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Optimization of the *Agrobacterium*-Mediated Transformation Process of Grapevine Cell Cultures and Evaluation of Its Effect on Secondary Metabolism

Optimización del Proceso de Transformación Mediada por *Agrobacterium* de Cultivos Celulares de Vid y Evaluación de su Efecto sobre el Metabolismo Secundario

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A mí Familia

A los que están

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Abbreviations

mha: million of hectares

mt: million of quintals

mhl: million of hectoliters

T-DNA: transferred DNA

***trans*-R**: *trans*-resveratrol

PAL: phenylalanine ammonia-lyase

C4H: cinnamate-4hydroxylase

4CL: 4-coumaroyl-CoA ligase

STS: stilbene synthase

sts: stilbene synthase gene

pal: phenylalanine ammonia-lyase gene

c4h: cinnamate-4hydroxylase gene

4cl: 4-coumaroyl-CoA ligase gene

tyr: tyrosinase gene

cpk: calcium-dependent protein kinase gene

NAA: α-naphthaleneacetic acid

BA: 6-benzyl-aminopurine

SCC: suspension-cultured cells

DW: dry weight

JA: jasmonic acid

MJ: methyl jasmonate

SA: salicylic acid

CD: cyclodextrins

MCD: methyl-β-cyclodextrin

DMCD: dimethyl-β-cyclodextrin

OMT: O-methyltransferase

GBD: GTPase binding domain

SCC: suspension-cultured cells

SAAT: sonication-assisted Ag*robacterium*-mediated transformation

GB5: Gamborg B_5

MS: Murashige and Skoog

YM: yeast extract-mannitol

OD: optical density

FW: fresh weight

PI: propidium iodide

FD: fluorescein diacetate

dpi: days after infection

SCV: sedimented cell volum**e**

CTAB: cetyltrimethyl ammonium bromide

DNA: deoxyribonucleic acid

RNA: ribonucleic acid

PCR: polymerase chain reaction

SSC: 0.3 M NaCl, 0.03 M sodium citrate

SAAT: sonication-assisted *Agrobacterium*-mediated transformation

SDS: sodium dodecyl sulfate

UV: ultra violet

Kna: kanamycin

Par: paramomycin

virG: virus induced reporter gene

eyfp: enhanced yellow fluorescent protein gene

***npt*II**: neomycin phosphotransferase gene

CaMV: cauliflower mosaic virus

Resumen

Los metabolitos secundarios en las plantas se producen en pequeñas cantidades cuando son inducidos por estrés. En concreto el resveratrol es un tipo de fenol natural que pertenece a la familia de los estilbenos que actúan como agentes defensivos. El *trans*-resveratrol, una de las formas isoméricas del resveratrol, está ampliamente presente en diferentes tejidos de la vid y en el vino. Este compuesto tiene capacidad antioxidativa, anticancerígena, anti-inflamatoria, capacidad de protección cardiovascular e incluso se ha descrito su utilización en enfermedades relacionadas con el envejecimiento. t*rans*-Resveratrol (*trans*-R) se puede producir mediante extracción directamente de los tejidos vegetales, mediante síntesis química o bien mediante procesos biotecnológicos.

Entre estos últimos la ingeniería genética ofrece la posibilidad de modificar plantas para incrementar la producción de estos compuestos. La biolistica y *Agrobacterium* han sido los dos métodos de transformación habitualmente utilizados para transformar tejidos de vid. Con ambos métodos ha sido posible obtener plantas de vid transformadas aunque en la actualidad la biolistica se está utilizando sobre todo para estudios funcionales mediante expresión transitoria de los transgenes.

La transformación de cultivos celulares no diferenciados puede constituir una interesante alternativa para la producción de compuestos del metabolismo secundario. Esto se podría conseguir mediante la sobre-expresión de genes o reguladores de las rutas biosintéticas. Asimismo, bloqueando genes competidores o silenciando genes implicados en su degradación.

El objetivo de este trabajo consiste en mejorar la bio-producción de *trans*-resveratrol en células cultivadas en suspensión (SCC) de *Vitis vinifera* cv Monastrell utilizando ingeniería genética. Con el fin de desarrollar un protocolo de transformación hemos diseñado una construcción utilizando el plásmido binario pMOG800 portando el gen de resistencia a antibióticos aminoglicosidos *npt*II y un gen marcador *eyfp*/IV2, interrumpido por un intrón para evitar su expresión en la bacteria, que codifica una proteína fluorescente bajo luz ultravioleta. Con esta construcción experimental hemos llevado a cabo diferentes experimentos para optimizar las condiciones de transformación.

En experimentos previos nos pareció que la kanamicina, el antibiótico aminoglicosido más ampliamente utilizado, no producía una selección adecuada. Por

ello realizamos una serie de evaluaciones de la viabilidad de las células cultivadas tras exponerlas a diferentes concentraciones de este antibiótico y pudimos comprobar que los SCC de vid no son sensibles al antibiótico kanamicina y ninguna de las concentraciones probadas produjo ningún efecto pernicioso sobre la viabilidad de las células sin transformar. En contraste el antibiótico paromomicina resultó mucho más efectivo disminuyendo la viabilidad de las células tratadas. Por esta razón este fue el antibiótico que se utilizó para seleccionar las células transformadas en todos los experimentos realizados a continuación. Hemos desarrollado un protocolo eficiente utilizando el método de Transformación Mediada por *Agrobacterium* y Asistida por Sonicación (SAAT de sus siglas en inglés) con cultivos celulares no diferenciados de Monastrell.

Algunos aspectos críticos del protocolo fueron el tiempo de exposición a SAAT que tuvo un óptimo alrededor de 4-5 minutos. Cuando no se aplicaron ultrasonidos a los cultivos el número de células expresando EYFP fue alrededor de 4 veces menor que con el tiempo óptimo de exposición y prácticamente no se obtuvieron microcallos transgénicos.

Por otra parte, la fase de crecimiento en que se encontraban las células en el momento de la infección fue también crítica, con un comportamiento óptimo de los cultivos que fueron infectados durante la fase exponencial, es decir alrededor de unos 7 días tras iniciar un cultivo con una densidad celular inicial de 20 g. L^{-1}. Esta densidad inicial produjo un mayor número de células expresando *eyfp*, así como microcallos fluorescentes, que cuando los cultivos se iniciaron con una densidad de 10 g. L^{-1}. Por otra parte la densidad celular de las muestras de cultivos que fueron infectados con la bacteria fue también crítica y solo se obtuvieron resultados cuando se infectaron muestras con 2 g de células, mientras que en las muestras con solo un gramo de células en muchos casos no se encontraron células flourescentes y en nunca dieron lugar a microcallos transgénicos en ninguno de los experimentos. Se comprobó que esto estaba relacionado con la densidad de siembra repartiendo los 2 g de células infectadas entre 4 o 6 placas Petri y obteniendo muchos mejores resultados cuando se utilizó una elevada densidad de siembra (500 mg/placa). La densidad de siembra tuvo, a su vez, un efecto sobre la cantidad de antibiótico necesaria para seleccionar los microcallos transgénicos siendo necesarios 30 mg L^{-1} de paromomicina para evitar el crecimiento de escapes en

la siembra de alta densidad mientras que tan solo 20 mg. L^{-1} evitaron la producción de escapes cuando las células se cultivaron con una densidad media (333,3 mg/placa). Concentraciones menores del antibiótico permitieron, en ambas densidades de siembra, la obtención de un mayor número de microcallos transgénicos pero acompañados de gran cantidad de escapes que en algunos casos fue imposible contar dado que su elevado número hizo que se fusionaran y formaran un callo continuo. En condiciones de selección óptimas los cultivos con alta densidad de siembra produjeron alrededor de 4 veces más células expresando *eyfp* y microcallos transgénicos que los de densidad de siembra media.

Como conclusión con este protocolo de transformación se consiguieron eficiencias optimas de transformación de más de 50 líneas transformadas por gramo de células cuando los cultivos celulares en suspensión, que se encontraban en la fase de crecimiento exponencial, se infectaron con cultivos de *Agrobacterium*, también creciendo exponencialmente, y después de tratar las células durante 5 min en un baño de ultrasonidos. Se seleccionaron los microcallos transgénicos tras sembrar las células con una densidad de 500 mg peso fresco de células/placa. No obstante, estas eficiencias de transformación se obtuvieron cuando no se aplicó una selección restrictiva (10 mg. L^{-1} de paromomicina) pero en estas condiciones se produjeron también una elevada cantidad de escapes. Con una selección mucho más restrictiva de 30 mg/L de paromomicina, que impidió la aparición de escapes, las eficiencias estuvieron alrededor de los 4 microcallos trasngénicos por gramo de células infectadas con *Agrobacterium*.

De esta forma se pudieron cultivar células expresando *eyfp* que fueron capaces de dividirse (se pudieron fotografiar en esas primeras etapas de divisiones celulares) y dar lugar a microcallos que eran fuertemente fluorescentes bajo luz ultravioleta. Fue posible diferenciar claramente, gracias a la fluorescencia de la proteína EYFP, los microcallos transformados de aquellos escapes que fueron capaces de crecer, generalmente próximos a los transformados, aprovechando probablemente la de-toxificación del medio producida por las células transgénicas.

La evaluación molecular de algunas de las líneas transgénicas producidas demostró la presencia de los dos genes marcadores al producirse amplificación en una PCR con cebadores específicos para cada uno de los transgenes. Además, encontramos que la mayoría de las líneas transgénicas contenían un número discreto de inserciones

del transgén, con 8 líneas de las 14 examinadas mediante Southern con solo una inserción y otras 4 con solo dos inserciones.

Los microcallos transgénicos fueron cultivados hasta que se obtuvo un volumen suficiente de material vegetal que permitiera iniciar un cultivo de células en suspensión. Cuando se dispuso de 500 mg de callo inició el cultivo con 2,5 ml de medio y se fue escalando progresivamente hasta disponer de un cultivo con 100 ml de medio.

Varias de las líneas transgénicas así producidas fueron cultivadas en presencia de diferentes concentraciones de antibiótico, desde 20 mg/L hasta 100 mg. L^{-1} de paromomicina. En todas las concentraciones ensayadas, incluso las más bajas (20 mg. L^{-1}) la línea silvestre fue incapaz de crecer mientras que las líneas transgénicas se comportaron como la silvestre, cuando esta se cultivó en ausencia de antibióticos, en todas las concentraciones de paromomicina ensayadas (20, 40, 60, 80 y 100 mg. L^{-1}).

Una vez optimizado el protocolo de transformación, se diseñó una nueva construcción utilizando el plásmido que habíamos utilizado en los experimentos anteriores pero clonando una unidad de expresión compuesta por el promotor 35S del virus del mosaico de la coliflor, la secuencia del gen de la estilbeno sintasa y el terminador NOS del gen de la nopalina sintasa de *Agrobacterium tumefaciens*. Se encargó la síntesis de esta unidad de expresión que se clonó en un sitio único de restricción EcoRI en el plásmido pMOG800-eyfpIV2-nptII entre ambos genes marcadores. Al tratarse de un sitio de restricción único se obtuvieron ambas orientaciones de la unidad de expresión de *sts*. Los dos plásmidos obtenidos se introdujeron mediante electroporación en la cepa LBA4404 de *Agrobacterium tumefaciens*. Aunque teóricamente las dos orientaciones deberían funcionar igual obtuvimos líneas transformadas con ambas y todas fueron evaluadas.

Con esta nueva construcción se obtuvieron nuevas líneas celulares transgénicas de Monastrell, en las que el objetivo era sobre-expresar el gen de la estilbeno sintasa. Se evaluaron 40 de estas líneas transgénicas, la mitad obtenida con cada una de las cepas de *Agrobacterium* con las dos posibles orientaciones de la unidad de expresión del gen *sts* clonadas dando lugar a los plasmidos pMOG800-stsF y pMOG800-stsR.

Antes de evaluar la producción de *trans*-resveratrol se llevó a cabo un estudio del crecimiento celular de las líneas transgénicas comparadas con las células silvestres. Se comprobó una ligera disminución en el crecimiento celular y un consecuente retraso en

la Ta, o momento en que los nutrientes pasaron a resultar limitantes, de 10 a 14 dias para las células silvestres y las transgénicas, respectivamente. Sin embargo, la viabilidad y la morfología celular resultaron ser idénticas en ambos tipos celulares.

Se determinó su capacidad de producción de *trans*-R tras elicitarlas solamente con ciclodextrinas o bien con la combinación de ciclodextrinas (CD) y jasmonato de metilo (MJ). La producción de *trans*-R fue mayor cuando se utilizó el tratamiento combinado (ciclodextrinas y jasmonato de metilo). Siete de 20 líneas transformadas con la construcción en orientación F (RB-35S-sts-Nos-LB) presentaron un incremento significativo en la producción extracelular de *trans*-R, mientras que tan solo en 3 de 20 líneas transformadas con la construcción en orientación R encontramos un incremento significativo. Además, la producción más elevada se dio en las líneas obtenidas con la orientación F.

Una línea transgénica (F2) fue capaz de producir los niveles más elevados de *trans*-R en comparación con las células no transgénicas así como con otras líneas transgénicas. Esta mayor producción se detectó tanto en el *trans*-R extracelular como en el intracelular. Resulta de interés destacar que esta producción de *trans*-R se mantuvo constante hasta después de 24 meses de sub-cultivos continuos demostrando la estabilidad del transgén.

Además, evaluamos el perfil de expresión endógena del gen *sts* en células no transgénicas así como la expresión endógena del gen *sts* junto a la del transgén en la línea transgénica F2. Este estudio se realizó examinando la evolución de la expresión con el tiempo a las 6, 24, 72 y 144 h desde la aplicación de CD y/o MJ al cultivo. El perfil de expresión siguió una evolución similar tanto en las células silvestres (expresión del gen *sts* endógeno) como en la línea transgénica (expresión simultanea del gen *sts* endógeno y del transgén). Sin embargo, los niveles de expresión del gen *sts* en la línea transgénica fueron mucho más elevados que en las células no transgénicas, cuando ambos tipos celulares habían sido elicitados, lo que probablemente está relacionado con la mayor producción de *trans*-R en esta línea transgénica.

Como conclusión en este trabajo hemos desarrollado el primer protocolo de transformación de células no diferenciadas de *Vitis vinífera* cv Monastrell que de forma consistente permite producir microcallos transgénicos que pueden ser convertidos en SCC transgénicos. Estas líneas SCC son capaces de crecer normalmente en elevadas

concentraciones del antibiótico paromomicina que resultan letales para las células silvestres. Este protocolo ha sido utilizado para la introducción de genes implicados en el metabolismo secundario tal como el gen de la estilbeno sintasa, clave en la producción de resveratrol.

Las líneas SCC transgénicas de *V. vinífera* cv. Monastrell transformadas con el plásmido pMOG800-sts obtenidas utilizando este protocolo han sido elicitadas con CD y/o MJ demostrándose que la utilización combinada de ambos elicitores incrementa significativamente la producción de *trans*-R en algunas de las líneas obtenidas con respecto a las células silvestres elicitadas de la misma forma. Por tanto, las líneas SCC transgénicas podrían constituir una herramienta de interés para una producción sostenible de elevados niveles de *trans*-R.

Summary

This work aims to improve the level of trans-resveratrol bio-production of suspension-cultured cells of Vitis vinifera cv Monastrell using genetic engineering. For this purpose, we have developed an efficient protocol using the Sonication-Assisted Agrobacterium-mediated Transformation method with undifferentiated Monastrell suspension-cultured cells. With this transformation protocol, transformation efficiencies were over 50 independent transformed cell lines per gram of cells when suspension-cultured cells, at the exponential phase of growth, were infected with Agrobacterium, which was also exponentially growing, and after treating the cells for 5 min in an ultrasound bath. We also found that cell plating density was a very important factor affecting transformation efficiency as well as the necessary concentration of antibiotic for the selection of the transformed microcalli. Results showed that high plating density (500 mg fresh weight of cells/dish) was the optimal choice for the transformation system. Paromomycin resulted much more effective than kanamycin for the selection of Monastrell transformed suspension-cultured cells.

Using this transformation protocol, new engineered Monastrell suspension-cultured transgenic cell lines with the stilbene synthase (*sts*) gene inserted were obtained. These transgenic cell lines were used for enhancing the *trans*-resveratrol production under elicitation with cyclodextrins alone or in combination with methyl jasmonate. The production of *trans*-resveratrol was greater when the combined treatment was used (cyclodextrins and methyl jasmonate). One transgenic cell line (F2) was able to produce the highest levels of extracellular *trans*-resveratrol compared with both, the non-transgenic cell line and other transgenic cell lines. Moreover, we tested the expression profile of endogenous and transgenic *sts* genes in transgenic *V. vinifera* cell line F2. The levels of *sts* gene expression in the transgenic cell line was much higher than in the non-transgenic cell line when both were elicited which is probably related to the larger production of *trans*-resveratrol in this transgenic cell line.

1. Introduction

1.1 Distribution of *Vitis vinifera* and worldwide wine production

Grapes (*Vitis vinifera*) are one of the most important fruits in the world, due to the production of wine, consumption as fresh fruits, beverages and raisins. The cultivation of grapes is widely spread around the world. Since 2000 the world's total vineyard surface area is decreasing, mainly due to the reduction of European vineyards (Figure 1.1). In 2015, the global area under vines is distributed along 7.5 million of hectares (mha), about 50% of the world's vineyard area is represented by 5 countries, Spain, China, France, Italy, Turkey (Figure 1.2). Among them, Spain is the country that has the largest extensions of vines worldwide. In 2015, the world production of grapes reached 75.7 million of quintals (mt) in 2015, and 40% of total world grape is produced in Europe. However, 72.6 mt of global grape production is available, and around 45% of this production is used for wine, 37 % as table fruit, 9% as dried fruit and 5% as musts and juices. In 2016, the global wine production was approximately 259 million of hectoliters (mhl) (Figure 1.3.), including sparkling and special wines, and excluding juices and musts.

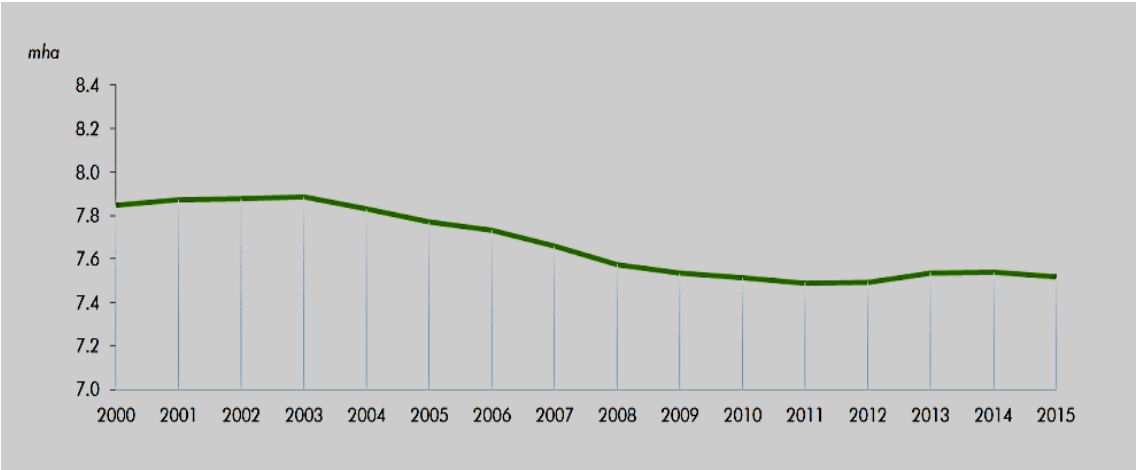


Figure 1.1. Evolution of the cultivable area of vineyards worldwide. (http://www.oiv.int/en/the-international-organisation-of-vine-and-wine).

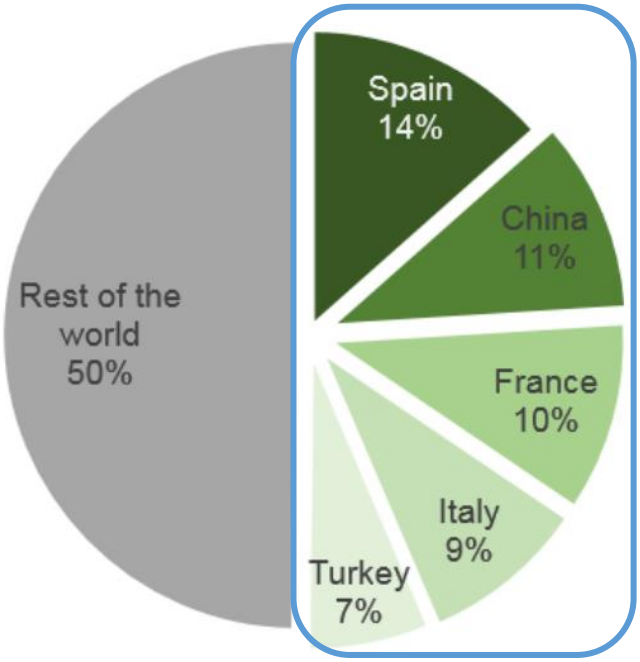


Figure 1.2. Distribution of cultivated grape vineyard surface along worldwide. (http://www.oiv.int/en/the-international-organisation-of-vine-and-wine)

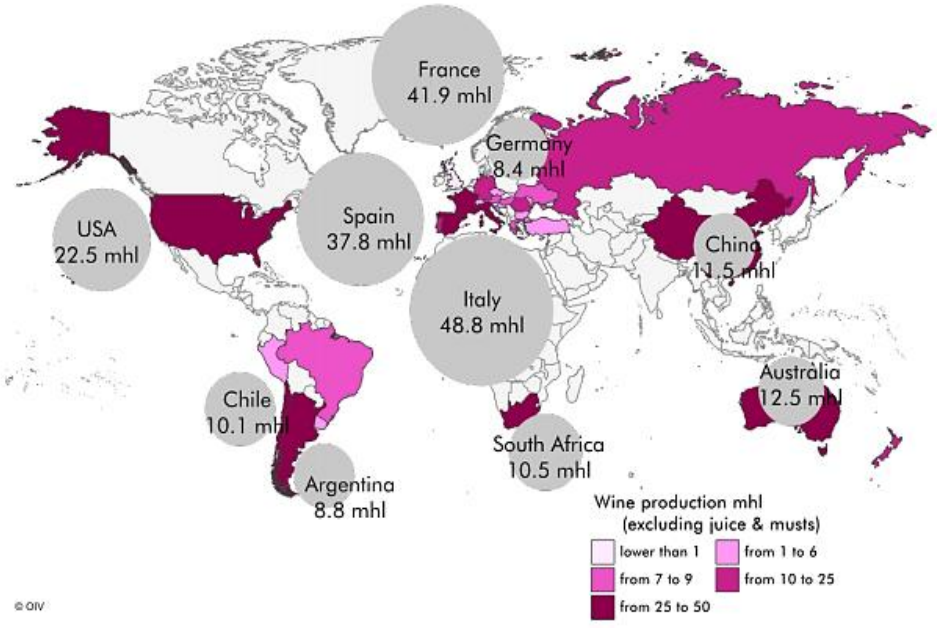


Figure 1.3 Wine production in 2016 expressed as mhl. (http://www.oiv.int/en/the-international-organisation-of-vine-and-wine)

In this thesis, we have used cell suspensions derived from immature grapes of *Vitis Vinifera*. cv Monastrell (Figure 1.4), which is a native red variety from the Spanish Mediterranean coast. Monastrell grapes exhibit thick-skinned berries, which allow them to thrive vigorously in warm and arid climates. Wines produced from this variety tend to be high content in alcohol and tannins, and also have a distinctive balsamic, blackberry taste, and mineral flavors. Wines are deeply colored, full-bodied and well-structured. Their tannin content, tight in texture and long lasting, is ideal for making red wine. This is the major variety in several Spanish DOs (Appellations) such as Almansa, Valencia, Jumilla, Yecla, Alicante, and Bullas (Moreno-Labanda et al., 2004).

Figure 1.4. Fruits of *Vitis Vinifera*. cv Monastrell.

1.2 Plant secondary metabolites

Secondary metabolites are produced in small amounts constitutively by plants or they are induced when plants are stressed with both biotic and abiotic elicitors (Goossens et al., 2003; Murthy et al., 2014). They have an unique and complex chemical structure (Verpoorte et al., 2000), and can be classified into three main groups

of compounds, which include alkaloids, terpenoids and phenolics. Specifically, phenolic compounds are a large class of secondary metabolites with have a low molecular weight which are mainly synthesized from the phenylpropanoid pathway, including simple phenols, benzoquinones, phenolic and phenylacetic acids, acetophenones, coumarins, flavonoids, naphtoquinones, anthraquinones, lignans, lignins and stilbenes (Bravo, 1998). Most of them have been widely used as drugs, cosmetics, agrochemicals, nutraceuticals, flavors, fragrances, coloring agents and food additives, because they have interesting biological activities.

