Molecular reprogramming in grapevine woody tissues at bud burst

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Authors: Henrique Noronha[1,2], Virginie Garcia[3], Angélica Silva[1,2], Serge Delrot[3], Philippe Gallusci[3*], Hernâni Gerós[1,2,4]

Affiliations:

[1] Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, Braga, Portugal.
[2] Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes e Alto Douro, Vila Real, Portugal.
[3] UMR EGFV, Bordeaux Sciences Agro, INRA, Université de Bordeaux, 210 Chemin de Leysotte, CS 50008, Villenave d'Ornon 33882, France.
[4] Centre of Biological Engineering (CEB), Department of Engineering, University of Minho, Braga, Portugal.

*Corresponding author
E-mail: philippe.gallusci@inrae.fr

Highlights:

-Bud burst in grapevine involves deep transcriptional changes in nearby woody tissues
-Cell division, cell wall metabolism and sugar mobilization pathways are upregulated
-Starch and soluble sugars of woody tissues are metabolized during bud burst
-*VviAMY2* is upregulated at bud burst and encodes a functional α-amylase
-Sugar mobilization is likely involved in wood secondary growth and bud development

Abstract

Perennial woody plants undergo a period of dormancy from the beginning of autumn until the end of spring. Whereas the molecular and physiological events that characterize dormancy release of buds have been described in detail, those occurring in woody tissues underneath the buds are mostly unknown. To bridge this gap, the mRNA populations of cane segments located underneath the bud were analyzed at bud dormancy (E-L 1) and at bud burst (E-L 4). They revealed an important reprogramming of gene expression suggesting that cell division, cell wall metabolism and the mobilization of sugars are the main metabolic and cellular events occurring in cane woody tissues at bud burst. Also, the upregulation of several genes of sugar metabolism, encoding starch- and sucrose-degrading enzymes and sugar transporters, correlates with the decrease in starch and soluble sugars in woody tissues concomitant with increased sucrose synthase and α-amylolytic biochemical activities. The latter is likely due to the *VviAMY2* gene that encodes a functional α-amylase as observed after its heterologous expression in yeast. Taken together, these results are consistent with starch and sugar mobilization in canes being primarily involved in grapevine secondary growth initiation and supporting the growth of the emerging bud.

Key-words: Bud break; Cell wall metabolism; RNA-seq; *Vitis vinifera*; Woody tissues; α-amylase

1. Introduction

Perennial woody fruit crops like apple, sweet cherry, olive and grapevine, cultivated in areas with seasons contrasted in terms of temperature and photoperiod, undergo a period of dormancy from the beginning of autumn till the end of spring. The induction and release of dormancy (bud set and bud burst) are finely tuned by complex environmental and endogenous signals [1].

Dormancy is induced by a decrease in photoperiod and temperature and the relative contribution of these cues differs among species [2]. Dormancy induction by the onset of short-days involves light perception by photoreceptors, the circadian clock and the flowering CO/FT signaling network [2]. Similarly, in grape, the genes *VviPHYA* and *VviPHYB,* which encode phytochromes, are down-regulated when buds are exposed to short-days [3,4]. Dormancy acquisition is also regulated by hormones, like ethylene, ABA and gibberellic acid, combined with genetic control [5,6].

Chilling exposure is a prerequisite for dormancy release before the plant is exposed to certain amount of heat that forces bud burst [7]. Grapevine requires at least 200 h of low temperatures to achieve a commercial acceptable bud break ratio, but as much as 1500 h at 4 ºC are needed for complete bud break [8,9]. A massive transcriptional reprogramming occurs in the emerging buds during developmental or forced bud burst that involves hormones like ABA, GA and ethylene [10,11,12,13,14,15]. In aspen, the transcription factor SHORT VEGETATIVE PHASE-LIKE and its downstream target TCP18, which are downregulated during chilling, are key components of dormancy release that regulate ABA and GA levels and their downstream pathways [7]. In grapevine, ABA levels decrease during bud burst likely due to the down regulation of *VviNCED1* and the concomitant up regulation of *VviA8H-CYP707A4*, involved in ABA synthesis and catabolism, respectively [14]. This decrease in ABA levels promotes gibberellin (GA) mediated-growth through the regulation of the expression of the genes *VviGA3ox*, *VviGA20ox*, and *VviGASA2*, which regulate endogenous levels of GA_1 [16,17]. Light is also an important signal that triggers bud burst in grapevine and accelerates vascular development and bud burst kinetics [18,19,20]. Noteworthy, two *CRY* photoreceptors and one *HY5* transcription factor that activate light-inducible genes are upregulated in dormant grapevine buds exposed to light [19].

While most studies on dormancy induction and release have been performed in the bud itself, the understanding of the physiological modifications that occur in nearby

103 woody tissues upon dormancy release and bud break is still limited. It has been widely
104 reported that starch accumulated seasonally in well-defined amyloplasts, decreases in
105 woody tissues in spring [21, 22, 23, 24, Reviewed by 25]. Because the phloem is
106 blocked during winter and early spring, the metabolic reactivation of the bud and leaf
107 primordial growth requires, besides carbon, the import of water and minerals that is
108 likely to occur through the re-establishment of xylem continuity between the bud and
109 the nearby cane [26, 27]. In agreement, the decrease in starch occurring in spring in
110 grapevine is accompanied by an increase in the sugar content of the xylem exudate,
111 which is thought to fuel early shoot growth [28, 29, 1]. Nonetheless, the transport
112 mechanism of sugars to the developing bud and their contribution for vegetative growth
113 is still a matter of debate. Carbon and energy sources are also required to initiate the
114 growth of the lateral meristems following spring cambial reactivation, including for cell
115 wall synthesis [30,31,31,32].
116 In the present work, the molecular basis of the physiological and structural
117 reorganization occurring in these tissues upon bud burst have been explored by
118 analyzing the mRNA populations of cane segments located underneath the bud at
119 dormancy (E-L 1) and bud burst (E-L 4) stages. The results highlight that, among the
120 physiological processes initiated at these developmental stages, cell division and cell
121 wall metabolism are the most important, together with the mobilization of sugars.
122 Additionally, biochemical evidence suggests that *VviAMY2* is a key enzyme involved in
123 cane starch mobilization at this stage. Taken together results are consistent with sugar
124 mobilization in canes being primarily involved in wood growth initiation but also to fuel
125 early growth of the buds.
126
127 **2. Material and Methods**
128 **2.1 Plant material**
129 Woody canes of cv. Vinhão were collected in November from a commercial vineyard in
130 Ponte de Lima, Portugal, and stored at 4 ºC until the beginning of the experiment in
131 March. Mature, lignified shoots of similar diameter (~2 cm) were cut into single bud
132 segments of approximately 15 cm. The cutting basal end was inserted into wet floral
133 foam and the top end was sealed with paraffin film to prevent dehydration. They were
134 then placed in a growth chamber to force bud burst (23 ºC, 12/12 h photoperiod). After
135 18 d (E-L 4; bud burst) in the growth chamber, 3 cm segments just below the bud (Fig.
136 1) were cut, frozen in liquid N_2,ground to a fine powder in a IKA A11 basic analytical

mill and kept at -80 ºC. Control samples were collected just before the beginning of the experiment (dormancy; E-L 1). At each sampling date, 3 or 4 independent cane segments were collected and pooled in 3 groups (biological replicates). The modified E-L system was used to classify the bud developmental stages [34].

2.2 Library preparation and sequencing

Total RNA was isolated following GRS Total RNA Kit-Plant (Grisp) with some adjustments. To 100 mg of frozen tissue, buffer R1 was added together with 1 M DTT. The remaining protocol was conducted following the manufacturer's instructions. RNA concentration and purity were determined in a Nanodrop (Thermo Fisher Scientific Inc.) and its integrity was assessed in a 1% agarose gel. First strand cDNA synthesis was performed with the Xpert cDNA Synthesis Mastermix kit (Grisp), following the manufacturer's instructions.

RNA degradation and contamination were monitored on 1% agarose gels, purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), integrity and quantitation were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. The purification step with AMPure XP system (Beckman Coulter, Beverly, USA) was applied to select cDNA fragments of preferentially 150~200 bp in length. Before sequencing, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The library preparations were sequenced on an Illumina platform and 125 bp/150 bp paired-end reads were generated.

2.3 Gene expression data analysis.

All data analyses were performed according to the Novogene analysis workflow. Briefly, raw reads were cleaned by removing reads containing adapter, reads containing poly-N and low-quality reads. Quality control was assessed with Q20, Q30 and GC content on the clean data. *Vitis vinifer*a reference genome and gene model annotation file were obtained from EnsemblPlant release 40 (http://plants.ensembl.org/Vitis_vinifera/Info/Annotation/). Paired-end clean reads were mapped using TopHat v2.0.12 [35]. Gene expression level was quantified with HTSeq

v0.6.1 [36]. Differential expression analysis of the two conditions with biological replicates was performed using the DESeq R package (1.18.0) [37]. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value < 0.05 and $|\log_2 FC| > 1.0$ found by DESeq were assigned as differentially expressed. Gene Ontology enrichment analysis of differentially expressed genes was analysed by the GOSeq R package and GO terms with corrected P-value less than 0.05 were considered significantly enriched. All the gene models were automatically categorized according to the MapMan ontology (x4.1) with Mercator tool [38] and MapMan standalone software (v3.5.1) was used to explore the data.

2.4 Starch quantification

Storage starch in the 3 cm segments nearby the bud was quantified following the method of Smith and Zeeman [39] adapted by Silva et al. [40]. An aliquot of 50 mg lyophilized cane tissues was extracted 3 times with 5 mL 80% ethanol to remove soluble sugars, and the starch grains were gelatinized by autoclaving. Starch was enzymatically degraded by α-amylase (AMY; 1U, Sigma–Aldrich) and β-glucosidase (10U, Sigma–Aldrich) in a medium containing 200 mM sodium acetate (pH 5.5) and glucose was quantified by the DNS method [41].

