

**PRODUCTION OF STILBENES IN TRANSGENIC PLANTS AND THE
METHOD OF PRODUCING THEREOF**

FIELD OF INVENTION

5 This invention relates to transgenic plants and plant materials. In particular, the present invention is related to the production of resveratrol and other stilbenes in plants.

BACKGROUND OF INVENTION

Cancer is the largest single cause of death in both men and women and
10 chemo-prevention of cancer is one of the most direct ways to reduce morbidity and mortality. Cancer-preventive agents include nonsteroidal anti-inflammatory drugs, eg. indomethacin, aspirin, piroxicam and sulindac, all of which inhibit COX. In the search for new cancer preventive agents, over the past 30 years, thousands of plant samples and extracts were studied and hundreds of these extracts were evaluated
15 on their potential to inhibit COX. In 1974, An extract from *Cassia quinquangulata* from Peru was identified as a potent inhibitor and the active ingredient was identified as resveratrol (3,5,4' -trihydroxy-trans-stilbene). In 1997, it was reported in Science Journal that resveratrol, a phytoalexin found in grapes and other foods was purified and shown to have cancer chemo-preventive activity in
20 assays representing three major stages of carcinogenesis. Resveratrol was found to act as an antioxidant and anti-mutagen and to induce phase II drug-metabolizing enzymes (anti-initiation activity); it mediated anti-inflammatory effects and

inhibited cyclooxygenase and hydroperoxidase functions (antipromotion activity) and it induced antiprogession activity of cancer. In addition, it inhibited the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture and inhibited tumorigenesis in mouse skin cancer model (Jang et al, 1997). These data and a host of other scientists around the world now strongly suggest that resveratrol, a common constituent in our diet merits investigation as a potential cancer chemopreventive agent in humans.

Alcohol, cardio-vascular diseases and the French paradox has been hotly researched and pursuit by the medical scientific communities around the world for the past 20 years. Numerous studies over the years have shown that comparing alcohol intake and ischemic heart disease have shown either an inverse relation or a U-shaped curve in which the equivalent of 2 drinks per day of any kind of alcohol is associated with a decreased incidence of coronary disease compared with no drinks, while higher doses result in an increased risk of infarction and stroke. The cardio-protective effects of most alcoholic beverages are probably due to an elevation of high density lipoprotein and the ability of alcohol to prevent platelet aggregation and increase fibrinolysis; however, there is an increased favorable effect from red wine. The unique cardioprotective properties of red wine reside in the action of flavonoids and stilbenoids which are minimal in white wine (with the exception of champagne). The best researched flavonoids are resveratrol and quercetin, which confer antioxidant properties more potent than alpha-tocopherol. Grape juice has about half the amount of flavonoids by volume as red wine. Resveratrol, however, being a phytoalexin, is not normally produced in grapes unless it is attacked or infected by microbial pathogens.

As resveratrol is a phytoalexin, it is produced by at least 72 plant species spreading over 31 genera and 12 families. The best studied plants that produces resveratrol are grapes and peanuts. The US and especially German Universities have been actively looking at the plant-pathogen interaction in the 2 plants described. Bayer AG, a giant chemical and pharmaceutical company have been actively sponsoring and working on this phytoalexin. They have isolated the genes (stilbene synthase) involved in resveratrol (phytoalexin) production and have shown that when expressed in transgenic plants, resveratrol can increase the resistance to pathogen attack on the plants. Bayer has also filed patents on the grape stilbene synthase gene that they have isolated. Fischer et al (1997) published a paper in The Plant Journal that over-expression of stilbene synthase gene in transgenic tobacco can lead to sterile pollen (due to the competition between chalcone synthase and stilbene synthase on the common precursor substrates).

Presently, there is also a company (PharmaScience) from Canada that is selling resveratrol in powder form and they claim that it is chemically synthesized. The Trade name is "Resverin". They can only supply in small quantity at a very high price. Also, during the past to years, there were also many reports that showed that resveratrol is a phytoestrogen. Hence, resveratrol has also been implicated to mimic estrogen action and hence, may have a potential in non-steroidal estrogen supplements and also may prevent osteoporosis.

One difficulty of trying to tap the antioxidant and antimutagenic benefits of such stilbenes is that they are often phytoalexins and are therefore only found in infected or wounded plants, and not found in healthy plants, even if the gene is

present naturally in the plant. Thus, although, for example, grapes have stilbene synthase (STS) genes and active STS enzymes, consumers typically do not benefit from consuming grapes, because resveratrol is not normally found in fresh, healthy grapes. There is therefore a need to produce plants that contain a high and
5 constitutive level of one or more of the desired stilbenes.

SUMMARY OF INVENTION

Accordingly, one aspect of the present invention is a transgenic plant in which at least one stilbene synthase (STS) gene construct is transformed therein, and with the constitutive production of the corresponding stilbene synthesized by
10 the transgenic STS enzyme, while maintaining fertility and normal physiological development. The plant is preferably a common vegetable that naturally produces high levels of the precursors for the transgenic STS in the edible portion, and more preferably one that does not contain an endogenous STS enzyme that can synthesize the same corresponding stilbene in its natural genome. The plant is
15 more preferably a leafy vegetable that is commonly eaten raw, but can be cooked if needed. An example of a STS gene is resveratrol (RV). An example of plant that is a suitable recipient plant for the transformation of resveratrol synthase gene is the red-leaf lettuce (*Lactuca Sativa*). Red-leaf lettuce is also referred to as red lettuce.

20 Thus, according to another aspect, the present invention is a transgenic plant in which at least one resveratrol synthase (RS) genes construct is transformed therein and with the constitutive production of resveratrol synthesized by the transgenic RS enzyme. The plant is preferably red-leaf lettuce.

According to yet another aspect, the present invention is related to an edible composition comprising portions of the transgenic plant, and preferably a drink developed from and comprising the juice of such a transgenic plant. Thus, the plant is preferably one that contains sufficiently large quantities of juices
5 containing the transgenic stilbene for the juice to be processed into a drink.

According to yet another aspect, the present invention is a method of producing a healthy transgenic plant containing a specific transgenic STS enzyme transformed therein. The transgenic plant further contains high and constitutive levels of the specific transgenic stilbene, while maintaining normal fertility and
10 normal physiological development. The method comprises (1) choosing a recipient plant that does not contain an endogenous stilbene synthase gene encoding the specific STS enzyme, but contains high levels of the precursors of the transgenic STS enzyme; (2) providing a genetic vector comprising an STS gene or a portion of an STS gene encoding an STS enzyme, the STS gene or the portion of the STS
15 gene being provided with a promoter suitable for constitutive expression of the STS enzyme in the recipient plant; (3) transforming the genetic vector into the recipient plant and (4) selecting and growing the transformed plant containing high and constitutive levels of the transgenic stilbene.

To check for endogenous stilbene synthase, oligonucleotides constructed
20 according to conserved regions of a known STS gene may be used as a probe, followed by southern blot analysis of the genome of the candidate recipient plant.

