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Vitis vinifera terpenoid cyclases: functional identification of two sesquiterpene synthase cDNAs encoding (+)-valencene synthase and (−)-germacrene D synthase and expression of mono- and sesquiterpene synthases in grapevine flowers and berries

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Abstract

Valencene is a volatile sesquiterpene emitted from flowers of grapevine, *Vitis vinifera* L. A full-length cDNA from the cultivar Gewürztraminer was functionally expressed in *Escherichia coli* and found to encode valencene synthase (VvVal). The two major products formed by recombinant VvVal enzyme activity with farnesyl diphosphate (FPP) as substrate are (+)-valencene and (−)-7-*epi*-α-selinene. Grapevine valencene synthase is closely related to a second sesquiterpene synthase from this species, (−)-germacrene D synthase (*VvGerD*). *VvVal* and *VvGerD* cDNA probes revealed strong signals in Northern hybridizations with RNA isolated from grapevine flower buds. Transcript levels were lower in open pre-anthesis flowers, flowers after anthesis, or at early onset of fruit development. Similar results were obtained using a third probe, (−)-α-terpineol synthase, a monoterpenol synthase. Sesquiterpene synthase and monoterpene synthase transcripts were not detected in the mesocarp and exocarp during early stages of fruit development, but transcripts hybridizing with *VvVal* appeared during late ripening of the berries. Sesquiterpene synthase transcripts were also detected in young seeds.

Keywords: *Vitis vinifera*; Vitaceae; Grape vine; Wine flavour and aroma; Terpene cyclase; Floral scent; Nootkatone; Nutraceutical

1. Introduction

Low-molecular-weight terpenoids, including a large array of monoterpenes, sequiterpenes, and norisoprenoids, are commonly found as volatiles emitted from flowers, fruits and leaves of plants. These compounds are widely recognized by humans as important fragrance, flavour and aroma compounds (Schrader and Berger, 2001) and can present desirable quality traits for plant breeding in agriculture, horticulture, and viticulture. The sesquiterpene valencene (**1**) (Fig. 1) is a major volatile emitted from flowers of white and red varieties of grapevine, *Vitis vinifera* L. (Buchbauer et al., 1994a,b; 1995). Valencene (**1**) is also a characteristic fruit flavour and aroma component in some citrus species, and is the likely precursor in the formation of nootkatone (**3**), a valuable compound associated with grapefruit (*Citrus x paradisi*) aroma (Sharon-Asa et al., 2003). Other terpenoid volatiles, mainly monoterpene alcohols such as linalool, geraniol, nerol and terpineol, have often been described as flavour and aroma compounds of grapevine berries and wine (e.g. Marais, 1983; Strauss et al., 1986;

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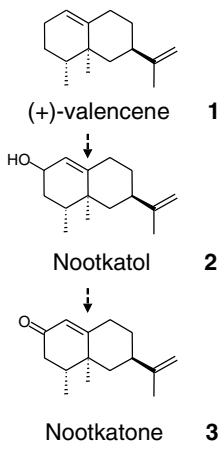

Fig. 1. Structure of (+)-valencene (**1**) and related sesquiterpenoids. Valencene (**1**) is the proposed biosynthetic precursor for the flavour compound nootkatone (**3**) via nootkatol (**2**).

Wilson et al., 1986; Park and Noble, 1993; Mateo and Jimenez, 2000). Despite the prominence of terpenoid volatiles in grapevine flowers and berries, little is known about the biochemical and molecular regulation of terpenoids in this economically important plant. Recently, Luan and Wüst (2002) showed that the exocarp and mesocarp of grapevine berries contribute to monoterpene formation via the 2C-methyl-D-erythritol 4-phosphate pathway. In earlier work, geranyl diphosphate synthase, a prenyl transferase for the formation of the immediate precursor of monoterpenes, was characterized from grapevine cell cultures (Clastre et al., 1993). Recently, we have initiated a project that is aiming at the discovery and functional characterization of genes of terpenoid formation in grapevine and described the first monoterpene synthase in *V. vinifera* (Martin and Bohlmann, 2004). Enzymes of the large family of plant terpenoid synthases (TPS) are critical for the generation of structural diversity of monoterpenoids, sesquiterpenoids, and diterpenoids (Bohlmann et al., 1998; Davis and Croteau, 2000). To our knowledge, an enzyme or gene that is specific to the formation of a sesquiterpene volatile in grapevine has not been reported to date. In this paper, we describe a pair of grapevine full-length cDNAs encoding two different sesquiterpene synthases, the multi-product (+)-valencene synthase (*VvVal*) and a (−)-germacrene D synthase (*VvGerD*), and expression of TPS transcripts in vegetative tissues and in flower buds, open flowers, and fruits of wine grape cultivars.