Stilbenes are polyphenols characterized by a 1, 2-diphenylethylene backbone, which occur in a number of plant species, and they play an essential role in plant defense reactions. Resveratrol (3, 4′, 5-trihydroxystilbene) is a type of natural phenol which exists as two isomers: *cis*- and *trans*-resveratrol (*trans*-R) (Fan et al., 2010). This compound can be either free or bound to glucose to form *cis*- or *trans*-piceid. Resveratrol was firstly detected from the roots of *Veratrum album* L. var. grandiflorum (Takaoka, 1940), and later isolated from the roots of *Polygonum cuspidatum* (Vaz-da-Silva et al., 2008), a plant used in traditional Chinese medicine.

1.3 Distribution and chemistry structure of *trans*-resveratrol

trans-R, a natural phytoalexin, is a polyphenol belonging to stilbene family, which act as defense agent. More than 70 plant species are able to produce *trans*-R (Figure 1.5), including bryophyte, pteridophyte and angiosperm plants, such as grapevine, pine, peanut, blueberry, cranberry, red currant, lingonberry, bilberry, strawberry, pistachio and jackfruit (Fernández-Mar et al., 2012; Jeandet et al., 2012; Li et al., 2015; Lim et al., 2015; Lu et al., 2016).

The grapevine and its products, especially wine, are one of the most important food sources for humans. In grapevine, *trans*-R was first detected in 1976 (Langcake and Pryce, 1976) in response to various biotic and abiotic stresses including pathogen infection, mechanical wound, ultraviolet radiation or chemical compounds (King et al., 2006). It is widely distributed in different tissues of grape, such as berry skin, seeds, stem phloem, leaves, axillary buds, roots and flesh (Wang et al., 2010). The grapevine and wine display a high variability in relation to *trans*-R concentration, which is

depending on different factors, genetic background, grape variety, climate conditions, agronomic and geographic factors, and plant stress conditions (Fernández-Mar et al., 2012). Moreover, wine type, winemaking, and fermentation are also able to modify the amount of *trans*-R in wine. Additionally, it was reported that the amount of *trans*-R in both berry skin and seeds in winemaking grapes is higher than in table grapes, and in red grapes than in green ones (Li et al., 2006). Red wine has the highest *trans*-R content compared to white and rose wines (Pervaiz, 2003).

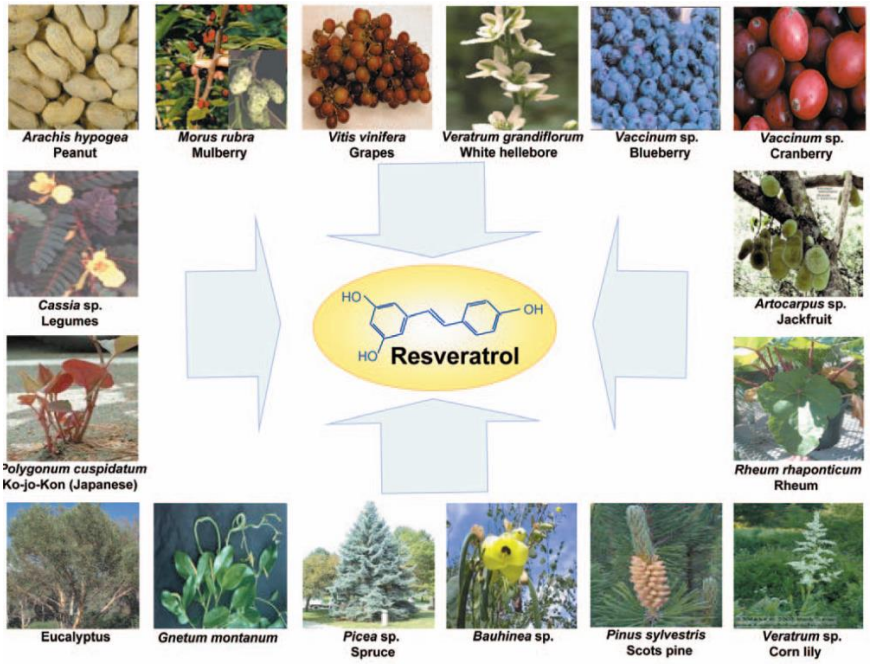
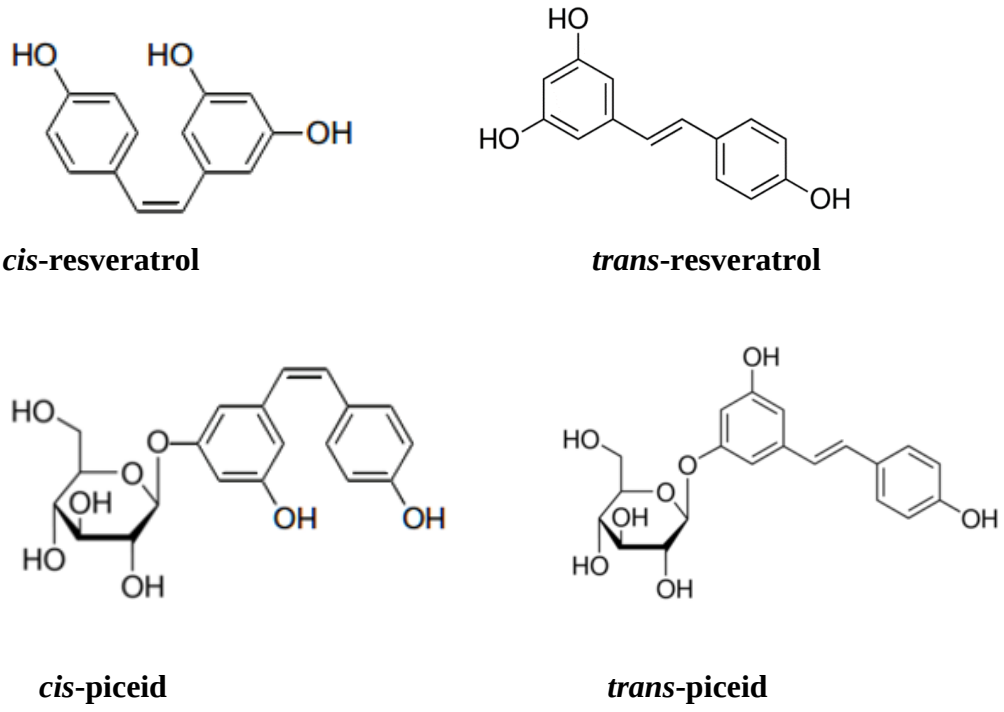


Figure 1.5. Plants which are able to produce naturally *trans*-R.

Resveratrol consists of two aromatic rings bridged by ethylene while the carbon atoms of aromatic ring are further attached to three hydroxyl groups (Murtaza et al., 2013). Due to the ethylene group between the aromatic groups, resveratrol is able to be catalyzed by glucosyltransferase in order to generate the corresponding *trans*- and *cis*-piceid (Almagro et al., 2013). In nature, resveratrol exists in its free and glycoside forms, and both also have two different configurations, namely *trans*- and *cis*-isomer forms (Figure 1.6). The *trans*-R possesses more stability than *cis*-R, which can be isomerized from *trans*-R in the presence of ultraviolet light (Lu et al., 2016).

In addition, *trans*-R is converted to pterostilbene (3,5-dimethoxy-4′- hydroxy-*trans*-stilbene) through a methylation and oxidation-reduction reaction, and can be also hydroxylated to synthesize oxyresveratrol (2,3′,4,5′-tetrahydroxy-*trans*-stilbene). A

class of oligomers with different degree of polymerization, namely viniferins including dehydrodimers (ε-viniferin, δ-viniferin), dehydrotrimers like α-viniferin, a cyclic resveratrol tetramer (β-viniferin) and more highly polymerized oligomers such as γ-viniferins (Langcake and Pryce, 1977a; Langcake and Pryce, 1977b; Pezet et al., 2003; Vitrac et al., 2005) (Figure 1.7).



***cis*-resveratrol** ***trans*-resveratrol**

***cis*-piceid** ***trans*-piceid**

Figure 1.6 Chemical structures of the *cis*- and *trans*-resveratrol and *cis*- and *trans*-piceid.

1.4 Importance of *trans*-resveratrol and its derivatives on human health

The biological activities of *trans*-R and its derivatives have been extensively studied for their promising pharmacological and nutritional values (Fernández-Mar et al., 2012). Pharmacological studies have demonstrated that resveratrol and its derivatives have antioxidant effects, anticancer activity, antidiabetic activity, neuroprotective activity, anti-inflammatory, cardio vascular protection, anti-platelet aggregation effect (De La Lastra and Villegas, 2005; De la Lastra and Villegas, 2007; Fernández-Mar et al., 2012), and more recent data indicated that *trans*-R could be used

in age-related human diseases (Marchal et al., 2013). Moreover, oxyresveratrol, piceatannol and pterostilbene, have a higher antioxidant activity and so, they are more effective than *trans*-R itself (Figure 1.7).

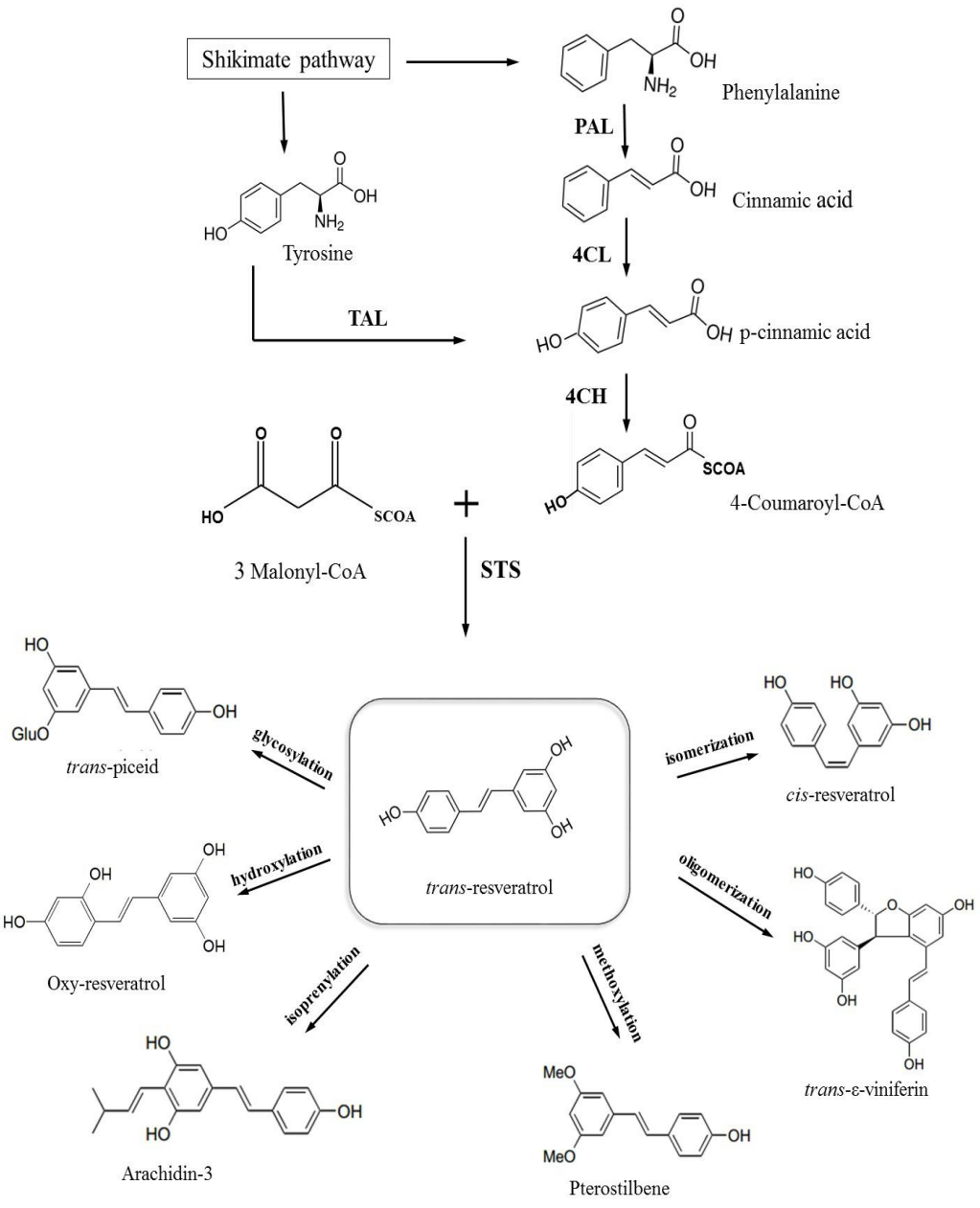


Figure 1.7. Biosynthetic pathway and derivatives of *trans*-resveratrol

One of the most striking biological activities of *trans*-R is its anticancer properties via the three major stages to inhibit carcinogenesis including initiation, promotion and progression (Aziz et al., 2003a).

In fact, *trans*-R promote cancer cell apoptosis, interfere cell cycle progression and inhibit cell proliferation by altering gene expression which can control the mechanism behind small RNA expression, and through regulating a series of transcription factors (Jang et al., 1997; De La Lastra and Villegas, 2005; Mei et al., 2015; Lu et al., 2016).

1.5 Production of *trans*-resveratrol and derivatives

trans-R and its derivatives have a wide range of biological activity, particularly related to the human health. The use of *trans*-R from plants is increasing due to the high demand of these compounds as ingredients for the cosmetic, nutraceutical and pharmaceutical industries, and natural production of *trans*-R and derivatives is insufficient to meet this current market demand. For this reason, it is important to develop efficient methods to obtain them commercially. At present, the methods used for obtaining *trans*-R and derivatives are divided into three types: extraction from plant raw materials, by chemical synthesis and by biotechnological processes.

1.5.1 Extraction from plant raw materials

Although grapevine, peanut and *P. cuspidatum* are the major sources of *trans*-R, they produce low levels of *trans*-R. Several efficient technologies have been used for the *trans*-R extraction from plant raw materials, for instance, using solvent, enzymatic (Xiang et al., 2004), and alkaline extractions (Su et al., 2004) or ultra-sonication-assisted (Cho et al., 2006), microwave-assisted, and supercritical fluid extractions (Pascual-Martí et al., 2001). Compared to conventional solvent extraction using water, methanol or ethanol, these new extraction methods not only greatly increased the yield, but also reduced the cost of solvents and time of extraction. However, in order to obtain sufficient amount of *trans*-R, these technologies need a high amount of plant raw materials for producing a pile of waste material, and therefore, they are not environmentally friendly. Consequently, these technologies have not been widespreadly applied to the industrial production of *trans*-R.

1.5.2 Chemical synthesis

The production of *trans*-R using chemical synthesis includes three steps: the skeleton of tolylene, *cis*-*trans*-isomerization and deprotection reaction. There are three main chemical synthetic methods used to obtain *trans*-R: (1) Wittig and Horner-Wadsworth-Emmons condensations respectively, with appropriated phosphonium ylides, which are often used in the Wittig reaction. (Morenno-Manas and Pleixats, 1985; Jeandet et al., 1991; Orsini et al., 1997) or phosphoryl stabilized carbanions (Maryanoff and Reitz, 1989) and aldehydes; (2) Perking reaction starting from benzaldehydes and various substituted hydroxyphenylacetic acids (Fan et al., 2010), and (3) the Heck reaction which is a palladium-catalyzed olefination of aryl or vinyl halides well suited for hydroxystilbene synthesis due to its remarkable chemoselectivity and amenability to a wide range functional groups (Ferré-Filmon et al., 2004). However, the synthetic sequence is not only rather long including multiple steps, but also use of polluting metal catalysts, consequently leading to low product yields and environmental contamination (Jeandet et al., 2014).

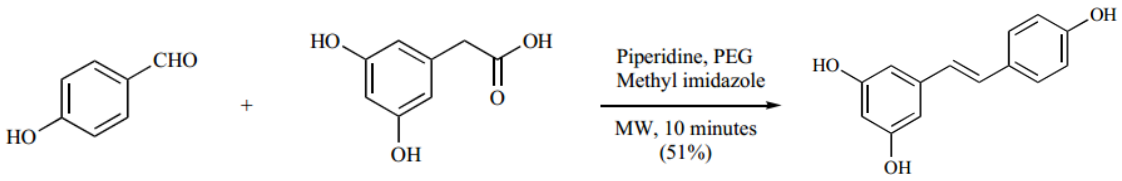


Figure 1.8 Perkin reaction to synthesize *trans*-resveratrol (Fan et al., 2010).

1.5.3 Biotechnological production of *trans*-resveratrol

Low yields of *trans*-R extracted from plants or obtained by chemical synthesis are achieved and so, they do not meet the increasing market demand. In the case of the chemical synthesis, the stereo-specificity, the strict conditions of the reactions and high costs represent the main difficulties for producing this bioactive compound. When *trans*-R is extracted directly from plant raw materials, the most important problems are

associated to the seasonal nature of cultivated plants, slow growth, extract heterogeneity due to their geographical localization and environmental conditions, cultivation and other limitations which result in a short supply of plants raw materials as a source of some valuable bioactive compounds. Additionally, some plants are difficult to propagate and in some cases, the risk of plant extinction as is the case of taxol, an antitumor drug extracted from the bark of *Taxus* trees after they are growing for 60 years, and so, (Oksman-Caldentey and Inze, 2004; Ramirez-Estrada et al., 2016), it is now being produced by plant cell cultures (Malik et al., 2011). For these reasons, the biotechnological production of *trans*-R is undoubtedly regarded as an attractive alternative to its production from plants or chemical synthesis.

Resveratrol and its derivatives are synthesized through the shikimate pathway (Figure 1.7) being the aromatic amino acids phenylalanine and tyrosine its initial precursors in the phenyl propanoid pathway. In this way, phenylalanine or tyrosine are converted into cinnamic acid or *p*-hydroxy-cinnamic acid (*p*-coumaric acid) respectively by the action of phenylalanine ammonia-lyase (PAL) or tyrosine-lyase (TAL), respectively. If cinnamic acid is obtained from phenylalanine, it is hydroxylated by cinnamate-4-hydroxylase (C4H) to produce *p*-coumaric acid. Then, this compound is transformed to *p*-coumaroyl-CoA in a reaction catalyzed by 4-coumaroyl-CoA ligase (4CL). Finally, *p*-coumaroyl-CoA is condensed with 3 molecules of malony-CoA to synthesize *trans*-R using stilbene synthase (STS). Furthermore, *sts* gene has been overexpressed in *V. amurensis.* cells to improve *trans*-R and derivatives production in cell culture systems (Aleynova et al., 2016; Kiselev and Aleynova, 2016), which suggest that genetic engineering in this field is feasible and effective.

1.5.3.1 *trans*-Resveratrol production using plant *in vitro* cultures

Plant *in vitro* cultures only need a basal mineral medium with a carbon source, vitamins and growth regulators which, in general, can be provided at low cost. The growth cycle of organs, tissues and cells *in vitro* is produced within weeks rather than months or years as occurs in plants cultivated in the field or in greenhouse. Such *in vitro* cultures are independent of seasonal, geographical and environmental fluctuations, and they are normally free of bacteria and virus because they are growing under aseptic and

controlled conditions. Additionally, *in vitro* cultures can be also used to conserve endangered plant species for future generations.

Currently, there is high market demand for *trans*-R and its derivatives and the natural content of these bioactive compounds in plants are insufficient to meet the demand (Mei et al., 2015). Another option is to use chemical synthesis, but this approach presents challenges because it requires a complex procedure and requires the use of toxic organic solvents and heavy metals which can result in environmental contamination (Murthy et al., 2014). An alternative to chemical synthesis, plant cell cultures, hairy roots tissues, and metabolic engineering can be combined to biosynthesize *trans*-R and its derivatives on a large scale and with high-yield. Therefore, plant *in vitro* cultures constitute an effective technology for the sustainable production of these secondary metabolites. In addition, plant *in vitro* cultures are not only able to produce high levels of these valuable natural products under sterile conditions, but also they can be used for studying both the biosynthesis pathways and the plant defense mechanisms at molecular level.

Therefore, various plant species have been cultivated *in vitro* and they have been used to obtain *trans*-R and derivatives (Kouakou et al., 2006; Keskin and Kunter, 2010; Jeandet et al., 2014; Yang et al., 2015). In fact, cell cultures and hairy root cultures obtained from grapevine and peanuts have been used to produce *trans*-R and derivatives (Donnez et al., 2009; Hasan et al., 2012; Almagro et al., 2013; Jeandet et al., 2014; Phippe et al., 2016). These *in vitro* cultures are able to produce high levels of *trans*-R and its derivatives, because their biosynthesis is induced by the presence of different abiotic and biotic stresses (Vuong et al., 2014). Grapevine and peanut genotype, culture medium and culture conditions, elicitors and other critical parameters can influence the production levels of *trans*-R and its derivatives in *in vitro* cultures. Consequently, many researchers are focused on studying these factors in order to improve the production of *trans*-R and its derivatives in these plant *in vitro* cultures (Jeandet et al., 2014; Vuong et al., 2014; Lu et al., 2016).

1.6 Strategies to increase the *trans*-resveratrol production

Several strategies have been developed in order to improve the production of *trans*-R and its derivatives, especially those which involve elicitation, optimization of the culture conditions, factors associated to cell growth and metabolic engineering. The development of techniques could enhance the levels of *trans*-R and its derivatives, decreasing the cost and increasing their yield and efficient production compared to their extraction from plant raw materials or their chemical synthesis.

1.6.1 Elicitors

In recent decades, extensive studies have been focused on improving *trans*-R production using different elicitors. Elicitors constitute a traditional and effective technique to increase the amount of secondary metabolites in plant *in vitro* cultures, including *trans*-R and its derivatives. Elicitors are defined as molecules which, introduced in small concentrations to a living cell system, induce or improve the biosynthesis of specific compounds related with defense. The biotic elicitors can be obtained from bacteria, fungal, virus, insects, herbivores as well as some components derived from the hydrolysis of the cell wall. The abiotic elicitors include heavy metals, chemical compounds and physical factors (Lu et al., 2016). Amongst all these elicitors, the most commonly used to enhance the production of *trans*-R and its derivatives are ultraviolet light, components of polysaccharide or oligosaccharide nature, fungal infection, heavy metals, cyclodextrins (CD) and signal molecules such as jasmonic acid (JA), MJ, salicylic acid (SA), chitosan, β-glucan, alone or in combination. Of all of them stand out for their importance JA, MJ, SA and CD. JA is one of the most important signal molecules involved in plant response to damage and pathogen infection. MJ, which is the methyl ester of JA has higher biological activity than JA, and it has been used to induce secondary metabolite production (such as taxol and taxanes, ginsenosides, indole alkaloids, and some phenolic compounds like rosmarinic acid and *trans*-R and its derivatives) in plant cell cultures (Jeandet et al., 2014). In this way, the highest levels of *trans*-R were detected in *V. vinifera* cell cultures treated with 100 μM MJ combined with 50 mM CD, reaching a final concentration of around 3000 mg L^{-1}, which means 4.8-fold higher than those levels found when cells were elicited with CD alone (Belchí-Navarro et al., 2012).

SA is also a signal molecule which plays a crucial role in the field of plant resistance. It is known that this molecule induces the biosynthesis of secondary metabolites, and more specifically is able to increase the accumulation of compounds of flavonoid nature (Almagro et al., 2013). Therefore, it has been employed as elicitor to enhance the production of *trans*-R and derivatives. In fact, Xu et al. (2015) studied the combined treatment of SA and UV-C in *V. vinifera* L. cv Cabernet Sauvignon cell cultures and they observed a high concentration of extracellular *trans*-R (2.33 mg L^{-1}), which was 1.56 fold higher than those cell cultures elicited only in the presence of UV-C. Moreover, these authors also observed that only SA was able to enhance the intracellular *trans*-R production.

CD are cyclic oligosaccharides obtained from the hydrolysis of starch by the action of cyclodextrin-glycosyltransferase from *Bacillus*. CD not only act as strong elicitors of the biosynthesis of *trans*-R, but also are able to form complex with *trans*-R increasing its solubility in aqueous solutions (Lucas-Abellán et al., 2007). Moreover, Bru Martínez et al. (2009) observed that *V. vinifera* cv Monastrell cell cultures elicited with randomly methylated β-cyclodextrins (MCD) were able to accumulate 5000 mg L^{-1} of *trans*-R outside the cells.

In other works, in which chitosan, coronatine, laminarin, β-glucan, amberlite, heavy metals are used, separately or in combination, were less effective to induce the production of *trans*-R and its derivatives that when CD and MJ were used in different *Vitis* sp. cell cultures (Aziz et al., 2003b; Ferri and Tassoni, 2011; Vuong et al., 2014; Almagro et al., 2015; Lu et al., 2016). Therefore, the joint action of CD with MJ was the most effective strategy used for the production of *trans*-R and derivatives.