For precursor level analysis, biochemical tests, such as HPLC, may be used. 4-Coumaroyl-CoA and malonyl-CoA are two precursors common to many

stilbene synthase enzymes. Alternatively, precursors levels may be inferred from the level of other intermediates of the same biochemical pathway, as illustrated in the example below.

The STS gene in the genetic vector may be a cDNA obtained from mRNA
5 or genomic DNA isolated from plants containing the appropriate STS gene. Any plant that can synthesize the stilbenes of interest may be a candidate donor plant. The STS gene or cDNA of the STS gene of interest may be obtained using oligoprimers homologous to conserved region of known STS genes. The isolated STS DNA may be cloned into a conventional genetic vector with a conventional
10 selectable marker (e.g. an antibiotic resistance gene) and a conventional promotor that can cause constitutive expression of the inserted STS DNA in the recipient plant.

In the specific preferred embodiment of this method, the genetic vector carries an RS gene or a portion of an RS gene encoding an RS enzyme resulting in
15 high and constitutive levels of transgenic resveratrol being expressed in the transgenic plant. The precursors available for the transgenic RV enzyme are used naturally by the non-transgenic plant for anthocyanin production. The recipient plant is of a species that has a red colour, indicating high levels of naturally-occurring anthocyanins and its precursors.

20 In another preferred embodiment, the recipient plant may be regenerated by tissue culture methods, and the callus analyzed for precursors levels. Callus and plantlets that are found to express high and constitutive levels of the precursors of the transgenic enzyme are selected. This allows the transformed plants (that have

begun to express the transgenic enzyme and thus begun to deplete precursors for the biosynthesis of the transgenic stilbene) to maintain good health during the course of tissue culturing, even though they grow into mature plants.

As used herein, STS genes refer to the family of genes that encode various
5 STS enzymes. The STS enzymes catalyze the synthesis of different members of the stilbene family. Resveratrol synthase (RS) gene refers to a specific member of the STS gene family that encodes the resveratrol synthase enzyme (RS enzyme). The RS enzyme catalyzes the conversion of 4-coumaroyl-CoA and malonyl-CoA to 3,4',5-trihydroxy-trans-stilbene (resveratrol).

10 Redness is determined in a general manner and may be observable by the eye and generally accepted and well known by one in the art. Example of plants that are commonly regarded as "red" include red-leaf lettuce, purple cabbage, red-beet root, red amaranthus, red sugar cane.

"Healthy" as used herein refers to a general state of health that is within the
15 normal range of that species as observable according to appearance, such as size and colour.

"Fertile" as used herein refers to the ability of the species to form viable seeds.

BRIEF DESCRIPTION OF DRAWINGS

20 Figure 1 shows the last step in the biosynthetic pathway of resveratrol and naringenin chalcone.

Figures 2A-D are genetic maps of plasmid pBI 121 carrying the R1 STS gene (Figure 2A), R65 STS gene (Figure 2B), R14 STS gene (Figure 2C) and R17 STS gene (Figure 2D).

Figure 3 is the Hydropathy curves of (a) *Vitis vinifera* cv. Optima STS (pSV21), (b) *Arachis hypogaea* STS (arqresol), (c) *Pinus sylvestris* STS (PSTS1) and (d) *Phalenopsis* sp. BS (pBibsy811).

Figures 4A-D show the 4 existing grape STS cDNA, pSV21, pSV25, pSV368 and VVLSTS as aligned using ClustralW. The primers used for isolating full-length *Vitis vinifera* cv. Red Flame STS genes are indicated in the boxed-in portion.

Figure 5 are the restriction maps of (a) grape STS (pSV21, pSV25, pSV368 and VVLSTS) (b) grape STS introns (Vst1-1 and Vst2-1) (c) *Vitis vinifera* cv. Red Flame cDNA (R1, R5 and R8) (d) *Vitis vinifera* cv. Red Flame gDNA (G13, G14 and G17). Boxed-in regions are introns.

Figure 6 is a DNA alignment between putative grape STS cDNA R6, gDNA G14 and grape STS VVLSTS.

Figure 7 is a DNA alignment between putative STS cDNA R12, gDNA G13 and grape STS pSV21.

Figures 8A-E are HPLC elution profiles for the analysis of RV in the control (Figure 8A) and the transgenic lines containing plasmids G14 (Figure 8B), G17 (Figure 8C), R1 (Figure 8D) and R15 (Figure 8E). The RV peaks are indicated by the arrows.

DETAILED DESCRIPTION

The following example is used to illustrate the various aspects of the present invention.

PRODUCTION OF RESVERATROL IN RED-LEAF LETTUCE

5 Resveratrol synthase (RS) gene from *vitis vinifera* cv. Red Flame is the stilbene synthase gene used in this example as the member of the STS gene family for cloning and transformation. For ease of description and understanding, RV produced by the transgenic RS enzyme and stilbene produced by the transgenic STS enzymes are referred to as transgenic RV and transgenic stilbene respectively.

10 Red leaf lettuce is chosen as the recipient plant for the RS gene in this example because it contains high levels of anthocyanin pigments for which 4-coumaroyl-CoA & malonyl-CoA are precursors. For example, Naringenin chalcone is an intermediate of the anthocyanin biosynthetic pathway, in which 4-coumaroyl-CoA and malonyl-CoA are precursors. 4-coumaroyl-CoA and malonyl-CoA are also

15 known precursors for resveratrol (RV), as shown in Figure 1. Thus, there is plenty of precursors for the conversion to RV by RS within the transformed lettuce. Furthermore, the lettuce species was tested for the presence of RV-related genes in their natural genome by hybridizing with oligonucleotides that represent the homologous regions of various known RV genes. Southern blot analysis showed

20 no hybridization occurring, indicating that there is no endogenous RS gene in lettuce making it a suitable recipient plant according to the present invention. As a result, no competition is expected between transgenic RS and endogenous RS,

such that high and constitutive levels of RV may be attained in the transgenic red-leaf lettuce.

To start off, some red grapes (cultivar red flame) were obtained from the local supermarket. We UV the grapes for 10 minutes to induce the transcription of the resveratrol synthase transcript and waited for 12 hours and extraction mRNA
5 from the UV irradiated grapes. We made oligoprimers 5' and 3' of the Stilbene synthase gene and through reverse PCR, pulled out the cDNA sequence of the genes. We also extracted genomic DNA of the red flame grapes and again through PCR isolated the genomic stilbene synthase genes (with 2 introns). The stilbene
10 synthase is a multi-gene family. Hence we mapped, sequenced and characterised all of them.

We chose 2 full length cDNA clones (named R1 and R65) and 2 genomic clones (named G14 and G17) and ligated them into an expression vector (pBI 121) driven by the Cauliflower Mosaic Virus (CaMv) 35S promoter. Clones R65, G14
15 and G17 were ligated into pBI 121 through the BamH1 and Sac1 restriction sites, while clone R1 was ligated into pBI 121 through the BamH1 and EcoR1 restriction sites. This plasmid expression vector was then transformed into Agrobacterium (strain LBA4404). The Agrobacterium that carries the pBI121 with the RS gene is selected through Kanamycin resistance selection. Hence, we obtained 4 different
20 Agrobacterium colonies carrying the 4 different constructs as shown in Figures 2A-D.

