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ORIGINAL PAPER

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Resveratrol glucoside (Piceid) synthesis in seeds of transgenic oilseed rape (*Brassica napus L.*)

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Abstract Resveratrol is a phytoalexin produced in various plants like wine, peanut or pine in response to fungal infection or UV irradiation, but it is absent in members of the *Brassicaceae*. Moreover, resveratrol and its glucoside (piceid) are considered to have beneficial effects on human health, known to reduce heart disease, arteriosclerosis and cancer mortality. Therefore, the introduction of the gene encoding stilbene synthase for resveratrol production in rapeseed is a tempting approach to improve the quality of rapeseed products. The stilbene synthase gene isolated from grapevine (*Vitis vinifera L.*) was cloned under control of the seed-specific napin promotor and introduced into rapeseed (*Brassica napus L.*) by *Agrobacterium*-mediated co-transformation together with a ds-RNA-interference construct deduced from the sequence of the key enzyme for sinapate ester biosynthesis, UDP-glucose:sinapate glucosyltransferase (*BnSGT1*), assuming that the suppression of the sinapate ester biosynthesis may increase the resveratrol production in seeds through the increased availability of the precursor 4-coumarate. Resveratrol glucoside (piceid) was produced at levels up to 361 µg/g in the seeds of the primary transformants. This value exceeded by far piceid amounts reported from *B. napus* expressing *VST1* in the wild type sinapine background. There was no significant difference in other important agronomic traits, like oil, protein, fatty acid and glucosinolate content in comparison to the control plants. In the third seed generation, up to 616 µg/g piceid was found in the seeds of a homozygous T3-plant with a single transgene copy integrated. The sinapate ester content in this homozygous T3-plant was reduced from 7.43 to 2.40 mg/g. These results demonstrate how the creation of a novel metabolic sink could divert the synthesis towards the production of piceid rather than sinapate ester, thereby increasing the value of oilseed products.

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Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a phytoalexin, derived from the phenylpropanoid pathway. Several plant species like grape (Langcake and Pryce 1976), peanut and pine (Kindl 1985) synthesize resveratrol that is absent in members of the *Brassicaceae*. A correlation between fungal disease resistance and resveratrol production of grapevine cultivars has been reported (Langcake and McCarthy 1979; Stein and Blaich 1985). Resveratrol can also be induced by other stress factors like UV light (Schoeppner and Kindl 1978), wounding (Langcake 1981) and elicitor treatment (Melchior and Kindl 1991). The biosynthesis of resveratrol is catalysed by the enzyme stilbene synthase, which converts one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA into resveratrol. As part of the phenylpropanoid pathway, these precursor molecules are present in all plant species as substrate for chalcone synthase, the key enzyme of the flavonoid pathway (Rupprich and Kindl 1978).

The gene encoding stilbene synthase (VST1) in grapevine *(Vitis vinifera)* has been expressed in various plant species (e.g. Hain et al. 1993; Stark-Lorenzen et al. 1997; Szankowski et al. 2003). These studies were mostly initiated to increase fungal disease resistance in crop species. A relationship between resveratrol production and enhanced fungal resistance has been shown (Hain et al. 1993; Leckband and Lörz 1998; Hipskind and Paiva 2000; Liang et al. 2000). Contrasting results were obtained in kiwi, where the production of resveratrol did not lead to an increased disease resistance (Kobayashi

