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Homologous and heterologous expression of grapevine *E*-(β)-caryophyllene synthase (VvGwECar2)

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ABSTRACT

E-(β)-caryophyllene is a sesquiterpene volatile emitted by plants and involved in many ecological interactions within and among trophic levels and it has a kairomonal activity for many insect species. In grapevine it is a key compound for host-plant recognition by the European grapevine moth, *Lobesia botrana*, together with other two sesquiterpenes. In grapevine *E*-(β)-caryophyllene synthase is coded by the *VvGwECar2* gene, although complete characterization of the corresponding protein has not yet been achieved. Here we performed the characterization of the enzyme after heterologous expression in *E. coli*, which resulted to produce *in vitro* also minor amounts of the isomer α-humulene and of germacrene D. The pH optimum was estimated to be 7.8, and the K_m and K_{cat} values for farnesyl pyrophosphate were 31.4 μM and 0.19 s^{-1} respectively. Then, we overexpressed the gene in the cytoplasm of two plant species, *Arabidopsis thaliana* and the native host *Vitis vinifera*. In *Arabidopsis* the enzyme changed the plant head space release, showing a higher selectivity for *E*-(β)-caryophyllene, but also the production of thujopsene instead of germacrene D. Overall plants increased the *E*-(β)-caryophyllene emission in the headspace collection by 8-fold compared to *Col-0* control plants. In grapevine *VvGwECar2* overexpression resulted in higher *E*-(β)-caryophyllene emissions, although there was no clear correlation between gene activity and sesquiterpene quantity, suggesting a key role by the plant regulation machinery.

1. Introduction

Terpenoids build up the biggest class of metabolites in plants, being involved both in primary (Croteau et al., 2000) and in secondary metabolism (Zwenger and Basu, 2008). Humans have traditionally used them as fragrances and flavors, since many of them are present in plant essential oils. More recently, they were investigated for their role as high-value chemicals for obtaining materials (e.g. rubbers), pharmaceuticals and even biofuels (Bohlmann and Keeling, 2008).

In nature however, terpenoids are best-known for their multiple ecological roles: from direct defense against pathogens and insects (Hasegawa et al., 2010; Heiling et al., 2010) to indirect defense attracting enemies of herbivores (Turlings and Ton, 2006; Unsicker et al., 2009), but also attraction of pollinators (Dudareva and Pichersky, 2000), mutualistic fungi (Ditengou et al., 2015) and as signals for plant-plant communication (Arimura et al., 2000). Their role as semiochemicals is also well exploited by phytophagous insects, which often use them as kairomones to recognize and locate their host plants (Bruce et al., 2005).

All the classes of terpenes formally derive from the C_5 isomers isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), whose biosynthesis can take place in plants via two distinct pathways: the mevalonate pathway in the cytoplasm, and the MEP/DOXP pathway in the chloroplast (Rohmer, 1999). The condensation of C_5 isomers by the prenyl-transferase enzymes leads to the C_{10}, C_{15}, C_{20}, C_{30} and C_{40} precursors of mono-, sesqui-, di-, tri- and tetra-terpenes respectively.

In the last step, the precursors are converted to terpenes by the terpene synthase (TPS) enzymes, which in each species are usually coded by gene families of 20–150 members, as predicted in the

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genomes sequenced so far (Chen et al., 2011). *E*-(β)-caryophyllene is an almost ubiquitous semi-volatile sesquiterpene: in particular in grapevine (*Vitis vinifera* L., Vitaceae) it is emitted by leaves, tendrils and flowers (Matarese et al., 2014) and it was shown to be biologically active to the European Grapevine Moth, *Lobesia botrana* (Den. & Schiff.) (*Lepidoptera*, *Tortricidae*), eliciting electrophysiological responses on their antennae (Tasin et al., 2005), as well as being part of an attractive lure both under laboratory and field conditions (Tasin et al., 2006; Anfora et al., 2009). The lure included also the sesquiterpene *E*-(β)-farnesene and the homoterpene *E*-(4,8)-dimethyl-(1,3,7)-nonatriene (DMNT), which is derived from the sesquiterpene alcohol *E*-nerolidol.

L. botrana is a polyphagous insect, with a wide range of host plants, but it is considered the major pest of vineyards in Europe. It recently spread also in the Neotropical Region, raising alarm over American viticulture areas (Ioriatti et al., 2011). Larvae feed on grapes, allowing various fungi, as *Botrytis cinerea* to lead the whole grape cluster to rot (Fermaud and Le Menn, 1989).

In this work, we biochemically characterized the TPS enzyme responsible for most of the production of *E*-(β)-caryophyllene in grapevine (VvGwECar2). Then we created stable transgenic lines of *Arabidopsis thaliana* (L., Brassicaceae) and *V. vinifera*, and quantified the headspace emission of the volatile produced under heterologous and homologous overexpression *in planta*.

The obtained lines could be used in the future to better investigate the mechanisms of the host-finding behavior of *L. botrana* and other model systems sharing the same plant volatile signals. The possible implications for the management of *L. botrana* disrupting its long-range host recognition and the oviposition site selection by the use of transgenic/cisgenic plants with unbalanced production of kairomones are also discussed.

