In planta production of the highly potent resveratrol analogue pterostilbene via stilbene synthase and O-methyltransferase co-expression

Agnes M. Rimando1,†, Zhiqiang Pan1,‡, James J. Polashock2, Franck E. Dayan1, Cassia S. Mizuno1, Maurice E. Snook3, Chang-Jun Liu4 and Scott R. Baerson1,*

1United States Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit, University, MS, USA
2United States Department of Agriculture, Agricultural Research Service, Genetic Improvement of Fruits and Vegetables Laboratory, Chatsworth, NJ, USA
3United States Department of Agriculture, Agricultural Research Service, Genetic Improvement of Fruits and Vegetables Laboratory, Athens, GA, USA
4Biology Department, Brookhaven National Laboratory, Upton, NY, USA

Received 26 May 2011; revised 31 July 2011; accepted 10 August 2011.
*Correspondence (fax +662 915 1035; email scott.baerson@ars.usda.gov)
†Both authors contributed equally to this work.

Summary

Resveratrol and related stilbenes are thought to play important roles in defence responses in several plant species and have also generated considerable interest as nutraceuticals owing to their diverse health-promoting properties. Pterostilbene, a 3,5-dimethylether derivative of resveratrol, possesses properties similar to its parent compound and, additionally, exhibits significantly higher fungicidal activity in vitro and superior pharmacokinetic properties in vivo. Recombinant enzyme studies carried out using a previously characterized O-methyltransferase sequence from Sorghum bicolor (SbOMT3) demonstrated its ability to catalyse the A ring-specific 3,5-bis-O-methylation of resveratrol, yielding pterostilbene. A binary vector was constructed for the constitutive co-expression of SbOMT3 with a stilbene synthase sequence from peanut (AhSTS3) and used for the generation of stably transformed tobacco and Arabidopsis plants, resulting in the accumulation of pterostilbene in both species. A reduced floral pigmentation phenotype observed in multiple tobacco transformants was further investigated by reversed-phase HPLC analysis, revealing substantial decreases in both dihydroquercetin-derived flavonoids and phenylpropanoid-conjugated polyamines in pterostilbene-producing SbOMT3/AhSTS3 events. These results demonstrate the potential utility of this strategy for the generation of pterostilbene-producing crops and also underscore the need for the development of additional approaches for minimizing concomitant reductions in key phenylpropanoid-derived metabolites.

Keywords: stilbene, nutraceutical, antimicrobial, metabolic engineering, O-methyltransferase.

Introduction

Stilbenes comprise a relatively small family of polyphenolic secondary metabolites that share a common 1,2-diphenylethylene nucleus. Both monomeric and oligomeric stilbene structures have been identified from 33 unrelated plant families (Shen et al., 2009). The important protective roles played by plant stilbenes as antifungal phytoalexins and phytoanticipins and in mitigating UV irradiation-induced stress have been extensively studied, and additional roles as antifeedants and allelochemicals have also been proposed for these compounds (Chong et al., 2009; Jeandet et al., 2010). The biosynthetic pathway for stilbenes diverges from the general phenylpropanoid pathway through the action of stilbene synthases (STS), a family of homodimeric type III polyketide synthases predominately utilizing p-coumaroyl-CoA and cinnamoyl-CoA starter units for the generation of the parent stilbene resveratrol and pinosylvin, respectively (Shen et al., 2009; Jeandet et al., 2010).

Significant interest has also been generated in this family of compounds as nutraceuticals, particularly in the case of the monomeric trihydroxystilbene resveratrol (3,5,4’-trihydroxy-trans-stilbene), which was initially suggested to represent a key ingredient underlying the cardioprotective effects attributed to red wine (Baur and Sinclair, 2006). Subsequent studies have indicated that resveratrol possesses additional health-promoting activities, including anticancer, antioxidant, anti-inflammatory and neuroprotective properties (Pervaiz and Holme, 2009; Fulda, 2010). Coupled with resveratrol’s well-established properties as a potent antifungal agent, the significant nutraceutical potential of this compound has prompted a flurry of efforts to engineer its production in various plant species (Delanoues et al., 2009). Pharmacokinetic studies have, however, raised some concerns regarding the efficacy of orally administered resveratrol owing to its relatively poor bioavailability (e.g. Boocock et al., 2007). Consequently, methoxylated resveratrol analogues, which can possess greater oral bioavailability because of decreased phase II metabolism and increased absorption, have been attracting increasing attention as alternative chemopreventive agents (Rimando and Suh, 2008; Lin et al., 2009; Fulda, 2010; Kapetanovic et al., 2010).

One particularly promising dimethoxylated derivative of resveratrol, pterostilbene (3,5-dimethoxy-4’-hydroxy-trans-stilbene), is found in red sandalwood (Pterocarpus santalinus), Indian Kino (Pterocarpus marsupium), Vaccinium spp. berries, and at low levels in grape (Vitis vinifera) leaves and berries (Seshadri, 1972; Langcake et al., 1979; Maurya et al., 1984; Adrian et al., 2000;
Rimando et al., 2004). A significant body of evidence suggests that pterostilbene possesses similar anticancer, antioxidant, anti-inflammatory and neuroprotective activities as indicated for its parent compound resveratrol (Manickam et al., 1997; Sivula et al., 2001; Rimando et al., 2002, 2005; Satheesh and Pari, 2006; Suh et al., 2007; Joseph et al., 2008). Additionally, pterostilbene exhibits superior pharmacokinetic properties, including a three- to fourfold greater oral bioavailability as determined by equimolar administrations performed in parallel studies using animal models (Remsberg et al., 2008; Lin et al., 2009; Kapetanovic et al., 2010). Experiments performed in vitro also indicate significantly higher antifungal activity for pterostilbene compared with resveratrol against agriculturally significant fungal pathogens such as Botrytis cinerea and Plasmopara viticola (e.g. Adrian et al., 1997; Alessandro et al., 2000; Pezet et al., 2004); thus, substantial benefits could potentially be derived for both growers and consumers of pterostilbene-producing crop plants.

In this study, we demonstrate that a previously characterized O-methyltransferase from Sorghum bicolor (SbOMT3) is capable of utilizing resveratrol as a substrate, yielding pterostilbene as the major product via A ring-specific 3,5-bis-O-methylation. Taking advantage of this activity, a strategy for engineering the in planta production of pterostilbene was tested, involving co-expression of SbOMT3 with a stilbene synthase sequence (AhSTS3) within the same T-DNA, resulting in the successful generation of pterostilbene-producing tobacco and Arabidopsis transformants. Phenotypic analyses revealed a decrease in floral pigmentation in multiple, independent pterostilbene-producing transformants, indicating a significant depletion of hydroxycin- flavonols and coumaryl- and caffeoyl-conjugated polyamines in transformants. Phenotypic analyses also revealed a decrease in floral pigmentation in multiple, independent pterostilbene-producing tobacco events, which was further investigated by HPLC profiling of the major phenolic compounds in wild-type and tobacco events, which was further investigated by HPLC profiling of the major phenolic compounds in wild-type and SbOMT3/AhSTS3-expressing floral tissues. Substantial decreases were found in the levels of total anthocyanins, 3′-hydroxylated flavonols and coumaryl- and caffeoyl-conjugated polyamines in transformants, indicating a significant depletion of hydroxycinnamoyl-CoA precursors. This work demonstrates an efficient approach for generating stably transformed plants accumulating a natural product of considerable nutraceutical value and provides additional insights concerning the potential metabolic consequences of in planta pterostilbene production.

