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Francesca Giampieri,[†] Massimiliano Gasparrini,[†] Tamara Y. Forbes-Hernandez,[†,‡] Luca Mazzoni,[§] Franco Capocasa,[§] Silvia Sabbadini,[§] JosèM. Alvarez-Suarez,[∥] Sadia Afrin,[†] Carlo Rosati,[⊥] Tiziana Pandolfini,[#] Barbara Molesini,[#] JoséF. Sánchez-Sevilla,[▽] Iraida Amaya,[▽] Bruno Mezzetti,[§] and Maurizio Battino*[,†,○]

[†]Dipartimento di Scienze Cliniche Specialistiche ed Odontostomatologiche-Sez. Biochimica, Facoltàdi Medicina and [§]Dipartimento di Scienze Agrarie, Alimentari e Ambientali, UniversitàPolitecnica delle Marche, Via Ranieri 65, 60131 Ancona, Italy

[‡]Área de Nutrición y Salud, Universidad Internacional Iberoamericana (UNINI), Campeche C.P.24040, México

[∥]Grupo de Investigación en Biotecnología Aplicada a Biomedicina (BIOMED), Facultad de Ciencias de la Salud, Universidad de Las Américas, Campus Queri - Bloque 7 — Planta Baja, EC170125 Quito, Ecuador

[⊥]ENEA Trisaia Research Center, S.S. 106 km 419.5, 75026 Rotondella, Matera, Italy

[#]Dipartimento di Biotecnologie, Universitàdi Verona, Verona, 37129 Italy

[▽]Instituto Andaluz de Investigación y Formación Agraria y Pesquera, IFAPA-Centro de Churriana, Cortijo de la Cruz s/n, 29140 Málaga, Spain

[○]Centre for Nutrition & Health, Universidad Europea del Atlantico (UEA), 39011 Santander, Spain

Supporting Information

ABSTRACT: Food fortification through the increase and/or modulation of bioactive compounds has become a major goal for preventing several diseases, including cancer. Here, strawberry lines of cv. Calypso transformed with a construct containing an anthocyanidin synthase (*ANS*) gene were produced to study the effects on anthocyanin biosynthesis, metabolism, and transcriptome. Three strawberry *ANS* transgenic lines (ANS L5, ANS L15, and ANS L18) were analyzed for phytochemical composition and total antioxidant capacity (TAC), and their fruit extracts were assessed for cytotoxic effects on hepatocellular carcinoma. ANS L18 fruits had the highest levels of total phenolics and flavonoids, while those of ANS L15 had the highest anthocyanin concentration; TAC positively correlated with total polyphenol content. Fruit transcriptome was also specifically affected in the polyphenol biosynthesis and in other related metabolic pathways. Fruit extracts of all lines exerted cytotoxic effects in a dose/time-dependent manner, increasing cellular apoptosis and free radical levels and impairing mitochondrial functionality.

KEYWORDS: *anthocyanidin synthase, anticancer effects, apoptosis, flavonoids, RNA-seq, strawberry, transcriptome, ROS, mitochondrial functionality*

INTRODUCTION

Berry fruits are favored worldwide and are among the richest sources of bioactive compounds, including minerals, vitamins, fibers, and polyphenols.[1] Among berries, the strawberry (*Fragaria* × *ananassa* Duch.), one of the most commonly consumed, is a significant dietary source of vitamins and phytochemicals, mainly represented by flavonoids (mostly anthocyanins), proanthocyanidins, and ellagitannins.[1−3] Strawberry consumption has been widely associated with the prevention of several chronic pathologies, such as cardiovascular diseases, type 2 diabetes, and obesity, and also certain types of cancers.[1,4−6] In particular, the role of strawberry bioactive compounds on cancer prevention seems to involve different mechanisms, which have not yet been fully elucidated. Cellular antioxidant capacity has long been considered as a first barrier against early stages of the mutagenesis process.[1] Indeed, antioxidants scavenge reactive oxygen species (ROS), decreasing DNA oxidative damage and enhancing DNA repair.[7] In addition, several studies have underlined the ability of these compounds to modulate the cellular processes linked to cancer progression, such as cell proliferation, differentiation, apoptosis, cell cycle arrest, intracellular communication, and angiogenesis.[5,6] Therefore, the genetic improvement of strawberry fruit for increasing the content of bioactive compounds would be beneficial for human health. Modification of biosynthetic pathways through classical breeding or genetic transformation can lead to the development of plants with higher nutritional and phytochemical features.[8,9] In particular, engineering the flavonoid pathway has proven to be an effective tool to enhance fruit nutritional value and quality.[8] Flavonoids are a large group of polyphenols that are widespread among plants and are

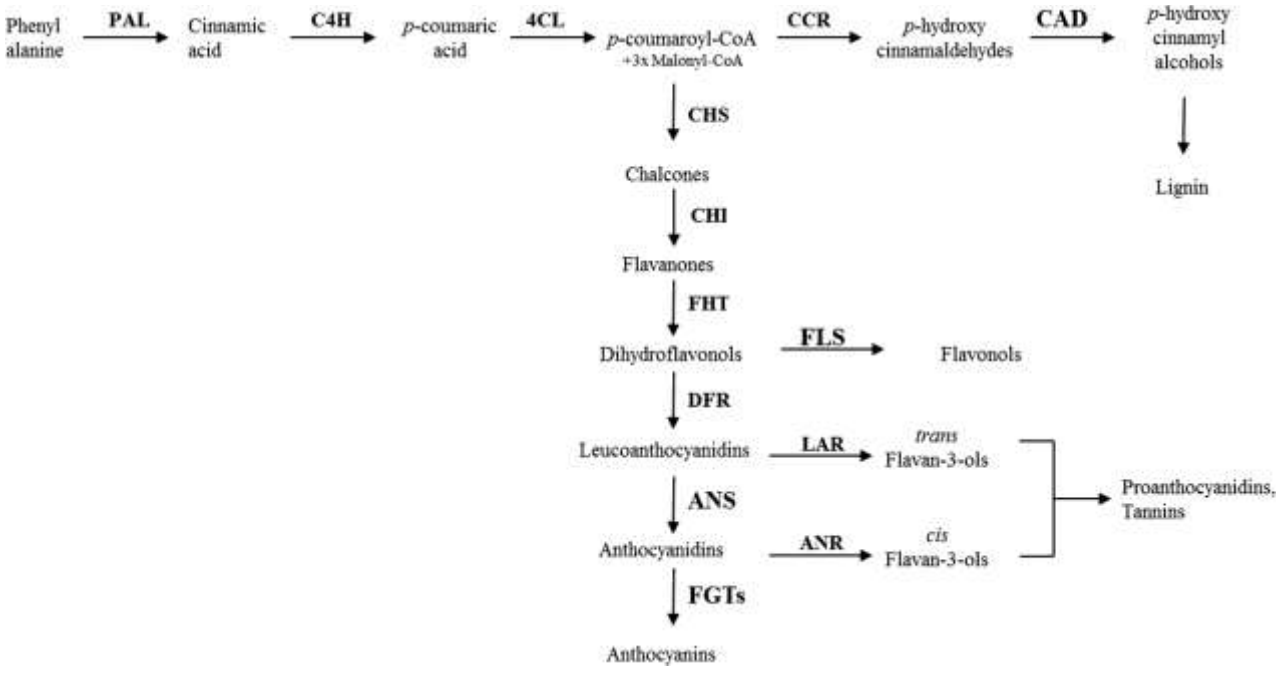

Figure 1. Polyphenol pathways in plants, leading to phenylpropanoids, lignin, tannins, and *fl*avonoids/anthocyanins. Major enzymes and classes of molecules are highlighted; enzymes cited in the text are highlighted in larger font. Abbreviations: 4CL, *p*-coumarate:CoA ligase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; C4H, cinnamic acid 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl-CoA reductase; CHS, chalcone synthase; DFR, dihydro*fl*avonol 4-reductase; FGTs, *fl*avonoid glycosyltransferases; F3H, *fl*avanone 3*β*-hydroxylase; FLS, *fl*avonol synthase; LAR, leucoanthocyanidin reductase; PAL, phenylalanine ammonia-lyase.

involved in many plant functions such as pigmentation, antioxidant capacity, protection from biotic and abiotic stresses, and plant-environment interaction; among them, the anthocyanin pigments are responsible for orange, red, and blue colors in plants.[10,11] Anthocyanin biosynthesis has been extensively studied in several plant species. and the corresponding structural genes have been isolated from many plants.[12] One of the last steps of this pathway involves *ANS*, catalyzing the formation of anthocyanidins before *fi*nal glycosylation steps (Figure 1). Since ripe strawberry fruits contain *fl*avonols, proanthocyanidins, and anthocyanins,[3,13] targeting *ANS* overexpression should direct *fl*avonoid *fl*ow toward anthocyanin biosynthesis, in competition with other polyphenol classes.