1.6.2 Optimization of culture conditions

The first essential step to produce high levels of *trans*-R is to establish a cell culture system, which includes the optimization of several important factors for each plant species, such as the selection of both the right culture medium and environmental culture conditions (Smetanska, 2008). In addition, the ability to produce *trans*-R and derivatives in a cell culture is dependent on the genotype. The influence of the genotype on the amount of *trans*-R and derivatives produced in response to elicitation has been recently evidenced (Zamboni et al., 2006; Liu et al., 2010), Zamboni et al. (2006)

observed that high extracellular *trans*-R levels were found in cell cultures elicited with dimethyl-β-cyclodextrin (DMCD) of *V. amurensis* (225.2 mg L^{-1}) and *V. riparia x V. berlandieri* (911.3 mg L^{-1}), whereas the lowest levels of *trans*-R were detected in *V. vinifera* cv Pinot Noir (0.51 mg $L^{-1)}$ and *V. vinifera* cv Merzling (4.31 mg L^{-1}). Similar results also was described by Liu et al. (2010), the callus of Zhi 168' (a hybrid of *V. monticola* × *V. riparia*) and 'Beta' (a hybrid of *Vitis labrusca*×*V. riparia*) produced much more *trans*-R than did two different *V. vinifera* cultivars after UV-C irradiation. In short, it is important to choose genotypes which have ability to produce higher levels of *trans*-R.

Culture conditions also play an important role in the production of *trans*-R and derivatives. The optimization of culture conditions depends on the cultivar used and includes the right selection of carbon and nitrogen source, growth regulators, basal mineral culture medium, pH, temperature, light and oxygen. Basal mineral culture medium is a determinant factor for growing plant *in vitro* cultures and influences the production and accumulation of secondary metabolites (Zhang and Zhong, 1997). The most commonly media used for grapevine cell cultures are Murashige and Skoog (MS) or Gamborg B5 (GB5) media, in some cases supplemented with Morel vitamins (Belchí-Navarro et al., 2012; Phippe et al., 2016). However, other culture media have been also used to growth *A. hypogaea* hairy root cultures (Hasan et al., 2012). Condori et al. (2010) reported that hairy root cultures of peanut cv Hull grown in modified MS media (MSV) produced high levels of *trans*-R and derivatives at all cell ages of the culture when compared to those obtained using GB5 media. In fact, after elicitation at day 9 of hairy root cultures, the levels of *trans*-R, *trans*-arachidin 1 and *trans*-arachidin 3 (420.7 mM, 107.0 mM and 124.1 mM, respectively) accumulated in MSV media was higher than those found in GB5 media (168.7 mM, 54.3 mM and 84.2 mM, respectively). Similarly, Yao et al. (2014) also observed that the selection of the culture medium could influence the extracellular level of *trans*-R in peanut hairy root cultures. Their results showed that basal salt (N_6) and GB5 media accumulated higher levels of *trans*-R (22.42 and 23.47 μg g^{-1}, respectively) than when 1/2 MS and MS media (11.38 and 16.25 μg g^{-1}, respectively) were used.

On the other hand, sucrose is the main carbon source used for plant *in vitro* cultures, and its concentration modifies the production levels of some interesting

compounds (Ferri et al., 2011). Thus, the accumulation of *trans*-R and its glucosides was notably improved using high levels of sucrose (30 g L^{-1} and 40g L^{-1}) compared with their production when low levels of sucrose (10 g L^{-1}) were used in *V. vinifera* cv Barbera cell cultures (Ferri et al., 2011). Moreover, cell cultures of *V. vinifera* cv Monastrell elicited with 100 μM MJ and 50 mM CD, produced the highest levels of *trans*-R (2714.9 mg L^{-1}) when these cell cultures were grown in the presence of 20 g L^{-1} sucrose, comparing in the presence of 10 and 30 g L^{-1} (Belchí-Navarro et al., 2012).

Hormone combination which is added to the culture media consists in an optimal mixture of auxins and cytokinins, which is crucial to promote both cell growth and the biosynthesis of secondary metabolites (Matkowski, 2008). Ho and Kuo (2010) tested the effect of various concentrations combinations of α-naphthalene acetic acid (NAA) and 6-benzyl-aminopurine (BA) on *trans*-R production, and they found that callus of *Vitis thunbergii* Sieb. et Zucc cultured in a culture medium supplemented with 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BA or 1 mg L^{-1} NAA and 1mg L^{-1} BA contained the highest levels of *trans*-piceid and *trans*-R, that is, 3616.36 and 209.29 mg kg^{-1} DW respectively.

Finally, light is the most important physical factor for the biosynthesis of some secondary metabolites when plant cell cultures are used (Yue et al., 2016). Most of grapevine cell cultures which produce *trans*-R are maintained in the dark to prevent anthocyanin biosynthesis (Santamaria et al., 2011; Belchí-Navarro et al., 2012; Yue et al., 2016). Other authors have described that the importance of the treatment with red light to enhance the amount of *trans*-R and its glycosylated forms in *V. vinifera* cell cultures (Tassoni et al., 2012). Bru Martínez et al. (2009) found that *V. vinifera* cv Monastrell cell cultures growing in darkness, and elicited with MCD produced 5027 mg L^{-1}, much more *trans*-R than the same cell cultures growing under a photoperiod of 16/8 h light/dark (101 mg L^{-1}). In addition, UV light has been considered a physical factor that induces stilbene biosynthesis in grapevines (Douillet-Breuil et al., 1999; González-Barrio et al., 2006). Thus, Tyunin and Kiselev (2016) observed that UV-C irradiation enhanced the levels of *sts* expression in *V. amurensis* cell cultures, and resulted in a significantly increase the accumulation of *trans*-R (0.0412% DW), which was 7.5 fold higher than in non-treated cells (0.0055% DW). On the contrary, *V. vinifera* cv Monastrell cell cultures treated with MCD and MJ, followed by short or long exposures to UV-C light, showed lower *trans*-R levels (5 min, 1.14 and 30 min, 0.20 mg *trans*-

resveratrol g^{-1} FW) than UV-unexposed cells (6.72 mg *trans*-resveratrol g^{-1} FW), so that in this case, UV-C light exposure was clearly detrimental to *trans*-R production since prolonged exposure to UV-C light (between 15 and 30 min) caused a drastic reduction in *trans*-R accumulation although no cell browning was observed (Almagro et al., 2011). However, when *V. vinifera* cv Monastrell cell cultures were jointly elicited with MCD and MJ and exposed to UV-A light, the maximal level of *trans*-R was found at long exposures (30 min, 8.26 ± 0.48 mg g^{-1} FW) although no significant differences were found with unexposed cells treated with the same chemicals elicitors (6.72 ± 1.03 mg g^{-1} FW) (Almagro et al., 2011). By the contrary, at short UV-A light exposures, a drop in the production of *trans*-R was observed, and this decrease was more drastic when cells were elicited with MCD and MJ jointly (15 min, 3.18 ± 0.62 mg g^{-1} FW) than in CD-treated cells (2.20 ± 0.15 mg g^{-1} FW) in comparison with unexposed-cells. In addition, when grapevine cell cultures were elicited only with MCD and exposed to UV-A during 30 min, a slight increase in the production of *trans*-R (4.50 ± 0.30 mg g^{-1} FW) was detected in comparison with unexposed CD-treated cells (3.15 ± 0.35 mg g^{-1} FW).

All these results suggested that long UV-A light exposures (30 min) only increased slightly the levels of *trans*-R when cell cultures were elicited only with CDs, and did not enhance *trans*-R levels when MJ was also present, so it seems that there is an antagonistic effect between MJ and UV-A light since short UV-A light irradiation decreased drastically the production of *trans*-R when cells were elicited in the presence of MJ (Almagro et al., 2011). Therefore, in some cases, UV light could also be used as elicitor, since it has been extensively reported its influence on the production of *trans*-R (Keskin and Kunter, 2008; Keskin et al., 2009; Keskin and Kunter, 2010; Xu et al., 2015; Zhan and Huang, 2015).

All these culture conditions alter both the growth of cell biomass and the expression of genes involved in the biosynthesis of *trans*-R and therefore, they should be optimized to achieve a high production of *trans*-R and its derivatives (Yue et al., 2016).

1.6.3 Effect of different factors associated to the cell growth on the production of *trans*-resveratrol

The production levels of *trans*-R using high yielding cell lines obtained from high-producing plants may vary. Variability which normally leads to a reduction in metabolite yield is often associated with subcultures, and it has been attributed to genetic changes by mutations produced in cells growing in the culture, or epigenetic changes, which are due to physiological conditions in which cells are grown. In addition, cell cultures are composed of low- and high-yielding and non-producing cells (Yue et al., 2016).

On the other hand, the average aggregate size of a culture often varies over the culture period, and can affect the production of secondary metabolites. Differences in cell growth kinetic of the cell cultures could lead to variability in *trans*-R production along different subcultures. Therefore, it is important to analyze these parameters before screening for high producing cell lines, and to establish a cell culture with a fast-growing and high-yield.

It is also important to consider the three different stages of growth in a *in vitro* culture system: the lag phase, the exponential phase and the stationary phase. Hairy root cultures of peanut cv Hull3 accumulated highest levels of *trans*-R (551.9 mM g^{-1} DW), *trans*-arachidin-1 (107nM g^{-1} DW) and *trans*-arachidin-3 (124 nM g^{-1} DW) in a modified Murashige and Skoog medium after 9 days of cultivation, which was found in the exponential phase (Condori et al., 2010). Krisa et al. (1999) also studied that piceid production was highest at first half of the exponential growth phase when *V. vinifera* was elicited with MJ. However, there is evidence that cell cultures at stationary growth phase produced higher levels of secondary metabolites than at the exponential phase (Ramirez-Estrada et al., 2016). For these reasons, in some cases cells used to produce *trans*-R are cultured in two-stages: firstly, they are cultured in an optimized medium for growing cell biomass, and when the cells are in the stationary phase, they are transferred to elicited medium (Ramirez-Estrada et al., 2016; Yue et al., 2016) to induce the production of *trans*-R.

Some research has shown that cell biomass accumulation is reduced after long time of cultivation and continuous subcultures in grapevine cell cultures, and in these

conditions, *trans*-R production was unstable or decreased (Dubrovina and Kiselev, 2012). In addition, long time of subcultures can lead to loss of cell characteristics due to genetic variation, such as changes in chromosome content, cell polyploidy level, DNA methylation or changes in cell morphology and in the biosynthetic capacity of a cell line. All these changes could reduce the level of protein biosynthesis, induce gene silencing and inhibit cell growth (Kiselev et al., 2013; Harding et al., 2015). A new strategy using the DNA demethylation agent, 5-azacytidine (200 mM) increased the accumulation of extracellular *trans*-R in *V. amurensis* cell cultures (Kiselev et al., 2011; Tyunin et al., 2012; Kiselev et al., 2013). To conserve the genetic characteristics of cell cultures, cryopreservation could be a method that allow maintaining the initial ability of these cells (Mustafa et al., 2011).

In conclusion, cell culture characteristics are a key factor during the process of production of *trans*-R, and it should be maintained in an optimal state to guarantee consistent biosynthesis of *trans*-R.

1.7. Genetic engineering

1.7.1 Grapevine transformation technologies

Grapevine is one of the most important fruit crop at a global scale, due to the production of wine, consumption as fresh fruits, beverages and raisins. A lot of strategies which include the development of transformation methods and the selection and regeneration systems for transgenic grapevines have been described in order to enhance the yield of existing cultivars as well as to develop new cultivars resistant or tolerant to biotic and abiotic stresses, and to increase the economic value traits like color, phenolic and flavonoid composition, and fruit quality (Reustle and Buchholz, 2009). Indeed, the increasing available knowledge in grapevine genetics, and the development of genetic transformation techniques can be used to study the functions of genes either through up-regulation of gene expression or gene silencing (Pereira, 2000). Genetic transformation technology also offers the possibility of adding or modifing single traits in grapevine cultivars without changing other genotype specific characteristics. Several techniques have been used to improve grapevine efficient transformation. Amongst them, the most successful have been *Agrobacterium*-mediated transformation (Franks et

al., 1998; Yamamoto et al., 2000; Iocco et al., 2001; Wang et al., 2005; Gago et al., 2011) and biolistic transformation (Torregrosa et al., 2002; Vidal et al., 2003; De la Torre et al., 2012; Sanjurjo et al., 2013).

1.7.1.1 Biolistic transformation in grapevine

Although *Agrobacterium*-mediated transformation is a widely used technique in plant genetic engineering, the use of other alternative methods such as biolistic has increased in recent decades. Biolistic or particle bombardment is a physical method commonly used to transfer genes into plant cells or tissues that employs DNA constructs that covering the surface of gold particles which are driven at high speed in target cells or tissues using a biolistic devices (Figures 1.9 and 1.10) (Taylor and Fauquet, 2002). Once transgene DNA is incorporated inside the cells, the DNA is eluted from the particles. If the foreign DNA reaches the nucleus, then transient or stable expression of the transgene can be produced. This physical method allows eliminating some of the limitations of *Agrobacterium*-mediated transformation since the specific mechanism of cell penetration is not required and the parameters are controllable. In fact, *Agrobacterium* technique requires the joint action of bacterial virulence factors and protein effectors of the plant cell or tissues, being a very slow and complex process. Although the proportion of transformants in both techniques is very low, the particle bombardment allows transforming the plant material with multicopies. In addition, plant species have different degrees of susceptibility to be transformed with *Agrobacterium*, therefore, different bacterial strains should be used depending on the species. With biolistic the use of antibiotics to eliminate *Agrobacterium* is not necessary, and thus, possible browning of cells and tissue death is avoided (Kikkert et al., 2004). However, the particle bombardment also has limitations, since the transferred DNA can be damaged and several copies inserted into plant cells generating unexpected effects.

In grapevine, several tissues have been found suitable for biolistic transformation: e.g. undifferentiated callus and cell suspensions (Torregrosa et al., 2002), and embryogenic cell suspensions (Kikkert et al., 1996). If the final goal is the regeneration of transgenic plants, embryogenic cell suspensions would be the most convenient tissue for biolistic experiments. However, biolistic methods can lead to multicopy number

compared with *Agrobacterium*-mediated transformation. Nowadays, biolistic is the method of choice for studying transient expression for functional analysis in grapevine.

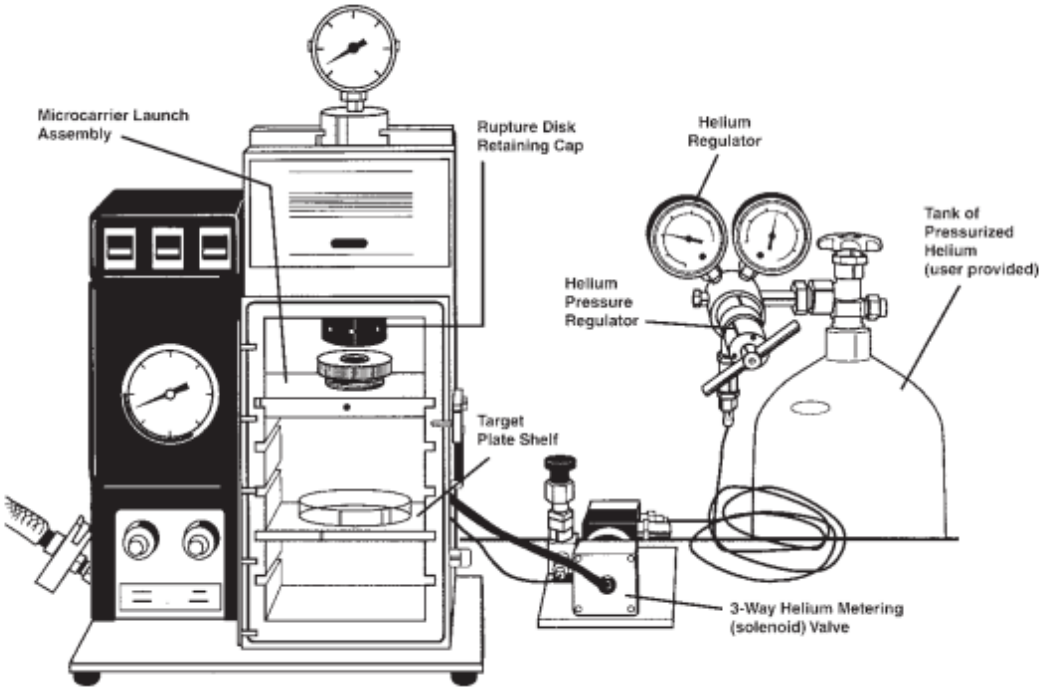


Figure 1.9 Components of the Biolistic® PDS-1000/He particle delivery system. (Drawing courtesy of Bio-Rad Laboratories, Hercules, CA.) (Kikkert et al., 2004)

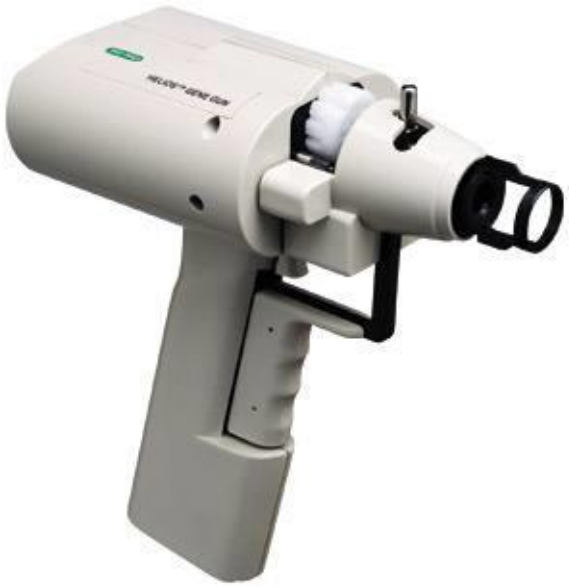


Figure 1.10 Helios® Gene Gun Systems (http://www.bio-rad.com)

The first application for functional studies was proposed by Torregrosa et al. (2002) and Verries et al. (2004). They described the transformation of cell suspensions of *V. vinifera* cv Cabernet Sauvignon for the analysis of the promoter region of alcohol dehydrogenase genes. Since then, cell suspensions of cultivar Chardonnay have largely been used for functional studies of genes involved in flavonoid biosynthesis (Jelly et al., 2014). More recently, leaf sections of cultivar Chardonnay and somatic embryos of cultivar Thompson seedless were subjected to particle bombardment for studying the regulation of the polygalacturonase-inhibiting protein defense gene (Joubert et al., 2013).

1.7.1.2 *Agrobacterium*-mediated transformation in grapevine

Agrobacterium tumefaciens has the natural ability to transfer a portion of its DNA, t-DNA (transferred-DNA) to the genome of a host plant (Sheng and Citovsky, 1996). Gene transfer from *Agrobacterium* to plant cells involves five essential steps: (a) induction of the bacterial virulence system, (b) generation of t-DNA complex, (c) transfer of t-DNA complex, (d) transport and integration of t-DNA complex into the plant genome, and (e) expression of t-DNA genes (Figure 1.11). This *Agrobacterium*-mediated transformation which is called *natural genetic engineering* (Hooykaas and Schilperoort, 1985; De La Riva et al., 1998), has been widely used to generate transgenic fruit plants in a high variety of fruit plant species, such as apple (Gilissen et al., 2005; Hutabarat et al., 2016; Liao et al., 2017), pear (Matsuda et al., 2009), banana (Kovács et al., 2013; Sreedharan et al., 2015), papaya (Suzuki et al., 2008), strawberry (Pantazis et al., 2013; Mercado et al., 2015), kiwifruit (Bulley et al., 2012), citrus (Dutt et al., 2015; Hao et al., 2016), mulberry (Chitra et al., 2014; Zhou et al., 2016), avocado (Raharjo et al., 2008; Palomo-Ríos et al., 2016), plum (Srivastava et al., 2011), apricot (Petri et al., 2008) and also, grapevine (Guan et al., 2013; Zhou et al., 2014; Dai et al., 2015; Cheng et al., 2016; Saporta et al., 2016).

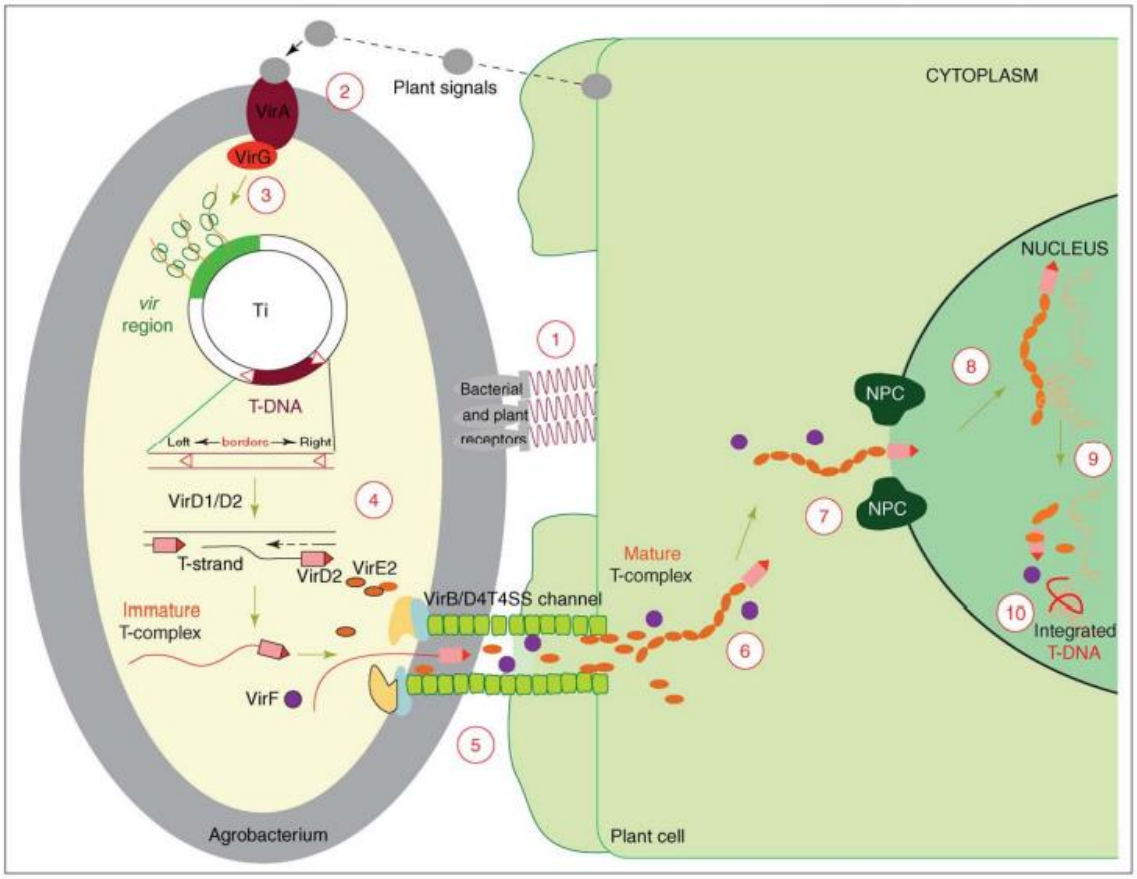


Figure 1.11. A model for the *Agrobacterium*-mediated genetic transformation (Tzfira and Citovsky, 2006)

Agrobacterium-mediated transformation of grapevine has been developed to introduce the genes of interest in different types of tissues including both embriogenic calli and embriogenic cell suspensions (Reustle and Buchholz, 2009), meristematic cell clusters (Xie et al., 2016). These target tissues used for transformation can be further developed into transgenic plants, if there are regeneration protocols available or can be used for the stable expression of transgenes without regeneration. Two *in vitro* culture techniques have been commonly used for regeneration of transgenic plants: organogenesis and embryogenesis (Rai and Shekhawat, 2014) although the most successful regeneration system used in grapevine has been via somatic embryogenesis using, as starting explants, leaves, ovaries, anthers, seeds, petioles, nodal sections and protoplasts (Saporta et al., 2016). Nevertheless, the most frequently and successfully target tissue used for grapevine transformation has been embryogenic tissues, specifically somatic embryos and embryogenic calli and cell suspensions. The

efficiency and reliability vary considerably from one genotype to another, and it is normally restricted to a few genotypes due to the complexity of grapevine transformation procedures. In fact, the process demands the induction and continuous maintenance of embryogenic cultures, which require a lot of manpower and work. Successful grapevine transformations have been achieved in some *Vitis* species, such as *V. rupestris, V. riparia* or *V. rotundifolia* and interspecific hybrids (Motioike et al., 2002; Saporta et al., 2016). Recent reports have mainly focused on obtaining transgenic grapevines which increase disease resistance, abiotic stress tolerance, and grape quality and yield by using *Agrobacterium*-mediated transformation, technology which in turn, increase the transformation efficiency and improve the different steps of the transformation procedure (Saporta et al., 2016).

