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Identification and expression analysis of genes associated with the early berry development in the seedless grapevine (*Vitis vinifera* L.) cultivar Sultanine

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ABSTRACT

Sultanine grapevine (*Vitis vinifera* L.) is one of the most important commercial seedless table-grape varieties and the main source of seedlessness for breeding programs around the world. Despite its commercial relevance, little is known about the genetic control of seedlessness in grapes, remaining unknown the molecular identity of genes responsible for such phenotype. Actually, studies concerning berry development in seedless grapes are scarce at the molecular level. We therefore developed a representational difference analysis (RDA) modified method named Bulk Representational Analysis of Transcripts (BRAT) in the attempt to identify genes specifically associated with each of the main developmental stages of Sultanine grapevine berries. A total of 2400 transcript-derived fragments (TDFs) were identified and cloned by RDA according to three specific developmental berry stages. After sequencing and in silico analysis, 1554 (64.75%) TDFs were validated according to our sequence quality cut-off. The assembly of these expressed sequence tags (ESTs) yielded 504 singletons and 77 clusters, with an overall EST redundancy of approximately 67%. Amongst all stage-specific cDNAs, nine candidate genes were selected and, along with two reference genes, submitted to a deeper analysis of their temporal expression profiles by reverse transcription-quantitative PCR. Seven out of nine genes proved to be in agreement with the stage-specific expression that allowed their isolation by RDA.

1. Introduction

Larger fruit size, higher fruit number per plant, better flavor, taste and nutritional quality, and reduced seed number and size are major goals in the genetic improvement of fruit crop species. Considering table-grapes (*Vitis vinifera* L.), seedlessness is one of the most appreciated quality traits. The understanding of genetic and molecular mechanisms driving seedlessness in table-grapes is justified by their economic relevance and most breeding programs have focused on the generation of new cultivars (cv) combining seedlessness with other quality traits such as larger berry size, muscat flavor or crispiness. The grapevine cv Sultanine, also known as Thompson Seedless, Sultanina or White Kishmish, is the world most important commercial seedless table-grape variety and it has been the main source of seedlessness in table-grape breeding programs [1–3].

Since the pioneer work of Stout [4], two different types of seedlessness mechanisms have been observed amongst grape genetic resources: parthenocarpy (like in cv Corinto) and stenospermocarpy (like in cv Sultanine). Parthenocarpy, or fruit development in the absence of pollination, yields small berries that completely lack seeds, employed mainly for the production of raisins. In stenospermocarpic varieties, pollination and fertilization occur normally, but the embryo and/or the endosperm abort 2–4 weeks after fertilization. As a result, seed development ceases, leaving only partially formed seeds or seed traces, while the ovary wall pericarp continues to grow to originate berries with sizes compatible with commercial requirements for fresh fruit consumption. The different hypothesis proposed for the genetic control of seedlessness in grapevine were further studied and revised [2,5–7].

A logical approach to look for genes possibly responsible for seedlessness would be the comparison of gene expression profiles

Abbreviations: BRAT, bulk representational analysis of transcripts; cDNA, complementary DNA; cv, cultivar; DDD, digital differential display; DP, differential product; DS, developmental stage; EST, expressed sequence tag; RDA, representational difference analysis; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TDF, transcript-derived fragment.

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between seeded and seedless varieties. Nearly identical genetic backgrounds are imperative for such strategy in order to avoid differential gene artifacts that, in fact, are not related to the seedless phenotype. This approach was employed by Hanania et al. [8,9] who were able to identify a Sultanine seeded-mutant. Through differential gene expression analysis between seeded and seedless lines of Sultanine, these authors were able to identify a gene coding for chloroplast chaperonin 21 (ch-Cpn21), whose silencing in tobacco and tomato fruits resulted in seed abortion. They concluded that the ch-Cpn21 protein was essential for seed development and its silencing was involved in seed abortion in stenospermocarpic grapes. Indeed, ch-Cpn21 is a strong candidate as key gene in the control of seedlessness in grapes. A suggestible final proof for such or any candidate gene would be the phenotype complementation of Sultanine grape or other seedless varieties.

Grapevine berry development is a unique process in plant biology. It has been intensely studied in seeded varieties due to their more prominent economic impact in wine making [10]. The dynamic and complex process of berry development is summarized in Fig. 1A and involves a cascade of biochemical changes [11]. Sizes, colors and general aspects of Sultanine berries along their development are represented in Fig. 1B. After anthesis, berries undergo a double sigmoidal pattern of growth divided into three distinct stages. The fruit-set stage involves rapid growth as the con-

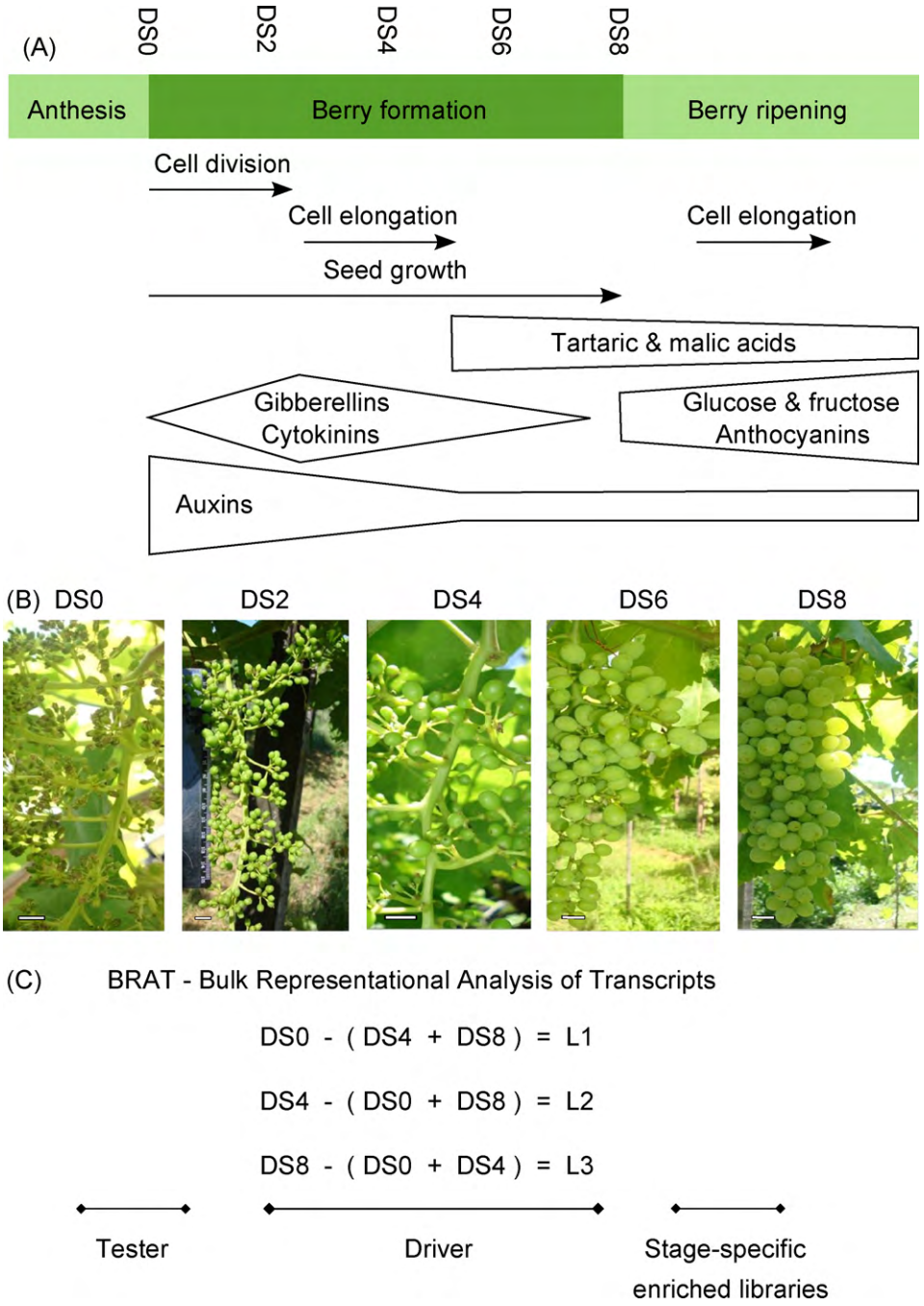

Fig. 1. (A) Representation of main grape fruit developmental stages from budburst until fruit harvesting (adapted from Carmona et al. [10]). Main fruit, cellular, biochemical and physiological aspects are indicated. (B) Sultanine grape seedless fruits at fruit-set (DS0) and at developmental stages (DS) of 2, 4, 6 and 8 weeks after DS0. Scale bars 1 cm. Pictures were taken by L.F. Revers at EMBRAPA Uva e Vinho in Bento Gonçalves, RS, Brazil, in January–February 2005. (C) The strategy of Bulk Representational Analysis of Transcripts (BRAT) employed to generate subtracted libraries from early developmental stages of Sultanine grape seedless berries.

sequence of cell division and elongation induced by the initial high auxin concentration and the increasing levels of gibberellins and cytokinins. Organic acids such as tartrate and malate accumulate in cell vacuoles, and major precursors of phenolic compounds are synthesized in this phase. Seeds are formed and fully maturated during this first stage of berry development. The véraison stage is characterized by slower growth and the initiation of berry softening, defining the transition between berry formation and ripening. Sugars and pigments begin to accumulate at véraison. Finally, the berry ripening phase is characterized by the attainment of final berry size and color, when sugar concentration increases, organic acid production decreases, and volatile secondary metabolites are synthesized, contributing to final berry flavor and aroma [11,12]. Recent and important works have focused on the transcriptome dynamics during grapevine berry development since most of the physiological and biochemical changes described are determined by gene transcriptional modulation [13–17].

