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Combining molecular and metabolomic analysis to evaluate transgenic *Vitis vinifera* plants expressing the *Vitreoscilla* haemoglobin (VHb)

L. Martinelli, I. Vaccari, L. Dalla Costa, V. Poletti, D. Masuero, and U. Vrhovsek

Fondazione Edmund Mach - Research and Innovation Centre, San Michele all'Adige, Italy

Summary

Transgenic *V. vinifera* 'Brachetto' plants expressing *Vitreoscilla stercoraria* haemoglobin gene (vhb) were obtained by transferring the pBI-VHb construct harbouring the *vhb* and the *nptII* genes, *via A. tumefaciens* EAH 105. From 18 distinct transgenic plantlets, 2 lines were randomly chosen and 11 and 14 plants respectively obtained by micropropagation were acclimated for 100 days in the greenhouse, together with 10 micropropagated plants of a wild-type control line. A data-driven assessment, aimed at evaluating all measurable low-molecular weight organic compounds in these transgenic plants was performed using UPLC-Q-TOF with an ESI interface. Comparisons between controls and transgenic lines and between the two transgenic lines detected significant differences in some metabolic patterns. For each line, a list of identified compounds was extracted from the 100 most significant biomarkers.

Key words: Gene transfer, metabolomics, UPLC/MS, *vhb* gene, *Vitis vinifera*, *Vitreoscilla stercoraria*.

Introduction

The *vhb* gene from *Vitreoscilla stercoraria* codes for a haemoglobin-like protein (VHb) with improved oxygen stress efficiency (Wakabayashi *et al.* 1986). Heterologous VHb expression in a wide variety of microorganisms enhanced growth rate, synthesis of various compounds and efficiency of metabolic pathways (Olano *et al.* 2008, Li *et al.* 2010) showing its role in oxygen intracellular diffusion and aerobic metabolism (Hofmann *et al.* 2009).

Vhb gene has been regarded as promising tool in metabolic engineering in industrial application for metabolite production from microorganisms (Bailey 1991, Zahng *et al.* 2007, Sanny *et al.* 2010). Moreover, when *vhb* gene was transferred to tobacco (Holmberg *et al.* 1997, Niklas *et al.* 1997), datura (Bülow *et al.* 1999), petunia (Mao *et al.* 2003), cabbage (Li *et al.* 2005), rice (Cao *et al.* 2004) and *Arabidopsis* (Wang *et al.* 2009), several intriguing physiological effects have been observed such as increased germination precocity, growth rate and synthesis of various compounds, suggesting its potential use to improve fine chemical production (Jokipii-Lukkari 2009, Wang 2009). VHb has also been shown to be an excellent breeding tool for the selection of hypoxia- and flooding-resistant plants in petunia (Mao *et al.* 2003) and for obtaining tolerance to submersion and reduced germination time in cabbage (Li *et al.* 2005).

Higher availability of cellular oxygen has been regarded as an explanation for the interesting results obtained in transgenic plants, being oxygen a limiting substrate in plant cell cultures and in enhanced metabolite synthesis (Zhang *et al.* 2007). Increased concentrations of oxygen as a consequence of *vhb* gene expression may involve a shift towards oxygene-related metabolism. However, the need for a more complex model to fully explain the regulation and action mechanism of VHb in the plant hosts is necessary as different results have been observed in various plants, such as in *Populus* spp. where plantlets expressing the *vhb* gene did not reveal significant variations in development habits and biomass production when compared to their wild-type counterparts (Häggman *et al.* 2003, Zelasco *et al.* 2006). Since heterologous *vhb* gene expression might produce controversial effects in the various plant species, beneficial role of the *vhb* gene should be analysed on a case-by-case basis (Jokipii-Lukkari *et al.* 2009).

Analysis of metabolite sets related to heterologous expression of the *vhb* gene would give a relevant contribution to the understanding of the role of this gene. Metabolomics has been successfully used in functional genomics to link gene sequences to functions and to characterise "silent plant phenotypes" or correlate functions with "orphan genes" (Hall 2006). Furthermore, relevant information has been obtained in studies of complex physiological perturbations, such as the response to biotic and abiotic stress, as in *Arabidopsis*, where a combination of transcriptomics with metabolomic approaches was able to detect marked changes in several primary metabolites (Nikiforova *et al.* 2004). In tomatoes, analysis of primary and secondary metabolites could also define the pleiotropic effects of a single gene mutation (Bino *et al.* 2005).

Metabolomics has also been successfully applied to analyse the function of specific genes in transgenic plants (Oksman-Caldentey and Saito 2005), in particular when no visible phenotypes were manifest, as in potato (Weckwerth *et al.* 2004) and tomatoes (Nunes-Nesi *et al.* 2005). Analysis of the overall metabolite composition of GM plants in comparison to conventional counterparts have also been proposed in risk assessment for detecting exogene unintended effects (Rischer and Oksman-Caldentey 2006).