Results and discussion

SbOMT3 catalyzes A ring-specific 3,5-bis-O-methylation of resveratrol

Previous studies carried out with S. bicolor have identified O-methyltransferase SbOMT3 as one of the enzymes likely participating in the biosynthesis of the allelopathic benzoquinone sorgoleone (Baerson et al., 2008a). Similarities between the 3,5-dihydroxy-1-hydroxyphenylethylene A ring substitution pattern of the widely studied stilbene resveratrol and the alkylresorcinol-ic substrates previously shown to be accepted by SbOMT3 would suggest the potential for resveratrol to also serve as a substrate for this enzyme. A ring-specific 3,5-bis-O-methylation of resveratrol would yield the dimethylether derivative pterostilbene, which (as discussed earlier) possesses significant potential as both a nutraceutical as well as a potent antimicrobial agent. Recombinant enzyme studies with SbOMT3, as well as two additional OMT enzymes previously isolated from S. bicolor (SbOMT1 and SbOMT2; Baerson et al., 2008a), were first carried out using resveratrol as substrate. In addition, the phenylpropanoid eugenol as well as the alkylresorcinol olivetol was tested, as these compounds had previously been determined to be the preferred substrates for SbOMT1 and SbOMT3, respectively. For these analyses, full-length open reading frames for all three OMTs were expressed in Escherichia coli as N-terminal polyhistidine fusions and radiometrically assayed in the presence of eugenol, resveratrol or olivetol (Figure 1). Surprisingly, significant activity was observed for both SbOMT1 and SbOMT3 with resveratrol as substrate, and for SbOMT1, activity levels were approximately threefold higher than levels where eugenol was provided as substrate. SbOMT1 had been previously identified as a possible eugenol OMT (EOMT), functionally related to an EOMT isolated from sweet basil and the (iso)EOMT isolated from Clarkia breweri (Wang et al., 1997; Gang et al., 2002). Given the present results, SbOMT1 could be more accurately considered as a potential stilbene OMT, functionally analogous to enzymes such as the pinoresinol-utilizing OMT (OsPOMT) identified from rice (Katsuyama et al., 2007). In contrast to SbOMT1, SbOMT3 was active with all three substrates, and the preferred substrate among those tested was clearly olivetol, where observed activities were approximately fivefold higher than those obtained with resveratrol. Previous studies using a panel of phenolic derivatives as substrates, including alkylresorcinols of varying side-chain lengths, demonstrated a clear preference of SbOMT3 for alkylresorcinolic-type substrates (Baerson et al., 2008a). This observed substrate preference for SbOMT3 is consistent with its proposed role within the sorgoleone biosynthetic pathway, which requires the O-methylation of

![Figure 1](image-url)
a 5-pentadecatrienyl resorcinol pathway intermediate produced via the action of an alkylresorcinol synthase (Cook et al., 2010). Recombinant SbOMT2, in contrast to SbOMT1 and SbOMT3, indicated no activity against any of the three substrates used in these experiments (not shown). Evaluation of steady-state kinetic parameters for reactions of SbOMT1 and SbOMT3 with resveratrol (Table 1) revealed that the $K_m$ of SbOMT1 for this substrate was approximately fourfold lower than that of SbOMT3, which appeared to account in large part for the higher catalytic efficiency ($k_{cat}/K_m$) observed for SbOMT1. Nevertheless, the catalytic efficiency observed for SbOMT3 with resveratrol was similar to that previously reported for its reaction with the preferred substrate olivetol (1.4 vs 3.1 $\text{w/s}$; Baerson et al., 2008a).

To examine the potential regioselectivity of SbOMT1 and SbOMT3 O-methylation of resveratrol, the reaction products from both enzymes were further analysed by gas chromatography/mass spectroscopy. Pronounced ring-specific O-methylation was observed for both enzymes, with SbOMT1 predominantly catalysing (B ring) 4′-O-methylation, yielding approximately 88% 3,5-dihydroxy-4′-methoxy-trans-stilbene, with the remaining three resorcinol methylether derivatives present in minor quantities (Figure 2). Importantly, a high degree of A ring-specific $\text{O}^\text{6}$-methylation was observed for SbOMT3, with pterostilbene constituting approximately 96% of the product obtained from resveratrol, and 3,5-dihydroxy-4′-methoxy-trans-stilbene also produced in small amounts (approximately 4%). Collectively, the results obtained from these recombinant enzyme studies (Figures 1 and 2) suggested that SbOMT3 could have utility for the metabolic engineering of pterostilbene production in transgenic organisms where resveratrol is available in adequate supply to serve as a substrate.

To examine the potential structural basis for the observed interaction of SbOMT3 with resveratrol, computational homology modelling and automated substrate dockings were also performed (Figure 3). Several previously determined plant type I OMT crystal structures (Zubieta et al., 2001, 2002; Liu et al., 2006a) provide a useful basis for an in silico homology-based structure–function analysis of OMT homologues. Among the crystallized proteins, the alfalfa (Medicago sativa) isoflavone 7-O-methyltransferase showed the highest relationship score with SbOMT3 (37% identity), and therefore, its crystal structure (PDB code: IFP2) was used to model SbOMT3. The SbOMT3 substrate docking model with resveratrol exhibited the best fit with the hydroxyl groups at the three and five positions of the A ring facing towards the methyl donor $\text{S}$-adenosyl-$\text{L}$-methionine (AdoMet) (Figure 3a). This binding pose is consistent with the observed regioselectivity of recombinant SbOMT3, preferentially O-methylating the 3- and 5-hydroxyl groups of the A ring of resveratrol (Figure 2). The 3- and 5-hydroxyl groups also

![Figure 3](image)

**Figure 3** Molecular modelling of the SbOMT3 active site docked with resveratrol. (a) Close-up molecular surface view of the SbOMT3 active site (coloured by atom type) in complex with resveratrol. The methyl donor AdoMet is coloured red. (b) Close-up view of the proposed binding mode of resveratrol within the SbOMT3 model. Resveratrol is coloured by atom type, methyl donor AdoMet is coloured red, and surrounding residues are shown in green. Numbering for amino acid residues shown were based on the deduced amino acid sequence of SbOMT3 (GenBank accession no. ABP01564).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/mg/h)</th>
<th>$k_{cat}$ (1/s)</th>
<th>$k_{cat}/K_m$ (w/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SbOMT1</td>
<td>16.9 ± 1.2</td>
<td>8.4 ± 0.1</td>
<td>1.0 x 10⁻⁴</td>
<td>5.9</td>
</tr>
<tr>
<td>SbOMT3</td>
<td>70.9 ± 9.2</td>
<td>8.3 ± 0.7</td>
<td>1.0 x 10⁻⁴</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**Table 1** Kinetic properties of SbOMT1 and SbOMT3

