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Piceid (Resveratrol Glucoside) Synthesis in Stilbene Synthase Transgenic Apple Fruit

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A stilbene synthase gene along with the selectable marker gene *bar* for herbicide resistance was transferred via *Agrobacterium tumefaciens* mediated transformation into apple (*Malus domestica* Borkh.) cvs. 'Elstar' and 'Holsteiner Cox'. The stilbene synthase catalyzes the conversion of 1 molecule of *p*-coumaroyl-CoA and 3 molecules of malonyl-CoA into 3,4′,5-trihydroxystilbene, commonly known as resveratrol. This phytoalexin has implications in both phytopathology and human health. Greenhouse-grown transgenic and nontransformed control plants were grafted onto dwarfing rootstock M27. Flowering and fruiting occurred within the following years, offering the opportunity to analyze transgenic apple fruit and fertility of transgenic plants as well as inheritance of the transgenes into the seedling progeny. Molecular analysis revealed that the stilbene synthase is expressed in transgenic plants and in the skin and flesh of transgenic apple fruit. After formation, resveratrol is modified by the addition of a hexose sugar. The resulting component was characterized as piceid. With the aim of characterizing the influence of the novel biosynthetic pathway on the accumulation of other phenolic compounds naturally present in apple fruit, the amounts of flavanols, flavonols, phloretin derivatives and hydroxycinnamic acids in wild type and transgenic fruit were determined by HPLC. In all investigated transformed lines that accumulated piceid, no negative correlation between levels of piceid and the above-mentioned compounds was observed, except for the flavonol contents, which slightly decreased. Inheritance of the transgenes was confirmed in the seedling progeny, which were obtained after pollination of transgenic plants with nontransgenic pollen and vice versa after pollination of nontransgenic plants with pollen obtained from transgenic plants. The fertility of stilbene synthase transgenic plants was demonstrated. To the authors' knowledge this is the first time that data are available on piceid synthesis in transgenic apple fruit and the effects of its accumulation on levels of other phenolic compounds present in the fruit.

KEYWORDS: ***Agrobacterium tumefaciens*; *bar*; flowering; grafting; herbicide resistance; *Malus domestica*; seedlings; *Vst*1**

INTRODUCTION

Stilbene synthase is an enzyme found in several unrelated plant species such as grapevine (*1*), peanut (*2*), and *Vaccinium* berries (*3*, *4*), where it is responsible for the synthesis of the phytoalexin resveratrol. It converts 1 molecule of *p*-coumaroyl-CoA and 3 molecules of malonyl-CoA into 3,4′,5-trihydroxystilbene, commonly known as resveratrol. The precursor molecules are present throughout the plant kingdom as substrates for the chalcone synthase, the key enzyme in the flavonoid pathway (**Figure 1**). The production of resveratrol is related to fungal infection (*5*, *6*) and abiotic stimuli (*7*, *8*) such as UV light (*1*, *9*) and ozone (*10*, *11*). Stilbenes, in general, and resveratrol, in particular, are biologically active compounds, which have antifungal activities against various pathogens (*12*), among them *Venturia inaequalis*, the causal agent of apple scab (*13*). Therefore, stilbene synthase genes have been transferred into tobacco (*14*), rice (*15*), kiwi (*16*), alfalfa (*17*), barley (*18*), wheat (*18*), a grapevine rootstock (*19*), and apple (*20*) to enhance resistance to fungal pathogens by endogenous enrichment of this stilbene. Exogenous application of resveratrol reduced postharvest decay in several fruit types such as tomatoes, grapes, and avocado pears (*21*). In addition to these implications in phytopathology, resveratrol and its glycosides have attracted

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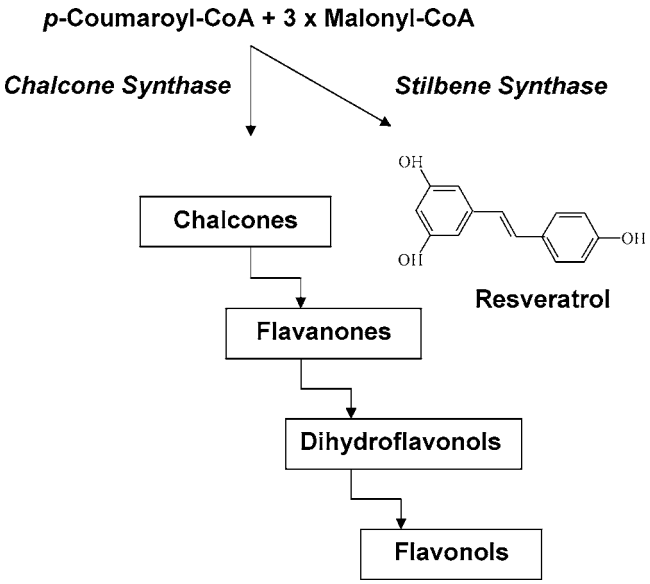

Figure 1. Scheme of the flavonoid pathway. The stilbene synthase uses the same substrates as the chalcone synthase but forms the stilbene resveratrol.

increasing interest as health-promoting agents because of their anti-inflammatory, estrogenic, antiplatelet, and anticarcinogenic activities (*22*−*26*). The biological and pharmacological activities of resveratrol are thought to be due to strong antioxidant properties. These facts stimulated research aimed at increasing the natural content of resveratrol in table grapes by UV irradiation of harvested fruits (*27*, *28*) or enabling transgenic fruit such as tomato to synthesize resveratrol (*29*) by expression of a recombinant stilbene synthase.