Despite knowledge of the *fl*avonoid pathway and the availability of tools, functional studies of *fl*avonoid pathway in strawberries have mostly been achieved by transient approaches rather than genetic engineering.[14,15] In this study, a 35S-driven *ANS* construct was introduced in the everbearing strawberry cultivar "Calypso" by *Agrobacterium tumefaciens*-mediated transformation.

This cultivar was chosen because it produces stable genetically modi*fi*ed plants[16,17] and is everbearing, thus yielding fruit immediately after plant acclimatization from in vitro conditions. The *ANS* gene from *Matthiola incana* (*MiANS*) was chosen since it displays limited homology to its strawberry homologue and also because a 35S-*MiANS* increased anthocyanin synthesis in *Forsythia.*[18] In this work, three *ANS* lines were generated and characterized by molecular and biochemical analyses in comparison to the wt control. Tests were also performed to evaluate the cytotoxic e*ff*ects of fruit extracts on HepG2, a human hepatic cancer cell line, in terms of apoptosis rate, ROS production, and mitochondrial functionality.

MATERIALS AND METHODS

Strawberry Genetic Transformation and Assessment of Transformed Lines. The binary vector pGP27*ans*, containing the *ans* coding sequence from *MiANS* under the control of 35S promoter, was used.[16] Transformation and regeneration experiments were performed using young expanded leaves from in *vitro* proliferating shoots of *Fragaria* × *ananassa* plants cv. Calypso as described.[17,18] *A. tumefaciens* (EHA105) containing the pGP27*ans* inoculation suspension was prepared in LB liquid medium supplemented with antibiotics (50 mg/L kanamycin, 50 mg/L rifampycin) and then incubated overnight at 29 °C on a shaker (100−150 rpm).

Transgenic State of 35S-*MiANS* Lines. Genomic DNA was extracted from 1 g of frozen leaves using the Nucleon PhytoPure system (GE Healthcare) according to the manufacturer's instructions. For PCR analysis, approximately 1 *μ*g of genomic DNA was used as template, and the following forward F 5′-CTTCGTCAACATGGTGGAGCACGACA-3′ and reverse R 5′-TGGAGATATCACATCAATCCACTTG-3′ primers were used. The resulting PCR product of 340 bp corresponded to a portion of CaMV 35S promoter.

For dot blot analysis, 5 *μ*g of denaturated genomic DNA was manually applied on Nylon Hybond-N+ membrane (GE Healthcare) and then *fi*xed by UV cross-linking. The membrane was incubated for 15 min in the denaturation solution (0.5 M NaOH, 1.5 M NaCl) and then washed for 1 min in 2XSSC. The DNA probe, corresponding to the 340 bp fragment of 35S promoter, was labeled with [^{32}P]dCTP using "Ready to go DNA labeling beads (-dCTP)" (GE Healthcare). Unincorporated nucleotides were removed with ProbeQuant G-50 micro columns (GE Healthcare). The membrane was hybridized overnight at 42 °C in ULTRAhyb bu*ff*er (Ambion). Labeled probe (106 cpm mL-1) was added to the hybridization bu*ff*er. The membrane was washed twice in 2X SSC containing 0.1% SDS for 5 min and twice in 0.1X SSC containing 0.1% SDS for 15 min at 42 °C. Autoradiography was then performed using Kodak X-AR5 *fi*lm.

For qPCR analyses, RNA was extracted from ripe fruits using a di*ff*erential 2-butoxyethanol precipitation-based method.[19] Before reverse transcription, RNA was treated with DNase I (Fermentas) to eliminate contaminating genomic DNA. First-strand cDNA

synthesis was performed using 750 ng of RNA in a final volume of 20 μL using the iScript cDNA synthesis kit (Bio-Rad), according to the supplier's protocol. Relative quantification of the *MiANS* gene was performed using the SsoAdvance Universal SYBR Green Supermix (Bio-Rad) and the comparative Delta-Ct method.[20] Expression data were normalized to the reference genes *DBP* and *FaGAPDH2*.[21] Primers used for the *MiANS* were qMiANS-F (5′-GCCCGAAAG-AAGAGCTGGAG-3′) and qMiANS-R (5′-CTCCCCAATCCAAAG-CTGCC-3′).

Identification of Genes Differentially Expressed in Transgenic Lines Overexpressing *ANS*. RNA extraction and RNA-seq from control and transgenic lines. From each transgenic ANS L5, ANS L18, and control lines 30 plants were grown in pots under greenhouse condition and using a standard soil-less strawberry cultivation system. All ripe fruit were harvested and divided in three replicates, ground using liquid nitrogen, and stored at −80 °C until further analysis. Total RNA was extracted from sampled strawberry fruits using a differential 2-butoxyethanol precipitation-based method.[19] Before reverse transcription, RNA was treated with DNase I (Fermentas) to eliminate contaminating genomic DNA. RNA quantity and quality were determined based on absorbance ratios at 260 nm/280 and 260 nm/230 nm using a NanoDrop spectrophotometer (ND-1000 V3.5, NanoDrop Technologies, Inc.). The integrity of the RNA samples was assessed by agarose gel electrophoresis and further verified using a 2100 Bioanalyzer (Agilent, Folsom, CA), and RIN values ranged between 7.1 and 7.4 for the nine samples.

For each of the 9 samples (3 lines with 3 biological replicates), one paired-end library with approximately 300 bp insert size was prepared using an in-house optimized Illumina protocol at the Centro Nacional de Análisis Genómico. Libraries were sequenced on Illumina HiSeq2000 lanes using 2 × 100 bp reads. An average of 37.7 million reads was generated for each sample, ranging from 33.12 to 42.71 M. Primary analysis of the data included base calling and quality control, with an assurance that <80% of all bases passing filter had a quality value of at least 30.

Mapping RNA-seq Reads to the Reference Genome, Generation of Read Counts, and Differential Expression Analysis. Raw RNA-Seq reads were processed to remove low-quality nucleotides and aligned to the *Fragaria vesca* reference genome assembly (*Fragaria vesca* Whole Genome v2.0.a1 Assembly & Annotation)[22] using the program HISAT2 2.1.0.[23] Default parameters of HISAT2 were used, allowing 40 multiple alignments per read and a maximum of 2 mismatches when mapping reads to the reference.

The aligned read files were processed by Cufflinks v2.2[9] essentially as previously described.[24] Reads were assembled into transcripts, which were classified as known or novel, their abundance was estimated, and tests for differential expression between the samples were performed. Normalized RNA-Seq fragment counts were used to measure the relative abundances of transcripts measured as Fragments Per Kilobase of exon per Million fragments mapped (FPKM).