![Figure 2](image)

**Figure 2** Analysis of recombinant SbOMT1 and SbOMT3 reaction products. Reaction products generated by recombinant SbOMT1 and SbOMT3 in assays using resveratrol as substrate were further characterized by GC-MS analysis to examine the distribution of mono-, di-, and trimethylether derivatives. The structures of the identified methylether derivatives produced by both enzymes are shown, and beneath, the corresponding percentages of total moles product formed by each enzyme are indicated.
approximate H279, consistent with the role of histidine as a general base in the deprotonation of hydroxyl groups, as seen in other type I OMTs (Zubieta et al., 2001, 2002; Liu et al., 2006a). In addition, the docking studies revealed that binding of resveratrol within the potential active site of SbOMT3 occurred predominantly through hydrophobic interactions with surrounding non-polar amino acids such as M187, M126, F25 (B) and M26 (B) within the predicted SbOMT3 dimer (Figure 3b), and the B ring of resveratrol likely undergoes π-stacking interactions with Y23 (B). Overall, the modelling studies predicted an active site cavity for SbOMT3 appearing well suited to accommodate the conformation of the non-physiological substrate resveratrol.

Engineering pterostilbene biosynthesis in tobacco and Arabidopsis

Based on the in vitro assays that demonstrated the ability of SbOMT3 to generate pterostilbene from resveratrol, a 2-gene strategy was devised for the production of pterostilbene in transgenic plants (Figure 4). The first transgene encoding a stilbene synthase (STS)-type polyketide synthase (PKS) enzyme would be capable of producing resveratrol in planta from the ubiquitously available precursors malonyl-CoA and coumaryl-CoA, which would then serve as substrate for SbOMT3, yielding pterostilbene. Stilbene synthase 3 (AhSTS3; GenBank accession no. P51069) from the peanut plant (Arachis hypogaea) represents one such type III PKS enzyme and was the first STS isolated from any plant species (Schröder et al., 1988). The occurrence of resveratrol in nature is limited to specific plant groups such as Vaccinium (e.g. blueberries and cranberries), Vitis (grapes) and Eucalyptus spp. (reviewed in Rimando et al., 2004); thus, for most plant species, the introduction of an STS in conjunction with SbOMT3 would be requisite for engineering in planta pterostilbene production.

To test this strategy, a binary vector was developed to simultaneously express both the peanut STS3 and SbOMT3 (Figure 5). For this approach, the complete open reading frame of AhSTS3 was positioned downstream of the CaMV 35S promoter and directly upstream of the polyadenylation region of the Agrobacterium tumefaciens nopaline synthase (NOS) gene. Similarly, the complete open reading frame for SbOMT3 was cloned downstream of the CaMV 35S promoter and directly upstream of the polyadenylation region of the Agrobacterium tumefaciens octapine synthase gene (OCS). Both transgene cassettes were cloned within the T-DNA borders of the binary vector pCB404 (Weyman et al., 2006).

Eight of the 15 tobacco events analysed were found to express both the 35S:STS and 35S:SbOMT3 transcripts (Figure 6a). As is typically seen in transformation experiments, significant variation was observed in steady-state transcript accumulation levels among events, and the levels of the 35S:AhSTS3 and 35S:SbOMT3 transcripts varied independently.

![Figure 4](image.png)

**Figure 4** Proposed in planta pterostilbene biosynthetic pathway created by co-expression of an STS with SbOMT3.

![Figure 5](image.png)

**Figure 5** pCRO1 binary vector developed for AhSTS3, SbOMT3 co-expression. The complete open reading frame of AhSTS3 was positioned downstream of the CaMV 35S promoter and directly upstream of the polyadenylation region of the Agrobacterium tumefaciens nopaline synthase (NOS) gene. Similarly, the complete open reading frame for SbOMT3 was cloned downstream of the CaMV 35S promoter and directly upstream of the polyadenylation region of the Agrobacterium tumefaciens octapine synthase gene (OCS). Both transgene cassettes were cloned within the T-DNA borders of the binary vector pCB404 (Weyman et al., 2006).
For example, the highest levels of 35S:SbOMT3 transcripts were observed in event T21; however, the levels of 35S:AhSTS3 transcripts in T21 were lower than the levels observed in all other events where 35S:AhSTS3 was detected. Importantly, significant accumulation of trans-pterostilbene was detected in events T6, T9, T19, T20 and T21, at levels ranging...
approximately from 22 to 52 μg/g fresh weight (Figure 6b). The levels of pterostilbene found in planta were approximately 8–12-fold higher than the corresponding levels of resveratrol observed (Figure 6b), with the exception of event T9 where accumulation of the two compounds occurred in roughly equimolar amounts. The latter observation could potentially be accounted for by the relatively low 35S:SBOMT3 transcript accumulation levels seen in event T9 (Figure 6a), which may have resulted in insufficient resveratrol O-methyltransferase activity. However, no clear-cut relationship was evident overall between 35S:AHSTS3/35S:SBOMT3 transcript levels and pterostilbene production, given that several of the transformants (T2, T15 and T16) expressed both transgenes at relatively high levels, yet failed to accumulate detectable levels of pterostilbene. Pterostilbene and resveratrol were not detected in wild-type plants (non-transformed cv. Wi-38). DNA gel blot analyses indicated the presence of approximately 1–4 integrated T-DNA copies per pterostilbene-producing event (Figure 6c), and no obvious relationship between T-DNA copy number and transgene expression or pterostilbene levels was discernible overall (Figure 6a–c).

In contrast to the results obtained with the tobacco transformants, real-time RT-PCR analyses indicated that both transgenes were expressed in all 13 pCRO1-transformed Arabidopsis events analysed (Figure 7a). The levels of 35S:AhSTS3 and 35S:SBOMT3 transcripts varied by approximately one order of magnitude among these events and exhibited less overall variation than was seen in tobacco (Figure 6a). As was the case for pCRO1-transformed tobacco, a significant proportion of the 35S:AhSTS3/35S:SBOMT3 expressers failed to produce pterostilbene at detectable levels, despite the fact that transgene transcript levels were in some cases comparable with those observed in certain pterostilbene-accumulating events (e.g. event A15 vs. pterostilbene-accumulating event A12—Figure 7a,b). The levels of pterostilbene detected within the 8 pterostilbene-positive events ranged from approximately 8–16 μg/g fresh weight, which was somewhat lower than the levels observed in tobacco (Figure 6b). Pterostilbene levels were also approximately five- to sevenfold higher than the corresponding levels of resveratrol detected within these events, suggesting somewhat less efficient turnover of resveratrol by SbOMT3 in Arabidopsis in comparison with tobacco (Figures 6b and 7b). Neither pterostilbene nor resveratrol were detected in the non-transformed controls (ecotype Col-0). DNA gel blot analyses indicated the presence of approximately 1–3 T-DNA copies per pterostilbene-producing Arabidopsis event (Figure 7c), and as was also observed for tobacco, these copy number values were not predictive for either transgene expression levels or pterostilbene content within a given event (Figure 7a–c).

