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Constitutive Expression of a Grapevine Stilbene Synthase Gene in Transgenic Hop (*Humulus lupulus* L.) Yields Resveratrol and Its Derivatives in Substantial Quantities

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Resveratrol, a well-known phytoalexin and antioxidant, is produced by the action of stilbene synthase (STS) in some plant species. Hop (*Humulus lupulus* L.) plants of the Tettnang variety were transformed with a gene encoding for STS from grapevine. Under the control of the constitutive 35S cauliflower mosaic virus promoter, expression of the transgene resulted in accumulation of resveratrol and high levels of its glycosylated derivatives in leaves and inflorescences. Piceid, the predominant derivative, reached a concentration of up to 560 μg/g of fresh weight (f.w.) in hop cones, whereas no stilbenes were detected in nontransformed controls (wild-type). In transgenic plants the amounts of α- and β-acids, naringenin chalcone, and prenylated flavonoids did not change significantly when compared with nontransformed plants. Transgenic plants showed normal morphology and flower development as did the nontransformed controls. The results clearly show that in hop constitutive expression of *sts* interferes neither with plant development nor with the biosynthesis of secondary metabolites relevant for the brewing industry. Since resveratrol is a well-known phytoalexin and antioxidant, *sts* transgenic hop plants could display enhanced pathogen resistance against microbial pathogens, exhibit new beneficial properties for health, and open new venues for metabolic engineering.

KEYWORDS: Phytoalexin; antioxidant; prenylation; flavonoid; *Agrobacterium tumefaciens*; tissue culture

INTRODUCTION

Resveratrol (3,4′,5-trihydroxystilbene) is a naturally occurring phytoalexin and antioxidant produced by a restricted number of plants such as grapes, peanuts, and mulberries (*1*−*4*). Since several plants, including grapevine, synthesize resveratrol when attacked by pathogens, stilbene-type phytoalexins have long been considered to play an important role in the defense response of plants against pathogens (*2*, *5*). Furthermore, the discovery of a relationship between moderate red wine consumption and a lowered incidence of cardiovascular disease, the so-called "French Paradox" (*6*, *7*), attracted interest in resveratrol from pharmacologists. The health-related properties of resveratrol were investigated intensively over the past two decades and numerous biological activities have been attributed to resveratrol (*8*).

Resveratrol accumulates by the action of stilbene synthase (STS). It is formed in plants from the common precursors malonyl-CoA and *p*-coumaroyl-CoA in a single enzymatic step (*9*) (**Figure 1**). Likewise, chalcone synthase (CHS), the key enzyme in flavonoid biosynthesis in plants, uses the same precursors and converts them into naringenin chalcone. Naringenin chalcone represents the central molecule that may be converted into a high number of different compounds like flavonoids, anthocyanidins, and condensed tannins, some of which are further decorated with glycoside residues, methyl groups, or prenyl side chains (*10*). Two prenylated flavonoids with interesting pharmacological properties are known from hop: xanthohumol (XN) and 8-prenylnaringenin (8-PN) (*11*). Both compounds derive from naringenin and have recently attracted much interest due to their anti-proliferative and phytoestrogenic activities, respectively (*12*). Other prenylated key compounds present in hop (α- and β-acids) are the well-known bittering compounds, adding taste and flavor to beer (*13*). The bittering acids derive from phloroglucinol precursors, which are synthesized by valerophenone synthase (VPS) from isovaleryl-CoA or isobutyryl-CoA in a reaction similar to that of CHS and STS (*9*). Because of their high similarity, CHS, STS,

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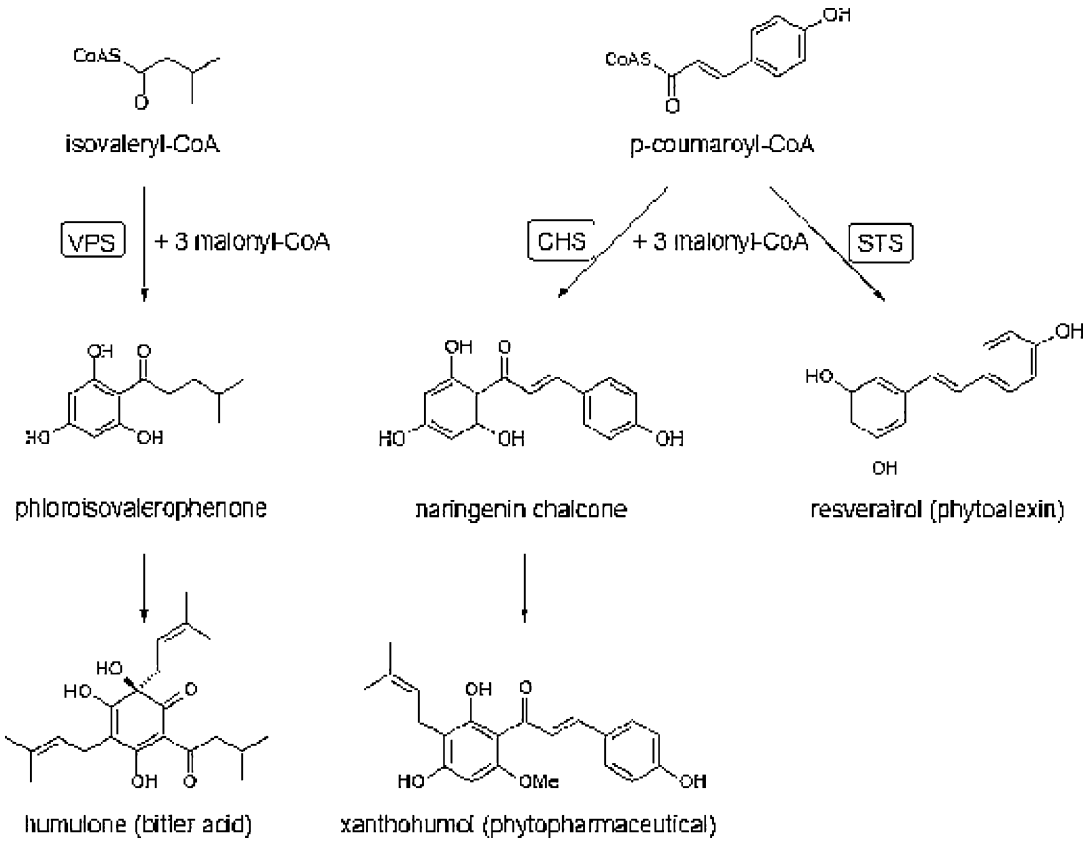