2.5 Cloning and biochemical activity of VviAMY2

The *pAG416GPD-VviAMY2* construct was prepared following the Gateway technique (Invitrogen) with the primers shown in Supplementary Table S1. Recombination sequences were introduced by PCR in the *VviAMY2* cDNA and the fragment recombined into the entry vector pDONR221 with the BP clonase enzyme. *VviAMY2* cDNA was then recombined into the *pAG416GPD* vector by the LR clonase enzyme. The *pAG416GPD-VviAMY2* plasmid was introduced in the CEN.PK113-5D yeast using the LiAc/SS-DNA/PEG method [42] and selected on solid synthetic medium [0.7% YNB (Alfa Aesar), 1.3% dropout (US Biological; without uracil), 0.5% ammonium sulphate, 2% glucose and 2% agar]. To study VviAMY2 amylolytic activity, control yeast harboring the pAG416GPD vector and expressing *pAG416GPD-VviAMY2* were grown in liquid synthetic medium, the OD_{640} nm adjusted to 1.0 and 0.1 and 10 µL aliquots were added to solid YPM media (1% yeast extract, 2% peptone and 0.5%

starch). After 24 and 72 h of growth at 30 ºC the plates were stained with iodine solution and starch degradation was inferred by the absence of starch-iodine staining.

2.6 Real-time PCR studies

Quantitative real-time PCRs were prepared with a Luminaris HiGreen qPCR Master Kit (Thermo Scientific) following the conditions optimized previously in a CFX96 Real-Time Detection System (Bio-Rad) [43]. Experiments were analyzed with the software Bio-Rad CFX Manager (Bio-Rad), using *VviGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *VviActin* as internal control. Gene expression was compared following the $2^{-\Delta\Delta CT}$ method [44]. The primers used to study gene expression are shown in Table S1.

2.7 *In silico* studies

To identify grapevine amylase genes, Arabidopsis sequences were blasted against the genome of *Vitis vinifera* (12X) using the online platform Phytozome (http://phytozome.jgi.doe.gov). Annotated genes were named following phylogenetic analysis using amino acid sequences from *V. vinifera*, *Arabidopsis thaliana* and *Solanum tuberosum* obtained from the National Center of Biotechnology (NCBI) and Uniprot. The alignment of sequences was performed with PRANKSTER [45] and Genedoc [46]. The phylogenetic tree was constructed using these alignments with PROTDIST, NEIGHBOR and RETREE from the PHYLIP software package [47] and Mega 4 [48].

2.8 Sugar extraction and quantification by high performance liquid chromatography

Sugar extraction was carried out as previously described [40]. Briefly, 100 mg of lyophilized cane tissue was mixed with 1 mL of ultrapure H_2O and 1 mL of chloroform. The biphasic mixture was vortexed for 5 min and incubated at 50 ºC for 30 min with continuous shaking. After incubation, samples were centrifuged at 14,500 *g* for 10 min at 4 ºC and the aqueous supernatant collected. The extracted sugars were passed through a PTFE 0.2 μm filter and quantified by HPLC-RI using a Rezex RCM–Monosaccharide Ca^{2+} (8%) column (Phenomenex) at a flow rate of 0.2 mL min^{-1} at 40 ºC, using water as the mobile phase. Sugars concentration of each sample was determined by comparison of the peak area and retention time with standard curves.

2.9 Enzymatic activities

For all enzymatic activities the same protein extraction procedure was used. Briefly, protein extraction was performed by mixing 100 mg of frozen cane tissue with 1 mL of 250 mM MES, 1 mM EDTA, 5 mM DTT, 1 mM PMSF and PEG 4000 and 2% (w/v) of insoluble PVPP. Following a vigorous homogenization, the samples were centrifuged at 13,500 *g* for 15 min at 4 ºC and the supernatants were used in the biochemical assays [40].

The amylolytic activity was measured as described by Mohamed et al. [49] with a reaction mixture composed of 25 µL of reaction buffer (pH 5.2, 50 mM Na-acetate, 10 mM $CaCl_2$), 50 µL of a 1% starch solution and 25 µL of protein extract. For total amylolytic activity, the assay was conducted at 47 ºC for 60 min. Regarding α-amylase activity, the extract was preheated at 70 ºC during 10 min to inactive heat sensitive enzymes and the assay was also conducted at 47 ºC for 60 min. Released sugars were quantified by the DNS method.

The sucrose synthase activity was measured as described by Pressey [50] using a reaction mixture composed of 50 mM Tris (pH 7.2), 250 mM sucrose, 10 mM UDP (uridine diphosphate), 10 mM NaF and 50 µL protein extract, in a final reaction volume of 100 µL. Controls lacking UDP were also performed. After incubation at 37 ºC for 60 min the reactions were terminated by addition of DNS and the reducing sugars were quantified.

2.10 Statistical Analysis

The results were analyzed by Student's t-test and by Analysis of Variances tests (one-way ANOVA) using Prism vs. 6 (GraphPad Software, Inc.). Statistical differences between sampling dates were marked with asterisks (*$P \leq 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$)

3. Results

3.1 Gene expression changes in cane at bud burst

To investigate the molecular mechanisms occurring in woody tissues in the vicinity of the emerging bud, shoots of *Vitis vinifera* (cv. Vinhão) were incubated at 23 ºC in a growth chamber (see material and methods) to force bud burst. RNA seq experiments were performed on triplicate samples of RNA isolated from 3 cm cane segments

sampled below the bud (Fig. 1) just before forcing bud burst (E-L 1; dormancy) and 18 d after, at the bud burst stage E-L 4. After trimming and removal of low-quality reads, total cleaned reads, ranging from 59.3 to 66.8 million, depending on the samples, were mapped to the grape reference genome [51,52], of which 78.1% to 79.5% were uniquely mapped (Table S2). Forty-seven percent of all identified grapevine genes were expressed (FPKM >= 3) in cane segments at E-L 1, while 52.8% were expressed at E-L 4 (bud burst; Table S3).

A total of 7,810 genes were considered differentially expressed (Log Fold change > 1.0; padj < 0.05) among which 5,016 were upregulated and 2,794 down regulated in E-L 4 conditions (Table S4). A Gene Ontology (GO) overrepresentation analysis was performed to gain insight into the regulatory pathways potentially affected. For upregulated genes, “cell wall metabolic processes” including cell wall organization and biogenesis, glucan metabolism, lignin, lignin precursors, pectin synthesis and cellulose metabolism, were overrepresented, together with genes involved in cellular carbohydrate metabolism and cell division (Fig. 2A and Fig. S2A). In contrast, genes belonging to functional categories corresponding to the translational machinery (ribosomal small RNAS, tRNA proteins or mRNA processing) were underrepresented in upregulated genes. Consistently for down regulated genes, those involved in DNA metabolism and replication that are important processes for cell division, were under-represented, while genes involved in vitamin, carotenoid and starch biosynthesis and ion transport were overrepresented (Fig. 2B and Fig. S2B).

MAPMAN tool [53] was then used to visualize gene expression data displayed on metabolic pathways (Fig. 3). Consistent with the GO analysis (Fig. 2 and Fig. S2A), several genes involved in cell wall metabolism were upregulated at E-L 4 condition (Fig. 3 and Table S5), including genes involved in cellulose and hemicellulose biosynthesis (bins 10.2 and 10.3), genes encoding cell wall proteins such as expansins (bin 10.7), or enzymes involved in cell wall metabolism such as pectin esterase (bin 10.5). Noteworthy, many genes involved in cell wall breakdown were also upregulated at E-L 4 (bin 10.6) as well as those of the lignin biosynthetic pathway (bin 16.10). In contrast, genes of the flavonoid (16.8) and terpene (bin 16.1) biosynthesis pathways were down-regulated, with the exception of a few genes involved in sterol synthesis. Of note, most genes of the starch biosynthesis pathways were also down regulated at E-L 4, whereas a subset of those involved in starch degradation and sucrose breakdown were

upregulated at this stage (Fig. 4A and 4B) consistent with the idea that sugar mobilization but not synthesis is an important process in canes at E-L 4.

3.2 Upregulated genes involved in starch metabolism encode active enzymes

The evolution of starch amount between EL1 and EL4 in cane segments located below the emerging bud was monitored (Fig. 5A). Starch concentration decreased by 27.5% at bud burst, from 18.6 ± 0.84 mg g FW^{-1} to 13.5 ± 0.06 mg g FW^{-1}in line with previous studies demonstrating stored starch mobilization during bud burst [54].

In total, the grapevine genome contains 5 *AMY* (Fig. S3) and 9 *BAM* (Fig. S4) genes of which 3 *AMY* and 4 *BAM* genes were identified as DEGs during bud burst (Fig. 5B). The results showed that from E-L 1 to E-L 4 the expression of *VviAMY2* and *VviBAM2* increased by 2.5 and 5-fold, respectively, but the converse was observed for *VviAMY1*, *VviAMY4*, *VviBAM1*, *VviBAM3* and *VviBAM5*.

The observed increase in *VviAMY2* and *VviBAM2* mRNA levels correlated with the two-fold increase in both total amylolytic and α-amylolytic activities (Fig. 5C). In addition, heterologous expression of the *AMY2* gene in *Saccharomyces cerevisiae* showed that encodes a functional enzyme. The amylolytic activity of the transformed yeast strains was assayed in plates supplemented with 0.5 % (w/v) starch. After 48 and 72 h of growth, iodine staining showed that yeast colonies expressing *VviAMY2* were able to degrade starch extracellularly (Fig. S5).

In contrast, other genes encoding enzymes involved in starch breakdown, including *VviGWD, VviLSF1*, which are involved in the phosphorylation and dephosphorylation of the granule, and *VviDPE,* that degrades maltose*,* were downregulated at bud burst (Fig. S6).

3.3 Soluble sugars present in cane woody tissues are metabolized at bud burst

The content in soluble sugars was also analyzed in canes segments located underneath the buds. All soluble sugars analyzed were significantly reduced from dormancy to bud burst, as follows: sucrose content was reduced 4-fold (from 18.19 ± 0.52 to 4.46 ± 0.40 mg g FW^{-1}), glucose content, 3.5-fold (from 5.19 ± 0.11 to 1.47 ± 0.13 mg g FW^{-1}), and fructose content 3-fold (from 8.21 ± 0.59 to 2.7 ± 0.45 mg g FW^{-1}; Fig. 6A).

The grape genome contains 5 *Susy* genes encoding the sucrose degrading enzyme sucrose Synthase [55], 3 of which, including *VviSusy1*, *-6* and *-5* were upregulated between E-L 1, and E-L 4 (Fig. 6B). Concomitantly, VviSusy activity measured in cane

protein extracts increased 2-fold (Fig. 6C). In contrast, the *VviSPS1*, *-4* and *-3* genes that encode sucrose-phosphate synthases, involved in sucrose synthesis, were downregulated (Fig. 6B).