Correspondence to: Dr. L. Martinelli, Fondazione Edmund Mach, IASMA Research Center, Via E. Mach, 1, 38010 San Michele all'Adige (TN), Italy. Fax: +39.0461.650872.

Current address: Museo delle Scienze, Via Calepina 14, 38122 Trento, Italy. E-mail: Lucia.Martinelli@mtsn.it

In grape, tissue culture and related applications, such as gene transfer, are generally hindered by low morphogenesis (MARTINELLI and GRIBAUDO 2009), thus enhanced metabolic activity would be an appealing trait to be transferred to this plant species in which increased cellular proliferation would be desirable. Furthermore, resistance to abiotic stresses, including drought and flooding, would be interesting, considering the different environmental conditions around the world for cultivation of this crop (CRAMER 2010).

Accordingly, *vhb* gene would provide grape with attractive traits and would be a significant case study for assessing the metabolic sets following its exogenous expression. Our study aims to evaluate the metabolomic changes of transgenic *V. vinifera* 'Brachetto' plants expressing the *vhb* gene as compared with the wild-type control. Our assessment was carried out integrating the molecular analysis with the metabolomic approach, being this latter an innovative aspect to be developed for a thorough transgenic plants evaluation.

Material and Methods

Plant material and gene transfer: Embryogenic calli of *V. vinifera* 'Brachetto', obtained and maintained as MARTINELLI *et al.* (2001) were co-cultured with *A. tumefaciens* EHA 105 carrying the pBI-VHb construct (ZELASCO *et al.* 2006), i.e. a pBI121-derived plasmid where the β-glucurunidase gene was replaced with a 440 bp fragment of the *vhb* gene from *Vitreoscilla stercoraria* (ATCC 15218). Gene transfer, antibiotic selection and plant regeneration were according to our protocols (DALLA COSTA *et al.* 2009, MARTINELLI *et al.* 1993). Putatively transgenic somatic embryos were recovered and germination was induced in 75 control and 225 transgenic ones, respectively. From these, emerging shoots were dissected and micropropagated. Twenty-one and 28 plantlets respectively from 2 lines resulting transgenic after molecular assays (C and F), and 28 plantlets from 1 wild-type line as control were acclimated *in vivo* in a growth chamber (94.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ cool white light and 16 h-light photoperiod, 25 °C and 70 % humidity): for each plant, the apical bud was dissected and planted on a 250 mL plastic glass with two 2 mm diameter holes on the bottom containing 50 g autoclaved turf and sealed with parafilm. During the following 60 d, holes were made in the latter until it was completely removed and glasses were kept on trays, where drops of water were provided daily. Then the plants were planted in 500 ml plastic pots containing 300 g "Terriccio Vegetal Radic" (Tercomposti SRL, Italy) with the addition of Master 15.5.30 (Valagro®, Chieti, Italy) for a further 3 months, watered weekly and sprayed with Switch® (Syngenta Crop Protection, Milano, Italy) Fludioxonil as an anti-fungicide treatment.

Molecular assays: Three and half months after beginning of acclimation, fully-expanded leaves were collected from each plant. Three of them were used for DNA and RNA assays and 10 for metabolomic analysis. DNA extraction was according to DALLA COSTA *et al.* (2010) and all the primers and probes were designed with Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

End-point PCR: End-point PCRs were performed according to DALLA COSTA *et al.* (2010) and primer pairs (Fw) 5'-TTGTTTGATATGGGTCGCCAAG-3' – (Rv) 5'-TTGACCGACAATCGGATAATGC-3' and (Fw) 5'-ACTGAAGCGGGAAGGGACTG-3' – (Rv) 5'-GGAGCGGCGATACCGTAAAG-3' were used to amplify a 177-bp sequence of the *vhb* gene and a 494-bp sequence of the *nptII* gene. *Agrobacterium* persistence was assessed by checking the amplification of a 542-bp sequence of the *wrbA* bacterial gene (primers: (Fw) 5'-ATGCGCACCCTGATAACGAGC-3' – (Rv) 5'-ACTCCTCTTACGGCCATATCG-3'). Bacterial lysate (95 °C, 10 min) of EHA105 pBI-VHb was used as control template. The PCR products were observed on 1.5 % agarose gels after Sybr Green staining (Invitrogen).

Southern blot: For Southern blots, specific probes for the *nptII* gene (734-bp) and the *vhb* gene (386-bp) were obtained according to Roche Diagnostics instructions with the PCR Digoxygenin labelling (PCR Dig Probe) Synthesis Kit using primer pairs (Fw) 5'-GATGGATTGCACGCAGGTTC-3' – (Rv) 5'-GGAGCGGCGATACCGTAAAG-3') and (Fw) 5'-CCTTCGCAAGACCCTTCCTCTA-3' – (Rv) 5'-ACCCAACAATTCTTGACCGACA-3'). For each sample, 10 μg of genomic DNA extracted from grape leaves were digested with the restriction enzyme *XmnI* (Promega) having a unique restriction site in the T-DNA sequence, outside the probe hybridization sites. Digestion products were precipitated, resuspended in 30 μl TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0) and electrophoresed on a 0.9 % agarose gel. Further membrane blotting and hybridization were performed following Roche users' manual. Autoradiographic film was exposed over night before development.