Concurrently, we also screened 5 different varieties of red-lettuce and assess their re-generation potential in tissue culture from cotyledon explants. The

red-lettuce variety Red salad Bowl was chosen because it showed the highest and fastest regeneration of plantlets produced from our tissue culture protocol.

The experiments were repeated, but this time the cotyledons were cut into squares of 2mm sq. in area and were incubated for 30 minutes in the Agrobacteria.

5 They were then rinsed and the tissue culture protocol that we established for red-salad bowl was followed. Plantlets regenerated were selected using Kanamycin at 150 mg/L concentration in the shoot induction medium. These plantlets were rooted and planted out and grown for 30 to 35 days before the leaves were harvested for RNA, DNA and extraction for resveratrol quantitation using HPLC.

10 Northern and Southern analyses showed that the stilbene synthase genes (R1,R65, G14 and G17) were expressed. Organic solvent extraction of the leaves samples were done according to reported protocols for resveratrol analysis and were analysed using HPLC and a pure sample of resveratrol bought from Sigma chemicals was used as a standard reference.

15 The experiment using tobacco plants that had been similarly transformed, selected and was also done to serve as positive controls as a normal green plant.

The data from HPLC quantification show that the transgenic red-lettuce are capable of producing high and constitutive amount of resveratrol (over 1ug/g fresh weight of leaf) as opposed to tobacco (best is around 0.36ug/g f.w.). The
20 transgenic red lettuce can produce up to 10 times the resveratrol as compared to transgenic tobacco when comparison is made using dry weight. Non transformed plants show no detectable resveratrol in them. A key observation and data obtained from our quantification of anthocyanin level of the transgenic red lettuce

when compared to the non-transformed control is that there is no significant difference in the anthocyanin level. This data shows that there is potential in escalating the resveratrol concentration of the red-lettuce to a much higher level if we were to further over express the resveratrol synthase (RS) gene expression in the vegetable. Methods of over-expression include the use of stronger constitutive
5 promoters or double promoters, use of the viral omega sequences for more efficient translation, and the use of other promoters like actin promoters.

This system also shows that it has the potential to be used in other coloured plants and fruits for high-level resveratrol yield. Assuming that we eat 200g of
10 vegetables daily, it will provide the resveratrol supply of >200ug into our body daily. Hence, we foresee the potential of this novel invention in paving the way for a new generation of vegetable nutraceuticals that have chemo-preventive ability against Cancer, Cardio-vascular and other potential diseases.

The following are the detailed procedures used to obtain the transgenic red-
15 leaf lettuce.

Choosing and Establishing Recipient Plant Material

4 varieties of red lettuces, namely, *Lactuca sativa* cv. Canasta, Lollo Rossa, Red Salad Bowl (Novartis seeds B.V, Holland) and Red Rapid (Known-you Seeds Co., Taiwan) were tested for their redness and re-generation ability in tissue
20 culture.

The whole process of re-generation were done as described in Curtis *et al.*, 1995, Methods in Molecular Biology, Vol. 44, Humana Press Inc. USA, pp.59-70,

with some modifications. 10 seeds from each variety were surface sterilized using 10% Clorox for 10 minutes and rinsed three times with sterile R.O. water. The seeds were then sowed in 50ml MS + B5 medium (Sigma Catalogue No.M-5519) in 250ml conical flasks and grew at $23\pm 2^{\circ}\text{C}$, 16 hours photoperiod, with light intensity of $18\mu\text{mol/s/m}^2$ (daylight fluorescent tubes) for 7 days.

The cotelydons of the 7-days old seedlings were excised, leaving the petiole intact but removed the apices of the cotelydons. Using a needle, the abaxial surface was poked repeatedly along the veins of the cotelydons. The cotelydons were floated on liquid UM medium (4.71g/L MS salts and vitamins, 30g/l sucrose, 2g/l casein hydrolysate, 2mg/l 2,4 dichlorophenoxyacetic acid (2,4-D, Sigma), 0.25 mg/l kinetin, 9.9 mg/l thiamine HCL, 9.5mg/l pyridoxine-HCL, 4.5mg/l nicotinic acid, 5.5g/l phtyagel, pH 5.8) for 10 minutes with their wounded surface in contact with the medium. The cotelydons were removed and immersed in UM agar medium. The explants were incubated for 2 days under the same conditions as for germinating seeds.

After 2 days in UM solid medium, the cotelydons were transferred to SI agar medium (4.71g/L MS salts and vitamins, 30g/l sucrose, 0.04 mg/l NAA (Naphthalene acetic acid) 0.5mg/l Benzyl amino purine (BAP), 500mg/l Carbeicillin, 100 mg/l cefotaxime, 150mg/l kanamycin sulfate, 5.5g/l phytigel, pH 5.8) with the abaxial surface in contact with the SI agar medium. The cotelydons were incubated as for germinating seeds and sub-cultured to fresh SI agar medium every 21 days.

After 49 days, the explants that produced callus and shoots were transferred to 50ml of SI agar medium with 0.11% (w/v) 2[N-morpholino]ethanesulfonic acid (MES).

Shoots that were approximately 1cm high were transferred to 250ml conical flasks each containing 50ml of rooting agar medium (4.71g/L MS salts and vitamins, 30g/l sucrose, 0.04 mg/l NAA (Naphthalene acetic acid), 150mg/l kanamycin sulfate, 5.5g/l phytigel, pH5.8). The shoots were incubated at the same conditions as germinating seeds.

Isolation of STS genes from grape and construction of genetic vector

10 Plant Material

Mature fruits of commercially available grapevine *Vitis* cv. Red Flame were used as plant material for the isolation of grapevine STS genes.

Total RNA and genomic DNA extraction

0.2g of *Vitis* cv. Red Flame skin tissues were ground in liquid nitrogen with the mortar and pestle. Both total RNA and gDNA were extracted using the same method described in Knapp and Chandless (RNA/DNA Mini-Prep from a Single Sample of Orchid Tissue. Bio Techniques, 21:54-56), with some modifications. 2ml of extraction buffer which contained 3% CTAB; 2% PVP; 1.42M NaCl; 20mM EDTA, pH 8.0; 100mM Tris, pH 8.0 and 5mM ascorbic acid were used to extract total RNA or gDNA of *Vitis* cv. Red Flame skin tissues. The samples were heated at 65°C for 15 minutes, followed by a chloroform extraction to get rid of proteinaceous substances. 1/5 volume of 5% CTAB (5% CTAB and 0.7M NaCl)

were added to the aqueous phase of the samples to remove polysaccharides and heated at 65°C for 15 minutes. Another chloroform extraction was performed. 2 volume of ice-cold, 100% EtOH were added to the aqueous phase of the samples and incubated at -20°C for 15 minutes. Total RNA and gDNA were pelleted after
 5 centrifuged for 15 minutes at 11,000 rpm, room temperature, using Eppendorf™ 5410C refrigerated centrifuge. The pellets were washed with 70% EtOH and dried in Eppendorf™ concentrator before dissolving in 50µl TE (10mM Tris-HCl, pH 8.0 and 1mM EDTA, pH 8.0). 3µl of the total RNA or gDNA isolated and 1µl of loading buffer (0.025% bromophenol blue, 0.025% xylene cyanol, 30% glycerol in
 10 1X TBE) were loaded onto 1% agarose gel together with 0.25µg each of lambda DNA/HindIII and phiX174 DNA/HaeIII markers. Horizontal gel electrophoresis was run at 100V for 1/2 hour. The quantity and the quality of total RNA or gDNA extracted were visualized and calculated using EtBr stain and Stratagene's Eagle-Eye II Junior documentation system.

