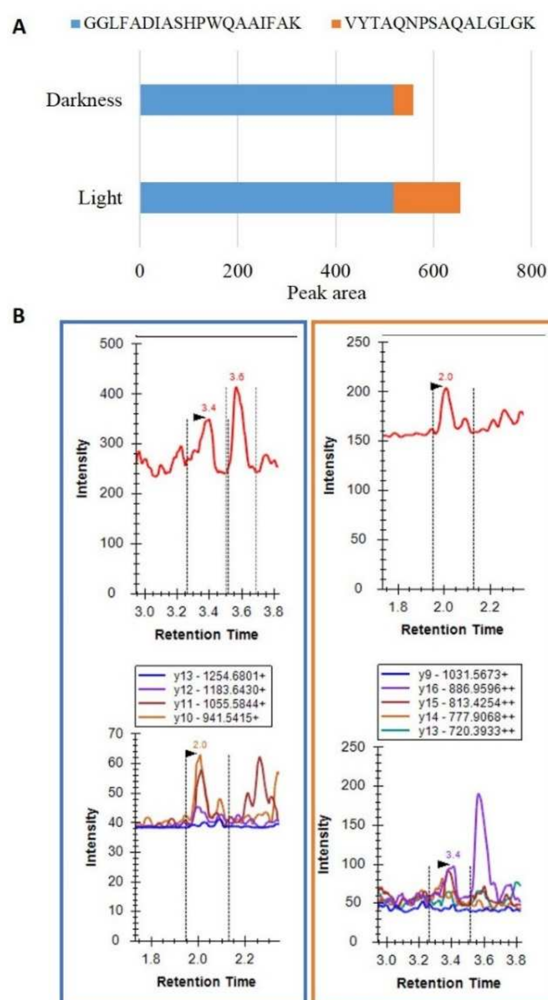

Figure 5. Peptide characterization. (A) Identification of the most intensive peptides in cell cultures under dark and light conditions. (B) Chromatogram, indicating specific retention time and intensity of each fragment ion.

Several factors might be responsible for this phenomenon, such as less differentiated cells and plastids (Michoux et al., 2013), low activity of the Prrn promoter in cell suspensions (Michoux et al., 2011), instability of the recombinant protein secreted to the medium due to protease release from disrupted cells (Hong et al., 2002), or aggregation occurring when the daughter cells do not separate in the cell division (Santos et al., 2016). Nevertheless, the rtPA yield was significantly higher than the tPA production in a hairy root system (Kang et al., 2011).

Unlike tPA, reteplase has no carbohydrate side chains and thus can be produced in *E. coli* cells, but most of the protein is present as an inclusion body, making its extraction and renaturation a time-consuming downstream process, involving solubilization and protein refolding and dialysis (Khodabakhsh et al., 2013). In contrast, as the target protein in our biotechnological system was tagged with 6-his, it was easily purified using a column containing TALON-Superflow® medium precharged with cobalts ions, and the active K2S protein was recovered according to the method previously developed by Abbdoli Nasab et al. (2016).

For the quantitative measurement of reteplase activity, the extracted native protein was obtained from a cell suspension growing in light, since the production was higher than in darkness. The crude extract was purified and concentrated as described in Material and Methods, and the use of the Plasminogen Activator Human Chromogenic Activity Assay Kit allowed activity against plasminogen to be measured throughout 9 h of incubation at 37°C. The behavior of the extracted enzyme (1460 µg) was comparable to the 2.5 UI mL^{-1} tPA standard curve (Fig. 6).

Further approaches to enhance the productivity of the K2S tobacco cell suspensions could be based on modifying the promoter and vector regulatory elements (Hong et al., 2002). Adding signal peptides like 33KDsp may improve secretion efficiency (Haung et al., 2015), minimize processing time and avoid proteolytic and oxidative degradation of recombinant proteins (Fischer, 1999). Fusion tags including HFB (Reuter et al., 2014) or zein-derived peptides could increase the protein accumulation (Joseph et al., 2012). The culture medium could be

optimized, e.g. by increasing aeration (Liu and lee, 1999) or adding growth medium supplements such as amino acids (Fischer et al., 1999), gibberellic acid, haemin (Tsoi and Doran, 2002), a carbon source (Santos et al., 2016), protease substrates like gelatin, biopolymers (Lee et al., 2002, Kwon et al., 2002) or bovine serum albumin (James et al., 2000; Baur et al., 2005). The results obtained demonstrate that the extraction of reteplase in native conditions preserves its biological activity and the biotechnological system could be successfully used for the production of this biopharmaceutical.

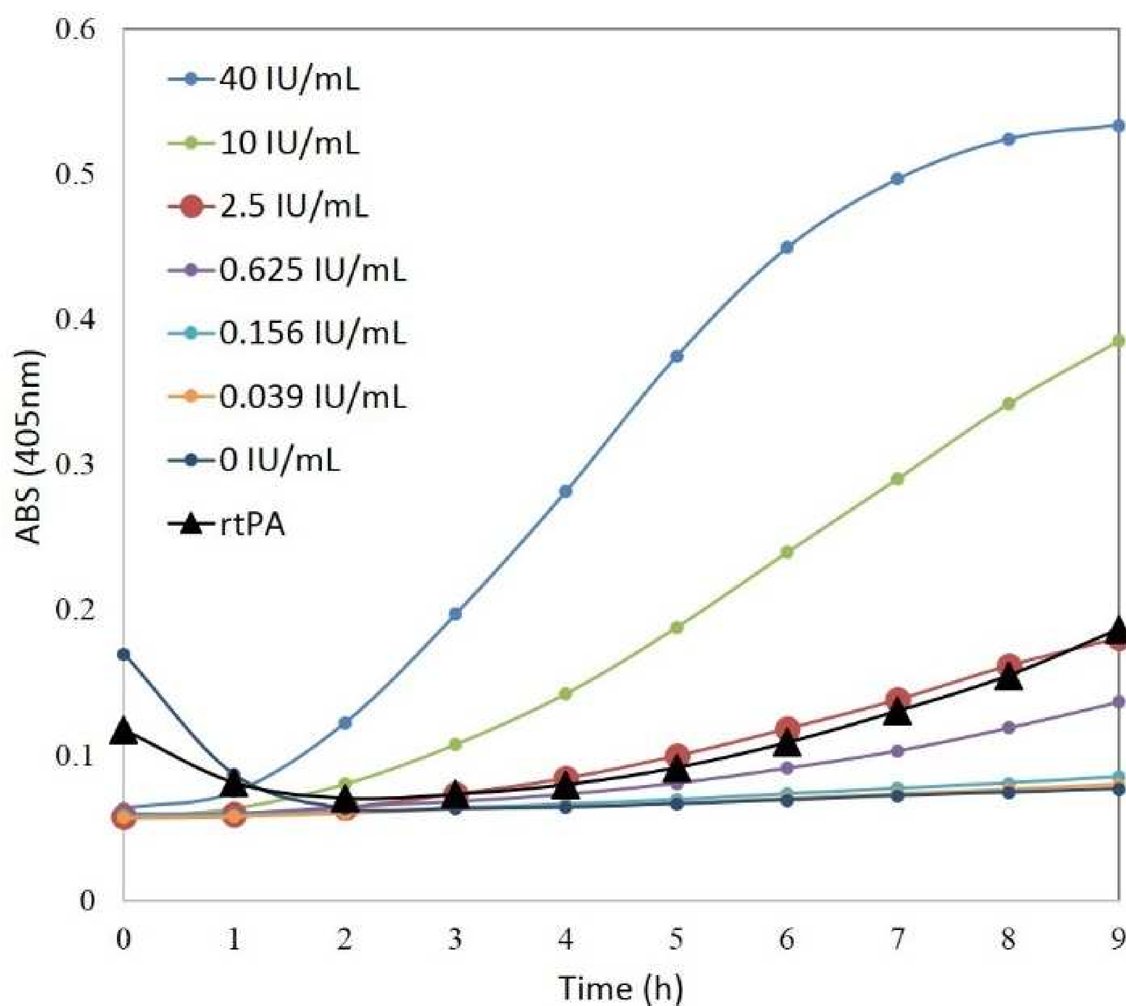

Figure 6. Quantitative determination of the native reteplase protein activity, extracted from the K2S cell suspension measured with Tissue type Plasminogen Activator Human Chromogenic Activity Assay Kit.

4. Conclusions

In this study we developed tobacco cell cultures derived from transgenic K2S plants for the viable production of the biopharmaceutical reteplase. Although the risk of transgene dispersion in transplastomic plants is low due to the maternal mode inheritance, the cell culture system guarantees transgene containment. Also, like other bioreactor systems, the target compounds can be produced under strictly controlled culture conditions, thus avoiding the risk of contamination with human pathogens associated with mammalian cells; it also has the capacity for proper protein folding. We have demonstrated the capacity of the transplastomic K2S cell cultures to produce active reteplase and the enhancing effects of light on its production, achieving a TSP content of 0.277% after a 2-week growth period. These results open a new avenue for the production of reteplase in transplastomic plant cell cultures as an alternative bio-sustainable system for biopharmaceutical production.

Author contributions

J.P., **P.C.** and **R.C.**, conceived and supervised the experiments. **M.A.**, obtained K2S plants. **R.B.-M.**, performed and developed the method for Mass Spectrometry. D.H., obtained the derived cell lines from K2S plants; developed the in vitro cultures, characterized and quantified the recombinant protein helped by **R.B-M**, and determine the reteplase activity. **J.P.**, **P.C.** and **D.H.**, wrote the manuscript. All authors contributed to results interpretation and reviewed the manuscript.

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General discussion

The **PhD thesis** deals with the important potential of Plant Cell Biofactories with the objective to demonstrate that biotechnological platforms based on plant cell and organ cultures constitute an alternative system to plant crops in the field for the production of pharmaceuticals; that they can be used to bioconvert foreign substrates into other compounds with improved biological activities or with high added value; and with the help of cutting edge technologies like metabolic engineering, they allow the design of new transgenic organisms with improved metabolic capacities. By gaining new insights into plant secondary metabolism, our study also aims to lay the groundwork for new approaches to optimized organisms for the biotechnological production of pharmaceuticals and biopharmaceuticals.

To achieve our general goal, we developed five paradigmatic examples of the biotechnological production of high added value compounds: **i)** centellosides, used to treat skin diseases used for dermatological disorders; **ii)** resveratrol and its derivatives piceatannol and pterostilbene, phenolic compounds with potent antioxidant activity and promising new therapeutical applications; and **iii)** the truncated human tissue plasminogen activator (K2S), one of the most important pharmaceutical proteins involved in the breakdown of blood clots in brain and heart blood vessels, as a good example of biopharmaceutical production (see **BOX 11**).

BOX 11

Compounds	Treatment
Centellosides	Skin diseases
Resveratrol Piceatannol Pterostilbene	Vascular diseases, cancers, viral infections, and neurodegenerative processes
Reteplase	Breakdown of blood clots

We thus developed several biotechnological platforms based on *in vitro* plant cell and organ cultures of *Centella asiatica*, tobacco and *Silybum marianum* species. *C. asiatica* plant cell cultures were established from the *in vitro* culture of explants derived from plant leaves, using an optimized medium for callus induction previously described by Bonfill et al. (2011). To obtain the cell suspensions, callus pieces were disintegrated and cultured in Murashige and Skoog (MS) medium 30 g L^{-1} sucrose + 2 mg L^{-1} 2,4-dichlorophenoxiacetic acid (2,4-D) + 0.1 mg L^{-1} benzylaminopurine (BA), shaken at 115 rpm at 25ºC in the dark (see **BOX 12**). The *C. asiatica*

cell line was subcultured routinely every 2 weeks, and several strategies were applied to increase the centelloside production based on elicitor treatments and feeding experiments with amyrin-enriched resins. Finally, the system was scaled up from shake flask to bioreactor level in optimal conditions (for details about the experimental part, see **Chapter 1**).

