Molecular Characterization of a Stable Antisense Chalcone Synthase Phenotype in Strawberry (*Fragaria* × *ananassa*)

STEFAN LUNKENBEIN,[†] HEATHER COINER,[†] C. H. RIC DE VOS,[‡] JAN G. SCHAART,[‡]
MARJAN J. BOONE,[‡] FRANS A. KRENS,[‡] WILFRIED SCHWAB,*,[†] AND
ELMA M. J. SALENTIJN[‡]

Biomolecular Food Technology, Technical University München, Lise-Meitner-Strasse 34,
85354 Freising, Germany, and Business Unit Genetics and Breeding, Plant Research International,
P.O. Box 16, 6700 AA, Wageningen, Netherlands

An octaploid (*Fragaria* × *ananassa* cv. Calypso) genotype of strawberry was transformed with an antisense chalcone synthase (*CHS*) gene construct using a ripening related *CHS* cDNA from *Fragaria* × *ananassa* cv. Elsanta under the control of the constitutive CaMV 35S promoter via *Agrobacterium tumefaciens*. Out of 25 transgenic lines, nine lines showed a reduction in *CHS* mRNA accumulation of more than 50% as compared to the untransformed cv. Calypso control. The antisense *CHS* construct was found to be integrated into the genome, with a copy number ranging from one to four. The pigmentation of the fruit was only affected when less than 5% of the control *CHS* expression level was detected. A stable antisense phenotype over a period of 4 years was obtained in the primary transgenic lines at a rate of 1:20. As a consequence of the reduced activity of CHS, the levels of anthocyanins, flavonols, and proanthocyanidins were downregulated and precursors of the flavonoid pathway were shunted to the phenylpropanoid pathway leading to highly increased levels of cinnamoyl glucose (520% of control), caffeoyl glucose (816% of control), and feruloyl glucose (1092% of control) as well as *p*-coumaryl alcohol (363% of control) and *p*-coumaryl-1-acetate (1079% of control), which occur only as trace components in untransformed control fruits. These results demonstrate that the introduction of an antisense *CHS* construct in strawberry results in an unpredictable biochemical phenotype, thereby confirming that CHS function is an important regulatory point of substrate flow between the flavonoid and the phenylpropanoid pathways.

KEYWORDS: Strawberry; *Fragaria* × *ananassa*; chalcone synthase; phenylpropanoid; *p*-coumaryl-1-acetate

INTRODUCTION

Strawberry belongs to the rose family (*Rosaceae*, subfamily Rosoideae, tribe Potentilleae) in the genus *Fragaria*. The four basic fertility groups in *Fragaria* are divided according to their ploidy level or chromosome number. *Fragaria vesca* is the most common native species, which contains 14 chromosomes and is diploid (*1*). The cultivated varieties of commercial strawberries usually recognized as *Fragaria* × *ananassa* are almost all octaploids, containing 56 chromosomes.

Strawberry is an important nonclimacteric soft fruit and is cultivated almost worldwide. Key fruit quality factors are taste, odor, textural properties, and color. The major pigments detected in strawberry are the anthocyanins pelargonidin-3-glucoside (purple) and cyanidin-3-glucoside (red) although minor amounts of pelargonidin-3-rutinoside, pelargonidin-3-glucoside-succinate,

and cyanidin-3-glucoside-succinate have also been detected (*2*, *3*) (**Figure 1**). These pigments belong to the flavonoids, a diverse family of secondary metabolites, which includes six major subgroups found in most higher plants: chalcones, flavones, flavonols, flavandiols, anthocyanins, and condensed tannins (or proanthocyanidins). A seventh group, the aurones, is widespread but not ubiquitous. In addition to playing key roles in pigmentation in flowers, fruits, seeds, and leaves, flavonoids function in signaling between plants and microbes, in male fertility, in stress responses, in defense, and in UV protection (*4*, *5*).

In strawberry, cDNA microarray analyses and metabolome profiling of fruit developmental stages using Fourier transform ion cyclotron mass spectrometry allowed the respective detection of ripening related genes and metabolites (*6*, *7*). In early stages of fruit development, strawberry fruits accumulate relatively large quantities of tannins (proanthocyanidins) and putative phenolic acids such as ellagic, hydroxybenzoic, vanillic, and gentisic acids. Between the turning and the red ripening stages of fruits, various types of phenolic and aromatic components