Notwithstanding all mentioned authors employed seeded grape varieties in their studies on grape berry development and, as far as we are concerned, there are no reports on transcript profiling of berry development in seedless grapevines. In the current work, a strategy based on Representational Difference Analysis (RDA) was used to identify moderately rare to very rare cDNAs associated with grape berry development in the stenospermocarpic Sultanine grape. In order to validate the strategy, nine candidate genes were selected and analyzed for their temporal transcriptional profile by reverse transcription-quantitative PCR (RT-qPCR). The results gathered on these genes are discussed in the light of previous reports and support the subtraction strategy employed in the present work to pinpoint stage-specific transcripts during seedless grape berry development.

2. Materials and methods

2.1. Plant material and RNA extraction

Grape berries from *V. vinifera* L. cv Sultanine were harvested from November 2004 to January 2005 (2004/2005) in open vineyards grown at Embrapa Uva e Vinho (Bento Gonçalves, RS, Brazil). Samples were collected in the morning hours from at least 12 independent plants, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Three distinct moments of berry fruit development were chosen to perform the RDA: fruit-set (DS0–BBCH code #71), characterized as the first stage of fruit development when young fruits begin to swell and flower remains are lost; 4 weeks after DS0 (DS4–BBCH code #75), when berries reach the size of peas and clusters are already hanging; and 8 weeks after DS0 (DS8–BBCH code #81), characterized by the beginning of ripening, when berries start to develop variety-specific color. For the reverse transcription-quantitative PCR (RT-qPCR) validation, we included other two stages of grape berry development: 2 weeks after DS0 (DS2–BBCH code #73), characterized by berries in pepper-corn size and clusters starting to hang; and 6 weeks after DS0 (DS6–BBCH code #77), when berries begin to touch each other. Berry growth stages were assumed according to the Extended BBCH Scale as reviewed by [18]. Total RNA was extracted with Purelink RNA Reagent (Invitrogen Life Technologies) and the miniprep protocol recommended by the manufacturer. RNA quality was assessed by agarose gel electrophoresis and spectrophotometry.

2.2. Full length cDNA synthesis

Complementary DNA was prepared using the SMART PCR cDNA Synthesis Kit (Clontech Laboratories), according to the manufacturer's instructions, in the presence of RNase OUT (Invitrogen Life Technologies). First-strand cDNA synthesis was performed with the Superscript III Reverse Transcriptase (Invitrogen Life Technologies) using 1 μg of total RNA as template. First-strand cDNA (2 μL) was used as template to synthesize the second-strand of cDNA. All RNA preparations were treated with DNase I (Fermentas) to eliminate residual DNA contamination.

2.3. Bulk representational analysis of transcripts (BRAT)

RDA was performed with total RNAs from the sampled stages of grape berry development identified by DS0, DS4 and DS8, as optimized by Pastorian et al. [19]. Approximately 2 μg of double-stranded cDNA were digested with the restriction enzyme MboI (New England Biolabs). Resulting products were purified using the GFX Kit (GE Healthcare) and ligated to adapters RBam12 (GATCCTCGGTGA) and RBam24 (AGCACTCTCCAGCCTCTCTCACCGAG). To provide sufficient amounts of starting material for RDA, cDNAs were diluted and amplified using the RBam24 as primer (25 cycles of denaturing at 95 °C for 45 s and annealing and extension at 70 °C for 3 min). Aliquots derived from the same driver cDNA synthesis reaction were used in all the remaining rounds of hybridization. Final PCR products were purified using the GFX Kit, resulting in the driver cDNA populations. To generate tester cDNA populations, a small aliquot of the drivers was completely digested with MboI to remove the 24mer primers that were incorporated into cDNA by PCR. After purification by GFX, the resulting products were ligated to different adapters, NBam12 (GATCCTCCCTCG) and NBam24 (AGGCAACTGTGCTATCCGAGGGAG). In order to isolate genes up-regulated in DS0, we used cDNAs from DS4 and DS8 (bulked) as driver and DS0 cDNAs as tester. In order to isolate genes up-regulated in DS4, we used cDNAs from DS0 and DS8 (bulked) as driver and DS4 cDNAs as tester and, finally, to isolate genes up-regulated in DS8, we used cDNAs from DS0 and DS4 (bulked) as driver and DS8 cDNAs as tester. To perform the first round of hybridization and amplification, generating the first differential product (DP1), driver and tester populations were mixed in the ratio of 50:1 in a 5 μL reaction at 67 °C for 24 h, and amplified by PCR with the NBam24 primer (7 cycles at 95 °C for 45 s and 70 °C for 3 min). PCR products were diluted and submitted to a new round of amplification to remove unwanted single-stranded cDNAs. After purification, DP1 products were digested with MboI to remove the NBam adapters before ligation to JBam12 (GATCCGTTCATG) and JBam24 (ACCGACGTCGACTATCCATGAACG) adapters. The second hybridization was performed at a ratio of 100:1. The second differential product (DP2) was obtained using the same procedure as for DP1, using JBam24 as primer instead of NBam24.

2.4. Cloning and sequence analysis

One hundred nanograms of the final RDA products (DP1 and DP2 fragments) were cloned into pGem T-Easy (Promega). *Escherichia coli* DH10B competent cells were transformed with the ligated products. Individual bacterial clones were grown in 96-deep-well plates. Plasmid DNA was prepared from 2400 selected clones and samples were sequenced using the automatic sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The quality established for sequence acceptance was defined by a Phred [20,21] equal or higher than 20 and a minimum length of 80 bp. Resulting cDNA sequences (without vector sequences) were then matched using the SisGen Automatized System of Sequence Analysis (http://genoma.embrapa.br/genoma/), a unified tool that dynamically integrates data from various databases including *V. vinifera* genome, *V. vinifera* peptide, *V. vinifera* mRNA, *Vitis* TIGR clusters, *Arabidopsis thaliana* proteins from MIPS, SwissProt, GO, non redundant database, NCBI and EST database. Sequence redundancy (*R*) was calculated according with the quantity of transcripts

Table 1
Gene-specific PCR primers used for RT-qPCR.

	Gene	Forward (F) and reverse (R) primer sequences
1	VvUBP1	F: GGGGTTTTGGGTTTGTTTCT
		R: GGCATCTGAATTTGGCTTGT
2	VvFS41	F: GAGACATCCTCACCCTGCTC
		R: GACTATGACTGTTTTATCCTGA
3	VvPHP1	F: CATCCATCACCAACCCATTT
		R: CCAACATGCAGTTCACCATC
4	VvRIP1	F: GGATCCTCGTTAAGGGATTTAGA
		R: CGCAAATTACCCAATCCTGA
5	VvP450	F: GCTCAACAGGGTCTTCTTTCC
		R: AACGGCGGGAGTAACTATGA
6	VvDOF1	F: GAGAGTTCGATTTCCGCTCA
		R: GAACCAGGCCTTGAAGTGTC
7	VvERF1	F: TTCATCGTCTCCGTCCTCTC
		R: TGGGTGTCTGAAATTCGGGA
8	VvCLP1	F: TCGATATCGGAGAAGAACACC
		R: CCGGGGATGAGGAGGACT
9	VvGID1L1	F: CCCACCGTGGAAGAAAAGTA
		R: TGAGGGGGTGTTCTCTTTTG
10	VvCNP21	F: GGGACAGAGGTGGAGTTCAA
		R: TTTCCTTGCTTGCCTCTGTT
11	VvACT	F: CTTGCATCCCTCAGCACCTT
		R: TCCTGTGGACAATGGATGGA
12	VvTUB	F: CAGCCAGATCTTCACGAGCTT
		R: GTTCTCGCGATTGACCATA

generated and the size (number of bases) that were added in mounting: $R = 1 - (c + s/i)$, where "c" refers to the number of contigs, "s" refers to the number of singletons, and "i" is the number of ESTs in each input. The novelty of sequences refers to percentage of sequences of each input not yet represented in clusters or singletons already submitted. Singleton and cluster consensual sequences were submitted and are available at the GenBank under accession number type dbEST: GR389936–GR390695.