BOX 12

Starting material	Derived in vitro culture (biotechnological platform)
In vitro C. asiatica plantlet cultures	Callus cultures → Cell suspension
In vitro tobacco plantlet cultures	Wild type and transgenic hairy roots → Transgenic callus → Transgenic cell cultures
S. marianum cell suspensions	Transgenic callus → Transgenic cell cultures
In vitro transplastomic tobacco plantlets	Transplastomic callus cultures → Transplastomic cell suspensión

In order to transfer the biosynthetic capacity of *t*-resveratrol and its derivatives to tobacco hairy roots and derived cell lines, leaf segments of *in vitro* tobacco plantlets were infected with a wild type *Agrobacterim rhizogenes* and several engineered *A. rhizogenes* strains (see **BOX 13**). As tobacco is an amenable plant species for genetic transformation, hairy roots began to appear at 2-3 weeks after the agroinfection. They were then excised from the explants and cultured individually to isolate the root lines. The hairy roots were cultured in hormone-free solid MS medium, which for the engineered root lines was supplemented with the selection antiobiotics and also with the antiobiotic claphoram to remove the remaining agrobacteria. The roots were then transferred to a liquid medium and cultured in a shaker at 115 rpm at 25ºC in the dark. Some selected transformed root lines were cultured in MS solid medium supplemented with 2.14 mg L^{-1} of naphtaleneacetic acid (NAA) and 0.215 mg L^{-1} of kinetin (KIN). After several subcultures, friable calli were obtained and disintegrated in liquid MS medium with the same hormones in order to obtain a fine cell suspension, which was subcultured every 12 days, shaken at 115 rpm at 25 ºC, in darkness (**BOX 12**) (see **Chapters 2** and **4**).

Transgenic cell lines of *S. marianum* were obtained as briefly described here. *Agrobacterium tumefaciens* C58C1 (pGV2260) carrying the binary plant expression vector pJCV52-STS3 (**BOX**

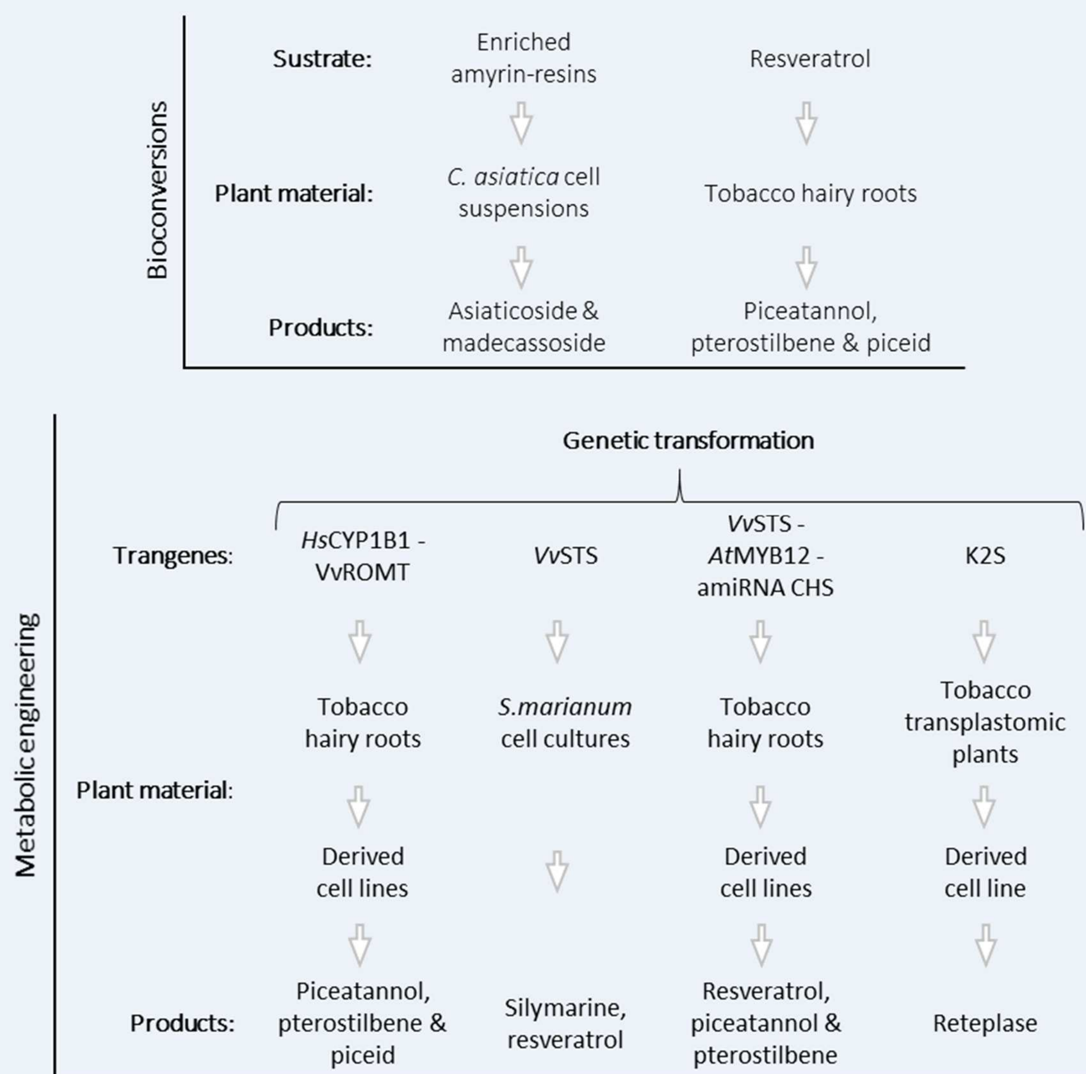
13) was employed to stably transform *S. marianum* cells as previously reported by Martínez-Márquez et al. (2014). The infected cell suspension was spread on Petri dishes containing solid MS medium (MS medium + 30 g L^{-1} sucrose + 1 mg L^{-1} 2,4-dichlorophenoxiacetic acid (2,4-D) L^{-1} + 0.5 mg L^{-1} benzylaminopurine (BA), + 10 g L^{-1} agar, shaken at 115 rpm at 25ºC in the dark), supplemented with claphoram and paromomycin (selection antibiotic). The Petri dishes were incubated at 25°C in the dark. The growing callus colonies were periodically subcultured with decreasing claphoram concentrations. To establish transgenic liquid cultures, callus tissue was transferred to fresh MS liquid medium and incubated under normal culture conditions (**BOX 12**) (see **Chapter 3**).

BOX 13

Transformation systems	Plasmids
Agrobacterium rhizogenes A4	pK7WG2_CYP1B1, pJCV52_ROMT, pJCV52_STS3, pJCV52_*At*MYB12, pDMDC32B_AtMiR390a-B/c_amiRNACHS
Agrobacterium tumefaciens C58C1 (pGV2260)	pJCV52-STS3
Biolistic	pKCZK2S

Transplastomic tobacco plants carrying the K2S gene were obtained as described previously by Abdoli-Nasab et al. (2013). In brief, sterile leaf fragments of tobacco plantlets were bombarded with microprojectiles carrying the K2S gene in the plasmid pKCZ (**BOX 13**). Leaf segments were subcultured by several rounds in MS medium supplemented with BA (3 mg L^{-1}), NAA (0.1 mg L^{-1}) and thiamine-ClH (1 mg L^{-1}), as well as the selection antibiotic spectinomicyn (500 mg L^{-1}). Green shoots were selected and subcultured several times to obtain homoplasmic plants. Leaf segments of the homoplasmic plants were cultured in MS medium supplemented with 11.5 µM of NAA and 1 µM KIN in order to dedifferentiate the tissues and obtain friable calli. Transplastomic cell lines were obtained by disintegration of the calli in MS liquid medium and cultured in an orbital shaker at 115 rpm, 25ºC, in the dark and light (see **Chapter 5**).

All the experiments carried out to achieve the final goal of this PhD thesis are summarized in **BOX 14**.



As previously mentioned, the biological activities of *C. asiatica*, together with the recent applications of plant stem cells as cosmetic ingredients (Morus et al., 2014), have generated a growing interest in its cell cultures as a new source of centellosides, the triterpenoid saponins responsible for these properties. Empirical approaches to improving secondary metabolite production in plant cell cultures can involve the combination of several strategies, including elicitor treatments, feeding experiments, permeabilizing treatments, etc. (Rao and Ravishankar, 2002). In this context, centelloside production can be enhanced in *C. asiatica* plant cell and hairy root cultures by elicitation with methyl jasmonate (MeJA), salicylic acid (SA), copper ions, and

yeast extract (YE), among others (Gallego et al., 2014). In this work (**Chapter 1**), we corroborated the positive effect of MeJA, SA, and YE on centelloside biosynthesis, and also demonstrated the effectiveness of the new elicitor coronatine (CORO), which increased the centelloside production five-fold more than MeJA. The effectiveness of CORO as an elicitor has been previously demonstrated for taxane production in *Taxus* cell cultures, driving a higher activation of taxane biosynthetic gene expression than MeJA and consequently an increased taxane yield (Cusido et al., 2014). Taken as a whole, the results demonstrated that CORO-elicited *C. asiatica* cell cultures could constitute a suitable platform for the biotechnological production of centellosides as well as a target ingredient for cosmetic products due to the high accumulation of these biologically active components.

Plant cell cultures also have the capacity to carry out regio- and stereoselective hydroxylations, hydrogenation, glycosidation, etc. of exogenous substrates, biotransforming them into other compounds with improved pharmacological actions (Ishihara et al., 2003). However, for industrial purposes, the substrates need to accumulate in high amounts in their natural sources or be chemically synthesized (Onrubia et al., 2013). In this scenario, we previously demonstrated the capacity of *C. asiatica* cell cultures to bioconvert up to 84% of the potential precursor α-amyrin into centellosides (Hernandez-Vazquez et al., 2010), although unfortunately, the high market price of this compound impedes the scale up of the process to an industrial level. However, sustainable sources of amyrins can be found in nature, such as Mexican copal or Manila elemi (MER) resins, which we selected to feed the *C. asiatica* cell cultures. The obtained results described in Chapter 1 demonstrate that Copal resin is highly toxic, unlike extracted Copal, which suggests that cleaning these resins with petroleum ether removes toxic compounds. While both extracted Copal (Ext Cop) and MER significantly enhanced the centelloside accumulation in the cell cultures, combining the precursors with elicitation generated a synergistic effect, and the centelloside production in cells treated with both Ext Copal and CORO increased more than 16-fold, compared with the 2.8-fold improvement achieved with the Ext Copal alone. This type of combined treatment has previously led to good results in, for example, improving taxane production in *Taxus* spp. cell cultures (Malik et al., 2011).

In the bioprocess based on plant cell and organ cultures, one of the last steps is to scale up the optimized process to bioreactor level. As mentioned, in this work (see **Chapter 1**), we used a 2 L CellBag with the treatment giving the best results in shake flasks: elicitation with CORO (1 μM) and feeding with MER (125 mg/L). MER was chosen as the precursor instead of Ext Copal, as it

does not require any preparation before its addition to the plant cell culture, always an advantage for industrial application. Under these conditions, the *C. asiatica* cell line grew very well, attaining a GI of 4.8 after 10 days of culture, with a final centelloside production of 7.3 mg g DW^{-1}, which was 4.2-fold higher than in shake flasks under the same conditions. The suitability of this system for scaling up centelloside production was thus demonstrated. We selected an orbitally shaken bioreactor with a 2 L CellBag because reusable models have several advantages over multiusable bioreactors: they require less sterilization work, are time- and cost-saving, and produce less waste and environmental contamination (Aranha, 2004; Rader and Langer, 2011). Until now, this type of bioreactor had not been tested for centelloside production.

In summary, the results obtained show that *C. asiatica* plant cell cultures are potentially a sustainable technology for the production of centellosides through the bioconversion of their natural precursors, amyrins. Moreover, the process was efficiently scaled up in an orbitally shaken bioreactor, improving the yields previously reached on a small scale, thereby achieving one of the main goals of this PhD thesis (see **Objectives** section).

As mentioned, plant cell cultures have also been used for biotransforming exogenous substrates by exploiting the regioselective and stereospecific properties of plant enzymes, as well as the vast potential of plants for biochemical reactions (Giri, et al., 2001)). Hairy root cultures obtained by genetic transformation of plant material with *Agrobacterium rhizogenes* can be an efficient alternative to plant cell cultures for bioconversions due to their genetic/biochemical stability, high growth capacity in hormone-free culture media and relatively low cost, and they have been successfully used for the heterologous production of known or new compounds from exogenous substrates with improved biological activities (Banerjee et al., 2012).