* To whom correspondence should be addressed. Tel: +49(0)8161-548-312. Fax: +49(0)8161-548-595. E-mail: schwab@wzw.tum.de.
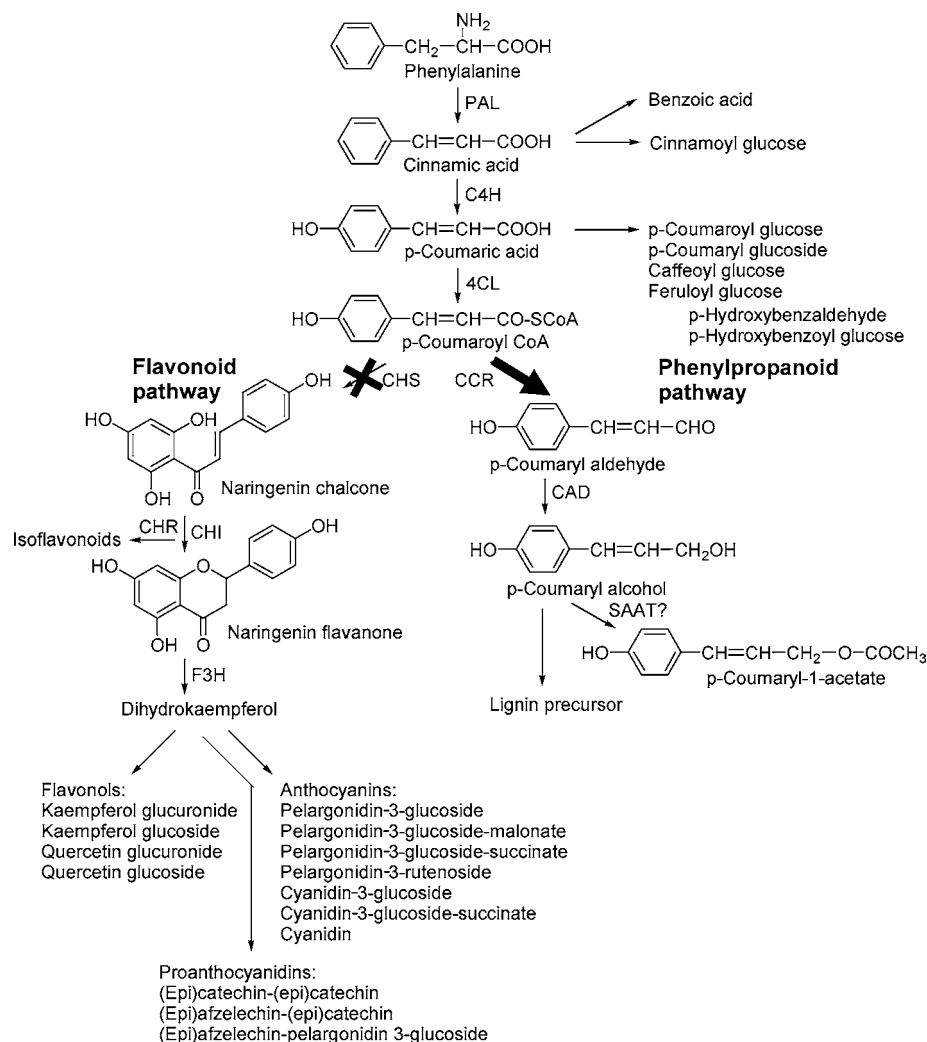[†] Technical University München.
[‡] Plant Research International.

Figure 1. Biosynthetic pathway leading to flavonols, proanthocyanidins, anthocyanins, isoflavonoids, phenylpropanoyl esters, *p*-coumaryl alcohol, and *p*-coumaryl-1-acetate. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, *p*-coumarate ligase; CHS, chalcone synthase; CHI, chalcone isomerase; CHR, chalcone reductase; F3H, flavanone 3-hydroxylase; CCR, coumaroyl CoA reductase; CAD, p-coumaryl aldehyde dehydrogenase; SAAT, strawberry alcohol acyl CoA transferase.

were observed, including conjugates with cinnamic acid and its derivatives *p*-coumarate, caffeate, ferulate, hydroxyferulate, and sinapate (*6*). Among the flavonoids detected in strawberry fruits are the flavonols dihydrokaempferol, dihydroquercetin, kaempferol glucoside, and quercetin glucoside and the anthocyanins leucocyanidin, pelargonidin-3-glucoside, pelargonidin, and cyanidin glucoside (*6, 8, 9*).

Strawberry is an economically important crop but displays only a short postharvest shelf life due to its rapid softening. In addition to breeding, genetic engineering constitutes a suitable new tool for improvements to the fruit. The genetic transformations of the cultivated strawberry (*Fragaria × ananassa* Duch.) and wild strawberry (*F. vesca*) have already been reported. Although they are considered to be relatively amenable to transformation using *Agrobacterium* (*10−15*), transformation frequencies are greatly influenced by the cultivar and the procedure used (*16, 17*). The technique is being used successfully to genetically engineer virus resistance (*18*). Other candidate traits for transformation include glyphosate resistance, broad spectrum fungal resistance through the use of the stilbene synthase gene and genes for systemic acquired resistance, nematode resistance through the use of transgenes producing protease inhibitors, and the manipulation of fruit softening by the antisense expression of a pectate lyase gene (*19−21*).

Chalcone synthase, or naringenin CHS, catalyzes the formation of naringenin, the precursor for several flavonoids derived from malonyl-CoA and *p*-coumaroyl-CoA, and is thus a key enzyme in the biosynthesis of flavonoids (**Figure 1**). CHS is a typical homodimeric plant polyketide synthase with two subunits of about 43 kDa (*22, 23*). Because the CHS from *Medicago sativa* was first crystallized for X-ray diffraction analysis (*23*), the evidence that CHS is closely related to other plant-specific polyketide synthases, including stilbene synthase (*24−26*), acridone synthase (*27*), and 2-pyrone synthase, is accumulating (*28*). CHS function is regarded as a regulatory focal point of substrate flow between the flavonoid pathway and the other competing directions of the phenylpropanoid pathway, leading to a diversity of phenolic compounds such as lignins and phytoalexins (*4, 29, 30*). Genes encoding CHS constitute a multigene family in which the copy number varies among plant species and functional divergence appears to have occurred repeatedly (*31−33*). The expression of the *CHS* gene in fruit tissue is developmentally regulated and associated with fruit coloring as shown in *Rubus* (*34*), apple (*35*), bilberry (*36*), grapevine (*37*), and strawberry (*7, 38*).