The significantly higher variation in 35S:AhSTS3 and 35S:SBOMT3 transcript levels observed among tobacco pCRO1 transformants, as well as the occurrence of apparent silencing of both transgenes in 7 of the 15 tobacco events analysed (and lack of detectable product formation in these events—Figure 6a,b), could both be explained, at least in part, by differences in the growth conditions employed for these experiments. For the entire duration of the experiment, Arabidopsis plants were maintained in growth chambers under controlled temperature and lighting conditions, whereas the tobacco transformants were initially maintained in growth chambers and then transferred to a greenhouse prior to sampling (see ‘Experimental procedures’). Importantly, the activity of the CaMV 35S promoter used to drive expression of both transgenes in pCRO1 (Figure 5) has been shown to be subject to environmental influences such as light intensity, temperature and photoperiod length (e.g. Aronen et al., 1995; Schnurr and Guerra, 2000; Down et al., 2001; Boyko et al., 2010); thus, it would be anticipated that introduction of tobacco pCRO1 transformants into a greenhouse environment, where significant fluctuations in both temperature and light conditions frequently occur, would give rise to increased plant–plant variability in 35S:AhSTS3 and 35S:SBOMT3 steady-state transcript levels. Moreover, the frequency of transgene silencing in plants has been shown in specific cases to increase in response to stress associated with conditions of elevated temperature or light intensity (e.g. Hart et al., 1992; Meyer et al., 1992; Neumann et al., 1997; Meza et al., 2001; Kotakis et al., 2010); therefore, the transfer of the tobacco transformants to a greenhouse, which involves adaption to higher light intensities as well as transient heat stress exposures, likely played a role in the occurrence of the seven tobacco events (events T3, T4, T8, T10, T11, T12 and T13—Figure 6a,b) where neither transgene-derived transcripts nor stilbenoid products accumulated to detectable levels.

Phenotypic analysis of pCRO1 transformants

Significantly, constitutive co-expression of SbOMT3 and AhSTS3 leading to pterostilbene production in both tobacco and Arabidopsis was not associated with major phenotypic effects on either vegetative or reproductive development (Figure 8a–d). One effect that was observed, however, within multiple, independent pCRO1 tobacco transformants was a clearly discernible reduction in floral pigmentation (Figure 9a). This reduction in pigment content was not entirely unexpected, given that AhSTS3 expressed from the strong CaMV 35S promoter in pCRO1 transformants would likely compete with endogenous chalcone synthase (CHS) for available p-coumaroyl-CoA (Austin and Noel, 2003; Delaunois et al., 2009). The closely related CHS and STS type III PKS enzymes catalyse the formation of identical tetraketide intermediates using p-coumaryl-CoA as the starter substrate, yet form different products owing to cyclization occurring via a C6/C1 Claisen condensation for CHS and a C2/C7 aldol condensation for STS (Tropf et al., 1994). CHS produces 2’,4’,4”,6’-tetrahydroxychalcone, which serves as a biosynthetic precursor to flavonoids as well as anthocyanin pigments responsible for many of the colours associated with flowers, fruits, and other plant structures (Winkel-Shirley, 2001). Additionally, as p-coumaryl-CoA is produced by the general phenylpropanoid pathway, a significant diversion of this metabolite towards pterostilbene production by AhSTS3 could potentially impact other pathways reliant on hydroxycinnamyl-derived precursors (Ferrer et al., 2008).

To gain additional insight into the effects of AhSTS3/SbOMT3 overexpression on metabolism, the levels of major phenolic compounds associated with flowers of Nicotiana spp. (Snook et al., 1988, 1992; Nugroho and Verpoorte, 2002) were profiled by reversed-phase HPLC using extracts prepared from mature petals of wild-type (cv. Wi-38) and representative pCRO1 transformant flowers (Figure 9b–d). The analysis of anthocyanins (monitored at 525 nm) revealed a single major peak representing >90% of total anthocyanins in petals of both control and pCRO1 transformant flowers (data not shown). Consistent with the observed differences in colour intensity (Figure 9a), the total anthocyanin content determined for pCRO1 transformants was approximately one-half of that found within wild-type plants (Figure 9b).
A comparison of the levels of other major phenolics identified in flower petals, which include caffeoylquinic acid isomers, flavonol glycosides and phenylpropanoid polyamine conjugates (Figure 9c,d), revealed either somewhat more modest changes or no difference between the two groups; however, some significant trends were observed. For example, the flavonoids present in corollas of tobacco flowers occur as paired glycosides of the flavonols quercetin and kaempferol, differing structurally by the presence of an additional 3’ hydroxyl group in the quer-
cetin aglycone (Snook et al., 1992; Winkel-Shirley, 2001). Interestingly, the pCRO1 transformants exhibited reduced levels of the quercetin derivatives quercetin-3-rutinoside (rutin) and quercetin-O-glucosyl-rutinoside, which were approximately 30% and 37% lower than levels observed in non-transformed controls, respectively. In contrast, nearly identical levels of the corresponding kaempferol derivatives were found in the two groups, suggesting the occurrence of a specific limitation in flavonoid 3’ hydroxylase (F3’H) activity in pCRO1 transformants.

Figure 7 Analysis of Arabidopsis pCRO1 transformants. (a) Relative 35S:SbOMT3 and 35S:AhSTS3 transcript levels were determined by quantitative real-time RT-PCR in leaf samples collected from 15 independent Arabidopsis transgenic events using gene-specific primers. Data were normalized to an internal control (18S rRNA), and the ΔΔCT method was used to obtain the relative expression level for each transcript, expressed as mean ± SD from assays performed in triplicate. Samples from wild-type plants (non-transformed ecotype Col-0) were included as controls. (b) Pterostilbene and resveratrol levels were determined by GC-MS analysis of extracts prepared from aliquots of all tissue samples used for real-time RT-PCR analysis (panel a). Data are expressed as mean ± SD from four measurements. (c) Genomic DNAs isolated from the eight identified pterostilbene-accumulating events (A1, A3, A4, A5, A9, A10, A12, A13) and non-transformed controls were digested with either BamHI or SphI and then probed using radiolabelled SbOMT3 coding sequences. B, BamHI; S, SphI.
This likely accounts for the reduction in total anthocyanins observed as well (Figure 9b), given that biosynthesis of cyanidin-3-rutinoside, the predominant anthocyanin produced by tobacco flowers (Aharoni et al., 2001), involves the precursor dihydroquercetin, which is also the immediate biosynthetic precursor to quercetin. In the majority of plant species, dihydroquercetin is produced via F3"H activity using dihydrokaempferol as substrate, and this enzyme is also thought to play a key role in determining cyanidin-3-rutinoside levels in tobacco (Nakatsuka et al., 2006, 2007).