Fruit Quality and Antioxidant Analyses on Strawberry Fruits. For fruit analysis and cell treatment, a hydrophilic extract was done for each strawberry line as previously reported.[6] Total polyphenol content (TPC) of these extracts was determined following the Folin-Ciocalteu method.[25] Total flavonoids (FLAVO) were determined by a colorimetric method described previously,[26] while for anthocyanins (ANTHO) quantitation a modified pH differential method was employed.[27] For the determination of the TAC, the Ferric Reducing Antioxidant Power was performed according to the protocol proposed by Deighton et al.[28]

Cell Culture. HepG2 cells were kindly provided by the Biological Research Laboratory of the Sevilla University (Spain) and were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere with 5% CO_2. For cell treatment, a concentrated supernatant of the fruit extracts from Calypso control and ASN lines was used, dried through a rotary evaporator, and stored in aliquots at −80 °C. The cells were incubated for 24 h with a wide range of concentrations of the different strawberry lines (0−5000 μg/mL). The cultivar Calypso was used as control.

Cell Apoptosis Rate. The apoptosis rate was measured using a Tali Apoptosis assay kit-Annexin V Alexa Fluor 488 (Invitrogen, Milan, Italy), as previously described.[29] Samples were analyzed with the Tali Image-Based cytometer, and the percentage of apoptotic, dead, and live cells was determined on the basis of the respective fluorescence histogram compared with that of the control.

Cellular ROS Concentration. Intracellular ROS generation was determined by CellROX Orange Reagent according to manufacturer's instructions. Cells were analyzed with the Tali Image-Based cytometer. The results were expressed as the percentage of cells with increased ROS levels related to the control.

Mitochondrial Functionality. Oxygen consumption rate (OCR) in HepG2 was measured in real-time using a XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica MA, USA) as previously reported.[30] Cells were seeded for 16 h in the XF-24 plate (30×10^4/well) and incubated for 24 h with the different concentrations of extracts. The medium was then replaced with XF-24 running media, and the plate was incubated at 37 °C for 60 min in the XF Prep Station incubator (Seahorse Bioscience, Billerica MA, USA). After incubation, the plate was transferred to the XF24 Extracellular Flux Analyzer, and after an OCR baseline measurement a profiling of mitochondrial function was performed by sequential injection of three compounds that affect bioenergetics, as follows: 55 μL of oligomycin (2.5 μg/mL) at injection A, 61 μL of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (3 μM) at injection B, 68 μL of rotenone/antimycin (1 mM) at injection C. Maximal respiratory capacity (MRC) was measured by calculating the mean OCR after injection of FCCP minus the rotenone/antimycin values. Data are expressed as pmol of O_2 consumed per minute.

Statistical Analyses. All results are expressed as means ± SD. Statistical analysis was performed using the one-way ANOVA and Tukey's Test correlations; $p \leq 0.05$ was considered significant. For analysis of differential gene expression, confidence intervals for FPKM estimates were calculated using a Bayesian inference method, and significance scores were corrected for multiple testing using the Benjamini-Hochberg correction.[31]

RESULTS

Production and Characterization of New "Calypso" *MiANS* Lines. Leaf discs of cultivar "Calypso" were infected with *A. tumefaciens* carrying the pGP27 binary vector. Only 2−3 subcultures on the same proliferation medium supplemented with antibiotics were enabled to select the most stable regenerants with solid-green tissues, which were transferred on rooting medium. At the end of the selection procedure, three 35S-*MiANS* (ANS L5, L15, and L18) lines able to root in the presence of the selection marker were acclimatized and further characterized in molecular and biochemical studies. PCR and dot blot analyses performed on the 35S CaMV promoter region confirmed the transgenic state of these lines (Figure 2). Analysis of MiANS gene expression by qRT-PCR was performed in ripe fruits of transgenics and control lines. Quite surprisingly, none of the transgenic lines expressed the MiANS transgene.

Phenolic Content and Antioxidant Capacity of Strawberry Extracts. Significant differences ($p < 0.05$) among control and ANS lines were outlined in terms of phenolics concentrations and TAC (Table 1). ANS L18 line had the highest ($p < 0.05$) TPC, even not significantly different from the control 'Calypso', while the lowest TPC was found for ANS L15 ($p < 0.05$). ANS L18 also had the highest concentration ($p < 0.05$) of FLAVO, followed by ANS L15, which presented a similar content to the control. The lowest FLAVO concentration was detected for ANS L5. ANS L15 had the highest total anthocyanin content, followed by ANS L18 and ANS L5 which were statistically similar to the control.

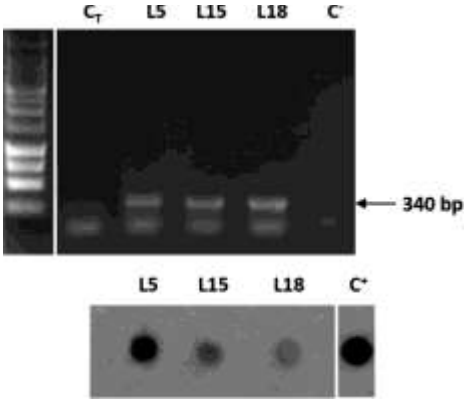

Figure 2. Molecular characterization of the transgenic state of 3 "Calypso" ANS lines. PCR (upper panel) and dot blot (lower panel) analyses were carried out on genomic DNA analyzing the presence of the 35SCaMV promoter. Ct untransformed plants; C^- no-template control; and C^+ purified 35SCaMV promoter.

Finally, ANS L18 selection showed the highest TAC value but similar to the control, while lines ANS L5 and ANS L15 had fruits with lower TAC than the control fruits (Table 1).

Transcriptome Analysis of Transgenic *MiANS* Lines. Two independent "Calypso" transgenic lines (ANS L5 and ANS L18) were selected for a global analysis of gene expression by RNA-seq in comparison to wt plants. RNA extracted from ripe fruits of ANS L5 and ANS L18 lines and control "Calypso" were used in biological triplicate for Illumina pair-end RNA-sequencing. Over 339 million reads 100 bp-long were generated, and after removal of adaptor sequences and low-quality reads, 329.6 million high-quality clean reads (97% of the raw data) remained. We first performed an alignment of sequencing reads to the *Fragaria vesca* Whole Genome v2.0.a1 Assembly & Annotation,[22] the Genome Database for Rosaceae [www.rosaceae.org], as the reference genome using HISAT2.[23] Between 77.39% and 79.14% of reads were paired in sequencing for each of the 9 samples, and an average of 78.15% of filtered paired reads were mapped to the reference genome. Key metrics for the assessment of the quality of mapping reads to the reference genome were extracted from the HISAT2 output and log files (Table S1).

After mapping the RNA-Seq reads to the reference genome, transcripts were assembled, and their relative abundances were calculated using Cufflinks.[31] A total of 35,237 gene/transcripts from the two transgenic lines and the control *Fragaria* × *ananassa* "Calypso" cultivar were predicted based in the reference model and 21,600, 21,313, and 21,638 were expressed in ripe fruits of control, ANS L5, and ANS L18 lines, respectively (genes with normalized reads lower than 0.1 fragments per kilobase of exon per FPKM were considered as not expressed).

Differential gene expression between pairs of different lines was calculated using the ratio of FPKM values of every gene in each line. Two thousand eighty-nine predicted genes were differentially expressed between all possible comparisons (Table S2). When compared to the control, a total of 898 genes were down-regulated in ANS transgenic lines. Of these, 283 were statistically significant in both ANS L5 and ANS L18 lines, 199 only in ANS L5, and 426 only in ANS L18. A total of 1,016 genes were up-regulated in ANS transgenic lines compared to the control, with 239 significantly up-regulated in both transgenic lines, 510 only in ANS L5, and 267 only significant in ANS L18. The observed ratios (log2 fold change) of differential expression ranged from −8.3 to 5.05 with positive and negative values indicating up- and down-regulation in the transgenic lines compared to the control (Table S2). Only 175 genes were differentially expressed between the two transgenic lines. Observed differential expression ratios between the transgenic lines ranged from −4.29 to 2.78.[32] Among the 2,089 differentially expressed transcripts, 1,927 corresponded to annotated genes in the genome sequence of the *Fragaria vesca* gene model v.2.0a1,[22] while 162 were potential new *loci*. Among these 162 novel *loci*, 59 corresponded to *Fragaria vesca* predicted genes annotated recently in the NCBI, and the remaining 103 transcripts have not been previously annotated.