2. Results

2.1. Identification of grapevine full-length sesquiterpene synthase cDNAs

Plant genome and EST databases are a valuable resource for the mining of genes of secondary metabolism including genes of terpenoid metabolism (e.g. Lange et al., 2000; Gang et al., 2001; Aubourg et al., 2002; Dudareva et al., 2003). Full-length cDNA sequence databases and full-length cDNA clone collections are a particularly powerful resource for functional genomics (Seki et al., 2002), because they allow for the identification of complete open reading frames (ORFs) for improved gene annotation and for expression of recombinant proteins and their functional characterization. In order to identify *TPS* genes in grapevine, in silico screening was performed in a full-length cDNA sequence database (http://www.vitigen.com) developed for the grapevine cultivar Gewürztraminer (Driesel et al., 2003). The database contained entries from an array of grapevine organs and tissues, including leaves, stems, tendrils, and developing berries. Using known TPS as search sequences, two full-length sesquiterpene synthase-like cDNA clones were identified, *VitiM4670* (VvVal) and *VitiM1610* (VvGerD), which resembled members of the *TPS-a* group of plant terpene synthases (Bohlmann et al., 1998; Aubourg et al., 2002) (Fig. 2). The *VitiM4670* (VvVal) cDNA (1918 bp) contains an ORF of 1671 bp for a predicted protein of 556 amino acids with a molecular mass of 64.4 kDa and a calculated isoelectric point of 5.77. The *VitiM1610* (VvGerD) cDNA (1911 bp) has an ORF of 1674 bp for a predicted protein of 557 amino acids, molecular mass of 64.3 kDa, and an isoelectric point of 4.54. The deduced amino acid sequences of VvVal and VvGer are 59% identical. VvVal and VvGerD also showed high amino acid identity (55% and 54%, respectively) with *Gossypium arboreum* δ-cadinene synthases (GenBank accesion X96429), and with *Citrus sinensis* valencene synthase (GenBank accession AAQ04608) (52% and 54%, respectively) (Fig. 2). The amino acid sequences of VvVal and VvGerD contain the conserved features of plant sesquiterpene synthases including the active site DDxxD motif (Starks et al., 1997; Whittington et al., 2002) and the RPx_8W motif (Bohlmann et al., 1998).

2.2. Functional characterization of VvVal and VvGer

Functional identification of the two enzymes encoded by cDNAs *VitiM4670* (VvVal) and *VitiM1610* (VvGerD) was accomplished by expression of the recombinant proteins in *E. coli* and in vitro enzyme assays. Both enzymes were active with farnesyl diphosphate (FPP) as substrate but not with geranyl diphosphate or geranylgeranyl diphosphate. Terpenoid products were identified by gas chromatography and mass spectrometry (GC-MS). The major sesquiterpene product of VvVal was identified as (+)-valencene (**1**) (49.5% of total product), followed by (−)-7-*epi*-α-selinene (35.5%) (Fig. 3) along with five minor products (Table 1). Identification of stereochemistry of (+)-valencene (**1**) formed by VvVal enzyme activity was based on comparison of