Apple has long been recognized as a great source of antioxidants. The synthesis of resveratrol in transgenic apple would expand the antioxidant capacity and can therefore be regarded as an additional factor improving the "inner quality" of the fruit.

In previous studies (*20*) we have produced transgenic apple plants expressing the stilbene synthase gene from grapevine (*Vitis vinifera* L.) under the control of its own wound-, pathogen-, and UV-inducible promoter. Here we report on the analysis of transgenic apple fruit concerning the expression of the stilbene synthase, the synthesis of resveratrol, and the influence of stilbene accumulation on metabolic profiles of other phenolic compounds. In addition, the inheritance of the transgenes was investigated in a seedling progeny.

To our knowledge this is the first time that transgenic apple fruit synthesizing the foreign phytoalexin piceid (resveratrol glucoside) has been produced.

MATERIALS AND METHODS

Plant Material. Transgenic 'Elstar' and 'Holsteiner Cox' apple (*Malus domestica* Borkh.) plants harboring the stilbene synthase gene (*Vst*1) from *V. vinifera* and the *bar* gene from *Streptomyces hygroscopicus* (*30*) were previously produced (*20*). The *Vst*1 gene is controlled by its own UV-, wound-, and pathogen-inducible promoter (*1*), whereas the *bar* gene is driven by the *nos* promoter. The *bar* gene confers resistance against the herbicide phosphinotricin, the active agent in Basta and Liberty.

The in vitro plants were cultured under continuous selective pressure with 10 mg/L glufosinate ammonium at 25 °C, with a 16-h photoperiod, and were subcultured every 4 weeks. Shoots were allowed to root [MS salts and vitamins (*31*), 3% sucrose, 1.5 μM IBA, 10 mg/L glufosinate ammonium, 0.8% plant agar, pH 5.8] for 4 weeks. Rooted plants were acclimatized in the greenhouse and allowed to grow on their own roots or grafted onto M27 to encourage early flowering. Transgenic 'Elstar' and 'Holsteiner Cox' flowers were hand-pollinated with pollen of nontransgenic 'Remo', and vice versa nontransgenic 'Remo' flowers were pollinated with pollen obtained from transgenic plants. Apples were harvested from the transgenic 'Elstar' line and from six 'Holsteiner Cox' lines as well as from nontransgenic 'Remo' plants. Seeds were surface sterilized with sodium hypochlorite, dried, and put on wet filter paper for several weeks until they started to germinate. The seeds were then put in soil and grown under greenhouse conditions.

DNA Analysis. Genomic plant DNA was isolated using the CTAB extraction method of Doyle and Doyle (*32*). To detect the transgenes in the apple genome, PCR analyses were performed. The primers used for the amplification of a specific *Vst*1 fragment were 5′-TCT TTA AGA GCC TTC AAT GC-3′ and 5′-TCT ACC AGT CTG ATT ATG CTG A-3′. For indication of the presence of the *bar* gene, the primers 5′-GCA GGA ACC GCA GGA GTG GA-3′ and 5′-AGC CCG ATG ACA GCG ACC AC-3′ were used. Amplifications were performed in 25-μL volumes using a thermal cycler (Biometra) under the following conditions: 94 °C for 1 min, followed by 30 cycles at 94 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 5 min. As a positive control the plant transformation vector pHKvst (*20*) was used.

Southern blot analyses were performed to verify the integration of the transgenes, to determine the respective copy number, and to characterize T-DNA segregation in the progeny plants. Twenty micrograms of genomic DNA was digested with 20 units of *Eco*R1 (recognizes one restriction site between the right border and the coding region of the *Vst*1 gene) at 37 °C overnight followed by the addition of another 10 units of the enzyme. After an additional 4 h, the DNA was fractionated in a 1% agarose gel and blotted on a positively charged nitrocellulose membrane (Boehringer Mannheim, Germany). The membranes were hybridized with the digoxygenin-labeled PCR probes of the *Vst*1 and the *bar* gene (for programs, see above). All steps were performed following the supplier's instructions (Roche, Mannheim, Germany).

