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Contrasting grapevines grafted into naturalized rootstock suggest scion-driven transcriptomic changes in response to water deficit

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ARTICLE INFO

This work is dedicated to the memory of our brilliant and enthusiastic colleague, Professor Dr. Nicolás Franck, our friend and mentor. His human and scientific legacy will continue through his impact on those who knew him.

Keywords:
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ABSTRACT

Viticulture is facing water deficit problems related to climate change, new extremes in heat and precipitation regimes and drought events. Rootstocks use was assessed as strategy for enhancing performance of Cabernet Sauvignon (CS) and Syrah (Sy) under water deficit. Vines were grafted onto naturalized grapevines selected from hyper-arid Chile, and compared to own-grafted and commercial Ruggeri 140. Plants were submitted to optimal (100 % ETc) and deficit (30 % ETc) irrigation throughout two seasons at field conditions. Functional traits along both seasons were determined. Water deficit reduced all growth and physiological traits especially in CS. R32 rootstock induced significantly higher values for most traits irrespective of cv and seasons associated to higher root growth. Transcriptomic analysis was further performed in both cultivars grafted over R32 rootstock by RNA-Seq, determining that gene up-regulation extent was higher in Sy. More stable transcriptional landscape was determined in CS than Sy, which might be linked to its hydric strategy. Unexpectedly, major differences in transcriptional behaviour were detected in R32 rootstock, revealing major transcriptional changes occurring at root level, suggesting scion-driven transcriptional regulation in response to stress. Finally, R32 rootstock can be considered for both near iso and anisohydric grapevines as adaptive strategy for climate constrains.

1. Introduction

Water scarcity is one of the main challenges that viticulture has to deal with under the current global climate change (GCC) scenario (Hannah et al., 2013). Current models have also determined that in coming years the average global temperature will increase by almost 2–5 °C, while rainfall will vary depending on the specific region. Recent studies have shown that variations in precipitation regimes will be the most threatening environmental factor in Mediterranean regions (Núñez et al., 2011). Grapevine growth and yield can be seriously reduced under water deficit and therefore may respond at many levels of organization, from the molecular and physiological levels to the level of the whole plant, where stomatal control of transpiration is one of the major strategies by which vines cope with water stress (Chaves et al., 2003; Marguerit et al., 2012). However, the response mechanisms, in terms of signalling and gene functioning, remain elusive and need further studies (Serra et al., 2013). Thus, genotype-architecture-physiology-environment interaction will be a key factor to GCC challenges, since the plant response strategies to water stress will determine its productivity, and by selecting genotypes that can withstand a region's

Abbreviations: 140Ru, 140 Ruggeri rootstock; ABA, abscisic acid; Bp, base pairs; CPM, counts per million; CS, Cabernet Sauvignon; cv, cultivar; DEG, differentially expressed gene; FC, fold change; FDR, false discovery rate; GCC, global climate change; GO, gene ontology; g_s, stomatal conductance; PCA, principal component analysis; P_n, net photosynthesis; qPCR, quantitative PCR; R32, rootstock genotype 32 (also for R50, R65, R70); RNA-Seq, RNA sequencing; RsCSA, rootstock cross sectional area; RtA, root area; ShLA, shoot leaf area; ShLN, shoot leaf number; Sy, Syrah; TkCSA, trunk cross sectional area; Ψx, stem water potential

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new climate regime, growers could ideally continue to grow at current locations (Wolkovich et al., 2017).

Grapevines display huge variability in responses to water deficit, indeed some genotypes display "near-isohydric" strategy that decrease their stomatal conductance to keep leaf water potential, maintaining their leaf area, whereas other genotypes display “near-anisohydric” strategy maintaining their evapotranspiration level in water stress, which results in a reduction of leaf water potential and compensate the flux by a reduction in leaf area (Schultz, 2003; Chaves et al., 2010; Lovisolo et al., 2010; Hochberg et al., 2013). In vines, abscisic acid (ABA) acts to reduce water loss and increase stress tolerance, in addition to reducing stomatal conductance and limiting leaf area expansion. Indeed, ABA tightly influenced the stomatal conductance of Cabernet Sauvignon (near-isohydric), whereas in Syrah (near-anisohydric) an ABA-related stomatal closure was induced to maintain high levels of water potential, showing that a soil-related hormonal root-to-shoot signal causing stomatal closure superimposes on the putatively variety-induced anisohydric response to water stress (Tramontini et al., 2014).