2. Results and discussion

2.1. Bacterial VvGwECar2 expression and characterization

In grapevine five genes are known to code for β-caryophyllene synthases (Martin et al., 2010) but only one (*VvGwECar2*) is actually expressed in all plant tissues and therefore accounts for most of the volatile production in vegetative parts and berries (Matarese et al., 2014), although in flowers other two genes (*VvGwECar1* and *VvPNECar2*) seem to play a big role.

Considering the importance of terpenes production in grapevine, we decided to biochemically characterize VvGwECar2. We expressed the recombinant enzyme fused with C-terminal His-tag into *E. coli* BL21, Rosetta and RIPL strains. RIPL gave the highest amount of protein visible on SDS-PAGE both in the soluble and in the insoluble fraction, so it was used for all the subsequent expressions. After purification with IMAC resin, each batch of the fusion protein was quantified with Bradford method (Bradford, 1976) and an aliquot was loaded on SDS-PAGE where it appeared with a band with a predicted molecular weight of about 67 kDa (Fig. 1).

To the purified recombinant VvGwECar2 protein we added farnesyl pyrophosphate (FPP) as the only substrate, since in functional assays other authors (Martin et al., 2010) reported no activity of this enzyme with geranyl pyrophosphate (GPP) or geranylgeranyl pyrophosphate (GGPP) that sometimes are accepted as alternative substrate although with a low efficiency (Cai et al., 2002). As frequently observed with other TPS enzymes, we found that more than one sesquiterpene product was formed (Fig. 2). Our enzyme catalyzed the cyclization of FPP to both β-caryophyllene and α-humulene, plus a small amount of germacrene D. Our GC-MS analyses on the pentane layer above the enzyme buffer provided an evidence of a 70:23,5:6,5 ratio (mass of component), for β-caryophyllene, α-humulene, and germacrene D, respectively. This is in agreement with what reported in Martin et al., 2010.

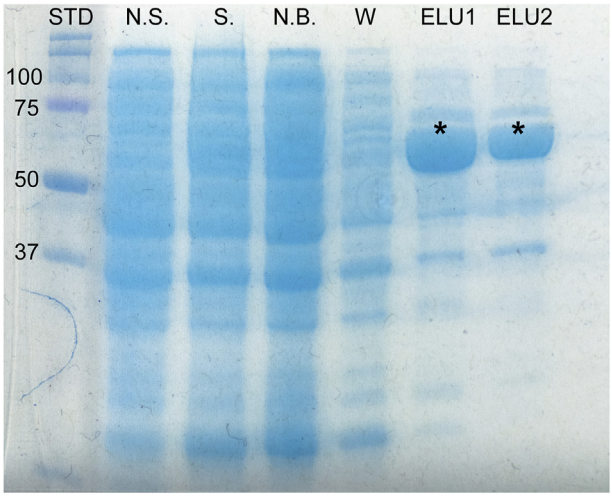

Fig. 1. SDS-PAGE from *E. coli* lysis to protein purification. STD = protein standard, N.S. = non-soluble total proteins, S. = soluble total proteins, N.B. = proteins not bound to IMAC resin, W. = protein washed out from resin, ELU1 and 2 = protein elution fractions. Asterisks indicate the band corresponding to *E*-(β)-caryophyllene synthase.

A substrate saturation and a time course were initially done to understand which concentration of the recombinant FPP was saturating under our conditions, and to estimate the length of the subsequent assays. The protein proved to be linearly stable even after overnight (15 h) reaction, and 100 μM FPP was chosen as a condition which would be saturating under any possible variation of enzyme quantity, due to variations in purification yields among the different batches (Fig. 3A, B).

The pH optimum was determined with assays from pH 4 to pH 9, with a peak of activity at pH 7.8, out of which the protein looses most of the catalytic activity (Fig. 3C). This result is in accordance with the values found for other TPS enzymes and suggests that the enzyme is cytosolic, which is not surprising considering that this sesquiterpene pathway derives from the mevalonate pathway, which typically occurs in the cytoplasm. The values for K_m (31.4 μM), k_{cat} (0.19 s^{-1}) and efficiency (k_{cat}/K_m 6.1 mM^{-1} s^{-1}) differ significantly from the ones reported for sesquiterpene synthases characterized so far. Further experiments are needed to clarify if this is a general property of grapevine TPS or not.

2.2. Plant VvGwECar2 overexpression

It is generally known that *in vitro* conditions can be very different from *in vivo* conditions, in terms of cofactors presence/absence, substrate concentration, pH and post-translational modifications. For this reason, we decided to carry out *VvGwECar2* overexpressions in plants, and check for headspace sesquiterpene production *in vivo*. *A. thaliana* has been proven to emit sesquiterpenes under genetic transformation when enzymes were targeted in the plastids (Aharoni et al., 2003) and mitochondria (Kappers et al., 2005), demonstrating that a pool of FPP exists in both cell compartments. However, in grapevine the main localization of FPP biosynthesis is the cytoplasm, with C_5 monomers isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) being supplied both from the cytosolic mevalonic acid (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway (Schwab and Wüst, 2015). For this reason, sesquiterpene synthases are believed to be cytosolic enzymes, although recently in grape berries a bifunctional nerolidol/linalool synthase was found to be