Expression Analysis. Specific mRNAs of the transgenes in apple fruit were checked via reverse transcriptase (RT)-PCR. Because the *Vst*1 gene is controlled by the inducible *Vst*1 promoter, the fruit material was UV-treated using a transilluminator (TFX-35C, Vilber Lourmat). Irradiation parameters were as follows: wavelength, 254 nm; irradiation distance, 5 cm; and irradiation time, 5 min. Total RNA was isolated using the RNeasy Mini Kit from Quiagen. cDNA generation was done as follows: 5 μg of total RNA and 100 pmol of oligo dT-primer were mixed and made up to 13 μL with DEPC−H_2O. After 10 min of incubation at 70 °C, 4 μL of the 5xRT-Puffer (Promega), 1 μL of rNAsin (Promega), and 10 mM dNTPs were added and incubated for 1 min at 37 °C. Reverse transcription occurred by adding of 1 μL of RNA-dependent DNA polymerase MMLV-RT (Promega) within 1 h at 37 °C. Primer sequences for PCR and the reaction conditions for detecting the transgenes were the same as mentioned above.

Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit was used as an endogenous control because it should be expressed in both transgenic and untransformed control tissues. A specific fragment with the size of 310 bp was amplified by using the primers 5′-CGG CAC CGT GGC TAC AGT AT-3′ and 5′-CAC GAA TCC ATG CTC CAA CT-3′. Themocycling conditions were as follows: 94 °C for 3 min, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 50 °C, and extension for 90 s at 72 °C and with a final extension for 3 min at 72 °C.

Analysis of Accumulation of Phenolic Compounds. Harvested fruit material was UV-treated to induce the stilbene synthase promoter as described above. After 24 h, the material was frozen in liquid nitrogen and subjected to analysis. As a control also non-irradiated fruit from transgenic and nontransgenic plants was used.

HPLC analysis to detect piceid in unripe fruit was performed according to the method of Szankowski et al. (*20*). Peak identification is published elsewhere (*20*).

Analysis of mature fruit was done as follows: samples were lyophilized and ground in a mortar or in a ball mill, depending on the available amount. The extraction of phenolic compounds was done with methanol (100%) containing 6-methoxyflavone as an internal standard for 30 min in a cooled water bath during sonication. After centrifugation, the supernatant was evaporated and the residue redissolved in small quantities of methanol and injected for HPLC analysis.

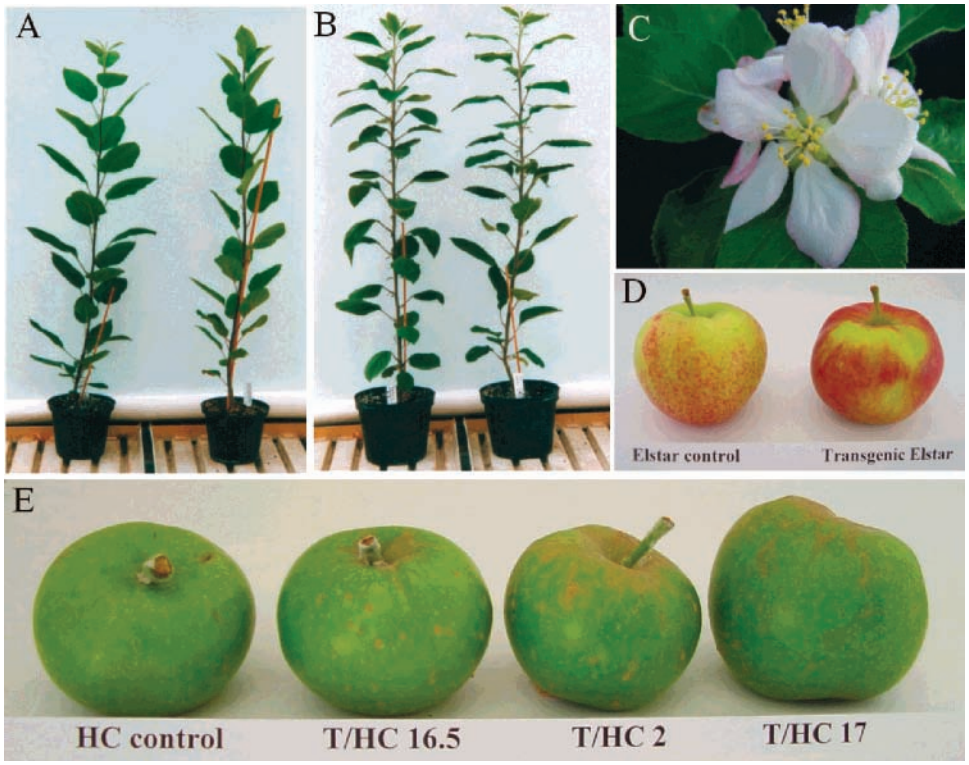

Figure 2. Greenhouse-grown transgenic 'Elstar' (**A**) and 'Holsteiner Cox' (**B**) plants (transgenic on the left and controls on the right) and their fruit (**D**, **E**) in comparison to their respective controls; (**C**) transgenic 'Holsteiner Cox' (T/HC) flower.