An additional effect of AhSTS3/SbOMT3 overexpression observed was a reduction in the levels of all four of the major phenylpropanoid polyamine conjugates present in flowers (Figure 9c,d). For the caffeoyl-conjugated polyamines detected (monocaffeoyl-putrescine, caffeoyl-dihydrocaffeoyl-spermidine and dicafeoyl-spermidine), the levels of pCRO1 transformants were reduced by approximately 28%–34% compared with those of the non-transformed controls and tri-p-coumaryl-spermidine levels were reduced by approximately 23%. A reduction in the levels of these compounds in AhSTS3/SbOMT3 overexpressing tobacco could also be anticipated, given that their associated hydroxycinnamoyl moieties are derived from p-coumaryl- and caffeoyl-CoA, both of which could potentially become limiting owing to a significant diversion of p-coumaryl-CoA towards pterostilbene production (Ferrer et al., 2008; Bassard et al., 2010). Phenylpropanoid-conjugated polyamines belong to a ubiquitous plant-specific family of phenolic acid-conjugated polyamines and arylmonoamines collectively referred to as ‘phenylamides’, which typically accumulate to high levels in the reproductive organs of angiosperms (Edreva et al., 2007; Bassard et al., 2010). Importantly, current evidence points towards a significant role in specific developmental transitions, defence responses and abiotic stress responses for these compounds; therefore, a reduction in the plant’s capacity to synthesize phenylamides could potentially impact a number of critical processes. Furthermore, flavonoids and anthocyanins represent economically important classes of compounds because of (i) their association with quality characteristics such as the fragrance and colour of fruits, vegetables and flowers, (ii) their potential nutraceutical value as anticarcinogenic, anti-inflammatory and cardiovascular health-promoting dietary agents, (iii) their function in plants as attractants for pollinators, UV protectants and several other critical biological roles (Winkel-Shirley, 2001; He and Giusti, 2010; de Pascual-Teresa et al., 2010). A concomitant reduction in the levels of flavonoids and anthocyanins, as observed in the present work (Figure 9a–d), could therefore also pose a challenge for generating viable stilbenoid-producing transgenic crops. Supplemental strategies, such as increasing phenylpropanoid precursor levels or down-regulating competing, non-essential pathways could likely be employed to circumvent these limitations (e.g. Howles et al., 1996; Nakatsuka et al., 2007).

This study extends upon previous efforts aimed towards engineering the in planta production of pterostilbene (Baerson et al., 2008b; Schmidlin et al., 2008), an important nutraceutical/antifungal compound with potential benefits to both human health and agriculture (Rimando et al., 2004; Rimando and Suh, 2008; Fulda, 2010). The model species used in this work represent two agronomically important taxonomic families, Brassicaceae and Solanaceae, which include major crop...
species members that could be similarly amenable to the present approach. Moreover, the fact that resveratrol production has been successfully engineered in diverse plant species (Delaunois et al., 2009) suggests that methods for directing in planta pterostilbene production could be broadly applicable as well.

The pterostilbene accumulation levels obtained for pCRO1 transformants of both plant species could likely be significantly increased through the use of alternative gene promoters and other genetic elements incorporated within enhanced transgene expression cassettes. Other STS or O-methyltransferase enzymes could also potentially be identified possessing more favourable steady-state kinetics or that are encoded by sequences more efficiently/stably expressed within a given target species. Alternative STS-type PKS enzyme sequences have been isolated from Vitis vinifera (grapevine), Vitis pseudoreticulata (Chinese wild grape), Pinus sylvestris (Scots pine), Parthenocissus henyana (silvervine creeper) and S. bicolor, which have been used successfully for directing resveratrol production in various stilbenoid- and non-stilbenoid-producing plant species (Melchior and Kindl, 1991; Fliegmann et al., 1992; Wiese et al., 1994; Serazetdinova et al., 2005; Yu et al., 2005, 2006; Liu et al., 2006b; Wang et al., 2007; Fan et al., 2008). Moreover, an alternative resveratrol-utilizing OMT (ROMT) was previously identified from V. vinifera, which was used successfully for generating pterostilbene when tested via Agrobacterium-mediated transient expression in N. benthamiana leaves (Schmidlin et al., 2008).

A systematic comparison of various STS/OMT combinations in, for example, stably transformed Arabidopsis plants would undoubtedly provide information useful for guiding further efforts towards engineering high-level pterostilbene production in crop species.
Experimental procedures

Heterologous expression and purification of recombinant OMTs

DNA manipulations and *E. coli* transformation protocols used for recombinant OMT experiments were performed according to standard procedures (Sambrook et al., 1989), and *E. coli* overexpression vectors containing the open reading frames (ORFs) for *SbOMT1*, *SbOMT2* and *SbOMT3* have been previously described (Baerson et al., 2008a). For recombinant protein production, *E. coli* cultures were grown at 37 °C to an optical density of 0.6 at 600 nm, induced with 0.5 mM IPTG, and then allowed to grow for an additional 5 h at 25 °C. Cells were harvested by centrifugation at 3000 × g for 20 min at 4 °C, washed with cold 0.9% NaCl, and then collected by re-centrifugation at 3000 × g. Pellets were reuspended in cold lysis buffer (50 mM Tris–HCl, pH 7.5, 1 mM NaCl, 5 mM imidazole, 10% glycerol, 1 μg/mL leupeptin) and extracted using a French press (Thermo IEC, Needham Heights, MA) at a pressure of 10 000 kPa. Benzonase (25 U/mL) and 1 mM PMSF were added immediately to the lysate. After 15-min incubation at room temperature, lysates were centrifuged at 15 000 × g for 20 min, and the supernatant was loaded onto His GraviTrap columns (Amersham Biosciences, Piscataway, NJ) activated with 2 mL of 50 mM Tris–HCl, pH 8.0, 500 mM NaCl and 5 mM imidazole). The column was washed with 4 mL buffer A following each 2 mL of supernatant added. Once sample loadings were complete, the columns were washed with 8 mL of buffer A, followed by 8 mL of buffer B (20 mM Tris–HCl, pH 8.0, 500 mM NaCl and 100 mM imidazole) to remove nonspecifically bound proteins. Recombinant proteins were then eluted with 2.5 mL of elution buffer (20 mM Tris–HCl, pH 8.0, 500 mM NaCl and 250 mM imidazole). Columns were washed with 10 mL of wash buffer C (20 mM Tris–HCl, pH 8.0, 500 mM NaCl and 1 M imidazole), followed by 10 mL of distilled water after each use, to remove contaminating proteins. Recombinant protein-containing fractions (250 mM imidazole) were desalted on a PD-10 column equilibrated with cold desalting buffer (20 mM Tris–HCl, pH 7.5, 10 mM DTT, 10% glycerol). Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). All recombinant proteins were at least 95% pure, as estimated by SDS–PAGE (not shown). Enzyme preparations were stored at −80 °C prior to use.