Figure 1. Biosynthetic routes to important phytopharmaceuticals and bittering acids in hop. Introduction of a foreign STS into hop leads to synthesis in a single step of the phytoalexin resveratrol. Potential competition for the substrate *p*-coumaroyl-CoA by the biosynthetic pathways to flavonoids (naringenin chalcone) and stilbenes (resveratrol) is evident. VPS, valerophenone synthase; CHS, chalcone synthase; STS, stilbene synthase.

and VPS, among a few others, are classified as type III polyketide synthases (*14*). In contrast to CHS, which is ubiquitous in plants, STS is not present in hop, whereas VPS has been found only in hop (*15*).

The various biological activities of stilbenes make it desirable to enhance their production by metabolic engineering. Additionally, the expression of only a single transgene is sufficient to produce resveratrol (and its derivatives) in plants (*16*). Interestingly, enhanced disease resistance of *sts*-transgenic plants against various pathogens was observed to be correlated with enhanced stilbene content (*16*−*21*). Furthermore, antioxidant levels increased in tomatoes transformed with the *sts* gene (*22*).

Here we report on the first transformation of hop (*Humulus lupulus* L.) with the *vst1* gene from grapevine expressed under control of the constitutive 35S cauliflower mosaic virus promoter. The impact of this transformation on the secondary metabolism of hop has been analyzed with particular focus on resveratrol derivatives and on important hop flavonoids like naringenin−chalcone, xanthohumol (X), desmethylxanthohumol (DMX), and 8-prenylnaringenin, as well as α- and *β*-acids.

MATERIALS AND METHODS

Plant Transformation, Regeneration, and Cultivation of Transgenic Plants. Transformation of internodal stem segments of Tettnang hop was performed as described previously (*23*) (T-DNA 4528 bp). Transgenic shoots were regenerated and identified by triplex-PCR (*24*). As positive controls for *vir*G and *npt*II, the binary vector was used. From each of the transgenic plants, ten clones were produced in tissue culture and eventually transferred to the greenhouse. Greenhouse-grown plants were cut back to their rootstocks by late December of each year and vernalized at 2 °C in the dark without further treatment until March of the following year. Then shooting was induced by transferring them to standard growing conditions either in the greenhouse or transplanted to a containment facility outdoors. This cultivation cycle was repeated in subsequent years to give rise to the following "generations". At least every spring and fall of each year, the stability of the integration of the transgene in the new growth was verified by triplex-PCR. Independent of the time and from which plant part they were harvested in all samples, the transgene was verified. Samples were taken from plants grown in the greenhouse and in the hop yard.

Southern Analysis. Hop DNA was extracted from leaves according to the protocol described previously (*25*). DNA (20 μg) was digested over night with 20 units of *Hin*dIII, which did not have a recognition site within the T-DNA. Digested DNA was purified by precipitation with 1/10 volume of 3 M NaAc, pH 5.2, and 2 volumes of ethanol for 30 min. After centrifugation at 15 000 rpm for 30 min, the supernatant was discarded; the DNA pellet was washed with 70% ethanol and air-dried before it was resuspended in 20 μL of ddH_2O. Digested DNA (20 μg) was separated on a 0.8% agarose gel at 10 V/cm. Prior to blotting, the separated DNA was depurinated in the gel for 20 min in 0.25 M HCl, denatured in 1.5 M NaCl, 0.5 M NaOH for 20 min, and neutralized in 1.5 M NaCl, 1 M Tris/HCl, pH 7.2, for 10 min. The gel was blotted with 20× sodium chloride/sodium citrate (SSC) buffer onto HYBOND N+ membranes (Amersham, München, Germany) overnight by descending transfer as described in ref *26*. Blotted DNA was cross-linked to the membrane by UV light using the Stratagene UV crosslinker (Stratagene, Amsterdam, The Netherlands) and prehybridized for 3−4 h at 47 °C in a solution containing 50% formamide, 5× Denhardt's solution, 5× SSC, 1% SDS, and 20 μg/mL salmon sperm DNA (denatured at 100 °C for 5 min). For radioactive detection of the transgene, 500 ng of purified vector DNA containing the transgene was labeled radioactively with ^{32}P-dCTP according to the manufacturers protocol using the HEXA-LABEL DNA-labeling kit (Fermentas, St. Leon Rot, Germany). Unincorporated nucleotides were removed by spin-column purification. The labeled probe (1 Mio. counts per mL) was then added to the hybridization solution and hybridization was performed overnight at 47 °C. The hybridized membrane was washed