2.5. RT-qPCR validation

Reverse transcription was performed with 1 μg of total RNA from each sample using M-MLV Reverse Transcriptase (Promega) according to the manufacturer's instructions. Design of oligonucleotides was performed with Primer3 (http://frodo.wi.mit.edu/cgi bin/primer3/primer3_www.cgi) with the criteria to amplify PCR products from 100 to 250 bp (Table 1). PCRs were performed with 10 μL out of 1/100 dilutions of single-stranded cDNAs, using the SYBR Green PCR Master Mix (Applied Biosystems) with a 7500 Sequence Detection System (Applied Biosystems) apparatus. Actin (VvACT) and α-tubulin (VvTUB [22]) gene sequences were used as internal controls to normalize the amount of mRNA present in each sample, using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen [23].

3. Results and discussion

3.1. Identification of differentially expressed genes during the early berry development in Sultanine grape

In order to identify genes differentially expressed during the early developmental stages of Sultanine grape berries, we designed and employed a modified strategy for the construction of subtracted libraries. Basically, the RDA method was performed as described by Hubank and Schatz [24] and optimized by Pastorian et al. [19] with modifications. During the harvest season (2004/2005), fruits from the same clonal plants were collected in five developmental stages (DS) as indicated in Fig. 1A and B. Total RNA samples were purified from first year's berries at DS0, DS4 and DS8 and pooled in three ways for the production of three distinct subtracted libraries according to Fig. 1C. Similar cDNA quantities from DS4 and DS8 were pooled as "drivers" to subtract the DS0 "tester" cDNA population, producing the first subtracted library (L1). Likewise, DS0 and DS8 cDNAs were pooled to subtract DS4 tester cDNAs to produce L2 and, finally, L3 was obtained by the subtraction of DS8 tester cDNAs with the equivalent pool of DS0 and DS4 driver cDNAs.

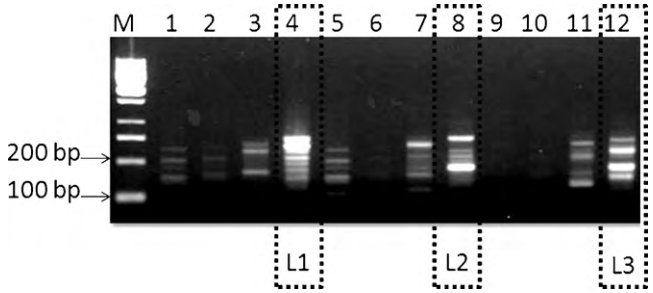

Fig. 2. Differential cDNA products obtained by BRAT. Lanes 1, 5 and 9 represent tester cDNAs, 2, 6 and 10 represent driver cDNAs, 3, 7 and 11 are the differential products after the first subtraction (DP1) and 4, 8 and 12 are the differential products after the second subtraction (DP2) representing libraries L1, L2 and L3, respectively. M indicates the 100 bp-DNA ladder (Invitrogen).

Although DS2 and DS6 were not employed for the construction of subtracted libraries, RNAs from all stages were later employed for the validation of selected candidate genes by RT-qPCR. The method employed was designed to optimize the subtraction towards stage-specific transcripts by pooling groups of samples (driver) to subtract the sample of interest (tester). By doing so, and avoiding complete and repetitive reciprocal subtractions that would lead to pairwise-specific transcripts, we drastically reduced the number of libraries. Such strategy allowed us to minimize costs, time and yielded consistent results concerning differentially expressed genes. We named this RDA strategy as Bulk Representational Analysis of Transcripts, or BRAT (Fig. 1C).

A smear of cDNA products was clearly observed in the starting material and after the first BRAT subtraction. After two cycles of RDA, using different tester and driver ratios, a different pattern was evident, especially in the DP2 (Fig. 2). The resulting BRAT fragments with lengths ranging from 100 to 500 bp were purified and cloned into pGEM-T Easy and a total of 2400 clones were sequenced and analyzed (Table 2). The number of valid ESTs from the DS0-, DS4- and DS8-enriched libraries passing sequence quality cut-off was 1554 (64.75%). The assembly of these ESTs yielded 504 singletons and 77 clusters, with an overall EST redundancy of 67.57%. To better illustrate the distribution of ESTs amongst developmental berry stages of cv Sultanine, we used a differential digital display (DDD) method [25] to construct a diagram of Venn (Fig. 3). In accordance with the designed strategy, most clusters/singletons were specific to only one developmental stage, as indicated in Fig. 3 and in Table 2. Throughout the present technical strategies we were

Table 2
Distribution of ESTs derived from BRAT analysis of Sultanine grape berries.

Sultanine library	RDA-BRAT			Total
	DS0	DS4	DS8	
Sequenced clones	768	864	768	2400
ESTs > 80 bp	538	457	559	1554
Unigene set	175	169	441	581[a]
Singletons	158	132	426	504[a]
Clusters[a]	17	37	15	77[a]
Redundancy (%)	81.92	77.98	67.26	67.57[a]
Novelty (%)	46.34	60.13	51.16	86.75[a]

Only ESTs longer than 80 bp and with a Phred ≥ 20 were included.

[a] Numbers represent scores after final clustering of all sequences. The term "cluster" is here and in the text referred as non-singletons, i.e., groups formed by more than one sequence read.

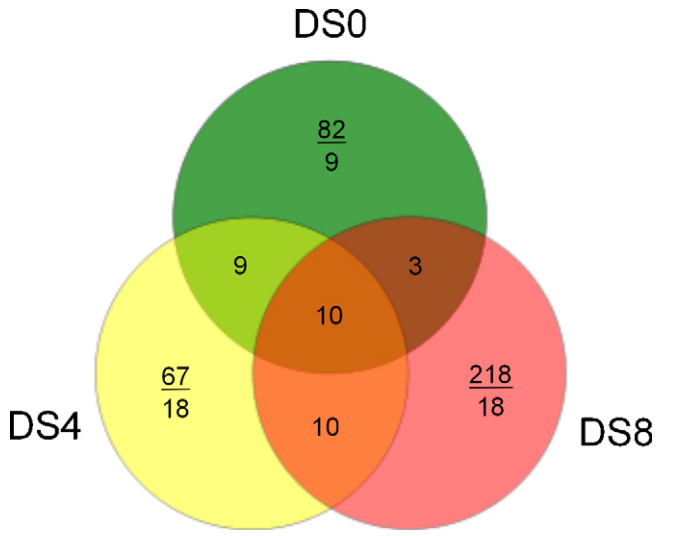

Fig. 3. Venn diagram representing the distribution of transcripts from DS0, DS4 and DS8 of Sultanine berries. Underlined numbers refer to transcript singletons and unmarked numbers refer to transcript clusters (non-singletons after final clustering).

able to identify 91 genes from the seedless grapevine cv Sultanine that are specific to the fruit-set (DS0) stage of berry development and, respectively, 82 and 236 genes whose expressions are specific to the developmental stages known as 4 (DS4) and 8 (DS8) weeks after DS0.

Amongst the 581 unigenes of the resulting BRAT analysis, we selected nine genes from the most populated clusters or based on their predicted function or commitment in fruit development (Table 3). All these genes were further analyzed by RT-qPCR in order to validate the results obtained by the BRAT method.

3.2. Stage-specific genes identified in the early berry development of seedless grapevine cv Sultanine

RT-qPCR has been readily integrated to validate the results of many primary differential gene expression screening methods like cDNA arrays, differential display PCR (DD-PCR), serial analysis of gene expression (SAGE), subtraction hybridization, RDA and cDNA-AFLP [26,27]. Before assaying candidate genes, four genes previously assumed as constitutively expressed in grapevine [22] were assayed with samples from the five DS of Sultanine berries from the harvest season (2004/2005). In our experiments, while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and a SAND-family member proved not to be constitutively expressed (data not shown), actin and α-tubulin (VvACT and VvTUB, respectively) exhibited the best results, as shown in Fig. 4K. Their expressions showed the minimal variation in every DS tested when normalized to each other and, therefore, both were used as internal controls to normalize the amount of mRNA for all candidate genes present in each sample, according to the $2^{-\Delta\Delta Ct}$ method [23].

Seven out of the nine candidate genes selected from the BRAT-derived EST collection proved to be in agreement with their commitment in a specific DS, at least in what concerned steady-state mRNA levels.

3.2.1. Sultanine berry genes specific to fruit-set (DS0) and 2 weeks after DS0 (DS2)

Amongst 91 TDFs specific to DS0 (Fig. 3 and Table 2), we selected three candidates for further analysis by RT-qPCR. A putative gene coding for an oligouridylate binding protein (VvUBP1) was identified and confirmed to be mostly expressed in the initial DS0 and

Table 3
Selected genes putatively involved in the regulation of early berry development in grapevine cv Sultanine identified by BRAT analysis.

