**Title**

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

**Authors**

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**Abreviations**

CA coniferyl alcohol

Cd methylated β-cyclodextrins

CHS chalcone synthase

MJ methyl jasmonate

Sm silymarin

STS stilbene synthase

*t*-R *trans*-resveratrol

Tx taxifolin

**Abstract**

The growing demand for *t*-resveratrol for industrial uses has generated considerable interest in its production. Heterologous resveratrol production in plant cell suspensions, apart from requiring the introduction of only one or two genes, has the advantage of high biomass yield and a short cultivation time, and thus could be an option for large scale production. *Silybum marianum* is the source of the flavonolignan silymarin. Phenylpropanoid synthesis in cultures of this species can be activated by elicitation with methyl jasmonate and methylated β-cyclodextrins, with products of the pathway (coniferyl alcohol and some isomers of the silymarin complex) being released to the medium. Given that stilbene synthase shares the same key precursors involved in flavonoid and /or monolignol biosynthesis, we explored the potential of metabolically engineered *S. marianum* cultures for *t*-resveratrol production. Cell suspensions were stably transformed with *Vitis vinifera* stilbene synthase 1 and the expression of the transgene led to extracellular *t*-resveratrol accumulation at the level of milligrams per litre under elicitation. Resveratrol synthesis occurred at the expense of coniferyl alcohol. Production of silymarin was less affected in the transgenic cultures, since the flavonoid pathway is limiting for its synthesis, due to the preferred supply of precursors for the monolignol branch. The fact that the expressed STS gene took excessively produced precursors of non-bioactive compounds (coniferyl alcohol), while keeping the metabolic flow for target secondary compounds (i.e. silymarin) unaltered, opens a way to extend the applications of plant cell cultures for the simultaneous production of both constitutive and foreign valuable metabolites.

## 1 Introduction

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

Lignin, stilbene and flavonoid synthetic pathways share the same precursor molecules (Fig.1). The first step in phenylpropanoid biosynthesis is the deamination of L-phenylalanine to *t*-cinnamic acid, catalyzed by phenylalanine ammonia lyase (EC 4.3.1.5). Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (EC 1.14.13.11) to give 4-coumaric acid, which is then activated by 4-coumaroyl:CoA ligase (EC 6.2.1.12) and the resulting 4-coumaroyl-CoA enters the flavonoid, stilbenoid and monolignol pathway.

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

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

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

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

The first gene transfer experiments were performed with a complete STS gene from *Arachis hypogea* introduced into tobacco [13], leading to *t*-R accumulation after induction

with short-wavelength ultraviolet (UV) light, a well-known elicitor of *t*-R synthesis [14]. It has also been shown that the heterologous expression of two grapevine STS genes, *Vst1* and *Vst2*, in tobacco confers a higher resistance to *Botrytis cinerea* infection [15]. Since this pioneering work, STS genes have been transferred to a number of crops, as well as to yeast and bacteria [6].

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

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

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

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

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

## 2 Materials and methods

### 2.1 VvSTS3 cloning and construction of the binary vector

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

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

### 2.2 Plant and bacterial culture

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

*A.tumefaciens* C58C1 (pGV2260) containing the binary plasmid was grown on solid LB medium at 28 ºC for 48 h. A single colony was inoculated in 50 ml of liquid LB medium

that contained 100 mg $l^{-1}$ rifampicyn and 75 mg $l^{-1}$ spectinomycin, and was incubated overnight at 28 ºC with shaking (180 rpm). 50 ml of LB medium, supplemented with 75 mg $l^{-1}$ spectinomycin, was inoculated with 1 ml of the 1-day-old culture and grown at 28 ºC and 180 rpm overnight. The bacterial medium was removed by centrifugation at 14,000 rpm for 3–5 min and the resulting bacterial pellet was washed and suspended in MSS medium.

### 2.3 Stable transformation of suspension cultures

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

In brief, exponentially growing cell suspensions (3 days after subculture) were supplemented with 200 mg $l^{-1}$ acetosyringone and co-cultured with the bacteria suspended in plant liquid medium (final bacterial $OD_{600}$ in co-culture 0.1). The *Agrobacterium*-infected culture was incubated in darkness at 25 °C at 100 rpm for 2 days. The infected cell culture was then centrifuged at 3000 rpm for 5 min and the pellet was washed three times with fresh culture medium.