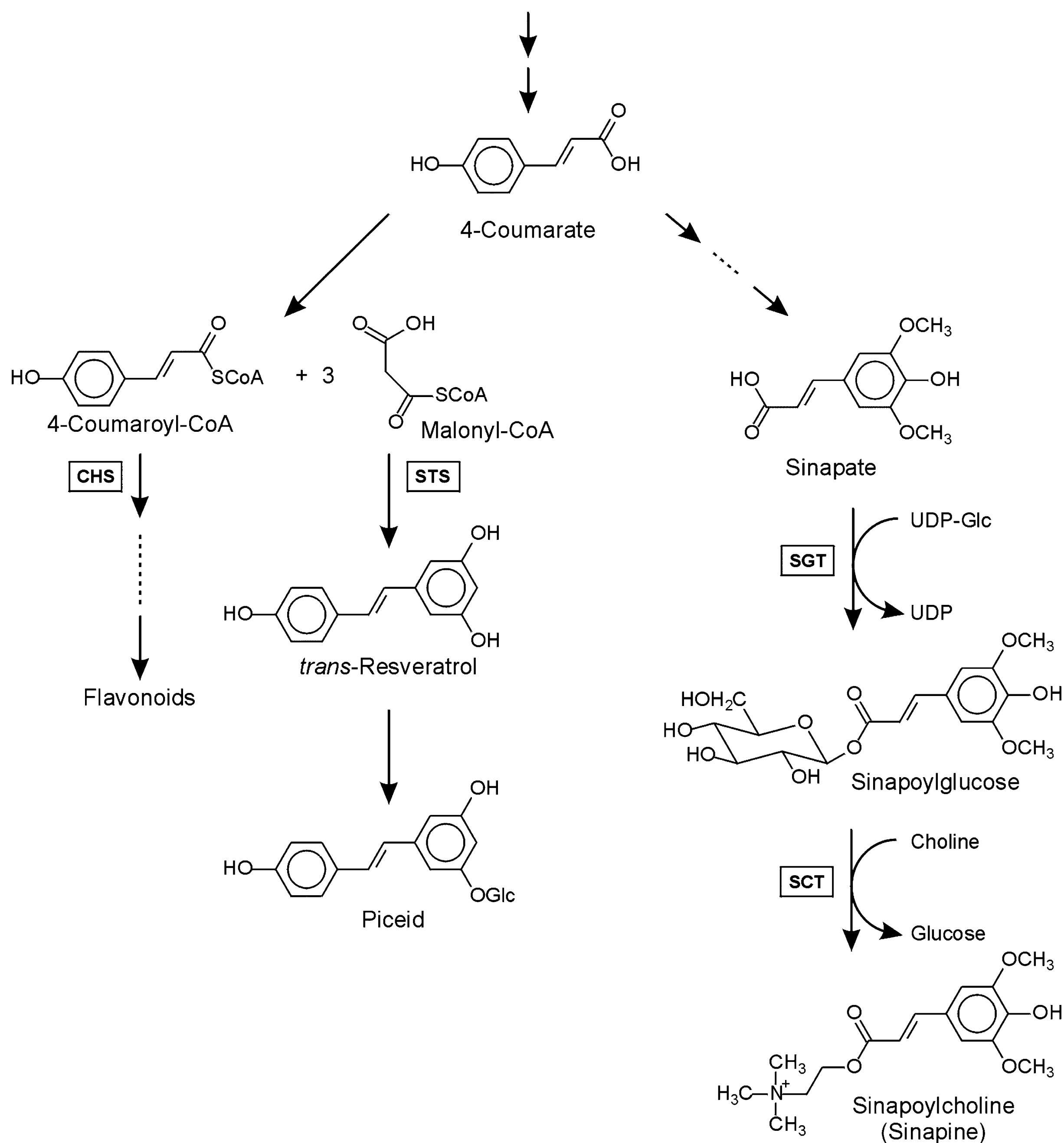

Fig. 1 Resveratrol and sinapate ester biosynthetic pathway in oilseed. The enzymes involved in sinapate ester metabolism are UDP-glucose (*UDP-Glc*): sinapate glucosyltransferase (*SGT*; EC 2.4.1.120), 1-*O*-sinapoyl-*β*-glucose: choline sinapoyl-transferase (*SCT*; EC 2.3.1.91). Resveratrol synthesis is catalysed by stilbene synthase (*STS*); the first step in flavonoid biosynthesis is catalysed by chalcone synthase (*CHS*)

et al. 2000). However, there are alternative reasons for producing resveratrol in transgenic plants, such as using this compound as nutraceutical or beneficial food component. Resveratrol and its glucoside (piceid) are known for their positive effects on human health. It has been shown that they reduce coronary heart disease mortality and arteriosclerosis (Manna et al. 2000), inhibit low-density lipoprotein oxidation (Manna et al. 2000) and eicosanoid synthesis (Pace-Asciak et al. 1995). An anti-cancer effect has also been reported (Jang et al. 1997).

In plant phenylpropanoid metabolism, 4-coumarate forms an important branching point, feeding biosyntheses of stilbenes, flavonoids and sinapate esters (Fig. 1). Seeds of *Brassica napus* accumulate mainly sinapoylcholine (sinapine) and sinapoylglucose accompanied by smaller amounts of other, not yet characterized, sinapate conjugates (Strack et al. 1983; Boucherau et al. 1991; Lorenzen et al. 1996). The total amount of sinapate esters reaches 1–2% of seed dry matter (Bell 1993). As energy-rich acyl donor, sinapoylglucose, which is

formed from UDP-glucose and sinapate by the enzyme UDP-glucose:sinapate glucosyltransferase (SGT; EC 2.4.1.120), plays a key role in sinapate ester metabolism (Fig. 1). In developing seeds, this glucose ester is transacylated by the enzyme sinapoylglucose:choline sinapoyltransferase (SCT, EC 2.3.1.91) to sinapine. During germination, sinapine is hydrolysed by a specific esterase (sinapine esterase, SCE; EC 3.1.1.49). The liberated sinapate is again conjugated by SGT to sinapoylglucose, which is converted to sinapoylmalate by the enzyme sinapoylglucose:malate sinapoyltransferase (SMT). Sinapoylmalate accumulates in epidermal and sub-epidermal tissues as an important UV protecting substance (Landry et al. 1995). Sinapate esters reduce the quality of the rapeseed meal, because they contribute to the bitter taste, astringency and dark colour of rapeseed products (Kozlowska et al. 1990; Naczk et al. 1998; Shahidi and Naczk 1992). In addition, sinapate esters may form complexes with proteins, thus lowering the nutritional value of the protein products (Kozlowska et al. 1990; Shahidi and Naczk 1992).

Since resveratrol and sinapate esters depend on the precursor 4-coumarate (Fig. 1), it was assumed that resveratrol production in the seeds of rape following transformation with the *VST1* gene could be enhanced by simultaneously suppressing the sinapate ester pathway. This hypothesis was supported by the fact, that transgenic rapeseed expressing a *SGT-ds-RNAi*-construct showed reduced levels of all sinapate containing compounds (Hüsken 2004). Plasmid constructs designed for seed-specific expression of *VST1* and silencing of *BnSGT1* by dsRNAi were used for *Agrobacterium*-mediated co-transformation. The present work was aimed at regenerating transgenic *Brassica napus* plants expressing the *VST1* gene and the *SGT-ds-RNAi* construct, and to identify transgenic lines with high levels of resveratrol and reduced levels of sinapate esters.