It has previously been demonstrated that *t*-piceatannol (*t*-Pt), a hydroxylated derivative of *t*-resveratrol (*t*-R), is more bioavailable than *t*-R and has higher anti-cancer and cancer chemopreventive activity (Szekeres et al., 2011; Piotrowska et al., 2012). Also, *t*-pterostilbene (*t*-Pt), a methoxy derivative of *t*-R, showed more potent antiproliferative effects than the latter against human colon cancer cells, even when administered at half-concentration (McCormack et al., 2013). Both *t*-Pt and *t*-Pt are scarcely distributed in nature and constitute prime targets for biotechnological production. Thus, given the advantages of hairy root cultures for carrying out biotransformations (as described in **Chapter 4**), we selected a biotechnological platform based on

a model system, tobacco hairy root cultures, to investigate its capacity to bioconvert exogenous *t*-R into the desired products *t*-Pn and *t*-Pt.

When wild type tobacco hairy roots were fed with exogenous *t*-R, a small part of the substrate was bioconverted into *t*-Pn and *t*-Pt, reaching levels of up to 2 mg L^{-1} and 0.06 μg L^{-1}, respectively. The results showed the capacity of constitutive, unspecific hydroxylases and methoxylases from tobacco to biotransform exogenous *t*-R into the target compounds, albeit at a low rate: 0.4% for *t*-Pn and even lower for *t*-Pt. The established hairy root cultures also showed the capacity to produce piceid, a glucosylate derivative of *t*-R, achieving levels near to 1 mg L^{-1}. Taking into account that *C. asiatica* cell cultures were able to bioconvert up to 84% of the precursor α-amyrin into centellosides (Hernandez-Vazquez et al., 2010), our results suggest that unspecific tobacco enzymes have a limited capacity for the heterologous production of *t*-Pn and *t*-Pt from *t*-R as an exogenous substrate, in comparison with the inherent specific capacity of *C. asiatica* cells to biotransform amyrins, which are the natural endogenous substrates for centelloside biosynthesis (**BOX 15**).

A limitation of bioconversion processes is they necessarily require an exogenous precursor/substrate. To overcome this handicap, and considering the current interest in *t*-R and its derivatives, we targeted these compounds by designing an approach that takes advantage of the potential of the metabolic engineering toolbox for transferring a complete or partial metabolic pathway from a plant species to a more amenable host (Miralpeix et al., 2013) (see **Chapter 2**).

As mentioned, tobacco is a model plant system easily transformed by *A. rhizogenes* to produce hairy root cultures and this trait may be harnessed for the heterologous expression of foreign genes harbored in engineered *A. rhizogenes* (Vasilev et al., 2014). We therefore used the same biological platform as before, tobacco hairy root cultures, but in this case, as well as the T-DNA of *A. rhizogenes*, the cultures harbored the following genetic constructions: the *Vitis vinifera* stilbene synthase (STS3) gene, for heterologous *t*-R production (Hain et al. 1990); the transcription factor (TF) *At*MYB12 from *Arabidopsis thaliana* to generate a holistic response in the phenylpropanoid pathway and to coordinate the up-regulation of multiple steps (Pandey et al., 2015); and an artificial micro RNA for chalcone synthase (amiRNA CHS) to limit the phenolic flux through the endogenous CHS enzyme, which competes for the phenylpropanoid precursors with the imported STS enzyme used for the flux deviation. Each gene was introduced alone or in combination with the others.

BOX 15 : Summary of the bioconversion results achieved in the biotechnological platforms designed.

Biotechnological platform	Substrate	Production (µg/L)	Chapter
C. asiatica cell cultures (SF, control conditions)	Amyrine-enriched resins	Centellosides (2829)	1
C. asiatica cell cultures (SF, CORO-elicited)		(6125)	
C. asiatica cell cultures (Bioreactor, CORO-elicited)		(10160)	
Tobacco hairy roots (wild type)	*t*-Resveratrol	*t*-Pn (1509), Pi (1065)	4
Tobacco hairy roots (HsCYP1B1 type)		*t*-Pn (7311), Pi (1070)	
Tobacco hairy roots (VvROMT type)		*t*-Pn (2000), Pi (291), *t*-Pt (2.5)	
Tobacco trangenic cell cultures (HsCYP1B1)		*t*-Pn (942)	

Coronatine (CORO); shaken flaks (SF); *t*-piceatannol (*t*-Pn); *t*-pterostilbene (*t*-Pt); Piceid (Pi)

As explained before, wild tobacco hairy roots can bioconvert *t*-R into other derivatives, such as piceid, *t*-Pn and *t*-Pt (see **Chapter 4**). Engineered hairy root cultures with *Vv*STS3 gene were also able to produce *t*-R in amounts correlated with the expression level of the *Vv*STS3 gene in the different transgenic hairy root lines obtained.

The genetic transformation of plants with STS genes has led to interesting results (Fischer and Hain, 1994; Hain and Grimming, 2000), and is still being studied intensively (Delaunois et al., 2009). In this research pipeline, the first experiments were performed by Hain et al. (1990), who expressed the STS gene from *Arachis hypogea* in tobacco plants, leading to resveratrol accumulation after induction with short-wavelength ultraviolet (UV) light. Heterologous expression of the STS gene has subsequently been reported in several edible plant species, including tomato, kiwifruit, apple, lettuce, barley and wheat, as a defense against plague attack, based on the phytoalexin activity of resveratrol (Delaunois et al., 2009 and references therein).

In the same way, several metabolic engineering strategies have been developed for *t*-R production in microorganisms, but unlike plant systems, these involve the introduction of a complex set of genes. In this context, the entire resveratrol pathway has been induced in modified yeast *Yarrowia lipolytica* (ATCC 20362 strain), including the genes encoding phenylalanine/tyrosine ammonia lyase (PAL/TAL), cinnamate-4-hydroxylase (4CH), *p*-coumaroyl-CoA ligase (4CL), and stilbene synthase (STS) (Huang et al. 2006). More recently, Trantas et al. (2009) constructed the complete resveratrol biosynthetic pathway in *S. cerevisiae* to produce resveratrol from the precursor phenylalanine (10 mM), reaching a content of 0.29 mg/L *t*-R after 120 h of cultivation. Higher yields have been achieved in engineered *Escherichia coli* strains; for instance, Katsuyama et al. (2007) reported a production of 171 mg/L of *t*-R. However, the production of *t*-R in microorganisms always requires the addition of precursors that they do not naturally contain (Jeandet et al., 2012).

As mentioned in the introduction, overexpression of transcription factors (TF) could represent a holistic approach to boosting a complete metabolic pathway (Capell and Christou, 2004). In this context, the transcription factors of the MYB family exert a tight control on flavonoid biosynthesis (Mehrtens, et al., 2005, Stracke et al., 2007). Specifically, it has been demonstrated that ectopic expression of TF *At*MYB12 increases the flavonoid content of tomato leaves and fruits (Pandey, 2015; Luo et al., 2008). In tobacco plants, its expression causes a strong transcriptome perturbation by up-regulating genes encoding enzymes downstream in the phenolic pathway, which increases flavonoid contents as well as the expression of several genes encoding upstream enzymes involved in the biosynthesis of common precursors in the stilbene and flavonoid biosynthetic pathways (Misra et al., 2010).

In the transgenic hairy root lines harboring the TF *At*MYB12, a high positive correlation between the transcription factor expression and increased flavonoid contents was observed, probably because of the up-regulation of the CHS gene. The expression of the upstream gene phenylalanine ammonia lyase (PAL) was also enhanced, leading to a higher accumulation of total phenolic compounds compared with the control roots (see **Chapter 2**).

Major metabolic differences due to the presence of the TF *At*MYB12 were analyzed using an NMR-based metabolomics platform. H-NMR fingerprinting coupled to principal components analysis (PCA) allowed the identification of notable perturbations in the transgenic root metabolome due to the TF *At*MYB12 compared with wild type hairy roots (Control line).

Transgene expression especially affected phenolic acids, amino acids, sugars and organic acids. Particularly noteworthy was the enhanced accumulation of two acids from the carboxylic acid cycle (CAC), fumaric and malic acids, as well as a high amount of GABA in the transgenic roots. In relation with phenolic acids, the metabolomics results also showed that together with high levels of cinnamic and *p*-coumaric acid, which are involved in the biosynthesis of stilbenes and flavonoids, the transgenic roots accumulated enhanced levels of caffeic and sinapic acid, which could be involved in lignin biosynthesis. These results agree with those reported by Misra et al. (2010) for transgenic tobacco plants and allows to asign *At*MYB12 TF a similar role both in hairy roots and whole plants

STS heterologous expression can alter flower morphology, and cause male sterility in tobacco and petunia (Fischer et al., 1997). It is known that sterility may also be linked to a competition for substrates between STS and endogenous chalcone synthase (CHS), because fertility can be restored by adding exogenous flavonol in tobacco (Fischer et al., 1997). As mentioned, STS and CHS are both involved in the polyphenol pathway, and it is reasonable to assume that the expression of exogenous STS may lead to a competition for a substrate. For this reason, we transformed the tobacco hairy root lines with amiRNA CHS to arrest the flavonoid biosynthetic pathway. Our results show that roots transgenic for amiRNA CHS achieved the lowest levels of flavonoids by inhibiting the CHS gene expression. This confirms that this branching point for stilbene biosynthesis was blocked, thus resulting in a greater flow of precursors for the production of *t*-R and its derivatives (see **Chapter 2**). Unfortunately, the transgenic root lines carrying the amiRNA CHS also showed a low *Vv*STS gene expression and stilbene production was lower than in the other root line types.

We have previously discussed that elicitation can improve the heterologous production of a target compound in plant cell cultures (Mehrotra et al., 2010; Exposito et al., 2010).

A positive effect of a combined elicitor treatment with β-methyl cyclodextrin (MBCD) and methyl jasmonate MeJA on *t*-R production has been previously reported both in non-transgenic and transgenic grapevine cell cultures (Jeandet et al., 2014; Martinez-Marquez et al., 2016) and also confirmed in transgenic *S. marianum* cell suspensions elicited with MBCD (see **Chapter 3**).

Taking this into account, we elicited the engineered hairy roots with 50 mM MBCD and 100 µM of MeJA. The obtained results showed that elicitation significantly increased the release of *t*-R

and its derivatives *t*-Pn and *t*-Pt to the liquid medium, whereas piceid remained mainly inside the roots; however, the system was not effective for improving the total stilbenoid contents of the root cultures, perhaps due either to a lack of sufficient precursor availability or to the limitation impossed by the amount of total STS protein expressed.

Considered as a whole, our results show the suitability of the engineered hairy root cultures for the heterologous production of *t*-R and its derivatives, as well as the effectiveness of *At*MYB12 in boosting the phenolic pathway in these systems and the efficiency of the amiRNA CHS in blocking competitive pathways (**BOX 16**). Therefore, one of the main goals of this PhD thesis, the heterologous production of *t*-R in tobacco hairy root cultures, was accomplished. However, these results also show the extreme complexity of biotechnological systems based on *in vitro* cultures of seed plants for metabolic engineering approaches.

BOX 16 : Summary of the heterologous stilbene production achieved.

Biotechnological platform	Genes inserted	TST (μg/L)	Sm (μg/L)	Chapter
Tobacco hairy roots (wild type)	T-DNA (pRi)	ND	ND	2
Tobacco hairy roots (transgenic)	T-DNA + *Vv*STS	(57) *t*-R + *t*-Pt + *t*-Pt + Pic		
	T-DNA + *Vv*STS + *At*MYB12	(469) *t*-R + *t*-Pt + *t*-Pt + Pic		
	T-DNA + *Vv*STS + *At*MYB12 + amiRNA CHS	(2.5) *t*-R + *t*-Pt + *t*-Pt + Pic		
S. marianum cell cultures	———	ND	(7000)	3
S. marianum cell cultures (transgenic)	*Vv*STS	(12000) only *t*-R	(6900)	

Total stilbenoids (TST): *t*-resveratrol (*t*-R) + *t*-piceatannol (*t*-Pn) + *t*-pterostilbene (*t*-Pt) + piceid (pic); Silymarin (Sm). ND, not detected.

Another aim was to transfer the stilbenoid pathway for *t*-R production to other plant cell cultures with the natural capacity to produce other important pharmaceuticals, thereby increasing their

range of therapeutic properties (see **Objectives** section). Accordingly, we obtained *S. marianum* cell cultures harboring the *Vv*STS3 gene. Under elicited conditions, *S. marianum* cell cultures, which are not a model plant system, produce silymarin (Sm), a flavonolignan prescribed for the treatment of chronic liver disease (Fraschini et al., 2002; Kren and Walterova, 2005), and more recently for the prevention of recurrent hepatitis C in liver transplant recipients by the European Medicines Agency (EMEA, 2010).