15 Primers determination

All existing genes sequences of *Vitis* cv Optima STS (pSV21, pSV25 and pSV368), *Vitis* cv. Lambruscoa Foglia Frastagliata STS (VVLSTS), *Phalenopsis* sp. BS (pBibsy811 and pBibsy212), *Arachis hypogaea* (peanut) STS (arqresol and a00769) and *Pinus sylvestris* (Scots pine) STS (PSTS1 and PSTS2) were obtained
 20 from GenBank in the website of National Center for Biotechnology Information (NCBI). All homology searches were performed using ClustalW Multiple Sequence Alignment of BCM Search Launcher from Human Genome Center, Baylor College of Medicine, Houston TX.

The primers used for isolation of full length grapevine STS genes were determined by multiple alignment of existing pSV21, pSV25, pSV368 (Melchior and Kindl, 1991) and VVLSTS (Sparvoli *et. al.*, 1994) as indicated in Figures 4A-D.

- 5 After determination of the primers sequences and the melting temperatures of the primers, they were custom synthesized commercially by Gibco BRL Custom Oligonucleotide Synthesis Service, L.T.I., U.S.A.

Reverse transcription of total RNA

- 10 1µg of UV-induced *Vitis* cv. Red Flame skin total RNA leaves total RNA was used as templates to allow the annealing of the 3' primer – 35GSTS2a at 65°C for ten minutes. After the primer was annealed, the total RNA was reverse transcribed with 200 units/µl SuperscriptTMII reverse transcriptase (Gibco BRL, LTI, U.S.A), SuperscriptIITM 1X reaction buffer, 10mM DTT and 200µM dNTP in the final volume of 50µl. The reverse transcription was carried out in Perkin Elmer
- 15 GeneAmp PCR system 2400 at 42°C for 1 hour, then SuperscriptTMII reverse transcriptase was inactivated at 70°C for 15 minutes.

- 20 The cDNA was purified through Tris-buffered phenol and chloroform:isoamyl alcohol (24:1) extraction. The aqueous phase was then precipitated with 1/10 volume of 3M sodium acetate and 2.5 volume of ice-cold, 100% EtOH. The pellet was dissolved in 20µl of sterile milli-Q water.

Polymerase chain reaction of cDNA and gDNA

Both 20µl of cDNA and 20ng of gDNA of *Vitis* cv. Red Flame skin, were amplified by Polymerase Chain Reaction (PCR) in Perkin Elmer GeneAmp PCR system 2400. The PCR reaction at a total volume of 50µl included 5 units/µl *Thermus flavus* (*Tfl*) DNA polymerase in 1X *Tfl* reaction buffer provided (Promega, U.S.A), 200ng/µl each of 5' primer 35GSTS1 and 3' primer 35GSTS2a, 200µM dNTP, 1.5mM magnesium sulfate and topped up with sterile milli-Q water.

PCR amplification for grapevine STS genes was done by holding at 92°C for 5 minutes, followed by 40 cycles of denaturing time of 1 minute at 92°C, annealing at 55°C for 2 minutes and an extension time of 2 minutes at 72°C. Further extension at 72°C for 6 minutes completed the PCR.

5µl of PCR reaction were separated and quantified by Horizontal gel electrophoresis in 1% agarose. After determining the presence of the desired MW fragments, the rest of 45µl PCR reaction were selective precipitated with 1/10 volume of 10X STE (10mM Tris.Cl, pH 8.0; 100mM NaCl and 1mM EDTA, pH 8.0), 1/10 volume of 4M NH₄OAc and 2.5 volume of ice-cold, 100% EtOH. The pellets were dissolved in TE for ligation into pGEM-T (+) vector.

Cloning into pGEM-T(+)

The cDNA and gDNA PCR products of *Vitis* cv. Red Flame skin were cloned into pGEM-T(+) vector using the pGEM-T(+)TM vector system kit

(Promega, U.S.A). The cDNA and gDNA PCR products in the ratio 1:3 (vector:insert) were ligated into pGEM-T(+) vector using 3 units/ μ l of T4 DNA ligase, T4 DNA ligase 1X buffer in the total volume of 10 μ l and incubated at 16°C overnight.

5 After ligating, 10 μ l of the ligation reaction mix were transformed into 200 μ l of XL1-Blue competent cells (Stratagene, U.S.A). They were put in ice for 10 minutes, followed by 5 minutes at 37°C, then back in ice for 1 minute. 1ml of plain LB broth was added and incubated at 37°C for 1 hour. After 1 hour recovery time, the cells were collected through centrifugation at 11,000rpm for 30 seconds,
10 then resuspended in 50 μ l plain LB broth. 50 μ l were used to spread onto 1.5% LB agar plates with 100 μ g/ml ampicillin. These plates were incubated at 37°C overnight.

The positive clones with the correct size inserts were selected using restriction enzymes *Sall* and *ClaI* (NEB Biolabs, U.S.A) digest following the
15 manufacturer's recommended conditions after plasmid miniprep.

Characterization of putative *Vitis* cv. Red Flame STS genes

After the putative *Vitis* cv. Red Flame STS genes had been isolated, they were characterized by restriction enzyme mapping (Figure 5), sequence analysis (Figures 6 and 7) and plotting of hydropathy curves (Figure 3).

20 Restriction enzyme mapping

Restriction enzyme mapping of the existing grapevine STS genes (pSV21, pSV25, pSV368 and VVLSTS) was identified using the website Webcutter 2.0.

According to the restriction enzyme maps obtained, *Pst*I, *Kpn*I, and *Hind*III (NEB, U.S.A.) were used to digest the putative clones of *Vitis* cv. Red Flame STS genes at the manufacturer's recommended conditions. After digestion, the reactions were analyzed on 1% agarose gel using Horizontal gel electrophoresis running at 100V for 45 minutes. Gels were viewed using Eagle-Eye II Junior documentation system (Stratagene, U.S.A.)