Since sugars were being degraded/mobilized after bud burst the expression of key genes coding for sugar transporters of the major facilitator superfamily (MFS) and of the SWEET family was analysed (Fig. 7). Results showed that *VviHT1, -2, -13* and *VviSWEET2b, -3, -4, -10, -12, -17a* were up regulated while *VviSUC12*, *-11* and *VviSWEET1* and *-17d* were down regulated.

3.4 Transcriptional reprograming of cell wall and cell division related genes during bud burst

Genes encoding cell wall structural proteins such as expansins but also enzymes involved in primary and secondary cell wall synthesis were upregulated at E-L 4 (Fig. 8). To get a more precise insight, we analyzed the different members of the complex gene families encoding these different proteins. Among the 29 expansin genes found in the grape genome (20 *EXPA*, 4 *EXPA*, 1 *EXLA* and 4 EXLB; [56]), 14 were upregulated (*VviEXPA4*, *-5*, *-6*, *-11*, *-13*, *-14*, *-16*, *-18*, *-19*, *VviEXPB2*, *-3* and *-4*, *VviEXLA1* and *VviEXLB1*) and 6 downregulated (*VviEXPA2*, *-12*, *-17* and *VviEXLB2*, *-3* and *-4*; Fig. S7A) at E-L 4. In addition, several genes involved in the synthesis of both primary and secondary cell wall including *VviCesa1*, *-2*, *-3*, *-4*, *-7*, *-8 -10* involved in the synthesis of cellulose were upregulated during bud burst (Fig. 8 and Fig. S7B).

The synthesis of secondary cell wall is controlled by a complex three-layered regulatory network of NAC-MYB transcription factors [57,58]. Here, it was found that the VNSs transcription factors (*VviNAC012*, *-057*, *067*, *-002* and *-049*; Fig. 9A), considered as the first-layer master switches, and *VviMYB46/83* (Fig. 9B), the second-layer master switch, were upregulated in canes at E-L 4 [57]. The steady-state transcript levels of *VviMYB20*, *VviMYB85* and *VviMYB103,* which are TFs involved in the third layer regulation of secondary cell wall synthesis, were also increased (Fig. 9C). Furthermore, genes involved in the synthesis of lignin precursors were upregulated (Fig. 8), together with isoforms of Cinnamoyl-CoA Reductase, *VviCCR1*, *-2*, *-3* and *-4*, which is an important control in regulating lignin biosynthesis (Fig. S8A; [59]). Furthermore, *VviABCG29-like1*, *-like1* and - *like 3*, which encode ABCG-like transporters involved in the export to the apoplast of lignin building blocks (monolignols) were upregulated at E-L 4 (Fig. S8B; [60]). Active cell wall metabolism has been associated with cell

growth and/or cell division. Consistently, several genes involved in the control of mitosis are also differentially regulated between E-L 1 and E-L 4 (Fig. 10). Among them several cyclins (bin 31.1), histones (bin 28.1) and CDC genes (bin 31.2) were found to be up regulated (Figure 10).

4. Discussion

In the present study, we analyzed the transcriptome reprogramming that occurs during bud burst in grape canes located underneath the bud. Results show that among all grape genes 1/4th was differentially regulated (7,810) from dormancy to bud burst in canes located below the emerging bud. A majority of the genes that were upregulated (5,076) are involved in cell wall biosynthesis, cell mitosis and to a lower extent in starch and sucrose mobilization. However, many genes related to cell wall metabolism and cell division were overrepresented in the upregulated DEGs indicating that, concomitantly to the induction of bud development, wood growth is also initiated in canes and it is most likely sustained by the mobilization of stored sugars.

4.1 Sugar mobilization in canes at bud burst

The decrease in starch, sucrose, glucose, fructose and raffinose contents observed in canes at bud burst, correlated with transcript levels of key genes and with modifications at the protein activity, which suggests that carbon reserves are being used to fuel spring growth. From 7 amylase genes identified as DEGs in the present study, only two - *VviAMY2* and *VviBAM2* - were upregulated during bud burst, which correlated with the increase of total amylolytic and α-amylolytic activities measured in protein extracts from cane segments below the bud. Interestingly, none of these proteins is predicted to be targeted to the amyloplast but to the secretory pathway (VviAMY2) or to the cytosol/secretory pathway (VviBAM2; Table S6). *AtAMY1*, which is homologous to *VviAMY2* (Fig. S2), is targeted to the apoplastic space in senescent leaves, and several promoter elements of this gene are putatively involved in the control of gene expression by ABA [61], a hormone involved in leaf abscission and aging [62]. Accordingly, the *VviAMY2* promoter region also contains several ABA responsive cis-elements (Table S7).

After the heterologous expression of *VviAMY2* in yeast, starch degradation was observed in the culture media, supporting that VviAMY2 is secreted to the extracellular space. Thus, it is tempting to speculate that VviAMY2 in woody tissues could recycle

nutrients from the apoplast, as previously observed in senescent leaves [61] and germinating seeds [63].

The observed decrease in the amount of soluble sugars at bud burst is in line with a previous report in walnut parenchyma cells during early spring that showed that starch, sucrose, glucose and fructose decreased during early-spring [64]. The decrease in the sucrose amount correlated with the observed increase in *VviSusy* transcripts (*VviSusy1, VviSusy5 and VviSusy6*) and with a subsequent increase in Susy biochemical activity in cane extracts at bud burst. Of note, one of the degradation products of sucrose by Susy is UDP-glucose, a precursor of cellulose and of xyloglucan hemicelluloses synthesis. Furthermore, UDP-glucose is also a precursor of nucleotide-sugars used for the synthesis of hemicelluloses xylan and mannan and pectins [65]. Thus, the sucrose catabolism is likely to play an important role in the synthesis of cell wall polysaccharides associated with spring growth.

4.2 Cell wall metabolism and lateral growth in grapevine canes during spring

During spring, primary growth occurs in the apical meristems, whereas secondary growth takes place in lateral meristems and promotes enlargement of roots and stems, as previously described for grapevine canes [56,66,67,68]. Consistently, our data suggest that cell division is occurring in cane wood tissues at bud burst concomitantly to sugar mobilization. This conclusion is strongly supported by the demonstration that upregulated genes at E-L 4 are significantly enriched in genes involved in DNA replication, and several processes related to mitosis including M Phase establishment, nuclear division, or cell division. In addition, genes upregulated at E-L 4 were also significantly enriched in genes encoding enzymes of the cellulose synthase complex (Cesa), and expansin proteins that regulate cell wall expansion and cell enlargement in a pH-dependent manner. Although expansins have been mainly associated with primary cell wall growth, *PttEXP1*, *PttEXP2*, *PttEXP8* and *PttEXPB1* are expressed in the stem cambial region in Aspen, suggesting a role also in secondary cell wall differentiation [69]. Furthermore, the expression of the expansin sub-family EXLB is restricted to woody tissues [56]. In agreement, our results showed that all members of *VviEXLB* are expressed in grapevine canes, consistent with a role of the corresponding proteins in secondary cell wall formation or modification.

A role for the genes *Vlexp1*, *-2* and *-3* in grape berry softening was proposed in Kyoho grapes [70]. Interestingly, two of their homologues in grapevine, *VviEXPA1* and

VviEXPA14, were also upregulated at E-L 4. Furthermore, *VviEXPA13* and *VviEXLA1* were also involved in cell wall loosing during berry development [71]. In the present study, the upregulation of these genes following bud burst, suggests that expansins are involved in cell expansion and/or division in grapevine woody tissues during spring.

A three-layer regulatory system of NAC-MYB regulatory factors was identified as an important network that regulates the formation of secondary cell wall of woody plants [57,58]. Our results also show a coordinated upregulation at bud burst of genes *VviVNDs* and *VviMYB46/83* that encode the main transcriptional regulators involved in the control of secondary cell wall metabolism. The NAC transcription factor SND1 (SECONDARY WALL-ASSOCIATED NAC-DOMAIN PROTEIN 1) is a key switch in the control secondary cell wall synthesis [72] that can directly bind the cis-regulatory regions of *MYB46* and *MYB83* genes to activate their expression [73,74,75]. It can also act as a direct regulator of other involved in lignin biosynthesis including ferulate 5-hydroxylase (F5H) [76]. Furthermore, it is described that *MYB46* and *MYB83* overexpression enhances lignin, cellulose and xylan biosynthetic pathways. *MYB46/MYB83* homologous in Eucalyptus (*EgMYB2*) can bind specifically to promotors of lignin biosynthesis genes such as CCR and CAD (Cinnamyl Alcohol Dehydrogenase). Their function appears to be redundant and highly conserved in woody species as showed in *Populus*, *Eucalyptus* and *Pinus taeda* [58,74,77]. This coordinated upregulation of the secondary cell wall synthesis machinery is fundamental for the differentiation of xylem vessels that occurs in woody tissues during spring [78]. Thus, the observed transcriptional reprogramming of genes involved in secondary cell wall synthesis in grapevine woody tissues is likely to play an important developmental role at the onset of bud burst promoting cell division and seasonal growth.

4.3 Carbon mobilization to the growing bud

Carbon made available in the cane at end of dormancy may also play an important role in bud growth and development. Phloem sieve elements play an important in carbohydrate transport during early-spring [79], which, in turn, requires the involvement of transporters for sugar loading and unloading. Accordingly, the observed transcriptional reprogramming (mostly upregulated) of the expression of different sugar transporter genes of the MFS and SWEET family during the transition from dormancy to bud burst, suggests that mediated transport of sugars plays important roles in sugar mobilization to the emerging bud. Nonetheless, in walnut, upregulation of hexose

transporters during early-spring correlated with carbohydrate transport through the xylem [64], thus we may not exclude that the observed transcriptional changes of sugar transporters in grapevine canes play the same role. Interestingly, two SWEET transporters from *Arabidopsis thaliana* (AtSWEET11 and -12) are localized in the xylem vessels and are fundamental for a correct development of the secondary cell wall [80].