Due to the high proportion of non-transformed escapes to kanamycin when cell suspensions were employed as plant material reported in the literature [30, 31], we previously checked the resistance of *S.marianum* cells to this antibiotic and percentages of viability up to 80% were scored even at 500 mg $l^{-1}$. When testing paromomycin, however, cell viability was lost at 50 mg $l^{-1}$ (personal observations). Therefore, for the selection of transformants, the infected cell suspension (2.5 ml) was spread on petri dishes containing solid (10 mg $l^{-1}$ agar) MSS medium supplemented with 300 mg $l^{-1}$ cefotaxime and 60 mg $l^{-1}$ paromomycin. Plates were incubated at 25°C in the dark. Periodical subcultures of the growing callus colonies were carried out at decreasing cefotaxime concentrations. After 3 months, cefotaxime was eliminated from the medium, and the transformed cells were allowed to grow in the MSS selection medium with 60 mg $l^{-1}$ paromomycin.

To re-establish liquid cultures, callus tissue was transferred to fresh MSS medium (with 60 mg $l^{-1}$ paromomycin.) and incubated under normal cultivation conditions. Subculture was repeated two times at 14-day intervals in the same medium. Subsequent subculturing was carried out into fresh MSS medium (without paromomycin).

### 2.4 Elicitation and metabolite analyses

For the elicitation experiments, 3 g wet weight 14-day old cells were transferred to 100 mL flasks containing 20 mL medium and incubated for three days prior to the addition of 30 mM final concentration Cd (chemically pure heptakis (2,6-di-O-methyl)-β Cd) purchased from Duchefa (Spain) or 100 µM MJ (Sigma-Aldrich, Spain). The elicitation conditions were selected based on previous results for Sm production in S.marianum cultures [22, 32]. Flavonolignans and *t*-R were extracted from the biomass with 80% methanol at 60ºC for 4 h. Extracts were filtered, dried in vacuo at below 40 ºC and resuspended in 1 mL methanol. The culture medium of cell cultures was separated from the biomass by filtration and flavonolignans and *t*-R were extracted three times with two volumes of ethylacetate. The combined extracts were dried *in vacuo* at below 40 ºC and resuspended in 1 mL methanol as above.

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

### 2.5 STS3 transgene analysis

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

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

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

## 3. Results

Initial transformation of *S. marianum* cell cultures with *A. tumefaciens* C58C1 (pGV2260) harboring pJCV52-STS3 was assessed by antibiotic resistance selection. After 4 weeks on the selection medium, paromomycin-resistant callus colonies were individually transferred to new plates with fresh selection medium. In the selection medium, the non-transformed material did not grow and turned brown, while transformed calli grew vigorously. Within 2–3 months of the initial transformation, sufficient callus material was obtained to check for plant genome T-DNA integration of the STS3 gene by PCR amplification using a primer pair specific for VvSTS3 under the control of P35S. Three *S. marianum* transgenic

calli were selected, and, as shown in Fig. 2A, the STS gene was present in all the transgenic clones, but not in the wild type.

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

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

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

The total accumulation of *t*-R in STS3-transformed cultures in the presence of elicitors after different times is shown in Fig.4 A. A significant amount of *t*-R was obtained from the extracellular medium of 30 mM Cd -treated cultures, increasing from the beginning of elicitation up to about 12 mg $l^{-1}$ at 76 h. A decrease in production was observed at 168 h.

Unlike Cd alone, treatments with MJ alone or MJ + Cd did not improve *t*-R production (Fig 4 B). The promoter used to overexpress the *STS* gene was the strong constitutive CaMV35S promoter.Probably, since enzyme production was constitutive and MJ did not alter its expression, MJ alone or in combination were ineffective. A progressive deceleration after 48 h was also observed, probably due to a toxic effect, since necrosis of cultures was visible after a week of treatment.

Flavonolignan accumulation in STS3-transformed cultures treated with Cd was compared with non-transformed control cultures under the same elicitation conditions. Fig 5A shows the chromatogram profiles, taken at A208, of medium extracts of non- transformed control cultures and of the transformed STS.A line after 48 h in the presence of 30 mM Cd. The relative percentage increases of CA and flavanolignan production both in non-transformed and STS- transformed cultures 24 and 48 h after Cd treatment are shown in Fig. 5B.