1.7.1.2.1 *Transgenic grapevine plants obtained via* Agrobacterium*-mediated transformation*

Some studies described successful procedures for obtaining transformed grapevine plants. In fact, Zhou et al. (2014) got *V. vinifera* cv Thompson Seedless transgenic lines overexpressing an ubiquitin ligase gene obtained from a Chinese wild grape which exhibited an increased resistance to powdery mildew compared with non-transgenic plants. Subsequently, Dai et al. (2015) obtained *V. vinifera* cv Chardonnay transgenic lines with a stilbene synthase gene using *Agrobacterium*-mediated transformation, which exhibited high levels of stilbenes and had an enhanced powdery mildew resistance. In addition, Cheng et al. (2016) and Dai et al. (2016) obtained *V. vinifera* cv Thompson Seedless transgenic plants overexpressing stilbene synthase and *V. vinifera* cv Red Globe transgenic plants which overexpressed a pathogenesis-related protein, with an increased resistance to powdery mildew. Similarly, somatic embryogenic calli induced from anther of *V. vinifera* cv Thompson Seedless was transformed with a thaumatin-like protein gene via *Agrobacterium*-mediated transformation (He et al., 2016), and the resulting transgenic plants had an improved downy mildew resistance. Xie et al. (2016) improved the regeneration system via organogenesis using as target tissue for transformation meristematic cell clusters (Mezzetti et al., 2002) in different grapevine cultivars, such as cv Chardonnay, Thompson Seedless, Red Globe, Cabernet Sauvignon, amongst others, which have been

transformed by *Agrobacterium* which improves greatly the processing efficiency, obtaining the highest transformation rate in the cultivar Chardonnay (more than 51.7%). In addition, this protocol is simple and reduced greatly the period from transformation to plant regeneration compared with the transformation when using embryogenic cultures as target tissue. Guan et al. (2013) used shoot tips and/or single-bud-internodes containing shoot apical meristems obtained from a Chinese wild grape, as initial transformation material for producing plants overexpressing glyoxal oxidase. Saporta et al. (2016) reviewed some recent works on grapevine breeding using *Agrobacterium*-mediated transformation. Their main objective was to improve fungi and virus resistance, cold tolerance and some attempts related to modification of berry color were also made through grapevine genetic transformation. They concluded that most transgenic grapevine plants were obtained after transformation of embryogenic cell masses with *Agrobacterium*.

1.7.1.2.2 Transgenic non-embriogenic undifferentiated suspension cells obtained via *Agrobacterium*-mediated transformation

Plant suspension cells are an useful model system due to their properties of rapid cell proliferation and good growth characteristics (Mustafa et al., 2011). Also their relatively simple transformation, has allowed their use for transferring genes in various plants species. Grapevine suspension cells have been used to produce bioactive secondary metabolites, such as stilbenes (Donnez et al., 2009; Jeandet et al., 2014; Phippe et al., 2016), anthocyanins (Ananga et al., 2013), and viniferins (Giovannelli et al., 2014). *Agrobacterium*-mediated transformation could be an easy way to incorporate key genes of these metabolic pathways into grapevine cells, thereby improving the production of these interesting metabolites. Up to now, transgenic grapevine suspension cells obtained via *Agrobacterium*-mediated transformation has been mainly focused on increasing the stilbene production, mainly resveratrol although other attempts have been carried out to enhance resveratrol derivatives.

It is possible to enhance the production of *trans*-R and its derivatives through genetic transformation of cell suspension cultures, by overexpressing genes and regulators of their biosynthetic pathway, by blocking competing genes or by silencing genes involved in their degradation. Moreover, the production of these bioactive

compounds can also be increased using transgenic plant *in vitro* cultures or microorganisms which have incorporated homologous or heterologous genes from the biosynthetic pathway of stilbenes.

1.7.2 Metabolic engineering of *trans*-resveratrol and derivatives

1.7.2.1 Ectopic production of stilbenes using genetic engineering in plant *in vitro* cultures

Metabolic engineering has been used in plant cell cultures and hairy root cultures for increasing the production of stilbenes and thus, meet the demand of the industry (Table 1). Thus, Aleynova et al. (2016) studied the effect of overexpressing of *sts* genes, specifically *vasts1*, *vasts2*, and *vasts7* genes on *trans*-R biosynthesis in *V. amurensis* cell cultures under the control of the double cauliflower mosaic virus (CaMV) 35S promoter. These authors observed an increase in the amount of *trans*-R production (0.36, 0.48 and 0.60 mg. L^{-1}, respectively) which was correlated to an up-regulated expression of transgenic *sts* gene (Table 1.1). Similarly, Kiselev and Aleynova (2016) increased the production of *trans*-R in cell cultures of *V. amurensis* overexpressing of *vasts7* gene reaching levels of 2.9 mg. L^{-1} of *trans*-R. On the other hand, the effect of overexpressing calcium-dependent protein kinase, *cpk* gene on *trans*-R production in *V. amuresis* cell cultures was studied by Aleynova et al. (2014). These authors showed that the overexpression of *vacpk20* triggered a rise in the production of *trans*-R reaching the maximal levels of 33.9 mg. L^{-1}. In the same way, Aleynova et al. (2015) showed that the overexpression of *vacpk29* gene also enhanced the production of *trans*-R (1.39 mg. L^{-1}) compared to control calli (0.79 mg. L^{-1}). All these data suggested that the *vacpk20* and *vacpk29* genes could be implicated in *trans*-R biosynthesis as positive regulators.

It has been also reported that *rol* gene could improve the production of some secondary metabolites in plant *in vitro* cultures (Almagro et al., 2013). In fact, the transformation of *V. amurensis* callus with *rolB* gene increased *trans*-R production more than 1000-fold (3.15% DW) in transgenic calli as compared to control line (0.003% DW) (Kiselev et al., 2007). Dubrovina et al. (2010) also showed that the overexpression of *rolC* gene stimulated *trans*-R accumulation, being 3.7 and 11.9-fold greater in two transformed *V. amurensis* cell lines.

On the other hand, *V. vinifera* cv Monastrell and *V. vinifera* cv Gamay cell cultures transformed with human cytochrome P450 hydroxylase 1B1 (*Hscyp1b1*) were elicited with 50 mM MCD and 0.1 mM MJ, and these cultures accumulated 20 mg/L and 10 mg. L^{-1} of piceatannol, respectively, that is 7 and 2.5-fold higher compared to non-transformed *Vitis* cell lines (Martínez-Márquez et al., 2016). In addition, these cell cultures were also transformed with O-methyltransferase gene (*Vvromt*), and the levels of pterostilbene was of the order of micrograms per liter. It shows that genetic engineering would be a feasible strategy to increase stilbene biosynthesis in grapevine cell cultures.

Hairy root cultures, obtained by genetic modification using *Agrobacterium rhizogenes*, could also represent a valuable technique for the production of *trans*-R and its derivatives. Hairy root cultures from peanut (*A. hypogaea*) were treated with sodium acetate, and the accumulation of *trans*-R increased 60-fold after 24 h of elicitation (98 μg. mg^{-1} DW (Medina-Bolivar et al., 2007). In addition, other stilbenes, including *trans*-pterostilbene, were also detected in the culture medium (237 ng *trans*-pterostilbene mg^{-1} DW). In a similar way, Kim et al. (2008) analyzed the concentration of *trans*-R in peanut hairy roots transformed with five different *Agrobacterium rhizogenes* strains, being *A. rhizogenes* R1601 the most effective strain for the production of *trans*-R (1.5 mg. g^{-1} DW). Halder and Jha (2016) also obtained 30 peanut hairy root culture lines infecting with three different *A. rhizogenes* strains (LBA9402, A4 and R1000). The highest levels of *trans*-R (1.21 mg. g^{-1} DW) were found in that hairy root line transformed with the R1000 strain, being 19-fold higher the production of *trans*-R than those non-transformed roots.

Moreover, Yang et al. (2015) were able to detected production levels of 40.29 mg *trans*-R L^{-1}, 56 mg *trans*-arachidin1 L^{-1} and 148 mg *trans*-arachidin-3 in peanut hairy root cultures treated with 9 g/L CD and 100 μM MJ. In the same way, peanut hairy root cultures treated with sodium acetate also produced high levels of *trans*-R (393.2 nM. g^{-1} DW), arachidin-1 (107.0 nM. g^{-1} DW) and arachidin-3 (124.1 nM. g^{-1} DW) as compared with non-treated hairy root cultures (Condori et al., 2010). Tisserant et al. (2016) obtained *V. vinifera* cv Pinot Noir hairy root cultures infected with *A. rhizogenes* ATCC 15834, and one hairy root culture line could produce 160 mg. L^{-1} *trans*-R which was accumulated in culture medium when it was treated with 50mM MCD and 100 μM MJ,

that is, 570-fold increase in comparison to control. All these studies showed that *Agrobacterium* strain selection and elicitation are crucial factors for biosynthesis of *trans*-R and its derivatives in hairy root cultures.

Recently, other authors have stably transformed *Silybum marianum* cell cultures with *V. vinifera sts*3 and the expression of this transgene led to extracellular *trans*-R accumulation when these transgenic cell lines were treated with MCD alone or in combination with MJ (Hidalgo et al., 2017). Moreover, the highest levels of *trans*-R accumulated in the culture medium were obtained with 30 mM MCD-treated cell cultures at 76 h of the treatment (12 mg. L^{-1}).

1.7.2.2 Heterologous production of stilbenes using metabolic engineering in microorganisms

Metabolic engineering of microorganisms has been used to enhance a large number of plant natural products. Microorganisms, yeasts and bacteria, do not possess the genes encoding enzymes responsible of the biosynthetic pathway for the *trans*-R and its derivatives, so it is necessary to introduce a tailored vector either containing the entire pathway that uses aromatic amino acids (L-phenylalanine or L-tyrosine), or precursors of biosynthetic pathway (p-coumaric acid) as substrates or harboring the specific genes from phenylpropanoid and stilbene pathways, such as *pal*, *c4h*, *4cl*, and *sts* (Donnez et al., 2009). There are many successful examples in the past decades using this strategy to produce *trans*-R and derivatives (Table 1.2).

One of the most successful report that used two recombinant *Saccharomyces cerevisiae* yeasts transformed with two key enzymes of *trans*-R biosynthetic pathway, *4cl* from *Arabidopsis thaliana* and *sts* from *V. vinifera*, using *p*-coumaric acid as precursor, was carried out by Sydor et al. (2010) (Table 1.2). Using these two yeast strains, they were able to produce high levels of *trans*-R (391 and 292 mg/L). Similarly, using the same procedure with other different yeast strain, WAT11, Wang and Yu (2012) obtained low levels of *trans*-R, which showed that yeast strain nature is crucial to obtain high levels of this bioactive compound. In other cases, researchers have used the strategy of transforming different yeast strains (FY23, CEN.PK 113-3b, and W303-1A)

Table 1.2 Ectopic production of stilbenes using genetic engineering in plant *in vitro* cultures.

Plant species	Type of culture	Genes/strain	Elicitor	Production	Reference
V. amurensis	Cell cultures	*sts 1/ sts 2/ sts 7*	-	0.36 /0.48/0.60 mg *trans*-R g^{-1} DW	Aleynova et al., 2016
V. amurensis	Cell cultures	*sts 7*	-	2.9 mg *trans*-R L^{-1}	Kiselev and Aleynova, 2016
V. amurensis	Callus	*Vacpk20*		33.9 mg *trans*-R L^{-1}	Aleynova et al., 2014
V. amurensis	Callus	*Vacpk29*		1.39 mg *trans*-R L^{-1}	Aleynova et al., 2015
V. amurensis	Callus	*rolB*	-	3.15% *trans*-R DW	Kiselev et al., 2007
V. amurensis	Callus	*rolC*	-	14.3 mg *trans*-R L^{-1}	Dubrovina et al., 2010
V. vinifera cv Monastrell	Cell cultures	*Hscyp1b1*	MCD + MJ	20 mg *trans*-piceatannol L^{-1}	Martínez-Márquez et al., 2016
V. vinifera cv Gamay	Cell cultures	*Hscyp1b1*	MCD + MJ	10 mg *trans*-piceatannol L^{-1}	Martínez-Márquez et al., 2016
A. hypogea	Hairy root cultures	*ATCC 15834*	Sodium acetate	98 μg *trans*-R mg^{-1} DW 237 ng *trans*-pterostilbene mg^{-1} DW	Medina-Bolivar et al.,2007
A. hypogea	Hairy root cultures	R1601		1.5 mg *trans*-R g^{-1} DW	Kim et al., 2008
A. hypogea	Hairy root cultures	*R1000*	-	1.21 mg *trans*-R g^{-1} DW	Halder and Jha, 2016
A. hypogea	Hairy root cultures	ATCC 15834	CD + MJ	40.29 mg *trans*-R L^{-1} 56 mg *trans*-arachidin1 L^{-1} 148 mg *trans*-arachidin-3 L^{-1}	Yang et al., 2015
A. hypogea	Hairy root cultures	ATCC 15834	Sodium acetate	393.2 nM *trans*-R g^{-1} DW 107.0 nM *trans*-arachidin1 g^{-1} DW 124.1 nM *trans*-arachidin3 g^{-1} DW	Condori et al., 2010
V. vinifera cv Pinot Noir	Hairy root	ATCC 15834	MCD + MJ	165 mg L^{-1} *trans*-R	Tisserant et al., 2016
Silybum marianum	cell cultures	*sts* 3	MCD	12 mg L^{-1} *trans*-R	Hidalgo et al., 2017

using the same genes (*4cl* and *sts*) but obtained from different plants (Table 1.2), and when these strains were fed with *p*-coumaric acid. In these cases, they obtained low levels of *trans*-R (0.00145, 5.8 and 3.1 mg. L^{-1}, respectively). In the same way, when two different yeast strains were fed with the precursor tyrosine, Wang et al. (2011) and Shin et al. (2012) also obtained low levels of *trans*-R (1.9 and 5.8 mg. L^{-1}). These results strongly support the importance not only the nature of the yeast strain used but also the plant source from which these genes are obtained.

On the other hand, a lot of works have been described using genetic engineering to transform the bacteria *E. coli*. In this way, Lim et al. (2011) reported an accumulation of *trans*-R above 2.3 g. L^{-1} using the engineered *E coli* strain BW27784 harboring *4cl* from *A. thaliana* and *sts* from *V. vinifera*, when this strain was cultured in YM9 medium in the presence of 15 mM *p*-coumaric acid and cerulenin. Moreover, this strain provided the best results in terms of *trans-R* production, in comparison with the productivity obtained from all engineering microorganisms during this decade. Similar studies carried out by Watts et al. (2006) using the same engineered *E coli* strain BW27784 harboring the same genes (*4cl* and *sts*) but from different plants, and only fed with p-coumaric acid, led to low levels of *trans-R* and derivatives (104.5 mg *trans*-R L^{-1} and 10 mg piceatannol L^{-1}). In these conditions and using *E coli* strain BL21, Zhang et al. (2015) and Beekwilder et al. (2006) obtained 80.52 and 16 mg L^{-1} of *trans-R*, respectively, Katsuyama et al. (2007) used *E. coli* strain BLR (DE3), which produce 171 mg. L^{-1} *trans-R.*

On the other hand, Chien et al. (2016) reported that the recombinant *E. coli* which contains the *tal* gene from *Rhodotorula glutinis*, *4cl* from *A. thaliana* and *sts* from *V. vinifera* was able to produce 1105 mg. L^{-1} of *trans*-R, using tyrosine as initial precursor. Other researchers which cloned *tal*, *4cl* and *sts* from various plant species in several *E. coli* strains and used culture media enriched with only tyrosine (Wu et al., 2013) or tyrosine and glucose (Wang et al., 2011) or glucose or ethanol (Li et al., 2015), obtained low levels of *tran*s-R production. Consequently, these results suggested that the source of the *tran*s-R biosynthetic pathway genes, the construct designed to express them, host strains, and the precursors used can modify the efficiency of the process to obtain *tran*s-R using the engineered microorganisms. Moreover, some studies have been focused on

the production of *tran*s-R derivatives. In fact, Katsuyama et al. (2007) obtained transgenic *E. coli* BLR strain overexpressing *pal* from *R. rubra*, *4cl* from *L. erythrorhizon*, *sts* from *A. hypogaea* and *pmt* from *O. sativa*. These *E. coli* transgenic lines were able to produce around 5.8 mg. L^{-1} pterostilbene. In addition, a tyrosinase (*tyr*) from *Streptomyces avermitilis* was expressed in *E. coli* to produce piceatannol (77.4 μM) in the presence of *tran*s-R (Lee et al., 2015). In the same way, Kang et al. (2014) obtained engineered *E. coli* harboring *tal* gene from *Saccharothrix espanaensis*, *4cl* gene from *Streptomyces coelicolor*, *sts* gene from *A. hypogaea*, *sbomt1* and/or *sbomt3* genes from *Sorghum bicolor*, which was able to produce methylated resveratrol analogs (pinostilbene, 3,5-dihydroxy-4'-methoxystilbene, 3,4'-dimethoxy- 5-hydroxystilbene, and 3,5,4'-trimethoxystilbene), in a simple sugar medium culture without any feeding precursors. Therefore, it is possible that genetic engineering of microorganisms could represent the new approach for the production of *tran*s-R and derivatives which have higher bioactivity than *tran*s-R.

On the other hand, Kang et al. (2015) have reported the only event of transformation of a fungus being able to produce 0.92 μg. g^{-1} *tran*s-R which was secreted by the recombinant fungal *Tremella fuciformis* (*Tro*1*)*, which in turn, had been transformed with *4cl* and *sts* from *V. vinifera*.

Table 1.2 Heterologous production of stilbenes using metabolic engineering in microorganisms

Microorganism	Species/strain	Genes	Origin of genes	Precursor	Production	reference
Yeast	Brazilian sugar cane-fermenting yeast	*4cl, sts*	*4cl /A. thaliana* *sts /V. vinifera*	*p*-coumarate	391mg *trans*-R L^{-1}	Sydor et al. 2010
	S. cerevisiae/ CEN.PK2-1	*4cl, sts*	*4cl /A. thaliana* *sts /V. vinifera*	*p*-coumarate	292mg *trans*-R L^{-1}	Sydor et al. 2010
	S. cerevisiae/ WAT11	*4cl, sts*	*4cl /A. thaliana,* *sts /V. vinifera*	*p*-coumaric acid	14.4mg *trans*-R L^{-1}	Wang and Yu 2012
	S. cerevisiae/ FY23	*4cl, sts*	*4cl /Poplar hybrid* (*P. trichocarpa×P.deltoides*), *sts /V. vinifera*	*p*-coumaric acid	0.00145mg *trans*-R L^{-1}	Becker et al. 2003
	S. cerevisiae/ CEN.PK 113-3b	*4cl, sts*	*4cl /N. tabacum* cv. Samsun *sts /V. vinifera*	*p*-coumaric acid	5.8mg *trans*-R L^{-1}	Beekwilder et al. 2006
	S. cerevisiae/ W303-1A	*4cl, sts*	*4cl /A. thaliana* *sts/A. hypogaea*	*p*-coumaric acid	3.1mg *trans*-R L^{-1}	Shin et al. 2011
	S. cerevisiae/ WAT11	*4cl, sts, tal*	*4cl /A. thaliana,* *sts /V. vinifera*	tyrosine	1.9mg *trans*-R L^{-1}	Wang et al. 2011
	S. cerevisiae/ W303-1A	*pal, c4h,* *4cl, sts*	*pal /R. toruloides,* *c4h and 4cl/A. thaliana,* *sts /A. hypogaea*	tyrosine	5.8mg *trans*-R L^{-1}	Shin et al. 2012
Bacteria	*E. coli/ BW27784*	*4cl, sts*	*4cl /A. thaliana* *sts /V. vinifera*	*p*-coumaric acid and cerulenin	2390 mg *trans*-R L^{-1}	Lim et al. 2011
	E. coli/ BW27784	*4cl, sts*	*4cl /A. thaliana* *sts /A. hypogaea*	*p*- coumaric acid	104.5mg *trans*-R L^{-1} 10 mg piceatannol L^{-1}	Watts et al. 2006
	E. coli/ BL21	*4cl,sts*	*4cl/A. thaliana* *sts/ A. hypogaea*	*p*-coumaric acid	80.52 mg *trans*-R L^{-1}	Zhang et al. 2015
	E. coli/ BL21	*4cl, sts*	*4cl/N. tabacum* cv. Samsun *sts/V. vinifera*	*p*-coumaric acid	16 mg *trans*-R L^{-1}	Beekwilder et al. 2006
	E. coli /	*4cl, sts*	*4cl/L. erythrorhizo,*	*p*-coumaric acid	171 mg. *trans*-R L^{-1}	Katsuyama et al.

	BLR (DE3)		*sts/A. hypogaea*			2007b
	E. coli	*tal, 4cl, sts*	*tal/ R. glutinis* *sts/ V. vinifera* *4cl/A. thaliana*	tyrosine	1105 mg *trans*-R L^{-1}	Chein et al. 2016
	E. coli	*tal, 4cl, sts,*	*tal/R.glutinis,4cl/Petroselinum crispum, sts/V. vinifera matB and matC /R. trifolii*	*L*-tyrosine	35.02 mg *trans*-R L^{-1}	Wu et al. 2013
	E. coli/ CEN.PK102-5B	*tal, 4cl, sts*	*tal/Herpetosiphon aurantiacus* *sts/ V. vinifera* *4cl/ A. thaliana*	Glucose ethanol	415.65 mg *trans*-R L^{-1} 531.41 mg *trans*-R L^{-1}	Li et al. 2015
	E. coli/ BL21	*tal, 4cl, sts*	*tal/S.espanaensis NRRL* *4cl/A.thaliana* *sts/A. hypogaea*	glucose and tyrosine	114.4 mg *trans*-R L^{-1}	Wang et al. 2010
	E. coli/ BLR	*pal, 4cl, sts pmt*	*pal/R. rubra,* *4cl/L. erythrorhizon* *sts/ A. hypogaea,* *pmt/ O. sativa*	tyrosine	37 mg *trans*-R L^{-1} 5.8 mg pterostilbene L^{-1}	Katsuyama et al. 2007a
Fungi	*Tremella fuciformis Tro1*	*4cl, sts*	*4cl and sts/ V. vinifera*	*p*-coumaric acid	0.92 μg *trans*-R g^{-1}DW	Kang et al. 2015

2. Objetivos

Objectives

The aim of this work was to improve the production of *trans*-resveratrol using two different strategies: the establishment of transgenic cell lines which overexpressed the stilbene synthase, *sts* gene, involved in *trans*-resveratrol biosynthesis, and using elicitation with cyclodextrins and/or methyl jasmonate in these transgenic cell lines. For this, it is needed to develop a stable and efficient transformation protocol of Monastrell suspension cultured cells using Sonication-Assisted *Agrobacterium*-mediated Transformation, and optimizing all the factors affecting transformation efficiency (Chapter1). This stable and efficient transformation protocol will be used to transfer *sts* gene to Monastrell cells, in order to obtain different transgenic cell lines, which will be elicited with cyclodextrins and/or methyl jasmonate. These transgenic cell lines will be screened in order to determine if they are able to produce higher levels of *trans*-resveratrol in comparison to non-transgenic cell lines (Chapter 2).

3. Chapter 1

Agrobacterium-mediated transformation in suspension-cultured cells of Vitis cv. Monastrell: Determination of Critical Parameters

Abstract

Although some works have explored the transformation of differentiated, embryogenic suspension-cultured cells (SCC) to produce transgenic grapevine plants, to our knowledge this is one of the first reports on the efficient transformation of dedifferentiated *Vitis vinifera* cv Monastrell SCC. This protocol has been developed using the sonication-assisted *Agrobacterium*-mediated transformation (SAAT) method. A construct harboring the selectable *nptII* and the *eyfp*/IV2 marker genes was used in the study and transformation efficiencies reached over 50 independent transformed SCC per gram of infected cells. Best results were obtained when cells were infected at the exponential phase. A high cell density plating (500 mg/dish) gave significantly better results. As selective agent, kanamycin was inefficient for the selection of Monastrell transformed SCC since wild type cells were almost insensitive to this antibiotic whereas application of paromomycin resulted in very effective selection. Selected *eyfp*-expressing microcalli were grown until enough tissue was available to scale up a new transgenic SCC. These transgenic SCC lines were molecularly and phenotypically evaluated demonstrating the presence and integration of both transgenes, the absence of *Agrobacterium* contamination and the ability of the transformed SCC to grow in highly selective liquid medium. The methodology described here opens the possibility of improving the production of high added value metabolites.