Materials and methods

Plasmid and bacterial strains

The vector pPSty5 carries the stilbene synthase gene (*Vst1*) from *Vitis vinifera L.* (Melchior and Kindl 1990) under the control of the napin promoter and *the NPTII*-gene conferring kanamycin resistance under control of the CaMV35S-promoter (Fig. 2). The full-length cDNA of the *SGT* gene from *Brassica napus* (*BnSGT1*) has been isolated (Milkowski et al. 2000) and a cDNA fragment covering the first 213 bp of the *BnSGT1* coding sequence was used to construct a suppression cassette under control of the seed-specific napin promoter which was finally inserted into binary vector pLH7000 (Hausmann 1999) resulting in the *dsRNAi* vector pLH-SGT-GUS (Hüsken 2004). *Agrobacterium tumefaciens* strain ATHV C58-C1 (Hellens et al. 2000) containing the binary vector pPSty5 (P. Jorasch, Universität Hamburg, unpublished data) and *Agrobacterium tumefaciens strain* AGL1 (Hellens et al. 2000) carrying the binary suppression plasmid pLH-SGT-GUS were used in the co-transformation experiments for the production of transgenic *Brassica napus* plants.

Plant material

Spring rapeseed (cv. Drakkar) with ‘00’-quality was used. Seeds were sterilized for 30 min in a 5% sodium hypochlorite solution and rinsed thrice in sterile distilled water. They were germinated on 1/2 MS-medium (Murashige and Skoog 1962) in a growth chamber at 20°C with a day length of 16 h.

Co-transformation, selection and regeneration

Agrobacterium-mediated transformation and plant regeneration were modified according to De Block et al. (1989). Hypocotyl segments were co-inoculated with ATHV C58C1 carrying the binary plasmid pPSty5 and AGL1 carrying the binary plasmid pLH-SGT-GUS. Transformed hypocotyl segments were regenerated in two steps on selective medium with 25 mg/l Kanamycin (pPSty5), 5 mg/l PPT (pLH-SGT-GUS) and 50 mg/l Kanamycin (Duchefa, Haarlem, The Netherlands), 10 mg/l PPT (Duchefa, Haarlem, The Netherlands). Three to six weeks after selection, calli with small shoots were regenerated. Shoots were separated and transferred to MS-medium (Murashige and Skoog 1962) for further regeneration and rooting. Surface-sterilized seeds of cv. Drakkar were germinated in vitro and plants obtained were propagated. These plants were transferred to the greenhouse together with the transformants (T1) and used as controls.

Fig. 2 Schematic drawing of the T-DNA region of the binary plasmid pPSty5. Sequences of functional importance are left border (*LB*) and right border (*RB*); CaMV 35S promoter (*p-35S*) and CaMV 35S terminator (*t-35S*). The seed-specific suppression cassette consists of a napin promoter fragment (*p-nap*) and the *VST1* gene from *Vitis vinifera*. Kanamycin resistance is conferred by the *nptII* gene from transposon Tn5 encoding neomycin-phosphotransferase. Recognition sites are shown for restriction endonucleases *Bam*HI (*B*) and *Pst*I (*P*)

Plant cultivation in the greenhouse and analysis of morphological traits

Putative transgenic plants (T1-plants) propagated in vitro were transferred to the greenhouse and cultivated at 16-h light with a photon flux density of 200–900 μmol m^{-2} s^{-1} and 8-h dark conditions. The main shoot of each plant was selfed for seed production (T2-seeds). Selected T2-plants were selfed to obtain T3-seeds and selected T3-plants were selfed to obtain T4-seeds. The transgenic T1-, T2- and T3-plants were compared to non-transformed control plants in a completely randomized design.

NPT-II-ELISA-test

Leaf tissue (50 mg) of putative transgenic plants was used. The NPT-II-ELISA-test was performed as described by the manufacturer (Agidia, Elkhart, USA). The absorbance of the reaction mixture was measured at room temperature at 450 nm. The standard curve was made following the manufacturer's instructions.

PAT-ELISA-test

Leaf tissue (100 mg) of putative transgenic plants was used. The PAT-ELISA-test was performed as described by the manufacturer (Steffens, Ebringen, Germany). The absorbance of the reaction mixture was measured at room temperature at 625 nm. The standard curve was made following the manufacturer's instructions.

DNA extraction, PCR and Southern blot analysis

To detect the introduced transgenes with PCR in the putative regenerated transgenic plants, DNA samples were extracted from leaf tissue using the DNAeasy-Mini-kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A primer pair (*PAT*-fw 5′-ATG GGC CCA GAA CGA CGC CC-3′; *PAT*-rev 5′-GCG TGA TCT CAG ATC TCG GT-3′ and *NPTII*-fw 5′-ATC GGG AGC GGC GAT ACC GTA-3′; *NPTII*-rev 5′-GAG GCT ATT CGG CTA TGA CTG-3′) for amplification of the 498 bp *PAT*-fragment (pLH-7000-SGT-GUS resistance marker) and of the 700 bp *NPTII*-fragment (pPSty5 resistance marker) were designed and used for PCR. Southern blot analysis was performed to verify the integration of the transgenes and to determine the respective copy number. For Southern blot analysis, DNA samples were extracted from leaf tissue of primary transgenic plants (T1) according to the DNAeasy-Maxi-kit (Qiagen, Hilden, Germany). Five microgram genomic DNA was digested with *Eco*RI and separated by 0.8% agarose gel electrophoresis. Separated DNA fragments were transferred onto a positively charged nylon membrane (Amersham Biosciences, Freiburg, Germany). Probes (*PAT*- and *NPT-II*-resistance marker) were labelled by PCR (primer sequences see above) with digoxigenin using a DIG-HyPrime-kit (Roche, Mannheim, Germany), and a DIG Nucleic Acid Detection Kit (Roche, Mannheim, Germany) was used for filter hybridization and detection according to the manufacturer's instructions.