The phenolic compounds were separated on a column (250 × 4 mm i.d.) prepacked with Hypersil ODS, 3-μm particle size, following a stepwise gradient using mixtures of solvent A (formic acid, 5% in water) and solvent B (methanol, gradient grade) from 95:5 (v/v) to 10:90 (v/v) with a flow rate of 0.5 mL/min (*33*). The gradient profile (% B in A) used was as follows: 0−5 min, isocratic, 5% B; 5−15 min, 5−10% B; 15−30 min, isocratic, 10% B; 30−50 min, 10−15% B; 50−70 min, isocratic, 15% B; 70−85 min, 15−20% B; 85−95 min, isocratic, 20% B; 95−110 min, 20−25% B; 110−140 min, 25−30% B; 140−160 min, 30−40% B; 160−175 min, 40−50% B; 175−190 min, 50−90% B. For the HPLC determination of the dihydrochalcone glycoside phloridzin the extract was diluted by 200 with methanol and the phloridzin was analyzed using a short column (12.5 × 4 mm i.d.) prepacked with LiChrospher 100 RP18, 5-μm particle size, and a gradient range from 40 to 90% aqueous methanol.

Hydroxycinnamic acids (*p*-coumaric acids and caffeic acid derivatives), the dihydrochalcone glycosides, the flavonols (quercetin glycosides), and the resveratrol glucoside piceid were detected at 280 nm, whereas the monomeric flavan-3-ols (catechin, epicatechin), the procyanidins (B2, B5, C2, E-B5), and the dihydrochalcone phloretin were estimated at 640 nm after postcolumn derivatization with 4-dimethylaminocinnamic aldehyde (DMACA) (*34*). Peak identification is published elsewhere (*35*, *36*). The identification of piceid is published (*37*).

Leaf Paint Assay. The activity of the *bar* gene in vegetative tissues of the seedling progeny was determined by checking the plant's capacity for detoxifying the herbicide glufosinate ammonium. Basta was applied onto the three youngest fully emerged leaves at a concentration of 600 mg/L glufosinate ammonium. Only half of the leaf was painted with Basta to prevent leaves from falling off due to necrosis. Plants were scored for susceptibility or resistance to the herbicide 1 week after treatment. The experiment was repeated three times, so that in total nine leaves per plant were treated.

RESULTS

Flowering and Fruiting of Transgenic Apple Plants. We have previously produced nine transgenic 'Holsteiner Cox' lines and one transgenic 'Elstar' line with a wide variation of copy numbers (one through six) of the stilbene synthase gene (*Vst*1) from grapevine, responsible for resveratrol synthesis, and the *bar* gene from *Streptomyces hygroscopicus* (*20*), conferring resistance against herbicides with the active agent phosphinotricin. Expression of the transgenes in the vegetative tissues was proven by RT-PCR, and the functionality of the proteins was checked by detection of a resveratrol glucoside (piceid) in leaf material and by herbicide treatments of transgenic plants. All lines, except one, showed expression of the transgenes (*20*).

Rooted plants were transferred to greenhouse conditions and grafted onto dwarfing rootstock M27 to encourage early flowering. The first plants flowered within the first and second years after grafting and continued to produce flowers in the following years. Some plants grown on their own roots flowered 4 years after potting. Plants of the transgenic 'Elstar' line (harboring four copies of the *Vst*1 gene) and of five transgenic 'Holsteiner Cox' lines (T/HC 2, 12, 17, 27, and 50, all harboring two copies of the transgenes, except line 50, which carries one copy) as well as of the respective nontransformed control plants of the cvs. 'Elstar' and 'Holsteiner Cox' were successfully pollinated and fruited. Transgenic lines showed no obvious differences in morphology, growth habit, leaf shape (**Figure 2A**,**B**), flower morphology and color (**Figure 2C**), or fruit shape and size (**Figure 2D**,**E**) in comparison to control plants and control fruit.

Expression of Recombinant *Vst*1 Gene in Fruit Tissues of Different Ripening Stages and in Different Fruit Tissue Types. In previous studies (*20*) we have demonstrated that the stilbene synthase promoter is UV light inducible in transgenic apple tissue as it is in *Vitis*. To check whether the stilbene synthase gene and the *bar* gene are expressed in fruit tissues, unripe as well as mature apple fruit was UV-irradiated and used for RNA isolation. Because the number of apples was limited, experiments with 'Holsteiner Cox' had to be performed with different transgenic lines. RT-PCR results from unripe fruit of two transgenic 'Holsteiner Cox' lines (T/HC 2 and T/HC 50), the transgenic 'Elstar' line, and a nontransformed control fruit