From the data shown in Fig. 5A and B, particularly those concerning CA accumulation, it can be recognized that most of the precursors (i.e. p-coumaric acid) for the monolignol pathway have been redirected by the STS protein to the stilbene pathway. Overall production of flavonolignan was less affected in transgenic cultures, since in control cultures the flavonoid pathway was already limiting for Sm synthesis due to the preferred supply of precursors for CA synthesis (more than 20 mg $l^{-1}$ was produced in Cd treated cultures [32]; see the peaks for CA in the chromatogram of Fig. 5A).

## 4 Discussion

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

Resveratrol, like many plant bioactive metabolites, accumulates in low quantities in plant cell cultures. The wide benefits of this compound and its application potential, has generated considerable interest in the last years, and attempts to implement the heterologous biosynthesis of *t*-R have been reported, both in microorganisms and whole

plants [38, 8]. Notably, heterologous production in plants, has the advantage of requiring the introduction of only one or two genes, since the other genes from the phenylpropanoid pathway are already present in the plant kingdom [39]. The engineering of r *t*-R in plants has led to an increase in antioxidant activities and in disease resistance in the transgenic plants and extended the postharvest shelf life of fruits [39, 40].

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

Reports in the literature concerning *STS* gene overexpression in plant cell suspensions are scarce. To our knowledge, heterologous synthesis of *t*-R in plant cell cultures has only been demonstrated in cultured cells of tobacco expressing a STS gene after elicitation with a crude preparation of *Phytophtora megasperma,* with a maximum *t*-R production of 50 ng $g^{-1}$fresh weight [10]. Very recently, Aleynova et al. [42], reported that overexpression of full-length *Vitis amurensis* STS1, *Va*STS2, and *Va*STS7 genes under the control of the double CaMV 35S promoter in grape cell cultures increased stilbene production up to 3.4 times (up to 6.12 mg $l^{-1}$).

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

There are many reports on the extracellular *t*-R accumulation in *Vitis* sp cultures treated with MJ, and/or overall, with Cd [reviewed in 44]. Similarly, in the current study, 10 mg $l^{-1}$ *t*-R was released to the culture medium in the presence of 30 mM Cd, although the *t*-R productivity in transgenic *Silybum* cultures was very far from that reported for *V.vinifera* cultures in which more than 3 g $l^{-1}$ *t*-R accumulated in the presence of 50 mM Cd + 100 µM MJ [45]. Nevertheless, levels were higher than in elicited transgenic tobacco suspensions or in STS-overexpressing *V. amurensis* cultures, as mentioned above.

We have previously published that elicitation treatments are required for the accumulation of phenylpropanoid–like compounds in *S. marianum*. The monolignol pathway was preferently induced by elicitors, with CA being massively accumulated in the extracellular medium [23]. Thus, monolignols compete with the flavonoid pathway for

precursors, which is subsequently the rate-limiting branch for Sm biosynthesis in cell cultures.