In order to further investigate the potential functions of differentially expressed genes in strawberry metabolism, we analyzed the 522 genes differentially expressed between the ANS lines and the control using the metabolic pathways facilitated by the MapMan software (Figure 3). A total of 476 differentially expressed genes were annotated in the *Fragaria vesca* gene model v2.0a, and 408 (85.71%) were located in different MapMan Bins (Figure 3a).[32]

The MapMan Bins more represented, in addition to categories Bin35 not assigned (125 genes) and Bin26 miscellaneous (44 genes), were Bin29 protein (38 genes), Bin27 RNA (34 genes), Bin34 transport, (26 genes), Bin17 hormone metabolism (22 genes), Bin30 signaling (20 genes), Bin33 development (13 genes), and Bin20 stress (13 genes); but according to the Wilcoxon Rank-Sum Test[33] the most significant Bins were Bin29.5.1 protein.degradation.subtilases (4 up-regulated genes, p = 0.014), Bin26.9 misc.glutathione S transferases (5 down-regulated genes, p = 0.021), Bin34 transport (26 genes, 18 up-regulated, p = 0.022), Bin21.3 redox heme (2 down-regulated genes, p = 0.024), Bin26.11 misc. alcohol dehydrogenases (2 down-regulated genes, p = 0.024), Bin21 redox (1 up-regulated and 8 down-regulated, p = 0.025), Bin26.16 misc.myrosinases-lectin-jacalin (3 genes up-regulated, p = 0.027), and Bin29.5.11 protein.degradation.ubiquitin (12 genes, 10 down-regulated, p = 0.029).

The endogenous strawberry *ANS* gene (gene32347-v1.0-hybrid) was not differentially expressed between lines, maintaining high levels of expression in the three lines:

Table 1. Phytochemical Analyses (Total Phenolics, Total Flavonoids, and Total Anthocyanins) and Total Antioxidant Capacity (TAC) of Strawberry Fruit Extracts

	total phenolics mg GA/g FW	total flavonoids mg Cat/g FW	total anthocyanins mg Pg/g FW	TAC μmol Tx/g FW
Calypso wt	2.68 ± 0.27ab	0.86 ± 0.00b	0.29 ± 0.00b	13.37 ± 0.19a
ANS L5	2.23 ± 0.17ab	0.60 ± 0.02c	0.29 ± 0.01b	10.49 ± 0.51b
ANS L15	1.59 ± 0.11b	0.92 ± 0.09b	0.44 ± 0.01a	9.24 ± 0.38b
ANS L18	3.09 ± 0.45a	1.23 ± 0.06a	0.33 ± 0.01b	13.66 ± 0.76a

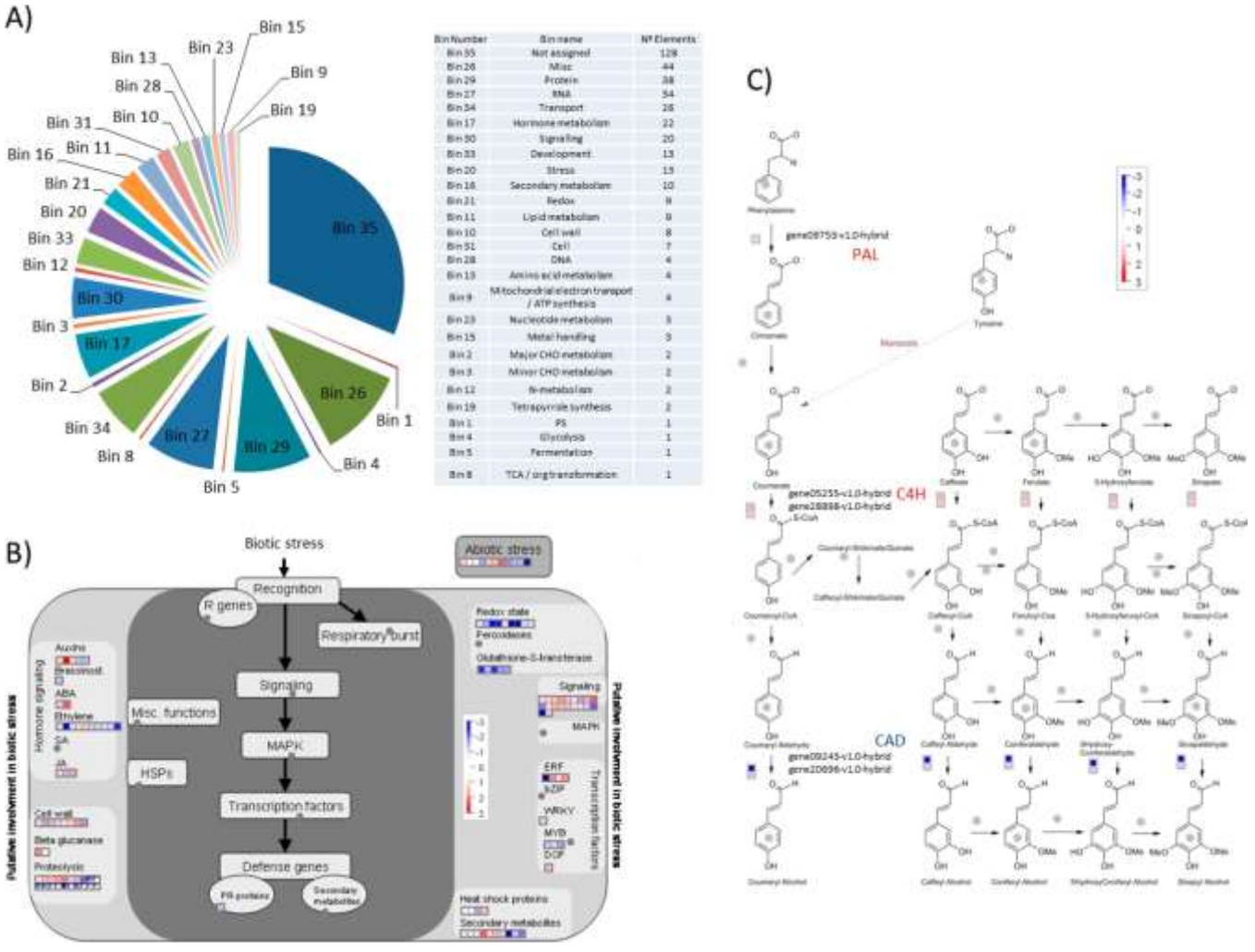

Figure 3. Overview of the effect of *MiANS* ectopic expression on biological processes of strawberry fruit using the MapMan software. a) Distribution of the 408 differentially expressed genes according to Bin groups of *Fragaria vesca* (*F.vesca*_m266.m02). b) MapMan scheme of biotic and abiotic stress categories. c) MapMan schema of genes differentially expressed in the phenylpropanoids pathway. Average Log2 fold change values of differentially expressed genes in ANS lines are represented as colored squares. Significantly decreased and increased transcripts are shown in blue and red, respectively.

1,108.6, 961.15, and 1,037.27 FPKM in control, ANS L5, and ANS L18, respectively.