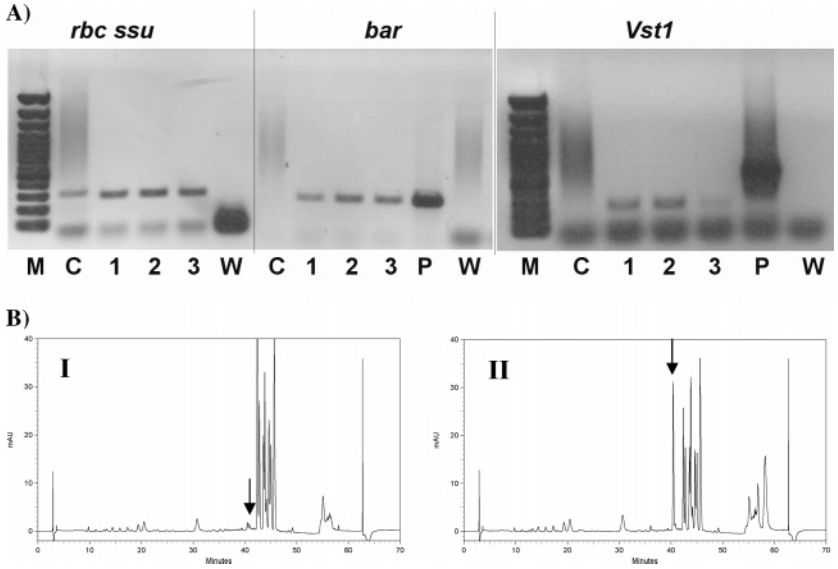

Figure 3. Transgene expression in apple fruit tissues. (**A**) RT-PCR analysis of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*rbc ssu*)-, *bar*-, and *Vst*1-mRNA accumulation in unripe transgenic apple fruit and the respective untransformed control fruit. Fruit tissues were UV irradiated before RNA isolation. An expected signal of 310 bp resulting from *rbc ssu* was obtained for all samples, whereas a 264 bp signal resulting from the *bar* gene was obtained for only the three transgenic lines (1–3). The expected size of the amplified *Vst*1 cDNA fragment was shortened by the length of the intron and amount to 255 bp. Fragments of the correct size were amplified in cDNA samples of transgenic fruit. No fragment was obtained when cDNA of untransformed control fruit was used. Lanes: M, molecular weight markers (100 bp+, Fermentas); C, nontransgenic control fruit (cv. 'Elstar'); 1, transgenic 'HC' line 2; 2, transgenic 'HC' line 50; 3, transgenic 'Elstar' line; P, plasmid pHK*vst*; W, water. (**B**) UV-induced resveratrol-glucoside (piceid) production in young transgenic apple fruit ('Elstar'): chromatographic profiles of HPLC analysis from non-irradiated (I) fruit and 5 min long UV-treated fruit (II). The piceid peak is indicated by arrows.

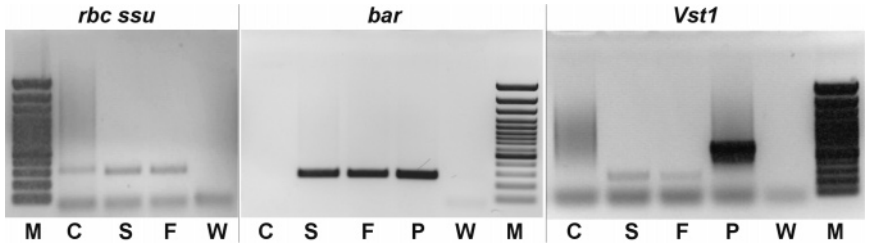

Figure 4. RT-PCR analysis for detecting ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*rbc ssu*)-, *bar*-, and *Vst*1-mRNA accumulation in skin and flesh of UV-treated mature transgenic apple fruit (transgenic 'Elstar' line). Lanes: C, nontransgenic control fruit (cv. 'Elstar'); S, skin of transgenic apple fruit; F, flesh of transgenic apple fruit; P, plasmid pHK*vst*.

of cv. 'Elstar' are shown in **Figure 3**. Using ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*rbc ssu;* endogenous control gene) specific primers, amplification products with the size of 310 bp were obtained from each cDNA template, whereas expected signals of 264 bp using *bar* gene specific primers were obtained only for the three transgenic lines (**Figure 3**).

Primer binding sites of the *Vst*1 gene are flanking an intron, so that DNA derived PCR products could be distinguished from RNA (cDNA) derived products by their size. When RT-PCR was performed on cDNA samples of transgenic fruit tissues, the expected fragment with the size of 255 bp was obtained for all three lines, whereas plasmid positive control led to the amplification of a 611 bp fragment, because the intron is included in the product (**Figure 3**). Amplification was negative when RNA of nontransformed fruit was used as template.

Analysis of cDNA generated from mRNA isolated separately from flesh and skin (taken from the vicinity of the irradiated surface) of a mature apple fruit indicated accumulation of specific *Vst*1 and *bar* mRNA in both fruit tissues (**Figure 4A**) independent from the developmental stage of the fruit.

Determination of *trans*-Resveratrol Glucoside (Piceid) in Transgenic Fruit. Tissues of unripe and mature apple fruit from individual *Vst*1 transgenic lines as well as from nontransformed control fruit were analyzed by HPLC for the accumulation of resveratrol. To induce the *Vst*1 promoter, tissues were subjected to UV irradiation. Previous preliminary results (unpublished) using UV-treated leaves of the transgenic 'Elstar' line indicated a time-dependent increase of the piceid concentration. Levels remained stable between 16 and 32 h after UV exposure; therefore, an incubation time of 24 h was chosen. Because of material limitations, analyses of unripe and mature fruit tissues were performed with different transgenic lines. The accumulation of a resveratrol derivative, namely, *trans*-resveratrol-3-*O*-β-D-glycopyranoside (piceid), was detected in fruit tissues. There was a strong correlation between induction of the *Vst*1 promoter and accumulation of the resveratrol glucoside in young (**Figure 3B**) as well as in ripe fruit (**Figure 5**). Neither resveratrol nor any of its derivatives were found in nontransformed control fruit, before or after UV irradiation. Line 27 failed to accumulate the stilbene (**Figure 5**). Interestingly, piceid was detected in unripe, green fruit material, but not in mature, red-pigmented fruit material of the transgenic 'Elstar' line.