two times for 10 min with 2× SSC/0.1% SDS and once with 1× SSC/0.1% SDS at the hybridization temperature. Afterward, the membrane was air-dried for 5 min and wrapped into SARAN. Depending on the labeling intensity of the probe, hybridized membranes were exposed to X-ray film for 10−72 h before being developed.

RNA Isolation. RNA was isolated from lupulin glands of wild-type and transgenic hop using a modified protocol of (*27*) designed for tissues with high levels of phenolic compounds and polysaccharides. This procedure utilized a lysis buffer with an increased concentration of guanidine isothiocyanate (4 M) in combination with 20% sarkosyl. Lupulin glands were obtained by removing them from bracteoles of dried hop cones (<8% humidity) stored at 8 °C. Lupulin glands were pulverized directly in the tube at room temperature. Until RNA isolation proceeded, the tubes containing approximately 50 mg of crushed tissue were stored in liquid nitrogen to prevent RNA degradation.

RT-PCR. Reverse transcription and amplification of the resulting cDNA molecules was carried out using the Qiagen OneStep RT PCR Kit (Qiagen, Hilden, Germany). The *sts*-specific primer pairs (Stsn1.s, 5′-ATGGCTTCAGTTGAGGA-3′; and Stsn4.a, 5′-TTGGAAGAGTG-GTCGTTCAAT-3′), were used to detect the presence of corresponding transcripts. To check for DNA contamination in RNA preparations, all reactions (1× QIAGEN OneStep RT-PCR Buffer, 400 μM dNTP, 0.6 μM each primer) were divided into two halves. An enzyme mix containing reverse transcriptase and polymerase, as well as template DNA, was added to one half, and reverse transcriptase reaction was performed for 30 min at 50 °C. Subsequently, the enzyme was inactivated by heat (94 °C, 15 min). To the remaining half, template DNA and enzyme mix (see above) was added in which the reverse transcriptase previously had been heat inactivated for 15 min at 94 °C. Amplification was carried out in all samples for 30 cycles (1 min at 94 °C, 30 s at 53 °C, 1.5 min at 72 °C). The amplification was stopped using a final elongation step of 10 min at 72 °C before cooling down to 8 °C for hold. All PCR products were stored at −20 °C until further analysis.

HPLC Analysis of Flavonoids and Hop Acids: **Extraction and Analysis of Stilbenes.** Extraction and analysis of prenylated flavonoids, hop acids, and glycosylated flavonols was performed as described previously (*13*). Leaves and inflorescences of transgenic plants and wild-type Tettnanger were harvested from plants grown outdoors in the season of 2004 and 2005. Samples were extracted either directly, stored at −20 °C in the freezer, or dried at room temperature in darkness. Plant tissue was homogenized in a MIXER MILL (Retsch, Haan, Germany) and extracted with MeCN (1:3, w/v) at room temperature. Extracts were diluted with water to 70% MeCN, chilled for 1 h at 20 °C, and subsequently cleared by centrifugation (13 000 rpm, 5 min). Supernatants were dried in vacuum, and residues were resuspended in 30% MeCN with 0.1 M HAc. Lupulin glands were mechanically detached from the epidermis by means of a fine needle, transferred to MeCN, and treated as described before.

Sample aliquots were analyzed in HPLC (Shandon Hypersil ODS, 5 μm, 250 mm × 4 mm column, diode array detection, flow rate 1.3 mL/min) using a gradient of solvent A (4% MeOH, 4% MeCN, 0.1 M HAc) and solvent B (MeCN). The gradient cycle consisted of an initial 15 min (100% solvent A), followed by a linear increase of solvent B to 20% in 25 min, then kept isocratic for 10 min.

Commercially available resveratrol (Sigma-Aldrich, Munich, Germany) and piceid (purified from *Fallopia japonica* rhizomes) were used as standards in HPLC, UV, MS, and ^{1}H NMR. Defined amounts of *trans*-piceid and *trans*-resveratrol were analyzed in HPLC for quantification of natural compounds in the samples. Calibration curves for detection at 320 nm were linear in the range between 4 and 1200 ng per injection. This allowed detection of both compounds down to a concentration of approximately 1 ppm in plant extracts. Extraction efficiency for *trans*-piceid was 81.5%, as was defined by adding specific amounts of the reference to tissue of wild-type Tettnanger, extracting the sample in the usual manner, and quantifying *trans*-piceid in HPLC.