Sequence analysis

The sequences of the putative *Vitis* cv Red Flame STS genes were analyzed using dideoxy nucleotide chain termination method. Sequenase™ Version 2 sequencing kit (Amersham-Pharmacia, Sweden) was used for the sequencing reaction with forward primer (ssDNA sequencing) and reverse primer (dsDNA sequencing). The reactions were labeled using 35S-dATP (NEN, U.S.A.). A 6% polyacrylamide gel was ran at 50W using the Sequencing Apparatus S2 (L.T.I., Inc., U.S.A.). Autoradiography was performed by exposing to Kodax MR Bio-Max film in a Kodax intensified screen cassette for approximately 16 hours. The films were then developed with Kodax Developer and Kodax Fixer. The sequences were read manually. The first 300 bases of the 5' sequences of clone R65 showed that it belongs to the PSV21 group of grape STS gene.

20 Hydropathy curves

Hydropathy curves were plotted for *Vitis* cv. Optima STS (pSV21), *Phalenopsis* sp. BS (pBibsy811), *Arachis hypogaea* STS (arqresol) and *Pinus sylvestris* STS (PSTS1) using the hydropathy plot website maintained by

Biochemistry and Molecular Biology at Pennsylvania State University. The hydropathy curves were plotted based on Kyte and Dolittle method.

Cloning *Vitis* cv Red Flame STS genes into expression vector

4 clones of *Vitis* cv. Red Flame STS genes were cloned into expression
5 vector pBI 121. Clones R65, G14 and G17 were cloned into pBI 121 vector through the BamH1 and Sac1 restriction sites. Clone R1 was cloned into pBI 121 through the BamH1 and EcoR1 restriction sites respectively. The genes were driven by a constitutive CaMV 35S RNA promoter. The cloned vectors are shown in Figures 2A-2D.

10 Transformation of plasmid containing STS gene constructs into *Agrobacterium* LBA4404

1. Prepare sufficient YEP for liquid culture and plating and restreak of transformants. Requires 5 ml liquid culture per transformation, 1ml for outgrowth, 20-40ml for plates. (YEP: 10g Bacto-peptone, 10g Bacto-yeast
15 extracts, 5 g NaCl; for solid 10g/l phytagar).
2. Grow *Agrobacteria* LBA 4404 colony O/N in 2ml YEP at 28°C.
3. Add to 50ml of YEP in reserved 250 ml flask and shake at 250rpm to OD of 0.5 to 1 at wavelength of 600nm.
4. Chill culture on ice for 5 min. Spin ~5,000rpm for 5 min.
- 20 5. Carefully decant supernatant and resuspend tube in 1 ml of 0°C, 20mM CaCl₂. Resuspend gently and strictly at 0°C.

6. Dispense 0.1 ml aliquots into prechilled microfuge tubes.
7. Add 1ug of DNA to cells, mix gently but thoroughly, then freeze in dry ice-EtOH bath.
8. Place cells in 37⁰C bath, 5 min.
- 5 9. Add 1 ml YEP and incubate with gentl shaking 2-4 hr.
10. Centrifuge for 45 sec at 4000rpm in a microfuge. With pipet tip discard all but 100-200 ul of medium, resuspend cells in remaining medium by pipeting and/or vortexing and plate on 25ug/ml Kanamycin. Incubate at 28⁰C. Colonies should appear in 2-3 days.

10 Transformation of Red Lettuce by Agrobacterium

1. Lettuce seeds, Var Red Salad Bowl were surface sterilised with 10%w/v chlorox for 10 minutes and rinsed 3 times with clean distilled water.
2. Seeds were than germinated in germination medium contained in 9 cm diameter Petri dishes (30 seeds/dish). Incubated at 24⁰C, 16 hr light at 15 18umol/s/m² intensity.
3. Agrobacterium tumefaciens strain LBA4404 containing the binary vector pBI121 constructs (R1, R65, G14 and G17) were grown in LB medium with pH7 and Kanamycin sulfate at 50 mg/L and 2 mg/L tetracycline-HCL for 1 day in a shaker at 210rpm.

4. Pour 20ml aliquots of UM agar medium into 9 cm diameter Petri dishes and allow to solidify.
5. Soak one sterile 7cm diameter Whatman filter paper in liquid UM and place onto the surface of the UM agar medium.
- 5 6. Excise the cotyledons from 7 day old seedlings, leaving the petiole intact, but remove the apices of the cotyledons. Score the abaxial side using a fine needle. Using a scapel, make shallow cuts (1mm apart) transversely across the surface of the cotyledons. Float the cotyledons for 10 mins in an Agrobacterium liquid culture. Controls are done similarly except agrobacteria is not used.
- 10 7. Remove the cotyledons and blot dry with sterile filter paper and transfer to the prepared UM dishes (10 cotyledons per dish). Incubate for 2 days under the same conditions as for germinating seeds.
8. Set up test plates as follow:
9. A. Control explants without agrobacterium inoculation on:
 - 15 i. SI medium
 - ii. SI medium + 100 mg/L kanamycin sulfate;
 - iii. SI medium + 150 mg/L Kanamycin sulfate
- B. Explants inoculated with Agrobacterium on:
 - i. SI medium + 100mg/L Kanamycin sulfate and:

ii. SI medium + 150mg/L kanamycin sulfate.

10. Transfer the explants to SI medium, submerging the petiolar ends of the cotyledons into the medium to a depth of about 2mm. Incubate as for germination of seeds and subculture to fresh SI agar medium every 17 days.
- 5 11. After 40 days, transfer those explants that have produced callus and shoots to a 250ml capacity flasks, each containing 60 ml of SI agar medium with 0.11% w/v Carbeicillin. 4 explant per flask, incubate at high light intensity of 80 μ mol/s/m₂.
12. Transfer shoots when approx. 1cm high to the rooting medium, incubate at
10 high light intensity of 80 μ mol/s/m₂.
13. When rooted, carefully remove plants from the containers, wash away the agar and transfer the plants into 10 inch pots filled with vermiculite. Enclose the plants in clear polyethylene bags for 3 days and remove them. The plants are then grown under full sunlight and fertilised with Graviota fertilizer. After 35
15 days, these plants were assayed for Northern, Southern and also extracted using organic solvent for HPLC analyses for resveratrol yield.

Selection of transgenic plants

After piercing, the cotyledons with agrobacteria carrying the constructs. The cotyledons were then immersed in UM agar medium (~15 cotyledons/plate)
20 and incubated at 23 $\pm$ 2°C, 16 hours photoperiod, with light intensity of 18 μ mol/s/m² (daylight fluorescent tubes) for 2 days.

After 2 days, the cotelydons were placed on SI agar medium with abaxial surfaces in contact with the medium. The cotelydons were grown in the conditions mentioned above. For the color selection, the cotelydons were sub-cultured into fresh SI agar medium every 21 days. Small shoots of ~1mm and that were red in color were discarded while the pink and green plantlets were placed in UM agar medium for 2 days. Pink or green plantlets that turned red at this stage were also discarded, while those remained pink or green, were subcultured into fresh SI agar medium. Those plants that were ~1cm in height were placed into rooting agar medium.