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

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

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**Legends for figures**

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

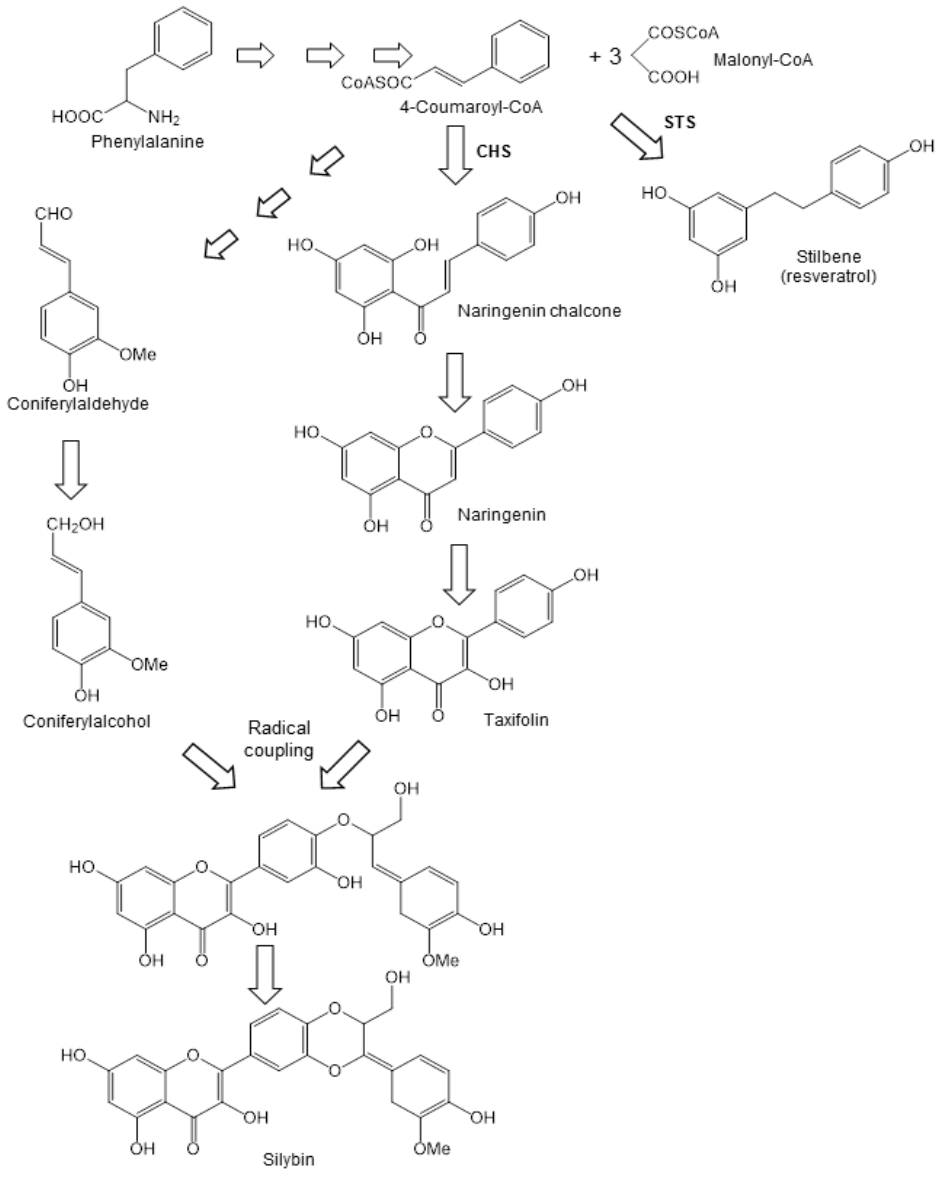

Fig. 2A PCR amplification products from genomic DNA of non-transformed and three transgenic cell lines of *Silybum marianum* suspension cultures. Amplification was carried with specific primers for *VvSTS3*.

Fig. 2B. Relative quantitative expression (ratio of gene to actin) of *VvSTS3* in non-transformed and STS transformed *S. marianum* suspension cultures after treatment with MJ or Cd for 24 h. Data represent mean of triplicate cultures ± SD.

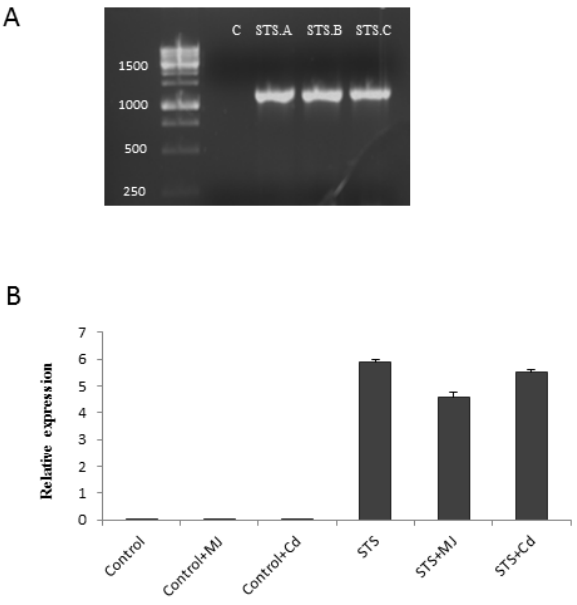

Fig. 3. Chromatograms (A306) of medium extracts of non-transformed (solid line) and STS-transformed *S.marianum* cultures (dotted line) treated with 30 mM Cd for 48 h. Inset: mass spectra of the peak (dotted line) at a retention time of 21 min.(Sb: silybin; IsoSb: isosilybin).