For compound purification, 9 g of dried hop cones were defatted with *n*-hexane (3 × 15 min), then extracted with 70 mL of MeOH (50 °C, 3 × 15 min). Filtered supernatants were evaporated to dryness under reduced pressure. The residue was resuspended in MeOH, absorbed on 6 g of silica gel (mesh 80 μm), dried under vacuum, and successively eluted with 20 mL portions of *n*-hexane, CH_2Cl_2, EtOAc, and MeOH. The EtOAc fraction contained resveratrol; its glucosides were detected in the MeOH fraction. Both fractions were further purified by TLC (KG 60 F254, Merck, Schwalbach, Germany) in benzene/2-butanone/MeOH/water (55:22:20:3). Final purification was carried out by HPLC as described above. NMR experiments were performed in a Varian Unity Inova spectrometer (Palo Alto, CA), operating at 500 MHz for ^{1}H. APCI(−)−LC−MS experiments were performed on a Finnigan (Hybaid Instruments, Waltham, MA) TSQ 700 for piceid. The compound from peak A was analyzed using ESI on a Platform 2 (Micro).

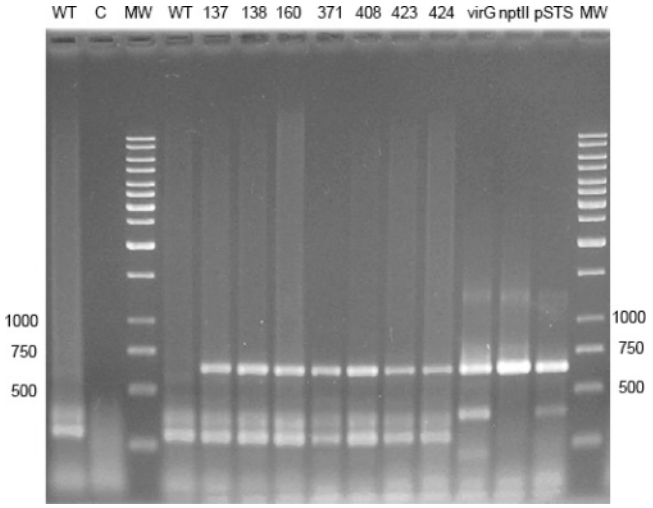

Figure 2. Triplex PCR for identifying transgenic plants. The presence of transgenes is shown in shoots of all seven transgenic plants 2 years after genetic transformation. WT, nontransformed control; C–, negative control (no template); MW, molecular weight standard; 137–424, seven transgenic plants analyzed; VirG, *vir*G control (390 bp); nptII, *npt*II control (640 bp); pSTS, positive control for binary plasmid (*npt*II product). The double band appearing at 290 and 330 bp shows amplification of hop chitinase, documenting the presence of hop DNA in all samples analyzed.

RESULTS

Plant Transformation and Molecular Characterization of Events. In two independent experiments, we transformed 2000 internodal stem segments of the hop genotype "Tettnanger" by cocultivation with *Agrobacterium tumefaciens* carrying a binary vector. Its T-DNA harbored the *vst1* cDNA sequence from grapevine (*28*) under of the control of the 35S promoter of cauliflower mosaic virus (CaMV). Selection of transgenic tissue was facilitated by the marker gene (*npt*II; kanamycin resistance) controlled by the promoter of nopaline synthase (*nos*) (schematic representation **Figure 3A**). Transformation was carried out in two consecutive experiments (A and B) with 1000 explants each. Overall 430 explants (22%) regenerated on selective medium. A total of seven transgenic plants were identified (0.4%) by triplex-PCR. These plants were numbered and had originally emerged from the following sources: explants 137, 371, and 424 (experiment A; one cluster of regenerates from one stem segment), 138 and 423 (experiment A; regenerates from stem segments from a single Petri dish different from the former), and 160 and 408 (experiment B; individual regenerates from one stem segment each, originating from two separate Petri dishes) (**Figure 2**).

The molecular background of the seven transgenic plants was analyzed further by Southern analysis. For this purpose, genomic DNA had been digested by *Hin*dIII, not cutting within the incorporated T-DNA, and was probed with the labeled transgene. (**Figure 3B**). Four independent transgenic events were