Qualitative RNA analysis: total RNA was extracted from leaves of the same plantlets used for Southern Blot assays, using the Qiagen RNeasy Kit according to the manufacturer's instructions. Retrotranscription was performed with the Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, following Invitrogen instructions. Each reaction contained 5 μg of template RNA, 200 ng of random primer examers and 1 μl of 10 mM dNTPs, in a total reaction volume of 20 μl. 2 μl of the obtained first strand cDNAs were used as amplification template in standard end-point PCRs (performed as above detailed) of the *vhb* and the *nptII* genes. The *Vitis* endogenous gene coding for actin (AF369525) was used as positive transcription control and amplified with primers (Fw) 5'-CATGCTATCCTTCGTCTTGAC-3' - (Rv) 5'-TCAGGCAGCTCATAGTTCTTC-3'.

Quantitative RNA analysis: Total RNA was extracted from the leaves of 9 plants acclimated *in vivo* from both transgenic lines C and F using the Spectrum Plant Total RNA kit (Sigma). Retrotranscription was performed with the Superscript III Reverse Transcriptase (Invitrogen) according to Invitrogen's instructions. Each reaction contained 5 μg of template RNA, 1 μL of 50 μM $oligo(dT)_{20}$ and 1 μL of 10 mM dNTPs, in a total reaction

volume of 20 µL. Quantitative Real-time PCR (qPCR) reactions were performed in duplicates in 96-well reaction plates on the iCycler iQ Thermocycler (Biorad) in a 25 µL final volume containing 100 ng cDNA, 1X SYBR GreenER qPCR SuperMix for iCycler instrument (Invitrogen), and 200 nM of each primer. The primer sequences for *nptII* and *vhb* exogenes were designed as following: *nptII*_for 5'-CTTGCCGAATATCATGGTGGAA-3', *nptII*_rev 5'-GGTAGCCAACGCTATGTCCTGA-3'; *vhb*_for 5'-AGCGCATTATCCGATTGTCG-3', *vhb*_rev 5'-ACGCGTCCAAAATGTCATCG-3'. The primers sequences of *actin* and the *gapdh*, chosen as reference housekeeping genes, have already been published (GATTO *et al.* 2008 and REID *et al.* 2006). The thermal protocol used was as follows: 2 min at 50 °C (UDG incubation), 10 min at 95 °C (UDG inactivation and DNA polymerase activation) followed by 40 cycles consisting of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing/extension). Amplicon specificity was checked at the end of the PCR reaction with melting curve analysis covering the range 55-95 °C. Gene expression data were analysed according to the relative quantification model published by Hellemans *et al.* (2007). For each amplification, the slope, PCR efficiency ($E = 10^{(-1/slope)}$) and respective standard errors were calculated by means of a linear regression curve generated from the Cq (quantification cycle) and quantity values of a cDNA dilution series. The Cq reference used for the conversion of Cq value in relative quantities (RQ) was the average Cq value of the Cqs obtained for all the samples amplified in the reaction.

Metabolomic analysis: Sample preparation: Leaves of 11 biological plant repetitions for each genetically modified line and control sample were used in the metabolomic experiment. To 0.2 - 0.3 g of each repetition, 40 mL of methanol/chloroform/water (10:3:1) and 50 µL of internal standard (4-hydroxystilbene, 200 mg·L^{-1}) were added. Each sample was blended in a food mill for 30 s and then centrifuged at 6,500 rpm for 7 min. Supernatant was filtered through 0.45 µm filter (Sartorius, Germany) and the filtrate was concentrated to a small volume (0.5 mL) under reduced pressure on a rotavapor (Büchi, Germany) at 38 °C, brought to a volume of 5 mL with methanol/chloroform/water (10:3:1), filtered through a 0.22 µm PVDF filter (Millipore, Bedford, MA) into HPLC vial and analysed using UPLC.

Analysis: The analysis was carried out as described in THEODORIDIS *et al.* (2012). Waters Acquity UPLC was coupled to a Synapt HDMS QTOF-MS (Waters, Manchester, UK) *via* an electrospray interface (ESI), operating in W-mode and controlled by MassLynx 4.1. An ACQUITY UPLC 1.8 µm 2.1 x 100 mm HSS T3 column (Waters) at 30 °C was eluted with a gradient program starting from 100% A (water, 0.1 % formic acid) from 0 till 6 min and then increasing linearly over 56 min to 100 % B (methanol, 0.1 % formic acid), then held isocratic until 60 min. The flow rate was 0.3 ml·min^{-1}. Samples (control, line C and line F) were randomised before being injected into the UPLC-HRMS in positive and negative ionisation mode. The injection volume was 5 µL and the samples were kept at 4 °C throughout the analysis. The data were processed with MassLynx software (Waters, Manchester, UK). For each line, a list of the 100 most significant biomarkers was extracted (VIP list) and compared to the spectral library developed in-house in order to identify the compounds on the base of their retention time (Rt) and accurate mass.