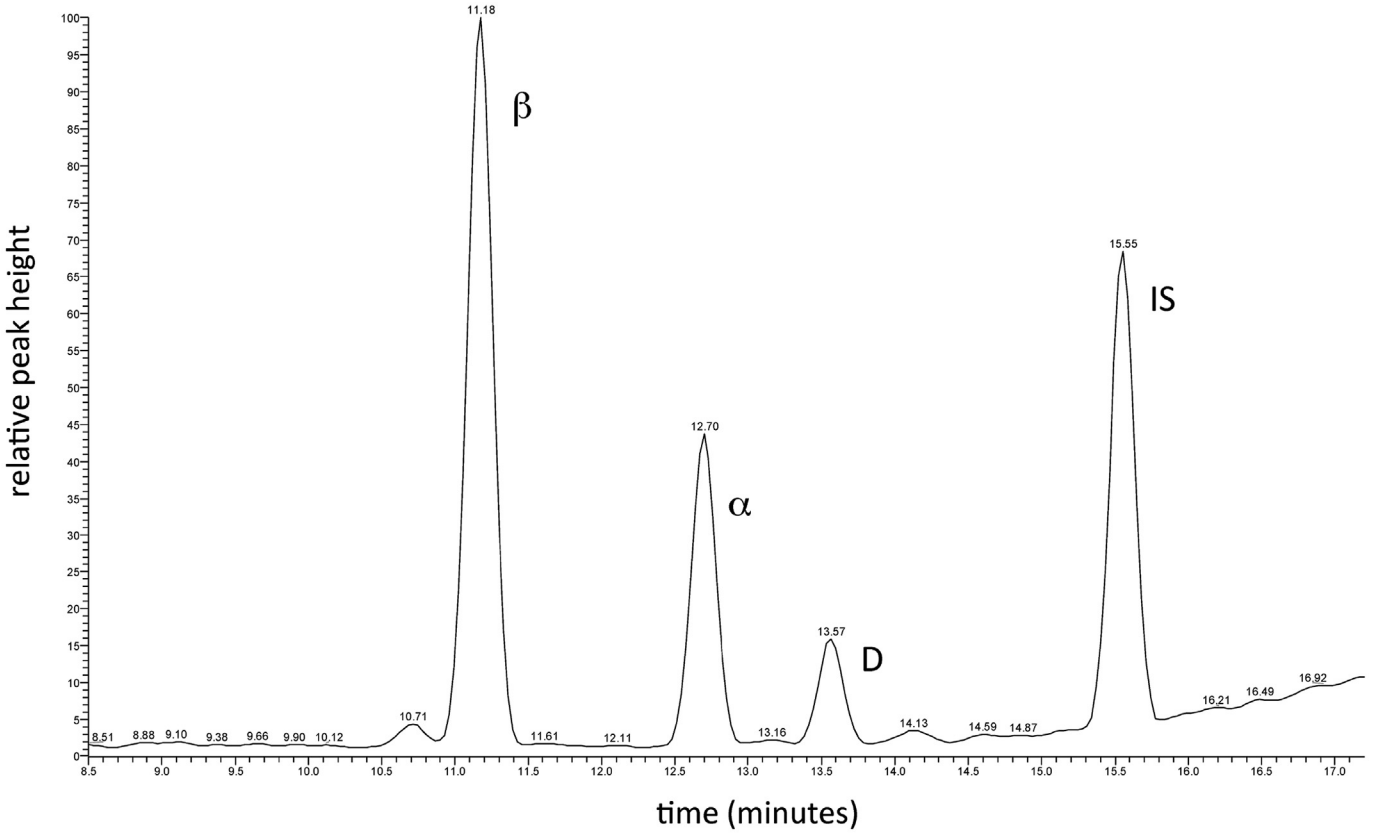

Fig. 2. Chromatogram of an enzyme assay. The three products and the internal standard are visible. β = *E*-(*β*)-caryophyllene, α = humulene, D = germacrene D, IS = internal standard.

located only in the plastid and acting as a linalool synthase *in vivo* (Zhu et al., 2014). Given that *VvGwECar2* protein sequence apparently lacks any import signal, and that its subcellular localization prediction is cytoplasmic (SherLoc, Shatkay et al., 2007), we considered our protein expression very likely to be cytosolic.

The coding sequence of the gene was moved without his-tag into pB2WG7 plant overexpression vector (Karimi et al., 2007) under the control of cauliflower mosaic virus (CaMV) 35S promoter, and used to transform *Col-0* wild type plants. Screening of transformed lines were done with glyphosate selection on seeds, followed by PCR on genomic DNA to check for the presence of the insertion and also RT-PCR using total RNA from 7-day-old seedlings, to confirm the presence of the gene transcript (data not shown).