Keywords: *eyfp*; *npt*II; paromomycin; plating density; SAAT

3.1 Introduction

Suspension-cultured cells (SCC) have been used as a convenient tool for the production of high added value plant-derived metabolites, and it is considered an attractive alternative to the extraction of these compounds from plant raw material (Cai et al., 2012; Skrzypczak-Pietraszek et al., 2014), since secondary metabolites are produced at higher levels and with different profiles in cell cultures compared to parental plants (Cai et al., 2011). Moreover, SCC can be easily used to study cell growth and production kinetics in order to optimize ideal conditions for large-scale production of these metabolites within a short (about 2–4 weeks) cultivation time (Srivastava et al., 2011). Grapevine (*Vitis vinifera* L.) is one of the most widely consumed fruit in the

world as food and beverage, and has one of the highest phenolic compound contents when compared to other fruits and vegetables (Burin et al., 2014). It has been demonstrated that some of the biological effects associated with the consumption of phenolic compounds are due to their antioxidant, anti-inflammatory, antibacterial and anti-carcinogenic activities (Burin et al., 2014; Lago-Vanzela et al., 2014).

There are several strategies to increase some of the high added value secondary metabolites of grapevine SCC such as elicitation (Santamaria et al., 2011; Belchí-Navarro et al., 2012; Vuong et al., 2014), precursor feeding (Riedel et al., 2012), *in situ* product removal (Vuong et al., 2014), and cell immobilization (Iborra et al., 1994). Amongst these different strategies, the use of biotic or abiotic elicitors is one of the most effective and favorable procedures to enhance the productivity of bioactive secondary metabolites, reducing the time to attain high product concentrations and increasing volumetric productivity (Cai et al., 2011; Cai et al., 2012).

Genetic transformation of grapevine has been achieved by *Agrobacterium*-mediated transformation (Franks et al., 1998; Yamamoto et al., 2000; Iocco et al., 2001; Wang et al., 2005; Gago et al., 2011), particle bombardment (Torregrosa et al., 2002; Vidal et al., 2003; De la Torre et al., 2012; Sanjurjo et al., 2013), and transient transformation of protoplasts (Jardak et al., 2002; Hichri et al., 2010).

Agrobacterium-mediated transformation has been widely used and is compatible with the regeneration of transgenic plants from a widely variety of cultivars (Iocco et al., 2001). Regarding grapevine, a number of grapevine cultivars have been stably transformed using *Agrobacterium*-mediated transformation, and most progress has been achieved using embryogenic cell masses (Mauro et al., 1995; Das et al., 2002; Bouquet et al., 2008). The method must be available to integrate an over-expression of key genes of the pathway for targeting metabolic engineering approaches.

Since plant SCC are known as an useful model system due to its properties of relatively simple transformation, rapid cell proliferation and good growth characteristics (Mustafa et al., 2011), they have been used to transfer genes in various plants species. *Agrobacterium*-mediated transformation have been carried out using embryogenic cells of some plant species, such as banana (Ganapathi et al., 2001; Huang et al., 2007;

Ghosh et al., 2009; Chong-Pérez et al., 2012), lily (Qi et al., 2014) , sweet potato (Yang et al., 2011) and rice (Hoa and Bong, 2002).

Agrobacterium-mediated transformation (Wu et al., 2012) and particle bombardment (Vidal et al., 2003; Sanjurjo et al., 2013) have been reported as the most important procedures for the production of transgenic grapevine using embryogenic cells. However, few reports on the stable transformation of undifferentiated non-embryogenic SCC are available, although the production of bioactive metabolites from grapevine has been mostly achieved in non-transgenic undifferentiated SCC (Jeandet et al., 2014). In pioneer experiments, Torregrosa et al. (2002) and Verries et al. (2004) studied transient expression of gene promoters in Cabernet Sauvignon SCC after particle bombardment, and more recently, Jelly et al. (2014) reviewed the applications of transient expression assays in cell suspensions for functional analysis of different genes. All these attempts to transform grapevine SCC rely on transient expression of reporter genes and/or genes of interest but there is not a reproducible procedure to stably transform undifferentiated grapevine SCC. Only an old paper (Baribault et al., 1989) reported the *Agrobacterium*-mediated transformation of SCC from Cabernet Sauvignon. Although transgenic calli were reported in this study, no transformed SCC were obtained, and there have not been published later reports using that protocol. In this study, we aimed to optimize a transformation protocol for the efficient and stable expression of transgenes in *V. vinifera* L. cv Monastrell SCC using *Agrobacterium.*

3.2 Material and methods

3.2.1 Initiation and maintenance of *Vitis* SCC

V. vinifera L. cv Monastrell calli were established in 1990 as described by Calderón et al. (1993). Since then, calli have been maintained by transferring to fresh solid culture medium monthly, at 25ºC in the dark in 250 mL flasks containing 100 mL of Gamborg B_5 basal salt medium (Gamborg et al., 1968) supplemented with Morel vitamins (Morel, 1970), 0.25 g. L^{-1} casein hydrolysate, 20 g. L^{-1} sucrose, 0.2 mg. L^{-1} kinetin and 0.1 mg. L^{-1} 1-naphthaleneacetic acid (GB5-1). The pH was adjusted at 6.0.

In the present study, Monastrell SCC were initiated by adding 20 g friable callus in 250 mL Erlenmeyer flasks containing 100 mL of liquid growth medium (GB5-1).

Flasks were maintained in a rotary shaker (110 rpm) at 25°C in the dark. During initial experiments, Monastrell SCC were routinely maintained by periodical subcultures every 14-16 days by diluting with one volume of liquid medium and then, distributing into two flasks. Transformation experiments were carried out with 3-day-old cell cultures after diluting. However, in later experiments SCC were maintained by filtering and weighting 20 g of fresh weight cells that were added to 100 mL of GB5-1 in a 250 mL Erlenmeyer flask. Experiments described below were carried out to determine the right time for the different steps of the optimal transformation.

3.2.2 *Agrobacterium* culture and transformation procedure

Agrobacterium tumefaciens strain LBA 4404 harbouring the plasmid pMOG800 which contains the selectable marker gene *npt*II driven by the *nos* promotor and the visual marker *eyfp*/IV2 gene under the control of a 35S promoter was used in the present study. A single fresh bacterial colony was picked from the Petri dish and diluted in 10 mL yeast extract-mannitol (YM) liquid medium supplemented with 50 mg. L^{-1} kanamycin and 50 mg. L^{-1} streptomycin in a 100 mL Erlenmeyer flask. The *Agrobacterium* culture was incubated at 28°C overnight in the dark on a rotary shaker at 140 rpm. A certain volume of bacterial suspension was diluted in fresh YM liquid medium in 250 mL Erlenmeyer flask with the appropriate antibiotics until an optical density of 0.1 at 600nm (OD_{600}). After incubating the bacterial culture for about 10 additional hours, the OD_{600} value reached was between 0.6-0.9, and the culture was then centrifuged at 3800 *g* for 15 min at room temperature and the pellet re-suspended in 30 mL of GB5-1 without plant growth regulators or casein hydrolysate. The *Agrobacterium* suspension was supplemented with 100 mM acetosyringone and pH adjusted at 5.5 (GB5-2) in a 250 mL Erlenmeyer flask and cultured at 25°C and 140 rpm for 5 additional hours before infecting.

Vitis cells, both from 3-day-old half-diluted cultures or from cultures initiated by weighting 20 g of cells, were filtered using a vacuum pump in order to remove the culture medium. 2 g of fresh weight (FW) of cells were then transferred into 25 mL Erlenmeyer flasks with 10 mL of the bacterial culture, and they were maintained for 12 min in darkness on a shaker at 110 rpm. After infection, cells were filtered to remove the excess of bacterial culture without using pumping and were transferred to disposable

Petri dishes previously prepared with 2 layers of sterilized filter paper which were soaked with 2.5 mL of GB5-2. Cells were spread as uniform as possible on the filter paper and then co-cultured in darkness at 25°C for 3 days. After that, cells were washed with GB5-1 supplemented with 500 mg. L^{-1} cefotaxime for 2.5 min in continuous agitation. Cells were then filtered and re-suspended in 8 mL of GB5-1 supplemented with 300 mg.L^{-1} cefotaxime and various concentrations of kanamycine or paromomycin. These cultures were maintained for 3 days in darkness at 110 rpm to completely eliminate *Agrobacterium*. Finally, 2.5 mL of cell dilution was transferred to corresponding semi-solid GB5-1 medium, spreading cells as uniform as possible and sealing the Petri dishes with Parafilm.

After 6 weeks, when callus generated from cells reached 3-5 mm, those callus expressing *eyfp* were transferred onto fresh semi-solid GB5-1 medium supplemented with cefotaxime (300 mg. L^{-1}) and paromomycin (10 mg. L^{-1}).

3.2.3 Factors affecting transformation

To determine the effect of applying sonication-assisted *Agrobacterium*-mediated transformation (SAAT), after infecting *Vitis* cells for 12 min, the flasks were placed in a Raypa® Model UCI-150 ultrasound bath (R. Epinar, S.L. Barcelona, Spain) for 0, 2.5, 5 or 7 min followed by shaking (110 rpm) for 10 additional min.

Low and medium cell density cultures were initiated by weighting cells and inoculating 10 or 20 g of cells in 100 mL of GB5-1. 1 or 2 g of cells were infected with *Agrobacterium* 5, 7 and 14 days later corresponding (according to the cell growth curves, data not shown) to the end of the lag phase, the exponential phase or the beginning of the stationary phase.

To determine the possible effect of initial cell density n the recovery of transformed microcalli, 2 g of cells infected 7 days after initiating a medium cell density culture were sonicated for 5 min and uniformly spread in 4 (500 mg SCC/plate, high density plating) or 6 (333.3 mg SCC/plate, medium density plating) Petri dishes.

3.2.4 Selection of transgenic cell lines

Different concentrations of kanamycine (20, 40, 60, 80 and 100 mg. L^{-1}) or

paromomycin (5, 10, 15, 20 and 30 mg. L^{-1}) were tested to determine their effect on the viability of grapevine cells. Cell viability was assessed by incubating a small aliquot of grapevine SCC for 1–2 min in GB5-1 medium containing 0.01 % (p/v) propidium iodide (PI) for the selective labelling of dead cells, and 0.01 % (p/v) fluorescein diacetate (FD) for the selective labelling of live cells, as described by Duncan and Widholm (1990). Fluorescence was observed with a fluorescence microscope (DMRB, Leica Microsystems Inc. Wetzlar, Germany) using specific filters. Additionally, Petri dishes containing medium with different paromomycin concentrations were spread with two different cell densities as described above. In all cases cells were treated for 5 min with SAAT while being infected with *Agrobacterium*.

3.2.5 Evaluation of transformation experiments

Petri dishes were observed under a Leica MZ75 stereomicroscope equipped with a fluorescence EYFP Plus filter module, which contains a 480/40-nm excitation filter, a 505-nm LP dichromatic beam-splitting mirror, and a 510-nm LP barrier filter. A 50-W, high-pressure mercury vapor lamp provided illumination. The number of cells expressing *eyfp* was counted 15 and 25 days after infection (dpi) and the number of *eyfp*-expressing microcalli was determined 60 dpi, as well as the number of escapes (microcalli that did not express *eyfp*).

Putatively transformed microcalli were transferred to fresh medium, when they could be easily distinguished by the naked eye, in individual 4 cm diameter Petri dishes and numbered as independent cell lines. When there was enough callus material, 500 mg were transferred to 10 mL liquid GB5-1 to scale up the SCC.

3.2.6 Sedimented cell volume (SCV) determination

Seven-day-old cell cultures were filtered using a vacuum pump, drying cells as much as possible. 5 g of cells were weighted and transferred to 100 mL GB5-1 medium in a 250 mL Erlenmeyer flask. SCC was maintained under the same conditions described previously. SCV was determined by taking 4 mL of SCC to graduated tubes and keeping them at 4°C overnight in the refrigerator. The following day, pellet volume and total volume were read. SCV was expressed as the percentage of the total volume that is already occupied by cells. Each value was calculated as the average from four

measures taken from two different SCC.

3.2.7 Molecular evaluation of transgenic cell culture lines

Once that SCC for the putatively transgenic lines were established, samples from these cultures were collected one week after subculture and centrifuged at 12500 *g* for 10 min. The supernatant was removed and 200 mg cells were weighted, frozen in liquid nitrogen and stored at –80°C. Genomic DNA was extracted with a modified CTAB method following the protocol described by Japelaghi et al. (2011). DNA concentration and quality (A260/A230 and A260/A280 ratios) were determined with a spectrophotometer (NanoDrop 1000).

Specific primers for the amplification of a 700 bp fragment from the *npt*II gene (ntpII-F: 5'-gaggctattcggctatgactg-3'and ntpII-R: 5'-atcgggagcggcgataccgta- 3'), and a 856 bp fragment from the *eyfp* gene (*eyfp*-F: 5'-atggtgagccaagggcgagga-3' and eyfp-R: 5'-ggaccatgtgatcgcgcttc-3') were used. Also PCRs were run with specific primers for a 660 bp fragment from the *vir*G gene (Petri et al., 2004) to detect *Agrobacterium* contamination.

The reactions for *npt*II-fragment amplification were carried out in a total volume of 25µl, with 400 µM of each dNTP, 0.4 µM of each primer, 2.5 mM $MgCl_2$, 0.2 µg of genomic DNA, 0.5 units of Taq DNA polymerase (GoTaq® Promega, Madison, USA), and PCR buffer. The amplification of the *eyfp* fragment was carried out in 25µl PCR reaction containing 200 µM of each dNTP, 0.2 µM of each primer, 2.5 mM $MgCl_2$, 0.2 µg of genomic DNA, 0.5 units of Taq DNA polymerase (GoTaq® Promega, Madison, USA), and PCR buffer. The amplification reaction for *npt*II gene consisted of denaturation step 94˚C for 5 min, followed by 30 cycles for 30 sec at 60ºC for primer annealing, 45 sec at 72˚C for extension step and with the final extension step for 10 min at 72˚C. For *eyfp* the reaction included an initial cycle of 5 min at 94°C for denaturation, 35 cycles of 60s at 64 ºC for annealing and 60s at 72°C for extension, followed by final extension at 72°C for 10 min. The amplified DNA fragments were visualized under ultraviolet light after electrophoresis on 1.0% agarose gels containing TAE running buffer and RedSafe Nucleic Acid solution (RedSafe ™, iNtRon, Korea).

For Southern blot hybridization, genomic DNA (20 µg) was digested with *Eco*RI.

Fragments were separated on 1.0 % agarose gel and transferred to a positively-charged nylon membrane (Roche GmbH, Mannheim, Germany) by capillary blotting. Hybridizations were performed at 42°C in DIG-Easy (Roche GmbH, Mannheim, Germany) buffer with a digoxigenin-labeled *nptII* probe synthesized by PCR with the specific primers described above. The blots were washed twice at 23°C in 2×SSC (0.3 M NaCl, 0.03 M sodium citrate) plus 0.1 % (w/v) sodium dodecyl sulfate (SDS), and twice at 65°C in 0.5×SSC, 0.1 % SDS for 15 min each time. Hybridization was detected by using the chemiluminescent substrate CDP-Star (Roche GmbH, Mannheim, Germany), following the manufacturer's instructions.

3.3 Results

3.3.1 Effect of SAAT on T-DNA transfer

The effect of three different times in a sonication bath versus a non-sonicated control, while infecting grapevine cells with *Agrobacterium*, was evaluated. SAAT treatments significantly increased the number of *eyfp*-expressing cells 15 and 25 dpi ($P<0.01$) and microcalli ($P<0.05$). Figure 3.1A shows that the increase in the number of *eyfp*-expressing cells 15 and 25 dpi produced by the ultrasound bath had an optimum time at around 4 min. The number of cells initially expressing *eyfp* 15 dpi (Figure 3.1B and 3.2A, B) dramatically decreased at 25 dpi and even few of these were able to divide and produce transgenic microcalli (Figures 3.1B, 3.2D and 3.2F). Therefore, other factors that may be affecting the transformation of cells as well as their survival and ability to divide were studied.

3.3.2 Effect of initial cell density, physiological stage of cells and plating density on transformation efficiency

Cell cultures initiated from a low (100g. L^{-1}) or medium (200g. L^{-1}) cell density were infected at three different times during the cell growth cycle, representing the lag phase (5 days), exponential phase (7 days) and stationary phase (14 days) of SCC. Cell cultures initiated from 10 mg. L^{-1} had a significant larger number of cells expressing *eyfp* 15 dpi, however these transformations were mostly transient and 25 dpi, the number of cells expressing *eyfp* was larger in Petri dishes coming from cultures of

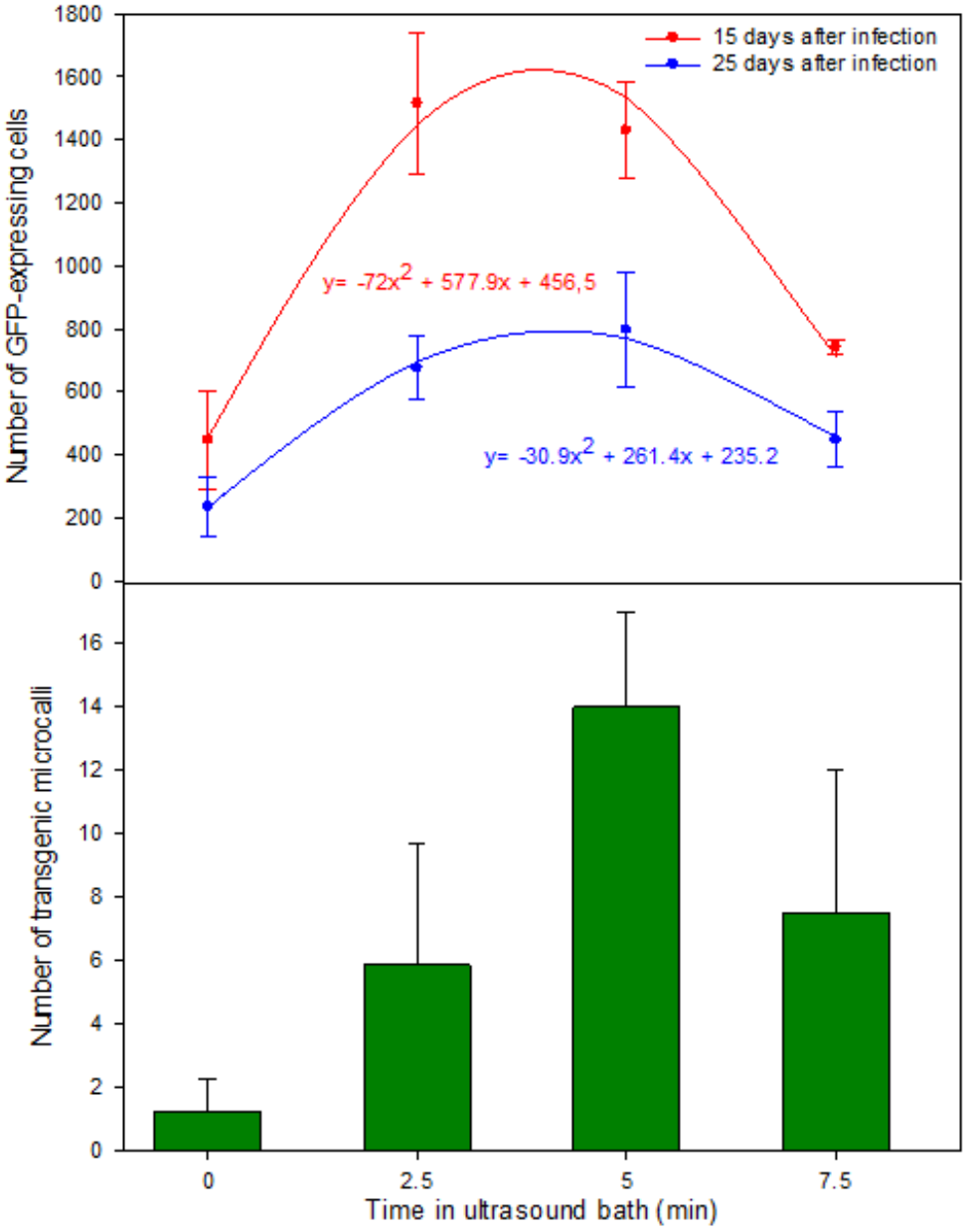


Figure 3.1 Effect of SAAT treatments on the number of *eyfp*-expressing cells (A) and microcalli (B). Experiments were repeated twice with four Petri dishes in each treatment.

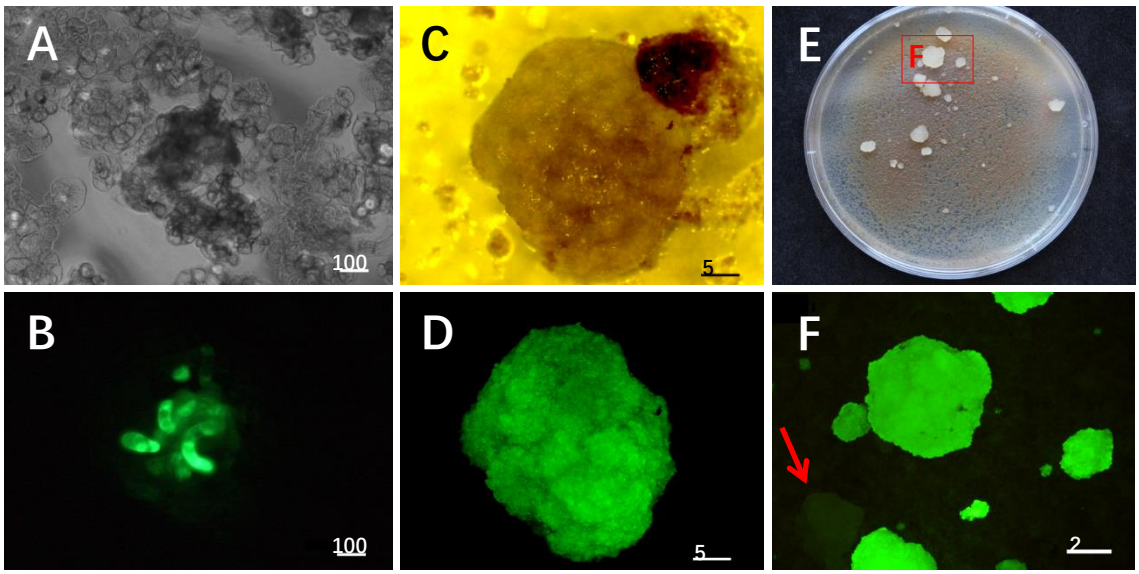


Figure 3.2 Transformation of grapevine SCC.

Top and bottom rows are photos taken with white or UV light, respectively. (A) Detail of a group of cells, (B) some of them expressing *eyfp* and starting first divisions. (C) Detail of a microcallus, notice the necrotic non-transformed area that does not express *eyfp* (D) as the rest of the callus. (E) Petri dish with transgenic callus developing in selective medium, (F) the square in E magnified and examined with UV light shows several transgenic callus and microcallus as well as a non-transformed escape (arrow).

medium cell density (Table 3.1). Also the number of *eyfp* positive microcalli (Figure 3.2D and F), recorded 60 dpi, was significantly larger in cultures of this density type (Table 3.1). There were large differences for all parameters recorded when infections at the three times of cell growth were compared. *Agrobacterium* transformation was much more successful when cells were in exponential phase, actively dividing. T-DNA transfer to cells in the lag phase was much scarcer, and did not occur at all when cells were already in the stationary phase. Finally, when the initial amount of infected cells was of 1 g, the number of transformed cells expressing *eyfp* was significantly much lower than when 2 g were used. Furthermore, transgenic microcalli were not obtained in any of the experiments with 1 g of cells, whereas this number was over 6 microcalli, as average from all treatments, when 2 g of cells were infected and was as high as 17.7 when 7-old-day grapevine SCC were used (Table 1).

Therefore, after these initial experiments 2 g of cells from a culture of 7-day-old

Table 3.1. Effect of different factors on Vitis cell culture transformation and transgenic microcalli production

Treatment			GFP+ cells 15 dpi*	GFP+ cells 25 dpi*	GFP+ microcalli*
Initial cell culture density (g. L^{-1})		Low (100)	41.0 ± 7.3a	8.5 ± 3.1a	1.4 ± 0.7b
		Medium (200)	15.8 ± 4.3b	11.7 ± 3.6a	5.0 ± 1.8a
Cell culture age (days)		5	6.9 ± 2.0b	2.9 ± 0.8b	0.7 ± 0.3b
		7	32.8 ± 5.5a	17.4 ± 4.4a	5.7 ± 1.9a
		14	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
Amount of infected cells (g)		1	13.4 ± 5.4b	0.8 ± 0.6b	0.0 ± 0.0b
		2	35.0 ± 5.5a	19.4 ± 4.2a	6.5 ± 1.9a
Low density (100g. L^{-1})	5d old	1 g cells	*n.d.*	0.0 ± 0.0b	0.0 ± 0.0a
		2 g cells	*n.d.*	2.5 ± 1.0a	0.9 ± 0.6a
	7d old	1 g cells	40.1 ± 11.4a	3.5 ± 2.3b	0.0 ± 0.0b
		2 g cells	41.9 ± 10.0a	28.1 ± 9.7a	5.1 ± 2.9a
	14d old	1 g cells	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
		2 g cells	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
Medium density (200g. L^{-1})	5d old	1 g cells	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0a
		2 g cells	13.7 ± 1.9a	9.0 ± 1.8a	2.1 ± 1.1a
	7d old	1 g cells	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b
		2 g cells	49.4 ± 9.4a	38.0 ± 9.5a	17.7 ± 4.9a
	14d old	1 g cells	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
		2 g cells	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a

*Values are means ± standard error. Means followed by different lowercase letters in the column and within each treatment are significantly different at $P<0.05$.

n.d.: not determined.

medium cell density (200g. L^{-1}) were used for subsequent experiments. To determine if this effect of the amount of cells was related to the plating density, an experiment was designed where 2 g of infected cells were plated in 4 (high plating density, 500 mg/plate) or 6 (medium plating, density 333.3 mg/plate) Petri dishes. Number of *eyfp*-expressing cells per gram of SCC were significantly (P<0.001) higher 15 and 25 dpi in high plating density than in medium plating density and, similarly, the number of *eyfp* positive microcalli, evaluated 60 dpi, were also significantly (P<0.001) larger (48.7 vs. 10.2 *eyfp* positive microcalli per gram of infected cells, see Figure 3.3).