Pioneer experiments in grapevine identified grapevine-specific factors in response to drought and salt stress (Cramer et al., 2007). These results demonstrated that many induced genes could be grouped according to their putative function, as transcription factors, ABA-responsive genes, proteins of reactive oxygen species and metabolic factors (Cramer et al., 2007). One of master phytohormones involved in the responses to drought is ABA (Le Henanff et al., 2013; Kuromori et al., 2018). In vines, stomatal conductance has been determined to correlate negatively with ABA concentrations in xylem flow, and ABA leaf concentrations are correlated with abundance of *VvNCED1*, which codifies the rate-limiting enzyme reaction in ABA biosynthesis (Speirs et al., 2013). Recent studies have also analysed transcriptome responses of grapevine rootstock and graft interface tissues after grafting of overwintering stems. Many genes were differentially expressed over time, and up-regulation of genes associated with cell wall synthesis, phloem and xylem development, and numerous graft interface-specific genes were identified (Cookson and Ollat, 2013).

A compelling strategy for overcoming abiotic constraints is the use of drought tolerant rootstocks (Stevens et al., 2010; Williams, 2010; Keller et al., 2012; Ollat et al., 2016; Warschefsky et al., 2016). The use of rootstocks has a profound effect on development since rootstocks are capable of influencing ecophysiological behaviour of scion and its berry quality (Pongrácz, 1983; Ibacache and Sierra, 2009; Marguerit et al., 2012; Tramontini et al., 2013; Ibacache et al., 2016; Lovisolo et al., 2016). Roots are essential for uptake and convey most of the water and nutrients required by shoots and synthesize the hormones needed for an adequate development of shoot system. Root water uptake capacity contrasts therefore the risk of plant tissues dehydration, concurrent to abiotic stresses (Aroca et al., 2011; Lovisolo et al., 2016). Accordingly, roots are essential for optimal plant productivity (Bechtold and Field, 2018). Several hypotheses have been proposed that explain scion vigour conferred by the rootstock, including alterations in water movement, hormone concentrations, nutrient acquisition and assimilation, and ultimately anatomy of graft union (Cookson and Ollat, 2013; Cochetel et al., 2017; Gautier et al., 2018; Gautier et al., 2019). Nonetheless, contrasting effects of rootstocks on grapevine responses to water deficit has been reported for different cultivars and climatic conditions (Keller et al., 2012; Tandonnet et al., 2018), but at spatial scale, root system architecture represents a highly dynamic physical network that facilitates plant access to a heterogeneous distribution of water in soil (Paez-Garcia et al., 2015; Feng et al., 2016). An appealing strategy for developing drought tolerant rootstocks is the use of grapevine naturalized genotypes, which have adapted to arid climatic conditions such as those thriving in a latitudinal gradient along the Atacama Desert (Bavestrello-Riquelme et al., 2012; Milla-Tapia et al., 2013). Since plants have survived harsh environmental conditions, naturalised and admixed genotypes are potential sources of new alleles, and are a unique source of diversity for grapevine rootstock breeding better adapted to the prospect of climate change (Bavestrello-Riquelme et al., 2012; Milla-Tapia et al., 2013).

To dissect how adaptive mechanisms to water stress of different rootstock genotypes affect plant performance, we studied response to deficit irrigation of Cabernet Sauvignon (near-isohydric) and Syrah (near-anisohydric) cultivars (Hochberg et al., 2013) grafted onto selected naturalized rootstocks and compared to both self-rooted plants and commercial 140 Ruggeri rootstock, which has been shown to increase drought tolerance in CS (Williams, 2010), and Sy (Stevens et al., 2010). Moreover, we determined specific responses for both cultivars over one naturalized rootstock by means of RNA-Seq analysis to study inducible changes triggered by water deficit.