Two phenylpropanoid units are needed for Sm biosynthesis, the flavonoid taxifolin (Tx) and the monolignol coniferyl alcohol (CA), and their oxidative coupling leads to Sm (Kim et al., 2003; Lee et al., 2003). Since STS shares the same key precursors employed for flavonoid and/or monolignol biosynthesis, the heterologous expression of *Vv*STS in *S. marianum* could find a suitable substrate for *t*-R production in what is otherwise a non-producing plant species. It would also allow the competition between the biosynthetic pathways to be studied.

The results obtained (see **Chapter 3**) show that the integration and expression of the *Vv*STS3 gene in the *S. marianum* genome equipped the cell suspension cultures for the heterologous production of up to 12 mg L^-1 of *t*-R, which was released to the culture medium only under elicitation [30 mM MBCD and 100 µM of MeJA]. This high *t*-R yield, compared with the production in the transgenic tobacco hairy root cultures (40 µg L^{-1}), suggests that a plant with a natural high phenolic production could also produce high levels of other phenolic compounds not found in natural conditions (**BOX 16**). The results also confirm the positive effects of elicitation for the heterologous production of a target compound in plant cell cultures (Mehrotra et al., 2010; Exposito et al., 2010), in contrast with the stilbene yields achieved in tobacco hairy root cultures (see **Chapter 2**).

There are many reports on the extracellular *t*-R accumulation in *Vitis* spp. cultures treated with MeJA, and/or MBCD (reviewed in Jeandet et al., 2014). Similarly, in the current study, 10 mg L^{-1} of *t*-R was released to the culture medium in the presence of 30 mM MBCD, although the *t*-R productivity in transgenic *Silybum* cultures was far from that reported for *V.vinifera* cultures, in which more than 3 g L^{-1} *t*-R accumulated in the presence of 50 mM MBCD + 100 µM MeJA (Martinez-Esteso et al. 2009). Nevertheless, levels were higher than in transgenic tobacco cell suspensions or in STS-overexpressing *V. amurensis* cultures (Hain et al., 1990; Aleynova et al., 2016).

As previously reported, elicitation treatments are required for the accumulation of phenylpropanoid-like compounds in *S. marianum.* The monolignol pathway was preferentially induced by elicitors, with CA accumulating massively in the extracellular medium (Belchi-Navarro et al., 2011). Thus, monolignols compete with the flavonoid pathway for precursors, which is subsequently the rate-limiting branch for Sm biosynthesis in cell cultures.

In the STS-transgenic *S. marianum* cultures, the CA contents decreased significantly, suggesting that *t*-R synthesis occurred at the expense of the monolignol due to the competition with the STS protein for precursors (*p*-coumaroyl CoA) (see **Chapter 3**). On the other hand, the Sm content was less affected, since the flavonoid branch in *S. marianum* cultures is the rate-limiting step (Torres and Corchete, 2016). Although there are no precedents in the literature concerning cell suspensions, competition for common precursors in transgenic plants is not unexpected; for example, a slight decrease in flavonols was seen in transgenic apple with heterologous expression of the STS gene (Rühmann et al., 2006). In contrast, substantial *t*-R production had no effect on the flavonol concentration in transgenic tomato plants (Giovinazzo et al., 2005; Schwekendiek et al., 2007).

From our results, we can conclude that *S. marianum* cell cultures represent a new heterologous host for *t*-R production. The fact that the newly expressed *Vv*STS3 gene took precursors of excessively produced non-bioactive compounds (CA), while keeping the flow for target secondary compounds (i.e. Sm) unaltered, opens a way to extend the applications of plant cell cultures for the simultaneous production of valuable metabolites, both constitutive and foreign. These findings fulfill another main goal of this PhD thesis (see **Objectives** section).

Although we achieved *t*-R production in different biotechnological systems based on plant cell and organ cultures, the levels of its derivatives *t*-Pt and *t*-Pt were very low. This fact, together with the low rate of bioconversion of *t*-R into its derivatives by the natural enzyme machinery of wild type tobacco hairy roots, prompted us to design new hairy root culture platforms for the bioconversion of *t*-R by heterologous expression of the human cytochrome P450 hydroxylase 1B1 (*Hs*CYP1B1) gene for the production of *t*-Pt and the resveratrol O-methyltransferase from *Vitis vinifera* (*Vv*ROMT). In both cases, the cultures were fed with exogenous *t*-R.

The results obtained (see **Chapter 4**) show that the tobacco hairy root cultures engineered to ectopically express the *Hs*CYP1B1 gene had the capacity to bioconvert *t*-R into *t*-Pn with a yield

of up to 7 mg L^{-1}, and others expressing the *Vv*ROMT gene were able to biosynthesize *t*-Pt, reaching a content of 2.6 µg L^{-1}. This concentration was 25-fold higher than those achieved in the control hairy roots, as mentioned above, but very low when compared with the production in the transgenic roots carrying the *Vv*STS genes together with the TF *At*MYB12, which reached a *t*-Pt production of up to 86 µg L-1 (**BOX 15**). This biotechnological system thus proved to be suitable for the production of *t*-Pn and, on a lower scale, *t*-Pt.

In a similar approach, Martinez-Marquez et al. (2016) recently reported a 200-fold enhancement of *t*-Pn production in grapevine cell cultures by the heterologous expression of the *Hs*CYP1B1 gene, and the presence of *t*-Pt exclusively in transgenic cell lines overexpressing the *Vv*ROMT gene but only when both cultures were elicited with MeJA and MBCD.

Transgenic hairy roots from a number of plant species have been previously used for the bioconversion of exogenous substrates. Through the heterologous expression of the hyoscyamyne-6β-hydroxylase gene from *H. muticus*, Häkkinen et al. (2005) obtained the alkaloid scopolamine after feeding tobacco hairy root cultures with its precursor hyoscyamine. Similarly, hairy root cultures of *Peganum harmala* expressing tryptophan decarboxylase of *C. roseus* produced high levels of serotonin (Karuppusamy et al., 2009), and *Beta vulgaris* hairy roots expressing the *p*-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) gene from *Pseudomonas fluorescens* produced vanillin when the cultures were fed with ferulic acid (Singh et al., 2015). Thus, our results confirm the capacity of the engineered hairy root cultures to biotransform exogenous substrates into target products with more interesting biological activities, which was one of the main objectives of this PhD thesis.

In this work (see **Chapter 4**), we also demonstrated the enhancing effects of methylated β-cyclodextrin (MBCD) on the bioconversion of *t*-R into *t*-Pn in the hairy root cultures. MBCD can act as a precursor solubilizer in biotransformation processes, such as the bioconversion of 17β-estradiol into 4-hydroxyestradiol in cell cultures of *Mucuna pruriens* or the bioconversion of coniferyl alcohol MBCD complex into podophyllotoxin in cell cultures of *Podophyllum hexandrum* (van Uden et al., 1994). In our experiment, considering the poor solubility of *t*-R in water, the solubilizing effects of MBCD may have contributed to the improved efficiency of the hairy root cultures in biotransforming *t*-R into *t*-Pn.

MBCD has also been used as a permeabilizing agent acting on plant cell membranes, thus promoting the release of plant secondary metabolites such as taxol in *Taxus* spp. cell cultures (van Uden et al., 1994). This effect could also be responsible for the higher extracellular *t*-Pn accumulation in the MBCD-treated cultures compared with the untreated control. In previous work (see **Chapter 2**), we already demonstrated an increase in the release of stilbenes to the culture medium in transgenic tobacco hairy roots carrying the *Vv*STS gene.

The membrane modification by MBCD may also facilitate the movement of substrates and products during the biotransformation processes and improve the uptake of *t*-R by the hairy roots, thus facilitating its metabolism inside the cells and its conversion to other stilbenoids like *t*-Pn. However, the positive effect of MBCD on the release of reaction products to the culture medium could negatively affect the production of the system if they are less stable in the medium. This may be the the explantion for *t*-Pt or piceid, which were absent in grapevine cell cultures even after elicitation with MeJA and MBCD (Martínez-Márquez et al., 2016). In the same way, treating the transgenic hairy root cultures carrying the *Vv*STS gene with MBCD decreased the total stilbene contents of the cultures (see **Chapter 2**).

As mentioned in the Introduction, plant cell cultures have been widely employed for the bioconversion of naturally abundant substrates to scarcer secondary metabolites with important biological activities (Giri et al., 2001; Cusido et al., 2014). However, according to our results, the tobacco transgenic cell cultures carrying the *Hs*CYP1B1 transgene had a much lower capacity to biotransform *t*-R into *t*-Pn than the parental transgenic roots. These results confirm those of Moyano et al. (2007), who reported that hairy roots were able to bioconvert hyoscyamine to scopolamine more efficiently than the corresponding derived cell lines in tobacco transgenic cultures heterologously expressing the hyoscyamine-β-hydroxylase gene from *Hyoscyamus muticus*. Perhaps due to ectopic *vs* constitutive gene sequence similarity, the introduction of transgenic copies of a gene would have resulted in reduced expression of the transgene as well as the endogenous gene.

Last but not least, this PhD thesis aims to demonstrate the capacity of tobacco cell cultures to produce recombinant biopharmaceuticals. As mentioned in the Introduction, the biopharmaceutical market is growing and it is expected that by 2020 the global sales of these products will be worth over $US 278.2 billion (Santos et al., 2016). To date, *Escherichia coli* is one of the most commonly used hosts to produce small recombinant proteins, owing to its short

production time, easy genetic transformation and feasibility for scaling up to bioreactor level for a high yield of biopharmaceuticals (Baeshen et al., 2015). Although biotechnological platforms based on transgenic *E. coli* cultures are simple and inexpensive, they can suffer endotoxin contamination and frequently fail to produce large and complex proteins. Also, as prokaryotic cells, they do not allow post-translational modifications such as protein folding and glycosylation, and as bacterial systems they can produce the target proteins associated with inclusion bodies, which are insoluble, inactive and require refolding (Santos et al., 2016).

Thrombolysis is the therapy of choice for Acute Myocardial Infarction (AMI) (Mandi et al. 2010). Tissue Plasminogen Activator (tPA), which derives from the fibrinolytic system of blood vessel endothelial cells, is a thrombolytic agent that activates plasminogen to form plasmin (Topol, 1997; Geng et al., 2010). Native tPA is a 70 kDa serine protease composed of 527 amino acid residues with five structural domains and 17 disulfide bonds (Qui et al., 1998; Geng et al., 2010). Chinese hamster ovary cells transfected with the tPA gene were developed to produce rtPA (Cartwright et al., 1992), but the process has the drawbacks of a high production cost, complex protein purification and the risk of contamination with human pathogens (Chu and Robinson, 2001).

Attracted by the simplicity and economy of production, investigators have made a number of efforts to produce recombinant tPA (rtPA) from bacteria, especially from *E. coli* (Sarmientos et al. 1989; Datar et al. 1993). Numerous strategies have been proposed to overcome the problems of low yield and the formation of inclusion bodies, which result in misfolding and in an inactive enzyme. The other major criterion is to synthesize the smallest molecule possible that retains activity, instead of full-length tPA. Different approaches for the production of rtPA and K2S are summarized in **BOX 17**.

Reteplase is a recombinant non-glycosylated form of human tPA (reteplase) produced commercially in *E. coli*. It has been modified to contain 355 (1–3 and 176–527) of the 527 amino acids of the original protein with a total molecular mass of 39 kDa. This deleted variant of tPA has lost the three structural domains of Kringle I, Finger, and EGF, while retaining the thrombolytic Kringle II plus serine protease domains, and contains 9 disulfide bonds (K2S) (Fathi-Roudsari et al. 2016). However, because of the deletion of three structural domains as well as the carbohydrate side chains, the plasma half-life of this mutated variant is longer than full length tPA, being 13–16 min (Baruah et al. 2006).

BOX 17 : Some examples of host organisms for the production of tissue plasminogen activator and main drawbacks of the systems.