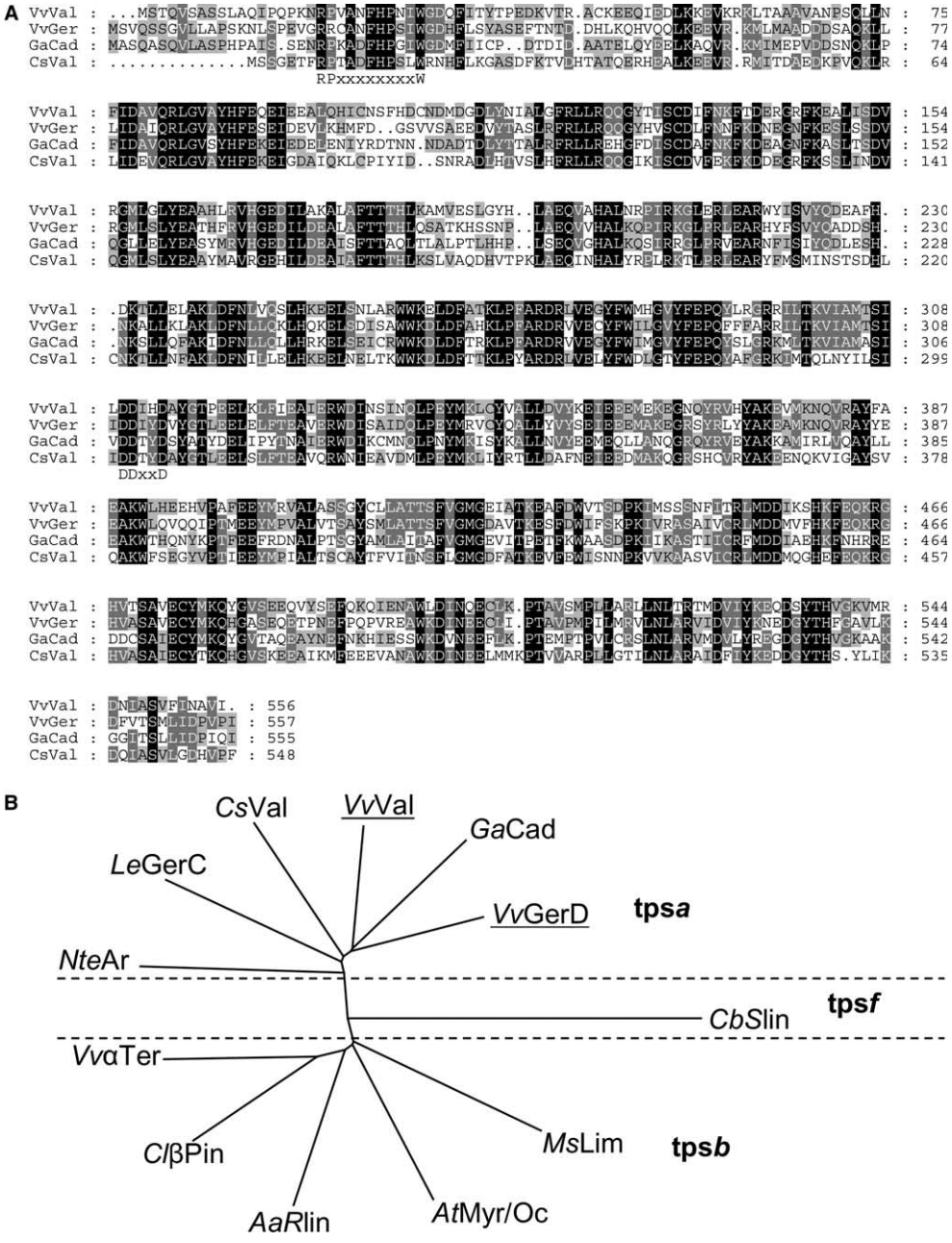

Fig. 2. Sequence comparison of grapevine sesquiterpene synthases. (A) Alignment of deduced amino acid sequences of grapevine sesquiterpene synthases VvVal and VvGer and two other sesquiterpene synthases. The alignment was created using Clustal X 1.83 software and edited using Genedoc software. GaCad: (+)-*δ*-cadinene synthase from *Gossypium arboreum* (Genbank accession number: X96429). CsVal: *Citrus sinensis* valencene*1* synthase (Genbank accession number: AAQ04608). Shading indicates levels of sequence conservation (100% conservation: white on black; 75% conservation: white on dark grey; 50% conservation: black on light grey). Known sesquiterpene synthase conserved motifs RPX_8W and DDXXD are indicated. (B) Phylogenetic tree of representative of monoterpene synthases and sesquiterpene synthases derived by neighbour joining analysis of amino acid sequences. Grapevine sesquiterpene synthases VvVal and VvGer cluster in the TPS-a subfamily of angiosperm sesquiterpene synthases. NteAr (*Nicotiana tabacum epi*-aristolochene synthase, Q40577), LeGerC (*Lycopersicon esculentum* Germacrene C synthase, AAC39432), CbSlin (*Clarkia breweri* S-linalool synthase, AAC49395), MsLim (*Mentha spicata* (−)-limonene synthase, A48863), AtMyr/Oc (*Arabidopsis thaliana* myrcene/ocimene synthase, AAG09310), AaRlin (*Artemisia annua*, *R*-linalool synthase AAF13357), ClSSin (*Citrus limon* (−)-SS-pinene synthase AAM53945), VvTer (*Vitis vinifera* α-terpineol synthase).

retention times and mass spectra with that of an authentic (+)-valencene (**1**) standard analysed on a Cyclosil-B GC column. The stereochemistry of (−)-7-*epi*-α-selinene (**4**) produced by VvVal was identified by comparison of retention times with those of a (+)-7-*epi*-α-selinene (**4**) standard (kindly provided by Dr. Wilfried König, Hamburg) and (−)-7-*epi*-α-selinene present in the essential oil of *Amyris balsamifera*. The major sesquiterpene product