Reduction of the CHS function using antisense technology has mainly been used to change flower pigmentation (*39−41*), although reduction of flower opening and alterations in flower

form have occasionally been observed (*40*). Antisense *CHS* transgenic plants have also been associated with abnormal pollen development in rice (*41*), enhanced adventitious root formation in walnut microcuttings (*42*), and increases in tannin accumulation and mean vestitol levels in glutathione-elicited root cultures of *Lotus corniculatus* (*43*). The phenomenon of the cosuppression of homologous genes was first described in petunias where the introduction of an additional *CHS* gene resulted unexpectedly in plants with totally white flowers (*44*−*46*). However, effects of CHS down-regulation on the levels of plant secondary metabolites other than flavonoids have not been reported, and no studies on *CHS* antisense phenotypes in fruit have been published until now.

Antisense technology has been used previously for the improvement of fruit quality traits in tomato (*47*, *48*) melon (*49*), and strawberry (*21*), among others. With respect to the improvement of crop traits, the phenotypic stability of antisense phenotypes is of crucial importance. In the present study, the ability of antisense technology to produce phenotypically stable gene silencing in octaploid *Fragaria* × *ananassa* was demonstrated using the strawberry *CHS* gene. The effect of the reduction in CHS function on the level of anthocyanins, flavonols, proanthocyanidins (all downstream of CHS), and phenylpropanoids (upstream of CHS) in ripe strawberry fruit was measured, providing for the first time detailed knowledge of the role of CHS in substrate flow between the flavonoid and the phenylpropanoid pathway in strawberry.

MATERIALS AND METHODS

Plant Material. The octaploid strawberry *Fragaria* × *ananassa* cultivar Calypso was used for transformation. Fruits used for genetic and molecular analyses were labeled with an adhesive tape just as they entered the turning stage and were harvested simultaneously in the ripe stage.

Chemicals. Except where noted, all chemicals, salts, solvents, and phenolic compounds were purchased from Sigma (Deisenhofen, Germany), Aldrich (Deisenhofen, Germany), Fluka (Deisenhofen, Germany), and Roth (Karlsruhe, Germany).

CHS Sequence and Antisense Fragment. The cDNAs with sequence homology to *CHS* were identified by sequencing a cDNA library of ripe strawberry fruit (*Fragaria* × *ananassa* cv. Elsanta), consisting of 1701 clones (*7*). Using the primers P8, 5′ -catg(ccatgg)-catggcaccttcact-3′, and M8, 5′-cg(ggatcc)aacttcgggca-3′, a 440 bp fragment was amplified from the 5′-end of cDNA (PRI clone C78) for antisense inhibition. The fragment was cloned in the antisense orientation between the constitutively expressed double CaMV 35S promoter and the Tnos terminator. Next, the expression cassette was cloned into the binary vector pBinplus (*50*) using the restriction enzymes PacI and AscI to generate the *CHS* antisense construct (*CHSas*).

Transformation of Strawberry. The strawberry cultivar Calypso was transformed with an *Agrobacterium tumefaciens* strain AGL0 (*51*) containing the *CHS* antisense construct in pBinplus. The plant transformation was performed according to Schaart et al. (*52*). Twenty-five transgenic kanamycin resistant lines harboring the CHS antisense construct were transferred to the greenhouse. Primary transgenic plants were kept in the greenhouse at 21 °C daily temperature and 18 °C night temperature with a day length of 16 h.

RNA Gel Blot Analysis. For RNA gel blot analysis, the total RNA was isolated from fruit tissue according to Schulz et al. (*53*). Six to 10 μg of glyoxal (1.5 M)-denatured total RNA were electrophoresed and transferred to a Hybond N$^+$ membrane (Amersham). Probes for hybridization probes were radioactively labeled with [^{32}P]ATP labeled via random prime labeling (Random Prime Labeling kit, Gibco). For quantification, RNA gel blots were exposed to a phosphorimager screen and subsequently scanned into a Bioimager device (Fujix BAS2000). Next, the signals were measured as photosimulated luminescence (OD per mm^2) using TINA software (Raytest, Straubenhardt, Germany). The

PRI cDNA clone C78 coding for CHS was used as a probe for RNA gel blot analysis. Two strawberry cDNA sequences coding for a ribosomal protein and a DNA binding protein were used as internal loading controls. The expression level quantification was determined relative to a loading control using the same RNA gel blot (after stripping off the first probe) for both probes.

DNA Gel Blot Analysis. Genomic DNA was isolated from young folded leaves harvested from greenhouse plants according to the method described by Doyle and Doyle (*54*) but with 1% (w/v) poly-(vinylpyrrolidone)-10 in the DNA extraction buffer. The DNA of untransformed control plants and of plants transformed with the *CHS* antisense construct was digested with EcoRI and NcoI. Following electrophoresis on a 0.8% agarose gel, the DNA was transferred to a Hybond-N membrane (Amersham) and hybridized with a [^{32}P]-labeled nptII DNA fragment. Polymerase chain reaction primers used to generate this 685 bp nptII fragment were prat69 nptII(+), tgggcacaa-cagacaatcggctgc, and prat70 nptII (−), tgcgaatcgggagcggcgatacc.