Kanamycin and phosphinothricin resistance of T2-seedlings

Primary transformants (T1-plants) were selfed to obtain T2 seeds. Seeds were sterilized for 30 min in a 5% sodium hypochlorite solution. Finally, the seeds were rinsed thrice in sterile distilled water. Fifty-five seeds per T1-plant were germinated on 1/2 MS-medium including 70 mg/l Kanamycin (Duchefa, Haarlem, The Netherlands) in a growth chamber at 20°C with a day length of 16 h. The development of antibiotic damage symptoms was scored 7 days post-treatment. After that, all T2-seedlings were transferred on 1/2 MS-medium including 10 mg/l PPT (Duchefa, Haarlem, Netherlands). The development of herbicide damage symptoms was scored 7 days post-treatment.

Resveratrol glucoside (piceid) and sinapate ester analysis

Grinded seed material (20 mg) was extracted with 1 ml Methanol/H_2O (4:1) in 2 ml-safe-lock tubes in the presence of zirconia beads (1 mm) using a bead beater (Bio Spec Products, Inc., Bartlesville, OK, USA). Reversed phase HPLC (Waters Separator 2795, Waters 2996 photodiode array detector and Waters 474 fluorescence detector) was carried out using a Nucleosil 5-μm C_{18} column (250×4 mm i.d.; Machery & Nagel, Düren, Germany). After centrifugation of the extracts 10 μl samples were injected. Separation was achieved using a 20-min linear gradient at a flow rate of 1 ml min^{-1} from 10 to 50% solvent B (acetonitrile) in solvent A (1.5% *o*-phosphoric acid in water). For detection of piceid, a Waters 474 fluorescence detector was used at 330 nm for excitation and 374 nm for emission and quantified by external standardization with piceid (Sigma, Taufkirchen, Germany). Piceid (*trans*-resveratrol-3-*O*-*β*-glucoside) from *Polygonum cuspidatum* was used as reference compound. Sinapate esters were detected using a photo diode array detector (PDA) at 330 nm and quantified by external standardization with 1-*O*-ß-D-sinapoylglucose, sinapine and sinapate, respectively. The total sinapate ester content (total SE) was calculated as sinapate equivalents.

Analysis of the seed quality traits

Seed samples were analysed for oil, protein, glucosinolate (GSL) and fatty acids by near-infrared reflectance

Table 1 Regeneration and transformation efficiency of *Brassica napus* T1-plants co-transformed with the pPSty5 + pLH-SGT-GUS plasmids

Experiment	Explants	25 mg Kan + 5 mg/l PPT	70 mg Kan + 10 mg/l PPT	ELISA[a] and PCR[a] positive	Transformation efficiency (%)
1	300	51	10	5	1.7
2	300	38	7	7	2.3
3	350	41	9	5	1.4
4	350	36	9	4	1.2
5	350	42	13	7	2.0
Sum/mean	1,650	208	48	28	1.8

[a]NPTII- and PAT-ELISA-Test and NPTII- and PAT-PCR

spectroscopy (NIRS) using the Raps2001.eqa. (Tillmann 2005).

Statistical analysis

All statistical parameters (mean, SD, χ^2) were calculated using the StatGraphics Plus for Windows 3.0 (STATISTICAL GRAPHICS CORP. 1997). For correlation analysis, Spearman rank correlation coefficients were used.

Results

Regeneration and transformation efficiency

A total of 48 NPT II- (70 mg/l kanamycin) and PPT-resistant (10 mg/l, Table 1) Drakkar plants were obtained from 1,650 explants. PCR analysis confirmed the presence of the *NPT II*- and *PAT*-marker genes in 28 of these plants, they showed a *NPT II*- and *PAT*-specific PCR-band of the expected size (Fig. 3) These plants also showed a NPT II- and PAT-ELISA signal (results not shown). The mean transformation efficiency was 1.8% with a range from 1.2 to 2.3% for the experiments (Table 1).

Piceid and sinapate ester content of T2-seeds

Twenty-eight NPT II- and PAT-ELISA-test positive and *NPT II*- and *PAT*-PCR-positive T1-plants were transferred to the greenhouse. The transgenic lines were fully fertile and normal in growth and morphology in comparison with the control plants (data not shown). There was no significant difference in other important agronomic traits, like oil, protein, fatty acid and glucosinolate content of the seeds (Table 2).