For kanamycin selection, after placing in SI agar medium for a week, the cotelydons were transferred to SI + 150µg/ml kanamycin sulfate agar medium. Subculturing was done every 4 weeks to SI + 150µg/ml kanamycin sulfate agar medium.

Using the procedures described above, Red Flame STS genes were successfully cloned into Red-lettuce and transgenic resveratrol produced in the transgenic plants. The results obtained by using the methods described above are shown below:

Recipient Plant Material

The 4 cultivars *Lactuca sativa* were tested for the anthocyanin level and regeneration ability *in vitro*. The amount of precursors for resveratrol (i.e. 4-coumaroyl-CoA and malonyl-CoA) can then be inferred from the anthocyanin levels, since 4-coumaroyl-CoA and malonyl-CoA are common precursors for these

two biosynthetic pathways. It is understood that the levels of 4-coumaroyl-CoA and malonyl-CoA may be determined directly by one skilled in the art, and is considered within the scope of the present invention.

Table 1 shows analysis of redness of cotyledons after 2 days in UM medium, color of calli after 14 days in SI medium and the re-generation ability after 37 days in SI medium for *Lactuca sativa* cv. Canasta, Lollo Rossa, Red Rapid and Red Salad Bowl.

From Table 1 as shown below, the cotyledon of *Lactuca sativa* cv. Red Salad Bowl was shown to be the most red in color and the *Lactuca sativa* cv. Lollo Rossa cotyledon was the least red, after 2 days in UM medium. Calli were formed after 2 weeks in SI medium showed that *Lactuca sativa* cv. Canasta, Lollo Rossa and Red Rapid had more light green calli than other colors calli. As for the *Lactuca sativa* cv. Red Salad Bowl, red calli was in higher percentage than other colors calli. Only *Lactuca sativa* cv. Red Rapid and Red Salad Bowl had calli of pink, dirty-red and white colors.

<i>Lactuca sativa</i> cv.	Redness of cotyledons	Calli						Re-generation ability
		Light green	Dark green	Red	Pink	Dirty red	White	
Canasta	++	+++	+	±	-	-	-	+
Lollo Rossa	+	+++	±	+	-	-	-	++
Red Rapid	+++	+++	±	++	+	±	±	+++
Red Salad Bowl	++++	+	+	+++ +	+	+	+	++++

Legends: ± means <30%; + means 30% - 50%; ++ means 50% - 70%; +++ means 70% - 90%; ++++ means >90%

Table 1

The 2,4-D (2,4-dichlorophenoxyacetic acid), an auxin, triggers the formation of anthocyanins. High anthocyanins production is one of the criteria for choosing the cultivar to be used for transformation. The higher the anthocyanins level, that means there are more substrates available (4-coumaroyl-CoA and malonyl-CoA). Therefore, when the STS genes are transformed into the red lettuce, they have ample substrates to use. This facilitates the color selection of transgenic plants, as the color change to light pink or dark pink will be more prominent if the untransformed portions of the cotyledons are red. Furthermore, the cotyledons of *Lactuca sativa* cv. Red Salad Bowl has a uniform color throughout the cotyledons (Figure 3a).

As for the re-generation ability, more than 90% of *Lactuca sativa* cv. Red Salad Bowl calli re-generated into plantlets. *Lactuca sativa* cv. Red Salad Bowl had the highest re-generation ability compared to *Lactuca sativa* cv. Canasta, Lollo Rossa and Red Rapid. *Lactuca sativa* cv. Red Rapid had approximately 70% to 90% calli re-generated into plantlets. This will shorten the time needed to select transgenic plants. Hence, *Lactuca sativa* cv. Red Salad Bowl is chosen as the plant materials for transformation of *Vitis vinifera* cv. Red Flame STS genes.

STS genes from Red Flame grape

20 Primers determination

ClustalW alignment of existing grape STS cDNA isolated from *Vitis vinifera* cv. Optima (pSV21, pSV25 and pSV368) and *Vitis vinifera* cv.

Lambruscoa Foglia Frastagliata (VVLSTS) showed that they were quite similar and shared high homology of 87.5% (Figure 4). Consensus regions at the 5' and 3' ends of the sequences were determined for isolating the full-length genes from *Vitis vinifera* cv. Red Flame. 5' primer determined was 5' GTC GAC CTT CCT CAA CTT AAT CTT 3' (designated as 35GSTS1) and 3' primer was 5' ATC GAT TTC CTT CAC TTA ATT TGT 3' (designated as 35GSTS2a). They were highlighted in red in Figure 4. 35GSTS1 contained a *Sall* linker while 35GSTS2a contained *Clal* linker, both at the 5' ends.

cDNA and gDNA putative clones of *Vitis vinifera* cv. Red Flame STS genes

10 *Vitis vinifera* cv. Red Flame cDNA of MW 1.3kb was obtained after RT and PCR. Out of 18 clones in pGEM-T(+), 12 clones contained insert sizes ranging from 0.9kb to 1.6kb after digesting with *Sall* and *Clal*. While for the gDNA, 1.6kb fragment was obtained. All the 18 clones that were digested with *Sall* and *Clal* contained insert sizes of 1.5kb to 1.6kb.

15 Restriction enzyme mapping

12 cDNA clones and 18 gDNA clones of *Vitis vinifera* cv. Red Flame were subjected to *PstI*, *KpnI* and *HindIII* digestion. 3 clones of cDNA of *Vitis vinifera* cv. Red Flame were found to have similar restriction enzyme maps as the existing grape STS genes (Figure 5a). The restriction mappings of the cDNA 3 clones, R1, R5 and R8 were shown in Figure 5c. The size of R1 was 1.3kb and it possessed 1 *KpnI* site and none of the *PstI* and *HindIII* sites. As for R5, it had 1.2kb size and 1 site each for *PstI*, *KpnI* and *HindIII* sites (Table 2). These 3 clones together with 3

other clones (R3, R6, R12), whose restriction enzyme maps did not show any similarity to that of the existing grape STS, were subjected to sequence analysis.

The MW of the inserts of the gDNA clones of *Vitis vinifera* cv. Red Flame was listed in Table 2. Out of 18 clones, 4 clones were of size 1.5kb and 5 clones were of size 1.6kb. All clones had the *PstI* and *KpnI* sites, while only 1 clone (G13) had the *HindIII* site. The restriction maps for G13, G14 and G17 were shown in Figure 5d and they were similar to each other. All of the 9 clones were subjected to sequence analysis.

Putative clones	Size (kb)	<i>Pst I</i>	<i>Kpn I</i>	<i>Hind III</i>
<u>cDNA</u>				
R1	1.3	X	√	X
R5	1.2	√	√	√
R8	0.9	√	√	√
<u>gDNA</u>				
G4	1.5	√	√	X
G5	1.5	√	√	X
G9	1.5	√	√	X
G11	1.5	√	√	X
G13	1.6	√	√	√
G14	1.6	√	√	X
G15	1.6	√	√	X
G16	1.6	√	√	X
G17	1.6	√	√	X

Table 2: The MW (in kb) and the presence of *PstI*, *KpnI* and *HindIII* in *Vitis vinifera* cv. Red Flame cDNA and gDNA restriction maps. Legend: √ - site present, X – site absent.