Several independent transgenic lines were obtained and grown in the greenhouse for four generations, until all the T-DNA insertions were at homozygosity. The plants were then analyzed for volatile organic compounds (VOCs) production with the closed loop stripping analysis (CLSA, Boland et al., 1984) and their emission was compared with that of control plants of the same age. In contrast with the behavior of the enzyme *in vitro*, we did not notice any trace of germacrene D production. On the other hand, a minor peak has been identified as thujopsene, which was never observed in the *in vitro* assays under all the conditions tested. We also observed a higher selectivity for *E*-(*β*)-caryophyllene production: the headspace of the modified plants was indeed dominated by α-humulene, thujopsene and *E*-(*β*)-caryophyllene, in a 1:0.75:5 mass ratio on average. However, we observed a great variation in sesquiterpenes quantity among the different lines, probably because of the difference in T-DNA copy number and expression. This variation seemed to affect mainly *E*-(*β*)-caryophyllene emission, while α-humulene and thujopsene were much less influenced (Fig. 4). In particular *E*-(*β*)-caryophyllene emission was estimated to range from 39 ng plant^{-1} day^{-1} to almost 100 ng plant^{-1} day^{-1}, determining a mass ratio variation from 1:0.75:3 to 1:0.75:8.

Compared to *Col-0* plants, whose emission range was much narrower, we observed an average of 8-fold increase in VOCs.

Finally, we inserted *VvGwECar2* into grapevine to obtain homologous expression, using the transformation protocol as described in Dalla Costa et al. (2014). Starting from 12 Petri dishes of embryogenic *calli* (var. "Brachetto Grappolo Lungo") we obtained after one year 20 independent transgenic lines growing on the selection marker, 10 of which were acclimatized in the greenhouse and analyzed for gene expression and sesquiterpene production. As controls, to exclude effects caused by the regeneration protocol, two independent lines obtained after transformation with just *nptII* gene were also tested. Overall, no differences in plant phenotypes or growth speed were observed. At the molecular level, expression level of the *VvGwECar2,* in both controls was very similar (Fig. 5), and kept as a reference for the overexpressing lines. In the overexpressing lines the expression variability was much higher than expected; indeed increase in expression levels ranged from 15-fold to 370-fold (Fig. 5). Such remarkable variability is unlikely caused by position effect of T-DNA on genome, while it is probably due to very different levels of chimerism in the obtained plants, a phenomenon that has been already observed and quantified in grapevine transformations previously (Dalla Costa et al., 2014).

Since a higher level of transcript does not necessarily lead to a higher level of active protein (Keurentjes et al., 2008), we decided to screen the plants with solid-phase microextraction coupled with gas-chromatography/mass spectrometry (SPME-GC-MS) to check

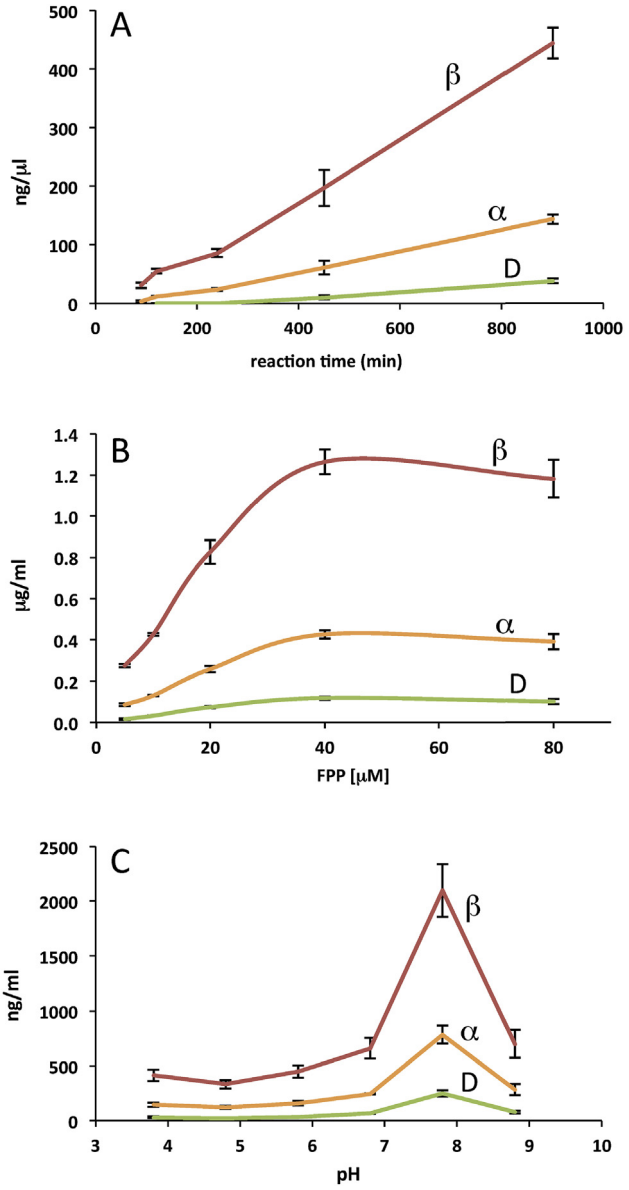

Fig. 3. Products concentration in the organic phase after enzyme assays, measured after a calibration line prepared with the pure standards (α, β) or relative to internal standard (D). (A) Linearity of the VOCs production after overnight reaction. (B) Substrate (FPP) saturation. (C) pH optimum. β = *E*-(*β*)-caryophyllene, α = humulene, D = germacrene D.