Clone name	Functional categories	Genoscope annotation	Best match in databases/species	Putative functional identification	Library	Chromosome location
VvUBP1	Nucleotide binding	GSVIVT00016402001	gi|52126053|gb|CV197216.1| (*Juglans regia*)	Oligouridylate binding protein-like	DS0	Chr.11
VvFS41	Protein biosynthesis	GSVIVT00025456001	gi|110705930|gb|EE083344.1| (*V. vinifera*)	Putative S1-like ribosomal protein	DS0	Chr.8
VvPHP1	No ontology found	No matches found	lcl|TC62058 (*Mus musculus*)	Heat shock protein 70	DS0	Chr.11
VvRIP1	No ontology found	Scaffold_2539	lcl|TC65996 (*V. vinifera*)	Ripening-induced protein	DS4	Chr.6
VvP450	ATP-dependent peptidase activity	GSVIVT00001811001	lcl|TC68767 (*Nicotiana tabacum*)	Cytochrome p450 like	DS4	Chr.18
VvDOF1	Regulation of transcription	GSVIVT00017546001	gnl|BL_ORD_1D|24989 (*A. thaliana*)	Dof zinc finger protein-like	DS4	Chr.17
VvERF1	Transcription factor activity	GSVIVT00032424001	gi|48428609|sp|Q39127| (*A. thaliana*)	Ethylene-responsive transcription factor TINY	DS4	Chr.4
VvCLP1	DNA binding and zinc ion binding	GSVIVT00031464001	lcl|TC56447 (*M. musculus*)	CONSTANS-like protein 2	DS8	Chr.11
VvGID1L1	No ontology found	GSVIVT00006107001	lcl|TC67464 (*M. musculus*)	Probable gibberellin receptor	DS8	Chr.14
VvCPN21	Protein folding	GSVIVT00016397001	gi|50660326|gb|AY680699.1| (*V. vinifera*)	*V. vinifera* chloroplast chaperonin 21 gene	[9]	Chr.11

DS2 stages (Fig. 4A) of berry formation. UBP1 is a heterogeneous-nuclear ribonucleoprotein (hnRNP) previously identified in plant nuclear extracts with a general role in pre-mRNA maturation. It is often associated to U-rich introns, suggesting that UBP1 is important to recruit the splicing machinery to pre-mRNAs. It was also proposed that these proteins function as a complex to stabilize mRNA by binding to U-rich sequences [28]. Plant UBP1 was additionally described to be important for alternative splicing [29] and for the formation of microRNAs (miRNAs) that may cause translational repression or mRNA cleavage [30].

The second candidate gene selected from the DS0 pool of TDFs was named VvFS41. It codes for a putative S1-like ribosomal protein that, in conjunction with rRNA molecules, make up ribosomal subunits involved in the cellular process of translation. They are also described to be responsible for the stabilization of highly compact rRNA structures by filling the gaps between RNA domains [31]. Our observations that VvFS41 and VvUBP1 are mostly expressed during DS0 and DS2 (Fig. 4B) are in accordance to the fact that it is exactly at these stages that most RNA and protein synthesis and processing occur, i.e., during cell division in berries (Fig. 1A). It is important to

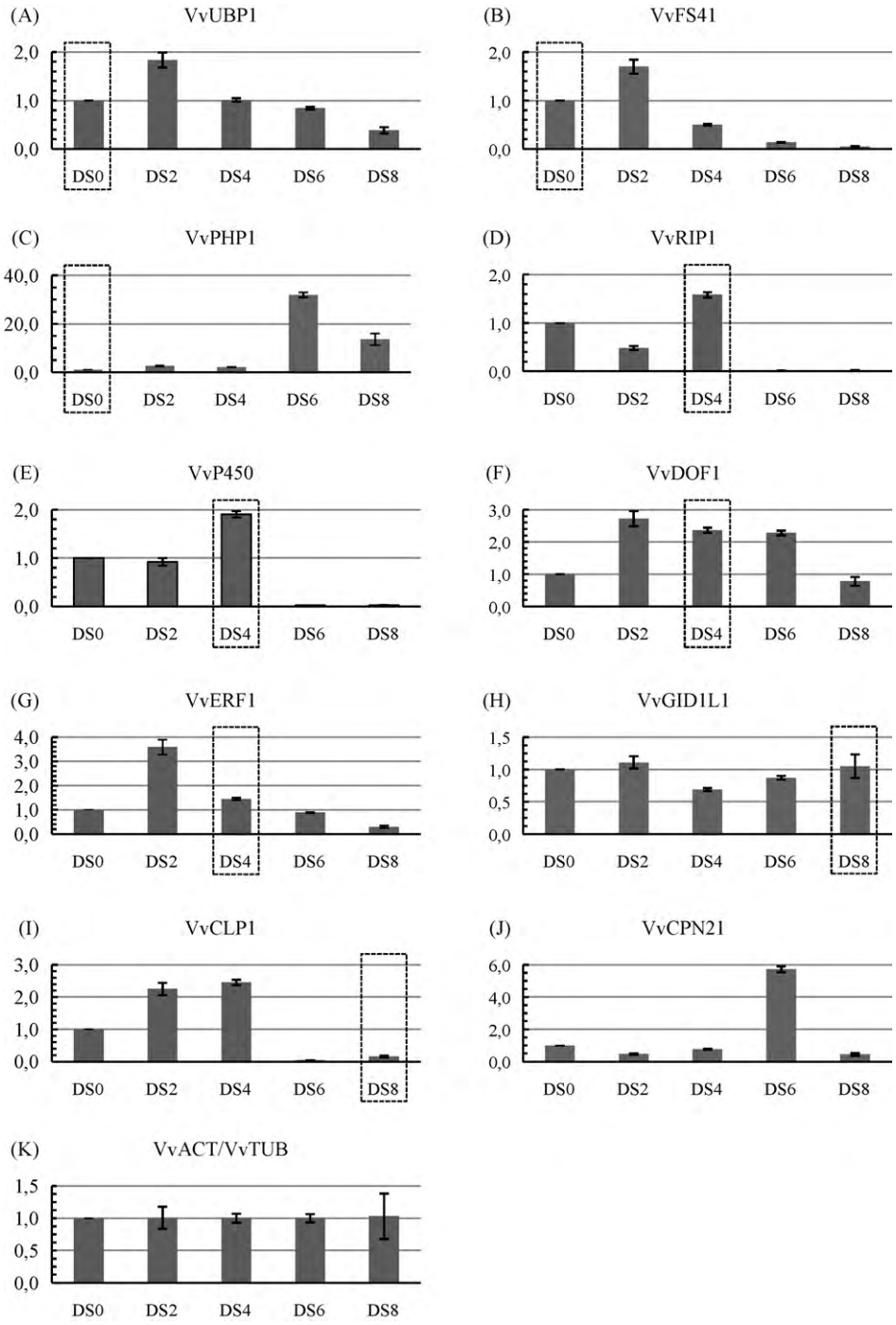

Fig. 4. Relative gene expression of VvUBP1, VvFS41, VvPHP1, VvRIP1, VvP450, VvDOF1, VvERF1, VvGID1L1, VvCLP1, VvCPN21 and reference genes VvACT and VvTUB measured by RT-qPCR at different fruit developmental stages during harvest 2004/2005. Developmental stages (DS0–DS8) are represented at the *X*-axis and relative expressions to DS0 stage at the *Y*-axis. Expressions of the reference genes VvACT and VvTUB (K) were used to normalize the expression values of all candidate genes. Bars marked by rectangles represent the developmental stage from which every candidate gene was originally isolated by BRAT (A–I). All data are presented as mean of four technical replicates.

mention that our choice to select these candidates was based on the fact that they belonged to some of the most populated clusters of TDFs obtained after RDA subtraction.

A gene encoding a heat shock protein 70 (Hsp70), putatively related to the plant hypersensitive response (HSR) and named VvPHP1, was the third candidate gene whose mRNA was amongst the richest ones during DS0 according to the BRAT analysis. RT-qPCR revealed however that VvPHP1 mRNA accumulated mostly at the DS6 and DS8 stages of grape berry development (Fig. 4C). Although we cannot explain such discrepancy, it is reasonable to believe that genes related to plant defense like Hsp70 would increase their expressions at the end of fruit and seed formation, after the burst of cell division and when summer temperatures tend to increase (Fig. 1A). It is also at these stages that fruits and seeds start accumulating more nutritious products therefore starting to attract insects and other herbivores. Within the diverse Hsp gene family, Hsp70 is the most studied member. The encoded and highly conserved 70 kDa protein-member plays a key role in the stress-response in plants [32], as it does in mammals [33]. It is reported that Hsp mRNA increases in the plant cytosol in response to different types of stresses, especially high temperatures [34,35]. It is believed that Hsp proteins function as chaperones of denatured proteins as well as assisting in the translocation and/or degradation of damaged proteins [36].