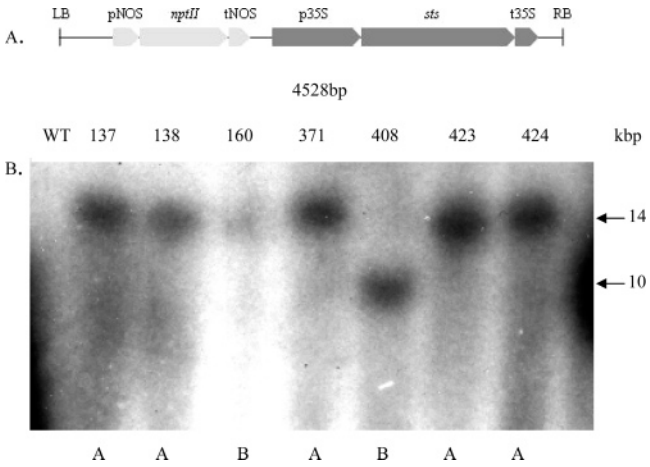

Figure 3. T-DNA and Southern blot analysis showing integration of the STS transgene in the hop genome. Schematic representation of T-DNA (A, drawn not to scale) showing selection marker (nopalin synthase promoter (pNOS) and terminator tNOS, neomycin phosphotransferase II (*npt*II)); 2× 35S promoter (p35S), stilbene synthase (*sts*), and 35S terminator (t35S). Seven transgenic plants were analyzed (lanes 2–8) together with one nontransformed control (wt, lane 1). Plants numbered 137, 371, and 424 originated from a single cluster of regenerates (experiment A), 138 and 423 from a separate Petri dish (experiment A), 408 from a single regenerate (experiment B), and 160 from a different explant (experiment B). Genomic hop DNA was digested with *Hin*dIII cutting outside the T-DNA region. The transgene was integrated only once into the genome of each plant. Only one hybridization signal was observed for each plant with approximate sizes of 10 kb (408) or 14 kb (137, 138, 160, 371, 423, and 424).

Table 1. Relation between Transgenic Events and Clone Designation as Deduced from Origin of Transgenic Plants and the Southern Analysis of DNA with the Transgene

event	experiment	transgenic line number
1	1	137, 371, 424
2	1	138, 423
3	2	408
4	2	160

identified according to their origin and Southern hybridization patterns: 137, 371, and 424 clones from event 1, experiment A; 138 and 423 from event 2, experiment A; 160 from event 3, experiment B; 408 from event 4, experiment B (**Table 1**). Each of the seven transgenic plants displayed only one hybridization signal with molecular sizes exceeding that of the inserted T-DNA (4528 bp) (**Figure 3**). Overall, the transformation efficiency of the two experiments was 0.2% (explant to field stage). In estimating the transformation frequency, it was assumed that plants 137, 371, and 424 or 138 and 423 would be clones.

The expression of the *sts* transgene was analyzed by reverse transcriptase PCR in ripe cones of all transgenic plants (**Figure 4**). In the transgenic plants, the expected amplicon size of 714 bp was detected. No signals were detected in the nontransformed controls.

Tissue Culture and Field Cultivation of Transgenic Plants. Each of the seven clones was multiplied in tissue culture in 2003 and consecutively transferred to the greenhouse. In June 2004, four replicas of each clone of transformants as well as three controls were planted in an outdoor containment facility. During September 2004, cones could be harvested from 15 of 31 plants (28 transgenics, 3 controls) grown outdoors. In 2005, nine additional controls were planted in the outdoor plot, and samples from all 40 plants were harvested at points of time throughout the growing season. All transgenic plants and nontransgenic controls had developed normally when their cones were harvested for chemical analysis in August and September 2005. To monitor stability of the transgene over time, triplex-PCR with DNA sampled from plant tissues was performed (last data point September 2006). All transgenic plants showed the presence of the selection marker (*npt*II), whereas the nontransgenic control did not, thus verifying the stability of the integration of the transgene (data not shown).

Chemical Analysis of Stilbenes in Transgenic Plants: Compound Identification. Implications for other metabolic processes due to the expression of the *sts* gene in transgenic plants were analyzed by metabolic profiling of transgenic hop plants and nontransgenic controls grown in the outdoor plot during 2004 and 2005. Compared with nontransgenic plants, extracts from bracts of transgenic plants showed at least six additional peaks (A−F) in HPLC analyses (**Figure 5**).

Their UV and migration characteristics were typical for stilbene derivatives. Purification and subsequent spectroscopic analysis using ^{1}H NMR and mass spectrometry led to the identification of *trans*- and *cis*-piceid (peaks B, D). *trans*- and *cis*-Resveratrol (peaks E, F) were identified by comparison with UV and chromatographic properties of a commercially available standard. Peak A contained a compound with an $[M - H]^+$ peak at *m*/*z* 405 and UV maxima at 229, 283, and 322 nm, characteristic for *trans*-astringin (*29*, *30*). The migration behavior of the compound in HPLC indicated higher polarity when compared to piceid. This was also consistent with the properties of astringin, which contains an additional hydroxyl group at C-3. Peak C also showed UV maxima typical for stilbenes (cis isomers) but could not be further characterized due to its low quantity in the available sample.

Quantification and Localization of Stilbenes. The quantification of resveratrol was hindered due to peak overlap with a flavonoid (peak 24) in HPLC and could only be estimated from comparison of peak ratios with and without addition of a standard. The amount of *trans*-resveratrol reached approximately 13 μg/g of dry weight (d.w.) in dried bracts and 2.7 μg/g of fresh weight (f.w.), respectively.