OMT enzymatic assays

Substrate specificities and kinetic parameters for recombinant OMTs were determined using a modified protocol based on Wang and Richersky (1999). All enzymatic assays consisted of 90 μL of assay buffer (250 mM Tris–HCl, pH 7.5, 10 mM DTT), 200 μL of purified enzyme preparation (200 μg protein/mL), 5 μL of a 10 mM substrate stock solution (dissolved in 100% ethanol) and 5 μL of 5 mM [14C]adenosyl-L-methionine (40–60 mCi/mL; ICN Biomedicals, Irvine, CA). Reactions were incubated for 30 min at 30 °C using a Thermomixer (Brinkman Instruments, Westbury, NY), and then quenched by addition of 25 μL of 6 N HCl. Radiolabelled products were subsequently extracted by the addition of 1 mL of hexane/ethyl acetate (1:1 v/v), and 300 μL of the (upper) organic phase was transferred to scintillation vials containing 5 mL of Ultima Gold scintillation fluid (Packard BioScience, Meriden, CT). Scintillation counts were carried out using a Tri-Carb 1600TR Liquid Scintillation Analyzer (Packard BioScience). Protein concentrations and time points used for activity measurements were controlled to ensure linearity of the assays. Kinetic parameters were determined from assays performed in triplicate as described previously, but with substrate concentrations ranging from 10 μM to 10 mM. Data from enzyme kinetics experiments were fit to the Michaelis–Menten equation using the SigmaPlot v. 9.01 enzyme kinetics module (Systat Software, Inc., Point Richmond, CA).

Regiospecific O-methylation of resveratrol by recombinant *SbOMT1* and *SbOMT3* was analysed using products formed during *in vitro* enzymatic assays performed as described earlier, except that 300 μL of the organic phase obtained from the final hexane/ethyl acetate extractions was instead dried to completion under a stream of nitrogen. The dried enzymatic products were then re-dissolved in a mixture of methanol/dichloromethane (1:1 v/v) and analysed by GC–MS on a Hewlett-Packard 5890 GC interfaced to a 5972 Mass Selective Detector (Agilent Technologies; Santa Clara, CA), using a J&W DB-5 capillary column (0.25 mm internal diameter, 0.25 μm film thickness, 30 m length; Agilent Technologies, Santa Clara, CA). The GC temperature programme was as follows: initial 180 °C, raised to 240 °C at a rate of 15 °C/min, then to 300 °C at the rate of 6 °C/min, and finally raised to 330 °C at the rate of 30 °C/min and held at this temperature for 2 min. The carrier gas was ultrahigh-pure helium at a flow rate of 1.0 mL/min. The inlet (splitless), GC interface, and ion chamber temperatures were 250, 250 and 250 °C, respectively. The sample injection volume was used 1.0 μL. Under these conditions, the following retention times were observed: 3’,4’,5-trimethoxystilbene (resveratrol trimethyl ether)—8.9 min, pterostilbene—9.3 min, 3,4-dihydroxy-5-methoxystilbene—9.9 min, 3,5-dihydroxy-4’-methoxystilbene—10.1 min, and resveratrol—10.6 min. Resveratrol and all methylether derivatives thereof were identified from their retention times and mass spectrum compared to standards, which were fully characterized by spectroscopic methods, including nuclear magnetic resonance spectroscopy.

Plant material and growth conditions

For real-time RT-PCR experiments and stilbene content determinations involving *Nicotiana tabacum* (Figure 6a,b), pcR01 transformant *T1* and wild-type (cv. Wi-38) seeds were first surface-sterilized by immersion in 70% ETOH for 1 min, flowed by treatment with 0.5x commercial bleach (3% sodium hypochlorite), 0.5% Triton X-100 for 10 min, and then finally rinsed four times in sterile distilled water. Sterilized seeds were then placed in Phytatray II culture vessels (Sigma-Aldrich, St. Louis, MO) containing germination medium (0.5 x Murashige and Skoog salts, 0.3% sucrose (w/v), adjusted to pH 5.7 with KOH), and the media used in vessels containing pcR01 transformant seeds was supplemented with 100 μg/mL kanamycin. Semi-solid media also contained 0.8% (w/v) agar. Culture vessels were maintained in growth chambers under a combination of cool-white fluorescent and incandescent lighting at an intensity of approximately 200 μmol/m2/s and a 16-h photoperiod. Seedlings (1–1.5 cm in height) were then transferred to soil-containing pots and maintained for an additional 2 weeks in growth chambers. Plants were then re-potted and transferred to a greenhouse with approximate day and night tempera-
tures of 30 and 23 °C, respectively, and natural lighting supplemented with artificial lighting to provide a total day length of 16 h and a minimum of 400 μmol/m²/s. Fully expanded leaves (30–35 cm in length) from 1-month-old plants were then harvested and immediately flash-frozen with liquid nitrogen and then stored at −80 °C prior to use. For phenotypic observations (Figure 8c,d), pCRO1 transformant T1 and wild-type tobacco seeds were surface-sterilized and sown in soil-containing pots, maintained in growth chambers for 2 weeks, and then transferred to a greenhouse as described earlier until primary inflorescences had completed development (approximately 9 weeks after initial planting). pCRO1 transformants were identified among the segregating T1 individuals by real-time RT-PCR analysis of flash-frozen leaf samples taken from each plant, using AhSTS3 gene-specific primers (described later).

For real-time RT-PCR experiments and stilbene content determinations involving Arabidopsis thaliana (Figure 7a,b), pCRO1 transformant T2 and wild-type (ecotype Col-0) seeds were surface-sterilized as described previously and then plated on Petri dishes containing semi-solid germination medium. Media used in plates containing pCRO1 transformant seeds also contained 50 μg/mL kanamycin. Petri dishes were then maintained in darkness at 4 °C for 72 h, transferred to growth chambers and then maintained at 21 °C under a combination of cool-white fluorescent and incandescent lighting at an intensity of approximately 150 μmol/m²/s and a 16-h photoperiod. Seedlings (2–3 leaf stages) were then transferred to soil-containing pots and returned to growth chambers for an additional 21 days. Fully expanded rosette leaves were then harvested and immediately flash-frozen with liquid nitrogen and then stored at −80 °C prior to use. For phenotypic evaluations (Figure 8a,b), pCRO1 transformant T2 and wild-type Arabidopsis seeds were surface-sterilized and sown in soil-containing pots, maintained in darkness at 4 °C for 72 h, and then transferred to growth chambers for a total of 5 weeks prior to evaluation, as described before. pCRO1 transformants among segregating T2 individuals were similarly identified by real-time RT-PCR analysis using AhSTS3-specific primers (described later).