3.2.2. Sultanine berry genes specific to 4 weeks after DS0 (DS4)

The RDA subtraction according to our BRAT strategy allowed us to rescue 82 classes of TDFs specific to DS4 (Fig. 3 and Table 2). RT-qPCR analysis proved that all four candidate genes elected from this group confirmed their higher steady-state mRNA levels around the middle stage of berry formation, especially at DS4 (Fig. 4D–G). The first candidate, named VvRIP1, is an ASR-like (abscicic acid stress ripening) gene putatively encoding a ripening-induced protein. ASR-like genes were reported to be involved in the transcriptional response to water stress and their expressions were also detected during fruit ripening in several plant species [37]. The *A. thaliana* genome lacks ASR-like genes and only one copy has been detected in grapes using Southern blot hybridization [38]. Interestingly in tomato, the ASR1 protein was localized in the nucleus and shown to bind a specific DNA sequence [39]. Moreover, ASR proteins from other species including grapevine were also localized in the nucleus, and it was proposed that these proteins may regulate specific promoters [38,40,41]. These findings suggest that ASR proteins are transcription factors whose likely targets are hexose transporters and abscisic acid (ABA) responsive genes. Since many ASR genes are themselves induced by ABA and regulate genes involved in sugar transport and ABA response, a cross-talk between plant hormones and sugars responding to water stress could be speculated. In fact, ABA and sugars act in a concerted way during developmental processes in plants [42]. Being highly expressed during DS4, we propose that VvRIP1 is not involved in the start of hexose synthesis since only during DS8 such compounds start accumulating in grape berries (Fig. 1A). Due to the strong effect of ABA on seed formation [43], VvRIP1 could be one of the factors involved in seed (miss) development in Sultanine grape as an ABA-responsive gene. Such speculation is corroborated by the fact that endosperm degeneration in Sultanine grapes occurs between the 3rd and 6th week after anthesis [4], exactly the period of the highest expression of VvRIP1.

A cytochrome P450 (CYP450) putatively encoded by one of the most populated TDF clusters (VvP450) was also one of the selected candidate genes at DS4. Our RT-qPCR analysis proved that VvP450 shows its higher steady-state mRNA level around the middle stage of berry formation (Fig. 4E), especially at DS4. CYP450 is known to belong to a very large and diverse superfamily of hemoproteins that use a plethora of both exogenous and endogenous compounds as substrates in enzymatic reactions. They are usually part of multicomponent electron transfer chains, called P450-containing systems that are known to lead to the synthesis of various fatty acid conjugates, plant hormones, defensive compounds, or bioactive substances, for instance. Terpenoids, which represent the largest class of characterized natural plant compounds, are often substrates for plant CYP450 [44,45]. In *A. thaliana*, Ito and Meyerowitz [46] described a mutant named 28-5 ap2-1 in which the overexpression of the CYP450-like enzyme CYP78A9 induced large and seedless fruits. Although the function of the proteins encoded by the CYP78A subfamily is still unknown, members of this family have been isolated as coding for floral- or meristematic-specific transcripts [46–48]. It has been speculated that one of the functions of the CYP78A subfamily members may be the production of signals that activates or enhances fruit development due to its wide involvement in synthesis or degradation of plant secondary products [46]. Therefore the large accumulation of VvP450 transcripts at DS4 is most probably related to the first step of berry cell elongation (Fig. 1A) as a consequence of the enhanced secondary metabolite production [10].

A cDNA putatively encoding a transcription factor homologous to the *A. thaliana* DOF zinc-finger protein family was also identified at DS4. DOF proteins share a DNA-binding domain of 52 amino acid residues that is structured as a Cys_2/Cys_2 Zn^{2+} finger [49] that recognizes cis-regulatory elements in the promoters of their target genes containing the common core 5′-AAAG-3′ [50]. According to the RT-qPCR analysis, VvDOF1 steady-state mRNA level was increased at DS2, keeping such high levels until DS6 (Fig. 4F). DOF-domain proteins were described to be involved in seed germination [51,52], stress-responses [53–55], light-responses [56–58], phytochrome signaling [59], responses to plant hormones including auxin [60,61] and gibberellin [62,63], and tissue specific expression in endosperms [64,65], leaves [56] or guard cells [66]. Therefore it is not surprising to detect high levels of DOF transcripts at the middle stage of berry formation in grapevine.

The last candidate gene selected amongst the most expressed ones at DS4 by BRAT was VvERF1, named after *V. vinifera* ethylene-responsive transcription factor (ERF) TINY-like. RT-qPCR analysis however showed that VvERF1 was actually more highly expressed at DS2 (Fig. 4G). Like in the case of VvUBP1, it is important to take into account that the DS4 “tester” cDNA population was subtracted with an equivalent mixture of DS0 and DS8 cDNAs as “drivers”, thus the relative amount of the transcripts in the final pool was an average of the original samples. Therefore the higher expression of VvERF1 in DS2 and DS4 is in agreement with the BRAT strategy. The ERF is a large family of transcription factors that belongs to the AP2/ERF superfamily which also contains the AP2 and RAV families [67]. It has been demonstrated that AP2/ERF proteins have important functions in the transcriptional regulation of a variety of biological processes related to growth and development, as well as various responses to environmental stimuli. While genes of the AP2 family have been shown to participate in the regulation of processes like flower development [68], spikelet meristem determinacy [69], leaf epidermal cell identity [70] and embryo development [71], the involvement of RAV family members was shown to be more related to ethylene [72] and brassinosteroid [73] responses. Likewise, after finding the first ERFs in tobacco, many proteins in the ERF family were identified and implicated in many cellular processes such as hormonal signal transduction [74], response to biotic [75,76] and abiotic stresses [77,78], regulation of metabolism [79,80], and in developmental processes [81,82] in various plant species.

3.2.3. Sultanine berry genes specific to 8 weeks after DS0 (DS8)

The highest numbers of stage-specific gene singletons (218) and clusters (18) resulting from our BRAT subtraction were found at

DS8 (Fig. 3 and Table 2). The first candidate gene chosen to validate the preferential presence of this group of TDFs was a probable gibberellin receptor similar to *A. thaliana* GID1L1. VvGID1L1 was present amongst the most populated clusters at DS8. Our RT-qPCR analysis showed that this class of transcripts was present at DS8 but it was also equally abundant at DS2 (Fig. 4H). As observed for VvUBP1 and VvDOF1, DS8 "tester" cDNA population was subtracted with an equivalent mixture of DS0 and DS4 cDNAs. Therefore the higher expression of VvGID1L1 at DS2 as well as at DS8 is in agreement with the BRAT strategy employed.

Biologically active concentrations of gibberellins in seeded grapevines were described to be high in flowers and in fruits during the formation phase (Fig. 1A), but then usually drop to lower levels over the course of berry development [83]. Nevertheless, according to Perez et al. [84], there is a second peak of active gibberellins at the start of the lag phase which coincides exactly with the beginning of DS8 (not represented in Fig. 1A), and it is 77 times higher in the seed when compared to the berry mesocarp. The presence of VvGID1L1 at DS8 (and DS2) in Sultanine berries corroborates this observation. Deluc et al. [15] showed that the transcript abundance of two putative gibberellin receptors, GIDL1 and GIDL2, increases during seeded berry development. The complexity of the gibberellin effects over grape berry and seed formation is evident when comparing these observations [15,84] with the knowledge that gibberellins take a prominent part in seedlessness [2,85,86] possibly in association with other growth regulating substances like auxins [87] or ethylene [88]. Treatment with gibberellins, besides delaying ripening, is effective in the promotion of seedlessness in seeded grapes, in the suppression of vestigial seed development in normally seedless grapes, in the increase of berry and cluster size and in the decrease of cluster compactness [89–91].

Another candidate gene, named VvCLP1, was also found amongst the most populated clusters at DS8 according to our BRAT methodology. Nevertheless, differently from VvGID1L1, it did not confirmed higher transcript abundance at this stage by RT-qPCR. On the contrary and most surprisingly, it exhibited the lowest level of steady-state mRNA exactly at DS8 (Fig. 4I). VvCLP1 putatively encodes a CONSTANS-like (COL) protein 2. The COL genes encode members of a family of plant zinc finger proteins arranged into three subgroups on the basis of variations within the B-box region. VvCLP1 would belong to the first subgroup whose members detain two B-boxes [92]. Numerous COL genes have been correlated to different aspects of plant flowering, especially in what concerns photoperiod, day length and the circadian clock [93,94]. While the *Arabidopsis* CONSTANS family is composed of at least 17 genes [95], preliminary grapevine genome analysis showed the presence of at least 14 putative COL-like genes in the grapevine genome [96]. Almada et al. [96] characterized two of these COL genes, demonstrating their diurnal expression pattern and the correlation to photoperiod control of bud dormancy and flowering induction. Therefore, the most probable reason for the observed inconsistency between BRAT and RT-qPCR results for VvCLP1 transcripts is the existence of similar sequences of different members of the grapevine COL gene family. In such a hypothesis, while BRAT allowed the total subtraction of VvCLP1 transcripts out of DS0 and DS4, remaining in excess in DS8, primers designed for VvCLP1 allowed the amplification of a specific gene member of the COL family that is not (or very lowly) expressed at DS8. As performed for all other genes selected, the primers designed for RT-qPCR of the VvCLP1 gene were tested against the grapevine genome, being specific to one gene sequence. Nevertheless it is reasonable that some sequence variations exist between the Pinot Noir genome available and the Sultanine genome.