Total Flavonol Content. Flavonols were measured in ripe fruits harvested from line 20, an untransformed control plant, and a transgenic control harboring an unrelated construct. Fruits were frozen in liquid nitrogen and ground to a fine powder. Hydrolyzed extracts were prepared essentially according to Hertog et al. (*55*), by adding 1 mL of 100% methanol [high-performance liquid chromatography (HPLC) grade] and 0.4 mL of 6 M HCl to 0.6 g fresh weight of frozen fruit powder. After incubation at 90 °C for 1 h, extracts were diluted with 2 mL of 100% methanol and sonicated for 5 min. Nonhydrolyzed extracts of line 17, line 20, and a control sample were prepared by adding 1.5 mL of 100% methanol to 0.5 g of ground frozen tissue, followed by sonication for 15 min. Samples were centrifuged at 2500 rpm for 5−10 min, and the supernatants were filtered (0.2 μm). Analysis was carried out using a W600 Waters system using a Luna C$_{18}$ column (150 mm × 4.6 mm, 3 μm; Phenomenex, United States) heated to 40 °C. A 5−50% acetonitrile gradient in 0.1% trifluoroacetic acid (1 mL min^{-1} flow rate) was used for separation. Samples were monitored continuously from 240 to 600 nm by a Waters 996 PDA detector. Data were analyzed using Waters Millenium^{32} software. Absorbance spectra and retention times of eluting peaks were compared with those of commercially available flavonoid standards (Sigma and Apin Chemicals, United Kingdom).

Identification of Metabolites−XAD Solid Phase Extraction of Strawberry Fruit. Amberlite XAD-2 polymeric adsorbent (20−60 mesh; Aldrich) used to fill a glass column (50 cm × 2.5 cm) was washed with methanol (100 mL) and conditioned with distilled water (200 mL). For the analysis of volatile and glycosidically bound compounds in strawberry fruits, 2 g of frozen ripe material was homogenized with 20 mL of water using an Ultra Turrax (T18 basic, IKA Works Inc., Wilmington, NC) and centrifuged (3500g, 10 min). The supernatant was loaded onto the XAD column, and the solid residue was re-extracted twice. After the column was rinsed with 100 mL of distilled water, volatiles were eluted with 50 mL of diethyl ether followed by a glycoside elution with 80 mL of methanol. The water phase from the diethyl ether extract was added to the methanolic extract, which was concentrated in vacuo to ca. 1 mL and used directly for liquid chromatography−ultraviolet−electrospray ionization−tandem mass spectrometry (LC-UV-ESI-MS^n) analysis. The diethyl ether extract was dried over anhydrous sodium sulfate, concentrated using a Vigreux column to ca. 1 mL, and pipetted into a gas chromatography (GC) vial, and the residual organic solvent was removed with a stream of nitrogen until the volume reached ca. 50 μL. For GC quantification, phenol (0.1 mg mL^{-1}) was added as an internal standard. Cinnamoyl glucose was synthesized according to Plusquellec et al. (*56*).

LC-UV-ESI-MS^n. The system used for LC-UV-ESI-MS^n analysis was a Bruker esquire 3000 plus mass spectrometer, equipped with an Agilent 1100 HPLC system, composed of an Agilent 1100 quaternary pump and an Agilent 1100 variable wavelength detector. The column was a Eurosher C18 column, with particle size 5 μm, 10 cm × 2 mm (Grom Analytik & HPLC GmbH, Rottenburg, Germany). The ionization parameters were as follows. The voltage of the capillary was 3074 V, and the end plate was set to −500 V. The capillary exit was −109.8 V, and the Octopole RF amplitude was 120 Vpp. The temperature of the dry gas (N$_2$) was 300 °C at a flow of 10 L min^{-1}. The full scan

mass spectra of the glycosides were measured from m z^{-1} 50 to 500 or 800 until the ICC target reached 20000 or 200 ms, whichever was reached first. Tandem mass spectrometry was performed using helium as the collision gas, and the collision energy was set at 1.00 V. Mass spectra were acquired in the negative and positive ionization mode. Autotandem mass spectrometry was used to break down the most abundant $[M + H]^+$, $[M - H]^-$, or $[M + HCOO]^-$ ions of the different compounds of the strawberry extracts. Identification of the glycosylated compounds was achieved using enzymatically produced reference compounds. The LC parameters went from 0% acetonitrile and 100% water (acidified with 0.05% formic acid) to 50% acetonitrile and 50% acidic water in 50 min, then in 20 min to 100% acetonitrile, where they were kept for 10 min before returning to 100% water and 0% acetonitrile in 5 min at a flow rate of 0.200 mL. The detection wavelength was 280 nm.

Capillary GC-MS. GC-MS analysis was performed with a Thermo Finnigan Trace DSQ mass spectrometer coupled to a Thermo Finnigan Trace GC with a split injector (1:20) equipped with Xcalibur software (version 1.4). The GC was fitted with a BPX5 20 M fused silica capillary column (30 m × 0.25 mm inner diameter; thickness of the film, 0.25 μm). The GC parameters were as follows: initial temperature of 40 °C for 3 min, increased to 250 °C at 5 °C min^{-1} intervals. The helium gas flow rate was 3 mL min^{-1}. The EI-MS ionization voltage was 70 eV (electron impact ionization), and the ion source and interface temperatures were kept at 230 and 240 °C, respectively. Compounds were identified by comparing their mass spectra and retention indices to the NIST mass spectra library and reference compounds. Statistical analyses of differences between the mean metabolite levels of individual plants were performed using a one-way analysis of variance. Significant differences between groups were determined by t-test using SigmaPlot 8.0 at a level of $p < 0.01$.