Quantification of *trans*-piceid in dried bracts of hop cones resulted in concentrations in tissue ranging from 2005 μg/g of d.w. in the transgenic plant 138 (event 2) to 2238 μg/g of d.w. in clone 424 (event 1). This is equivalent to 411 and 458 μg/g of f.w. of bracts, respectively. Comparison of clones from plants of the same transgene integration event showed similar variation in quantities, suggesting that the differences were of individual nature rather than a consequence of the transformation experiment. The cis form of piceid was present in significantly lower amounts reaching approximately 20% of the trans isomer. It can be assumed that both isomers are naturally present in plants and that the cis form is not an artifact caused by light-induced isomerization during preparation. Preparations from fresh plant tissue under low-intensity light contained *cis*-piceid as well. The overall concentration of stilbenes in bracts reached 480−560 μg/g of f.w. None of the stilbene derivatives were detected in HPLC analysis of nontransformed wild-type plants of Tettnanger.

Piceid was also detected in true leaves of transgenic hop plants, although to a much lesser extent. The amount of *trans*-piceid reached an average of 195 μg/g of d.w. in dried leaf tissue (mean value of events 137, 138, 160, 371, and 423). The high flavonoid content in these samples prohibited the quantification

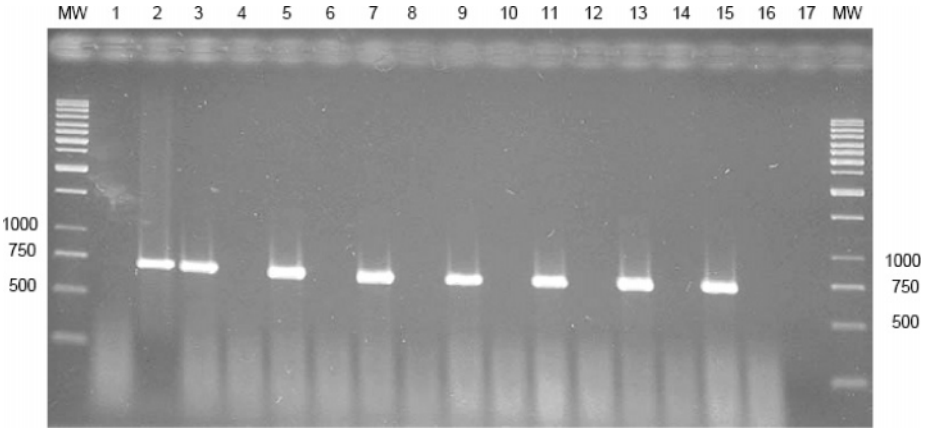

Figure 4. Expression of stilbene synthase in cones of transgenic plants and nontransformed controls. RT PCR products (714 bp) specific for stilbene synthase were obtained from cone RNA of seven transgenic hop plants. Lane 1, RNA, nontransgenic control; lane 2, plasmid DNA (PCR product); lanes 3–15 (odd numbers), RT products obtained from plant numbers 137, 138, 160, 371, 408, 423, and 424 separated by controls (reverse transcription inhibited by incubation on ice) (lanes 4–16; even numbers); lane 17, control without template; MW, molecular weight marker.

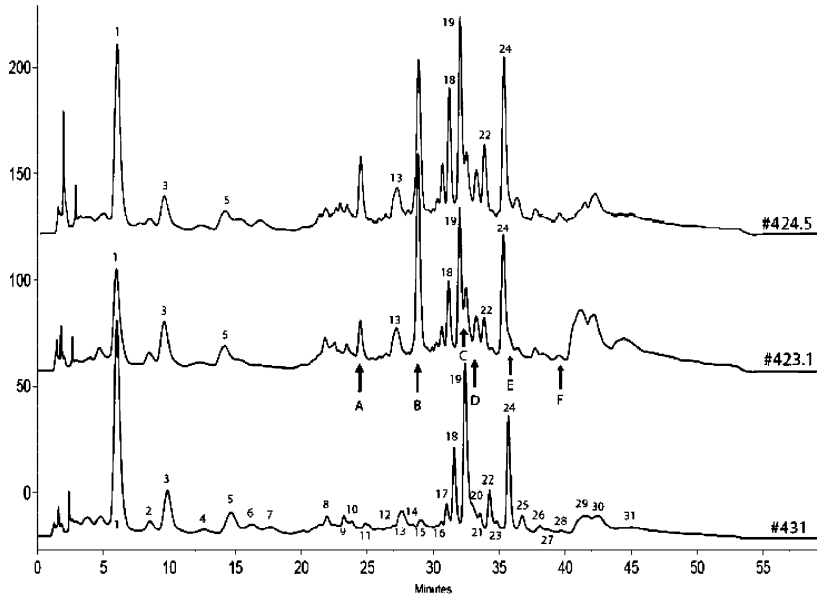

Figure 5. HPLC analysis of secondary metabolites in STS transgenic hop. Diagrams of HPLC (A_{320nm}) with MeCN extracts from bracts of nontransformed controls (Tettnanger 431) and transgenic clones 424 (event 1) and 423 (event 2). Peaks in the sample of the wild-type are numbered consecutively. A–F indicate peaks present only in transgenic plants.

of other stilbenes. Extracts from mechanically separated trichomes of leaves and bracts showed small amounts of *trans*-piceid besides the expected hop acids and prenylated flavonoids. However, they did not contain the flavonol glycosides typical for the leave extracts. These results indicate that the activity of stilbene synthase was constitutively expressed in all tissues as may be expected from a gene under the regulation of the 35S promoter.