Results and Discussion

Transgenic plant recovery: Putatively transgenic *in vitro* cultures and transgenic *in vivo* plantlets were compared with the wild-type counterpart. Within a one year-period following gene transfer, the calli did not exhibit differences in colour, volume and hydration and no enhanced growth or greater efficiency in terms of embryo regeneration was achieved. However, as regards embryo conversion into plantlets, efficiency was slightly higher in putatively transgenic cultures (10 % as compared to 7 % for the control). In grape, besides canonical embryo germination (separation of cotyledons with shoots merging from apical meristem and root elongation from basal meristem), massive proliferation from each somatic embryo followed by regeneration of adventitious shoots it is not infrequent (MARTINELLI and GRIBAUDO 2009). In the present work, the tendency to regenerate via organogenesis rather than germination was more marked in putatively transgenic cultures as compared to controls.

One shoot from single embryos (the first regenerated in the case of organogenesis) was dissected and micropropagated. Twenty putatively transgenic lines were obtained and tested for the presence of the *vhb* and *nptII* genes. End-point PCR detected both genes in 18 lines whilst, as expected, no PCR products were found for the controls, and persistent *Agrobacterium* contamination was excluded, since no amplification of the bacterial *wrbA* gene fragment was observed in the plant DNA samples (not shown).

Southern Blot assays on 6 randomly chosen transgenic lines performed with a *vhb*- and a *nptII*-probe confirmed the integration of both exogenes in the plant genomes. For both exogenes, different copy number insertions were assessed. For the *vhb* gene (Fig. 1), a single copy insertion was found in lines A, B, C and E while two and three copy

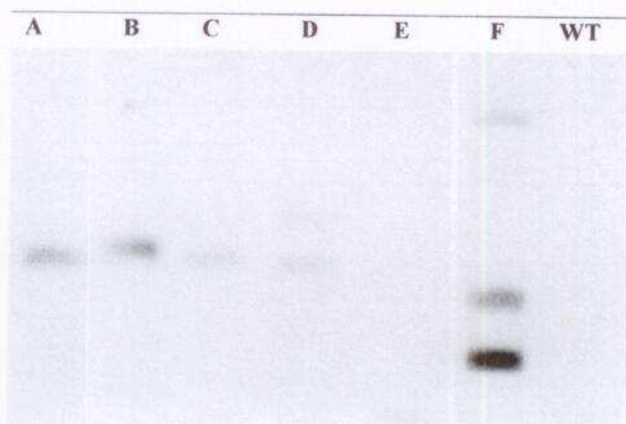


Fig. 1: Southern Blot analysis of the *vhb* exogene performed on 6 randomly chosen T_0 transgenic plants (lanes A-F) and a wild-type control (WT). DNA was digested with *XmnI* restriction enzyme.

insertions were found respectively in lines D and F. As for the *nptII* gene, single insertions were obtained for lines C and D, whilst two insertions were present in lines A, B, E and F (not shown).

In 4 lines, the expression of *vhb* and *nptII* genes was assessed after amplification of the cDNA obtained from the total RNA (Fig. 2). As expected, the *vhb* and *nptII* genes were expressed in the transgenic samples and in the bacterial lysate, but not in the wild-type control (Fig. 2A, 1B).

During micropropagation, the roots of the transgenic plants showed features we never observed in the wild-type controls or in the several transgenic grape plantlets obtained with various exogenes during many years of experience. The roots of *vhb* plantlets were visibly thicker and had an extremely high production of secondary roots and swollen tips (Fig. 3).

Exogene expression and metabolomic profiles in *in vivo* plants: Two lines (C and F) (respectively 21 and 28 replicates) and a wild-type control (28 replicates) were acclimated and kept in a greenhouse for 150 d to characterise the *vhb*-expressing grapevines in *in vivo* conditions. Some plants did not overcome the acclimation phase and the percentages of surviving plants were 19 %, 21 % and 14 % respectively for line C, line F and the control. Although we do not consider the number of samples adequate for conclusive evaluation, the phenotypes of wild-type and transgenic plants were visibly different, being these latter shorter and more branched and having shorter internodes (Fig. 4), features more evident in line F than in line C. Leaves from the surviving plants were collected for parallel analysis of exogene expression and metabolomic profiles.