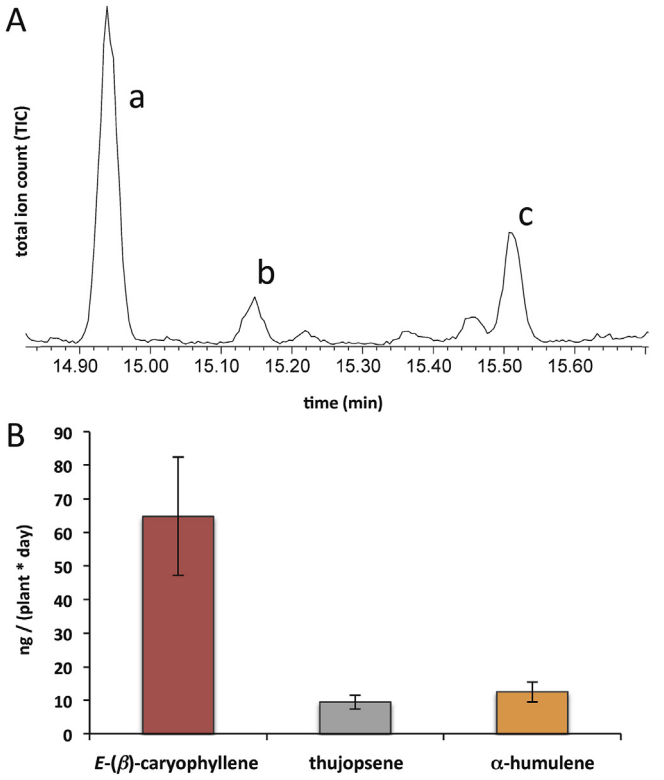

Fig. 4. GC-MS analysis of the headspace of transgenic *Arabidopsis* plants. (A) Chromatogram (*m*/*z* = 93) of the VOCs produced by *VvGwECar2* overexpression. The main peaks are *E*-(*β*)-caryophyllene (a), thujopsene (b) and α-humulene (c). The minor peaks are compounds released in low amounts from the plastic bag used to enclose the plants. (B) Differences of VOCs production among independent lines.

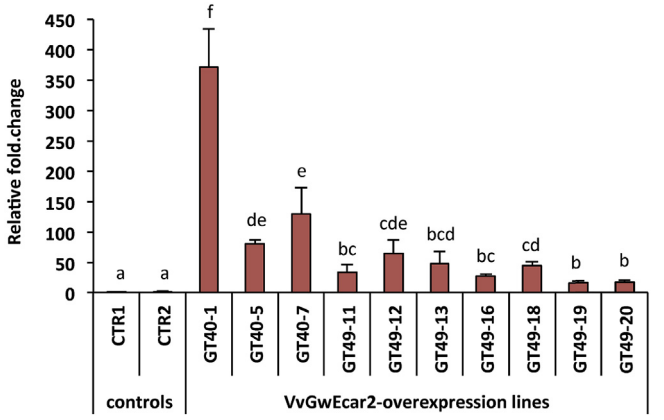

Fig. 5. *VvGwEcar2* relative expression obtained by RT-qPCR. Means of controls were set to 1. At least three biological replicates were used for each line. Letters on bars indicate different groups according to one-way ANOVA followed by Fisher LSD post hoc test ($P < 0.05$).

for changes in sesquiterpenes production compared to control plants (Fig. 6). SPME was chosen because it allows a very low detection threshold, considering that sesquiterpenes are usually not covering more than 1% of total amount of VOCs emitted by grapevine (Matarese et al., 2014). As expected, young leaves of control plants emitted *E*-(*β*)-caryophyllene in the range of 5 ng/g of fresh weight, while the transgenic lines showed a strong increase, ranging from an average of 7 up to 20 ng/g, 4-fold more. Also in this case we observed both a higher selectivity for *E*-(*β*)-caryophyllene production rather than α-humulene, and a high variability between lines. A similar variability was observed on the very small α-humulene content, which ranged from 0.5 ng/g to 1.5 ng/g (fresh weight) among the different lines, at most 3-fold more the wild-type plants. In contrast with Arabidopsis, we could not detect any trace of thujopsene: this sesquiterpene seems to be a byproduct of the enzyme outside the homologous expression system, and it was indeed never found in earlier grapevine headspace collections that we performed.

Linearity of SPME fiber's response within this range of concentrations was tested with a calibration line from pure standards, which was also used for the quantifications.

Surprisingly, there is almost no correlation between gene expression data and volatiles quantification in the transgenic lines, suggesting a deeper regulation of either the enzyme activity or the

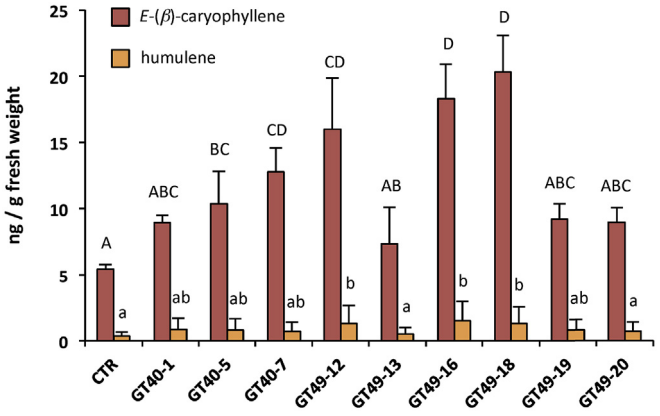

Fig. 6. Volatile collection and analysis by SPME-GC-MS from young grapevine leaves taken from acclimatized plants in the greenhouse. Leaves were grounded in liquid nitrogen and the obtained powder (at least 0.5 g) was weighted. Letters on bars indicate different groups according to one-way ANOVA followed by Fisher LSD post hoc test ($P < 0.05$).

substrate availability in the cytoplasm. For future works a co-expression of a FPP-synthase may lead to a more efficient flux of substrate in the pathway and thus a higher production of sesquiterpene, as suggested recently by the work of Bruce et al. (2015).