**Binary vector construction**

To construct an AhSTS3 expression cassette, the GUS open reading frame (ORF) contained within the binary vector pBI121 (Jefferson et al., 1987) was replaced with the AhSTS3 ORF. Briefly, pBI121 was digested with SacI, treated with T4 DNA polymerase (New England Biolabs, Ipswich, MA) per manufacturer’s instructions and then digested with Smal. The vector backbone fragment (lacking GUS) was then gel-purified and re-circularized with T4 DNA ligase to generate the intermediate vector pBI/GUS- (not shown). The AhSTS ORF, containing flanking XbaI and BamHI restriction sites, was amplified via PCR using plasmid-based AhSTS3 sequences as template with the following primers: forward, 5'-CTCTAGAAGGAGGATAATAACAT GGAAGGGGGGAATTCCGAAG-3', and reverse, 5'-CGGATCTTTATATGGG-3'. The amplified fragment was then digested with XbaI and BamHI, gel-purified, and ligated to similarly prepared pCB404 (Weyman et al., 2006), yielding the intermediate vector p35S/OMT (not shown), containing the complete CaMV35S promoter::SbOMT3::OCS terminator expression cassette. The complete SbOMT3-containing cassette was then amplified using p35S::OMT as template with the following primers: forward, 5'-TGCGGCGCCCTCAGCGCGCGCATCGAGAATATTCCAT CCGC-3', and reverse, 5'-CGGCAGCGAGTATGAGAC. The PCR primers were designed to introduce 5'-flanking Ntot-BbvCI-AscI restriction sites and a 3'-flanking Ntot restriction site to the amplified SbOMT3 cassette. The amplified CaMV35S promoter::SbOMT3::OCS cassette was then digested with Ntot and ligated to Ntot-digested pCB404, resulting in the intermediate binary vector pCBOMT3 (not shown). The CaMV35S promoter::AhSTS3::NOS terminator expression cassette, containing flanking BbvCI and Ascl restriction sites, was then generated by PCR amplification using p35S/STS as template, with the following primers: forward, 5'-GCCTCAGCAGGTTCCAGAT TAGCTTTC-3', and reverse, 5'-CGCGGCGCGGATCTAGTAACA TAGATGACCGG-3'. The resulting PCR product was then digested with BbvCI and Ascl, gel-purified and ligated with similarly prepared pCBOMT3, yielding the final pCRO1 binary vector containing both AhSTS and SbOMT3 expression cassettes, as confirmed by DNA sequence analysis (Figure 5). All DNA manipulations involved in the construction of pCRO1 involved standard cloning procedures (Sambrook et al., 1989).

**Plant transformation**

For the generation of tobacco transformants, the plasmid pCRO1 (Figure 5) was first transformed into the A. tumefaciens strain EHA105 (Hood et al., 1993) using the freeze-thaw method devised by An et al. (1988). Recombinant A. tumefaciens strains harbouring pCRO1 were then co-cultivated with leaf disc explants from tobacco (cv. Wi-38), and transgenic plants were generated under kanamycin selection from independent calli using the method described by Horsch et al. (1988). For the generation of Arabidopsis (ecotype Col-0) transformants, pCRO1 was first transformed into the A. tumefaciens strain LBA4404 (Hoekema et al., 1983) as described earlier and then, T1 seed populations were generated using the ‘floral-dip’ method as previously described (Clough and Bent, 1998). T1 transformants were identified by plating surface-sterilized T1 seeds on germination medium supplemented with 50 μg/mL kanamycin, as described previously.

**Quantitative real-time RT-PCR analysis**

Total RNAs prepared for use in real-time PCR assays were isolated from flash-frozen, pulverized leaf samples using the Trizol reagent (Invitrogen Corp., Carlsbad, CA), with an additional homogenization step of 30 s at 25 000 rpm using a handheld homogenizer. The RNA recovered was then re-purified with a RNeasy Plant Mini-Kit (Qiagen Inc., Valencia, CA) per manufacturer's instructions. RNA recovery and purity were determined spectrophotometrically, and sample integrity was assessed by agarose gel electrophoresis. All real-time PCR reactions were performed in triplicate using a GenAmp 7300 Sequence Detection System (Applied
biosystems, Foster City, CA). First-strand cDNAs were synthesized from 2 μg of total RNA in a 100-μL reaction volume using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems) per manufacturer’s instructions. Independent PCR reactions were performed using the same cDNA for both the gene of interest (AhSTS3 or SbOMT3) and 18S rRNA, using the SYBR Green PCR Master Mix (Applied Biosystems) with the following gene-specific primer pairs: SbOMT3-forward, 5'-CCTGCAGAGGACGTTGAAGAAG-3'; reverse: 5'-CTGCCAGGACAAATGTGTGT-3'; 18S rRNA-forward, 5'-GGCTGAAAGCATGATACCC-3'; and reverse, 5'-TCGGCATCGTTTAGTGTT-3'. Primers were designed using Primer Express software (Applied Biosystems) and the Amplify program (Engels, 1993). A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the GeneAmp 7300 sequence detection system. A negative control reaction in the absence of template (no template control) was also routinely performed in triplicate for each primer pair. The change in fluorescence of SYBR Green I dye in every cycle was monitored by the GenAmp 7300 system software, and the threshold cycle (CT) above background for each reaction was calculated. The CT value of an arbitrary calibrator (e.g. the tissue sample from which the largest CT value of 18S rRNA was subtracted from that of the gene of interest) was used to calculate the fold change in expression level relative to the calibrator. The fold change in expression level relative to the calibrator was expressed as \(2^{-\Delta\Delta CT}\).

**DNA gel blot analysis**

For T-DNA copy number estimates, genomic DNAs were extracted from young leaves harvested from pCR01 tobacco and Arabidopsis transformants, as well as wild-type (cv. WI-38, eco-type Col-0) seedlings using a DNeasy Plant Mini-Kit (Qiagen, Inc., Valencia, CA) per manufacturer’s instructions. Ten-microgram aliquots of genomic DNA were digested with either BamHI or SpH1, size fractionated on 0.8% (w/v) agarose gels and then transferred to nylon membranes. Radiolabelled SbOMT3 coding sequences were used as probe, prepared with [α-32P]dCTP (6000 Ci/mmol and 20 mCi/mL; Perkin-Elmer, Waltham, MA) and a Rediprime II DNA labelling kit (GE Healthcare, Piscataway, NJ), per manufacturer’s instructions. Membranes were hybridized at 65 °C for 16 h, washed twice for 10 min in 2X SSC, 0.2% SDS at 55 °C and then twice for 10 min in 0.2X SSC, 0.2% SDS at 65 °C, followed by two additional washes for 20 min in 0.1X SSC, 0.1% SDS at 65 °C and then finally subjected to autoradiography for approximately 18 h. Restriction endonuclease digestions and DNA gel blotting procedures were performed according to standard protocols (Sambrook et al., 1989).