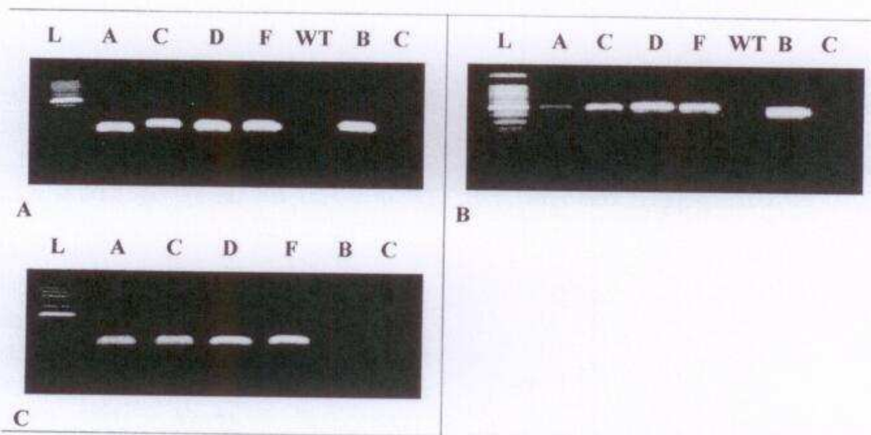


Fig 2: Qualitative expression analysis of *vhb* (**A**) and *nptII* (**B**) exogenes and actin endogene (**C**) in 4 transgenic lines (**A, C, D, F**). L = ladder (Promega 100-bp); WT = wild type plant; B = bacterial lysate; C = negative control (bidistilled sterile water).

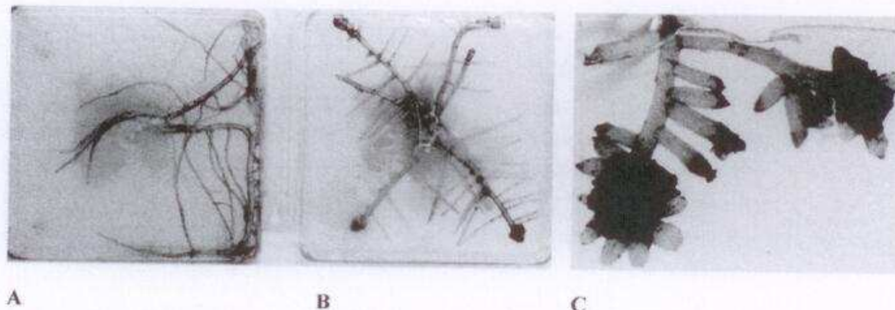


Fig. 3: Aspect of roots during micropropagation. Examples of root features during a 2 month micropropagation period for the control (**A**) and transgenic (**B**) plantlets. Roots of VHb plantlets were thicker, overproduced secondary roots, had swollen tips, often noticeably, as in **C**.