2. Materials and methods

2.1. Plant material, drought stress conditions and morpho-physiological measurements

Field experiment was conducted during two growing seasons (2014/15 and 2015/16) at Las Cardas experimental Station (30°13′ South, 71°13′ West) of University of Chile under semiarid climate conditions. Cultivars Cabernet Sauvignon (CS, near-isohydric) and Syrah (Sy, near-anisohydric) were grafted onto four naturalized genotypes (R32, R57, R65 and R70) selected in northern Chile for their tolerance to water deficit (Bavestrello-Riquelme, et al., 2012; Milla-Tapia et al., 2013), and also to commercial tolerant rootstock Ruggeri 140 (140Ru) and self-grafted vines (RF). Vines were planted in 35 L pots with a soil – ground sheet – perlite mixture (1:1:1) in a 1 × 3 m spacing within north-south oriented rows. After the first season, vines were conducted in a VSP trellis and winter-pruned to two cordons with four spurs carrying four buds each. Two irrigation treatments were applied: full irrigation (T100) and 30 % irrigation (T30) via a drip irrigation system in both cultivars and randomly distributed within 4 blocks. For RNA-Seq experiments, R32 rootstock and both cvs leaf tissues were selected from irrigation treatments with *three biological replicates*.

Morphological measurements were performed at a two-week interval along both growing seasons: shoot growth on one representative shoot per vine (shoot length, leaf number per shoot [L/Sh] and area [ShLA, calculated as L/Sh x average individual leaf area that was estimated with an allometric equation relating leaf area to leaf length and width]), trunk (TkCSA) and rootstock (RsCSA) cross sectional area (estimated from perimeter assuming a circular section). Each month, root traits (area [RtA], volume [RtV] and length [RtL]) were also estimated from images obtained from accession tubes inserted in each pot with a rootscan Imager (CID BioScience) and analyzed with the Rootsnap 1.3.2.25 software. Physiological traits were measured each second week: net photosynthesis (P_n) and stomatal conductance (g_s; Li-Cor 6400 XTR IRGA); maximum quantum efficiency (Fv/Fm; Hansatech FMS2 fluorometer) and midday stem water potential (Ψx; pressure chamber PMS 1505D).

2.2. Total RNA Isolation and cDNA Synthesis

Leaf and root tissues were sampled at the maturation stage for RNA extraction, one set for each treatment, grinded under liquid nitrogen and collected after grinding. For each analysis, 50 − 100 mg of combined leaf tissue were processed. Total RNA was extracted using the 2 % CTAB Method and Lithium Chloride precipitation proposed by Zeng and Yang (2002), further purified with the Ultra Clear RNA Isolation Kit (MOBIO) and treated with RQ1 RNase-Free DNase Promega). RNA purified and stored in DEPC water was treated at -80 °C. After gel verification and quantification analysis, two different pools will be composed (drought and control, biological triplicates) using the same quantity from each plant tissue (2ug total RNA from each condition) and sent to Macrogen, Inc. (Seoul, South Korea) for Illumina Paired-end

sequencing.

2.3. High-throughput Illumina RNA sequencing and data analysis

RNA samples from treated tissues were treated and a library construction and deep sequencing for each sample was performed by contract at a Macrogen facility using HiSeq 2500 system platform (Seoul, South Korea). The Illumina NGS workflows included 4 basic steps from sample preparation, for library construction RNA was extracted from a sample, performing quality control (QC), and passed sample was proceeded with the library construction. The sequencing library was prepared by Tru-Seq RNA kit (Illumina Inc.) following the manufacturer's protocol. Supplemental Table 1 Table shows obtained raw data statistics for sampled tissues.

2.4. Differentially Expressed genes comparison

Sequencing quality of each library was analysed by means of FastQC v0.11.5 software. After this quality analysis, trimming process was carried out by Trim galore v0.4.3 software to remove sequence adapters and filtering by sequence quality with default parameters. Following this procedure, trimmed reads were mapped to the reference genome PN40024 (Jaillon et al., 2007) through STAR software (Dobin et al., 2012). Results are shown in Supplemental Table 2 for Cabernet Sauvignon, and Supplemental Table 3 for Syrah.

Once reads were mapped against reference genome, SAM formats were transformed to Bam formats and thereafter ordered by SamTools software (Li et al., 2009). These procedures generated map files in bam format, to perform reads counts by each individual gene by using HTSeq software (Anders et al., 2015). Then, calling normalization procedure was performed to trimmed mean M value (TMM) using edgeR software (Robinson et al., 2010).

DEG analysis was performed by edgeR, using filter parameters of FDR < 0,05 and > 2-fold change, comparing differential expressed genes between both irrigation treatments and combinations of tissues in both leaves and roots of cultivars grafted on R32 (see Supplemental Fig. 1). Lists of genes with significant DE between conditions were compared using Venn diagrams between cultivars and tissues (http://bioinfogp.cnb.csic.es/tools/venny/). Then further analysis included gene ontology with such lists, zooming at unique genes in each cultivar separately with AgriGO tool (http://systemsbiology.cau.edu.cn/agriGOv2/). Once Gene Ontology (GO) analysis was performed, a reduction of redundancy in GO terminology was carried out by means of REVIGO tool (http://revigo.irb.hr/).