Among the down-regulated genes, the gene family of oxidoreductases appeared particularly over-represented, with more than 15 differentially expressed genes: two 1-amino-cyclopropane-1-carboxylate oxidases (gene11421-v1.0-hybrid, gene01733-v1.0-hybrid), two 2-aminoethanethiol dioxygenase-like (gene01257-v1.0-hybrid, gene30244-v1.0-hybrid), two alcohol dehydrogenases (gene30068-v1.0-hybrid, gene30069-v1.0-hybrid), two cinnamyl alcohol dehydrogenases (*CAD*) (gene20696-v1.0-hybrid, gene09243-v1.0-hybrid), 3 glutathione S-transferases (gene23386-v1.0-hybrid, gene22014-v1.0-hybrid, gene28763-v2.0.a2-hybrid), or 2 cytochrome p450s (gene24010-v2.0.a2-hybrid, gene12222-v1.0-hybrid) (Table S2). The top 25 down-regulated genes in both ANS transgenic lines are listed in Table 2. Not only oxidoreductases but also other noncatalytic genes related to oxygen and hypoxic stress are over-represented, such as 2-aminoethanethiol dioxygenase-like protein, the universal stress protein A with a −7.65 average log2-fold ratio (about 200-fold change) in the two transgenic lines, and gene11086-v1.0-hybrid with the highest homology to the ethylene-responsive transcription factor ERF071, with a −4.74 average log2-fold change (over 22-fold lower expression) in the transgenic lines.

Among the 239 significantly up-regulated genes in both transgenic lines, 16 were related to transport (Table S2). As an example, the gene30398-v1.0-hybrid showed high similarity to the CSC1-like protein ERD4, a cation membrane transporter associated with responses to abiotic and biotic stresses in *Arabidopsis*.[34] The expression of this transcript was only 4-fold higher (1.9 log2-fold) in both transgenic lines compared to the control, a ratio lower than those observed for the majority of down-regulated genes. Among the remaining up-regulated genes, gene10501-v1.0-hybrid shares a similarity to the transcription factor (TF) MYC2, a positive regulator of flavonoid biosynthetic pathway in *Arabidopsis*. This MYC2 gene from *Fragaria vesca* was recently identified as a candidate transcription factor associated with the flavonoid pathway using a transcriptome coexpression network analysis.[35] However, the expression of this transcript was only 4- and 3-fold higher (2.02 and 1.02 log2-fold) in lines ANS L5 and ANS L18 than in the control. Three genes involved in the early steps of the flavonoid and phenylpropanoids biosynthetic pathways were up-regulated in transgenic lines: The gene09753-v1.0-hybrid with homology

Table 2. List of the Nine Not Expressed and 25 Significantly down-Regulated Genes in the Transgenic Lines Compared to the Control "Calypso"[a]

gene	locus	function	FPKM Calypso	FPKM ANS L5	FPKM ANS L18	Log2 fold change ANS L5-Calypso	Log2 fold change ANS L18-Calypso
-	Fvb2:7914125-7914293	transposase [Streptomyces sp. Root264]	30.7628	0	0		
-	Fvb4:20621477-20621866	—	8.58568	0	0		
-	Fvb2:21802458-21802983	PREDICTED: uncharacterized protein LOC103436258 [*Malus domestica*]	2.66298	0	0		
-	Fvb1:21326036-21329383	hypothetical protein [*Rosa rugosa*]	2.11122	0	0		
gene18418-v2.0.a2-hybrid	Fvb6:8290612-8293556	methyl-binding domain-containing protein 5-like	1.25556	0	0		
-	Fvb1:1175495-1176308	—	1.11479	0	0		
-	Fvb6:33640788-33641788	—	0.88808	0	0		
gene23648-v1.0-hybrid	Fvb7:5392782-5401030	r3h domain-containing protein 2	0.62875	0	0		
-	Fvb5:27372899-27374449	hypothetical protein [*Rosa rugosa*]	0.46945	0	0		
gene11662-v2.0.a2-hybrid	Fvb2:22385688-22388159	hypothetical protein L484_010421	393.909	2.45058	1.26506	−7.32859	−8.28251
gene30244-v2.0.a2-hybrid	Fvb6:10155927-10157253	2-aminoethanethiol dioxygenase-like	36.777	0.20744	0.161999	−7.46997	−7.82668
gene31571-v1.0-hybrid	Fvb6:4282270-4284805	universal stress protein a-like protein	1296.09	8.32588	9.51563	−7.28235	−7.08965
gene10190-v2.0.a2-hybrid	Fvb6:33310891-33312670	2-nonaprenyl-3-methyl-6-methoxy-benzoquinol hydroxylase	191.282	0.779799	2.58547	−7.93838	−6.20913
gene25051-v1.0-hybrid	Fvb5:23128943-23131402	wound-induced protein	265.616	1.72502	2.81281	−7.26659	−6.56118
gene30099-v1.0-hybrid	Fvb6:9568316-9570984	stress response protein nst1	153.086	1.25947	1.62338	−6.92539	−6.5592
gene25053-v2.0.a2-hybrid	Fvb5:23120719-23121770	protein pxr1-like	849.79	6.81071	12.5758	−6.96316	−6.07839
gene03436-v1.0-hybrid	Fvb3:13080909-13082172	ubiquitin carboxyl-terminal hydrolase 12	223.835	2.94351	3.43987	−6.24875	−6.02394
gene26386-v2.0.a2-hybrid	Fvb6:38003124-38006519	kelch repeat-containing At3g27220-like	12.2103	0.134064	0.261846	−6.50904	−5.54324
gene06111-v1.0-hybrid	Fvb4:16326997-16332090	aerobic magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase (aerobic Mg-protoporphyrin IX monomethyl ester oxidative cyclase) (probable)	1115.79	16.2531	22.6792	−6.10121	−5.62056
gene37914-v2.0.a2-hybrid	Fvb6:33302548-33310038	wound-responsive family protein	381.562	6.4705	6.76219	−5.8819	−5.81829
gene30740-v1.0-hybrid	Fvb3:2014022-2015903	zinc finger constans-like 11-like protein	19.1035	0.551092	0.649525	−5.1154	−4.87831
gene11086-v2.0.a2-hybrid	Fvb2:20577054-20578550	ethylene-responsive transcription factor erf071	87.878	3.31902	3.2601	−4.72667	−4.75251
gene30069-v1.0-hybrid	Fvb2:9552243-9555571	alcohol dehydrogenase	64.7559	2.42143	3.28471	−4.74108	−4.30117
LOC101300575	Fvb7:13280126-13289619	+PREDICTED: RNA-binding protein with multiple splicing 2-like [*Fragaria vesca* subsp. vesca]	203.593	15.0089	6.63686	−3.76181	−4.93905
gene05410-v1.0-hybrid	Fvb6:27600167-27607599	hepatoma-derived growth factor-related protein 2 (HRP-2) (probable)	9.53816	0.62768	0.522177	−3.92561	−4.1911
gene08771-v2.0.a2-hybrid	Fvb2:26222590-26223311	two-on-two hemoglobin-3	382.81	30.1417	20.1203	−3.6668	−4.2499
-	Fvb1:57343-59182	—	88.5017	5.14597	6.47378	−4.10419	−3.77302
gene10300-v1.0-hybrid	Fvb6:33696864-33699528	lob domain-containing protein 41-like	84.8063	6.59361	5.28445	−3.68503	−4.00435
gene19959-v1.0-hybrid	Fvb3:7773784-7777092	haloacid dehalogenase-like hydrolase domain-containing protein 3	189.239	12.7089	14.1686	−3.8963	−3.73944
gene30986-v1.0-hybrid	Fvb1:1996971-1998659	early nodulin-93-like	14.0498	0.765925	1.33062	−4.19721	−3.40038
gene32701-v1.0-hybrid	Fvb2:9485386-9487539	acyl-	10.838	0.669509	0.918558	−4.01685	−3.56058
gene31964-v2.0.a2-hybrid	Fvb2:8765322-8773421	extra-large guanine nucleotide-binding protein 1-like	101.254	8.70006	6.36928	−3.54081	−3.9907