Our main objective when developing the present work was to establish a first profile of gene expression during key stages of grape berry development in a seedless cv model like Sultanine. In doing so, and with a subtractive approach like BRAT, it was very tempting to speculate on the commitment of the selected candidate genes as related, at least in part, with the seedless phenotype. Hanania et al. [8] has suggested that a key protein determining seedlessness in grapes is the chloroplast chaperonin 21 (ch-Cpn21), identified after suppression subtractive hybridization analysis between seeded and seedless grapevine lines of cv Sultanine. These authors showed that ch-Cpn21 silencing in tobacco and tomato plants using VIGS resulted in growth reduction, chlorosis and seed abortion, reinforcing the idea of a key role for ch-Cpn21 in seed formation. From the data presented by Hanania et al. [8] it seems clear that down-regulation of ch-Cpn21 is correlated with the seedless phenotype, indicating that the absence and/or lower levels of ch-Cpn21 may lead to seed abortion. In the present study, we decided to check the transcript levels of the putative ch-Cpn21 gene (VvCPN21) along the stages of Sultanine berry development. As shown in Fig. 4J, the lower levels of VvCPN21 were observed exactly at the stages of early berry formation (DS0–DS4), when degeneration of seed endosperm takes place. Interestingly, the expression of VvCPN21 was highest only at DS6, when seeds would be already fully developed in normal, seeded grapes. The patterns of VvCPN21 mRNA accumulation measured in our analysis therefore fully support the conclusions of Hanania et al. [8].

A considerable number of works [13–17] have focused on gene expression profiling during fruit development in seeded grapevine cultivars. Through such works, authors were able to characterize critical genes whose expressions may program cellular metabolism mostly through hormonal signaling and transcriptional regulation. The study of seedless berry development or the establishment of a link between seeded and seedless grapes were not focused on those works. Hanania et al. [8] have successfully proved the commitment of ch-Cpn21 in the seedless phenotype after a subtractive screening between seeded and seedless grape berries, but these authors have not described gene expression profiles obtained.

At the best of our knowledge, the present work is the first to describe genes differentially expressed and specific to the main stages of berry development in a seedless grape cv like Sultanine. Employing the described BRAT strategy, we were able to identify 1554 TDFs derived from mRNAs that most specifically accumulated at the fruit-set stage and at 4 and 8 weeks after fruit-set. BRAT results were validated by seven out of nine selected TDFs via RT-qPCR. We therefore believe that the BRAT strategy is quite useful for screening sample-specific transcripts when multiple temporal (stage-specific), spatial (organ-specific) or signal-specific samples need to be assayed. Besides checking the relevance of BRAT-selected genes during Sultanine berry development, next step will be the comparison of Sultanine berries through the key developmental stages with berries from seeded varieties at equivalent stages.