Recombinant protein	Host organisms	Drawbacks	References
tPA	*E. coli*	Low yield, not glycosidation, inclusion bodies, misfolding.	Harris et al. 1986
	Asperigillu nidulans		Upsall et al. 1987
	Sacharomices cerevisae	Not correct glycosidation, misfolding.	Martegani et al. 1992
	Mammalian cells	High production cost, complex protein purification and risk of contamination with human pathogens.	Lubiniecki et al. 1990 Cartwright, 1992
	Tobacco plants (nuclear genomic transformation)	Transgene dissemination, contamination with agrochemicals and fertilizers, fluctuations in yield due to culture conditions and the impact of bacterial and fungal infections	Hahn et al. 2009 Masoumi et al. 2017
Reteplase	*E. coli*	Requires solubilization of inclusion bodies, refolding and dialysis of protein.	Youchun et al. 2003
	Transplastomic tobacco plants	Limited transgene dissemination, contamination with agrochemicals and fertilizers, fluctuations in yield due to culture conditions and the impact of bacterial and fungal infections.	Abdoli-Nasab et al. 2013
	Transplantomic tobacco cell cultures	Low production of the active protein	See chapter 5

Due to the clinical interest of tPA, our group recently obtained tobacco transplastomic plants harboring the K2S gene driven by the promotor Prrn for the production of reteplase (Abdoli-Nasab et al., 2013) and we optimized a purification system of the recombinant protein reaching a production of up to 30.6 µg/100 mg of leaf tissue (Abdoli Nasab et al., 2016). The production of recombinant proteins in transgenic plants, as a large-scale agricultural system, enjoys the

advantages of inexpensive growth media (requiring only water, minerals and sunlight), low capital equipment costs and easy scale-up. However, according to the US Agricultural Department, transgene dissemination and contamination of the human food chain are major concerns in the use of field-grown transgenic crops, especially when edible plant species are used as the host (Wilson and Roberts, 2012).

To avoid this problem, transplastomic plants have been targeted by metabolic engineering approaches to biopharmaceutical production, because maternal mode inheritance partially reduces transgene dissemination by pollen. Additionally, the high number of chloroplasts per plant cell and the high copy number of the plastid genome could permit a higher transgene expression and consecuently an improved production of the recombinant proteins (Bock, 2014).

Like production systems based on transgenic plants, plant cell cultures have the capacity for proper protein folding and can assemble complex recombinant proteins. Moreover, they combine these advantages with those of the original bioreactor systems, that is, total transgene containment, controlled and sterile growth conditions, chemically defined culture media and straightforward compliance with pharmaceutical good manufacturing practices (GMP), which enhances biosafety and productivity (Demain and Vaishnav, 2009; Santos et al., 2016).

Plant cell cultures also offer other intrinsic advantages over plant crops, such as a significantly shorter production time compared to the growth cycle of a whole plant. This allows proteins to be manufactured in days or weeks, a time-scale compatible with clinical demands, rather than months or years (Doran, 2000). Additionally, *in vitro* plant cell cultures are unaffected by field cultivation factors, such as climate, soil quality, season, day length and weather. Also, downstream processing and protein purification are simplified, especially when the target protein is secreted into the culture medium (Pham et al., 2012).

Continuing in this research pipeline, the final aim of this PhD thesis was to produce reteplase in a new biotechnological platform based on cell cultures derived from homoplasmic transplantomic K2S tobacco plants. As described periously, callus cultures were established from leaf explants of a homoplasmic transplastomic K2S plant and disintegrated to form the cell suspension, which was cultivated under dark and light conditions. A characteristic green color was observed in the callus and cell suspension grown under light conditions. After 12 days of culture, samples were taken and the growth capacity was measured as a growth index (harvested fresh weight/inoculum fresh

weight, GI) and biomass productivity (rx). Both the cell cultures reached a GI > 3, which represents a rx > 18.5 g L-1d^{-1} (see **Chapter 5**). These results show the high capacity of the system based on tobacco suspension cultures to produce biomass, which was not affected by the K2S gene expression. Similar results for growth capacity have been reported previously for tobacco cell cultures genetically designed for the heterologous production of the *t*-resveratrol derivative, *t*-piceatannol (Hidalgo et al., 2017), and for the production of scopolamine in transgenic tobacco cell lines carrying the *Hn*H6H gene (Moyano et al., 2007).

In contrast, K2S gene expression was 4.6-fold higher in the cell cultures growing under light than in the dark (See **Chapter 5**). These results could be related to the green color of this cell suspension, which is probably caused by a higher degree of chloroplast organization and/or their number per cell. Recently, Barreto et al. (2017) developed transgenic calli for the heterologous production of fragment C of tetanus toxin (TetC), and reported a positive correlation between green shoot development and TetC yield when the callus was cultivated in a temporary immersion culture.

The presence of reteplase was detected by mass spectrometry analysis. We detected 7 different reteplase peptides, 3 of which were proteotypic and exclusive to K2S, without any homology with related species. Each peptide was detected with at least 3 transitions, which gives specificity to the analysis. In all the K2S samples, the most intensive signals were detected for the peptides GTHSLTESGASCLPWNSMILIGK and VYTAQNPSAQALGLGK, at a retention time of 3.4 min and 2.0 min, respectively, confirming the capacity of the K2S transgenic cell line to biosynthesize the target biopharmaceutical.

The reteplase content reached by the tobacco cell suspension when cultivated in darkness was 0.083 % of the total soluble proteins, which increased up to 0.277% in light conditions, representing an increase of more than 3-fold (see **Chapter 5**). The achieved production was lower than those obtained by the transplantomic mother plant (approx. 1% of TSP) (Abdoli Nasab et al. 2016), but in this case the protein was harvested after a culture period of 8 weeks rather than the 2 week-culture period of the cell suspension.

In contrast with tPA, reteplase has no carbohydrate side chains and thus can be produced in *E. coli* cells, but most of the protein is present as an inclusion body, and therefore it needs to undergo extraction and renaturation. This is a long and time-consuming downstream process, including the

solubilization of inclusion bodies and the refolding and dialysis of protein (Khodabakhsh et al., 2013). However, in our biotechnological system, as the target protein was tagged with 6-his, it was easily purified using a column containing TALON-Superflow® medium precharged with cobalt ions, and the active K2S protein was recovered, following the method previously developed by Abbdoli Nasab et al. (2016).

For the quantitative measurement of the reteplase activity, the extracted native protein was tested by the plasminogen activator human chromogenic activity assay kit. The behavior of the extracted enzyme (1460 µg) was comparable to the 2.5 UI mL^{-1} tPA standard curve (see **Chapter 5**). These results demonstrate that the reteplase extraction in native conditions preserves its biological activity, and so the biotechnological system could be successfully applied for the production of this biopharmaceutical. Importantly, it would guarantee the transgene containment and, like other bioreactor systems, permit the production of reteplase under strictly controlled culture conditions, thereby avoiding the risk of contamination with human pathogens associated with mammalian cells.

Conclusions

Based on the results of the five case studies described in this PhD thesis, and considering that our main aim was to demonstrate the high potential of plant biofactories for multiple applications in the biotechnological production of pharmaceuticals and biopharmaceuticals, we can conclude:

1. Within the scope of the biotechnological production of centellosides (see **Chapter 1**), our results demonstrate that *C. asiatica* plant cell cultures have potential as a sustainable technology for the production of these bioactive compounds. We found that a combined treatment in which cell cultures are supplemented with the new elicitor coronatine and fed with natural amyrin-rich products, such as Copal extract or Manila elemi resin, significantly enhanced the centelloside production of the system. We also showed that scaling up the production system to a 2 L CellBag was easy and improved the growth and centelloside production capacity of the *C. asiatica* cell suspension.

2. In relation with the production of bioactive stilbenes, based on the results described in **Chapter 2**, we can conclude that STS-engineered hairy root cultures are suitable for the heterologous production of *t*-resveratrol (*t*-R) and its derivatives *t*-piceatannol (*t*-Pn) and *t*-pterostilbene (*t*-Pt). We also demonstrated the effectiveness of the *At*MYB12 TF in boosting the phenolic pathway and the amiRNA CHS in blocking competitive pathways. However, our results also indicate the strong metabolomic perturbations induced by *At*MYB12 TF and confirm the extreme complexity of metabolically engineering biotechnological systems based on seed plant *in vitro* cultures.

3. In the same research pipeline, and according with the results reported in **Chapter 3**, we can conclude that *S. marianum* cell cultures constitute a new heterologous host for *t*-R production. The fact that the newly expressed STS gene redirected precursor flux away from an unwanted, excessively produced non-bioactive compound (coniferyl alcohol), while keeping the flow toward target secondary compounds (i.e. silymarin) unaltered, opens a way to extend the applications of plant cell cultures for the simultaneous production of both constitutive and foreign valuable metabolites. These potential needs further exploration.

4. Looking for new sources of the scarce resveratrol-derived stilbenes, *t*-Pn and *t*-Pt, in **Chapter 4** we successfully developed a *t*-Pn-producing biotechnological platform based on metabolically engineered tobacco hairy roots heterologously expressing the *Hs*CYP1B1 gene for the bioconversion of exogenous *t*-R into the target compound in quantities near mg L^{-1}. Similarly, the developed system based on the heterologous expression of the *Vv*ROMT gene has potential as a biotechnological source of t-Pt after an optimization process. Finally, both untransformed systems were also able to biosynthesize *t*-Pn, *t*-Pt and piceid using the natural genetic competence of the host plant to perform non-specific hydroxylations, methoxylations and glycosylations, thus demonstrating the immense capacity of plant cells to carry out biotransformations and generate known or even new products.

5. Although there is only a limited risk for transgene dispersion in transplastomic plants due to the maternal inheritance mode, in **Chapter 5** we developed tobacco cell cultures derived from homoplasmic transplastomic K2S plants for the viable production of the biopharmaceutical reteplase. This system guarantees transgene containment and, like other bioreactor systems, permits the production of the target compounds under strictly controlled culture conditions, avoiding the risk of contamination with human pathogens associated with mammalian cells, as well as having the capacity for proper protein folding. In conclusion, we have demonstrated the potential of the transplastomic K2S cell cultures for producing active reteplase and the positive effects of light for enhancing production, achieving a content of 0.277% of TSP after a 2-week growth period. These results open a new avenue for the ecological and bio-sustainable production of this biopharmaceutical.

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Annexed 1.
Supplementary material for Chapter 2

Table S1. Sequences of the primers used to amplify the genes by PCR and qPCR

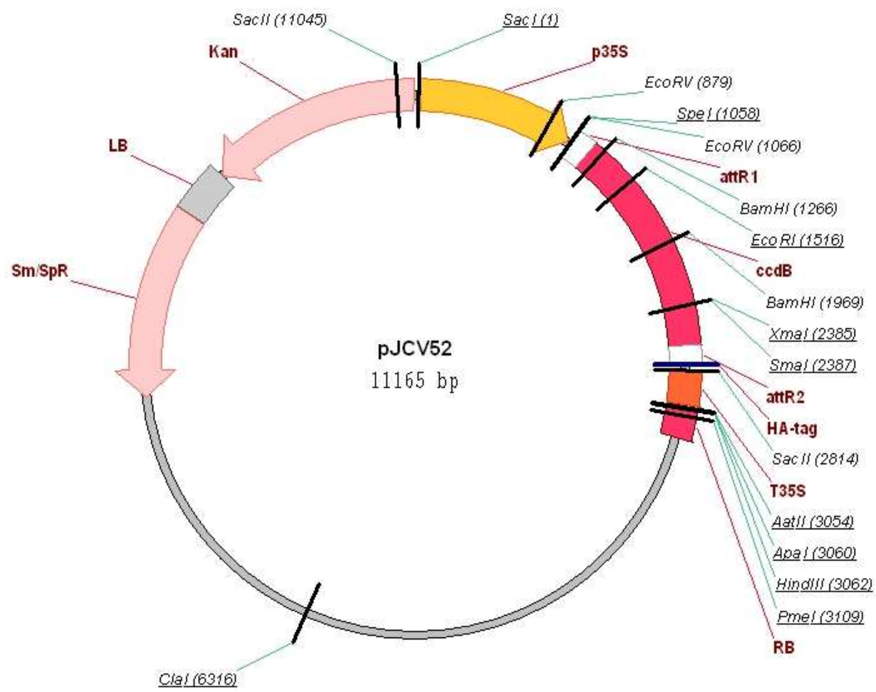
PCR			
Gene	Primer Sequence	Amplicon size bp	Reference
VvSTS-3	FW: ATGGCTTCAGTTGAGGAATTTAGA RV: TTAATTTGTAACCGTAGGAACGCT	1179	Hidalgo et al. (2007a)
AtMYB12	FW: CACCATGGGAAGAGCGCCATGTTG RV: TGACAGAAGCCAAGCGACCA	1117	This work
amiRNA-CHS	FW: ACGAAGAGGAGATGACGTGTG RV: GTTTTGCCTCGGCCTTTTTCA	287	This work
rolC	FW: TAACATGGCTAGAAGACGACC RV: AAACTTGCACTCGCCATGCC	534	Slightom, et al. (1986)
virD	FW: ATGTCGCAAGGCAGTAAGCCCA RV: GGAGTCTTTCAGCATGGAGCAA	438	Hirayama et al. (1988)
Program: 95ºC for 5 min, 30 cycles (95ºC, 60 s; 60ºC, 40 s; 72ºC, 90 s) and an extension cycle of 10 min at 72ºC			
qPCR			
Elongation factor (qPCR)	FW: TGGTCAGGAGATTGCGAAAGAGC RV: ACGCAAAACGCTCCAATGGTG	130	Hidalgo et al. (2007b)
STS (qPCR)	FW: AAGGGGAAAAAGCCACCACA RV: TTCGATGGTCAAGCCAGGTC	77	Hidalgo et al. (2007a)
AtMYB12 (qPCR)	FW: GAGGCCACGATCGGGAATAG RV: CAGAAGCCAAGCGACCAAAG	87	This work
amiRNA-CHS (qPCR)	FW: GAGATGACGTGTGTTCCTTCG RV: GAGGAAGAAGGAGAAGAGAAGGTG	73	This work
PAL (qPCR)	FW: TCGAAGATGAATTGAAGGCTGTTTT RV: GGAATAGCAGGGTTTCCACTTTCT	82	This work
CHS (qPCR)	FW: CAGCCGCGGTCATTATAGGT RV: AGAGTTTGGGCTGCAGAGAC	82	This work
Program: 95°C for 2 min, 40 cycles (95°C, 10 s; 60°C, 20 s; 72°C, 20 s) followed by a melting curve			