Sequence analysis

First 300bp sequence analysis and using BLAST program (website: <http://www.ncbi.nlm.nih.gov/BLAST/>), showed that the cDNA clones, R1 and R5

were 94.5% homology to pSV25 as shown in Table 3. But R5 had 85bp missing. For R3, R65 and R12, they were of the same sequence and shared 97.5% homology to pSV21. R6 was homologous to VVLSTS with 99% homology level but 82bp were missing as shown in Figure 6.

Putative clone Existing STS genes	cDNA		gDNA	
	Clones	%	Clones	%
pSV21	R3, R12	97.5	G13	93
pSV25	R1, R5	94.5	-	-
pSV368	-	-	-	-
VVLSTS	R6	99	G5, G9, G14, G17	99

5

Table 3: Homology level of *Vitis vinifera* cv. Red Flame cDNA and gDNA STS putative clones with existing grape STS genes (pSV21, pSV25, pSV368 and VVLSTS)

As for the gDNA clones of *Vitis vinifera* cv. Red Flame, G13 revealed 93% homology to pSV21 (Table 3). 4 gDNA clones (G5, G9, G14 and G17) as shown in Table 3, was 99% homology to VVLSTS. After the first 300bp sequencing analysis, these 4 gDNA clones were found to be of the same sequence.

Since clones that were homologous to pSV21 and VVLSTS were isolated from both cDNA and gDNA, alignment was done between these clones. In Figure 7, R12 and G13 were similar but not identical to each other. Same result was obtained for R6 and G14 (Figure 6).

Hydropathy curves

The hydropathy curves for *Vitis vinifera* cv. Optima STS (pSV21), *Phalenopsis* sp. BS (pBibsy811), *Arachis hypogaea* STS (arqresol) and *Pinus sylvestris* STS (PSTS1) shown in Figure 6 were similar to each other. They were divided into 3 main domains. The hydrophobic N-terminal (a.a. 1 to 127),
 5 hydrophilic middle portion (a.a. 128 to 313) and a mixture of hydrophobic and and hydrophilic C-terminal (a.a. 314 to 392). The a.a position is based on pSV21.

Clones pUCSTS-R1, R3 and R12 are full-length cDNA STS genes from *Vitis vinifera* cv. Red Flame. According to the sequence analysis, they are homology to pSV25 (R1) and pSV21 (R3, and R12). The MW of these cDNA
 10 clones do not correspond to the expected MW, which is in the range of 1.179kb to 1.237kb, when the 5' (35GSTS1) and 3' (35GSTS2a) primers are used. But, they correspond to the MW of pSV21, pSV25, pSV368 and VVLSTS, which are 1.323kb, 1.3kb, 1.251kb and 1.547kb respectively (Melchior and Kindl, 1991 and Sparvoli *et al.*, 1994).

15 Furthermore, the restriction maps of R1, R3 and R12 are not the same as that of the existing grape STS genes (Figure 5a and Figure 5c). These differences can be explained by the different cultivars used.

As for pUCSTS-R5 and R6, sequence analysis revealed that they have 85bp and 82bp deletion respectively. Although R5 is homologous to pSV25 and R6 is
 20 homologous to VVLSTS, this deletion causes a shift in the open reading frame. As translation uses codon of threes to make amino acids, a shift in the open reading frame, will affect the functionality of the proteins produced. Therefore, these 2 clones are considered as cloning artifacts.

gDNA clones pUCSTS-G5, G9, G13, G14 and G17 isolated are full-length STS genes from *Vitis vinifera* cv. Red Flame. The sizes of the clones which are in the range of 1.5kb to 1.6kb, correspond to the gDNA STS genes *Vst1* and *Vst2* isolated from *Vitis vinifera* cv. Optima (Wiese *et al.*, 1994).

5 Due to the sequences of *Vst1* and *Vst2* are not available except for the sequences of the introns, sequence analysis of gDNA STS clones cannot be compared to *Vst1* and *Vst2*. But, *Vst1* is 98% homology to pSV25 (Wiese *et al.*, 1994). Therefore, the gDNA STS clones can also be compared to pSV25. From the sequence analysis, the gDNA clones obtained are homologous to either pSV21
10 (G13) or VVLSTS (G5, G9, G14 and G17). This again can be explained by the different cultivars used. Another reason maybe the genes that are similar to pSV25 are not being isolated in this experiment.

Restriction maps of the gDNA STS clones from *Vitis vinifera* cv. Red Flame do not show any similarity to those of the existing STS genes (Figure 5a, 5b
15 and 5d). Different cultivars used maybe the reason for this result.

Due to the full sequences have not been sequenced for cDNA and gDNA STS genes of *Vitis vinifera* cv. Red Flame, the hydropathy curves for these clones of STS genes cannot be plotted. However, the hydropathy curves for *Vitis vinifera* cv. Optima STS (pSV21), *Phalenopsis* sp. BS (pBibsy811), *Arachis hypogaea* STS
20 (arqresol) and *Pinus sylvestris* STS (PSTS1), as shown in Figure 3, were similar to each other with 3 main domains. Preisig-Müller *et al.* (1997) showed that the N-terminals of STS and BS were responsible for the substrate recognition or specificity, while the C-terminals were responsible for the product formation.

STS(s) of different plants are quite conserved. Also, despite *Vitis vinifera* cv. Optima (pSv21) and *Arachis hypogaea* STS (arqresol) produced resveratrol, while *Pinus sylvestris* STS (PSTS1) produced pinosylvin as product (Schanz S., 1992 FEBS,313(1):71-74), the STS(s) between these 3 plants are similar.

- 5 STS and BS are conserved too. STS utilizes 4-coumaroyl-CoA and malonyl-CoA whereas BS utilizes m-hydroxyphenylpropionyl-CoA and malonyl-CoA, despite this, their hydropathy curves are similar. According to Fliegmann J. (1992, Plant Mol. Biol. 18:489-503), STS can utilize substrates other than their originally preferred ones, but in a lower rate (that is K_m value is lower). This may
- 10 provide the explanation of the similar hydropathy curves.

Analysis of transformed plants

Plants transformed with the various gene constructs were analyzed for RV concentrations. Results are shown in Table 4.

STS construct	Estimated resveratrol concentration ($\mu\text{g/g f.w.}$)	
	<i>Nicotiana tabacum</i>	<i>Lactuca sativa red lettuce</i>
G14	0.09	0.48
G17	0.27	1.09
R1	0.15	0.94
R65	0.36	0.40
Ctrl	0.00	0.00

Anthocyanin levels were also analyzed. Table 5 shows: anthocyanins level expressed as A_{530}/g for control and transgenic *L. sativa* cv Red Salad Bowl.