Quantification via LC-UV-ESI-MS^n. Metabolite levels in individual fruits harvested from the transgenic line 20 were independently determined. Control fruits from several untransformed Calypso plants were individually analyzed throughout the course of the experiment. Each purified sample was measured once with LC-UV-ESI-MS^n. Signals of the individual compounds were integrated in their $[M + H]^+$, $[M + Na]^+$, $[M - H]^-$, or $[M + HCOO]^-$ ion traces. Mean values of the signal intensities of the controls were set at 100%. Levels of the metabolites in fruits of transgenic line 20 were calculated as percent of the signal intensities in the controls.

Synthesis of the Reference Compound *p*-Coumaryl-1-acetate. *para*-Coumaryl alcohol (Chempur, Karlsruhe, Germany) (0.01 mmol) was peracetylated with acetanhydride (1 mg) and pyridine (50 mg) for 24 h at ambient temperature. After the addition of water (2 mL) and diethyl ether (1 mL) pyridine was removed from the organic phase by successive extractions (3×) with saturated CuSO$_4$ solution (1 mL). Crude *p*-coumaryl diacetate was recovered after evaporation of the diethyl ether. Regioselective hydrolysis of the diacetate was peformed according to Allevi et al. (*57*). Briefly, the diacetate (0.01 mmol) dissolved in acetone (40 μL) was added to phosphate buffer (200 μL; pH 7) containing a lipase preparation from *Pseudomonas cepacia*. The resulting suspension was shaken at 25 °C and monitored by GC-MS. After consumption of the starting diacetate, the reaction was quenched by adding ethyl acetate and the resulting mixture was filtered through silica gel. The organic layer was separated and dried, and the solvent removed in vacuo.

RESULTS

Sequence Analysis of CHS Genes from Strawberry. Several partial cDNA clones (PRI clone A135, C78, G110, G157, and G137) with homology to *CHS* were identified in a cDNA library of ripe strawberry fruit (*58*), and it was shown that strawberry *CHS* (AI795154) was expressed during the ripening process with a peak in expression at the turning stage of fruit ripening, the stage in which the red fruit color starts to develop (*67*). The highest nucleotide homology was found with *CHS* from *Rosa hybrida* (gi, 29420432; 90% identity) and with *CHS*6 from *Rubus idaeus* (gi, 22086371; 88% identity). At the
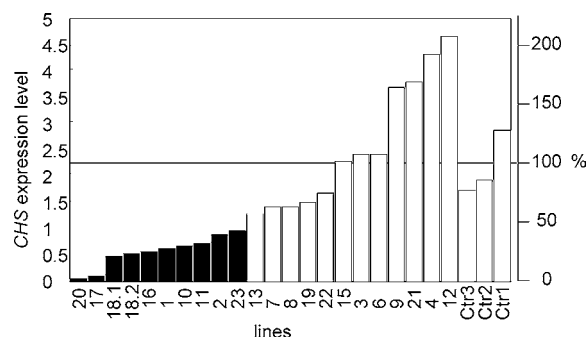

Figure 2. Relative *CHS* expression level in ripe fruit samples of transgenic lines (numbers) and three untransformed control lines (Ctr). Black bars represent a group of transgenic lines with a significantly reduced expression level of *CHS* relative to a ribosomal probe (group 1, $n = 10$, average = 0.56, and SD = 0.30), and white bars show a second group of control plants and transgenic plants with unaltered relative *CHS* expression levels ranging from 1.29 to 4.67 (group 2, $n = 15$, average = 2.5, and SD = 1.12). The 100% value represents the median of group 2. Group 1 of transgenic lines differed significantly from group 2 in a t-test ($P = 7.9E-0.6$).

amino acid level, homology with *R. hybrida* (BAC66467) and with *R. idaeus* (AAM90652) was 96%.

Expression Analysis and CHS Phenotype of Transgenic Plants. Antisense suppression is a commonly available method to obtain down-regulation of specific endogenous plant genes. To test the effectiveness of the antisense technology in strawberry, we transformed the octaploid *Fragaria* × *ananassa* cv. Calypso with a *CHS* antisense construct and 25 kanamycin resistant lines were transferred to the greenhouse.

The cultivar used for transformation, cv. Calypso, is vegatively propagated and produces fruits the whole year around. This enables the continuous analysis of primary transgenics to select chimeric plants and somaclonal variants. The 25 primary transgenic lines were monitored for *CHS* transcript abundance in ripe fruits and for a CHS deficiency phenotype.

The relative *CHS* transcription level among lines varied from 0.05 to 4.67 as compared with the loading control (**Figure 2**). Most plants, including the untransformed controls, ranged in expression level from 1.29 to 4.67 (white bars). A group of nine transgenic lines (black bars) showed a relative *CHS* expression level of less than 1 (**Figure 2**); that is, the expression levels of these nine lines were reduced to 43% or less of the control level. Two clones of the same line (line 18) showed similar expression levels suggesting that the antisense effect is transferred via clonal reproduction (**Figure 2**).

Southern analysis was performed on several lines to determine the number of transgene copies integrated into the genome. The hybridization patterns obtained using the nptII coding region as a probe indicated that in six of the lines (1, 4, 12, 17, 18, and 20), three, one, four, four, three, and three copies were present (**Figure 3**).