Influence of Piceid Production on Other Phenolic Compounds in Transgenic Apple Fruit. To clarify whether the novel biosynthetic pathway introduced in transgenic apple alters

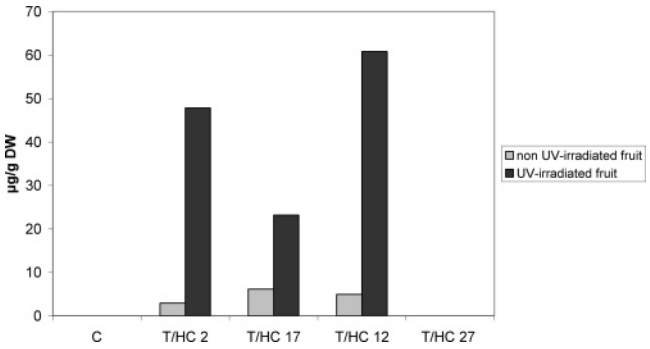

Figure 5. *trans*-Piceid production in ripe transgenic apple fruit of 'Holsteiner Cox'. Extracts for HPLC analysis were taken from non-UV-irradiated fruit and 24 h after 5-min exposure to UV light. No piceid was found in nontransgenic control fruit or in transgenic line 27. Levels of *trans*-piceid in fruit of the other transgenic lines increased after UV-mediated promoter induction. T/HC, transgenic 'Holsteiner Cox' fruit; C, control fruit (cv. 'Holsteiner Cox').

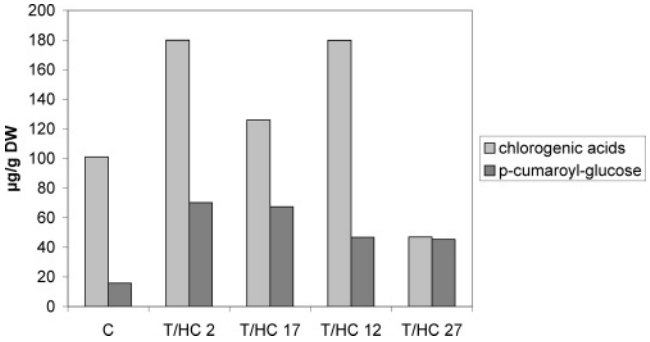

Figure 6. Hydroxycinnamic acids accumulation in transgenic and control apple fruit (24 h after UV treatment).

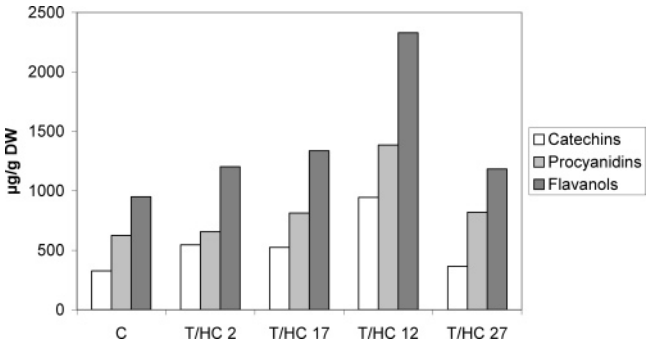

Figure 7. Accumulation of flavanols in 'Holsteiner Cox' wild-type and transgenic apple fruit (24 h after UV treatment).

the accumulation of other phenolic compounds present in apple fruit, we determined the amounts of several products of the flavonoid pathway in ripe fruit of the control and four transgenic 'Holsteiner Cox' lines. Potential alterations due to substrate competition between chalcone synthase and stilbene synthase were expected upon high stilbene synthase activity (*38*). Because resveratrol accumulation significantly increased after UV-mediated promoter induction (**Figure 5**), we compared results obtained from irradiated transgenic tissues with those obtained from irradiated control fruit tissues.