3. Conclusions

Despite being the plant with the largest TPS gene family known so far, very few TPS from *Vitis vinifera* have been extensively characterized so far (Lücker et al., 2004; Martin et al., 2010; Zhu et al., 2014; Drew et al., 2016). K_m and K_{cat} values for grapevine *E*-(β)-caryophyllene synthase appear indeed to be quite different from the ones reported for other sesquiTPS already characterized: further studies will clarify if this is a general propriety of grapevine TPSs or not. We noticed also differences in the enzyme behavior between three different conditions: *in vitro*, in plant in a heterologous system and in plant in the homologous system; this differences were both in different secondary products formation and in their relative abundance. Furthermore, we confirmed that a crucial role in defining the quantity of volatiles is played by the regulation of the flux in the pathway, since a great increase in one gene activity is not leading to an equivalent increase of the final product.

The availability of the genetic and metabolic tools presented in this work would accelerate also the creation of basic knowledge on the mechanisms underlying either production of volatiles in grapevine or host plant selection by phytophagous insects. This knowledge is the prerequisite to interfere with chemical, physiological and behavioural mechanisms involved in the *Vitis*-phytophagous insects, such as *L. botrana*, interactions and to develop novel sustainable control techniques (Ioriatti et al., 2011; Cattaneo et al., 2014). As a consequence, our study will pave the way to new possibilities for the control of the grapevine insect pests based on the manipulation of the semiochemicals scenario used by the insects during grapevine location.

4. Experimental

4.1. Cloning of the E-(β)-caryophyllene synthase into plant over-expression vectors

The coding sequence of the isoform VvGwECar2 was kindly provided by Prof. Bohlmann's laboratory into the bacterial expression vector pET28b+ with C-terminal His-tag. The vector was used as a template to amplify the coding sequence of the enzyme with Phusion High-Fidelity DNA-Polymerase (New England Biolabs) using the forward primer "attB-bCar for" (5′ – GGGGACAAGTTTG TACAAA AAAGCAGGCTTAA-CAATGTCTGTTCAGTCTTC AGTGG – 3′) and the reverse primer "attB-bCar rev" (5′ – GGGGACCACTTTGTACAAGA AAGCTGGGTT-CATATTGGCACAGAATCTATAAGC – 3′). PCR product was cloned directly into pDONR221 via BP-clonase reaction (Invitrogen), and transformed into *E. Coli* DH5α chemically competent cells. Plasmid minipreps from single colonies grown in 5 ml cultures were sequenced to check for their quality using M13(-20) for (5′ – GTAAAACGACGGCCAG – 3′) and M13 rev (5′ – CAGGAAA-CAGCTATGAC – 3′) primer pair. The coding sequenced was then moved into the gateway destination vectors pK7WG2D and pB2WG2 (Karimi et al., 2007) via LR reaction (Invitrogen). 1 μl of the reaction was used to transform chemically competent *E. Coli* TOP10 cells (Invitrogen), then plasmid minipreps from single colonies grown in 5 ml cultures were sequenced to check for their quality using my35Sprom for (5′ – CCACTA TCCTTCGCAAGACCC – 3′) and my35Sterm rev (5′ – GAAGTATTTTACAAATACAAA TACA-TACTAAGG – 3′) primer pair.

4.2. Heterologous expression and purification of E-(β)-caryophyllene synthase in E. coli

The plasmid pET28b+ containing the coding sequence of VvGwECar2 was transformed into chemically competent BL21-CodonPlus(DE3)-RIPL *E. coli* cells (Agilent Technologies) with heat shock method. A single colony was used to inoculate 5 ml of LB medium containing kanamycin (50 mg/L) and chloramphenicol (25 mg/L) at 37 °C overnight. 200 μl of this culture was added to 100 ml of fresh LB containing kanamycin and chloramphenicol at proper concentrations. Bacteria were grown at 28 °C until $OD_{600} = 0.4$, then they were grown at 16 °C until OD_{600} reached 0.7. Induction was achieved by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) 0.5 mM to the ice-cooled culture. Cells were maintained for 20 h at 16 °C and then harvested by centrifugation (2000*g* at 4 °C, 15 min).

Cell pellet was suspended in 2 ml in lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% v/v glycerol, pH 7.75), then 3 μl of a 0.1 M phenylmethanesulfonyl fluoride PMSF (Sigma) solution were added to prevent proteolysis during the next steps. Bacteria cells were treated with three freeze-thaw cycles of 5 min in liquid nitrogen and 5 min at 42 °C, 1 h of incubation at 4 °C with lysozyme (Sigma) 1 mg/ml, and shaked with TissueLyserII (Qiagen) using 0.5 g of glass beads (Sigma) and four cycles of 30 s at 30 Hz.