Table S2. Analysis of the Pearson lineal correlation between the different parameters studied. ccP, Pearson correlation coefficient. TF, transcription factor: PAL, phenylalanine ammonia lyase; CHS, chalcone synthase, TFC, total flavonoids content; Stilb., total stilbenes content.

Related variable	**ccP**	**p value**
TF / PAL	0.970	0.001
TF / CHS	0.967	0.002
TF / TFC	0.882	0.020
TF / Stilb.	0.888	0.018
Inh / CHS	-0.665	0.072
Inh / TFC	-0.626	0.085
CHS / TFC	0.858	0.001
CHS / TPC	0.435	0.209
STS / Stilb	0.816	0.025
TF / TPC	0.423	0.403

Table S3. Metabolites identified by relevant spectra (1D and 2D NMR).

Group of compounds	Metabolite	Chemical shift (ppm)	Multiplicity/coupling constant (Hz)
Amino acids	Proline	4.08/2.31	(dd, *J* = 8.6; 6.0)/(m)
	Alanine	1.49	(d, *J* = 7.2)
	Glutamine	2.12/2.45	(m)/(m)
	Leucine	0.96/0.98	(d,*J* = 6.2)/(d, *J* = 6.4)
	Threonine	1.33	(d, *J* = 6.8)
	Valine	1.00/1.01	(d, *J* = 6.6)/(d, *J* = 7.1)
Sugar	α-Glucose	5.19	(d, *J* = 3.7)
	β-Glucose	4.58	(d, *J*= 7.9)
	Sucrose	5.41/4.17	(d, *J*=3.8)/(d,*J* = 8.6)
Organic acids	γ-Amino-butyrate (GABA)	1.9/2.32/3.01	(m)/(t,*J* = 7.5)/(t,*J* = 7.5)
	Acetic acid	1.94	(s)
	Fumaric acid	6.53	(s)
	Malic acid	2.82	(dd, *J* = 16.9; 8.1)
Phenolic acids	Cinnamic acid	7.63/7.42/7.62/6.49	(m)/(m)/(d, *J* = 16)/(d, *J* = 15.7)
	Caffeic acid	7.12/6.89/7.06	(d, *J*= 2)/(d, *J* = 8.2)/(dd, *J* = 8.4; 2)
	Sinapic acid	6.93/7.46/6.37/3.88	(s)/(d, *J* = 16)/(d, *J* = 16)/s
	p-coumaric acid	7.50/6.89/7.59?/6.49	(d, *J* = 8.49)/(d, *J* =8.2)
Others	Inositol	4.08/3.61/3.44/3.25	(t, *J* = 2.82)/(t, *J* = 9.97)/(dd, *J* = 10; 1.5?)/(t, *J* = 9.03)
	Nicotine	8.69/8.66/8.09/2.76	(d, *J* = 2.0)/(dd, *J* = 4.8; 1.4)/(dt, *J* = 4.7; 1.6)/(s)



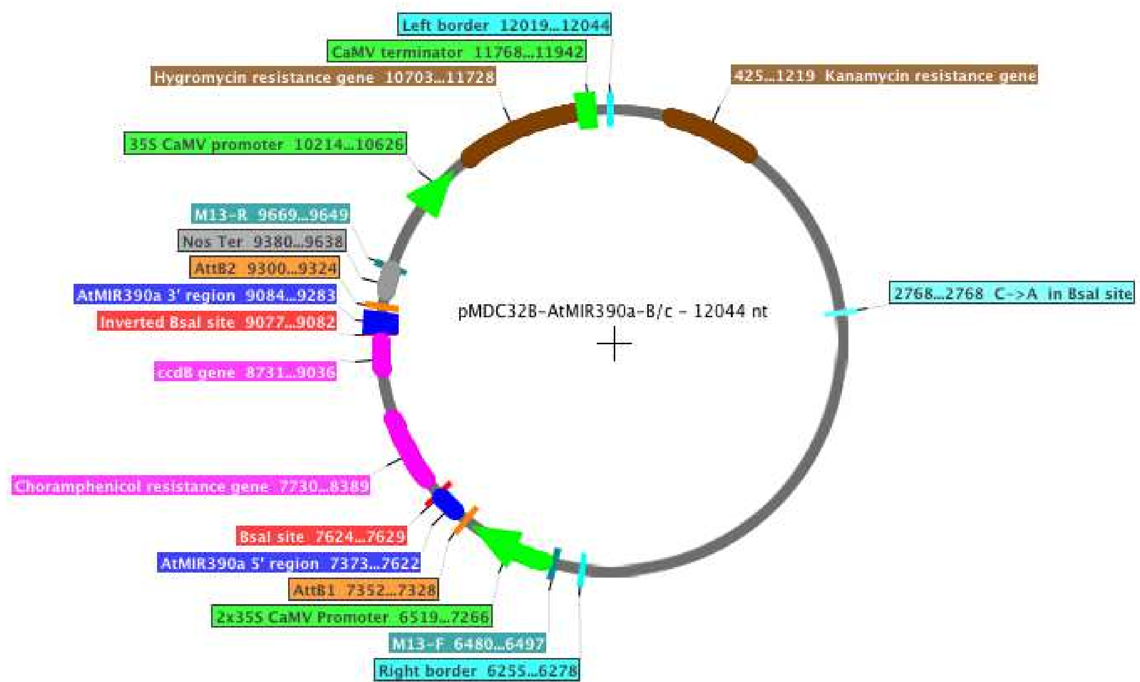


Fig. S1: Plant expression vectors pJCV52 (Karimi,et al., 2002) and pMDC32B-AtMIR390a-B/c (Carbonell et al., 2014)

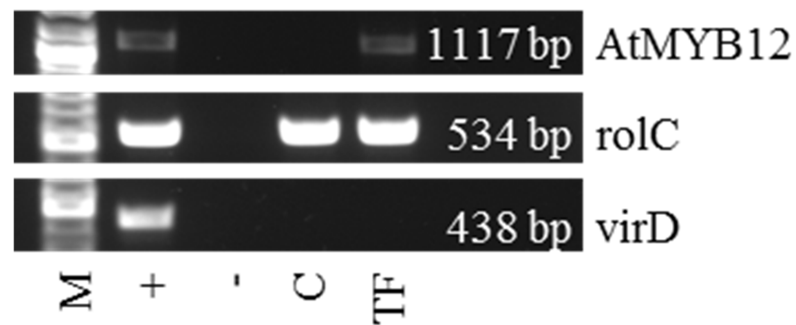


Fig. S2: PCR analysis of the genomic DNA of HR lines. (+) positive control (corresponding *A. rhizogenes* used for the infections), (-) negative control (DNA of *Nicotiana tabacum* wild type plant). C, wild type hairy root (control), TF, hairy root line carrying the AtMYB12 transcript

Supplementary information (1) for the design of amiRNA-CHS.

The amiRNA for *Nicotiana tabaccum* CHS, was designed from the gb|AF311783.1 sequence by the web tool: (http://p-sams.carringtonlab.org/amirna/designer) as described by Carbonell et al. (2014a). The optimal amiRNA CHS sequence result was:

P-SAMS P-SAMS amiRNA Designer P-SAMS syn-tasiRNA Designer FAQ About

Results

Optimal Results

amiRNA Optimal Result 1

amiRNA:	5' TTAATCATTGATTTTTCACAG 3'
amiRNA*:	5' GTGAAAAAGCAATGATTAACA 3'
Forward Oligo:	5' TGTATTAATCATTGATTTTTCACAGATGATGATCACATTCGTTATCTATTTTTTCTGTGAAAAAGCAATGATTAA 3'
Reverse Oligo:	5' AATGTTAATCATTGCTTTTTCACAGAAAAAAATAGATAACGAATGTGATCATCATCTGTGAAAAATCAATGATTAA 3'

TargetFinder Predicted Targets

Target accession:	gi|13925889|gb|AF311783.1| Nicotiana tabacum chalcone synthase mRNA, complete cds
Target description:	unknown
Score:	1
Coordinates:	228-248
Strand:	+
Target sequence:	GUGUGAAAAAUCAAUGAUUAA
Base pairing:	::::::::::::::::::::
amiRNA sequence:	GACACUUUUUAGUUACUAAUU

amiRNA optimal results:
amiRNA: 5' TTAATCATTGATTTTTCACAG 3'
amiRNA*: 5' GTGAAAAAGCAATGATTAACA 3'

Forward Oligo: 5'
TGTATTAATCATTGATTTTTCACAGATGATGATCACATTCGTTATCTATTTTTTCTGTGAAAAAGCAATGATTA
A 3'
Reverse Oligo: 5'
ATGTTAATCATTGCTTTTTCACAGAAAAAAATAGATAACGAATGTGATCATCATCTGTGAAAAATCAATGATTA
A 3'

TargetFinder Predicted Targets
Target accession: gi|13925889|gb|AF311783.1| *Nicotiana tabacum* chalcone synthase mRNA, complete cds
Score: 1
Coordinates: 228-248
Strand: +
Target sequence: GUGUGAAAAAUCAAUGAUUAA
Base pairing: :

amiRNA sequence: GACACUUUUUAGUUACUAAUU

Supplementary information (2). Sequence of *Nicotiana tabacum* chalcone synthase mRNA, complete cds

>gi|13925889|gb|AF311783.1| Nicotiana tabacum chalcone synthase mRNA, complete cds
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TACTCGGGCTCCGTCCATCGGTCAAGCGGTTCATGATGTACCAACAAGGTTGCTTTGCCGGTGGCACGGT
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CTGCAGCCCAAACTCTTCTCCCCGATAGCGAAGGCGCTATCGACGGTCACCTTCGTGAAGTTGGGCTTAC
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ATTGTTTATTTTGTTTCTTGTGGTTGAATTTATTTTGTTTGTAATGAATGTATTTGCTCTATTTTGCTATTTC
ATCTTGCAAATAATGAAATTTGTAATGTGAACTATTTAATCAAAGAACTGAATTTCTTTCTCTT

Supplementary information (3). Sequence of *Vitis vinifera* stilbene synthase clone

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AGCTACCCCCGACCACTGTGTCTACCAGTCTGATTATGCTGATTACTATTTCAGGGTCACTAAGAGCGAGCA
CATGACTGAGTTGAAGAAGAAGTTCAATCGCATATGTGACAAATCAATGATCAAGAAGCGTTACATTCACTT
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AGATTATTACTGCTGAGGTACCTAGACTTGGTAGGGATGCAGCATTGAAGGCTCTTAAAGAGTGGGGCCAA
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TGAGATCACTGTTGTTACATTCCGTGGGCCTTCCGAAGATGCTTTGGACTCTTTAGTTGGCCAAGCCCTTTT
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GTAACATGTCTAGTGCATGTGTGTTGTTTATTTTGGATGAGATGAGAAAGAAATCCCTAAAAGGGGAAAAA
GCCACCACAGGTGAAGGATTAGATTGGGGAGTACTATTTGGTTTTGGACCTGGCTTGACCATCGAAACTGT
TGTGCTGCATAGCGTTCCTACGGTTACAAATTAA

Supplementary information (4). Blast result for aligned sequences of VvSTS and NtCHS

Score	Expect	Identities	Gaps	Strand
493 bits(546)	5e-143	820/1170(70%)	26/1170(2%)	Plus/Plus

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Annexed 2.
Supplementary material for Chapter 4

Anexxed 2

Table S1. Sequences of the primers used to amplify the genes by PCR and qPCR.