Table 2. continued

gene	locus	function	FPKM Calypso	FPKM ANS L5	FPKM ANS L18	Log2 fold change ANS L5-Calypso	Log2 fold change ANS L18-Calypso
gene19672-v1.0-hybrid	Fvb2:8206790-8208188	nonsymbiotic hemoglobin	95.3655	6.76758	7.50647	−3.81676	−3.66726
gene10643-v2.0.a2-hybrid	Fvb5:12639162-12646638	senescence-associated family protein	170.117	11.8323	14.9128	−3.84572	−3.51191

[a]The gene number according to the *Fragaria vesca* Whole Genome v2.0.a1 (www.rosaceae.org). Transcripts detected by cuffllinks with no reference transcript predicted in the *Fragaria vesca* gene model are named by NCBI *Fragaria vesca* gene homologous (LOC-) or remain unnamed (−).

to phenylalanine ammonia-lyase (PAL) and the genes gene28898-v1.0-hybrid and gene05255-v1.0-hybrid, with homology to 4-coumarate ligase (C4H). The top 25 up-regulated genes in both ANS transgenic lines are listed in Table 3.

The overview of differentially expressed genes using MapMan supported previously observed results. In the secondary metabolism category (Bin16), where the flavonoids and phenylpropanoids pathways are included, two *CAD* genes were down-regulated (gene09243-v1.0-hybrid −2.867 and gene20696-v1.0-hybrid −1.112 average log2-fold change) in ANS lines. These two *CAD* genes and the PAL and CH4 up-regulated genes appear in the cell wall and secondary metabolism categories in Figure 3b and in the phenylpropanoids pathway (Figure 3c). Several transcription factors, including the putative transcription factor MYC2 TF (gene10501-v1.0-hybrid) were down-differentially expressed in ANS lines (Bin 27). The majority of differentially expressed genes was related to biotic or abiotic stress responses and presented much lower expression values in the ANS transgenic lines (Figure 3b).

Evaluation of Cytotoxicity of Strawberry Extracts. In the present work, a dose-dependent cytotoxic effect on cell viability was observed in cells treated with strawberry extracts of the three ANS lines and the control Calypso: indeed, cellular viability decreased with the increasing concentrations of the extracts, while apoptosis and death rate increased. In particular, treatment with 100 μg/mL (Figure 4a) resulted in a modest decrease in cellular viability and in a concomitant moderate increase in cellular death, higher in all ANS lines compared to control Calypso. Treatment with 1000 μg/mL (Figure 4b) led to a significant decrease ($p \leq 0.05$) in cell viability and a noteworthy increase in cellular death, especially for ANS L5 and ANS L18, while with 5000 μg/mL treatment (Figure 4c) a very strong reduction in cell viability was found for extracts of all ANS lines, compared to control Calypso.

These results were confirmed by the evaluation of intracellular ROS concentration: after treatment with 100 μg/mL fruit extracts, a slight increase of ROS concentration was found for all ANS lines, even if not significantly different from "Calypso", but different ($p \leq 0.05$) from untreated cells. A significant ($p \leq 0.05$) increase of intracellular ROS levels was registered for all the ANS lines with 1000 μg/mL of fruit extracts, compared to both Calypso and untreated cells. The rise of ROS levels was even more pronounced with 5000 μg/mL, especially for ANS L15 and ANS L18 lines (Figure 5).

Mitochondrial Functionality. Fruit extracts of ANS lines also affected negatively mitochondrial functionality (Figure 6). MRC was measured by calculating the mean OCR after injection of FCCP minus the rotenone/antimycin values. MRC increased in cells treated with extracts from the ANS lines compared to untreated cells: at a lower concentration (100 μg/mL), the MRC rate dramatically increased ($p \leq 0.05$) with ANS L15 and ANS L18. The same trend was observed with 1000 μg/mL extracts, while at 5000 μg/mL the maximal oxygen consumption rate decreased considerably, except for ANS L15.

DISCUSSION

During the last 30 years, strawberry breeding objectives have mainly focused on meeting aesthetic standards with eating quality, durable disease resistance, and health benefits.[2,36] All these traits equally received the highest priority, since markets have become more demanding about fruit quality. In the present study, transgenic strawberry lines ANS L5, ANSL 15, and ANSL 18 were obtained by genetic modification of the commercial variety "Calypso" using a construct that ectopically overexpressed the gene *MiANS*, involved in the metabolism of anthocyanins, from *Matthiola incana*. The effect of overexpressing this gene on fruit polyphenolic content and TAC was investigated, as well as the anticancer effect of fruit extracts.

Phytochemical analysis highlighted that ANS transgenic lines had a variable phenolic, flavonoid, and anthocyanin content with respect to the wt "Calypso". In particular, ANS L18 fruits had the highest levels of total phenolics and flavonoids, while those of ANS L15 had the highest anthocyanin concentration despite the lowest phenolic content. Interestingly, total antioxidant capacity positively correlated with total phenolics content, of which only a moderate percentage was represented by anthocyanins, as reported for other berry species.[28]

A puzzling evidence from early molecular PCR analyses was the lack of transgene expression despite the proof of transgene presence, which promoted deeper transcriptomic analyses. Transgene silencing and, more in general, variability of transgene expression have been widely reported in plant transformation experiments, as a result of multiple factors such as position effects (point of insertion and orientation of T-DNA), transgene copy number, sequence homology, and ploidy level of target plant species.[37] Transgene dosage was associated with silencing in *Arabidopsis*, where a regulator gene of the flavonoid pathway was silenced in homozygous progenies obtained by self-fertilization of transformants.[38] Also, the octoploid background of *F.* × *ananassa* might have played a role against *MiANS* expression, as shown in *Arabidopsis*,[39] especially if considering possible sequence homologies of conserved domains of structural flavonoid gene families.[40] Dot blot analysis (Figure 2, lower panel) pointed out likely differences in the transgene copy number in the three transgenic lines, with ANS L5 showing probable multiple insertions compared to ANS 15 and ANS 18 lines. Indeed, L5 had the same total anthocyanin levels as the wt control and the lowest flavonoid content among lines, indicating a possible, positive correlation between transgene dosage and silencing. The variation in phenolic phytochemicals among the three investigated lines points out the need of further analyses which go beyond the scope of the scope of the present investigation

Table 3. List of the Two Genes Not Expressed in the Control Calypso but in the Transgenic Lines and the Top 25 Significantly up-Regulated Genes in the Transgenic Lines Compared to the Control "Calypso"[a]