4.4 Conclusions

As a whole, our results showed that an important transcriptional reprograming occurs in woody tissues near the emerging bud that correlates with a decrease in the level of nonstructural carbohydrates. Upregulated genes code for key enzymes involved in cell wall synthesis and differentiation, cell division, starch breakdown and sucrose metabolism that are likely involved in secondary growth. In parallel, the upregulation of sugar transporter genes suggests that cane sugar reserves fuels bud grow. But further research is still needed no only to fully understand the source-sink relations occurring in woody tissues nearby the emerging bud, but also the structural modifications involved in grapevine secondary growth and the reestablishment of xylem and phloem continuity. From an agronomic standpoint, this knowledge may ultimately allow the optimization of some common practices in the field that modify source–sink relationships, including pruning.

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510

511 **Data Availability Statement**

512 The data that support the findings of this study are available in the European Nucleotide
513 Archive with the ENA study accession PRJEB43358.

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Figure legends:

Fig. 1. Experimental design**.** *Vitis vinifera* woody canes (cv. Vinhão) cut into single bud segments were incubated in conditions forcing bud burst. Cane segments below the bud

were collected just before forcing bud burst (E-L 1; dormancy) and after 18 d at 24 ºC (E-L 4; bud burst).

Fig. 2. Functional analysis of DEGs in grapevine canes after forcing bud burst. GO enrichment analysis of up-regulated genes at E-L 4 revealed overrepresented (A) and underrepresented (B) biological processes. The most significantly (corr. *p*-value<0.01) enriched GO terms in biological processes are represented with a $\log_2$ (enrichment fold).

Fig. 3. MapMan metabolism overview obtained from the DEGs in grapevine canes after forcing bud burst. Each square represents a gene. In blue, genes more expressed at E-L 4 (bud burst); in red, genes more expressed at E-L 1 (dormancy). See additional files for details about genes in each box.

Fig. 4. MapMan pathway for glucose (A) and starch (B) metabolism with DEGs. Each square represents a gene. In blue, genes more expressed at E-L 4 (bud burst); in red, genes more expressed at E-L 1 (dormancy). Bin numbers are shown in pink. See additional files for details about genes.

Fig. 5. Starch metabolism in grapevine canes after forcing bud burst. Starch quantification (A), FPKM values (mean ± SD) of DEGs of *α*- and *β-amylases* (B) and amylolytic activity (C). Results indicate the mean ± SD of three biological replicates. Asterisks denote statistically significant differences (***P* <0.01).

Fig. 6. Sugar metabolism in grapevine canes after forcing bud burst. Quantification of sucrose, glucose and fructose by HPLC (A). FPKM values (mean ± SD) of DEGs of of *VviSusys* and *VviSPSs* (B) and biochemical activity of Susy (C). Asterisks denote statistically significant differences (**P*≤ 0.05; *****P* < 0.0001).

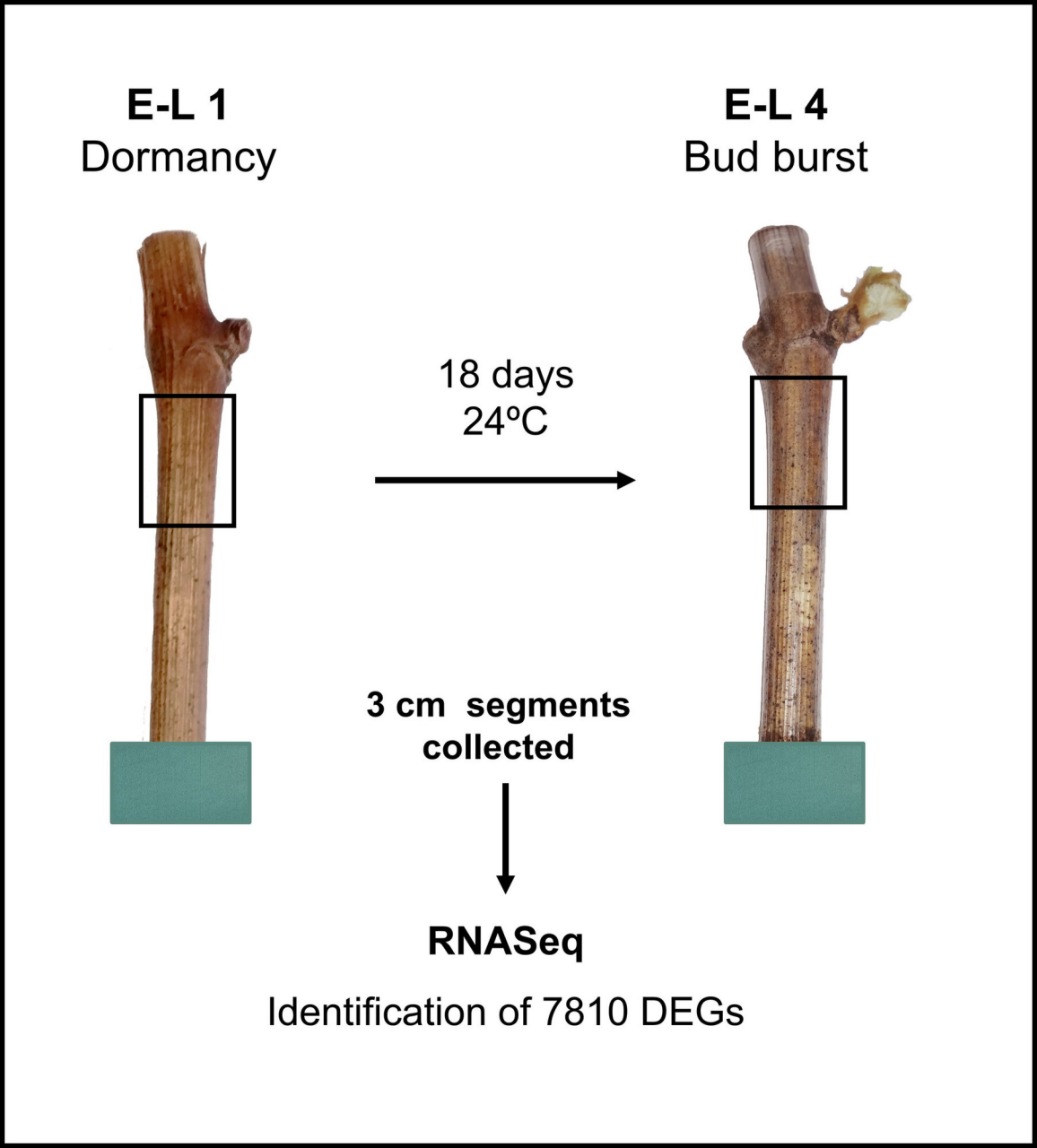
Fig. 7. Analysis of sugar transporters in grapevine canes after forcing bud burst. FPKM values (mean ± SD) of DEGs of *MFS sugar* transporters (A) and *VviSWEET* transporters (B).

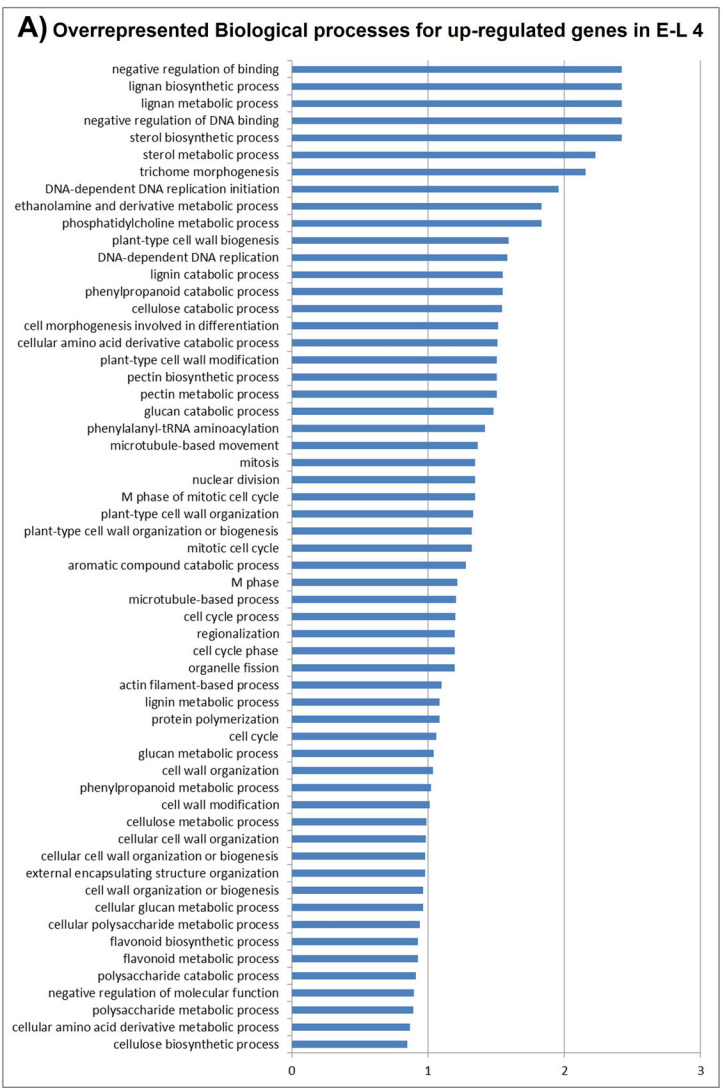
Fig. 8. Structure and composition of the primary and secondary cell wall of plants showing the MapMan visualization of DEGs. Each square represents a gene. In blue, genes more expressed at E-L 4 (bud burst); in red, genes more expressed at E-L 1 (dormancy). Bin numbers are shown in pink. See additional files for details about genes. PAL, phenylalanine ammonia-lyase; CAD, cinnamyl-alcohol dehydrogenase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; HCT, Shikimate O-

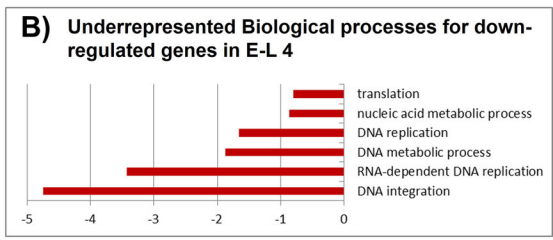
hydroxycinnamoyltransferase; C3H, coumarate 3-hydroxylase; CCoAOMT, Caffeoyl-CoA O-methyltransferase; CCR1, Cinnamoyl CoA reductase; F5H, ferulate-5-hydroxylase; COMT, catechol-O-methyltransferase. Image adapted from Loix et al. (2017) CC BY 4.0.