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References

[1] N. Loomis, J. Weinberger, Inheritance studies of seedlessness in grape, J. Am. Soc. Hortic. Sci. 104 (1979) 181–184.
[2] A. Bouquet, Y. Danglot, Inheritance of seedlessness in grapevine (*Vitis vinifera* L.), Vitis 35 (1996) 35–42.
[3] L. Costantini, J. Battilana, F. Lamaj, G. Fanizza, M. Grando, Berry and phenology-related traits in grapevine (*Vitis vinifera* L.): from quantitative trait loci to underlying genes, BMC Plant Biol. 8 (2008) 38.
[4] A. Stout, Seedlessness in grapes, New York Agr. Exp. Stat. Technol. Bull. 238 (1936) 1–65.
[5] F. Lahogue, P. This, A. Bouquet, Identification of a codominant scar marker linked to the seedlessness character in grapevine, Theor. Appl. Genet. 97 (1998) 950–959.
[6] J. Cabezas, M. Cervera, L. Ruiz-García, J. Carreño, J. Martínez-Zapater, A genetic analysis of seed and berry weight in grapevine, Genome 49 (2006) 1572–1585.
[7] N. Mejía, M. Gebauer, L. Muñoz, N. Hewstone, C. Muñoz, P. Hinrichsen, Identification of QTLs for seedlessness, berry size, and ripening date in a seedless × seedless table grape progeny, Am. J. Enol. Viticult. 58 (2007) 499–507.
[8] U. Hanania, M. Velcheva, E. Or, M. Flaishman, N. Sahar, A. Perl, Silencing of chaperonin 21, that was differentially expressed in inflorescence of seedless and seeded grapes, promoted seed abortion in tobacco and tomato fruits, Transgenic Res. 16 (2007) 515–525.
[9] U. Hanania, M. Velcheva, N. Sahar, M. Flaishman, E. Or, O. Degani, A. Perl, The ubiquitin extension protein S27a is differentially expressed in developing flower organs of Thompson seedless versus Thompson seeded grape isogenic clones, Plant Cell Rep. 28 (2009) 1033–1042.
[10] M. Carmona, J. Chaib, J. Martínez-Zapater, M. Thomas, A molecular genetics perspective of reproductive development in grapevine, J. Exp. Bot. 59 (2008) 2579–2596.
[11] B. Coombe, M. McCarthy, Dynamics of grape berry growth and physiology of ripening, Aust. J. Grape Wine Res. 6 (2000) 131–135.
[12] C. Conde, P. Silva, N. Fontes, A. Dias, R. Tavares, M. Sousa, A. Agasse, S. Delrot, H. Gerós, Biochemical changes throughout grape berry development and fruit and wine quality, Food 1 (2007) 1–22.
[13] F. Peng, K. Reid, N. Liao, J. Schlosser, D. Lijavetzky, R. Holt, J.M. Martínez Zapater, S. Jones, M. Marra, J. Bohlmann, S.T. Lund, Generation of ESTs in *Vitis vinifera* wine grape (Cabernet Sauvignon) and table grape (Muscat Hamburg) and discovery of new candidate genes with potential roles in berry development, Gene 402 (2007) 40–50.
[14] S. Pilati, M. Perazzolli, A. Malossini, A. Cestaro, L. Demattè, P. Fontana, A. Dal Rì, R. Viola, R. Velasco, C. Moser, Genome-wide transcriptional analysis of grapevine berry ripening reveals a set of genes similarly modulated during three seasons and the occurrence of an oxidative burst at *vèraison*, BMC Genome 8 (2007) 428.
[15] L. Deluc, J. Grimplet, M. Wheatley, R. Tillett, D. Quilici, C. Osborne, D.A. Schooley, K.A. Schlauch, J.C. Cushman, G.R. Cramer, Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development, BMC Genome 8 (2007) 429.
[16] S. Lund, F. Peng, T. Nayar, K. Reid, J. Schlosser, Gene expression analyses in individual grape (*Vitis vinifera* L.) berries during ripening initiation reveal that pigmentation intensity is a valid indicator of developmental staging within the cluster, Plant Mol. Biol. 68 (2008) 301–315.
[17] S. Zenoni, A. Ferrarini, E. Giacomelli, L. Xumerle, M. Fasoli, G. Malerba, D. Bellin, M. Pezzotti, M. Delledonne, Characterization of transcriptional complexity during berry development in *Vitis vinifera* using RNA-Seq, Plant Physiol. 152 (2010) 1787–1795.
[18] D. Lorenz, K. Eichhorn, H. Bleiholder, R. Klose, U. Meier, E. Weber, Growth stages of the grapevine: phenological growth stages of the grapevine (*Vitis vinifera* L. ssp. *vinifera*)—codes and descriptions according to the extended BBCH scale, Aust. J. Grape Wine Res. 1 (1995) 100–103.
[19] K. Pastorian, L. Hawel, C. Byus, Optimization of cDNA representational difference analysis for the identification of differentially expressed mRNAs, Anal. Biochem. 283 (2000) 89–98.
[20] B. Ewing, P. Green, Base-calling of automated sequencer traces using phred. II. Error probabilities, Genome Res. 8 (1998) 186–194.
[21] B. Ewing, L. Hillier, M. Wendl, P. Green, Base-calling of automated sequencer traces using phred. I. Accuracy assessment, Genome Res. 8 (1998) 175–185.
[22] K. Reid, N. Olsson, J. Schlosser, F. Peng, S. Lund, An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development, BMC Plant Biol. 6 (2006) 27.
[23] K. Livak, T. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[24] M. Hubank, D. Schatz, Identifying differences in mRNA expression by representational difference analysis of cDNA, Nucleic Acids Res. 22 (1994) 5640–5648.
[25] D. Murray, P. Doran, P. MacMathuna, A. Moss, *In silico* gene expression analysis—an overview, Mol. Cancer 6 (2007) 50.
[26] M. Polesani, F. Desario, A. Ferrarini, A. Zamboni, M. Pezzotti, A. Kortekamp, A. Polverari, cDNA-AFLP analysis of plant and pathogen genes expressed in grapevine infected with *Plasmopara viticola*, BMC Genome 9 (2008) 142.
[27] J. Díaz-Riquelme, D. Lijavetzky, J.M. Martínez-Zapater, M.J. Carmona, Genome-wide analysis of MIKCC-type MADS box genes in grapevine, Plant Physiol. 149 (2009) 354–369.
[28] M. Lambermon, G. Simpson, D. Wieczorek Kirk, M. Hemmings-Mieszczak, U. Klahre, W. Filipowicz, UBP1, a novel hnRNP-like protein that functions at multiple steps of higher plant nuclear pre-mRNA maturation, EMBO J. 19 (2000) 1349–1638.
[29] D. Staiger, L. Zecca, D. Wieczorek Kirk, K. Apel, L. Eckstein, The circadian clock regulated RNA-binding protein AtGRP7 autoregulates its expression by influencing alternative splicing of its own pre-mRNA, Plant J. 33 (2003) 361–371.
[30] M. Arteaga-Vazquez, J. Caballero-Perez, J. Vielle-Calzada, A family of microRNAs present in plants and animals, Plant Cell 18 (2006) 3355–3369.
[31] C. Gueydan, C. Wauquier, C. De Mees, G. Huez, V. Kruys, Identification of ribosomal proteins specific to higher eukaryotic organisms, J. Biol. Chem. 277 (2002) 45034–45040.
[32] V.B. Rajan, P. D'Silva, *Arabidopsis thaliana* J-class heat shock proteins: cellular stress sensors, Funct. Integr. Genomics 9 (2009) 433–446.
[33] K. Ohtsuka, M. Hata, Molecular chaperone function of mammalian Hsp70 and Hsp40—a review, Int. J. Hyperthermia 16 (2000) 231–245.
[34] S. Mishra, J. Tripp, S. Winkelhaus, B. Tschiersch, K. Theres, L. Nover, K-.D. Scharf, In the complex family of heat stress transcription factors, HsfA1 has a unique role as master regulator of thermotolerance in tomato, Genes Dev. 16 (2002) 1555–1567.
[35] M. Cronjé, I. Weir, L. Bornman, Salicylic acid-mediated potentiation of Hsp70 induction correlates with reduced apoptosis in tobacco protoplasts, Cytometry 61 (2004) 76–87.
[36] B. Bukau, A. Horwich, The Hsp70 and Hsp60 chaperone machines, Cell 92 (1998) 351–366.
[37] L. Maskin, G. Gudesblat, J. Moreno, F. Carrari, N. Frankel, A. Sambade, M. Rossi, N.D. Iusem, Differential expression of the members of the Asr gene family in tomato (*Lycopersicon esculentum*), Plant Sci. 161 (2001) 739–746.
[38] B. Cakir, A. Agasse, C. Gaillard, A. Saumonneau, S. Delrot, R. Atanassova, A grape ASR protein involved in sugar and abscisic acid signaling, Plant Cell 15 (2003) 2165–2180.
[39] Y. Kalifa, E. Perlson, A. Gilad, Z. Konrad, P. Scolnik, D. Bar-Zvi, Over-expression of the water and salt stress-regulated Asr1 gene confers an increased salt tolerance, Plant Cell Environ. 27 (2004) 1459–1468.
[40] H. Wang, C. Hsu, G. Jauh, C. Wang, A lily pollen ASR protein localizes to both cytoplasm and nuclei requiring a nuclear localization signal, Physiol. Plant 123 (2005) 314–320.
[41] C. Yang, Y. Chen, G. Jauh, C. Wang, A Lily ASR protein involves abscisic acid signaling and confers drought and salt resistance in *Arabidopsis*, Plant Physiol. 139 (2005) 836–846.
[42] R. Finkelstein, S. Gibson, ABA and sugar interactions regulating development: cross-talk or voices in a crowd? Curr. Opin. Plant Biol. 5 (2002) 26–32.
[43] A. Frey, B. Godin, M. Bonnet, B. Sotta, A. Marion-Poll, Maternal synthesis of abscisic acid controls seed development and yield in *Nicotiana plumbaginifolia*, Planta 218 (2004) 958–964.
[44] S. Tanabe, M. Ashikari, S. Fujioka, S. Takatsuto, S. Yoshida, M. Yano, A. Yoshimura, H. Kitano, M. Matsuoka, Y. Fujisawa, H. Kato, Y. Iwasaki, A novel cytochrome P450 is implicated in brassinosteroid biosynthesis via the characterization of a rice dwarf mutant, *dwarf11*, with reduced seed length, Plant Cell 17 (2005) 776–790.
[45] J. Ehlting, V. Sauveplane, A. Olry, J. Ginglinger, N. Provart, D. Werck-Reichhart, An extensive (co-)expression analysis tool for the cytochrome P450 superfamily in *Arabidopsis thaliana*, BMC Plant Biol. 8 (2008) 47.
[46] T. Ito, E. Meyerowitz, Overexpression of a gene encoding a cytochrome P450, CYP78A9, induces large and seedless fruit in *Arabidopsis*, Plant Cell 12 (2000) 1541–1550.
[47] J. Nadeau, X. Zhang, J. Li, S. O'Neill, Ovule development: identification of stage-specific and tissue-specific cDNAs, Plant Cell 8 (1996) 213–239.
[48] S. Zondlo, V. Irish, CYP78A5 encodes a cytochrome P450 that marks the shoot apical meristem boundary in *Arabidopsis*, Plant J. 19 (1999) 259–268.
[49] Y. Umemura, T. Ishiduka, R. Yamamoto, M. Esaka, The Dof domain, a zinc finger DNA-binding domain conserved only in higher plants, truly functions as a Cys2/Cys2 Zn finger domain, Plant J. 37 (2004) 741–749.
[50] M.Á. Moreno-Risueno, M. Martínez, J. Vicente-Carbajosa, P. Carbonero, The family of DOF transcription factors: from green unicellular algae to vascular plants, Mol. Genet. Genomics 277 (2007) 379–390.
[51] M. Papi, S. Sabatini, D. Bouchez, C. Camilleri, P. Costantino, P. Vittorioso, Identification and disruption of an *Arabidopsis* zinc finger gene controlling seed germination, Genes Dev. 14 (2000) 28–33.
[52] G. Gualberti, M. Papi, L. Bellucci, I. Ricci, D. Bouchez, C. Camilleri, P. Costantino, P. Vittorioso, Mutations in the Dof zinc finger genes DAG2 and DAG1 influence with opposite effects the germination of *Arabidopsis* seeds, Plant Cell 14 (2002) 1253–1263.
[53] B. Zhang, W. Chen, R. Foley, M. Büttner, K. Singh, Interactions between distinct types of DNA binding proteins enhance binding to ocs element promoter sequences, Plant Cell 7 (1995) 2241–2252.
[54] W. Chen, G. Chao, K. Singh, The promoter of a H2O2-inducible, *Arabidopsis* glutathione S-transferase gene contains closely linked OBF- and OBP1-binding sites, Plant J. 10 (1996) 955–966.
[55] H. Kang, R. Foley, L. Oñate-Sánchez, C. Lin, K. Singh, Target genes for OBP3, a Dof transcription factor, include novel basic helix-loop-helix domain proteins inducible by salicylic acid, Plant J. 35 (2003) 362–372.
[56] S. Yanagisawa, J. Sheen, Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression, Plant Cell 10 (1998) 75–89.
[57] S. Yanagisawa, Dof1 and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize, Plant J. 21 (2000) 281–288.