T2-seeds obtained from 28 T1-plants were analysed by HPLC for the accumulation of resveratrol and sinapate esters. The HPLC profiles from T2-seed extracts revealed a fluorescence signal with a shorter retention time than that of resveratrol (Fig. 4). This result suggested that the resveratrol has been modified by an endogenous glucosyltransferase activity to piceid. This was confirmed by HPLC-MS data (data not shown). The piceid content (Table 3) ranged from non-detectable amounts up to 361 μg/g seeds. T1-plants showed varying levels of total sinapate esters (Table 4). The average concentration of total sinapate esters of the control plants was 8.79 mg/g. The lowest control plant contained 7.28 mg/g total sinapate esters. The average concentration in the T1-plants was 6.14 mg/g and the lowest T1-plant contained 2.54 mg/g total sinapate esters. Among the transgenic T1-plants, a negative, not significant correlation between piceid and sinapate esters (Table 5) was found, which ranged from −0.34 to −0.36, and a high and significant correlation between sinapoylglucose and all other sinapate esters, which ranged from 0.88** to 0.97**. Furthermore, sinapine, the sum of other not characterized sinapate esters and the total sinapate esters content were highly correlated to each other. Table 6 shows the piceid content and sinapate ester composition of the three T1-plants with the highest piceid content. One of the T1-plants (1502.17) containing 361 μg/g piceid showed a sinapoylglucose content reduction to 0.48 mg/g, whereas the minimum of the controls

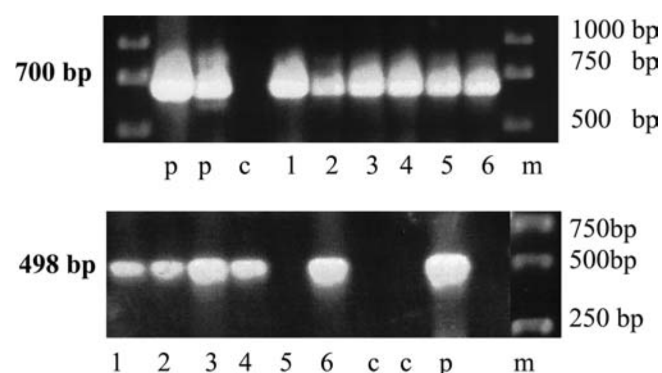

Fig. 3 PCR analysis of *Brassica napus* T1-plants co-transformed with pPSty5 and pLH-SGT-GUS plasmids. **a** Gel electrophoresis of PCR product from NPTII-gene with the expected length of 700 bp. **b** Gel electrophoresis of PCR product from PAT-gene with the expected length of 498 bp. *Lanes: 1–6* T1-plants; *c* untransformed control; *p* plasmid; *m* marker

Table 2 Comparison (mean ± SD) of seed quality traits between controls ($n = 30$) and transgenic T1-plants ($n = 28$) co-transformed with the pPSty5 + pLH-BnSGT-GUS plasmids

Trait	Control ($n = 30$)	1502 ($n = 28$)	Trait	Control ($n = 30$)	1502 ($n = 28$)
Oil (%)	44.9 ± 2.4	43.6 ± 2.9	18:1 (%)	68.5 ± 2.3	67.6 ± 2.9
Protein (%)	24.7 ± 1.9	25.7 ± 1.9	18:3 (%)	6.7 ± 0.8	6.9 ± 1.2
GSL (μmol/g)	22.4 ± 2.1	22.7 ± 3.2	22:1 (%)	0.0 ± 0.0	0.0 ± 0.0

GSL Glucosinolates, *18:1* oleic acid, *18:3* linolenic acid, *22:1* erucic acid

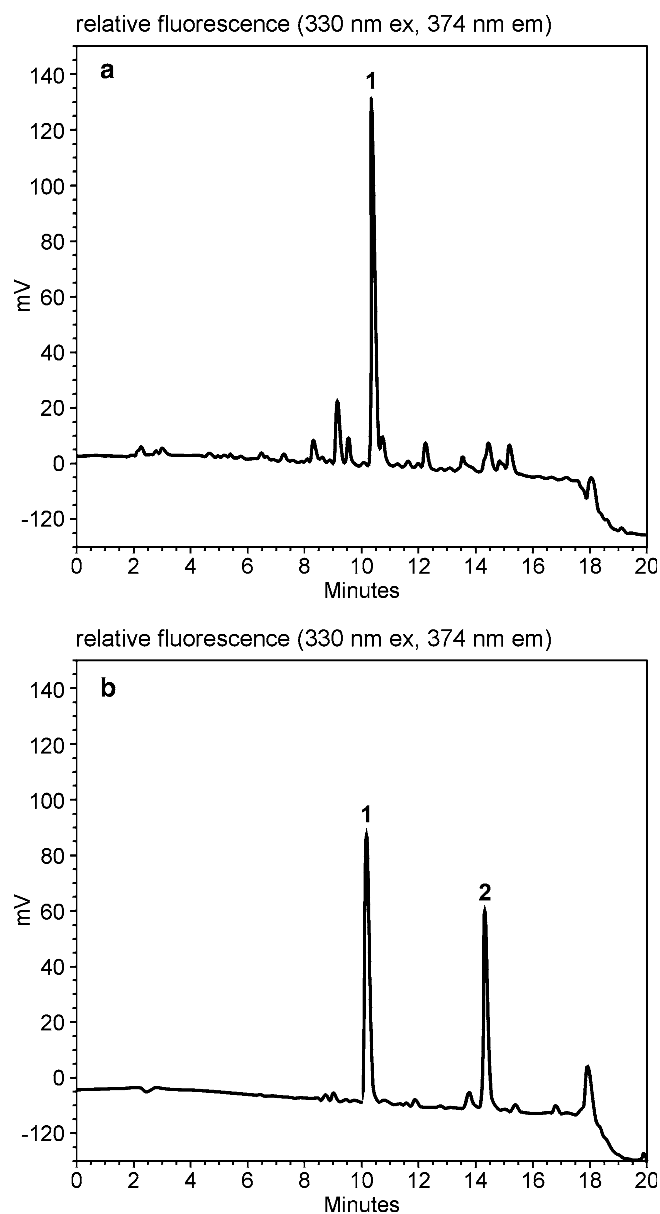

Fig. 4 Reversed-phase HPLC (fluorescence detection) of extracts (**a**) from transgenic seeds (T2). Elution pattern (**b**) of a mixture of piceid (*1*) and *trans*-resveratrol (*2*)

contained 1.21 mg/g. The sinapine content in seeds of this line showed a reduction to 5.00 mg/g, the total sinapate ester content was reduced to 4.00 mg/g.