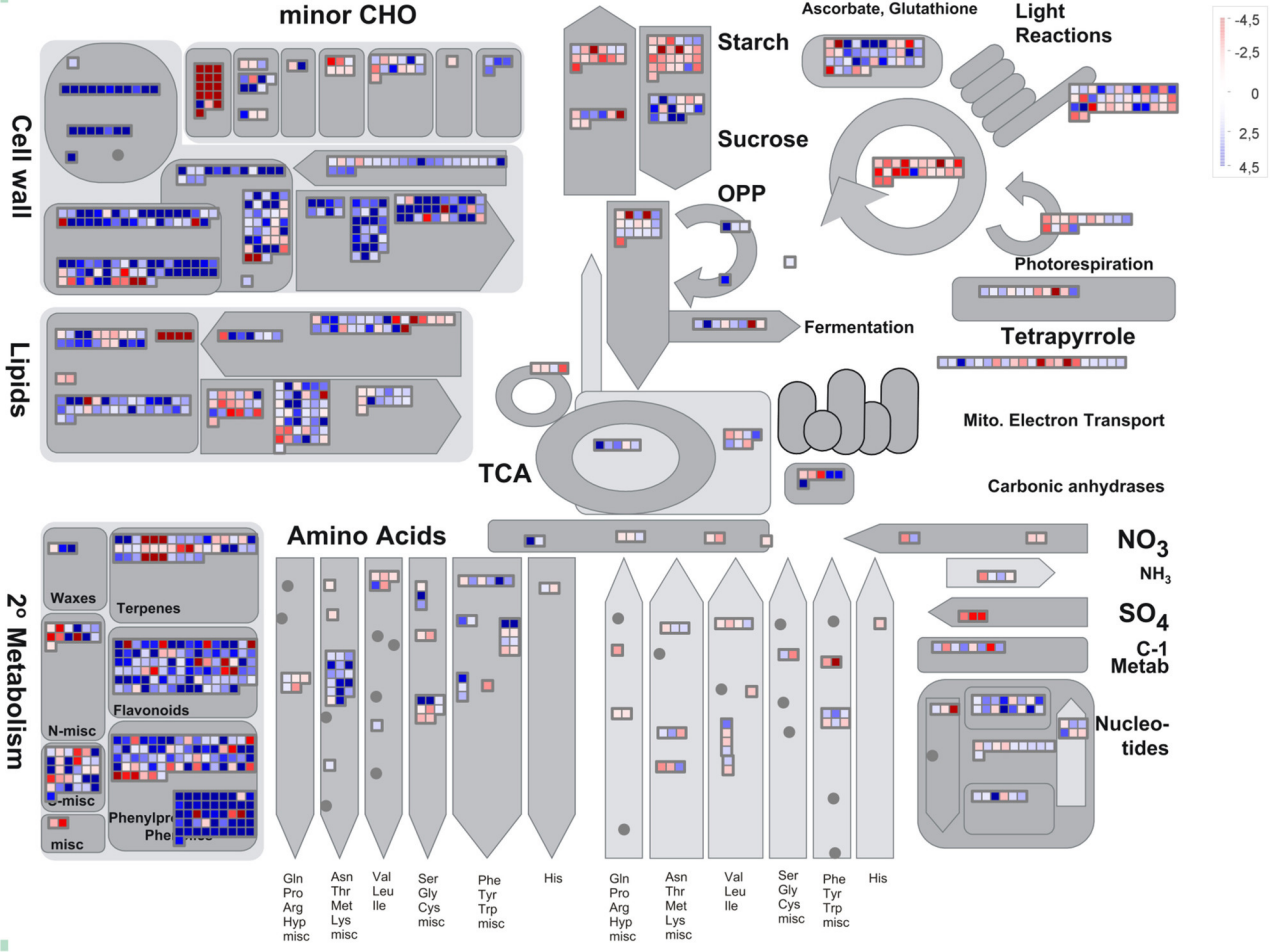
Fig. 9. Analysis of the regulatory network of secondary cell wall formation in grapevine canes after forcing bud burst. FPKM values (mean ± SD) of DEGs of the first-layer *VNSs* transcription factors (A), second-layer *VvMYB46/83* (B) and third-layer *VviMYB20*, *-20* and *-103* (C) master switches.

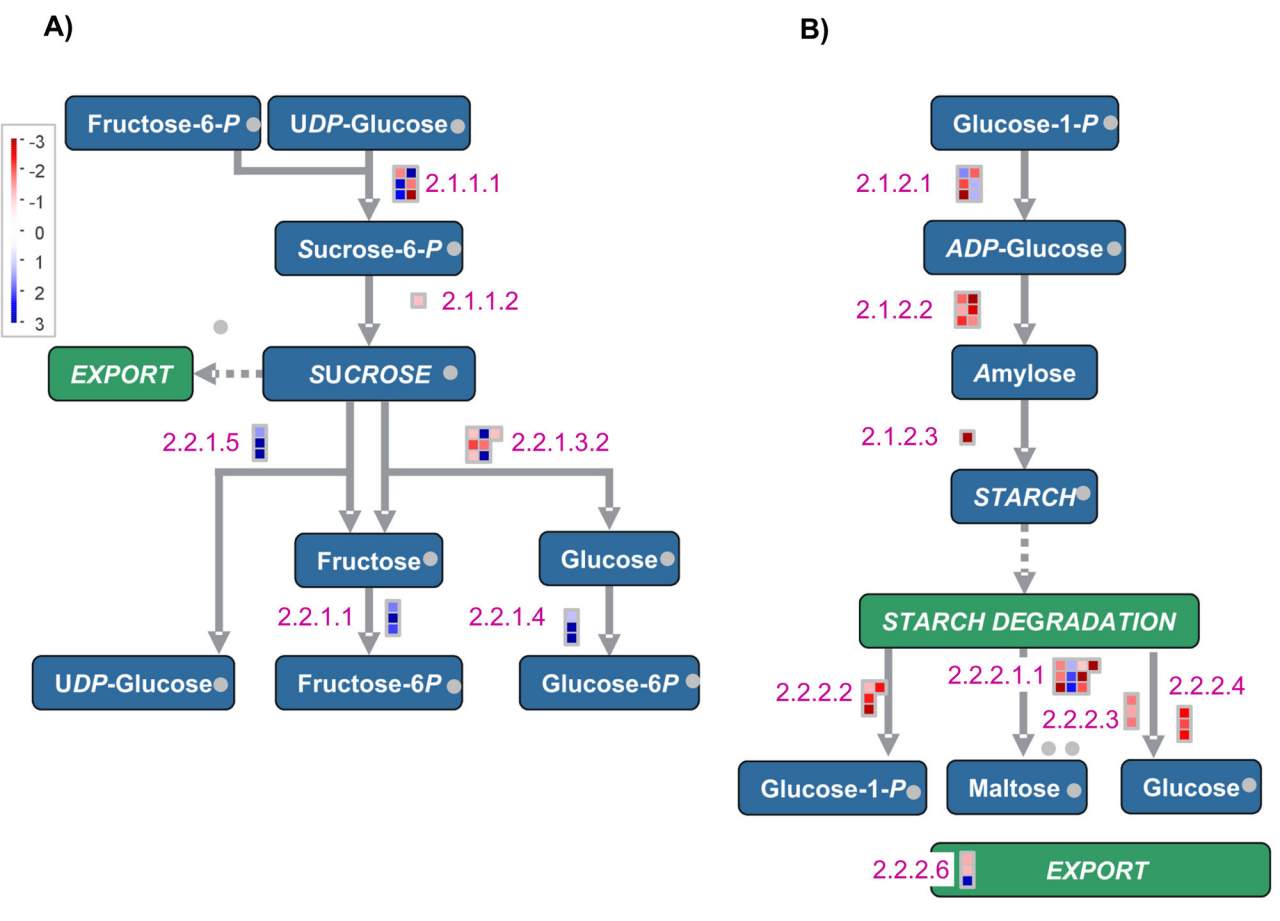
Fig. 10. MapMan visualization of cell cycle genes (bin 31.3) with DEGs. Each square represents a gene. In blue, the genes more expressed in the E-L 4 condition (bud burst); in red, the genes more expressed in the E-L 1 cane segments (dormancy). See additional files for details about genes.