Quantification of Bitter Acids. *Prenylated Flavonoids.* The analysis of prenylated flavonoids showed two major compounds, desmethylxanthohumol (DMX), a precursor for 8-prenylnaringenin (8-PN) and 6-prenylnaringenin (6-PN), and xanthohumol (X), a precursor for isoxanthohumol.

The levels of DMX varied from 0.4 to 0.9 mg/g of the dry weight of the hop cones (see **Table 3**). Except for clone 160, there were no significant differences between transgenic clones and the nontransformed control. The levels of X varied from 1.0 to 3.4 mg/g of the dry weight of the hop cones (**Table 3**). Clones 137, 371, 424, 138, and 423 showed no significant differences from nontransformed controls, whereas clone 160 was lower in content.

Hop Acids. The analysis of hop acids showed four major peaks corresponding to the α-acids (cohumulone, *n*-humulone, and adhumulone) and the *β*-acids (colupulone, *n*-lupulone, and adlupulone). Because no significant differences were found in the relative composition of α- and *β*-acids, the total α-acid and total *β*-acid levels were comparable (**Table 3**).

The levels of α-acids and *β*-acids varied from 10.9 to 51.4 mg/g and from 13.1 to 39.8 mg/g of the dry weight of the hop cones, respectively. Only clone 160 contained significantly lower levels of α- and *β*-acids than nontransformed control. Overall transgenic and nontransformed clones did not differ significantly in hop acids from each other.

Chemical Analysis of Flavonol Glycosides. Five major peaks corresponding to flavonol glycosides (kaempferol and quercetin derivatives) were detected in the chromatograms and characterized based on retention times, UV spectra, mass spectra, and comparisons with authentic standards. All five flavonol glycosides were detected in each chromatogram. The relative ratios of their respective peak areas were compared with each other in all samples (**Table 3**). Except for lower values in clone

Table 2. Effects of Stilbene Synthase Gene Expression in Plants Transformed with the *Vitis vinifera* Stilbene Synthase Gene (*vst*) under Control of Different Promoters

plant	promoter	observation	stilbene[a] content (μg/g of f.w.)	ref
apple	VST	accumulation of unidentified resveratrol-glycoside	*b*	*38*
apple fruit	VST	accumulation of piceid in fruit	*b*	*35*
kiwi	VST	no resistance	180	*39*
papaya	VST	increased resistance to *Phytophtora palmivora*	54	*40*
rice	VST	increased resistance to *Pyricularia oryzae* (rice blast)	*b*	*19*
sorghum	VST	increased resistance to *Botrytis cinerea* (greymold)	*b*	*17*
tobacco	VST	increased resistance to *Botrytis cinerea* (greymold)	*b*	*16*
tomato	VST	increased resistance to *Phytophthora infestans* (late blight), but not against *B. cinerea*, *Alternaria solani*	*b*	*18*
wheat	VST	increased resistance to *Oidium tuckeri* (powdery mildew)	*b*	*20*
wheat	VST	increased resistance to *Puccinia recondita*	35–190	*41*
Brassica napus seeds	napin	accumulation of piceid, no difference in other agronomic traits	361–620	*32*
alfalfa	35S	accumulation of piceid; increased resistance to *Phoma medicaginis*	10–15	*21*
hop	35S	accumulation of *trans*- and *cis*-piceid, resveratrol, and astringin	490–560	this study
poplar	35S	no increased resistance to *Melampsora pulcherrima* (rust)	309–615	*4*
tobacco	35S	altered flower morphology, male sterility	50–290	*33*
tomato	35S	increased antioxidant activity	26–53	*22*
pea	VST	inducible resveratrol accumulation	0.53–5.2	*42*

[a] Amount of resveratrol and resveratrol glycosides in μg per g of f.w. [b] Not determined.

Table 3. Content (mg/g) of Prenylated Flavonoids and Hop Acids in Dried Hop Cones from Nontransgenic Control and Seven Transgenic Plants from Four Independent Events

sample	DMX	X	total α-acids	total β-acids	rel content of totalflavonol glycosides (%)
event 1 exp 1 (cl 137)	0.61 ± 0.01	3.00 ± 0.01[a]	33.33 ± 1.14	33.23 ± 1.038	99 ± 28
event 1 exp 1 (cl 371)	0.70 ± 0.01	3.00 ± 0.010[a]	40.65 ± 0.94	30.00 ± 0.565	131 ± 15
event 1 exp 1 (cl 424)	0.88 ± 0.01	3.3 ± 0.007[a]	51.36 ± 1.09[a]	38.27 ± 0.917[a]	117 ± 20
event 2 exp 1 (cl 138)	0.86 ± 0.02	3.4 ± 0.024[a]	47.33 ± 1.41[a]	40.35 ± 0.850[a]	114 ± 11
event 2 exp 1 (cl 423)	0.81 ± 0.01	3.3 ± 0.014[a]	46.80 ± 0.82[a]	35.93 ± 0.427	117 ± 17
event 3 exp 2 (cl 408)	0.73 ± 0.01	2.24 ± 0.008	25.43 ± 0.21[b]	35.10 ± 0.457	133 ± 14
event 4 exp 2 (cl 160)	0.43 ± 0.01[b]	1.04 ± 0.008[b]	10.88 ± 0.13[b]	13.10 ± 0.260[b]	76 ± 12
nontransformed control	0.82 ± 0.02	2.2 ± 0.047	38.23 ± 0.91	28.24 ± 0.694	100 ± 25

[a] Significantly higher than control. [b] Significantly lower than control.