In fruit of piceid-synthesizing lines (T/HC 2, 12, and 17), all harboring two copies of the *Vst1* gene (*20*), the accumulation of hydroxycinnamic acids (chlorogenic acids and *p*-coumaroyl glucose; **Figure 6**), flavanols (catechins and procyanidins; **Figure 7**), and phloretin derivatives (**Figure 8**) was not

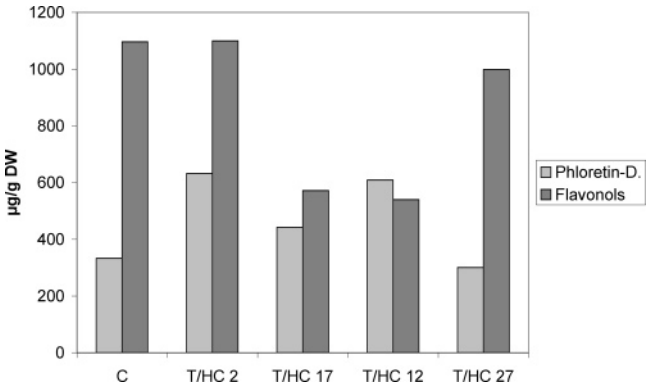

Figure 8. Levels of phloretin derivatives and flavonols in transgenic and control apple fruit of 'Holsteiner Cox' (24 h after UV treatment).

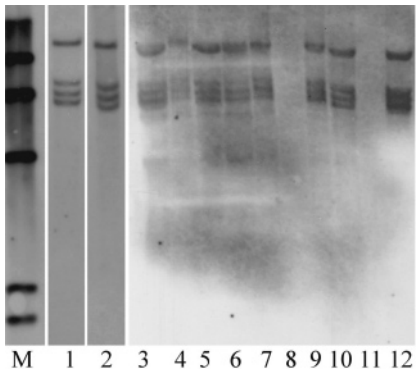

Figure 9. Southern blot hybridization analysis of the seedling progeny of transgenic 'Elstar' and 'Remo' (pollinated with pollen obtained from transgenic 'Elstar') to investigate the inheritance of the transgenes. All blots were hybridized with the DIG-labeled, PCR-generated *Vst*1 fragment. The parental line and the progeny plants contained four *Eco*R1 fragments. Lanes: M, molecular weight markers II, *DIG*-labeled (Roche); 3–7, Remo seedlings pollinated with transgenic 'Elstar' pollen; 10, parental transgenic 'Elstar' line; 1, 2, 9, 11, and 12, 'Elstar' seedlings.

negatively influenced in comparison to fruit of their nontransformed counterparts.

Amounts of flavonols in transgenic line 2 were comparable to values found in nontransformed control fruit and in transgenic fruit of line 27, which is unable to synthesize piceid, whereas transgenic lines 12 and 17 accumulated lower amounts of flavonols than did the control (**Figure 8**). No correlation was observed with the synthesis rate of piceid (**Figure 5**).

Inheritance of the Transgenes. Seeds were harvested from transgenic 'Elstar' and 'Holsteiner Cox' as well as from nontransformed 'Holsteiner Cox' and 'Remo'. Seeds from transgenic 'Elstar' and nontransgenic 'Remo' germinated, whereas seeds from 'Holsteiner Cox' were lost due to contaminations or failure of germination. No seedlings were recovered from that cultivar.

The inheritance pattern of the transgenes in the seedling progeny was determined using southern blot analysis. Two of 5 seedlings of the transgenic 'Elstar' line and 8 of 14 investigated seedlings of 'Remo' (pollinated with pollen of the transgenic 'Elstar' line) harbored the transgenes. In all cases both the *Vst*1 (**Figure 9**) and the *bar* genes (data not shown) were detected. Integration patterns of the transgenes were the same as in the parental line (**Figure 9**). The four *Vst*1 and *bar* copies were not separated from each other, revealing that they have been integrated close to each other on the same chromosome, probably as tandem repeats.

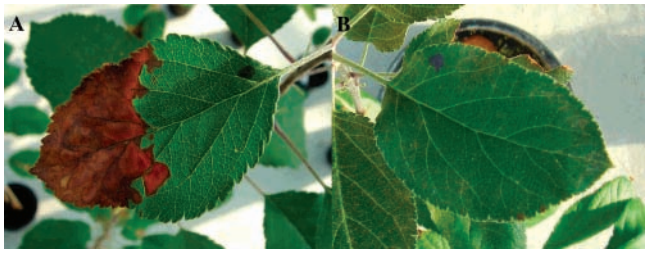

Figure 10. Effect of Basta painted on individual leaves of susceptible (**A**) and resistant (*B*) (*Vst*1/*bar* transgenic) progeny plants, 7 days after application.

Expression of the *bar* gene was investigated using leaf paint assays with the herbicide Basta. Results were consistent with integration analysis (**Figure 9**). All treated leaves of plants without transgene integration were severely damaged and exhibited strong necrosis (**Figure 10A**). In contrast, all leaves from transgenic progeny plants showed no symptoms (**Figure 10B**) and were therefore classified as resistant.

DISCUSSION

Piceid Synthesis in Transgenic Apple Fruit and Its Influence on Other Compounds of the Flavonoid Pathway. The recombinant expression of a stilbene synthase gene from grape causes the accumulation of a resveratrol derivative, piceid, in transgenic apple plants (*20*). In this study we have confirmed that piceid is also produced in transgenic apple fruit.

The juvenile phase of the tissue culture derived greenhouse plants was reduced by grafting onto dwarfing rootstock M27. The transgenic plants were phenotypically normal in the greenhouse as described for other transgenic apple plants (*39*−*41*) except endochitinase transgenic apple plants, which showed reduced vigor (*42*).