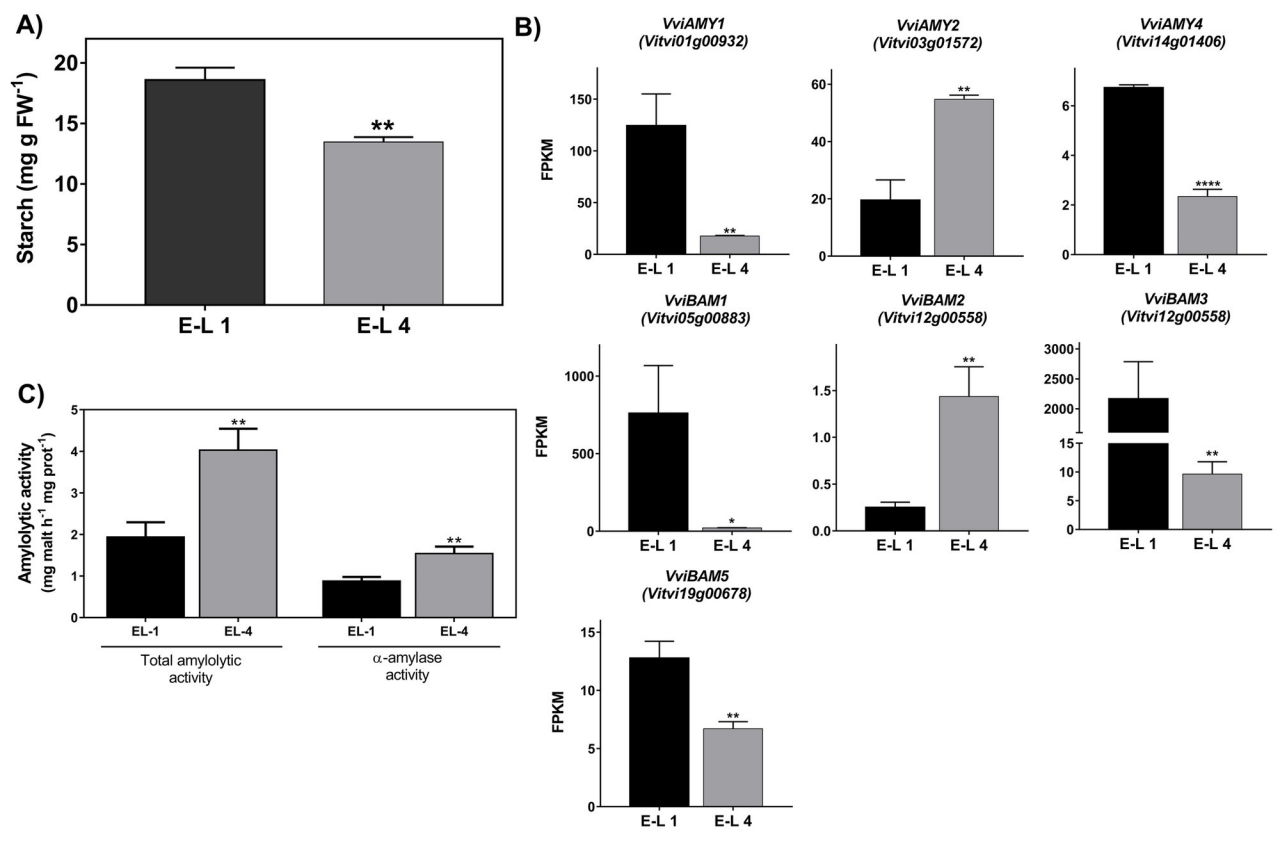

E-L 1
Dormancy
E-L 4
Bud burst
18 days
24ºC
3 cm segments
collected
RNASeq
Identification of 7810 DEGs

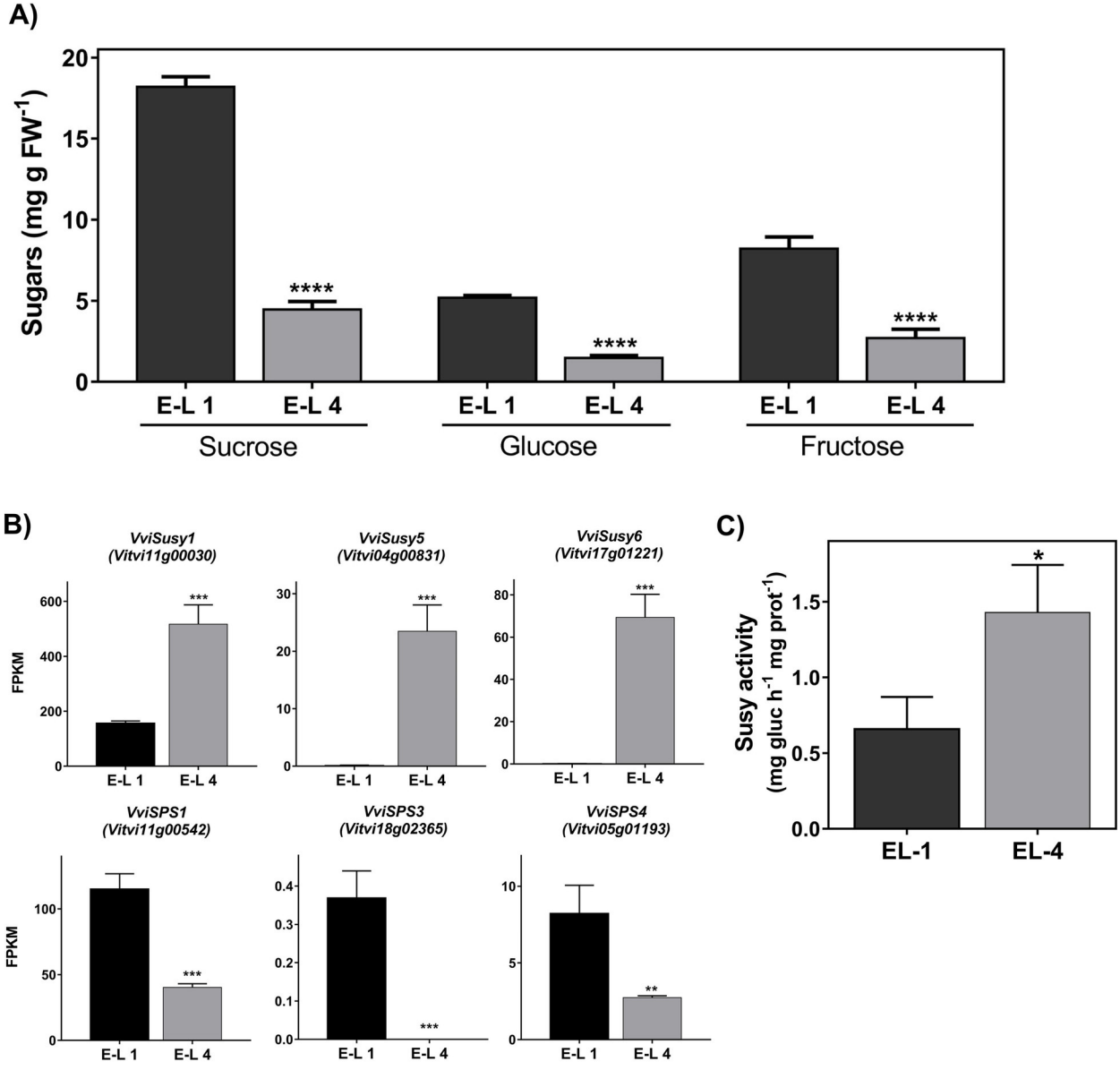

A) Overrepresented Biological processes for up-regulated genes in E-L 4
negative regulation of binding
lignan biosynthetic process
lignan metabolic process
negative regulation of DNA binding
sterol biosynthetic process
sterol metabolic process
trichome morphogenesis
DNA-dependent DNA replication initiation
ethanolamine and derivative metabolic process
phosphatidylcholine metabolic process
plant-type cell wall biogenesis
DNA-dependent DNA replication
lignin catabolic process
phenylpropanoid catabolic process
cellulose catabolic process
cell morphogenesis involved in differentiation
cellular amino acid derivative catabolic process
plant-type cell wall modification
pectin biosynthetic process
pectin metabolic process
glucan catabolic process
phenylalanyl-tRNA aminoacylation
microtubule-based movement
mitosis
nuclear division
M phase of mitotic cell cycle
plant-type cell wall organization
plant-type cell wall organization or biogenesis
mitotic cell cycle
aromatic compound catabolic process
M phase
microtubule-based process
cell cycle process
regionalization
cell cycle phase
organelle fission
actin filament-based process
lignin metabolic process
protein polymerization
cell cycle
glucan metabolic process
cell wall organization
phenylpropanoid metabolic process
cell wall modification
cellulose metabolic process
cellular cell wall organization
cellular cell wall organization or biogenesis
external encapsulating structure organization
cell wall organization or biogenesis
cellular glucan metabolic process
cellular polysaccharide metabolic process
flavonoid biosynthetic process
flavonoid metabolic process
polysaccharide catabolic process
negative regulation of molecular function
polysaccharide metabolic process
cellular amino acid derivative metabolic process
cellulose biosynthetic process
0
1
2
3


B) Underrepresented Biological processes for down-regulated genes in E-L 4
translation
nucleic acid metabolic process
DNA replication
DNA metabolic process
RNA-dependent DNA replication
DNA integration
-5
-4
-3
-2
-1
0

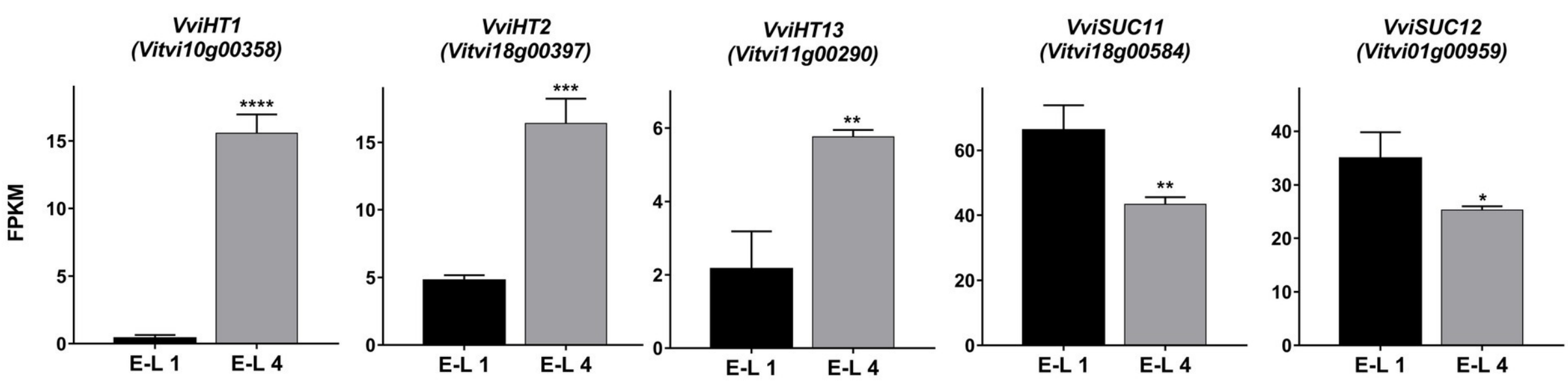

minor CHO
Cell wall
Lipids
2º Metabolism
Starch
Sucrose
OPP
Fermentation
TCA
Ascorbate, Glutathione
Light Reactions
Photorespiration
Tetrapyrrole
Mito. Electron Transport
Carbonic anhydrases
Amino Acids
NO3
NH3
SO4
C-1 Metab
Nucleo-tides
Waxes
Terpenes
Flavonoids
N-misc
misc
Gln Pro Arg Hyp misc
Asn Thr Met Lys misc
Val Leu Ile
Ser Gly Cys misc
Phe Tyr Trp misc
His
-4,5
-2,5
0
2,5
4,5