Transgene copy number in high piceid lines

The three transgenic T1-plants with the highest piceid content as determined in the T2-seeds were characterized for their kanamycin- and PPT-resistance segregation in T2-seeds and analysed by Southern blot hybridisation for the integration of the transgenes in T1-plants. When testing the resistance to kanamycin and PPT of T2 -seeds ($n = 55$ per line) it was found, that 9–12 from 55 seedlings of each line were not resistant to kanamycin (Table 7), indicating a 3:1 inheritance pattern, suggesting that each of the three transformants contains only one T-DNA locus. After transfer to PPT-medium these seedlings showed similar segregation patterns (Table 7). These results correspond to an integrated single copy for each resistance marker gene (NPT II, PPT) as also determined by Southern blot (Table 7, blot not shown).

Piceid and sinapate ester content in the offspring of selected transgenic lines

From each of the three transgenic lines with the highest piceid content and single copy integration for the pPSty5 and pLH-SGT-GUS plasmid 20 T2-seeds were sown together with cv. Drakkar control plants ($n = 22$) in the greenhouse. All T2-plants were normal during the vegetative and reproductive phase of their growth in the greenhouse. There was no significant difference in other important agronomic traits, like oil, protein, fatty acid and glucosinolate content of harvested T3-seeds in comparison with the control plants (data not shown). The average piceid concentration in the T2-plants (T3-seeds) was 310 μg/g for line 1502.15, 203 μg/g for line 1502.16 and 182 μg/g for line 1502.17. The highest piceid content (424 μg/g) was detected in the T2-plant 1502.15.7 (Table 8). The average total sinapate ester content of the control plants was 8.40 mg/g. The average concentration of the T2-plants was 2.80 mg/g for line 1502.15, 3.60 mg/g for line 1502.16 and 4.30 mg/g for line 1502.17. In the T2-plant 1502.15.7 with the highest piceid content the total sinapate ester content was reduced to 1.30 mg/g (Table 8). In comparison to the lowest control plant (7.48 mg/g), the reduction was 83%. The sinapine content in this T2-plant (T3 seeds) of line 1502.15 was 1.90 mg/g. This means a relative reduc-

Table 3 Variation of the piceid content (μg/g) in T1-plants (T2-seeds) co-transformed with pPSty5 + pLH-SGT-GUS plasmids and of control plants

Plasmid	*n*	Mean	SD	Minimum	Maximum
pPSty5 + pLH-SGT-GUS	28	107.73	116.15	0.00	361.00
Control plants	30	0.00	0.00	0.00	0.00

n Number of plants, *SD* standard deviation

Table 4 Variation of the total sinapate ester content[a] (mg/g) in T1-plants (T2-seeds) co-transformed with pPSty5 + pLH-SGT-GUS plasmids and of control plants

Plasmid	*n*	Mean	SD	Minimum	Maximum
pPSty5 + pLH-SGT-GUS	28	6.14	2.70	2.54	10.46
Control plants	30	8.79	0.70	7.28	10.33

[a]Calculated as sinapate equivalents (mg/g)
n Number of plants, *SD* standard deviation

Table 5 Spearman rank correlations (r_s) between sinapate esters (SE) and piceid in T1-plants co-transformed with pPSty5 + pLH-SGT-GUS plasmids ($n=28$)

	Sinapoylglucose	Sinapine	Other SE[a]	Total SE content[a]
Sinapine	0.93**			
Other SE[a]	0.92**	0.88**		
Total SE content[a]	0.97**	0.99**	0.94**	
Piceid	−0.34	−0.35	−0.34	−0.36

[a]Calculated as sinapate equivalents (mg/g)
**significant at $p<0.01$

Table 6 Total amount of piceid (μg/g) and sinapate esters (SE) (mg/g) in the T2-seeds of *B. napus* T1-plants co-transformed with pPSty5 + pLH-SGT-GUS plasmids and of a control plant

Line	Piceid	Sinapoylglucose	Sinapine	Other SE[a]	Total SE content[a]
Control[b]	0.00	1.21	7.44	0.72	7.28
1502.15	351	0.48	4.46	0.19	3.68
1502.16	335	0.30	4.38	0.11	3.44
1502.17	361	0.48	5.00	0.12	4.00

[a]Calculated as sinapate equivalents (mg/g)
[b]Control plant with the lowest total SE content (from $n=30$)

Table 7 Kanamycin and phosphinothricin (PPT) resistance of T2-seedlings and copy number as determined by Southern blot

Line	T2-seedlings ($n=55$)		Segregation	T2-seedlings ($n=55$)		Segregation	Copy number[b]	
	r	n.r.	(χ^2)	r[a]	n.r.[a]	(χ^2)	NPT II	PAT
1502.15	44	11	3:1 (0.7)	47	8	3:1 (3.2)	1	1
1502.16	46	9	3:1 (2.2)	47	8	3:1 (3.2)	1	1
1502.17	43	12	3:1 (0.3)	45	10	3:1 (1.3)	1	1

(χ^2) = 3.84 (5%, 1 DF) r/n.r. resistant/not resistant (70 mg/l Kanamycin)
[a]10 mg/l PPT
[b]Results of Southern blot

tion of 80% in comparison to the lowest control plant with 9.46 mg/g. The increase of the piceid content in the 60 T2-plants (T3-seeds) derived from 3 different T1-plants was inversely related to the total sinapate ester content with $r_s = -0.69$** (Fig. 5).