160, the content of flavonol glycosides of transgenic plants was found to be similar to nontransformed controls.

DISCUSSION

The gene for STS has been transferred to a number of plant species in which the transgene was expressed under the control of its own inducible promoter or of constitutive promoters (**Table 2**). Both strategies resulted in increased levels of stilbenes.

In transgenic hop described here, resveratrol was quantitatively glycosylated. This is similar to results reported from poplar (*4*), Rehmannia (*31*), and alfalfa (*21*). Piceid, the 3-*O*-glycoside of resveratrol, appears to be the predominant end product in most *sts* transformed plants. In our transgenic hop, piceid exceeded the unglycosylated resveratrol by a factor of 100 or more. In previous studies, the content of resveratrol was often estimated as resveratrol equivalents because of the formation of glycosylated and other derivatives as well as cis/trans isomerization. Therefore, quantitative comparison of individual compounds is rather difficult. However, based on total stilbene content, cones of S35-promoted transgenic hop plants reached the highest levels described so far. Only leaves of poplar (*4*) and seeds of *Brassica napus* (*32*) showed similar quantities. From transgenic plants under control of inducible promoters, significantly lower levels of stilbenes were reported (**Table 2**).

It was interesting to note that despite the constitutive expression of *sts* in hop, significant tissue-specific differences in stilbene concentration were observed. Leaves contained only about 1/10 of stilbenes present in mature bracts, and lupulin glands contained only trace amounts. This is similar to the findings in alfalfa where significant differences have been detected in the stilbene content of leaves, stems, and roots (*21*). In our hop expressing STS under a constitutive promoter, we did not observe any negative effects on plant development, growth, or morphology. Nevertheless, transformation events may have a negative impact on the general fitness of a plant. Fischer et al. (*33*) reported altered flower morphology as well as male sterility in transgenic tobacco (*sts* with S35 promoter). In a model for explaining male sterility, it was proposed that in plants transgenically expressing STS this enzyme would compete with CHS for common precursors (*34*). Such competition would explain the difference between protein content and resveratrol accumulation in leaves of *sts* transgenic poplar (*4*). In our results, the influence of transgenic *sts* on male fertility could not be observed because only female plants were transformed.

Transforming plants with STS may also influence other secondary metabolites. On the basis of grain yield and oil content in transgenic *Brassica napus* constitutively expressing *sts*, no negative effects on agronomically important traits were reported (*32*). Likewise, a high stilbene content in bracts of cones of transgenic hop did not impede other agronomically important flavonoids and hop acids. In contrast, most transgenic hop clones yielded even slightly higher amounts of these compounds when compared with nontransformed controls of Tettnanger.

In a study of resistance against infection with *Botrytis cineria* (*34*), it was stated that constitutive expression of *sts* was less effective than induced expression because after fungal infection the 35S promoter was down-regulated. Although it may be safer to control the *sts* transgene by its inducible promoter, it was pointed out that addition of 35S enhancer elements dramatically improved the ability of transgenic wheat to resist infection (*20*). These findings were supported by Hipskind and Paiva (*21*) who observed in transgenic alfalfa an increased resistance against *Phoma medicaginis* with S35-promoted *sts* transgenes. The effect of *sts* transfomation in our hop plants on pathogen resistance is currently under investigation.

Besides its value as a phytoalexin, resveratrol is also a useful antioxidant (*4*). Successful attempts to increase resveratrol levels have been shown in apple where the transgene was expressed under the control of its original promoter (*35*). Similar observations were made in tomato fruits when *sts* expression was controlled by the 35S promoter (*22*). Hop was not known to be a natural source for stilbenes until very low quantities (0.5 μg/g of d.w. of reveratrol and 2 μg/g of d.w. of piceid in hop pellets) were reported recently from several North American hop cultivars such as Tomahawk (*36*, *37*). Until now no stilbenes were found in European varieties. In our study on nontransformed Tettnanger, no stilbenes were found above the detection limit of approximately 0.5 μg/g of d.w. Furthermore, using stilbene synthase specific primers, nontransformed controls of Tettnanger did not show any signals in a PCR analysis of DNA (**Figure 2**) or RT PCR of RNA from cones (**Figure 4**), respectively.

However, the current study has shown that pharmacologically as well as agronomically important quantities of stilbenes in hop may be produced by genetic transformation. This approach could open new venues for metabolic engineering of secondary metabolites like flavonoids.

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