A)
Fructose-6-P
UDP-Glucose
2.1.1.1
Sucrose-6-P
2.1.1.2
EXPORT
SUCROSE
2.2.1.5
2.2.1.3.2
Fructose
Glucose
2.2.1.1
2.2.1.4
UDP-Glucose
Fructose-6P
Glucose-6P
-3
-2
-1
0
1
2
3
B)
Glucose-1-P
2.1.2.1
ADP-Glucose
2.1.2.2
Amylose
2.1.2.3
STARCH
STARCH DEGRADATION
2.2.2.2
2.2.2.1.1
2.2.2.3
2.2.2.4
Glucose-1-P
Maltose
Glucose
2.2.2.6
EXPORT

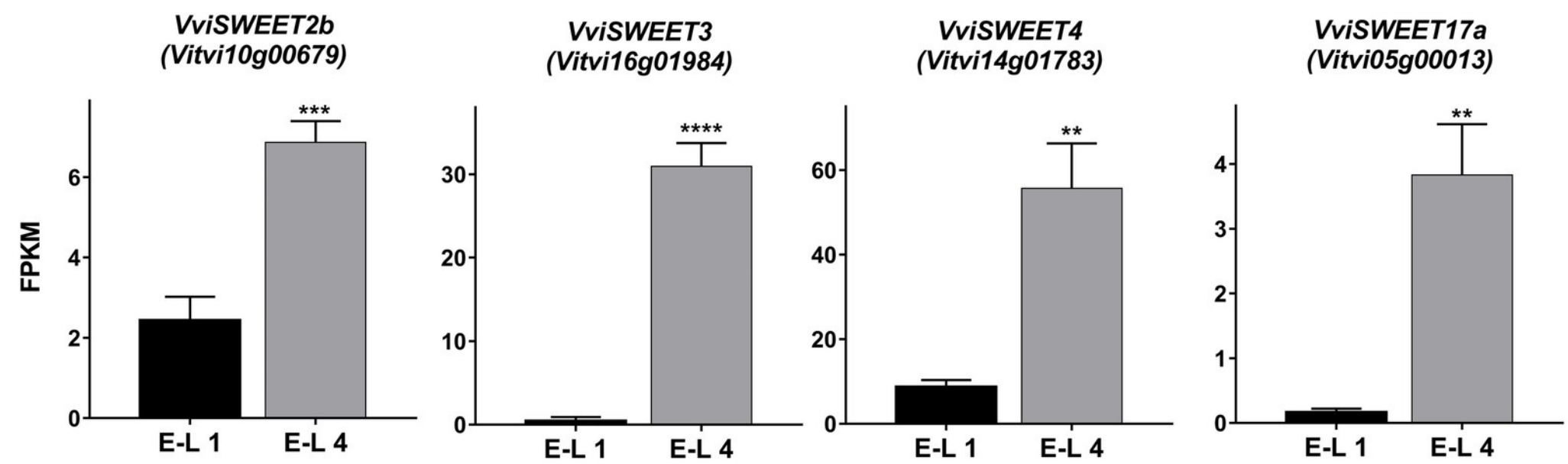

A)
Starch (mg g FW-1)
20
15
10
5
0
**
E-L 1
E-L 4
B)
VviAMY1
(Vitvi01g00932)
FPKM
150
100
50
0
**
E-L 1
E-L 4
VviAMY2
(Vitvi03g01572)
60
40
20
0
**
E-L 1
E-L 4
VviAMY4
(Vitvi14g01406)
6
4
2
0
****
E-L 1
E-L 4
VviBAM1
(Vitvi05g00883)
FPKM
1000
500
0
*
E-L 1
E-L 4
VviBAM2
(Vitvi12g00558)
1.5
1.0
0.5
0.0
**
E-L 1
E-L 4
VviBAM3
(Vitvi12g00558)
3000
2500
2000
15
10
5
0
**
E-L 1
E-L 4
VviBAM5
(Vitvi19g00678)
FPKM
15
10
5
0
**
E-L 1
E-L 4
C)
Amylolytic activity
(mg malt h-1 mg prot-1)
5
4
3
2
1
0
**
**
EL-1
EL-4
EL-1
EL-4
Total amylolytic activity
α-amylase activity

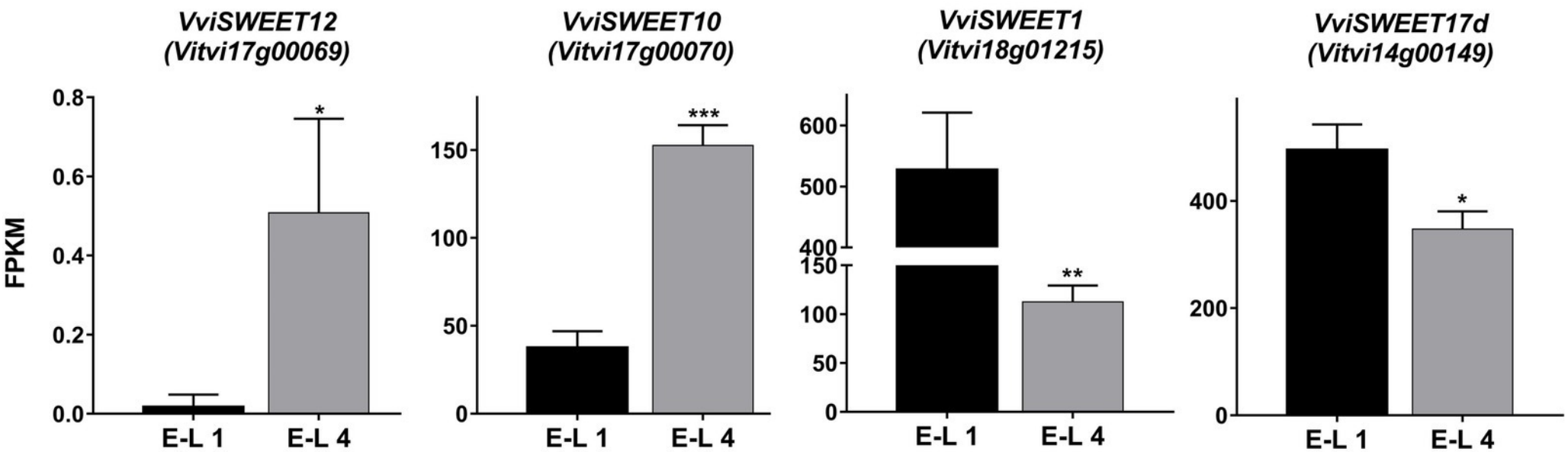

A)
Sugars (mg g FW-1)
20
15
10
5
0
****
****
****
E-L 1
E-L 4
E-L 1
E-L 4
E-L 1
E-L 4
Sucrose
Glucose
Fructose
B)
VviSusy1
(Vitvi11g00030)
VviSusy5
(Vitvi04g00831)
VviSusy6
(Vitvi17g01221)
FPKM
600
400
200
0
30
20
10
0
80
60
40
20
0
***
***
***
E-L 1
E-L 4
E-L 1
E-L 4
E-L 1
E-L 4
VviSPS1
(Vitvi11g00542)
VviSPS3
(Vitvi18g02365)
VviSPS4
(Vitvi05g01193)
FPKM
100
50
0
0.4
0.3
0.2
0.1
0.0
10
5
0
***
***
**
E-L 1
E-L 4
E-L 1
E-L 4
E-L 1
E-L 4
C)
Susy activity
(mg gluc h-1 mg prot-1)
1.5
1.0
0.5
0.0
*
EL-1
EL-4

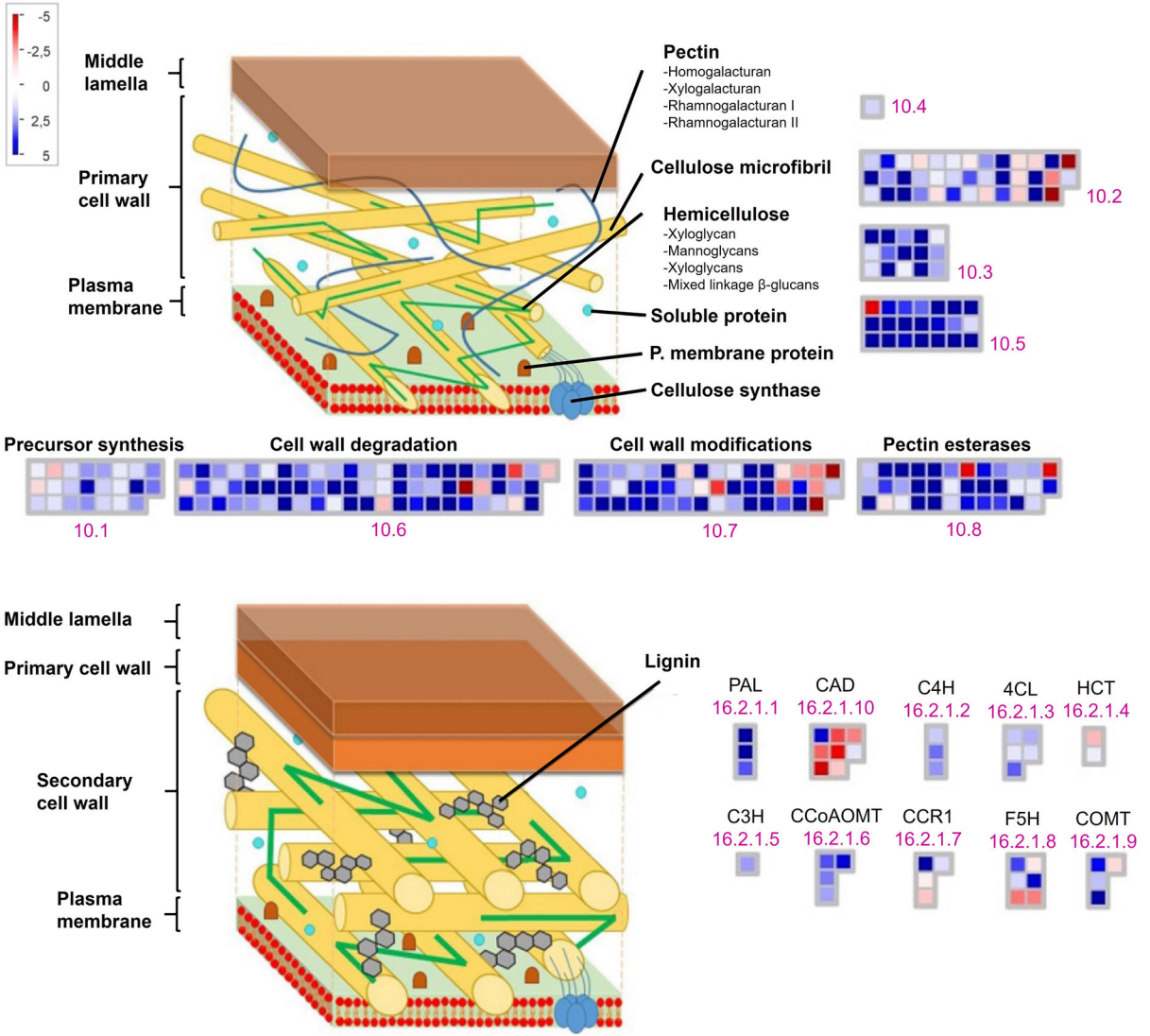
A)