[58] M. Papi, S. Sabatini, M. Altamura, L. Hennig, E. Schäfer, P. Costantino, P. Vittorioso, Inactivation of the phloem-specific Dof zinc finger gene DAG1 affects response to light and integrity of the testa of *Arabidopsis* seeds, Plant Physiol. 128 (2002) 411–417.
[59] D. Park, P. Lim, J. Kim, D. Cho, S. Hong, H. Nam, The *Arabidopsis* COG1 gene encodes a Dof domain transcription factor and negatively regulates phytochrome signaling, Plant J. 34 (2003) 161–171.
[60] A. De Paolis, S. Sabatini, L. De Pascalis, P. Costantino, I. Capone, A rolB regulatory factor belongs to a new class of single zinc finger plant proteins, Plant J. 10 (1996) 215–223.
[61] Y. Kisu, T. Ono, N. Shimofurutani, M. Suzuki, M. Esaka, Characterization and expression of a new class of zinc finger protein that binds to silencer region of ascorbate oxidase gene, Plant Cell Phys. 39 (1998) 1054–1064.
[62] K. Washio, Identification of Dof proteins with implication in the gibberellin-regulated expression of a peptidase gene following the germination of rice grains, Biochim. Biophys. Acta 1520 (2001) 54–62.
[63] M. Mena, F. Cejudo, I. Isabel-Lamoneda, P. Carbonero, A role for the DOF transcription factor BPBF in the regulation of gibberellin-responsive genes in barley aleurone, Plant Physiol. 130 (2002) 111–119.
[64] J. Vicente-Carbajosa, S. Moose, R. Parsons, R. Schmidt, A maize zinc-finger protein binds the prolamin box in zein gene promoters and interacts with the basic leucine zipper transcriptional activator Opaque2, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 7685–7690.
[65] M. Mena, J. Vicente-Carbajosa, R. Schmidt, P. Carbonero, An endosperm-specific DOF protein from barley, highly conserved in wheat, binds to and activates transcription from the prolamin-box of a native B-hordein promoter in barley endosperm, Plant J. 16 (1998) 53–62.
[66] G. Plesch, T. Ehrhardt, B. Mueller-Roeber, Involvement of TAAAG elements suggests a role for Dof transcription factors in guard cell-specific gene expression, Plant J. 28 (2001) 455–464.
[67] J. Riechmann, J. Heard, G. Martin, L. Reuber, C. Jiang, J. Keddie, L. Adam, O. Pineda, O.J. Ratcliffe, R.R. Samaha, R. Creelman, M. Pilgrim, P. Broun, J.Z. Zhang, D. Ghandehari, B.K. Sherman, G-.L. Yu, *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes, Science 290 (2000) 2105–2110.
[68] R. Elliott, A. Betzner, E. Huttner, M. Oakes, W. Tucker, D. Gerentes, P. Perez, D.R. Smyth, AINTEGUMENTA, an APETALA2-like gene of *Arabidopsis* with pleiotropic roles in ovule development and floral organ growth, Plant Cell 8 (1996) 155–168.
[69] G. Chuck, R. Meeley, S. Hake, The control of maize spikelet meristem fate by the APETALA2-like gene indeterminate spikelet, Genes Dev. 12 (1998) 1145–1154.
[70] S. Moose, P. Sisco, Glossy15, an APETALA2-like gene from maize that regulates leaf epidermal cell identity, Genes Dev. 10 (1996) 3018–3027.
[71] K. Boutilier, R. Offringa, V. Sharma, H. Kieft, T. Ouellet, L. Zhang, J. Hattori, C.M. Liu, A.A.M. van Lammeren, B.L.A. Miki, J.B.M. Custers, M.M. van Lookeren Campagne, Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth, Plant Cell 14 (2002) 1737–1749.
[72] J. Alonso, A. Stepanova, T. Leisse, C. Kim, H. Chen, P. Shinn, D.K. Stevenson, J. Zimmerman, P. Barajas, R. Cheuk, C. Gadrinab, C. Heller, A. Jeske, E. Koesema, C.C. Meyers, H. Parker, L. Prednis, Y. Ansari, N. Choy, H. Deen, M. Geralt, N. Hazari, E. Hom, M. Karnes, C. Mulholland, R. Ndubaku, I. Schmidt, P. Guzman, L. Aguilar-Henonin, M. Schmid, D. Weigel, D.E. Carter, T. Marchand, E. Risseeuw, D. Brogden, A. Zeko, W.L. Crosby, C.C. Berry, J.R. Ecker, Genome-wide insertional mutagenesis of *Arabidopsis thaliana*, Science 301 (2003) 653–657.
[73] Y. Hu, Y. Wang, X. Liu, J. Li, *Arabidopsis* RAV1 is down-regulated by brassinosteroid and may act as a negative regulator during plant development, Cell Res. 14 (2004) 8–15.
[74] M. Ohme-Takagi, H. Shinshi, Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element, Plant Cell 7 (1995) 173–182.
[75] S. Yamamoto, K. Suzuki, H. Shinshi, Elicitor-responsive, ethylene-independent activation of GCC box-mediated transcription that is regulated by both protein phosphorylation and dephosphorylation in cultured tobacco cells, Plant J. 20 (1999) 571–579.
[76] Y. Gu, C. Yang, V. Thara, J. Zhou, G. Martin, Pti4 is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase, Plant Cell 12 (2000) 771–786.
[77] Q. Liu, M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki, K. Shinozaki, Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*, Plant Cell 10 (1998) 1391–1406.
[78] J. Dubouzet, Y. Sakuma, Y. Ito, M. Kasuga, E. Dubouzet, S. Miura, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression, Plant J. 33 (2003) 751–763.
[79] P. Broun, P. Poindexter, E. Osborne, C. Jiang, J. Riechmann, WIN1, a transcriptional activator of epidermal wax accumulation in *Arabidopsis*, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 4706–4711.
[80] J. Zhang, C. Broeckling, E. Blancaflor, M. Sledge, L. Sumner, Z. Wang, Overexpression of WXP1, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*), Plant J. 42 (2005) 689–707.
[81] H. Banno, Y. Ikeda, Q. Niu, N. Chua, Overexpression of *Arabidopsis* ESR1 induces initiation of shoot regeneration, Plant Cell 13 (2001) 2609–2618.
[82] G. Chuck, M. Muszynski, E. Kellogg, S. Hake, R. Schmidt, The control of spikelet meristem identity by the branched silkless1 gene in maize, Science 298 (2002) 1238–1241.
[83] G. Symons, C. Davies, Y. Shavrukov, I. Dry, J. Reid, M. Thomas, Grapes on steroids. Brassinosteroids are involved in grape berry ripening, Plant Physiol. 140 (2006) 150–158.
[84] F. Perez, C. Viani, J. Retamales, Bioactive gibberellins in seeded and seedless grapes: identification and changes in content during berry development, Am. J. Enol. Viticult. 51 (2000) 315–318.
[85] C. Srinivasan, M. Mullins, Control of flowering in the grapevine (*Vitis vinifera* L.): formation of inflorescences in vitro by isolated tendrils, Plant Physiol. 61 (1978) 127–130.
[86] C. Ledbetter, C. Shonnard, Improved seed development and germination of stenospermic grapes by plant growth regulators, J. Hortic. Sci. Biotechnol. 65 (1990) 269–274.
[87] P. Kimura, G. Okamoto, K. Hirano, Effects of gibberellic acid and streptomycin on pollen germination and ovule and seed development in Muscat Bailey A, Am. J. Enol. Viticult. 47 (1996) 152–156.
[88] J.P. Nitsch, C. Pratt, C. Nitsch, N.J. Shaulis, Natural growth substances in Concord and Concord Seedless grapes in relation to berry development, Am. J. Bot. 47 (1960) 566–576.
[89] Y. Ben-Tal, Effects of gibberellin treatments on ripening and berry drop from Thompson seedless grapes, Am. J. Enol. Viticult. 41 (1990) 142–146.
[90] C. Agüero, A. Vigliocco, G. Abdala, R. Tizio, Effect of gibberellic acid and uniconazol on embryo abortion in the stenospermocarpic grape cultivars Emperatriz and Perlon, Plant Growth Regul. 30 (2000) 9–16.
[91] G. Fanizza, F. Lamaj, L. Costantini, R. Chaabane, M. Grando, QTL analysis for fruit yield components in table grapes (*Vitis vinifera*), Theor. Appl. Genet. 111 (2005) 658–664.
[92] F. Robson, M.M. Costa, S.R. Hepworth, I. Vizir, M. Piñeiro, P.H. Reeves, J. Putterill, G. Coupland, Functional importance of conserved domains in the flowering-time gene CONSTANS demonstrated by analysis of mutant alleles and transgenic plants, Plant J. 28 (2001) 619–631.
[93] S. Kim, S. Lee, H. Kim, H. Nam, G. An, OsMADS51 is a short-day flowering promoter that functions upstream of Ehd1, OsMADS14, and Hd3a, Plant Physiol. 145 (2007) 1484–1494.
[94] S. Wenkel, F. Turck, K. Singer, L. Gissot, J. Le Gourrierec, A. Samach, G. Coupland, CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of *Arabidopsis*, Plant Cell 18 (2006) 2971–2984.
[95] S. Griffiths, R.P. Dunford, G. Coupland, D.A. Laurie, The evolution of CONSTANS-like gene families in barley, rice, and *Arabidopsis*, Plant Physiol. 131 (2003) 1855–1867.
[96] R. Almada, N. Cabrera, J.A. Casaretto, S. Ruiz-Lara, E.G. Villanueva, VvCO and VvCOL1, two CONSTANS homologous genes, are regulated during flower induction and dormancy in grapevine buds, Plant Cell Rep. 28 (2009) 1193–1203.