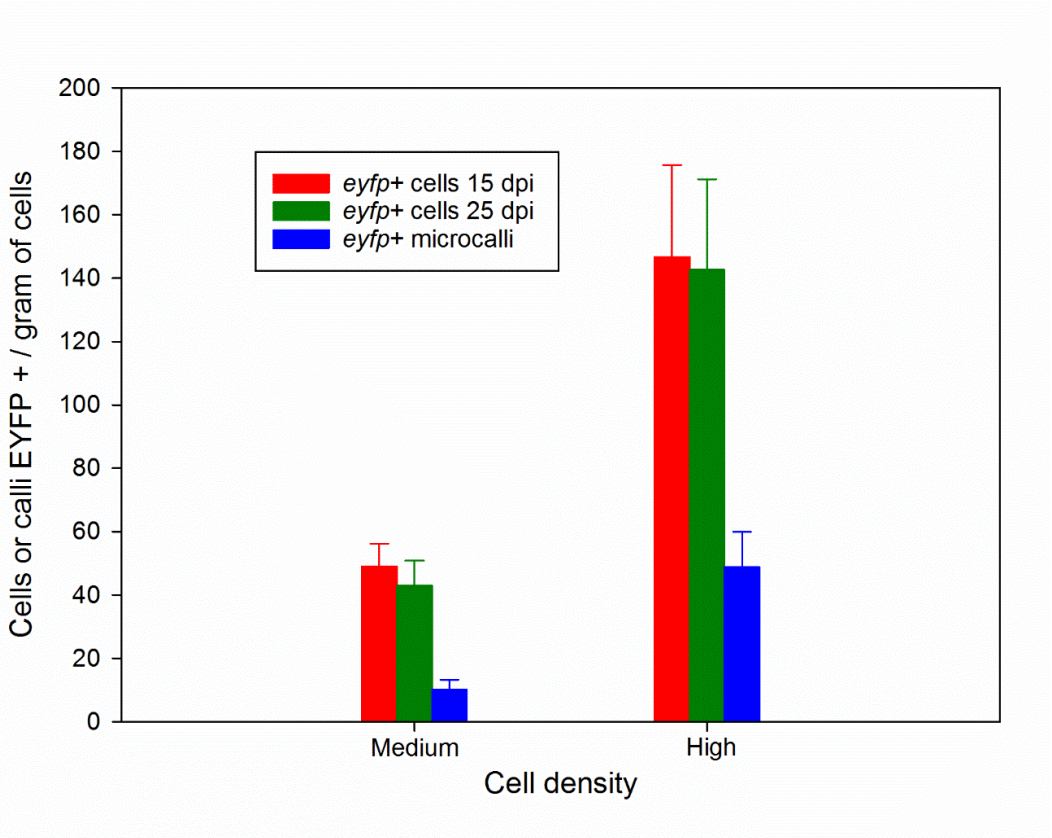


Figure 3.3 Effect of plating density on the number of *eyfp*-expressing cells 15 and 25 dpi (days after infection) and microcalli.

Two grams of infected cells were plated at two different densities: 333.3 mg/Petri dish (medium plating density) or 500 mg/Petri dish (high plating density). Experiments were repeated at least five times with a total number of 95 Petri dishes evaluated.

3.3.3 Effect of antibiotics on selection of transgenic cells

During previous experiments, we tried to select transgenic cells using kanamycin as a selective agent. However, even at relatively high concentrations it did not seem to have any pernicious effect on non-transgenic cell viability (Figures 3.4 A and B). The antibiotic was changed to paromomycin, another aminoglycoside antibiotic, obtaining completely different results (Figures 3.4 C and D). Several paromomycin concentrations were tested to determine their effect on non-transformed cell viability (Figures 3.4 E-J) and their ability to select transformed microcalli preventing the growth of non-transformed microcalli or escapes (Table 3.2). In Figure 4, the number of viable cells stained with FDA decreased as the concentration of paromomycin increased (Figure 3.4 F, H and J) and, in parallel, an increasing larger number of cells stained with PI, which stains nuclei of dead cells, were found with increasing paromomycin concentrations (Figures 3.4 E, G, and I).

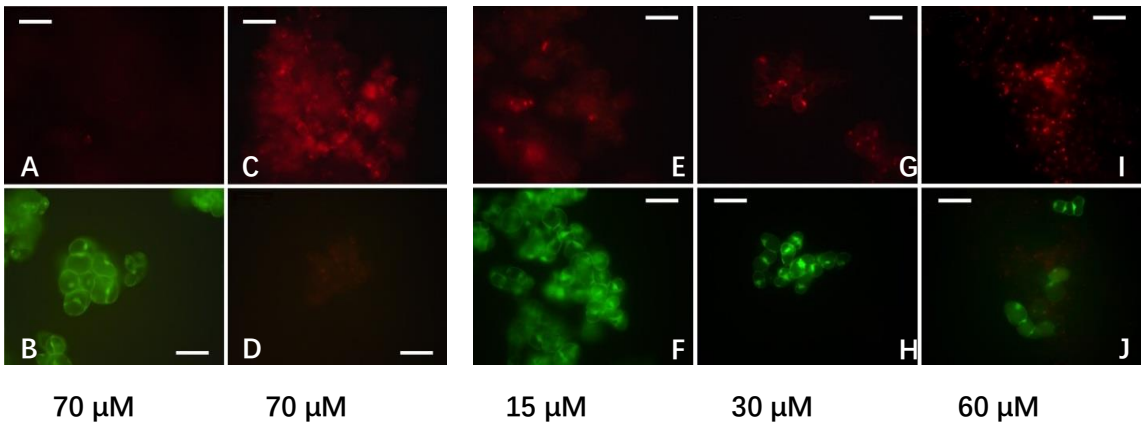


Figure 3.4 Evaluation of cell viability of wild type grapevine cells.
Cell viability was evaluated with fluorescein diacetate (FDA) whereas dead cells were identified with propidium iodide (PI). The effect of 70 µM kanamycin (Kan, A-B) and paromomycin (Par, C-D) was compared as well as the effect of different paromomycin concentrations (E-F) 15 µM, (G-H) 30 µM and (I-J) 60 µM on cell viability.

However, much lower concentrations of the antibiotic completely inhibited the development of transgenic calli in Petri dishes with both, high and medium cell densities. However, the antibiotic concentration necessary to completely inhibit growth of escapes was higher (30 mg. L^{-1}) when a high cell density per plate was used whereas a paromomycin concentration of only 20 mg. L^{-1} prevented non-transgenic calli when

infected cells were plated at medium density (333.3 mg/plate) and only a few transgenic calli were obtained (Table 3.2). Low paromomycin concentrations (5 mg. L^{-1} for high cell density and 10 mg. L^{-1} for medium cell density) allowed transgenic cell division and production of transgenic microcalli but also the number of escapes was overwhelmingly large. This made impossible to count them in most of the Petri dishes since they grew fast and soon, became a mass of cells covering most of the plate. With larger concentrations of paromomycin in both cases, the number of escapes was much more discrete still allowing the production of transgenic microcalli (Figures 3.2 C-F). The number of transgenic microcalli linearly decreased ($P<0.01$ in the case of medium plating density and $P<0.001$ for high plating density) with the increase in the paromomycin concentration, whereas the decrease in the number of escapes was more dramatic and totally inhibited at concentrations over 15 and 20 mg. L^{-1} of paromomycin for low and high densities plating, respectively (Table 3.2).

Table 3.2 Effect of paromomycin antibiotic on the production of transgenic calli and escapes.[a]

Paromomycin concentration (mg. L^{-1})	**Medium Cell Density[b]**		**High Cell Density[b]**	
	Transgenic calli[c]	Non-transgenic calli[c]	Transgenic calli[c]	Non-transgenic Calli[c]
5	35.7± 5.1	*nd*	*nt*	*nt*
10	7.6 ± 3.5	5.1 ± 3.4	115.4 ± 23.4	*nd*
15	10.4 ± 5.6	5.6 ± 2.8	*nt*	*nt*
20	0.0 ± 0.0	0.0 ± 0.0	56.4 ± 17.0	14.9 ± 4.5
30	2.0 ± 1.5	0.0 ± 0.0	8.6 ± 4.3	0.0 ± 0.0

nd: Non determined because in most Petri dishes they were too many to be counted.

nt: Non tested

*Each treatment combination was repeated at least twice with a total number of 87 Petri dishes evaluated.

†Two grams of infected cells distributed in six Petri dishes for medium and in four Petri dishes for high cell densities.

‡Mean±standard error.

3.3.4 Establishment of transformed SCC and molecular evaluation of transgenic cell lines

Transgenic microcalli were subcultured into fresh medium monthly until enough cell material was available to launch a new SCC. A minimum amount of 500 mg of callus was used to start a cell culture in 10 mL of culture medium that was scaled up until a regular cell culture was obtained. Then, DNA was extracted from the cells and a PCR was run for each of the transgenes included in the construction (*npt*II and *eyfp*) as well as for a *virG* gene specific of *Agrobacterium* to detect any bacterial contamination. Amplification of both transgenes was obtained in all lines tested whereas no amplification of *virG* was found in any of them (Figure 3.5A). Additionally, to demonstrate transgene integration, hybridization of a probe complementary to the sequence of the *npt*II gene was used in a Southern blot (Figure 3.5B). From 10 independent lines tested, as indicated for the different integration patterns, 7 had only one band, 2 lines presented two bands and 1 line showed three bands indicating that at least 1 to 3 integrations occurred in each transgenic line (Figure 3.5C).

3.3.5 Evaluation of transgenic SCC

Four different transgenic lines and a non-transformed Monastrell control SCC were grown in the presence of different paromomycin concentrations. Control cell cultures were able to grow only in the absence of antibiotic whereas in the five paromomycin concentrations tested (from 20 to 100 mg. L^{-1}) there were not increase in the SCV, and in some cases, this parameter even slightly decreased with time. However, the four transgenic lines grew in the presence of the highest paromomycin concentrations tested (40–100 mg. L^{-1}) as the non-transformed control did without antibiotic (Figure 3.6).

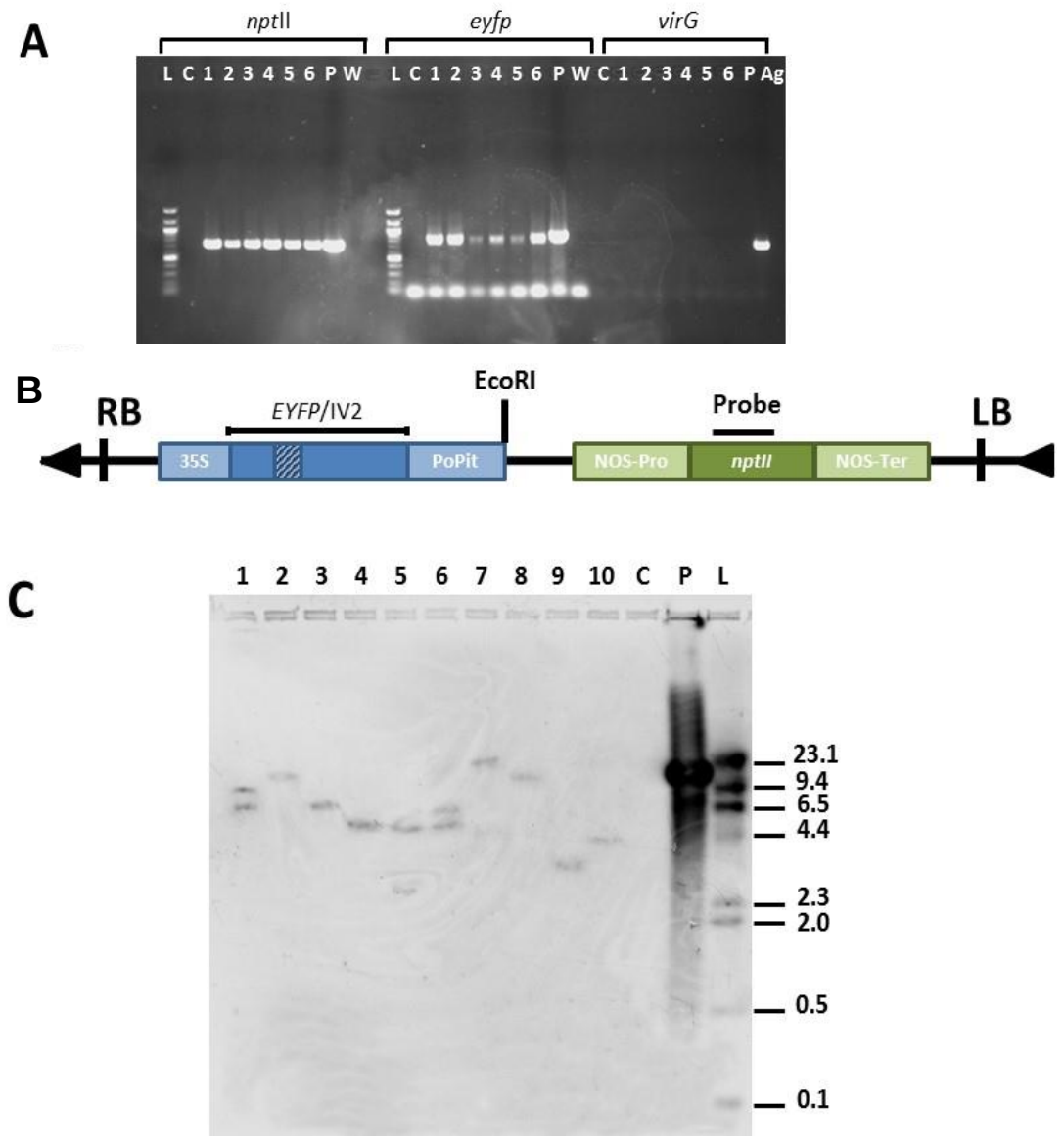


Figure 3.5 Molecular evaluation of transgenic SCC lines.

(A) PCR with primers for the amplification of fragments from *npt*II, *eyfp* and *virG* genes. L: 100bp ladder, C: wild type SCC negative control, P: pMOG800 plasmid, W: negative control, non-template sample (H_2O), Ag: DNA from *Agrobacterium*, lanes 1-6 transgenic SCC lines. (B) Representation of plasmid pMOG800 T-DNA between right (RB) and left (LB) borders, 35S and Nos promoters, PoPit and Nos terminators, EYFP/IV2, eyfp expression sequence interrupted by the IV2 plant intron, *npt*II gene. The T-DNA was digested at the EcoRI restriction site and hybridized with a probe complementary of a fragment from the *npt*II gene. (C) Southern blot to confirm integration of the T-DNA in the grapevine genome. L: digoxigenine marked ladder, P: pMOG800 plasmid, C: wild type SCC control, lanes $1^{-1}0$: transgenic SCC lines.

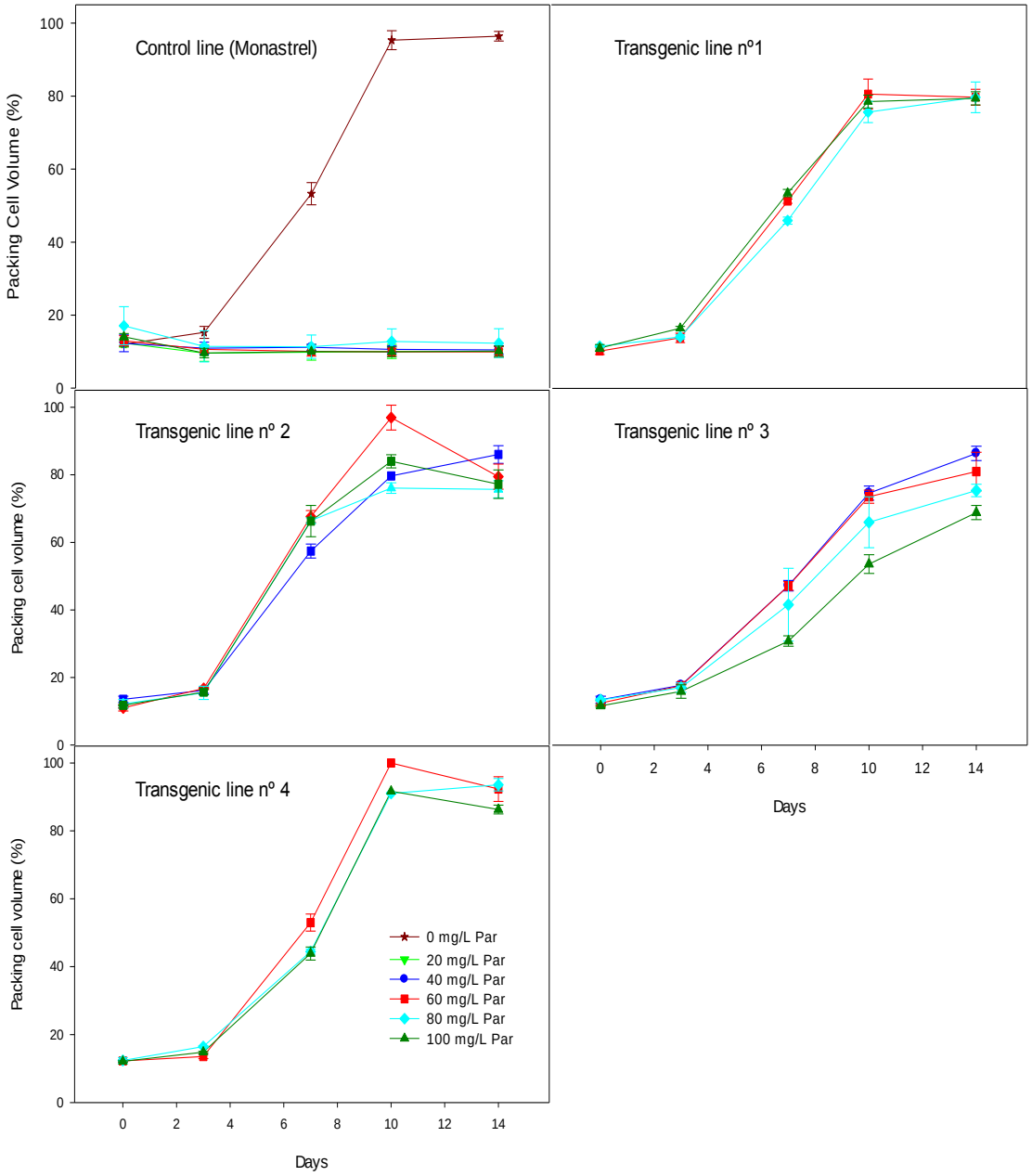


Figure 3.6 Phenotypic evaluation of transgenic SCC and a wild type SCC control. Evolution of the sedimented cell volume (SCV) of SCC with time in culture media supplemented with different paromomycin concentrations.

3.4. Discussion

The quality of grapevine, as with most plants, mainly depends on its metabolites. The production of these metabolites is especially sensitive to external conditions. In particularly, the chemical diversity of grapevine is mostly affected by secondary metabolites. These secondary metabolites consist of a wide array of species-specific chemicals and belong to different phytochemical groups such as alkaloids, terpenes, volatile oils, resins, cardiac glycosides, tannins, sterols, saponins and phenolics, many of which have proved to be of great value to the pharmaceutical, agrochemical, food and cosmetic industries. In general, secondary metabolites are known to play key physiological functions in plants including their adaptation to the environment, acquired resistance to pests and diseases, pollinator attractant capacity and the building of symbiotic relations with microorganisms. They are also very often crucial in the determination of the quality of food attributes (color, taste, and aroma) and colors and pigments of ornamental plants (Ali et al., 2010).

Differentiated cultures can be useful for studying tissue-specific biosynthetic pathways, which are not always expressed in cell suspension cultures. *In vitro* undifferentiated SCC are more convenient for the large-scale production of fine chemicals in bioreactors and for the study of cellular and molecular processes as they offer the advantage of being a simpler model system than plants. SCC contain a relatively homogeneous cell population, allowing rapid and uniform access to nutrients, precursors, growth hormones and signal compounds (Mustafa et al., 2011). The use of plant SCC is particularly promising because these biotechnologically engineered systems may represent a reliable alternative method of secondary metabolites production under controlled conditions.

To overcome the inability of *in vitro* cultured cells to synthesize high levels of secondary metabolites, different strategies have been considered: pathogen attack, environmental stresses, an exogenous supply of biosynthetic precursors or chemical elicitors and genetic manipulation (Kiselev, 2011).

Undifferentiated SCC from different plant species have been successfully transformed to produce secondary metabolites. For instance, tobacco BY-2 cell cultures

have been transformed to accumulate interleukin-10 (Bortesi et al., 2012) or *Vinca minor* cell cultures to produce an increase of the alkaloid vincamine (Verma et al., 2015) as well as other examples described by Verpoorte et al. (1999). However, to our knowledge there are not publications reporting the transformation of undifferentiated *Vitis* SCC with genes involved in the secondary metabolism, probably because there has not been an efficient transformation protocol until now.

In initial experiments, we detected a large variation within treatment and between experiments as can be noticed for the large standard errors recorded. This variability has been somehow reduced as the optimization of different parameters was done, however is still large. With optimized conditions, variability was obtained but differences between experiments were mainly the numbers of transgenic microcalli produced, whereas in initial experiments often no *eyfp*-expressing cells were seen.

One of the factors that were critical to produce more consistent results was the developmental stage of the SCC. In fact, infecting them at exponential phase, when they were actively dividing, produced much better results. This agrees with other authors that found a much larger transient transformation when cells were infected at the end of the exponential phase just before depletion of nutrients started (De la Torre et al., 2012). Other authors have used grapevine cells in the exponential phase although they did not compare with cells in other phases (Wu et al., 2012).

A significant increase was found in both, the number of *eyfp*-expressing cells and number of transgenic microcalli when SAAT treatments were applied. SAAT was first reported by Trick and Finer (1997), who used this technique for the production of transgenic soybean (*Glycine max*) and Ohio buckeye (*Aesculus glabra*) plants from SAAT-treated embryogenic cell suspensions, and transient expression of a foreign gene in different tissues of maize (*Zea mays*), cowpea (*Vigna unguiculata*), spruce (*Picea glauca*) and wheat (*Triticum aestivum*).

Plant tissue is damaged by sonication during SAAT and surface damage has been observed by scanning electron microscopy (Trick and Finer, 1997; Beranová et al., 2008), allowing the tissue to be much more easily transformed by *Agrobacterium tumefaciens* (Gaba et al., 2008). Micro-wounding probably accounts for the increase in the rate of transformation, as energy released from the cavitation of micro-bubbles

causes visible wounds within and on the tissue (Trick and Finer, 1997). Control (untreated) cells showed very low levels of transient or stable transformation. Transient *eyfp* expression increased with sonication treatment time up to 5 min, and then decreased. Larger times of exposure to ultrasound caused damage probably due to larger micro-wounds produced by the ultrasound (Gaba et al., 2008). Actually, times reported here are unusually large since long SAAT treatments reduced tissue culture responses (Trick and Finer, 1997).