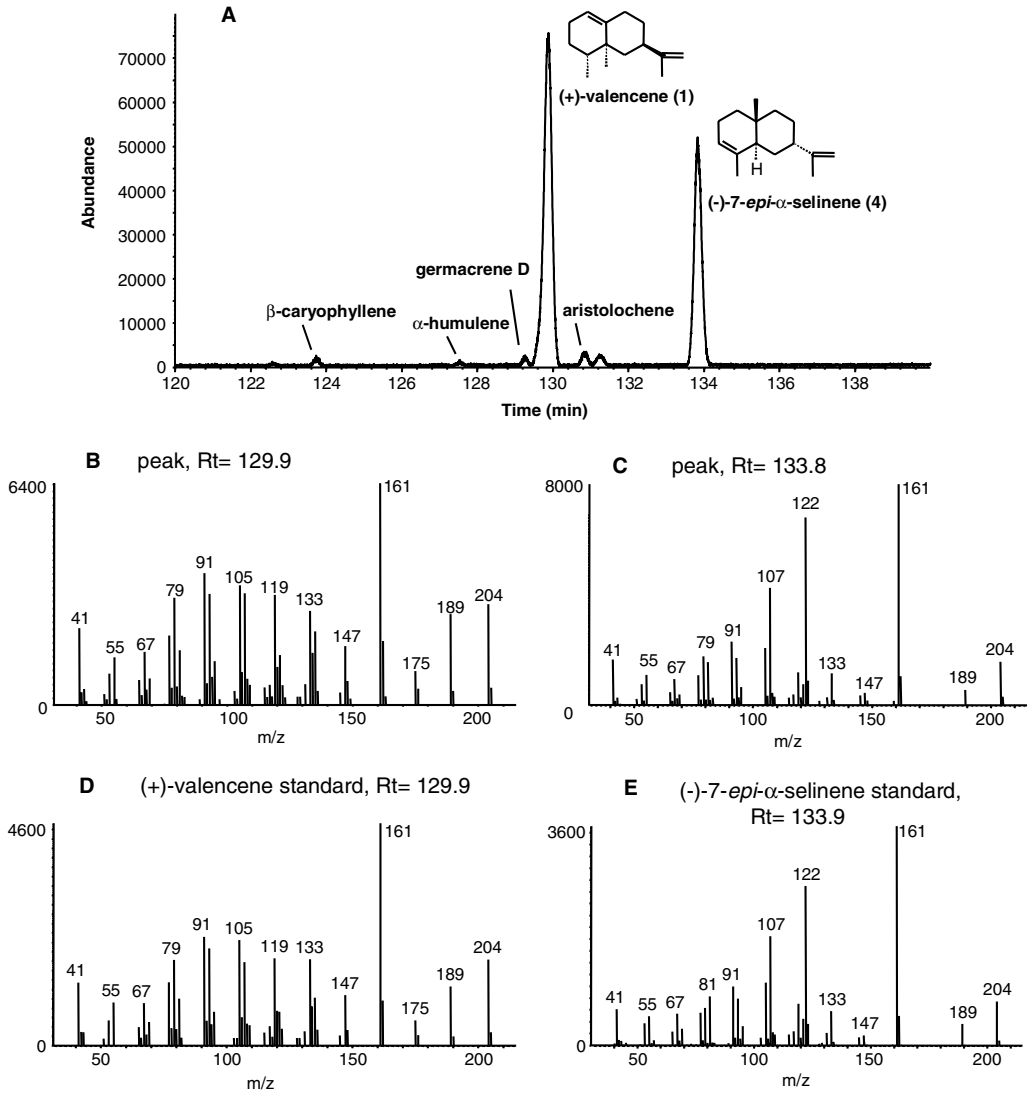

Fig. 3. GC-MS profile of products formed by the heterologously expressed sesquiterpene synthase VvVal. (A) Total ion chromatogram of the products formed with FPP as substrate. The main product peaks were identified as (+)-valencene (**1**) (B) and (−)-7-epi-α-selinene (**4**) (C). Identification was based on comparisons of retention times and mass spectra with those of authentic standards for (+)-valencene (**1**) (D), (+)-7-epi-α-selinene (**4**) (not shown), and (−)-7-epi-α-selinene (**4**) (E) from an *Amyris balsamifera* essential oil sample. Additional minor sesquiterpene products formed by the VvVal enzyme were identified as β-caryophyllene, α-humulene, germacrene D and aristolochene. A putative α-selinene product that was found using a DB-wax column but apparently coelutes with the valencene peak in the chromatogram shown here.

Table 1
Sesquiterpene products formed by recombinant VvVal using FPP as substrate

Sesquiterpene product	% of total product
(+)-valencene (**1**)	49.5
(−)-7-epi-α-selinene (**4**)	35.5
α-selinene (tentative)	7.0
aristolochene	3.58
E-β-caryophyllene	1.96
α-humulene	1.6
germacrene D (**5**)	0.85

Percent values of total product are the means of two independent enzyme assays analyzed on a DB-wax column.

of VvGerD was identified as (−)-germacrene D (**5**) (92%) along with a second product tentatively identified as δ-cadinene (8%) (Fig. 4). Stereochemistry of (−)-germacrene D (**5**) was determined as described before for poplar (−)-germacrene D (**5**) synthase (Arimura et al., 2004). Product profiles of VvVal and VvGerD were analyzed from two separate sets of assays, with or without silica/$MgSO_4$ purification of the pentane-extracted products of the aqueous assay mixture. Nearly identical product profiles were obtained with both assay systems indicating that product profiles were not affected by the silica/$MgSO_4$ step.

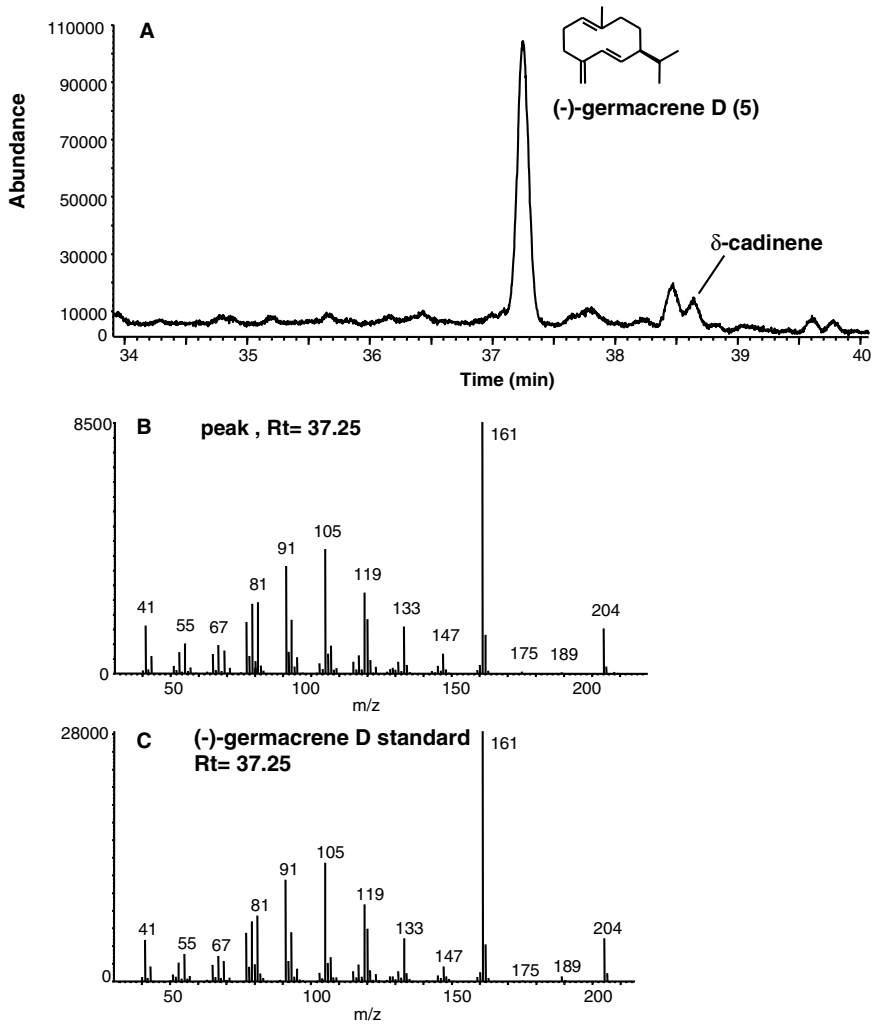

Fig. 4. GC-MS profile of products formed by the heterologously expressed sesquiterpene synthase VvGerD. (A) GC chromatogram of the products formed with FPP as substrate. The main product peak on the HP5 column was identified as (−)-germacrene D (**5**) by comparison of retention time and mass spectrum (B) with that of a (−)-germacrene D (**5**) standard (C).