Twenty T2-plants derived from T1-plant 1502.17 could be grouped according to their piceid and total sinapate ester content (T3-seeds). By analysing the kanamycin and PPT-segregation of the T3-seedlings of these T2-plants it could be shown that the T2-plants that

Table 8 Piceid (µg/g), sinapine and total sinapate ester (SE) content (mg/g) in T3-seeds of selected T2-plants with the highest piceid content co-transformed with the pPSty5 + pLH-SGT-GUS plasmid and of a control plant

Line	Piceid	Sinapine	Percent	Total SE[a] content	Percent
Control[b]	0.00	9.46	100	7.48	100
1502.15.7	424	1.90	20	1.30	17
1502.16.8	308	2.21	23	1.51	20
1502.17.20	326	2.10	22	1.44	19

[a]Calculated as sinapate equivalents (mg/g)
[b]Control plant with the lowest total SE content (from $n = 22$)

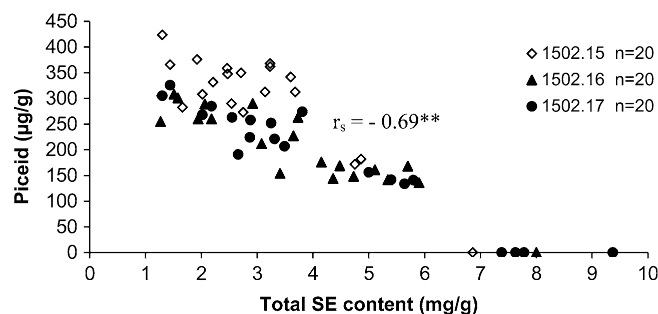

Fig. 5 Correlation between piceid and total sinapate ester (*SE*) content in T2-plants ($n = 60$) derived from three T1-plants with the highest piceid content

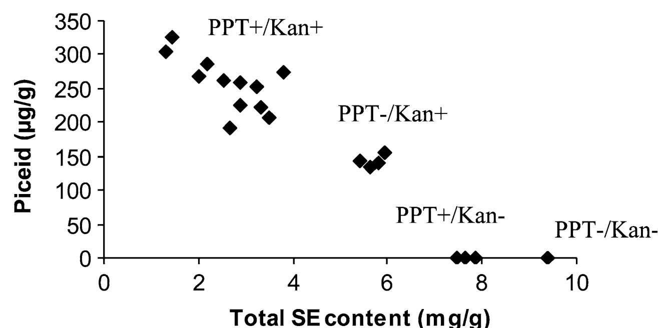

Fig. 6 Piceid (µg/g) and total sinapate ester (mg/g) content of T3-seeds obtained from T2-plants (line 1502.17, $n = 20$). *PPT* +/- Phosphinothricin resistant/not-resistant; *Kan* +/- kanamycin resistant/non-resistant

Table 9 Piceid (µg/g), total sinapate ester (SE) (mg/g) and sinapine content (mg/g) of T4-seeds of selected T3-plants with the highest piceid content, transformed with the pPSty5 + pLH-SGT-GUS plasmid and of control plants

Line	*n*	Mean	SD	Minimum	Maximum
Piceid					
Control	13	0	0	0	0
1502.15.7	20	388	81	221	616
Total SE[a]					
Control	13	8.65	0.60	7.43	9.93
1502.15.7	20	2.51	0.61	1.55	4.10
Sinapine					
Control	13	8.73	0.60	7.37	9.9
1502.15.7	20	3.64	0.88	2.26	5.94

[a]Calculated as sinapate equivalents (mg/g)
n Number of plants, *SD* standard deviation

were homozygous or hemizygous for both integrated genes (*VST1* + *SGT-dsRNAi*) had the highest piceid and the lowest total sinapate ester content (Fig. 6). The T2-plants that were homozygous or hemizygous for the *VST1* gene, but were lacking the *SGT-dsRNAi* gene had an intermediate piceid and total sinapate ester content. The T2-plants carrying only the *SGT-dsRNAi* gene had a lower total sinapate ester content than the plants which did not have either one of the two transgenes. These groups were showing the expected 9:3:3:1 segregation, respectively.