To our knowledge, this is the first time that SAAT has been successfully used to improve T-DNA transfer to undifferentiated *Vitis* cell cultures to produce transgenic SCC lines. The *nptII* gene confers resistance to aminoglycoside antibiotics such as kanamycin and paromomycin. In most studies on transformation of grapevine SCC kanamycin has been the most frequently used selection agent (Scorza et al., 1996; Franks et al., 1998; Vidal et al., 2003; Dhekney et al., 2009; Wu et al., 2012; Dabauza et al., 2015) although at very diverse concentrations from 10 to 100 mg. L^{-1}, probably depending on the *Vitis* genotype. In the present study we found that Monastrell cells are almost insensitive to kanamycin and the selection was carried out with paromomycin, since this antibiotic had been previously used by other authors to select transformed grapevine tissues (Mauro et al., 1995; Wang et al., 2005). Beside amynoglicoside antibiotics, other antibiotics, such as hygromycin, have been successfully used as selective agents in *Vitis* transformation experiments (Franks et al., 1998; Saporta et al., 2014; Dai et al., 2015). Sometimes, the effect of kanamycin and paromomycin on the transformation efficiency of grapes has been compared (Wang et al., 2005) finding that, even at low concentration (10 mg. L^{-1}), paromomycin had a much quicker effect on cell death than kanamycin, although best results were obtained at 20 or 25 mg. L^{-1} of paromomycin. Similar results were reported by Mauro et al. (1995), who found that paromomycin at 5 mg. L^{-1} induced cell death in 2 or 3 days of culture in different *Vitis* cultivars. Although the concentrations of kanamycin used in this study were relatively high, were unable to inhibit growth of non-transformed cells which formed callus on the Petri dishes. A similar result was found by Kikkert et al. (1996) who reported that kanamycin inhibited embryo development but not callus growth in Chancelor embryogenic SCC, while paromomycin inhibited both callus growth and embryo development. However, Colby and Meredith (1990) found that only 20 mg. L^{-1} of

kanamycin completely inhibited callus growth in three different *Vitis* cultivars (French Colombard, Cabernet Sauvignon and Thompson Seedless), whereas 50 mg. L^{-1} was the minimal killing concentration for Albariño cell suspensions (Saporta et al., 2014), suggesting a genotype-dependent kanamycin sensitivity.

The synergistic effect of plating density on transgenic cell viability and division found in this work agrees with previous observations of our group. For instance, microcalli cannot be isolated too early and need to be large enough before transfer to new Petri dishes or they stop cell division and growth. These results agree with evidences that feeder grapevine cell layers allow plating grapevine cells and protoplasts at low density (Yamakawa et al., 1985). These and other authors' results suggest the occurrence of certain chemical substances that promote colony formation at low cell density.

In conclusion, we have developed, to our knowledge, the first transformation protocol of undifferentiated *Vitis* cv Monastrell cells which consistently produces transgenic microcalli that can be converted to transgenic SCC being able to grow in really high concentrations of selection media, as a control SCC does without antibiotics. The process developed in this study is an efficient transformation system which can be used for the introduction of genes involved in the secondary metabolism in the grapevine cells.

4. Chapter 2

A new strategy to enhance the biosynthesis of trans-resveratrol by overexpressing stilbene synthase gene in elicited Vitis vinifera cell cultures

ABSTRACT

In this work, transgenic lines of suspension cultured cells of *Vitis vinifera* cv. Monastrell containing the plasmid pMOG800-*sts* have been obtained. The cell growth of these transgenic cell lines decreased slightly as compared to non-transgenic suspension cultured cells, while cell viability was not affected. In addition, the elicitation with cyclodextrins and methyl jasmonate enhanced the production of *trans*-resveratrol, observing the highest levels of this compound in *sts*-expressing transgenic *Vitis* suspension cultured cells with the *sts* expression cassette in the forwards orientation. Moreover, the forwards 2 (F2) transgenic cell line produced the greater levels of *trans*-resveratrol in comparison with the non-transgenic cell line. In fact, when suspension cultured cells were treated with both elicitors, the accumulation of *trans*-resveratrol outside the cells in the F2 transgenic suspension cultured cells increased twice (1458 mg. L^{-1}) as compared to non-transgenic cell lines (724 mg. L^{-1}). In both cases, the levels of *trans*-resveratrol detected in the treatment with cyclodextrins and methyl jasmonate were greater than the sum of the individual treatments, and therefore we observed a synergistic effect in the presence of both elicitors. Moreover, the expression profile of *sts* gene in transgenic *V. vinifera* cell lines was similar to the expression profile detected for the endogenous *sts* gene in non-transgenic *V. vinifera* cell lines, being the expression levels greater in the treatment with methyl jasmonate and cyclodextrins, which was related to the high levels of *trans*-resveratrol found in the presence of both elicitors.

Keywords: *Agrobacterium* system; stable transformation; stilbene synthase; *trans*-resveratrol; *Vitis vinifera* cell culture.

4.1 Introduction

Vitis vinifera is able to produce stilbenes, which are derivatives of the monomeric unit *trans*-resveratrol (3,5,4´-trihydroxystilbene, *trans*-R, (Jeandet et al., 2014)). This molecule is biosynthesized from phenylalanine which is converted to cinnamic acid by the action of phenylalanine ammonia lyase (PAL). The cinnamic acid is transformed to 4-coumaroyl-CoA in two reactions catalyzed by cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL). Then, stilbene synthase (STS) is able to condense three

malonyl-CoA units with one molecule of 4-coumaroyl-CoA to form *trans*-R (Wang et al., 2015).

Usually, *trans*-R and others stilbenes are biosynthesized in grapevine tissues in specific developmental stages (Hall and De Luca, 2007), and as a defense response against certain kinds of stresses (Pezet et al., 2004; Bru et al., 2006; Adrian and Jeandet, 2012). Besides its phytoalexin activity, *trans*-R has also been postulated to have beneficial effects on human health (Siemann and Creasy, 1992), becoming one of the most widely bioactive compounds studied. In fact, several studies have been devoted on the beneficial effects of *trans*-R on cardiovascular diseases (Bradamante et al., 2004), neurological system (Okawara et al., 2007), preventing carcinogenesis (Vang et al., 2011; Fernández-Mar et al., 2012), and obesity disorders (Baur et al., 2006), amongst others.

The signaling molecules related with defense responses can also be used in *Vitis* cell cultures to enhance *trans*-R production, the most commonly used being a JA-active derivative, MJ (Tassoni et al., 2005; Kiselev et al., 2007; Belhadj et al., 2008; Donnez et al., 2011) and SA (Kiselev et al., 2007; Laura et al., 2007). JA and MJ act as key compounds of the signal transduction pathway involved in the induction of the secondary metabolite biosynthesis which takes part in plant defense reactions (Creelman and Mullet, 1997; Chung et al., 2003). Thus, the production of secondary metabolites increases when plant cell cultures are elicited with JA and derivatives (Vasconsuelo and Boland, 2007; Sabater-Jara et al., 2010). In *Vitis*, *trans*-R and stilbene-related production is increased by adding JA or MJ (Tassoni et al., 2005; Belhadj et al., 2008). Actually, *V. vinifera* SCC treated with CD and MJ is a more efficient system than CD treatment alone for the production of *trans*-R (Bru et al., 2006; Belchí-Navarro et al., 2012; Almagro et al., 2014).

Due to the high value of this compound, great effort has been made to produce it in high amounts. Amongst the different strategies to obtain high levels of *trans*-R, the use of elicited suspension-cultured cells (SCC) of *V. vinifera* is considered a successful procedure to increase its productivity. In fact, the most significant success in increasing *trans*-R content from *Vitis* cell cultures has been reached using CD. CD are a family of compounds made up of glucose molecules bound together in a ring (cyclic

oligosaccharides), and they are produced from starch by enzymatic conversion. They have been used in the food, pharmaceutical, and chemical industries, as well as in agriculture and environmental engineering, since they are able to solubilize non-polar compounds in aqueous media. A typical CD contains several glucose monomers ranging from six to eight units in a ring, forming a cone shape. β-CD, which are seven-sugar ring molecules, have often been used for increasing *trans*-R in *Vitis* cell cultures (Morales et al., 1998; Bru et al., 2006). Under CD elicitation, grapevine cell cultures produced high levels of *trans*-R, which is secreted and accumulated outside the cells. This effect is due to the fact that CD chemically resemble the alkyl-derived pectic oligosaccharides released from the cell walls when a fungal attack is produced (Bru et al., 2006) and grapevine cells respond to their presence by synthesizing *trans*-R. Therefore, CD act not only as inducers of *trans*-R biosynthesis but also as promoters of complexes that remove *trans*-R from the culture medium, reducing both feedback inhibition and *trans*-R degradation, and allowing its accumulation in high concentrations (Almagro et al., 2011; Almagro et al., 2013).

This innovative procedure allows the direct extraction of this compound from the culture medium without biomass destruction (Belchí-Navarro et al., 2012). In relation to this work, a rational approach was carried out studying the global transcriptomic profiles in *V. vinifera* SCC elicited with CD and/or MJ using the GrapeGen microarrays from Affymetrix (Almagro et al., 2014). The elicitation with both elicitors strongly increased the expression of genes upstream of the *trans*-R biosynthetic pathway like shikimate kinase, chorismate synthase and mutase, and prephenate dehydratase, amongst others. In addition, some genes of the phenylpropanoid pathway were extensively up-regulated in *V. vinifera* SCC elicited with CD alone or in combination with MJ such as *pal, c4h, 4cl,* and *sts* genes (Almagro et al., 2015). Therefore, the overexpression of the *sts* genes could be a promising approach for increasing the biosynthesis of *trans*-R in *V. vinifera* SCC. In this study, a construct harboring a *sts* gene driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter, was introduced into *V. vinifera* SCC. From all the *sts* genes analyzed from the transcriptomic profile, which were over-expressed in the treatment with CD and/or MJ (Almagro et al., 2014), we have chosen specifically that *sts* gene which showed a high expression when

V. vinifera SCC were treated with CD alone or in combination with MJ, and this was used to transform *V. vinifera* SCC.

In this study, we have analyzed both the influence of CD and/or MJ on the production of *trans*-R in the engineered *V. vinifera* SCC, and the expression levels of *sts* gene in all treatments after 6, 24 ,72 and 144 hours of elicitation.

4.2 Material and Methods

4.2.1 Plant material

Vitis vinifera L. cv Monastrell calli were established and maintained in our laboratory as described by Calderón et al. (1993). *V. vinifera* SCC were initiated by transferring 20 g of friable callus into 250 mL flasks which containing 100 mL of Gamborg B5 liquid medium (Gamborg et al., 1968), and maintained in a rotary shaker at 110 rpm. These SCC were periodically subcultured every 8 days.

4.2.2 Determination of cell viability

In order to measure the cell viability, a small aliquot of SCC was incubated with culture medium containing 0.01 % (p/v) propidium iodide (Sigma-Aldrich, Spain) to detect dead cells as described by Almagro et al. (2015), and 0.1 % (p/v) neutral red (Sigma-Aldrich, Spain) for the selective labelling of live cells. Cell viability was analyzed with an optical microscope as described by Almagro et al. (2015).

4.2.3 Construction of transformation vectors

The plasmid pMOG800-*sts* was constructed by cloning cDNA from *sts* (GeneBank accession number XM_002264419.2) under the control of a CaMV 35S promoter in an *Eco*RI position between the selectable marker gene *npt*II driven by the *nos* promotor and the visual marker *eyfp*/IV2 gene (Figure. 4.1C). The plasmid was introduced in *Agrobacterium tumefaciens* (recently renamed to *Rhizobium radiobacter*) strain LBA4404.

4.2.4 *Agrobacterium* culture and transformation procedure

Agrobacterium culture and transformation of SCC was performed as described by Chu et al. (2016). Briefly, a bacterial colony was diluted in 10 mL yeast extract-mannitol liquid medium which contained 50 mg. L^{-1} kanamycin and 50 mg. L^{-1} streptomycin and incubated at 28 ºC overnight in the dark on a rotary shaker at 140 rpm. The bacterial culture was scaled up to 30 mL in Erlenmeyer flask from an optical density of 0.1 (OD_{600}) to 0.6-0.9 by incubating for more than 10 h. Then the bacterial culture was centrifuged at 3800 x *g* for 15 min, and the pellet re-suspended in 30 mL of GB5-1 (Chu et al., 2016). The suspension of *Agrobacterium* supplemented with 100 μM acetosyringone was cultivated as described by Chu et al. (2016). The cell transformation was carried out as described by Chu et al. (2016). The callus expressing *eyfp*/IV2 was relocated onto fresh semi-solid culture medium which contained paromomycin (20 mg. L^{-1}) and cefotaxime (300 mg. L^{-1}).

4.2.5 Molecular analysis of transgenic cell lines

Aliquots obtained from transgenic SCC were collected and centrifuged at 12500 *g* for 10 min. Then, 200 mg cells from the pellet were used for extracting genomic DNA as described by Japelaghi et al. (2011). In this study, specific primers have been used for the amplification of a 700 bp fragment from the *npt*II gene (*nptII*-F: 5'-gaggctattcggctatgactg-3'and *nptII*-R: 5'-atcgggagcggcgataccgta- 3'), an 856 bp fragment from the *eyfp*/IV2 gene (*eyfp*-F: 5'-atggtgagccaagggcgagga-3' and *eyfp*-R: 5'-ggaccatgtgatcgcgcttc-3'), and a 430 bp fragment from the 35S promoter to the *sts* gene (35S-F: 5'-ctctagaactagttatggcttcagttga-3'and *sts*-R: 5'-gcaccaggcatttctacacc- 3'). The reactions for *npt*II and *eyfp*/IV2 fragments amplification were carried out as described by Chu et al. (2016) while the amplification reaction for the 35S-*sts* fragment consisted of a denaturation step at 95 ºC for 3 min, followed by 35 cycles of 30 s at 35 ºC, 45 s at 58 ºC and 1 min at 72 ºC, followed by final extension at 72 ºC for 5 min.

For Southern blot hybridization, 20 μg of genomic DNA was digested with *EcoR*I, and the fragments were separated using a 1.0 % agarose gel and transferred to a positively-charged nylon membrane. DNA was hybridized at 42℃ in DIG-Easy buffer with a digoxigenin-labeled *eyfp*/IV2 probe, and the probe was obtained by PCR amplification with the specific primers as described above. The blots were performed as

described by Chu et al. (2016), and the detection was made with the chemiluminescent substrate CDP-Star (Roche GmbH, Mannheim, Germany), following the manufacturer's instructions.

4.2.6 Elicitation of *V. vinifera* suspension cultured cells

Elicitation experiments were made using 7 days old *V. vinifera* SCC. For that, 4 g of fresh weight (FW) of cells were added into 100 mL flask containing 20 mL culture medium supplemented with 100 µM MJ and/ or 50 mM CD for 144 h as described by Belchí-Navarro et al. (2012). After elicitation, cells and culture medium were used for *trans*-R quantification.

4.2.7 Quantification of trans-resveratrol in both culture medium and cells

The levels of *trans*-R in the spent medium were analyzed in a HPLC-DAD (Waters 600E, Waters 996) as described by Belchí-Navarro et al. (2012). In addition, the levels of *trans*-R inside the cells were also analyzed using a HPLC-DAD (Waters 600E, Waters 996) as described by Almagro et al. (2015). *trans*-R was identified and quantified by comparison with authentic standard (Sigma-Aldrich, Spain).

4.2.8 Gene expression analyses

4.2.8.1 RNA isolation, cDNA synthesis and real-time quantitative PCR

Elicited cells, as described in 2.6, were harvested from *in vitro* transgenic SCC as well as from non-transformed controls at different times after elicitor treatments (6, 24, 72 and 144 h). RNA was extracted from transgenic and non-transformed cells using Trizol Reagent (Invitrogen) following manufacter's instructions. RNAs were digested with DNase I (DNA-free Kit, Ambion, Austin, TX, USA) and quantified with a spectrophotometer Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, USA). cDNA was synthesized using RETROscript cDNA Synthesis Kit. The expression levels of *sts* gene (both endogenous and transgenic) were normalized with those of an elongation factor *ef1-α* gene (VIT_06s0004g03240) by RT-PCR using the GeneAmp 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA), and the SYBR Green Master Kit (Applied Biosystems). Primers used in this study for the *sts* gene were *sts*-F: 5'-cgccaggagataatcactgct-3'and *sts*-R: 5'-gcaccaggcatttctacacc- 3'.

They were used at final concentration of 300 nM with 2 µl cDNA and 1×SYBR Green. The steps of RT-PCR were: denaturation at 95 ºC for 10 min, amplification in a two-step procedure: 15 s of denaturation and 1 min of annealing and extension at 54ºC for endogenous *sts* and *ef*1-*α* for 40 cycles. Transcripts levels were expressed as relative increases to the non-elicited wild type control.

4.3 Results and discussion

4.3.1 Establishment of transgenic *V. vinifera* cv Monastrell SCC

The transformation protocol developed (Chu et al., 2016) has allowed the production of a large number of transgenic lines after infection of *V. vinifera* cv. Monastrell SCC with *A. tumefaciens* LBA4404 harbouring the plasmid pMOG800-*sts*. We regenerated cell lines with the *sts* expression unit cloned in a unique restriction site in the two possible directions: 35S promotor-*sts*-*Nos* terminator from right to left border (F) or the other way around (R). Therefore, we obtained two constructs which were used for transformation experiment to produce transgenic lines (Figure 4.2.1). In selection medium, the transformed callus grew vigorously. Expression of e*yfp*/IV2 visual marker was used in order to detect green fluorescence by examination under ultraviolet light of *V. vinifera* callus. Then, we initiated *V. vinifera* SCC with transformed callus which were used to perform the elicitation with CD and/or MJ. The presence or absence of transgenes in F and R transgenic cell lines was studied by PCR (Figure 4.1B). In order to confirm the integration of the transgenes, some PCR-positive transgenic lines were chosen for the analysis of Southern blot using an *eyfp*/IV2 probe (Figure 4.1C). Here, we have used *Eco*RI to digest DNA, and therefore one band should be predictable for each T-DNA integration. The results showed in Figure 4.1C indicated that a single integration pattern was detected in one line (lane 4), whereas multiple insertions of the transgene were found in the other three lines studied (Figure 4.1C, lanes 1, 2 and 3). However, in the non-transformed SCC no signal was found (Figure 4.1C, lane NC).

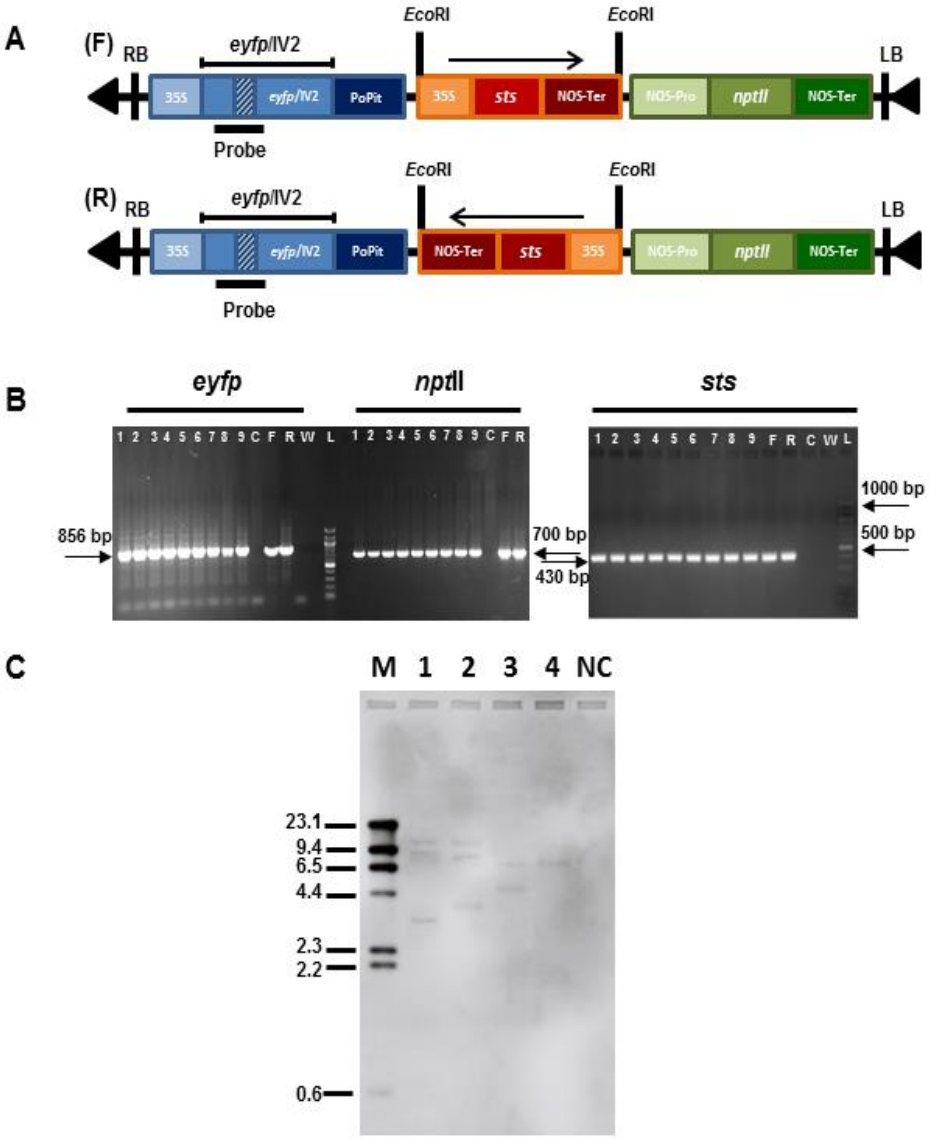
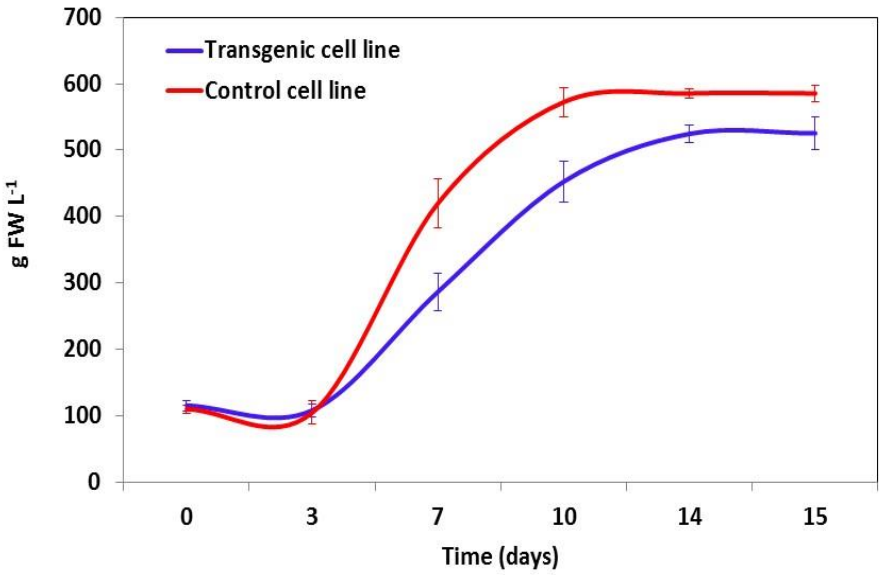


Figure 4.1 Molecular evaluation of transformed cell lines. (A) Representation of plasmid pMOG800-*sts*. Forward orientation (F) and Reverse orientation (R). (B) PCR with primers for the amplification of fragments from *npt*II, *eyfp*/IV2 and *sts* genes. L: 100 bp ladder, C: wild type SCC negative control, F and R: plasmids, W: negative control, non-template sample (water), lanes 1-9 transgenic cell lines. (C) Southern blot analysis of e*yfp* PCR-positive cell lines (lanes 1-4). M: Lambda *Hind*III Dig-labelled molecular marker (kb). NC: negative control (non-transformed cells).

4.3.2 Characterization of cell growth in both transgenic and non-transgenic V. vinifera cell lines

Once the SCC were well-established and prior to perform elicitation treatments, we characterized the cell growth in both transgenic and non-transgenic *V. vinifera* SCC. Non-transgenic *V. vinifera* SCC exhibited a typical S-shaped growth curve (Figure 4.2). The initial cell density was 100 g. L^{-1} which increased up to 585 g. L^{-1} at the end of the growth curve. In this curve, three growth phases were distinguished: the lag phase from the first to the third day of cultivation, the exponential phase from the fourth to the ninth day of cultivation and the stationary phase from the tenth to the fifteenth day of cultivation. With respect to the kinetic parameters T_a, which is the time when the decrease of nutrients provoke stress and cell death, was 10 days.



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On the other hand, when transgenic *V. vinifera* SCC were grown for 15 days, there was a small decrease of cell growth as compared to non-transgenic *V. vinifera* SCC (Figure 4.2). In fact, the growth curve was initiated with the same cell density as the control SCC (Figure 4.2) and the maximal biomass reached was of 520 g. L^{-1}, and T_a was 14 days (Figure 4.2). In contrast to our results, Martínez-Márquez et al. (2015) observed that transgenic *V. vinifera* SCC transformed with GFP had the same cell growth as the non-transformed cell line. An increase on cell growth was observed in cell

lines of *V. amurensis* with overexpression of *Vasts7* gene as compared with control cells (Kiselev and Aleynova, 2016). Therefore, the effect of gene overexpression on cell growth in *Vitis* SCC depends on gene type used for its transformation, the species used and the culture conditions.

On the other hand, although a slight decrease on cell growth was detected in transgenic *V. vinifera* cell lines, cell viability was not altered (Figure 4.3) and no significant differences were detected in the morphology of non-transgenic *V. vinifera* cell lines (Figure 4.4A) or transgenic *V. vinifera* cell lines (Figure 4.4B).