2.3. Transcripts of grapevine TPS genes in flower buds, flowers and young fruits

Valencene (**1**), together with other terpenoids, has been reported as a major volatile emitted from flowers of several grapevine cultivars (Buchbauer et al., 1994a,b, 1995). We tested expression of *VvVal* and *VvGerD* at four different developmental stages of grapevine flowers (Fig. 5) using the Chardonnay cultivar for Northern analysis of flowers and vegetative tissues. This cultivar was chosen because plants were conveniently available for sampling at the appropriate time of year. In addition to the two sesquiterpene synthases, we used a third grapevine *TPS* cDNA probe, *VvTer*, encoding a monoterpenol synthase for the formation of (−)-α-terpineol (Martin and Bohlmann, 2004). Probes for *VvVal*, *VvGerD* and *VvTer* showed little or no cross-hybridizations in dot blot tests (Fig. 5A). Grapevine flowers are relatively small (≈3 × 3 mm) and are organized in dense clusters of several hundred at different stages of development. From these clusters we selected flowers for Northern analysis that could be clearly distinguished as (i) flower buds within a few days before opening, (ii) open, pre-anthesis flowers, (iii) post-anthesis flowers, and (iv) set flowers at the onset of fruit development. All three TPS probes, *VvVal*, *VvGerD* and *VvTer*, revealed strongest hybridization signals with RNA from flower buds and strongly reduced transcript levels in pre- and post-anthesis open flowers and in set flowers at early fruit onset (Fig. 5B). While *VvGerD* revealed transcript levels that were similar in open pre-anthesis flowers, in flowers after anthesis, and in the early stages of fruit onset, transcripts were barely detectable with *VvVal* after anthesis and at early fruit onset. The *VvGerD* probe also detected transcripts in the vegetative tissues of young leaves, green stems and tendrils, while transcripts hybridizing with the sesquiterpene synthase *VvVal* or the monoterpene synthase *VvTer* appeared to be restricted to flowers.

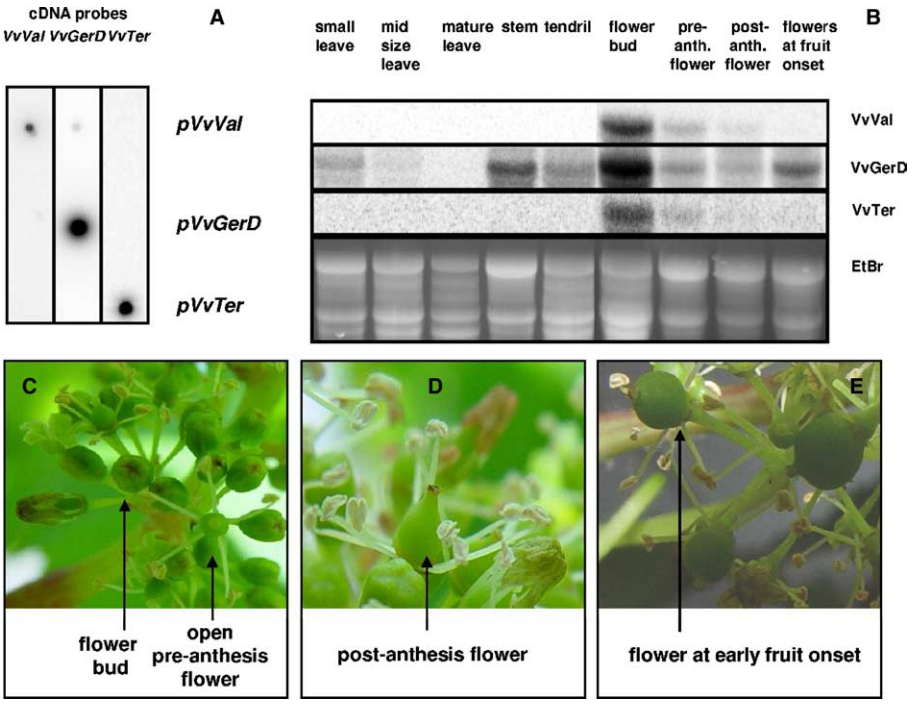

Fig. 5. Northern blot analysis of *TPS* transcripts in vegetative organs and flowers of grapevine. (A) Dot blots demonstrate lack of cross-hybridization of three grapevine *TPS* cDNA probes, *VvVal*, *VvGerD*, and *VvTer* with spotted cDNA plasmids. (B) Northern blot analysis of *VvVal*, *VvGerD* and *VvTer* with different vegetative organs and flowers using the grapevine cultivar Chardonnay. The ethidium bromide stained gel is shown on the bottom to compare for loading differences. (C–E) Images of flower buds, open pre-anthesis flowers, post-anthesis flowers, and flowers with early fruit onset used for Northern blot analysis.

2.4. Transcripts of grapevine TPS genes in developing berries

The flavours and aromas of grapevine berries and wines produced from aroma-rich cultivars such as Gewürztraminer are known to result from complex terpenoid profiles (Mateo and Jimenez, 2000; Girard et al., 2002). We followed expression of monoterpene synthases and sesquiterpene synthases over an extended time course of fruit development beginning immediately after fruit set and abscission of non-set flowers until late ripening over a period of three months from late July to early October 2002. Fruit samples were collected weekly from the Gewürztraminer cultivar. RNA was isolated from fruit tissues including skins. With the exception of the first two (July-23 and July-30) samplings, seeds were removed from fruits prior to RNA extraction. Seeds could not be completely removed from the July-23 and July-30 fruit samples due to the very small seed size. Using the three grapevine *TPS* probes, *VvVal*, *VvGerD* and *VvTer*, transcripts were detectable by Northern blot analysis only with the *VvVal* probe (Fig. 6). Transcripts became detectable in September, almost two months after flowering, then increased and reached a maximum at the final sample date in early October when the fruit was at peak maturity for wine making (Fig. 6A). In parallel, levels of fruit acids (mostly tartaric acid and malic acid), sugars (mostly glucose and fructose), and pH were monitored in berries as a measure of fruit ripening (Fig. 6C). The detection of *VvVal* transcripts coincided with the stabilization of acid levels in the fruits following a four-week period of rapid depletion. Initially, we also detected *TPS* transcripts probes in fruits collected at the beginning of fruit development in July. Upon closer inspection we found that *TPS* transcripts detected in these samples could be attributed, at least in part, to expression in seeds at this early fruit stage (Fig. 6B). *TPS* transcripts were not detected at later stages of seed development, however, overall RNA contents of seeds vanished and RNA quality became impaired near fruit maturity (September 17 to October 8).