Gene	Primer Sequence	Amplicon size bp	Reference
rolC	Sense 5'-TAACATGGCTAGAAGACGACC-3' Reverse 5'-AAACTTGCACTCGCCATGCC-3'	534	1
*Hs*CYP1b1	Sense 5´-CCTATGTCCTGGCCTTCCTT-3´ Reverse 5´-ACTCTGCTGGTCAGGTCCTT-3´	245	2
*Vv*ROMT	Sense 5´- CACCATGGATTTGGCAACG-3´ Reverse 5´-AGGATAAACCTCAATGAGGGA C-3´	1100	2
virD	Sense 5'-ATGTCGCAAGGCAGTAAGCCCA-3' Reverse 5'-GGAGTCTTTCAGCATGGAGCAA-3'	438	3
Elongation factor	Sense 5´- TGGTCAGGAGATTGCGAAAGA GC -3´ Reverse 5´- ACGCAAAACGCTCCAATGGTG -3´	130	This work
*Hs*CYP1b1	Sense 5´- TTTTCAGTGGGCAAAAGGCG -3´ Reverse 5´- GGTGAGCCAGGATGGAGATG -3´	82	This work
*Vv*ROMT	Sense 5´- CGATGATGATTTTCGCCCCG -3´ Reverse 5´- CTCCCATTCGTTCTCGTCCC -3´	50	This work

Table S2. Calibration curve

Sample Name	Analyte Peak Name	Analyte Peak Area (counts)	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Equation of curve
		x		y	
ppb0.39	*t*-Resveratrol	763.713396	0.39	0.256859	$y = 0.0009x - 0.3985$ $R^2 = 1$
ppb0.78		1439.95107	0.78	0.837166	
ppb1.56		2121.44183	1.56	1.42198	
ppb3.125		3803.43042	3.125	2.86536	
ppb6.25		8266.67191	6.25	6.69545	
ppb12.5		15317.5911	12.5	12.7461	
ppb25		29208.628	25	24.6666	
ppb50		58917.2495	50	50.1608	
ppb100		116942.662	100	99.9547	
		x		y	
ppb0.39	Piceid	1584.22714	0.39	0.85061	$y = 0.0003x + 0.3476$ $R^2 = 1$
ppb0.78		2992.22286	0.78	1.2976	
ppb1.56		4941.48679	1.56	1.91642	
ppb3.125		9168.62143	3.125	3.25839	
ppb6.25		19831.2368	6.25	6.6434	
ppb12.5		37930.255	12.5	12.3892	
ppb25		76705.5246	25	24.699	
ppb50		146961.793	50	47.0029	
ppb100		318774.849	100	101.548	
		x		y	
ppb0.39	*t*-Piceatannol	334.648096	0.39	0.756154	$y = 0.0014x + 0.3029$ $R^2 = 1$
ppb0.78		655.427093	0.78	1.19057	
ppb1.56		1204.09439	1.56	1.93361	
ppb3.125		1946.55837	3.125	2.93911	
ppb6.25		3581.88342	6.25	5.15377	
ppb12.5		8616.25	12.5	11.9716	
ppb25		17041.7668	25	23.382	
ppb50		39271.8422	50	53.4874	
ppb100		72724.1228	100	98.7907	
		x		y	
ppb0.39	*t*-Pterostilbene	278.803271	0.39	0.650916	$y = 0.0011x + 0.3497$ $R^2 = 1$
ppb0.78		1040.26607	0.78	1.47371	
ppb1.56		1566.57036	1.56	2.0424	
ppb3.125		3292.82357	3.125	3.90769	
ppb6.25		5130.94016	6.25	5.89385	
ppb12.5		10928.5934	12.5	12.1585	

ppb25		23437.2971	25	25.6746	
ppb50		42146.6475	50	45.8909	
ppb100		93992.4229	100	101.912	

Table S3. Transition and retention time of stilbenes

Stilbene	Q1 Mass (Da)	Q3 Mass (Da)	Retention time (min)
t-Resveratrol	227.100	184.700	3.10
Piceid	389.200	227.100	2.94
t-Piceatannol	243.000	200.700	3.03
t-Pterostilbene	255.100	239.800	3.37

Table S4. Growth Index (GI) of hairy root and cell cultures after a culture period of 28 days for roots and 12 days for cells. The results are the average of 3 biological replicates±SD.

Line	GI of hairy root after 28 days	GI of cells suspension after 12 days
Control 1	5,3 ± 0,21	10,1 ± 0,38
Control 2	5,1 ± 0,20	9,8 ± 0,33
CYP1B1 L8	4,7 ± 0,23	10,8 ± 0,35
CYP1B1 L27	5,2 ± 0,19	9,8 ± 0,32
VvROMT L3	4,8 ± 0,21	10,0 ± 0,30
VvROMT L7	5,0 ± 0,20	9,7 ± 0,37

Fig. S1

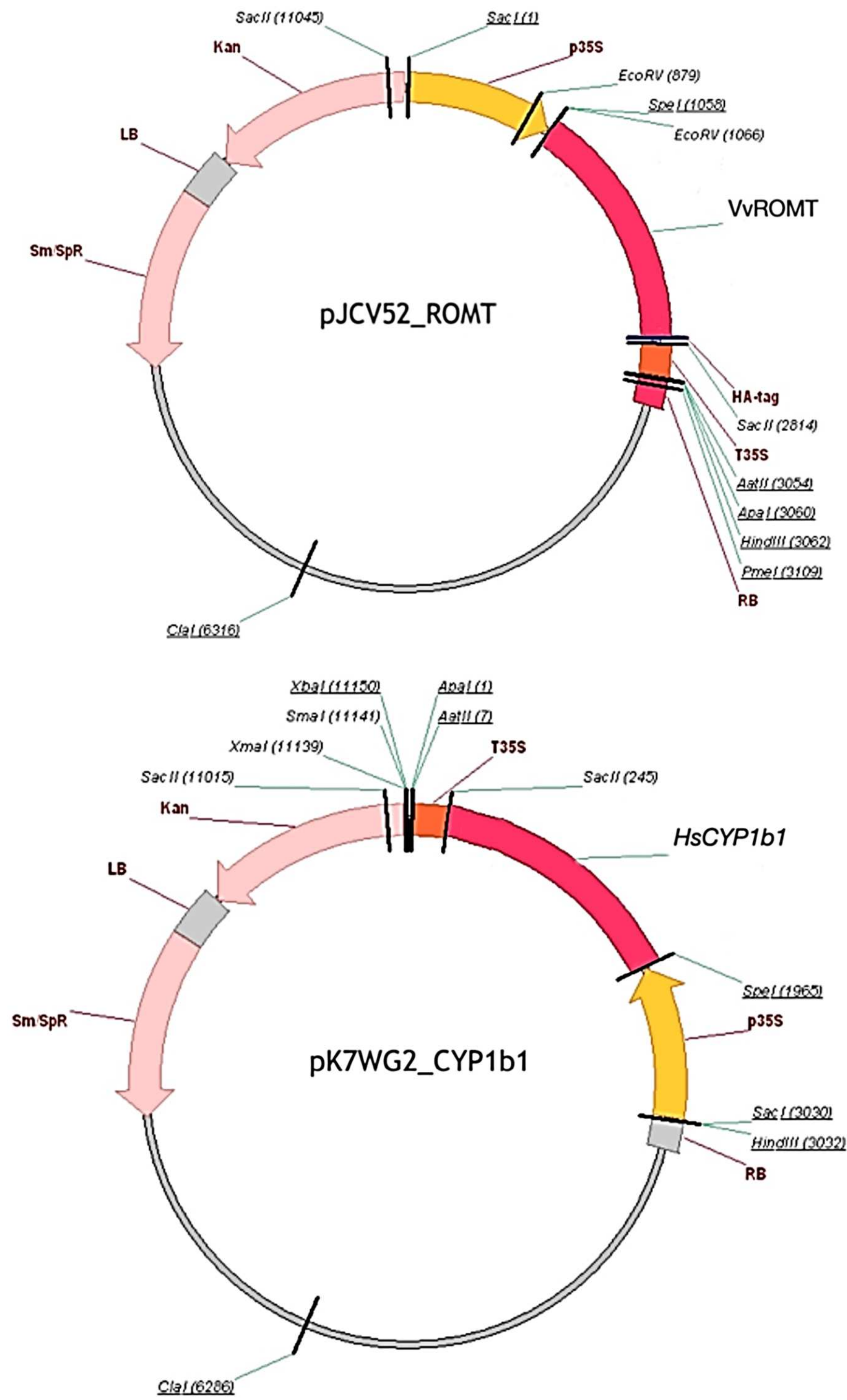
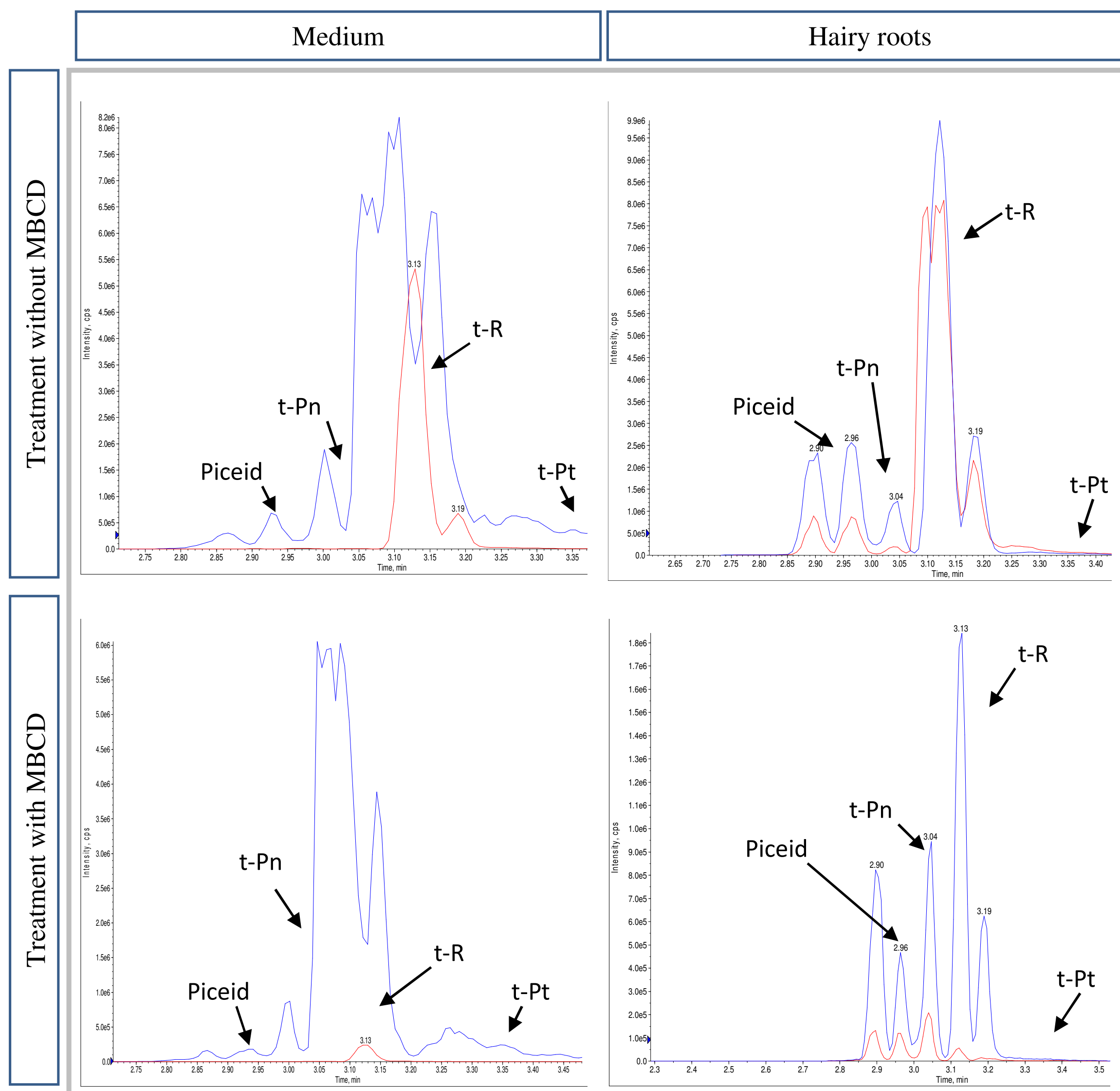