From T2-plant 1502.15.7 with the highest piceid content and single copy integration for the pPSty5 and pLH-SGT-GUS plasmids 20 T3-seeds were sown together with cv. Drakkar control plants ($n = 13$) in the greenhouse. All T3-plants were normal during the vegetative and reproductive phase of their growth. There was no significant difference in other important agronomic traits, like oil, protein, fatty acid and glucosinolate content of harvested T3-seeds in comparison with the control plants (data not shown). The average piceid concentration in the T3-plants (T4-seeds) was 388 µg/g (Table 9). The highest detected piceid concentration was 616 µg/g seeds. The average concentration of total sinapate esters of the control plants was 8.65 mg/g. The average concentration of the T3-plants was 2.51 mg/g (Table 9). In the T3-plant with the highest piceid content (616 µg/g) the total sinapate ester content was reduced to 2.40 mg/g. In comparison to the lowest control plant (7.43 mg/g), the reduction was 68%. In this T2-plant the sinapine content was reduced to 3.50 mg/g, which is a relative reduction of 52% in comparison to the lowest control plant with 7.37 mg/g. By analysing the PPT- and kanamycin-resistance of T4-seedlings ($n = 20$) from all T3-plants it was found that all 20 seedlings of each T3-plant were resistant to PPT and Kanamycin (data not shown), indicating the homozygosity of these T3-plants.

Discussion

The present study reveals that by creating the novel metabolic sink piceid the content of the undesirable sinapate esters is drastically reduced in transgenic seeds of oilseed rape as shown for segregating T2-plants (Fig. 6). This effect is obviously enhanced by the transgenic suppression of the endogenous BnSGT-gene (see Fig. 6). Since the content of sinapate or other SE (see 'other SE'

in Table 6) is also decreased in the transgenic lines, it appears that the common precursor 4-coumaroyl-CoA is used for enhanced production of piceid. Our results are supported by the observation of Orsini et al. (2003) who reported that the expression of the *VST1* gene alone (using plasmid pPSty5 as in our experiment) in cv. Drakkar has led to a maximum piceid content of 159 μg/g seeds among 23 transgenic lines analysed (T2-seeds). In the present experiments, a maximum piceid content of 321 μg/g in T2 seeds was found.

Among the transgenic rapeseed lines containing the *VST1* gene from *Vitis vinifera* and the *BnSGT1* suppression construct pLH-SGT-GUS one homozygous plant (T3-plant) with a single copy insertion was found to contain 616 μg piceid/g seeds. Resveratrol itself was not detected. When the *VST1* gene has been expressed in various plant species, an accumulation of resveratrol and/or piceid between 2 μg/g fresh weight leaves and 290 μg/g fresh weight leaves has been observed (Langcake and Pryce 1976; Fischer et al. 1997; Fettig and Hess 1998; Hipskind and Paiva 2000; Kobayashi et al. 2000).

Co-transformation and copy number

Komari et al. (1996) reported that tobacco and rice plants co-transformed with two separate T-DNAs either by single bacterial strain (containing two T-DNAs on separate plasmids) or by dual bacterial strain (each strain containing one T-DNA) infection contained one locus for each T-DNA. Linked loci were not favoured in either the dual method or the single strain method by using an octopine-type *Agrobacterium* for co-transformation. De Block and De Brouwer (1991) evaluated linkage of transgenes in *Brassica napus* transformants produced by the dual-infection method. They observed that linked loci were favoured. They used a derivative of a nopaline-type strain C58, and suggested that the use of nopaline-type strains in the dual method might favour linked co-transformation. Therefore, the use of C58C1 (nopaline-type) and AGL1 (succinamopine-type) in this experiment may be an important factor contributing to unlinked loci, i.e. progeny with a single copy integration for both T-DNAs segregating independently for both transgenes. The co-transformation efficiency in the present study was 1.8%, which is only half of that compared to single strain transformation experiments using the same SGT-plasmid in the same *Agrobacterium* strain and following the same transformation protocol as described in Hüsken (2004). Komari et al. (1996) reported that the frequency of co-transformation was relatively low using the dual method compared to the single-strain method.

There are conflicting reports about the relationship between transgene copy number and expression level. They have been shown to be negatively correlated, not correlated or positively correlated (Bauer et al. 1998; Hobbs et al. 1993; McCabe et al. 1999). Hipskind and Paiva (2000) reported that many of the higher piceid-accumulating transgenic alfalfa T1-plants contained only one copy of the *VST1* gene, as determined by Southern blot, while the lower accumulating lines contained mostly two or three copies. Reduced expression levels at higher copy numbers may be due to post-transcriptional-gene-silencing (Pickford and Cogoni 2003) or transcriptional-gene-silencing (Jakowitsch et al. 1999). This is in agreement with the present study, that the three selected T1-plants with the highest piceid content contained a single copy for the *VST1* gene.

Implications for seed quality improvement in rapeseed

It was shown, that it is possible to achieve a high amount of piceid (616 μg/g) and a drastical reduction of sinapate esters (2.40 mg/g) in seeds by co-transformation of rapeseed with *VST1* and silencing of *BnSGT1* using the seed-specific napin promotor. There was no indication that other important agronomic traits, like oil, protein, fatty acid and glucosinolate content of the seeds are affected by expressing the stilbene synthase gene and lowering the sinapate ester content. In tobacco and petunia, overexpression of stilbene synthase genes under control of a CaMV 35S promoter led to a substrate competition between stilbene and chalcon synthases causing male sterility (Fischer et al. 1997). In our experiment, there was no evidence for male sterility due to an overexpression of the stilbene synthase gene, which can be explained by the use of a seed specific napin promoter. Zum Felde (personal communication) studied the correlation of sinapate esters content with other seed quality traits in segregating doubled haploid winter rapeseed populations. He found no significant correlation between sinapate ester and oil, protein, fatty acid and glucosinolate content, respectively. Therefore, the high expression of piceid and the drastical suppression of sinapate esters in *B. napus* should not have any negative impact on other important seed quality traits.

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