3. Discussion

In this paper we provide a first characterization of sesquiterpene synthase cDNAs in grapevine, *V. vinifera*, which is economically one of most significant fruit plants worldwide. Terpenoid volatiles are important fruit aroma and flavour components in this species and contribute much to the quality of table grapes and wine (e.g. Marais, 1983; Strauss et al., 1986; Wilson et al., 1986; Park and Noble, 1993; Mateo and Jimenez,

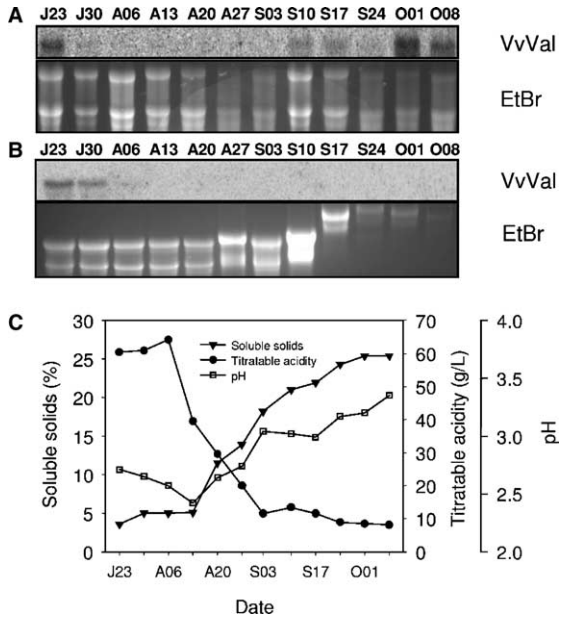

Fig. 6. Northern blot analysis of *VvVal* in grapvevine fruits. Fruits of the grapevine cultivar Gewürztraminer were harvested during 2002 on July 23 (J23), July 30 (J30), August 6 (A06), August 13 (A13), August 20 (A20), August 27 (A27), September 3 (S03), September 10 (S10), September 17 (S17), September 24 (S24), October 1 (O01) and October 8 (O08). (A) Total RNA for Northern analysis was isolated from mesocarp and exocarp after removal of all seeds, except for the July-23 and July-30 samples where seeds were too small for their complete separation from the mesocarp and exocarp tissues. (B) Total RNA was isolated from seeds. In (A) and (B), RNA blots were hybridized with the valencene synthase *VvVal* probe. The ethidium bromide stainings of RNA in agarose gels are shown to illustrate possible loading differences. RNA at late stages of seed development showed repeatedly unusual retardation in agarose gels. (C) Soluble solids, composed mostly of the sugars glucose and fructose, and titratable acids, mostly tartaric acid and malic acid, and the pH of juice pressed from fruits of the grapevine cultivar Gewürztraminer sampled weekly during 2002 on July 23 (J23), July 30, August 6 (A06), August 13, August 20 (A20), August 27, September 3 (S03), September 10, September 17 (S17), September 24, October 1 (O01) and October 8 (O08).

2000). In addition to the many reports on terpenoid profiles of wines and grapevine berries, previous studies also described the prominence of terpenoid volatiles in flowers of grapevines, with valencene (**1**) as a major component of the scent bouquet in both white and red grapevine varieties (Buchbauer et al., 1994a,b, 1995). In agreement with earlier reports of terpenoids in fruits and flowers, we found expression of *TPS* genes correlated distinctively with grapevine flowering and fruit ripening. Abundance of *TPS* transcripts detected with three *TPS* probes was associated with flower buds shortly before opening at bloom when the transcripts could be involved in the formation of flower volatiles. The expression of valencene synthase, *VvVal*, in flower buds and to lesser extend in open flowers suggests a specific role in flower volatile formation, because expression of *VvVal* was not found in other parts of the plants at the same time. While other *TPS* are also expressed in flower tissues, transcripts hybridizing with *VvGerD* were detectable also in some vegetative tissues and could function in the formation of volatile or non-volatile terpenoids in leaves and stem tissues. Terpenoid volatiles in flowers of grapevines could function in the attraction of insect pollinators of the small, green flowers which shed their calyptras entirely at bloom. As the flowers in a cluster bloom sequentially over several days, the scent of young flowers may attract insects to flowers at anthesis. However little seems to be known about insect pollination in grapevine which is thought to be primarily wind- or self-pollinating. Terpenoid volatiles of grapevine flowers could also provide protection of reproductive tissues against pathogens or small herbivores.

After fruit set, *TPS* transcripts were no longer detected in the developing berries, except in very young seeds, until the late stages of berry development, which is distinguished as the process of fruit ripening. Grapevine berries undergo considerable physical and biochemical changes during their development, particularly during ripening. The non-climacteric fruit develops with a double-sigmoidal growth pattern (Coombe and Bishop, 1980; Coombe, 1992). After anthesis and fruit set, size increase in young berries is due to both cell division and cell expansion (Harris et al., 1968). Early berry development is then followed by a lag phase of little or no growth. Resumption of growth after this lag phase coincides with a process called veraison at which a number of physiological and biochemical processes are initiated, including acceleration of berry growth, accumulation of sugar and anthocyanins, softening of berries and reductions in organic acids (Coombe, 1992). According to Wilson et al. (1984) levels of terpenes in fruits of the cultivar Muscat increased with sugar accumulation and several monoterpenes reached peak levels in the overripe fruit. We found expression of *TPS* during late stages of berry development several weeks after veraison when the organic acid levels had stabilized and the rate of soluble solids accumulation was high. The temporal patterns of *TPS* expression at the time of flowering and during late fruit development in Chardonnay and Gewürztraminer, respectively, are similar to those of several genes of anthocyanin formation in flowers and berries of the grapevine cultivar Shiraz, namely phenyalalanine ammonia lyase, chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, dihydroflavonol 4-reductase and leucoanthocyanidin dioxygenase (Boss et al., 1996). In addition to expression of *TPS* genes associated with flower volatiles and fruit ripening, there was also some distinct expression of *TPS* early on in seeds of developing grape berries. Transcripts in young seeds were first found when very young fruits could not be completely removed of their seeds and this finding was then confirmed with isolated seeds. Grapevine seeds are rich in phenolic metabolites and