The pigmentation of the fruit was only affected in plants where less than 25% of the control *CHS* mRNA level remained (lines 17, 18 and 20). A chimeric phenotype for pigmentation was observed in line 18 that showed a reduction of *CHS* mRNA accumulation to 21−24% of the control level (**Figure 4A**). Pink ripe fruits were observed in line 17 and line 20, both of which contained only 4 and 2% of the *CHS* mRNA amount found in the controls (**Figure 4B**,**C**). In lines 17 and 20, the effect on pigmentation was present in all fruits. After 1 year, the effect of *CHS* antisense inhibition on pigmentation disappeared in the primary transgenic line 17. A stable effect on the phenotype
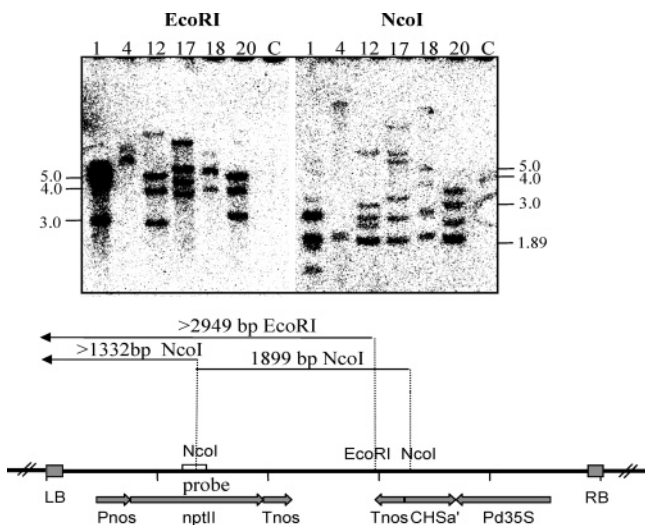
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Figure 3. Southern analysis of DNA of six transgenic lines (line 1, 4, 12, 17, 18, and 20) and an untransformed Calypso control (C) digested with NcoI and EcoRI, using a nptII fragment as a probe. The fragment between the right border (RB) and the left border (LB) of the binary vector (pBinplus) is integrated into the genome. Digestion with NcoI generates a hybridizing fragment that is internal to the borders (1899 bp) and a border fragment that is determined in size by the place of insertion in the genome. Only the EcoRI enzyme digestion generates such a border fragment.

over a period of 4 years was thus only observed in line 20 (primary transgenic line), returning a frequency of 1:20 for a stable antisense phenotype. Fruit firmness was not measured, but the rate of ripening and fruit size appeared to be normal, and also, the germination of seed was unaffected.

Chemical Analysis. The first committed step in the flavonoid biosynthesis is catalyzed by CHS, which produces naringenin chalcone by the condensation of three molecules of malonyl-CoA and one molecule of 4-coumaroyl-CoA. In this way, the CHS catalysis represents a branching point in the phenylpropanoid metabolism leading to flavonoids (flavonols, anthocyanins, and proanthocyanidins) (**Figure 1**), and when coacting with chalcone reductase (CHR), to isoflavonoids (not detected in strawberry).

In strawberry fruits, kaempferol is the main flavonol (*55*). HPLC-UV analyses of hydrolyzed extracts showed that this was also the case in our cultivar (4.7 mg/kg fresh weight quercetin; 7.3 mg/kg fresh weight kaempferol). The reduction of *CHS* mRNA accumulation in the *CHS* antisense line 20 resulted in levels of the kaempferol (3.4 mg/kg) and quercetin (3.4 mg/kg) being reduced by approximately half. The main anthocyanin detected in strawberry by HPLC-UV, pelargonidin-3-glucoside, was more strongly affected by the reduction in *CHS* mRNA accumulation. The remaining anthocyanin content of the stable *CHS* antisense phenotype (line 20) was limited to 8%, with the pink fruit color reflecting the residual CHS activity. In the unstable *CHS* antisense phenotype (line 17), the residual CHS activity led to a 13% reduction in anthocyanin content.

To perform a more detailed analysis of the levels of plant secondary metabolites, a XAD solid phase extraction was performed with fruits of transgenic line 20 and of control plants. GC-MS analysis of the obtained extracts showed that the concentrations of a number of volatiles produced by the transgenic fruits were not significantly different than the levels in control fruits (**Figure 5**). However, significantly lower amounts of methyl nicotinate as well as higher levels of *p*-coumaryl alcohol, benzoic acid, and *p*-hydroxybenzaldehyde were produced by fruits of line 20, and one compound occurred
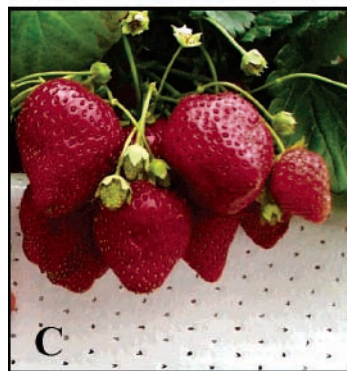

Figure 4. Phenotypes of *CHS* antisense lines. A chimeric phenotype of line 18 with pink sectors (**A**). Orange to pink fruit of line 20 (**B**). The bright red fruit color of the untransformed cv. Calypso control plants (**C**).

with a 10-fold higher abundance in antisense fruits than in control fruits. On the basis of its fragmentation pattern in the mass spectrum, we identified this substance as *p*-coumaryl-1-acetate. The structure was confirmed by comparing the chromatographic and mass spectral data with those of a synthesized reference compound (**Figure 6**).

We also analyzed the individual major flavonols, anthocyanins, and proanthocyanidins in control fruits and fruits of line 20 by LC-UV-ESI-MS^n (*2*, *8*) (**Table 1**). Levels of all flavonols, proanthocyanidins, and anthocyanins in line 20 fruits were lower than in controls (<100% of control), whereas glucose esters of *p*-coumaric, cinnamic, caffeic, and ferulic acids as well as *p*-coumaryl glucoside were significantly higher (>100 % of control). However, the concentration of *p*-hydroxybenzoyl glucose was slightly lower in the *CHS* antisense fruit.