The cell lysate was treated with DNaseI (1 μg/ml) for 30 min at 37 °C to reduce its viscosity and centrifuged at 4 °C at 13,000*g* for 5 min. The pellet was trashed while the supernatant, containing the soluble proteins, was used for purification, SDS-PAGE and protein quantification.

The soluble fraction of cell-lysate was combined with 80 μl of Profinity IMAC resin (Bio-Rad) and tilt-shaked at 4 °C for 1 h to bind the his-tagged protein. This was followed by two washing steps with 500 μl of washing solution (5 mM imidazole, pH 6.3) and three elution steps with 50 μl elution solution (0.3 M NaCl, 50 mM NaH_2PO_4, 0.4 M imidazole, pH 8.0). All the fractions were analyzed on glycine-SDS-PAGE and stained with Coomassie Brilliant-Blue (Bio-Rad). The three elution steps were mixed into one, and protein concentration was determined with the Bradford method: enzymes was used directly into assays, without being stored.

4.3. Enzyme assay

Each enzyme assay was done with the same batch of freshly-purified enzyme, 3 µl (about 0.5–1 µg, depending on the batch) per reaction in 2 ml glass vials (Agilent) in a total volume of 200 µl. Standard reaction buffer was 50 mM HEPES, 10 mM $MgCl_2$, 20 µM $MnCl_2$, 2 mM DTT, 10% v/v glycerol, pH 7.0, and reaction were carried out at 30 °C. To trap the volatiles produced, 200 µl of a pentane layer with 10% isooctane was put above the reaction. Cuparene was used as internal standard (IS), at a final concentration of 1 mg/L. Reactions were stop removing the pentane layer with a glass Pasteur pipette and transferring it into a new 2 ml glass vial. Time course was done at 0min, 90 min, 120 min, 240 min, 450 min and 900 min using 100 µM farnesyl pyrophosphate (FPP, Sigma) as substrate. pH optimum was determined from 4.0 to 9.0 after overnight reaction. K_m and K_{cat} values were determined after 11 h of reaction with FPP at 2.5 µM, 5 µM, 10 µM, 15 µM using Hanes plot. The values of enzyme concentration was derived from Bradford assay done on the elution batch before starting each enzyme assay, and the size of protein was estimated to be 67 kDa (including his-tag). Three technical replicates were conducted for each reaction.

4.4. Analysis of the products

A CTC CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a 10 µL syringe was used to inject the volatiles from the vials. 1 µL of sample was injected in splitless mode into a Trace GC Ultra Gas Chromatograph coupled to a Quantum XLS Mass Spectrometer (Thermo Scientific, Electron Corporation, Waltham, MA). Compounds were separated using a VF-Wax® column (100% polyethylene glycol; 30 m × 0.25 mm × 0.25 µm, Agilent J&W Scientific Inc., Folsom, CA). The GC oven parameters were as follows: initial temperature was 70 °C, maintained for 1 min, followed by an increase to 130 °C at a rate of 5 °C min^{-1}, the oven was then maintained at 130 °C for 1min, then a rate of 20 °C min^{-1} until 230 °C for 1 min; splitless time of 0.8 min and GC inlet temperature of 250 °C. Helium was used as carrier gas in constant flow mode at 1.2 mL min^{-1}. The total cycle time was 20 min. The MS detector was operated in SIM mode (*m/z* 45, 69, 93, 119, 132, 133, 145, 161) and in scan mode (mass range 40–450 *m/z*) with a 0.2 s scan time and the transfer line to the MS system was maintained at 250 °C. Products were identified by comparing the mass spectra with those found in the NIST database, and by injection of standards when available. Quantifications were performed after calibration lines were prepared from the same standards.

4.5. Grapevine transformation

The vector pK7WG2D containing *VvGwECar2* coding was use to transform via *Agrobacterium tumefaciens* embryogenic *calli* of *V. Vinifera* (variety "Brachetto Grappolo Lungo") as described in Dalla Costa et al., (2014). Transgenic plants were propagated and maintained *in vitro* until acclimatization in the greenhouse.

4.6. Arabidopsis thaliana transformation

The vector pB2WG7 with constitutive CaMV 35S promoter driving *VvGwECar2* expression was used to transform *A. thaliana* Columbia 0 plants via the floral dip method (Clough and Bent, 1998). Transformed plants were screened on solid MS media (Duchefa) with sucrose 15 g/L and 2,2 g/L Basal Salts including vitamins and supplemented with glyphosate (Sigma) 25 mg/L. After one week the resistant lines, bigger than wild-type and with visible first root, were transferred into soil and let grow in the greenhouse for four generations, until nearly 100% of the seeds were homozygous for T-DNA insertion.