powders and oils made from grapeseeds are valued as health-food products for their high contents of nutraceutical antioxidants. The finding of *TPS* transcripts in very young seeds warrants future analysis of seed terpenoids. A preliminary analysis of young Gewürztraminer seeds collected on 23 July 2002 revealed at least six different sesquiterpenes, with α-humulene and *E*-β-caryophyllene predominant in addition to lower levels of monoterpenes (not shown).

A valencene synthase was recently cloned from orange (*Citrus sinensis*) fruit peel (Sharon-Asa et al., 2003). In contrast to the apparently single-product orange enzyme, the stereoselective grapevine (+)-valencene synthase forms a second major product and several minor products with FPP as substrate. In future structure–function analysis, these two different valencene synthases will provide a useful pair of enzymes to identify characteristics of single-product and multi-product sesquiterpene synthases. Single product and multi-product valencene synthases also provide interesting new genes and enzymes for biotechnological applications, in particular for the formation of natural aromas in *E. coli* or yeast fermentation systems (Martin et al., 2003; Jackson et al., 2003; Reiling et al., 2004), or for the development of new fruit aromas and floral scents (e.g. Lewinsohn et al., 2001; Lavy et al., 2002; Lücker et al., 2004). In addition, expression of (+)-valencene synthase in suitable *E. coli* or yeast cells engineered for sesquiterpenoid formation (Martin et al., 2003; Jackson et al., 2003) could conceivably be employed for efficient functional in vivo cDNA screening targeted at the discovery of new enzymes specialized for the conversion of valencene (**1**) to nootkatone (**3**) (Fig. 1), a sequence of oxidations that is presumably catalyzed by cytochrome P450-dependent monooxygenase and alcohol dehydrogenase activities. Finally, very little is known about the cell and tissue organization of terpenoid flavour and aroma formation in grapevine berries (Luan and Wüst, 2002). The newly characterized grapevine *TPS* cDNAs will provide important probes for future localization studies in this system.

4. Experimental

4.1. Plant material

Tissues of *V. vinifera* cv. Chardonnay were harvested from plants grown outside under natural environmental conditions at the horticulture plant growth facilities of the University of British Columbia, Vancouver, BC. Fruits of *V. vinifera* cv. Gewürztraminer were sampled at vineyards of the Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, in Summerland, Okanagan, BC, Canada. Gewürztraminer fruit samples were harvested in intervals of one week from 23 July 2002 until 8 October 2002. At each sampling, five samples of 10–30 berries each were harvested, immediately frozen in liquid nitrogen and stored at −80 °C prior to RNA isolation. In order to monitor fruit ripening, soluble solids (mostly sugars), fruit acids and pH were measured according to Ough and Amerine (1988). A composite sample of 50–100 berries at each sampling was crushed using a mortar and pestle and the juice was pressed through cheesecloth. Juice analysis was for percent soluble solids using a digital refractometer (Atago, Japan) and for titratable acidity (TA) using an automatic titrator (Brinkmann Instruments, Canada). The titration was with 0.1 M NaOH to a pH endpoint of 8.1, and TA was expressed in tartaric acid equivalents.

4.2. Identification of full-length cDNA clones

The *V. vinifera* L., cv. Gewürztraminer full-length cDNA EST database of Vitigen AG (Siebeldingen, Germany) was screened using standard BLAST algorithms (Altschul et al., 1990) and known sesquiterpene synthase sequences (Aubourg et al., 2002). Two full-length cDNA clones, VitiM4670 (*VvVal*)) and VitiM1610 (*VvGerD*) were purchased from the VitiGen AG plasmid stock collection (Driesel et al., 2003).

4.3. Expression of sesquiterpene synthases cDNAs in E. coli and enzyme assays

The complete ORFs of VitiM4670 (*VvVal*)) and VitiM1610 (*VvGerD*) were amplified by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) with the *VvGerD* forward primer 5′-CAC CAT GTC TGT TCA GTC TTC AGG-3′ and reverse primer 5′-TCA TAT TGG CAC AGG ATC-3′, and with the *VvVal* forward primer 5′- CAC CAT GTC TAC TCA AGT CTC AGC-3′ and reverse primer 5′-TTA TAT TAC AGC GTT GAT GAA AAC AGA CG-3′. PCR reactions were performed in a PTC 100 thermocycler (MJ Research Inc., Waltham, MA). The PCR program used was a denaturing step at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and then 72 °C for 2 min. PCR products were directly cloned into the pET101/DTOPO directional expression vector (Invitrogen, Carlsbad, CA). The recombinant expression plasmids were rescued and maintained in *E. coli* TOP10 F′ cells, analyzed by PCR using insert and vector based primers, and inserts sequenced. Plasmids were transformed into *E. coli* CodonPlus BL21-DE3 RIL (Stratagene) cells for expression of TPS.