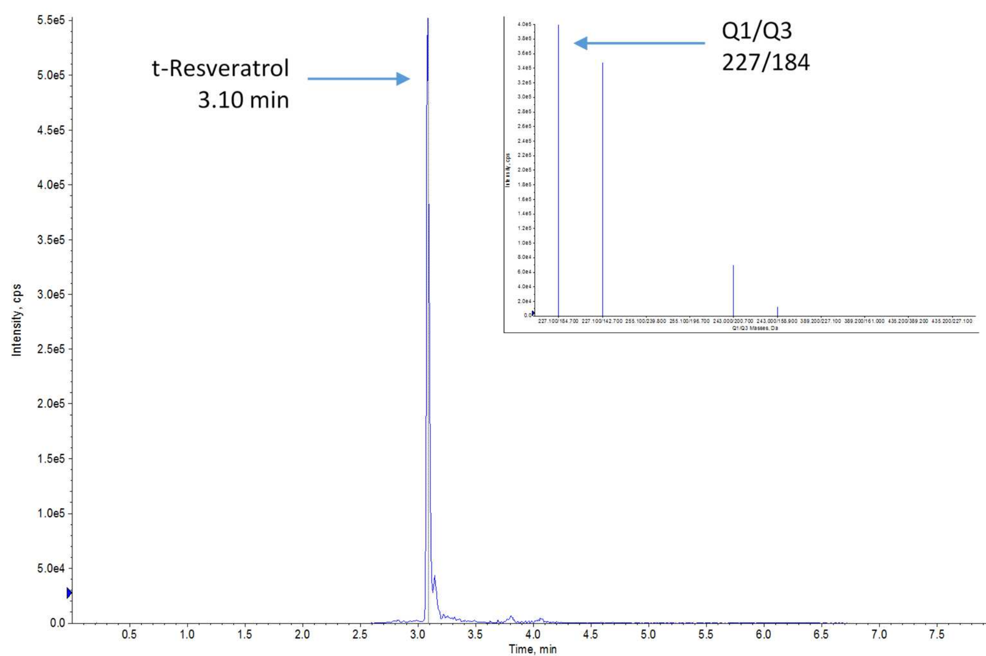
Fig. S1: Plant expression vector pK7WG2_CYP1B1 or pJCV52_ROMT [4].

Fig. S2

A



B



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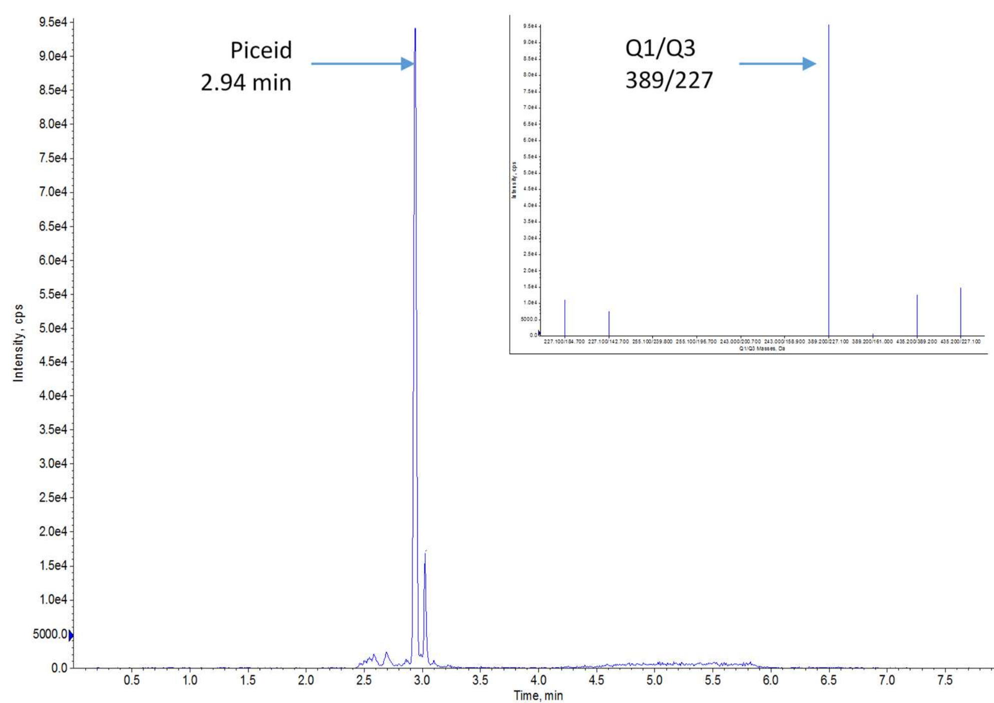

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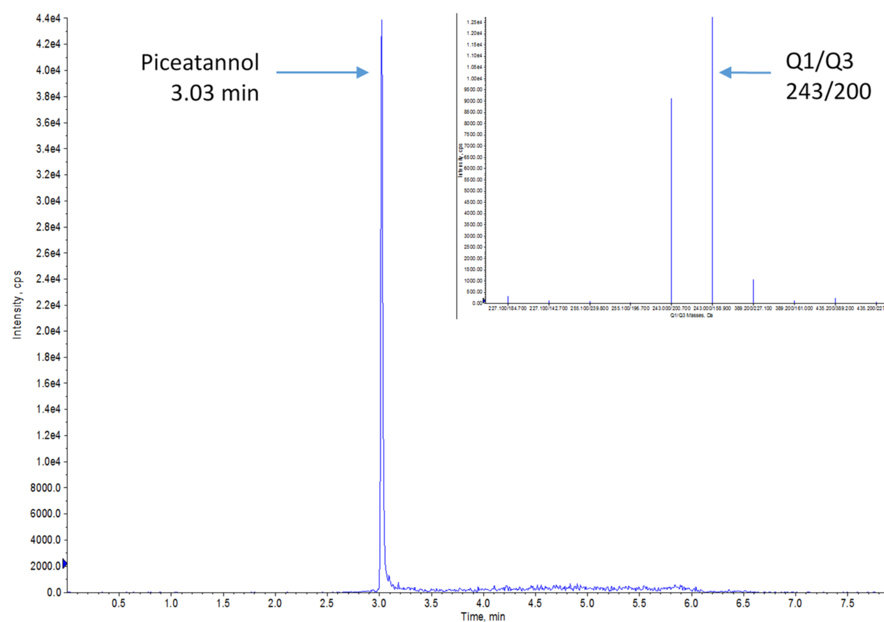


E

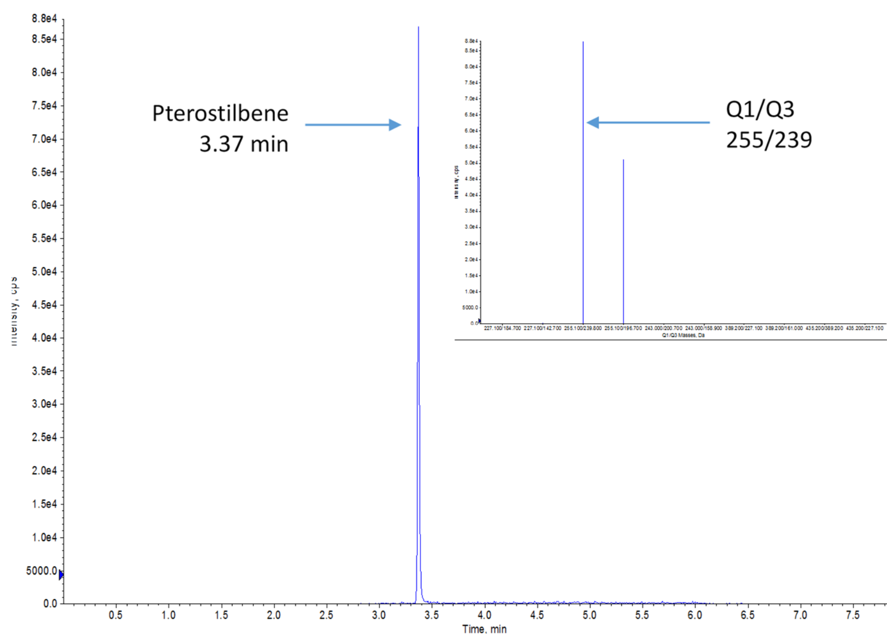


Fig. S2. (A).Total ion current chromatogram (TIC) of MRM from the most representative samples after 24 h of feeding assays, in red the control line and in blue the transgenic line. Extract ion chromatogram (XIC) and fragmentation pattern, (B) t-Resveratrol (Q1/Q3 227/184), (C) Piceid (Q1/Q3 389/227), (D) Piceatannol (Q1/Q3 243/200) and (E) Pterostilbene (Q1/Q3 255/239).

Unprocessed original scans for all of the blots in figure 3.

Fig. 3A

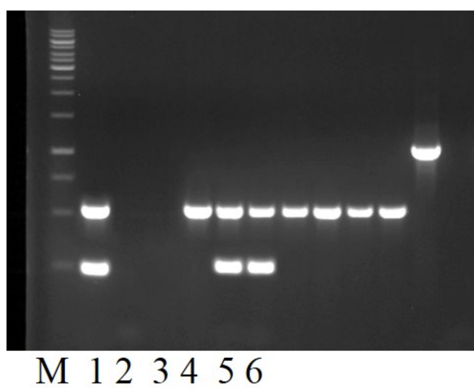


Fig. 3B

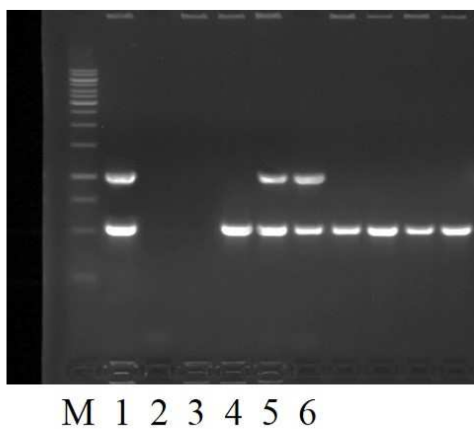
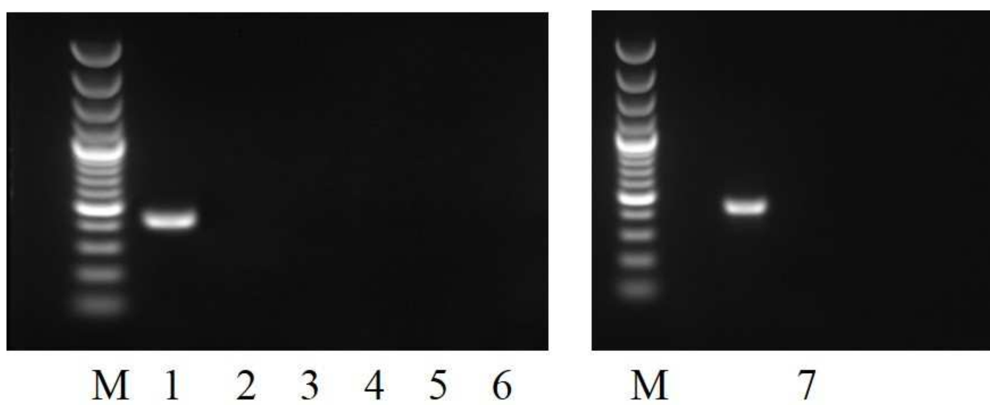


Fig. 3C



References

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4. Karimi M, Inzé D, Depicker A. Gateway vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci.* **7**(5):193-195.