4.7. RNA extraction and RT-qPCR

RNA was extracted with Spectrum™ Plant Total RNA kit (Sigma) from 7-day-old *Arabidopsis* seedlings and from young grapevine leaves in the greenhouse (second and third internode below the apex) following the manufacturer's guidelines. RNA quality and quantity was checked on spectrophotometer and 1% agarose electrophoresis before cDNA was retro-transcribed using SuperScript® III Reverse Transcriptase (Invitrogen) with Random Primers. A specific primer pair able to discriminate *E*-(β)-caryophyllene synthase from other TPS genes in the grapevine family (bCarRT for 5′ - GAAGTGAGGAAGATGCTAATGG – 3′ and bCarRT rev 5′ – AACGGAACCATCGAACATG – 3′) was created using the online tool Primique (Fredslund and Lange, 2007; http://cgi-www.daimi.au.dk/cgi-chili/primique/front.py), the pair had an efficiency of 91%. The qPCR was performed on an CFX96 thermocycler (Bio-Rad) using GAPDH and Actin (Reid et al., 2006) as reference genes. Real-time PCR was carried out with the following cycle: 95 °C 10′; 40 × (95 °C 30″, 60 °C 30″); The manufacturer's software (CFX Manager, Bio-Rad) was used to calculate primer pair efficiency (E) and the threshold cycle (Ct) mean and standard deviation for each sample. The analysis of relative quantification was performed using the workflow reported in Hellemans et al., 2007. For each primer pair used, Ct from control lines were used as reference to calculate the ΔCt. The geometric mean between the value E^ΔCt from Actin and GAPDH were used to calculate a normalization factor (NF), which was finally used to calculate the fold difference according to the formula: Fold Difference = E^ΔCt/NF.

4.8. Headspace collection and analysis from Arabidopsis plants

Headspace volatiles of *A. thaliana* plants were collected using the close-loop stripping analysis (CLSA) method (Boland et al., 1984; Abraham et al., 2014). Eight plants in five weeks old were put into a closed plastic cooking bag (45 × 55 cm, Toppits, Melitta, Sweden) where an air pump created a flow of activated-charcoal-cleaned air at 0.4 L/min. The air flow hit an adsorbent filter (ORBO 1103, Porapak Q (50/80) 150/75 mg, Supelco) for 6 h, before trapped compounds were eluted with 1 mL of dichloromethane, and concentrated under the hood 5 times. Three bags replicates were set up for each line tested. A Gerstel MPS autosampler was used to perform liquid injections of the extracts. A 7890A GC gas chromatograph (Agilent) coupled to a 5975D MSD mass spectrometer (Agilent) was used: compounds were separated using a HP-5MS column (5% phenyl methyl siloxane; 30 m × 0.25 mm × 0.25 µm, Agilent). The GC oven parameters were as follows: initial temperature was 50 °C, maintained for 1.5 min, then a rate of 7.5 °C min^{-1} until 250 °C maintained for 10 min; injection volume was 2 µl in splitless mode and with a GC inlet temperature of 280 °C. Helium was used as carrier gas in constant flow mode at 1.2 mL min^{-1}. The total cycle time was 38.17 min. The MS detector was operated in scan mode (mass range 20–400 *m/z*) and the transfer line to the MS system was maintained at 230 °C. Identification of the volatile compounds was made by injecting pure reference standards (Supplementary Fig. 1) and comparing retention index and mass spectra with the use of NIST database.

4.9. Volatiles collection and analysis from grapevine plants

Grapevine sesquiterpene collection was performed with a method adapted from Matarese et al., 2014. A CTC CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) with a single

magnetic mixer (SMM Chromtech) and SPME fibre conditioning station was used to extract the volatiles from the sample vial headspace. A Trace GC Ultra gas chromatograph coupled to a Quantum XLS mass spectrometer (Thermo Scientific, Electron Corporation, Waltham, MA) was used: compounds were separated using a VF-Wax® (100% polyethylene glycol; 30 m × 0.25 mm × 0.25 μm, Agilent J&W Scientific Inc., Folsom, CA). The GC oven parameters were as follows: initial temperature was 40 °C, maintained for 4 min, followed by an increase to 60 °C at a rate of 2 °C min^{-1}, the oven was then maintained at 60 °C for 1min, then a rate of 5 °C min^{-1} until 190 °C for 1 min and a rate of 10 °C min^{-1} until 230 °C maintained for 4 min; splitless time of 5 min and a GC inlet temperature of 250 °C. Helium was used as carrier gas in constant flow mode at 1.2 mL min^{-1}. The total cycle time was 50 min. The MS detector was operated in scan mode (mass range 40–450 *m/z*) with a 0.2 s scan time and the transfer line to the MS system was maintained at 250 °C.

SPME extraction was carried out with slight modification from the one described in Matarese et al., (2014). Half gram of leaf powder was added with 0.3 g of NaCl and 3 mL of freshly prepared citrate-phosphate buffer (0.1 M Na_2HPO_4, 50 mM citric acid, pH 5.0) and put into 20 mL glass headspace vials. Each sample was spiked with 50 μL of 2-octanol at 2.13 mg L^{-1} alcoholic solution as internal standard. Samples were kept at 60 °C for 20 min and then extracted for 35 min at 60 °C. The headspace was sampled using 2-cm DVB/CAR/PDMS 50/30 μm fiber from Supelco (Bellefonte, PA). The volatile and semi-volatile compounds were desorbed in the GC inlet at 250 °C for 4 min in splitless mode and the fibre was reconditioned for 4 min at 270 °C.

4.10. Data processing

For GC-MS data form the enzyme assay and SPME extractions, processing was carried out with XCALIBUR™ 2.2 software provided by the vendor. Identification of the volatile compounds was made by injecting pure reference standards (Supplementary Fig. 2) and comparing retention index and mass spectra with the use of databases NIST MS Search 2.0.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.08.002.

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