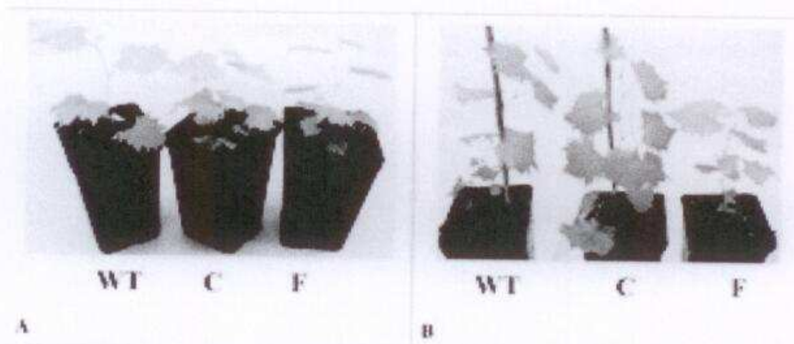


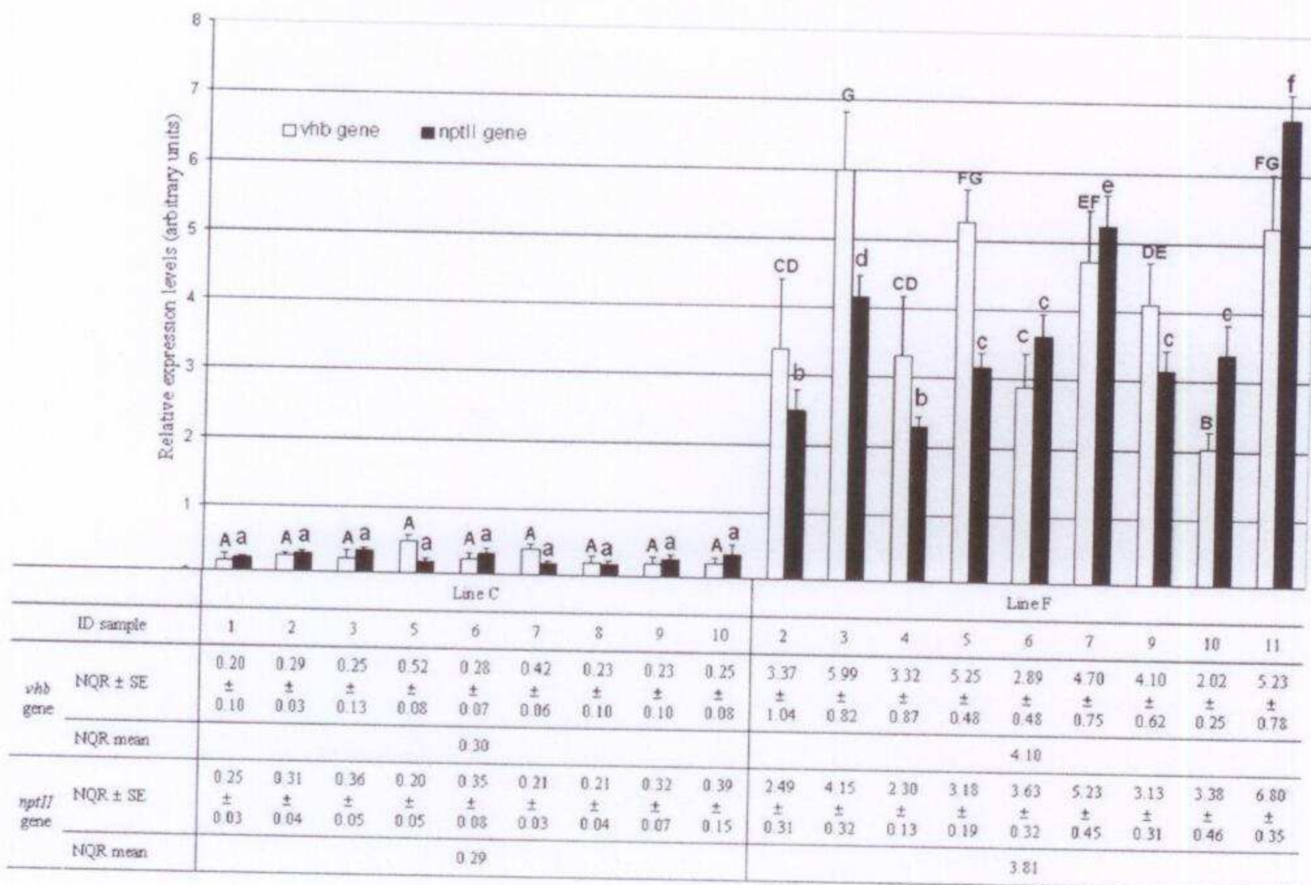
Fig. 4: Comparison of representative plants of the wild-type (WT) control and transgenic lines C and F after 79 (**A**) and 104 (**B**) days of acclimation in the greenhouse.

Vhb and *nptII* gene expressions were evaluated on 9 biological replicates of the two transgenic lines, C and F (Fig. 5). Worth stressing, the transcript profiles of the two exogenes were evaluated independently, as this analysis is suitable for comparing the expression of single specific genes in a group of samples. Accordingly, it is irrelevant that *vhb* and *nptII* are regulated by different promoters, respectively CaMV35S and NOS.

The normalised relative quantity (NRQ) of both exogene transcripts was shown to be 13 times higher in line F than in line C and, according to ANOVA ($P < 0.001$) and the Newman-Kleus post-hoc test, the 9 biological replicates of line C were all very significantly different from the replicates of line F, as shown by different letters. This finding may be considered to be an outcome of the higher level of exogene integration obtained for lines F (3 and 2 copies respectively for the *vhb* and *nptII* genes) as compared to the single-copy insertion for both exogenes detected in line C.

Furthermore, significant differences in both exogene expressions were found between replicates of line F, despite the fact that they were grown in the same controlled conditions (Fig. 5), possibly because of chimerical exogene integration within the mother plant tissues (DALLA COSTA *et al.* 2009; FLACHOWSKY *et al.* 2008) or epigenetic variations induced by environmental stimuli (DOWN *et al.* 2001; BOYKO *et al.* 2010). Lines C and F and the wild-type counterpart were subjected to metabolomic analysis. We successfully applied a holist-metabolomic approach to obtain a more in-depth understanding of the function of the heterologous VHb protein, such as up- and down-regulation of metabolites related to *vhb* expression (ZHANG *et al.* 2007, JOKIPII-LUKKARI *et al.* 2009). Our results are the outcome of a sound analysis, as 11 biological repetitions were performed for each variant.