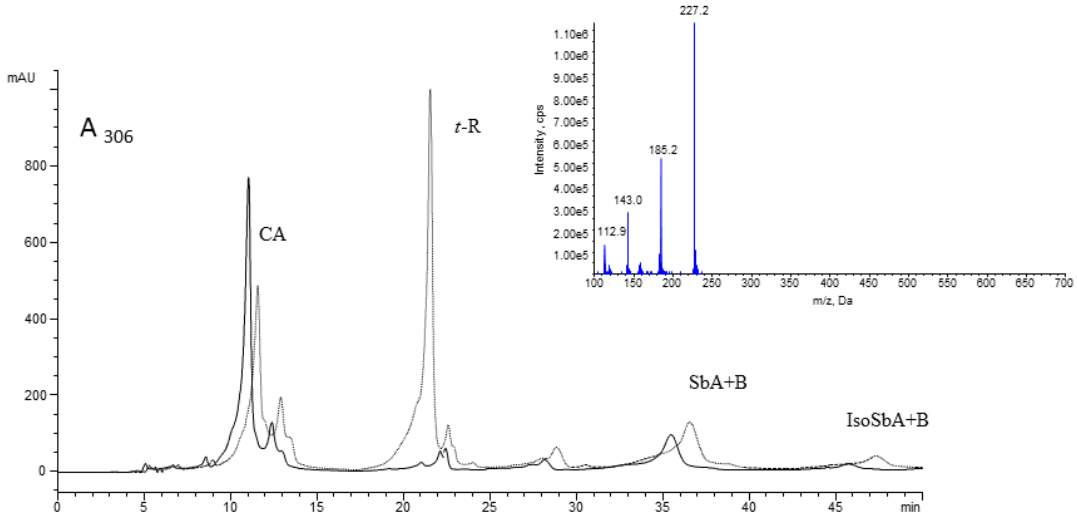

Fig. 4 A. Effect of Cd on *t*-R accumulation in the culture medium of *S.marianum* cultures transformed with *VvSTS3*. Cd (30 mM) were added at day 3 after subculture and samples were extracted at different incubation periods. Data are means ± SD from two independent experiments, each in triplicate.

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

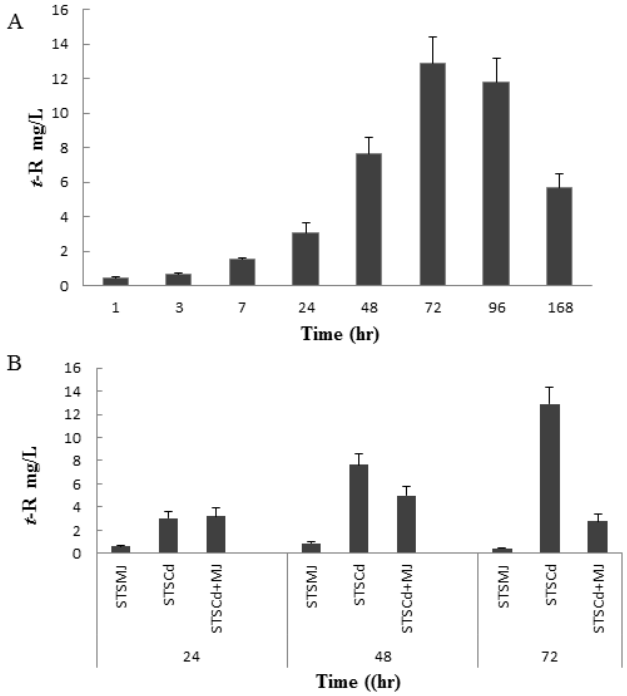

Fig. 5A. Chromatograms (A280) of medium extracts of untransformed and STS-transformed *S.marianum* cultures treated with 30 mM Cd for 48 h.

Fig. 5 B. Percentaje of increase of CA and Sm accumulation in non-transformed and STS-transformed *S. marianum* cultures treated with 30 mM Cd for 24 or 48 h. Data represent mean of triplicate cultures ± SD.