DISCUSSION

For the first time, we have demonstrated the transformation of the octaploid strawberry with an antisense *CHS* gene construct using a ripening-related *CHS* cDNA from *Fragaria × ananassa* cv. Elsanta under the control of the constitutive CaMV 35S
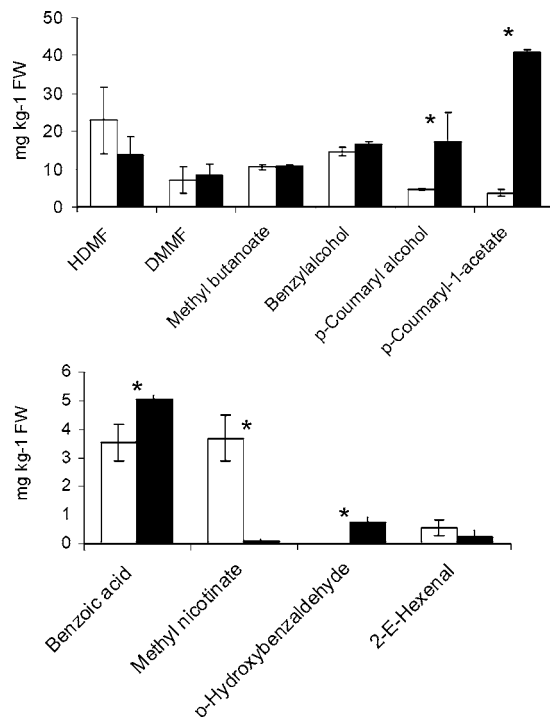
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Figure 5. Quantitative GC-MS analysis of volatiles obtained from fruit of *CHS* antisense line 20 (black bars) and untransformed cv. Calypso control plants (white bars). Asterisks (*) indicate that the data are significantly different from the data of control (CC) fruit ($p < 0.01$). HDMF, 4-hydroxy-2,5-dimethyl-3(2H)-furanone; DMMF, 2,5-dimethyl-4-methoxy-3(2H)-furanone.
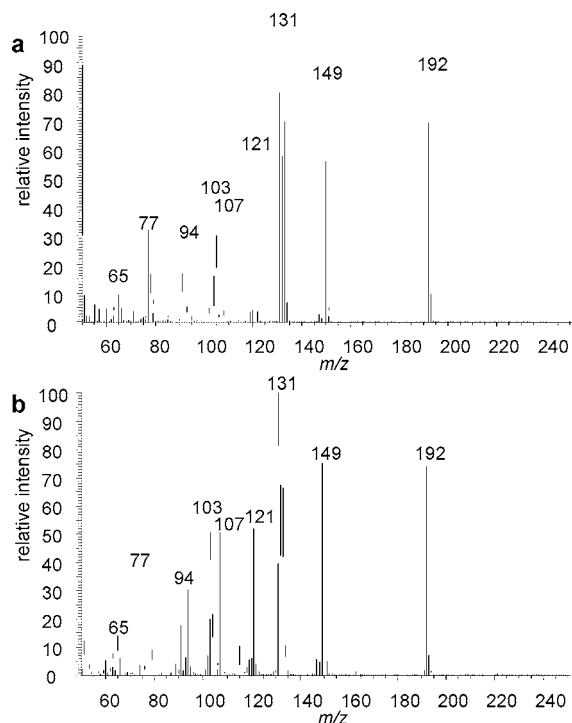

Figure 6. Electron impact mass spectrum of a compound formed predominantly in fruit of *CHS* transgenic line 20 (**a**) and mass spectrum of the synthesized *p*-coumaryl-1-acetate (**b**).

Table 1. HPLC-UV-ESI-MS[n] Analysis of Ripe Fruit Samples[a]

	retention time (min)	% of control	refs
pelargonidin-3-rutenoside	21.0	0.1	*69, 70*
pelargonidin-3-glucoside-malonate	24.6	6.1	*69, 70*
pelargonidin-3-glucoside	20.7	7.5	*69–71*
cyanidin-3-glucoside	19.5	13.9	*69, 70*
cyanidin	44.2	17.8	*69*
(epi)afzelechin-pelargonidin-3-glucoside	19.6	2.2	*71*
(epi)catechin-(epi)catechin (isomer 1)	19.9	8.5	*72*
(epi)afzelechin-(epi)catechin (isomer 1)	22.1	9.9	*72*
(epi)catechin-(epi)catechin (isomer 2)	20.7	14.0	*72*
(epi)afzelechin-(epi)catechin (isomer 2)	23.6	25.5	*72*
kaempferol-glucuronide	32.7	10.2	*3*
kaempferol-glucoside	31.6	37.6	*3*
quercetin-glucoside	29.5	55.0	*3, 69*
quercetin-glucuronide	30.8	58.8	*3, 69*
4-hydroxybenzoyl glucose	16.6	40.4	*73*
p-coumaroyl glucose	21.6	173.5	*69*
p-coumaryl glucoside	22.2	218.3	*69*
cinnamoyl glucose	29.7	519.6	*74*
caffeoyl glucose	18.9	815.9	*69*
feruloyl glucose	22.7	1 092.4	*69*

[a] Metabolites were determined in three single fruits from untransformed control plants and three single fruits from transgenic line 20. Standard deviations of the detector responses for the metabolites ranged from 5 to 50% in the controls and from 1 to 15% in the transgenic fruits. Levels in the transgenic fruits were calculated and expressed as percent of the concentration in untransformed cv. Calypso control lines.

cell suspension cultures, where CHS activity correlates with anthocyanin synthesis (*61*, *62*), and in fruit, where anthocyanin biosynthetic genes are coordinately expressed during coloration (*35*, *36*).