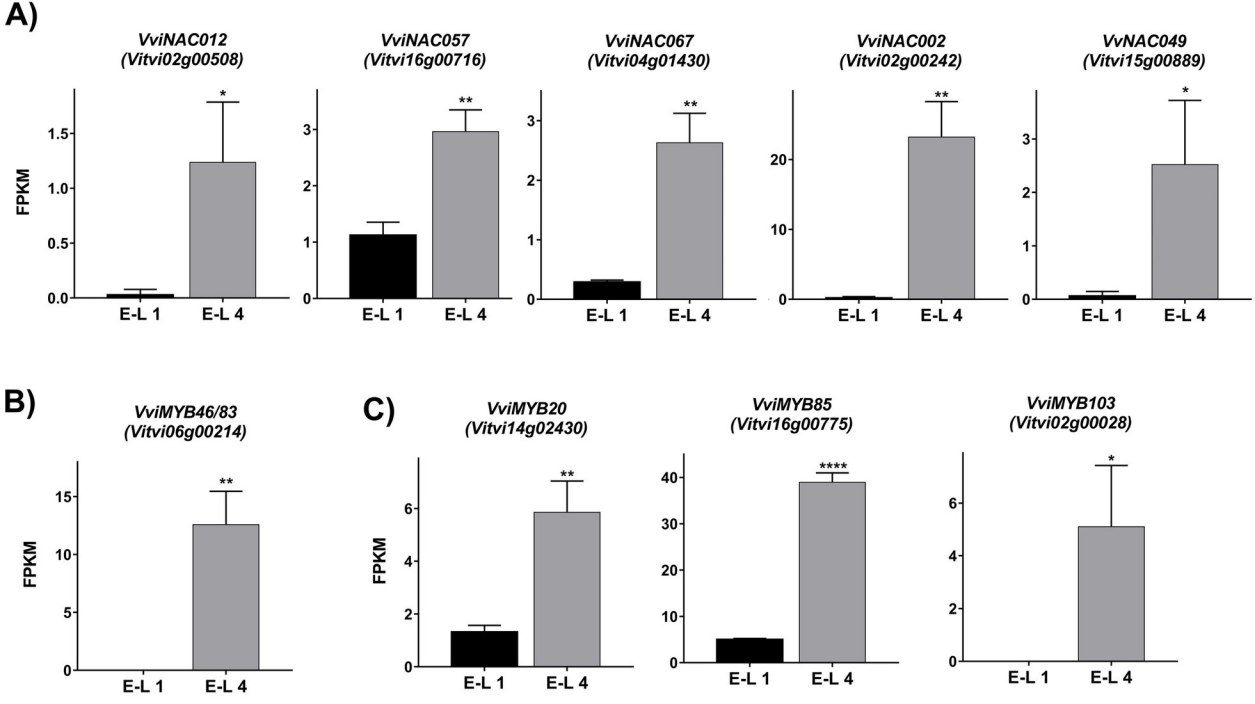

VviHT1
(Vitvi10g00358)
VviHT2
(Vitvi18g00397)
VviHT13
(Vitvi11g00290)
VviSUC11
(Vitvi18g00584)
VviSUC12
(Vitvi01g00959)
FPKM
E-L 1
E-L 4

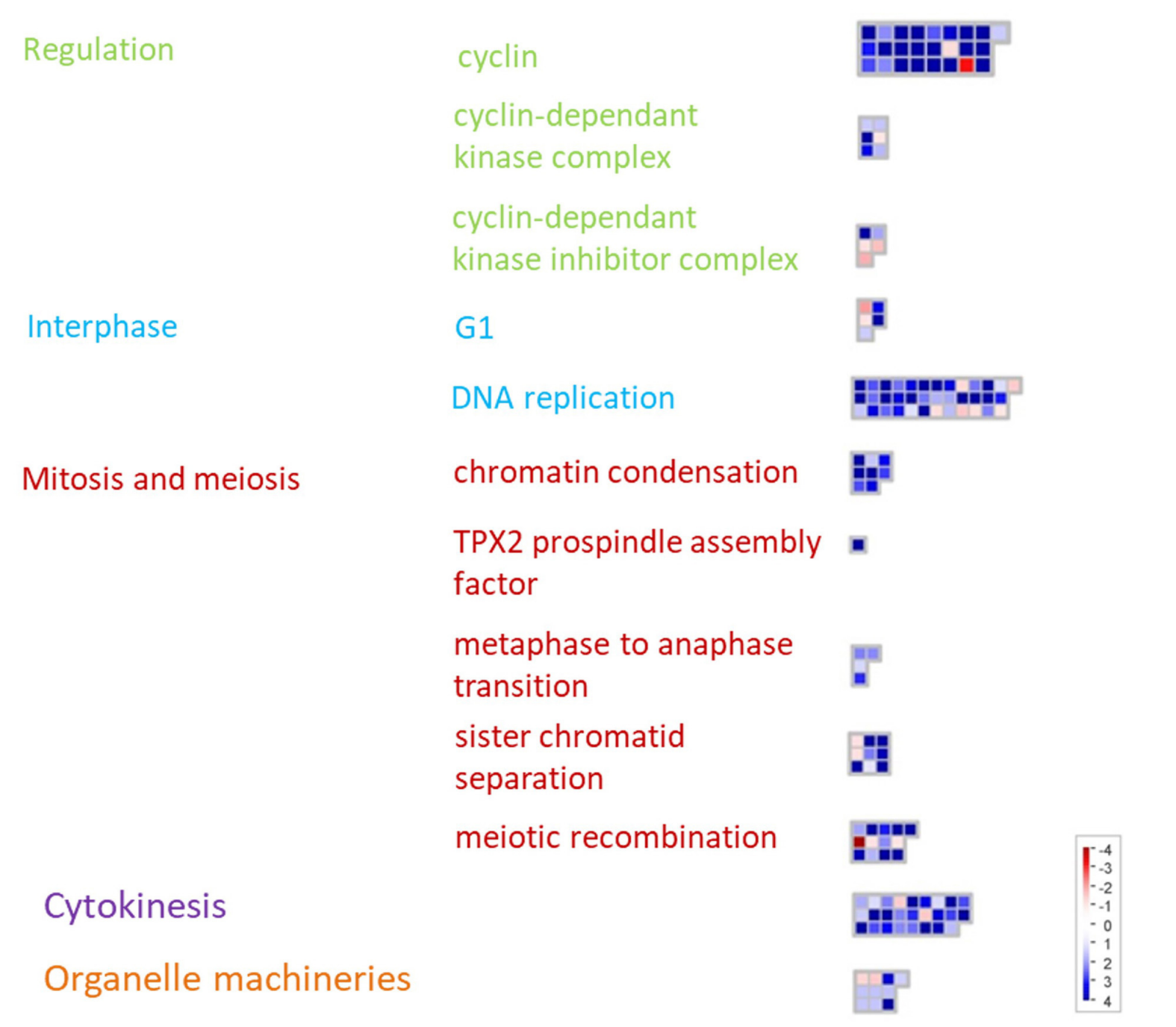
B)


VviSWEET2b
(Vitvi10g00679)
VviSWEET3
(Vitvi16g01984)
VviSWEET4
(Vitvi14g01783)
VviSWEET17a
(Vitvi05g00013)
FPKM
E-L 1
E-L 4


VviSWEET12
(Vitvi17g00069)
VviSWEET10
(Vitvi17g00070)
VviSWEET1
(Vitvi18g01215)
VviSWEET17d
(Vitvi14g00149)
FPKM
E-L 1
E-L 4


-5
-2,5
0
2,5
5
Middle lamella
Primary cell wall
Plasma membrane
Pectin
-Homogalacturan
-Xylogalacturan
-Rhamnogalacturan I
-Rhamnogalacturan II
Cellulose microfibril
Hemicellulose
-Xyloglycan
-Mannoglycans
-Xyloglycans
-Mixed linkage β-glucans
Soluble protein
P. membrane protein
Cellulose synthase
10.4
10.2
10.3
10.5
Precursor synthesis
Cell wall degradation
Cell wall modifications
Pectin esterases
10.1
10.6
10.7
10.8
Middle lamella
Primary cell wall
Secondary cell wall
Plasma membrane
Lignin
PAL 16.2.1.1
CAD 16.2.1.10
C4H 16.2.1.2
4CL 16.2.1.3
HCT 16.2.1.4
C3H 16.2.1.5
CCoAOMT 16.2.1.6
CCR1 16.2.1.7
F5H 16.2.1.8
COMT 16.2.1.9


A)
VviNAC012 (Vitvi02g00508)
VviNAC057 (Vitvi16g00716)
VviNAC067 (Vitvi04g01430)
VviNAC002 (Vitvi02g00242)
VvNAC049 (Vitvi15g00889)
FPKM
E-L 1
E-L 4
B)
VviMYB46/83 (Vitvi06g00214)
C)
VviMYB20 (Vitvi14g02430)
VviMYB85 (Vitvi16g00775)
VviMYB103 (Vitvi02g00028)


Regulation
cyclin
cyclin-dependant kinase complex
cyclin-dependant kinase inhibitor complex
Interphase
G1
DNA replication
Mitosis and meiosis
chromatin condensation
TPX2 prospindle assembly factor
metaphase to anaphase transition
sister chromatid separation
meiotic recombination
Cytokinesis
Organelle machineries
-4
-3
-2
-1
0
1
2
3
4