The total number of features (the amount of signals associated to a specific compound) detected using an untargeted metabolomic approach is reported in Tab. 1. Considering all features, comparing line C to the control, 45.7 % (negative ionisation mode) and 46.1 % (positive ionisation mode) were shown to be up-regulated, whilst the percentage of up-regulated features was slightly lower for line F as compared to the control (37.6 % and 41.6 % for respectively negative and positive modes). Moreover, a comparison between the two transgenic lines has been carried out to verify if the different VHb expression (*i.e.* 13 times higher in line F than in line C, Fig. 5) would strongly affect their metabolomic profiles. Actually, line F showed a percentage of up-regulated features of 41.2 % (negative) and 44.5 % (positive) compared to line C. A similar trend has been found when the 100 most significant biomarkers-VIP (the most modulated ones) have been evaluated (Tab. 2). VIP Identification was performed using a database created in-house where retention times, exact masses and mass spectra of standards were compared to those of the sam-



		Line C									Line F								
	ID sample	1	2	3	5	6	7	8	9	10	2	3	4	5	6	7	9	10	11
vhb gene	NQR ± SE	0.20 ± 0.10	0.29 ± 0.03	0.25 ± 0.13	0.52 ± 0.08	0.28 ± 0.07	0.42 ± 0.06	0.23 ± 0.10	0.23 ± 0.10	0.25 ± 0.08	3.37 ± 1.04	5.99 ± 0.82	3.32 ± 0.87	5.25 ± 0.48	2.89 ± 0.48	4.70 ± 0.75	4.10 ± 0.62	2.02 ± 0.25	5.23 ± 0.78
	NQR mean	0.30									4.10								
nptII gene	NQR ± SE	0.25 ± 0.03	0.31 ± 0.04	0.36 ± 0.05	0.20 ± 0.05	0.35 ± 0.08	0.21 ± 0.03	0.21 ± 0.04	0.32 ± 0.07	0.39 ± 0.15	2.49 ± 0.31	4.15 ± 0.32	2.30 ± 0.13	3.18 ± 0.19	3.63 ± 0.32	5.23 ± 0.45	3.13 ± 0.31	3.38 ± 0.46	6.80 ± 0.35
	NQR mean	0.29									3.81								

Fig. 5: Expression levels of *vhb* and *nptII* genes in 9 biological replicates of lines C and F. NRQ: Relative Quantity Normalized to the geometric mean of two control genes (*gapdh* and *actin*). Error bars show the standard error (SE) of the NRQ values calculated according to the propagation of error (HELLEMANS *et al.* 2007). Expression data were evaluated with analysis of variance and Newman-Keuls post-hoc test (different letters identify significantly different groups) using STATISTICA software (StatSoft) version 9.1.

Table 1

Total number of extracted features and percentages of up-regulated features for lines C and F as compared to the control and line F as compared to line C

	No. of up-regulated features	Total number of features	% up-regulated
C *vs.* control			
negative	1104	2418	45.66
positive	2297	4979	46.13
F *vs.* control			
negative	905	2409	37.57
positive	2074	4983	41.62
F *vs.* C			
negative	882	2139	41.23
positive	1821	4094	44.48

Table 2

Percentage of up-regulated features for lines C and F as compared to the control and line F as compared to line C in the first 100 more significant biomarkers

	No. of up-regulated features	Total number of features
C *vs.* control		
negative	48	100
positive	42	100
F *vs.* control		
negative	25	100
positive	29	100
F *vs.* C		
negative	28	100
positive	23	100

Table 3

Top ten up- and down-regulated features

C *vs.* control	Rt (min)	Mass	F vs control	Rt (min)	Mass	F vs C	Rt (min)	Mass
negative mode			negative mode			negative mode		
up-regulated	20.7	379.1598	up-regulated	55.69	1183.533	up-regulated	36.28	679.1958
	11.47	631.1132		52.38	685.2995		36.28	453.1326
	1.09	563.8246		27.80	483.2137		29.28	227.0700
	55.69	1183.533		57.27	819.5299		33.12	453.1341
	34.29	465.2673		1.59	320.9967		50.21	311.1685
	55.68	681.2314		27.38	445.2057		48.94	721.3652
	14.75	373.1137		55.68	681.2314		46.53	517.3529
	1.07	433.8667		15.30	495.0432		48.94	675.3605
	24.07	525.1979		55.69	613.2446		52.17	481.2572
	26.41	509.2234		15.30	479.0774		15.61	477.1231
down-regulated	60.09	896.5637	down-regulated	57.63	1548.07	down-regulated	18.27	611.1389
	17.54	175.0599		1.49	387.1111		15.95	881.1939
	59.50	808.5151		55.30	849.4948		16.80	881.1927
	50.70	469.3327		31.07	515.078		14.09	611.1403
	56.68	762.9679		23.09	429.2104		45.28	319.1380
	56.57	764.9586		30.68	433.0744		44.89	319.1388
	2.41	87.0077		59.50	808.5151		13.43	609.1238
	12.56	685.1199		45.29	361.1700		15.19	897.1890
	60.53	503.4127		60.09	896.5637		12.07	309.1192
	56.58	130.9432		56.68	762.9679		18.15	289.0692
positive mode			positive mode			positive mode		
up-regulated	1.33	187.0034	up-regulated	13.91	129.1077	up-regulated	13.9	189.1528
	1.34	216.0374		1.33	187.0034		17.73	231.2071
	12.07	284.1048		56.20	557.6008		18.77	333.1524
	56.89	645.2469		56.19	575.6129		48.94	699.3555
	51.97	311.1663		55.67	1185.547		48.94	358.1624
	1.36	202.0732		1.36	202.0732		55.44	393.2957
	55.67	1185.547		1.50	455.1164		13.99	144.1028
	14.73	413.0857		55.67	673.2914		13.99	102.0558
	55.68	592.2688		55.68	592.2688		55.67	1185.5470
	1.07	96.9726		56.89	645.2469		54.60	315.2292
down-regulated	51.35	131.0863	down-regulated	28.75	410.0361	down-regulated	14.08	307.0815
	59.04	866.525		53.77	541.3857		13.57	595.1445
	61.05	404.7613		51.59	309.1857		17.40	323.1097
	60.08	806.5679		53.78	380.2012		12.07	287.1164
	43.15	265.1778		57.34	769.4647		13.46	289.1244
	59.37	770.5709		60.09	410.2625		39.03	225.1950
	28.75	410.0361		53.71	330.2781		39.03	247.1770
	54.83	265.2589		24.56	687.0767		39.03	471.3655
	59.04	1564.138		56.68	263.2371		39.03	449.3831
	59.49	730.5397		57.42	512.3065		14.21	144.1012