Nine out of 25 primary transgenic lines of the octaploid strawberry contained significantly reduced levels of *CHS* transcript, ranging from 43 to only 2% of the untransformed control level of *CHS* transcript in the most severe case (line 20). Although *CHS* mRNA was successfully down-regulated in the regenerated plants, complete suppression of anthocyanin biosynthesis was not achieved. It is assumed that either the residual activity of CHS or the additional isoenzymes, which have been recently described, are responsible for this observation (*31*, *63*). However, isoenzymes of CHS have not been analyzed and the expression levels have not been determined up to now.

We observed two types of pigment changes, namely, a chimeric phenotype with *CHS* transcript levels of 21−24% of control (line 18) and pink fruits with *CHS* mRNA amounts of only 2−4% of controls (line 17 and line 20). These lines 18, 17, and 20 contained three, four, and three transgene copies, respectively. Thus, the transgene copy number does not correlate with the level of expression or phenotype. Assuming stable integration of the antisense *CHS* genes in each line, this difference in pigmentation indicates that the integrated antisense *CHS* gene(s) are expressed in fundamentally different ways. This may be due to the position effect where bordering sequences have the potential to influence gene expression in a qualitative way like affecting spatial distribution of gene expression (*39*, *59*). A stable effect on the phenotype over a period of 4 years of vegetative maintenance was only observed in line 20.

Strongly reduced levels of *CHS* transcripts led to the depletion of anthocyanins, flavonols, and proanthocyanidins, but the pools were affected differently (**Table 1**). The pathways probably compete for the substrate dihydrokaempferol, a downstream metabolite of naringenin. Therefore, the effect on the product ratios depends on the kinetics, intracellular localization, and organization of the involved enzymes (*4*). Levels of anthocya-

promoter and the Tnos terminator via *A. tumefaciens*. We tested the stability of the transformation by monitoring the changes in the levels of *CHS* transcripts, which can easily be detected phenotypically by the loss of pigments (*39*, *59*, *60*). Similar phenotypic effects have been previously reported in strawberry

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nins were much more down-regulated in line 20 than those of the proanthocyanidins or flavonols. Some of the metabolites recently observed in strawberry fruit, like pelargonidin-3-glucoside-succinate, could not be detected in the cv. Calypso, indicating the cultivar-dependent occurrence of some compounds.

In addition, the careful characterization of the metabolites of transgenic line 20, the stable phenotype, showed that concomitant with the depletion of the flavonoids, anthocyanins, and proanthocyanidins was the accumulation of metabolites of the phenylpropanoid pathway, specifically, *p*-coumaryl alcohol, which increased by a factor of 3.6, and *p*-coumaryl-1-acetate, which increased by a factor of 10.6 (**Figure 5**). These compounds are putative precursors of lignin biosynthesis and have never before been detected in strawberry (*64*). The nonfunctional CHS reduces the flux toward the production of anthocyanins, proanthocyanidins, and flavonols, making intermediates available for the phenylpropanoid pathway (**Figure 1**). Recently, a similar result was obtained by antisense suppression of the flavanone 3-hydroxylase (*f3h*) (*65*). Transgenic anti-*f3h* flowers showed dramatically reduced anthocyanin levels and were significantly more fragrant than controls. Analyses of fragrance compounds in these transgenic plants revealed 5−7-fold increases in levels of the phenylpropanoids methyl benzoate and 2-hydroxy methyl benzoate, as compared to controls.

In our experiments, the amounts of *p*-coumaroyl glucose, *p*-coumaryl glucoside, cinnamoyl glucose, caffeoyl glucose, and feruoyl glucose were also increased confirming the shunting of the intermediates to the phenylpropanoids. In maize, the accumulation of maysin, a C-glycosyl flavone and an important component of corn earworm (*Helicoverpa zea* Boddie) resistance, correlates with the activity of functional CHS, whereas a lack of CHS increases chlorogenic acid, a product of the phenylpropanoid pathway (*30*). In our study, only the concentration of *p*-hydroxybenzoyl glucose, a putative descendant of the phenylpropanoid pathway, was slightly reduced.

A cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) gene from strawberry have been already described (*58*, *66*). The corresponding enzymes are involved in the biosynthesis of *p*-coumaryl alcohol, and the following conjugation of the alcohol with acetyl-CoA to *p*-coumaryl-1-acetate is probably performed by strawberry alcohol acyltransferase (SAAT), an enzyme recently characterized from strawberry (*67*). The significantly reduced level of methyl nicotinate, a known strawberry volatile in the transgenic line, remains unknown, but there is obviously a close relationship with the phenylpropanoid pathway (*68*).

In this study, we demonstrated that a lack of functional CHS increases *p*-coumaryl-alcohol and *p*-coumaryl-1-acetate accumulation in strawberry, most likely through the redirection of intermediates originally destined for anthocyanin, proanthocyanidin, and flavonol biosynthesis to the phenylpropanoid pathway. Down-regulation of CHS not only affected the levels of compounds downstream of the pathway but also the amounts of metabolites upstream of the CHS branching point. This unexpected result demonstrates that both the regulation of secondary metabolism and the accumulation or depletion of secondary metabolites in transgenic plants are insufficiently understood.

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