gene	locus	function	FPKM Calypso	FPKM ANS L5	FPKM ANS L18	Log2 fold change ANS L5- Calypso	Log2 fold change ANS L18- Calypso
LOC101309210	Fvb2:4163541-4163651	PREDICTED: uncharacterized protein	0	300.281	722.358		
LOC101305487	Fvb6:8133970-8134148	PREDICTED: putative disease resistance protein RGA1	0	4.41385	7.72632		
gene25979-v1.0-hybrid	Fvb5:8738308-8739784	transcription factor pre6-like	0.280215	9.29161	3.16352	5.05132	3.49693
gene16792-v1.0-hybrid	Fvb6:372582-375231	auxin efflux carrier component 8	0.360304	2.08762	2.92196	2.53457	3.01965
gene32514-v1.0-hybrid	Fvb5:1583051-1585784	cytochrome p450 86a8	0.541584	2.2016	3.20719	2.0233	2.56605
gene08549-v1.0-hybrid	Fvb2:27282549-27287361	synaptotagmin-2	0.160791	0.75243	0.736002	2.22637	2.19452
gene32054-v1.0-hybrid	Fvb5:2420323-2421765	at5g41810 k16l22_9	0.528799	2.18381	2.38776	2.04606	2.17487
gene15206-v1.0-hybrid	Fvb2:27829214-27831854	jacalin-related lectin 3	0.752413	3.31142	3.06639	2.13785	2.02694
gene38037-v1.0-hybrid	Fvb6:37181681-37184844	probable galactinol--sucrose galactosyltransferase 5	1.12782	4.67457	4.82047	2.0513	2.09564
gene32077-v1.0-hybrid	Fvb5:2527687-2530129	PREDICTED: uncharacterized protein LOC101310991	2.78962	10.3997	12.5671	1.8984	2.17151
gene20922-v1.0-hybrid	Fvb7:18182014-18183845	uncharacterized acetyltransferase at3g50280	0.876011	3.04501	3.94046	1.79743	2.16934
gene05139-v1.0-hybrid	Fvb4:22426085-22427850	jacalin-related lectin 3-like isoform x1	2.38862	9.93688	8.81138	2.05661	1.88319
gene05107-v1.0-hybrid	Fvb4:22259171-22262816	glycine-rich cell wall structural isoform x8	312.135	1605.33	922.051	2.36263	1.56268
gene31335-v1.0-hybrid	Fvb3:20097219-20099079	9-*cis*-epoxycarotenoid dioxygenase chloroplastic-like	2.04895	9.42847	6.74679	2.20214	1.71931
gene30398-v1.0-hybrid	Fvb3:2852992-2858810	csc1-like protein erd4	12.4416	48.3887	47.5414	1.95949	1.93401
gene05747-v1.0-hybrid	Fvb1:10864195-10869562	calcium-transporting atpase plasma membrane-type-like	9.18777	28.2564	43.8008	1.62079	2.25317
gene25112-v1.0-hybrid	Fvb5:6756185-6758954	bidirectional sugar transporter sweet4	7.88641	29.5209	29.5019	1.90429	1.90337
gene16444-v1.0-hybrid	Fvb6:2147903-2168896	Staphylococcal secretory antigen ssaA2, Precursor (probable)	10.4596	33.6403	44.4744	1.68536	2.08814
gene21073-v1.0-hybrid	Fvb1:21340114-21344317	19770330 homeodomain-like superfamily protein isoform 2	0.28554	0.824954	1.32397	1.53062	2.2131
gene35995-v1.0-hybrid	Fvb2:26804035-26807213	abc transporter g family member 14	0.400913	1.45599	1.46781	1.86064	1.8723
gene37042-v1.0-hybrid	Fvb5:2948134-2950451	u-box domain-containing protein 19-like	2.96015	8.8908	12.8287	1.58664	2.11564
gene26870-v1.0-hybrid	Fvb5:23648297-23659126	peptide transporter PTR5 (probable)	0.97319	3.24036	3.69191	1.73536	1.92357
gene11628-v1.0-hybrid	Fvb2:22192620-22196106	PREDICTED: uncharacterized protein LOC101310190	0.394861	1.26389	1.46316	1.67845	1.88967
gene11154-v1.0-hybrid	Fvb2:20970132-20972797	wat1-related protein at4g08300-like	3.33859	10.2172	12.6943	1.6137	1.92687
gene23986-v1.0-hybrid	Fvb1:10541913-10544199	subtilisin-like protease	0.343999	1.2255	1.11445	1.83289	1.69586
gene13272-v1.0-hybrid	Fvb7:21186297-21193933	low-temperature-induced 65 kDa protein (probable)	33.5826	112.271	111.5	1.74119	1.73126
gene16256-v1.0-hybrid	Fvb1:16822476-16841892	abc transporter g family member 11-like	0.622216	1.93356	2.20648	1.63577	1.82626

[a]The gene number according to the *Fragaria vesca* Whole Genome v2.0.a1 (www.rosaceae.org). Transcripts detected by cufflinks with no reference transcript predicted in the *Fragaria vesca* gene model are named by NCBI *Fragaria vesca* gene homologous (LOC).

focused on health effects of strawberry extracts produced, to elucidate the effect of *MiANS* insertion in "Calypso".

Our transcriptomic analysis on *ANS* and control lines suggests that the ectopic expression of the heterologous structural gene *MiANS,* encoding a late enzyme of the flavonoid biosynthetic pathway, had an impact in the biotic and abiotic stress and in the secondary metabolism of strawberry fruit. Based on the low similarity between the endogenous and the *MiANS* gene, it is anticipated that no interference in the RNA-seq analysis can be expected, like for example mapping of reads from *MiANS* transgene expression in transgenic lines. In concordance with this, expression of the *FvANS* gene was not affected in the transgenic lines.

The expression of genes encoding enzymes of two early steps on the phenylpropanoids pathway, PAL and C4H, was significantly up-regulated in transgenic lines. Furthermore, the expression of two *CAD* genes, also involved in the phenyl-propanoid pathway, was largely reduced in ANS lines. The first

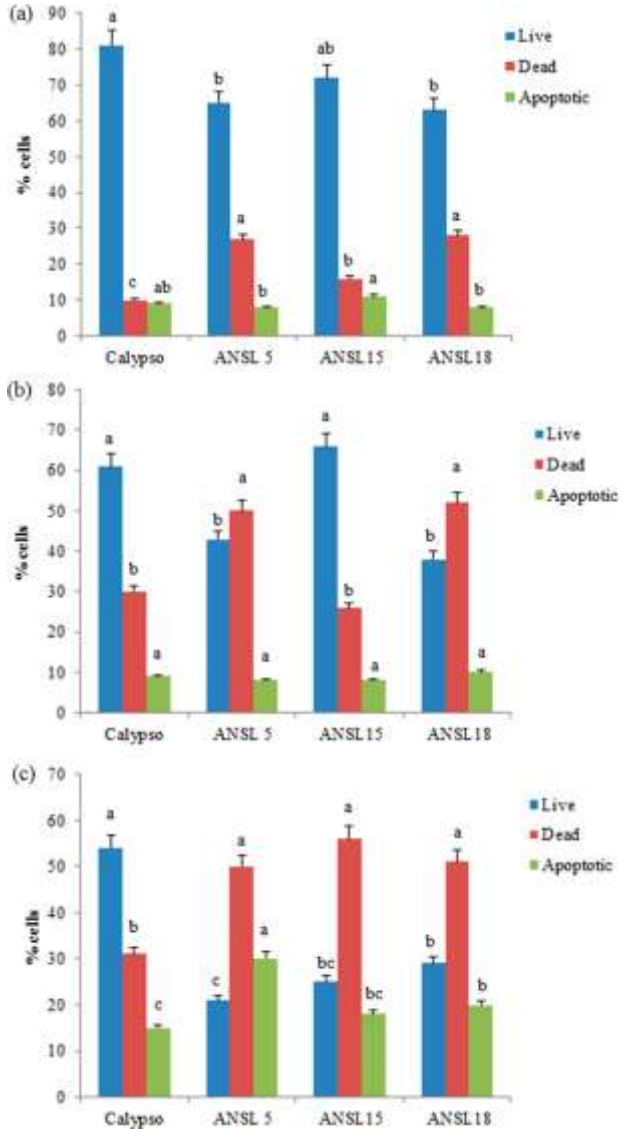

Figure 4. Percentage of live, dead, and apoptotic cells after the treatment with (a) 100 μg/mL, (b) 1000 μg/mL, and (c) 5000 μg/mL of fruit extracts of ANS lines and "Calypso" control. Columns belonging to the same set of data and labeled with di*ff*erent letters are signi*fi*cantly di*ff*erent from the control "Calypso" ($p < 0.05$).