**Pterostilbene, resveratrol content determinations**

Mature tobacco and Arabidopsis leaves were ground in liquid nitrogen; then, 100 mg of frozen, powdered tissue aliquots was mixed with 500 μL of extraction solution (MeOH:acetone/H2O/acetic acid; 40:40:20:0.1 v/v/v), vortexed for 20 s and then sonicated for 20 min. Samples were then centrifuged for 10 min at 13 000 g, and the supernatants were transferred to GC vials. The remaining pellets were then re-extracted as aforementioned, and the supernatants from the second extraction were combined with those from the first extraction. The combined supernatants were then dried to completion using a vacuum centrifuge, re-dissolved in 400 μL of distilled, de-ionized H2O, and then extracted three times with 500 μL ethyl acetate. The ethyl acetate extracts were then transferred to GC vials and dried to completion under a stream of nitrogen. The dried extracts were then treated with N-O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)/N,N-dimethylformamide (1:1 v/v) and heated at 70 °C for 40 min prior to GC-MS analysis.

GC-MS analysis was performed with a JEOL GCMate II System (JEOL USA Inc., Peabody, MA) using a J&W DB-5 capillary column (0.25 mm internal diameter, 0.25 μm film thickness and 30 m length; Agilent Technologies, Foster City, CA). The GC temperature program was as follows: initial 190 °C, raised to 239 °C at a rate of 20 °C/min and held at this temperature for 1 min, raised to 242 °C at a rate of 0.2 °C/min, and then finally raised to 300 °C at a rate of 30 °C/min and held at this temperature for 0.5 min. The carrier gas was ultrahigh-pure helium at a flow rate of 1.0 mL/min. The inlet (splitless), GC interface and ion chamber temperatures were 250, 250 and 230 °C, respectively. The sample injection volume was 2.0 μL. The retention times for pterostilbene and resveratrol under these conditions were 13.5 min and 16.5 min, respectively. Pterostilbene and resveratrol contents in tobacco and Arabidopsis samples were monitored using selected ion monitoring at m/z 328, 313 and 297 for pterostilbene, and m/z 444, 429 and 373 for resveratrol. The levels of both compounds in the samples were quantified with calibration curves of external pterostilbene and resveratrol standards.

**HPLC analysis of phenolic compounds in flower petals**

The major phenolic compounds (flavonols, phenylpropanoid polyamine conjugates, caffeoylquinic acid isomers and anthocyanins) in wild-type tobacco (cv. WI-38) and transformant T9 flower petals were analysed by reversed-phase HPLC as previously described (Snook et al., 1988, 1992; McMullen et al., 2001). Petals from mature flowers were harvested from approximately 2-month-old greenhouse-grown plants, flash-frozen in liquid nitrogen, lyophilized and then hand-pulverized using a mortar and pestle. For the analysis of flavonols, phenylpropanoid polyamine conjugates and caffeoylquinic acid isomers, powdered tissue aliquots were placed in tubes containing 3 mL of chrysir-MeOH solution (2.0 mg/3 mL; chrysir recrystallized from amyl alcohol), sonicated for 20 min and then filtered through 0.45-μm nylon filters. The filtered extracts were then analysed by reversed-phase HPLC using a Hewlett-Packard 1050 diode array HPLC system (Hewlett-Packard Co., Palo Alto, CA), equipped with an Altex Ultrasphere C18 column (5-μm particle size, 4.6 mm internal diameter and 250 mm length; Beckman Instruments, Norcross, GA). A H2O/MeOH linear gradient from 10% to 100% MeOH in 35 min was used (each solvent contained 0.1% H3PO4), with a flow rate of 1 mL/min. The same procedure was followed for the analysis of anthocyanins, except that samples were extracted in 1% HCl-MeOH (v/v). Flavonols, phenylpropanoid polyamine conjugates and caffeoylquinic acid isomers were monitored at 340 nm and anthocyanins at 525 nm. Compounds were identified by a combination of UV spectra, retention time correlation with standards and HPLC-MS (Snook et al., 1988, 1992). HPLC-MS was performed using a ThermoQuest Finnigan LCQ Duo mass spectrometer (Thermo Fisher Scientific, Waltham, MA) operated in the electrospray ionization mode, equipped with a Spectra System AS3000 auto-injector (Spectra Systems, Providence, RI).
a Spectra System P2000 binary gradient pump and a Spectra System UV 6000LP photodiode array detector. The column used for the RP-HPLC analyses described earlier was also used for HPLC-M5, with a H2O/MeOH linear gradient from 10% to 100% MeOH in 60 min, and flow rate of 0.2 mL/min. Both solvents contained formic acid at 0.1% (v/v), and elution was monitored at 340 nm. Chlorogenic acid was used to tune the instrument for caffeoyl compounds (phenylpropanoid polyamine conjugates and caffeoylquinic acid isomers) while rutin was used for flavonols. Phenylpropanoid polyamine conjugates and caffeoylquinic acid isomers was quantified using chlorogenic acid's response factor, while rutin was used for flavonols. Relative anthocyanin levels were determined from the ratios of average peak areas obtained at 525 nm and expressed as percentage of wild type. All analyses were performed using 3 biological replicates comprised of tissue pooled from 3 to 4 different T1 generation pCRO1-9 and wild-type plants.

Homology modelling and automated substrate docking

All computational studies were carried out using a Silicon Graphics Octane-2 workstation. Resveratrol chemical structure was sketched using the program Sybyl 7.2 (Tripos L.P., St. Louis, MO), and the molecule was minimized for 1000 steps each of steepest descent followed by conjugate gradients. Docking was performed within the SBOMT3 model active site (Baerson et al., 2008a) using the program GOLD v.3.1.1 (CCDC Software Ltd, Cambridge UK). The active site pocket was defined within 15 Å around the N+2 atom of H332. The default set parameters were selected, and the early termination criterion box was unchecked. A total of 25 independent generic algorithm (GA) runs were performed, and the GOLD score was used to evaluate binding poses. The 25 docking poses of resveratrol were also visually inspected for hydrophobic and hydrophilic interactions between ligand and enzyme.

Substrates, chromatography standards

Eugenol, olivetol, trans-resveratrol, chlorogenic acid, rutin (quercetin-3-O-rutinoside) and chrysin were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Nictoflorin (kaempferol-3-O-rutinoside) was purchased from Indofine Chemical Co. (Hillsborough, NJ). Synthesis of trans-pterostilbene was performed as previously described (Rimando et al., 2005).

Acknowledgements

We thank Julie Blessitt, Melanie Mask, Binh Chung, Susan Watson and Gloria Hervey for their excellent technical support. We are also grateful to Dr. Joachim Schröder for generously providing the AhSTS53 cDNA clone used for this work.

References