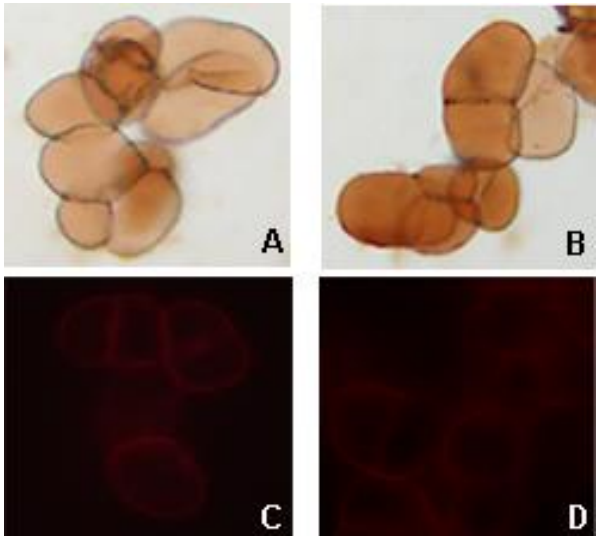

Figure 4.3 Cell viability (assessed by neutral red and propidium iodide as described in Material and Methods) in elicited *V. vinifera* SCC for 144 h. A and C) non-transgenic cells; B and D) transgenic cells.

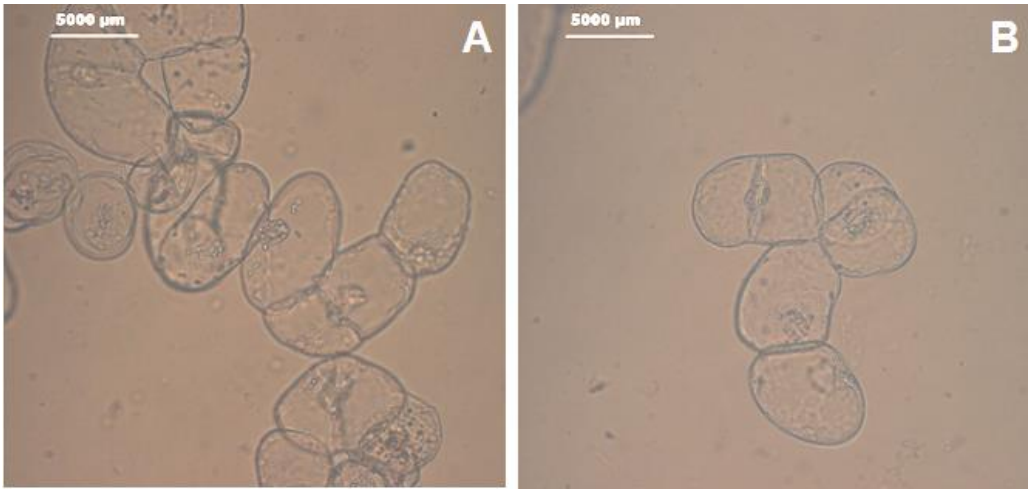

Figure 4.4. Cell morphology in *V. vinifera* SCC. A) non-transgenic cells; B) transgenic cells.

4.3.3 Effect of sts gene overexpression on *trans*-resceratrol production in transgenic and non-transgenic V. vinifera cell lines treated with cyclodextrins and/or methyl jasmonate

CD can act as elicitors in different plant SCC because they are able to induce the biosynthesis of secondary metabolites in different SCC (Almagro et al., 2012; Briceño et al., 2012; Almagro et al., 2014) and its combination with MJ led to high increases in the production of *trans*-R in *V. vinifera* SCC (Lijavetzky et al., 2008; Belchí-Navarro et al., 2012). For this reason, we analyzed the effect of CD and/or MJ on extracellular *trans*-R production in transgenic and non-transgenic *V. vinifera* SCC after 144 h of cultivation (Figure 4.5).

Twenty independent transgenic SCC from each of the two different orientations (F and R) were evaluated in the presence of CD and MJ separately or in combination. Figures 4.5A and B show extracellular *trans*-R production of all F and R transgenic SCC overexpressing the *sts* gene normalized with respect to the levels of production of non-transgenic SCC. As can be seen in Figure 4.5A, the addition of CD and MJ to F1, F2, F3, F4, F9, F10 and F12 transgenic SCC provoked a significant enhancement of the extracellular biosynthesis of *trans*-R while the levels of *trans*-R in CD-transgenic SCC were lower or in the same order than those found in CD-non-transgenic SCC. However, using the second orientation (R), only a significant enhanced *trans*-R production was observed in the R4, R5 and R11 transgenic SCC treated with both elicitors (Figure

4.5B). Moreover, the highest levels of *trans*-R were detected in *sts*-expressing transgenic *Vitis* SCC with the *sts* expression cassette in the F orientation (RB-35S-*sts*-*nos*-LB) (Figure 4.5A). The F2 transgenic SCC produced the greater amount of *trans*-R in comparison with the non-transgenic SCC (803 mg. L^{-1}, Figure 4.5A). For this reason, we selected this F2 cell line to analyze the levels of *trans*-R both inside and outside the cells at 144 h. As shown in Figure 4.6A, no extracellular *trans*-R was found in control and MJ-treated SCC, and not significant differences were observed on *trans*-R production when non-transgenic and F2 transgenic SCC were elicited with CD alone (Figure 4.5A, around 480 mg. L^{-1}). In contrast, the addition of CD and MJ increased the accumulation of *trans*-R in the spent medium of F2 transgenic SCC increased twice (1458 mg. L^{-1}) as compared to non-transgenic SCC (724 mg. L^{-1}, Figure 5A). In both cases, the levels of *trans*-R produced when SCC were treated with both elicitors was greater than the sum of the individual treatments, and therefore we detected a synergistic effect on *trans*-R production in the presence of CD and MJ in both non-transgenic and F2 transgenic SCC. Similar results were found by Almagro et al. (2014) and Lijavetzky et al. (2008) who observed a synergistic effect on *trans*-R production in the presence of CD and MJ in non-transgenic *V. vinifera* SCC.

Although this is the first study on the production of *trans*-R in elicited transgenic *V. vinifera* SCC, there are some research which determine the levels of *trans*-R in transgenic plants (Kiselev, 2011). Coutos-Thévenot et al. (2001) showed that the maximal levels of *trans*-R (around 350 μg. g dry $weight^{-1}$ (DW)) were found in plants of 41B rootstock transformed with a chimeric gene which contained *sts1* and an alfalfa PR10 promoter. Similarly, an increase of *trans*-R levels (400 μg. g^{-1} FW) was observed in transgenic plants of *Nicotiana tabacum* which overexpressing *sts1* and *sts2* (Hain et al., 1993).

On the one hand, the production of plant metabolites in transformed plant *in vitro* cultures can be increased using *rol* genes located in R_i-plasmids of *A. rhizogenes* (Palazón et al., 1998). Thus, Kiselev et al. (2007) observed that the *rolB* gene increased both the expression levels of *sts* and *pal* genes as well as the levels of *trans*-R (31.50 mg. g^{-1} DW) in callus cultures of *V. amurensis*. More recently, Kiselev and Aleynova (2016) have obtained transgenic *V. amurensis* SCC with overexpression of the *VaSTS7* gene, observing that this overexpression increased the *trans*-R content in transgenic

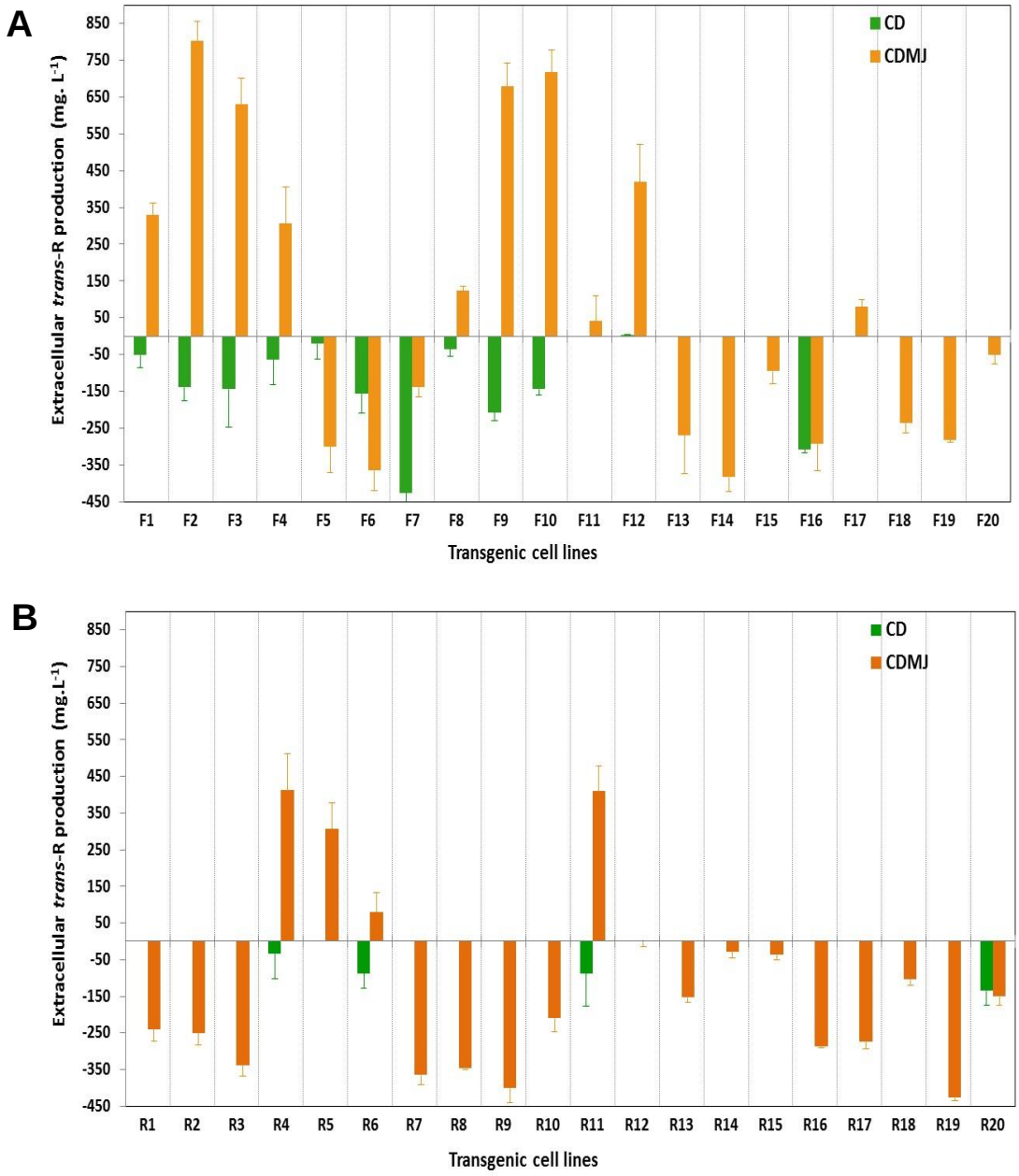

Figure 4.5 Effect of 50 mM CD separately or in combination with 100 µM MJ on extracellular *trans*-R production. A) F transgenic *V. vinifera* SCC after 144 h of cultivation (35S promotor-*sts*-*Nos* terminator from right to left border); B) R transgenic *V. vinifera* SCC after 144 h of cultivation (35S promotor-*sts*-*Nos* terminator from left to right border). Data were normalized with the *trans*-R production obtained from control cell lines. Experiments were repeated three times. Data are the mean ± SD of the replicates.

SCC until 6 times as compared with non-transgenic SCC, as occurs in our cultures. However, our total *trans*-R levels are higher than those found in 41B rootstock *Vitis* plants, *N. tabacum* plants, *V. amurensis* callus or *V. amurensis* SCC, indicating that our transgenic *V. vinifera* SCC treated with CD and MJ could be used as a successful system for increasing *trans*-R production in a more environmentally friendly and economical way.

On the other hand, the maximal levels of *trans*-R were detected in CD-treated cells (8.36 ± 0.69 µg. g FW^{-1} that means 1.67 mg. L^{-1}) or CD and MJ-treated cells (4.95 ± 1.20 µg. g^{-1} FWthat means 0.99 mg. L^{-1}) after 144 h of treatment in F2 transgenic cell line (Figure 4.6B). It was almost twice as high as the maximal values found in the non-transgenic SCC in the same conditions (3.16 ± 0.31 µg. g FW^{-1} that means 0.63 mg. L^{-1} and 3.22 ± 0.17 µg. g^{-1} FW that means 0.64 mg. L^{-1} in CD and CD+MJ-elicited SCC, respectively). Moreover, the intracellular levels of *trans*-R found when SCC were elicited with CD and/or MJ after 144 h of cultivation (Figure 4.6B) was three order of magnitude lower than the extracel|u|ar levels found in culture medium (Figure 4.6A). This is due to CD form inclusion complexes with *trans*-R, allowing its release and accumulation outside the cells (Belchí-Navarro et al., 2012).

The instability of SCC during long successive subcultures is an often found and widely recognized phenomenon. However, there are notable exceptions as the production of shikonin and berberine in *Lithospermum erythrorhizon* and *Coptis japonica* SCC, respectively (Whitmer et al., 2003). Similarly, we did not detect a decrease in the production of *trans*-R in our experiments during 24 months of continuous subculturing (data not shown). Moreover, (Dubrovina and Kiselev, 2012) also showed that the decrease in biosynthetic capability of *trans*-R of *V. amurensis* transgenic calli can be reversed using MJ or SA.

4.3.4 Effect of cyclodextrins and methyl jasmonate separately or in combination on the expression of endogenous and transgenic *sts* gene in transgenic and non-transgenic *V. vinifera* cell lines

The expression of the endogenous *sts* gene was determined in non-transgenic *V. vinifera* SCC treated with CD and/or MJ after 6, 24, 72 and 144 h of treatment

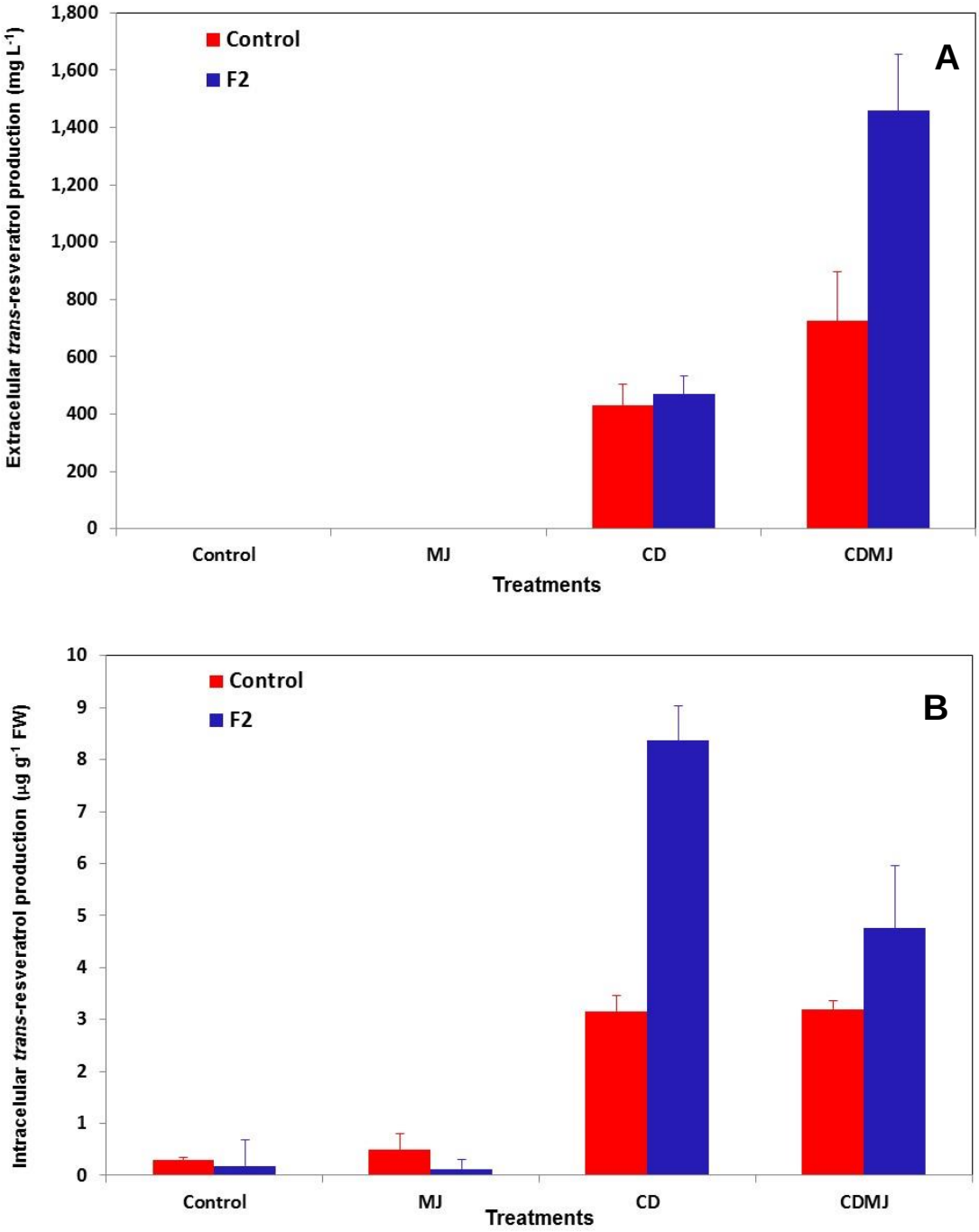

Figure 4.6 Effect of 50 mM CD and/or 100 µM MJ on extracellular production of *trans*-R (A) and intracellular production of *trans*-R (B) in control and F2 transgenic *V. vinifera* SCC after 144 h of cultivation. Experiments were repeated three times. Data are the mean ± SD of the replicates.

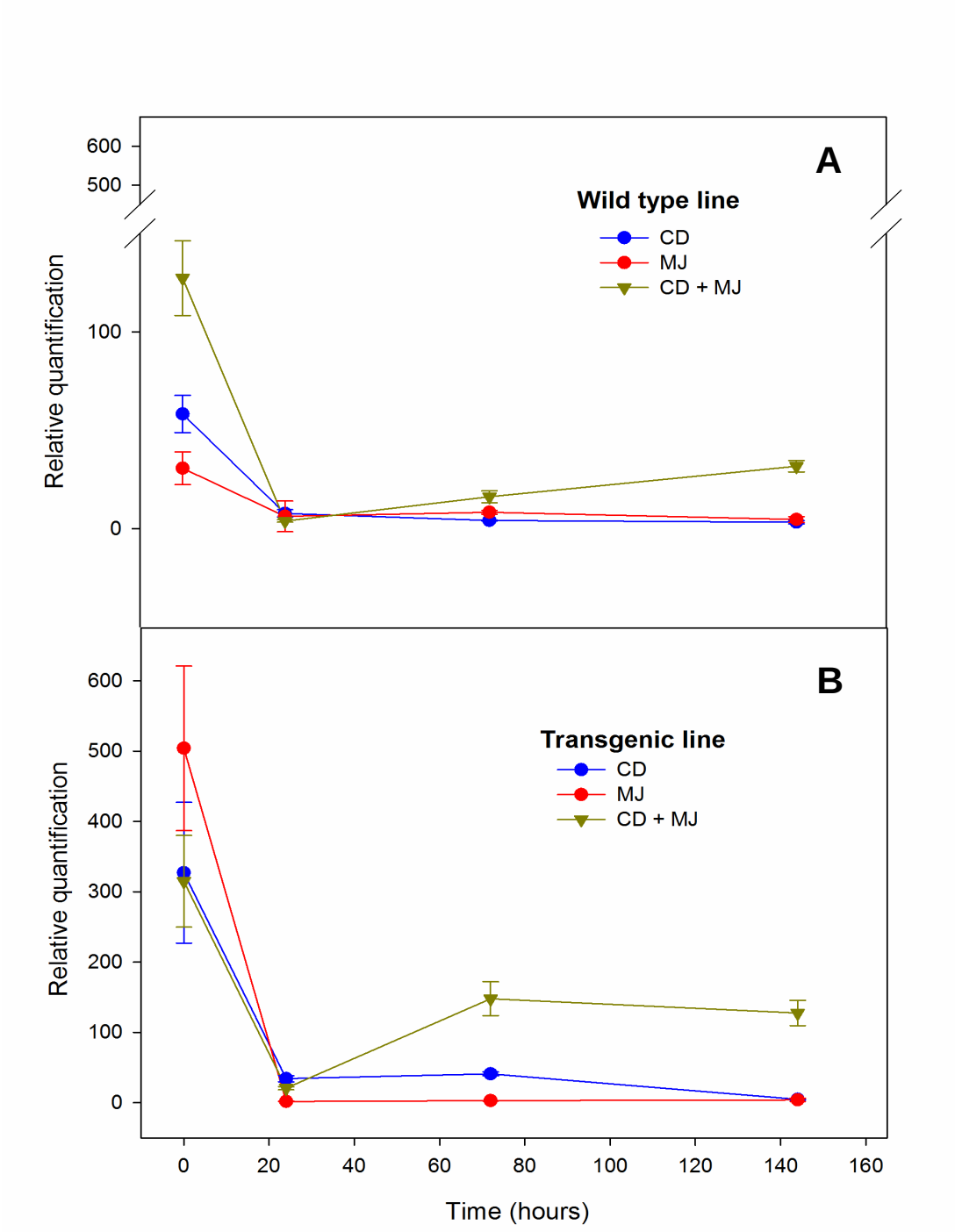

Figure 4.7 Relative quantification of endogenous (A) and transgenic (B) expression of *sts* gene in *V. vinifera* SCC treated with CD, MJ or CD+MJ. The levels of *sts* gene expression were analyzed by real-time quantitative PCR. Levels of transcripts were calculated using the standard curve method with grapevine *ef*α1 gene as internal control. The values were normalized with respect to the expression of control cells. Experiments were repeated three times. Data are the mean ± SD of the replicates.

(Figure 4.7A). As can be observed from Figure 4.7A, *sts* gene was induced by treatment with CD and/or MJ. The expression of the *sts* gene was synergistically increased by the presence of CD and MJ at 6 h of cultivation while the expression values with MJ or CD alone were similar. Levels of *sts* transcripts decreased at 24 h of cultivation and after that there was a slight but progressive increase when elicited simultaneously with CD and MJ until 144 h (Figure 4.7A). Moreover, the expression profile of *sts* gene (endogenous + transgenic) in the F2 transgenic *V. vinifera* SCC (Figure 4.7B) was similar to the expression profile detected for the endogenous *sts* gene in non-transgenic *V. vinifera* SCC (Figure 4.7A), although the levels of *sts* expression were much higher in transgenic lines. In fact, the expression levels of *sts* gene in transgenic cell line were higher at 6 h of cultivation in all treatments tested and then, quickly decreased. In addition, *sts* gene expression was measured in elicited conditions with the simultaneous application of CD and MJ increasing its levels until 72 h of cultivation and then, it was maintained until the end of the experiment. The high levels of *sts* gene expression were directly related to *trans*-R biosynthesis in non-transgenic and transgenic cell lines, and therefore, correlated to defense responses. In agreement with our results, Lijavetzky et al. (2008) and Pietrowska-Borek et al. (2014) also found high levels of *sts* transcripts levels in elicited *V. vinifera* SCC with CD.

4.3.5 Conclusions

In conclusion, our results show that transgenic SCC of *V. vinifera* cv. Monastrell with contain the plasmid pMOG800-*sts* treated with CD and MJ produce high levels of *trans*-R cells, being the concentration of *trans*-R in transgenic cell lines higher than in wild type cell lines. Therefore, elicited transgenic SCC of *V. vinifera* cv. Monastrell could be used for a sustainable high-level production of *trans*-R.

5. Conclusions

Conclusions

We have developed for the first time, a transformation protocol to be applied to undifferentiated *Vitis vinifera* cell cultures which is based on the use of a Sonication-Assisted *Agrobacterium*-mediated Transformation method and on the optimization of all the factors which control the transformation efficiency (specifically plating cell density and the selection media with antibiotics). The efficient transformation system developed in this study could be used for the introduction of genes involved in the secondary metabolism in the grapevine cells.

Using this protocol, we have obtained more than 50 transgenic suspension-cultured cells of *Vitis vinifera* cv Monastrell which contain the plasmid pMOG800-eyfp-*sts*. Using elicitation with cyclodextrins and/or methyl jasmonate as the second strategy to improve the production of *trans*-resveratrol, we have obtained a transgenic cell line which produced high levels of *trans*-resveratrol, being its extracellular concentration higher than those found in non-transgenic cell lines. Therefore, elicited transgenic suspension-cultured cells of *Vitis vinifera* cv Monastrell could be used for the sustainable production of a high-level of *trans*-resveratrol.

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