Transgene expression in transgenic apple fruit was first reported by James et al. in 1996 (*43*) and later in 1999 by Yao et al. (*41*). In both cases an antibiotic resistance gene or the GUS reporter gene was used. Dandekar et al. (*44*) produced transgenic apple fruit modified in the capacity to synthesize endogenous ethylene. To our knowledge this is the first time that resveratrol production was achieved in transgenic apple fruit. As we have already found for leaf tissues of *Vst*1 transgenic apple plants (*20*), a glucoside resveratrol derivative characterized as piceid (*trans*-resveratrol-3-*O*-β-D-glycopyranosid) was detected in fruit, as well. Glycosylated forms of resveratrol also occur in *Vitis* (*45*) and were found in transgenic kiwi (*16*) and alfalfa (*17*), whereas both the free and the glycosylated forms were detected in transgenic tomato fruit (*29*). Glycosylation is often associated with the necessity for storage.

Piceid accumulation occurred in fruit skin and flesh and was independent from the ripening stage in transgenic 'Holsteiner Cox' fruit, but was restricted to unripe fruit in the case of 'Elstar'. No piceid was found in red-pigmented mature 'Elstar' fruit, despite the fact that specific *Vst*1-mRNA was detected.

Results from only one transgenic 'Elstar' line do not allow us to clarify whether this is a general phenomenon in 'Elstar' or whether it is specific to that line. But because the color development was the main obvious phenotypical change, one could speculate that the failure of piceid accumulation is probably a consequence of the concomitant rise of anthocyanin accumulation in ripening apples during color development. The stilbene synthase and the chalcone synthase, key enzymes of the polyphenolic pathway, utilize the same substrates *p*-coumaroyl-CoA and malonyl-CoA such that a differential carbon folding results in the synthesis of stilbenes and chalcones, respectively (**Figure 1**). Substrate limitations due to resveratrol synthesis might impair the accumulation of other compounds of the flavonoid and phenylpropanoid pathway (*38*), and, vice versa, strong chalcone synthase activity may negatively influence resveratrol synthesis as might be the case in the transgenic 'Elstar' line during color development.

In transgenic 'Holsteiner Cox' apple fruit no such negative correlation between resveratrol/piceid synthesis and accumulation of flavanols, hydroxycinnamic acids, and phloretin derivatives was observed, indicating no substrate limitations.

In transgenic tomato expressing the stilbene synthase gene cinnamic and coumaric acids are present at lower levels than in the wild type, suggesting that in this case the first precursors in phenylpropanoid biosynthesis are most utilized (*29*). A main difference compared to our study was the use of the 35S promoter, causing constitutive expression of the *Vst*1 gene. In addition, the amounts of resveratrol/piceid were much higher.

Fertility of Stilbene Synthase Transgenic Apple Plants. In this study we have shown that the transgenes (*Vst*1 and *bar*) were inherited into the progeny of 'Remo' pollinated with pollen from transgenic 'Elstar' plants. The constitutive expression of the stilbene synthase gene, under the control of an enhanced cauliflower mosaic virus (CaMV) 35S promoter, induced male sterility in tobacco and caused modifications in flower morphology and color (*38*). Therefore, the stilbene synthase is also an interesting candidate to induce male sterility to prevent transmission of transgenes by pollen. The stilbene synthase gene in apple is driven by its own inducible *Vst*1 promoter from *Vitis*. Although small amounts of *trans*-piceid were constitutively present in plants without promoter induction by UV irradiation (probably due to abiotic and/or biotic stresses), fertility was not negatively influenced. This indicates that the use of a stilbene synthase as a candidate to induce male sterility in order to close a potential escape route for transgenes into the environment necessitates a strong constitutive promoter.

No seedlings were recovered from transgenic 'Holsteiner Cox'. Many seeds were lost due to contaminations. The remaining seeds did not germinate, obviously because of the triploid character of this cultivar and the accompanied production of aneuploid progenies. Those could be saved only through in vitro embryo rescue (*46*). Therefore, this cultivar was unsuitable for inheritance analysis.

Conclusions. We have shown that stilbene synthase transgenic plants are phenotypically normal, at least under greenhouse conditions, and flowered within the first and second years after grafting and in subsequent years under greenhouse conditions. Transgenic apple fruit was phenotypically indistinguishable from nontransgenic fruit of the same cultivar. The stilbene synthase gene is expressed in fruit skin and flesh after promoter induction. The introduction of the novel pathway does not dramatically influence the accumulation of other phenolic compounds naturally present in apple fruit. Resveratrol is modified by the addition of a sugar in both vegetative and fruit tissues. Because of the high antioxidant activity of resveratrol, its synthesis in apple contributes to the "inner quality" of the fruit and might also have positive effects on fruit conservation during storage (*21*).

ABBREVIATIONS USED

DW, dry weight; HC, 'Holsteiner Cox'; IBA, indole-3-butyric acid; MS, Murashige and Skoog; RT-PCR, reverse transcriptase Polymerase Chain Reaction.

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