Sample	A_{530}/g
PBI (control)	0.0102
G14	0.0133
G17	0.0048
R1	0.0133
R65	0.0047

Table 5

5 HPLC analysis of RV of different transgenic plants is shown in Figures 8A-E. The method of HPLC analysis is as follows:

Resveratrol extraction from putative *L. sativa* cv Red Salad Bowl and *N. tabacum* cv Xanthi

Resveratrol was extracted from putative transgenic *L. sativa* cv Red Salad Bowl and *N. tabacum* cv Xanthi as described in Hain R.(1990, Plant. Mol. 10 biol.15:325-335) and Celotti E. (1996, J. Chromatogr. A.730;47-52) with some modification.

5g of fresh leaves from putative transgenic *L. sativa* cv Red Salad Bowl and *N. tabacum* cv Xanthi were ground in liquid nitrogen with mortar and pestle 15 until powdery. Before the powder started to thaw, 1ml/g fresh weight of methanol (MeOH) was added for extraction at room temperature for 24 hours. After MeOH extraction, the slurry was centrifuged at 7,000rpm for 15 minutes to remove the cell debris. 2 volumes of milli-Q water were added to the supernatant. This

solution was mixed with 9ml ethyl acetate for 15 seconds. The tubes were cooled top 4°C for 3 minutes, then placed in -20°C for 5 minutes. The cooling of the tube improved the separation between the organic phase and the water phase. The organic phase was recovered while the aqueous phase was further extracted twice
5 with 6ml ethyl acetate. The organic phase was recovered and anhydrous sodium sulphate was added to remove any traces of water. The water phase was used for anthocyanin determination. The organic phase was concentrated in Eppendorf™ vacuum concentrator. 50µl of MeOH were added to the dried samples.

HPLC analyzes

10 The putative transgenic samples were analyzed using Shimadzu model CBM-10A reverse-phase HPLC system (Japan). 6ng/µl of chemically synthesized *trans*-resveratrol (Sigma, U.S.A.) were used as the standard. 50µl of the extracted samples were run through C18 column (125mm X 5mm) with water:glacial acetic acid:acetonitrile (75:5:20) as the mobile phase. The flow rate was set at 0.5ml/min
15 and diode array UV detector (SPD-M10AVP) was set at 306nm. The retention time of resveratrol was about 17 minutes.

Anthocyanins determination using visible light spectrophotometry

The method used for the determination of anthocyanins in putative transgenic *L. sativa* cv Red Salad Bowl and *N. tabacum* cv Xanthi was as
20 described in Mancinelli (1990) with some modification. Extraction method followed that of resveratrol determination because the anthocyanins dissolved into the water phase while the resveratrol dissolved into the organic phase. 1ml of the water phase was read by Du® 650 spectrophotometer (Beckman, U.S.A.) in a light

path 10mm cuvette. The absorbances at 530nm and 657nm were determined and the anthocyanins level was calculated by the formula $(A_{530} - 0.25A_{657})/(\text{fresh weight in gram})$. The anthocyanins concentration was expressed as A_{530}/g . The absorption peak of anthocyanins was measured at absorbance 530nm. As for the
5 absorption peak at 657nm, it measured the degraded products of chlorophyll in acidic MeOH.

CLAIMS



1. A red transformed plant comprising a genetic vector transformed therein, said
vector comprising isolated or synthetic DNA encoding a stilbene synthase
5 gene.
2. A red transformed plant according to Claim 1 wherein said genetic vector is a
plasmid and said DNA is an isolated or a synthetic grapevine DNA.
3. A red transformed plant according to Claim 1 wherein said genetic vector is a
plasmid and said stilbene synthase gene is resveratrol synthase gene.
- 10 4. A red transformed plant according to Claim 1 wherein said transformed plant is
red-leaf lettuce.
5. A red transformed plant according to Claim 1 wherein said transformed plant is
capable of producing viable seeds.
6. A transformed plant transformed with a stilbene synthase gene or a portion of a
15 stilbene synthase gene, said stilbene synthase gene encoding a specific stilbene
synthase enzyme, said specific stilbene synthase enzyme synthesizing
constitutive levels of a specific stilbene in said transformed plant, said
transformed plant in the natural untransformed state containing no endogenous
stilbene synthase gene capable of synthesizing said specific stilbene while
20 containing high levels of precursors for said stilbene synthase gene.



7. A transformed plant according to Claim 6 wherein said stilbene synthase gene is resveratrol synthase gene, said specific stilbene synthase enzyme is resveratrol synthase, and said specific stilbene is resveratrol.
8. A transformed plant according to Claim 6 wherein said transformed plant is
5 red-leaf lettuce.
9. A method of producing a transgenic plant containing a specific transgenic stilbene synthase (STS) enzyme transformed therein, said transgenic plant obtained from a recipient plant comprising:
 - 10 a) selecting a recipient plant containing no endogenous stilbene synthase gene encoding said specific stilbene synthase in its natural genome, said recipient plant further containing high levels of the precursors of the transgenic STS enzyme;
 - b) providing a genetic vector comprising an STS gene or a portion of an STS gene encoding said specific STS enzyme, said STS gene or the portion of
15 said STS gene being provided with a promoter suitable for constitutive expression of said specific STS enzyme in the recipient plant;
 - c) transforming the genetic vector into the recipient plant and
 - d) selecting and growing the transformed plant containing high and constitutive levels of the transgenic stilbene.



10. A method according to Claim 9 wherein said selecting step further comprises growing said recipient plant as callus culture in a tissue culture system, and analyzing said callus culture for the levels of precursors for said STS enzyme.
11. A method of producing a transgenic plant containing a transgenic resveratrol synthase (RS) enzyme, said transgenic plant obtained from a recipient plant comprising:
- 5 a) selecting a recipient plant that does not contain any endogenous RS gene in its natural genome but contains high levels of 4-coumaroyl-CoA and malonyl-CoA;
- 10 b) providing a genetic vector comprising an RS gene or a portion of an RS gene encoding an RS enzyme, the RS gene or the portion of the RS gene being provided with a promoter suitable for constitutive expression of the RS enzyme in the recipient plant;
- c) transforming the genetic vector into the recipient plant and
- 15 d) selecting and growing the transformed plant containing high and constitutive levels of the transgenic resveratrol.
12. A method of producing a transgenic plant containing a transgenic resveratrol synthase (RS) enzyme, said transgenic plant obtained from a recipient plant comprising:



- a) selecting a recipient plant having a natural genome with no endogenous RS gene present, the edible portion of said recipient plant further containing high and constitutive levels of anthocyanin;
- b) providing a genetic vector comprising an RS gene or a portion of an RS gene encoding an RS enzyme, the RS gene or the portion of the RS gene being provided with a promoter suitable for constitutive expression of the RS enzyme in the recipient plant;
- c) transforming the genetic vector into the recipient plant and
- d) selecting and growing the transformed plant containing high and constitutive levels of resveratrol in the edible portion.

13. A method according to claim 12 wherein said recipient plant is red-leaf lettuce.