ples. The top 10 up- and down-regulated compounds for each combination are reported in Tab. 3. Of the list of 100 most significant biomarkers, we were able to confirm the identification of 7 compounds for line C and 11 for line F, as reported in Tab. 4. Most of these were down-regulated.

Similarly, in hybrid aspen, the concentration of quercetin derivatives and apigenins was found to be lower in VHb lines as compared to the control (HÄGGMAN *et al.* 2003). In *Arabidopsis*, on the contrary, it was shown that the *vhb* transgene did not change the chemical composition but affected the content of at least 10 secondary metabolites, 2 of them being shown to have significantly increased (WANG *et al.* 2009). Finally, in C and F lines we found the presence of 3 common compounds, namely quercetin 3-rutinoside, isohramnetin 3-rutinoside and methyleptecanoate (Tab. 4, underlined).

The remaining biomarkers were unidentified. This fact is yet the main constraint of untargeted plant metabolomics, as structural elucidation of the majority of plant metabolites is expected to take years before being identified. Furthermore, the lack of the commercially available standards is a major drawback for identification of biomarkers. As a rule, unidentified compounds were labelled as "known unknowns" and with the integration of databases, some of these will be identified in the near future, as an increase in metabolomic studies will produce the knowledge necessary for recognising key molecular events related to specific gene expression.

Conclusion

In our study, metabolomics was successfully applied to assess plants produced from two distinct lines of *V. Vinifera* transgenic plants expressing the heterologous *Vhb* gene. Comparing controls to transgenic lines and comparisons between the two transgenic lines detected significant differences in some metabolic patterns. For complete recognition of all pathways which might be influenced by *vhb* gene expression, further studies and greater knowledge are needed. Indeed, at present, many metabolic compounds are still unknown, whereas their identification is still required in order to recognise the metabolic pathways in which they are involved. This type of research results of great interest, offering the chance to study the behaviour of the biological system as a whole (holistic approach) and providing additional data and insights to our current knowledge.

Moreover, the synergy of molecular genetics and metabolomics is emerging as a significant biological research methodology in terms of identifying gene functions. In some plant species this approach has already been pursued for analysing the genes involved in metabolic changes, whilst in grape, it is still at an early stage. We hope that our study will make a useful contribution towards the development of valuable analytical tools for functional genomics studies for this important fruit crop.

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Table 4

Compounds identified among 100 VIPs

	Mass	Compound	Ionisation mode	
Line C vs. control Rt (min)				
1.24	106.0506	Serine	positive	down-regulated
1.31	120.0666	threonine, homoserine	positive	down-regulated
29.17	609.1476	quercetin 3 rutinoside	negative	down-regulated
30.23	303.0146	ellagic acid	positive	up-regulated
31.28	593.1493	kaempferol 3-rutinoside	negative	down-regulated
31.72	623.1604	isorhamnetin 3-rutinoside	negative	down-regulated
58.71	285.2780	Methyleptadecanoate	positive	down-regulated
Line F *vs.* control Rt (min)				
1.41	135.0294	L-threonic acid	negative	down-regulated
1.49	341.1074	Disaccharide	negative	down-regulated
1.49	387.1111	Threalose	negative	down-regulated
15.30	311.0393	trans-caftaric acid	negative	down-regulated
29.17	609.1476	quercetin 3 rutinoside	negative	down-regulated
31.07	447.0908	kaempferol 3-glucoside	negative	down-regulated
31.04	303.0502	morin dihydrat	positive	down-regulated
31.72	623.1604	isorhamnetin 3-rutinoside	negative	down-regulated
53.12	253.2172	palamitoleic acid	negative	up-regulated
56.68	263.2371	ethyl linoleate	positive	down-regulated
58.71	285.2780	Methyleptadecanoate	positive	down-regulated

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