Recombinant *E. coli* cells were grown in 25 ml LB with ampicillin at 37 °C to an OD_{600} of 0.7, followed by addition of 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and cultivation overnight at 20 °C. Cells were centrifuged, resuspended in 2 ml assay buffer (15 mM Mopso pH 7.0, 10% glycerol, 5 mM DTT), and sonicated

as described (Fäldt et al., 2003). Cell lysates were cleared by centrifugation and the supernatant was assayed for TPS enzyme activity. Assays were done in volumes of 1 ml with 300 μl of cell extracts, 10 mM $MgCl_2$, and 46 μM FPP (Echelon Bioscience Inc., Salt Lake City, UT), overlaid with 1 ml pentane, at 30 °C for 1 h. Assays were extracted twice with 1 ml of pentane and pentane extracts were passed through silica columns overlaid with $MgSO_4$ (Steele et al., 1998). Columns were rinsed with Et_2O (1.5 ml). Assays were also extracted with Et_2O (1 ml) and ether extracts passed over silica columns. In a second set of assays, instead of using silica columns overlaid with Na_2SO_4, pentane extracts were washed with 2 ml of water. Pentane- and ether-extracts were carefully concentrated on ice under nitrogen. As a negative control for terpene synthase enzyme assays, 300 μl of cell extracts were heat-denatured for 3 min prior to enzyme assays. Cell extracts were also assayed with the alternative substrates GPP and GGPP (Echelon Bioscience Inc., Salt Lake City, UT) but no activity was found.

4.4. Gas chromatography–mass spectrometry

The pentane and ether extracts were analysed on an Agilent 6890 Series GC System (Agilent Technologies Canada Inc., Ottawa, Ontario) coupled to an Agilent 5973 Network Mass Selective Detector (70 eV) using three columns of different polarities, a DB-WAX column (0.25 mm × 0.25μm × 30 m; J & W Scientific, Palo Alto, CA), a CyclosilB column (0.25 mm × 0.25 μm × 30 m; J & W Scientific), or a HP5 column (0.25 mm × 0.25 μm × 30 m; Agilent Technologies). Splitless injections (3 μl) were done at an injector port temperature of 200 °C with a flow of 1 ml min^{-1}. The temperature program for sample analysis with the DB-wax column was: 40 °C for 1 min, followed by an increase of 0.8 °C min^{-1} to 180 °C, and subsequently ramped to 250 °C with 15 °C min^{-1} with a 10 min hold at 250 °C. For the CyclosilB column, the temperature program was: 40 °C for 1 min, followed by a ramp of 0.9 °C min^{-1} to 165 °C, and subsequently ramped to 250 °C with 20 °C min^{-1} with a 10 min hold at 250 °C. For the HP5 column, the temperature program was from 70 to 200 °C with 3 °C min^{-1}, and subsequently ramped to 300 °C with 20 °C min^{-1} with a 2 min hold at 300 °C. MS parameters were set to scan between 40 and 300 amu. All products were identified using authentic standards and/or Wiley library matches (Palisade Corporation, Newfield, NY). The authentic standard for (+)-valencene (**1**) was from Bedoukian Research Inc. (Danbury, CT), (+)-7-epi-α-selinene (**4**) was kindly provided by Dr. Wilfried König (Universität Hamburg, Germany), and (−)-7-epi-α-selinene (**4**) was present in an *Amyris balsamifera* essential oil sample (Applied Essential Oil Research, New Albany, IN). Stereochemistry was determined using a CyclosilB column and by comparing retention times of enzyme assay products with those of authentic standards. Stereochemistry of (−)-germacrene D (**5**) was identified by GC-FID as described previously (Arimura et al., 2004).

4.5. RNA isolation and Northern analysis

All samples were ground using a mortar and pestle in liquid nitrogen. Where possible, seeds were carefully removed from fruits prior to grinding tissues to a very fine powder. RNA isolation was carried out as described (Kolosova et al., 2004). RNA gel blot analysis was performed by separating 10 μg of total RNA on 1.2% agarose gels under denaturing conditions using formaldehyde and transfer of RNA overnight by capillary action onto a Nitrocellulose membrane (Hybond N^+, Amersham Biosciences Corp, Baie d'Urfé, Québec, Canada). Ribosomal RNA stained with ethidium bromide was visualised using a ChemiImager 5500 with AlphaEaseFC™ software (Alpha Innotech Corporation, San Leandro, CA). Labelling of cDNA probes was done using a StripEZ™ DNA StripAble DNA probe synthesis kit (Ambion Inc., Austin, TX) with (α-^{32}P)-dATP (3000 Ci/mmol, 10 mCi/ml; Perkin–Elmer Applied Biosystems, Streetsville, Missisauga, Ont., Canada) according to the manufacturers' recommendations. Unincorporated dNTPs was removed using a gel filtration column (MicroSpin S-300 HR columns; Amersham Biosciences). After 1 h of prehybridisation of membranes at 65 °C in hybridisation buffer (0.05 M $Na_4P_2O_7$, 0.115 M NaH^2PO^4, 7% SDS, 1 mM EDTA, 100 μg ml denatured salmon sperm DNA), membranes were incubated in hybridisation buffer with heat-denatured cDNA probes for 16 h at 65 °C. Membranes were rinsed with 10 ml wash buffer (0.05 M $Na^4P^2O^7$, 0.115 M NaH^2PO^4, 1% SDS, 1 mM EDTA) and washed twice in 40 ml wash buffer at 65 °C for 30 min. Hybridization signals were detected using a Storm 860 phosphoimager (Amersham Biosciences).

4.6. Sequence analysis

Deduced amino acid sequences were generated using the entire open reading frame within EditSeq 5.00 (DNASTAR Inc., Madison, WI). Amino acid alignments were made with ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/) and visualized with GeneDoc (http://www.psc.edu/biomed/genedoc/). Phylogenetic trees were generated using the nearest neighbor joining method through ClustalX and visualized with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

4.7. GenBank accession

The sequences of VitiM4670 (VvVal) and VitiM1610 (VvGerD) have the GenBank accession numbers AY561843 and AY561842, respectively.

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