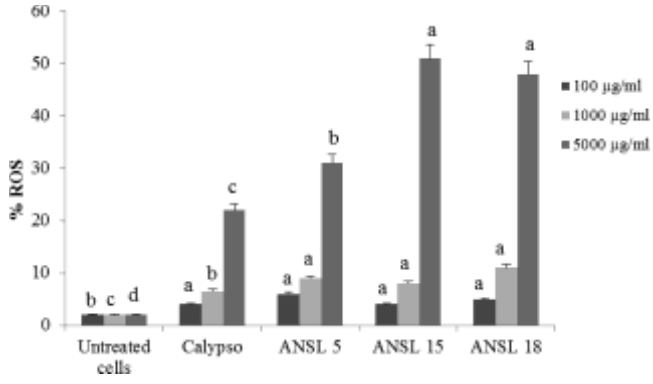

Figure 5. Percentage of intracellular ROS in untreated cells and after treatment with 100 μg/mL, 1000 μg/mL, and 5000 μg/mL of ANS lines and Calypso control strawberry extracts. Columns belonging to the same set of data and labeled with di*ff*erent letters are signi*fi*cantly di*ff*erent from the control ($p < 0.05$).

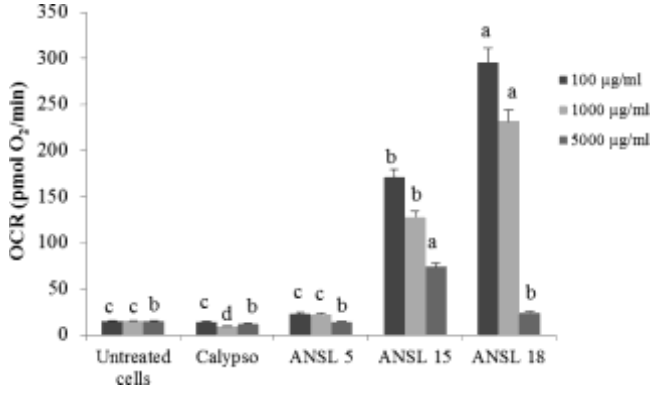

Figure 6. Maximal respiratory capacity monitored in untreated cells, control cells (treated with "Calypso" extracts), and in cells treated with extracts of ANS lines, at di*ff*erent concentrations. Columns belonging to the same set of data and labeled with di*ff*erent letters are signi*fi*cantly di*ff*erent from the control ($p < 0.05$).

part of the pathway is common to the *fl*avonoids pathway (Figures 1 and 3). We could hypothesize that these changes in expression could result in an increased *fl*ux to the *fl*avonoid pathway and in particular to anthocyanin biosynthesis in ANS lines, resulting in a reduction of common substrates for phenylpropanoid biosynthesis. Substrate *fl*ow between the *fl*avonoid and the phenylpropanoid pathways was shown to occur studying the e*ff*ect of *CHS* silencing in strawberry fruit.[9] Phenylpropanoids also contribute to fruit antioxidant capacity and a reduction in the *fl*ux to this biosynthetic pathway could explain why TAC was not signi*fi*cantly increased in ANS transgenic lines. Our global analysis of gene expression highlighted a large reduction in the expression of a reduced number of genes, such as a number of oxidoreductases that could be related to response to biotic and abiotic stresses. A higher content of TPC, FLAVO, and ANTHO in ANS transgenic lines could result in an adjustment of the cellular redox homeostasis through reduced expression of these stress related genes.

The consumption of fruits and vegetables is associated with a decreased risk of developing many chronic degenerative diseases, including cancer. Berries exert anticancer activities thanks to the wide range of bioactive compounds they contain: these substances inhibit cell proliferation, induce apoptosis, regulate angiogenesis, modulate metabolizing enzymes and cell signaling, and a*ff*ect gene expression.[41] In the present work, the cytotoxic and pro-apoptotic e*ff*ects of strawberry extracts of ANS transgenic and control lines were evaluated on human liver cancer cells, highlighting a dose-dependent cytotoxic e*ff*ect in cells treated with these three ANS strawberry lines. At lower concentration, the three ANS lines had a similar e*ff*ect than the control, while at medium and higher concentrations, the pro-apoptotic e*ff*ects were more pronounced in the three selections. Our results are in accordance with previous studies, in which di*ff*erent strawberry cultivars have shown to inhibit proliferation in diverse cancer cell lines, including HepG2, to di*ff*erent levels.[9,42] The varying magnitude of the inhibitory e*ff*ects on cancer cell proliferation indicates that there is a potential to select cultivars with increased levels of health-promoting compounds that could be used as components in food products. In addition, the study of management factors in

traditional and organic cultivation and in the breeding strategy might be helpful in elucidating which compounds exhibit the antiproliferative activity against cancer cell growth.[43]

One of the mechanisms through which many phytochemicals could induce apoptosis is the activation of the intrinsic pathway, which involves a variety of cellular stimuli including ROS generation. Our results confirmed that the treatment of the three ANS lines with fruit extracts induced intracellular ROS production, at a higher level compared with fruit extract of the control line, and in a dose-dependent manner, showing that the production of ROS could be a realistic pathway through which this fruit could inhibit cell proliferation and induce apoptosis, as previously reported for other dietary polyphenols.[44−46]

Finally, the deleterious effects of fruit extracts from ANS lines, and to a lesser extent also from control, especially at higher concentrations, on cancer cells were confirmed by mitochondrial functionality evaluation: even if the respiratory chain is not the major source of energy in cancer cells, it was possible to note the negative effect of treatment with strawberry ANS extracts also on these organelles. In particular, at lower and medium concentrations, MRC values increased, especially with ANS L15 and ANS L18 lines, because treatment led to moderate cell apoptosis and death and cells tried to counteract this condition increasing their mitochondrial respiration. At higher concentrations, MRS considerably decreased, because of the high presence of apoptotic and dead cells, which presented faulty mitochondria.

In conclusion, the modification of phenolic metabolism brought about by ectopic ANS transgene insertion altered the content of strawberry bioactive compounds with possible positive effects on human health, as evidenced by the experiments on human hepatic cancer cells. This investigation is of particular interest to better define biotechnology strategies for validating gene/enzyme role in metabolic pathways and for increasing fruit nutritional quality of elite strawberry cultivars. This work showed the alterations to flavonoid content and described extensive modifications in transcript expression patterns due to *MiANS* transformation, demonstrating the benefits for human health for the increased cytotoxic effect on human liver cancer cells HepG2.

AUTHOR INFORMATION

Corresponding Author

*Phone: +39 071 2204646. Fax: +39 071 2204123. E-mail m.a.battino@univpm.it. Corresponding author address: DISCO, Facoltàdi Medicina, UniversitàPolitecnica delle Marche, Via Ranieri 65, 60131 Ancona, Italy.

ORCID

Massimiliano Gasparrini: 0000-0001-9738-2663

JosèM. Alvarez-Suarez: 0000-0001-8509-1498

Maurizio Battino: 0000-0002-7250-1782

Author Contributions

M.B. and B.M. conceived and designed the research. T.J.F.H., M.G., L.M., J.M.A.S., S.A, F.C., S.S., and F.G. performed fruit and cellular analysis. C.R. assembled the ANS construct. T.P. and J.S. characterized GM lines. S.S. and I.A. performed the RNA-Seq study. F.G., R.C.I., R.R., S.A., and J.C. wrote the manuscript. Critical revision was done by C.R., B.M., and I.A.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ANS, anthocyanin synthase; ANTHO, anthocyanins; C4H, 4-coumarate ligase; CHS, chalcone synthase; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; FLAVO, total flavonoids; FPKM, fragments per Kilobase of exon per Million fragments mapped; MiANS, Mathiola incana; MRC, maximal respiratory capacity; OCR, oxygen consumption rate; PAL, phenylalanine ammonia-lyase; ROS, reactive oxygen species; TAC, total antioxidant capacity; TF, transcription factor; TPC, total polyphenol content

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