2.5. Total RNA Isolation and cDNA Synthesis for Gene expression analysis by qRT-PCR

Tissues were sampled at maturation stage for RNA extraction, one set for each treatment, grinded under liquid nitrogen and collected after grinding. For each analysis, 50 − 100 mg of combined leaf tissue were processed. Total RNA was extracted using the 2 % CTAB Method and Lithium Chloride precipitation proposed by Zeng and Yang (2002), further purified with the Ultra Clear RNA Isolation Kit (MOBIO) and treated with RQ1 RNase-Free DNase Promega). RNA purified and stored in DEPC water was treated at -80 °C. First strand DNA was synthesized from 2 µg of total RNA using reverse inversion components of the high capacity RNA kit for cDNA (Thermo Fisher Scientific).

Primer pairs for quantification of various gene products by real-time PCR are shown in Supplementary Table. After diluting cDNA to a concentration of 10 ng / µL 5 µL were placed in each reaction in a final volume of 20 µL as described previously (Morales et al., 2017). Thermocycling conditions were as follows: initial enzyme activation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 5 s, hybridization at 60 °C for 10 s, and extension at 72 °C for 10 S, followed by a gradient of melt (Melt curve) at a resolution of 0.5 °C and soak time 5 s. The reactions were carried out on the AriaMx Real Time PCR System. Amplicon specificity from each set of primers was determined by fusion gradient. All cDNA samples compared for gene expression levels were assayed in a single batch for each pair of primers and each set of assays was run in triplicate, additionally non-RT and NTC controls were included.

2.6. Statistical Analysis

Analysis of variance (mean separation via Tukey's test) and principal component analysis were performed using the Infostat statistical software. Relative changes in gene expression were determined using the Pfaffl method (Pfaffl, 2006), which considers the efficiency of amplification for each primer sets, control, treatments and *Ubiquitin* as normalizing housekeeping gene. To visualize the results, the maximum expression of each gene was taken as a unit, then transformed into the Log_2 RATIO to be studied by an analysis of variance (ANOVA) using the statistical package Infostat V1.1. The differences were significant if $P < 0.05$. A test of LSD Fisher stock was applied to observe differences between cultivars and rootstocks in stress treatments.

3. Results and Discussion

3.1. Changes in morphological and physiological parameters under water stress

Considering scions' growth, Sy at full irrigation (T100) exhibited higher shoot leaf number (ShLN; Fig. 1A), shoot leaf area (ShLA; Fig. 1B), and cross-sectional area of the trunk (TkCSA; Fig. 1D) than CS, irrespective of treatment and season. TkCSA was reduced by 30 % of deficit irrigation (T30) during both seasons, whereas ShLN and ShLA were reduced by T30 during both seasons for Sy but only during the second season for CS (Fig. 1). Deficit irrigation also reduced root area only during the second season in both cultivars, without significant differences between cultivars (Fig. 1F). The same seasonal effect was observed in other trait related to rootstock growth, cross sectional area (RsCSA; Fig. 1G), without differences between CS and Sy. Also, root volume and length did not exhibit significant differences between cultivars and seasons (data not shown). Deficit irrigation (T30) also reduced midday stem water potential (Ψx) in both cultivars for both season but to a higher extent during the second season (Fig. 1E). In terms of gas exchange, T30 reduced net photosynthesis (P_n) and stomatal conductance (g_s) of both cultivars during both seasons but no significant differences between Sy and CS were observed for these traits (Fig. 1D, H respectively). Rootstocks did not significantly modify physiological traits (data not shown) but had significant effects on rootstock and shoot growth, which did not interact with irrigation treatment, when analysing each cultivar separately.

Effects on rootstock growth were observed in root area (RtA CS season 1; Fig. 2B) and trunk (TkCSA; all but Sy season 1 Fig. 2D) under T30, in which the rootstock R32 was always ranked within the significantly higher levels (Fig. 2). Although rootstocks only affected TkCSA for Sy during the first season, they significantly affected ShLA in both seasons and cultivars (Fig. 2A). Accordingly, the rootstocks enabling development of a higher leaf area (as assessed by ShLA) under deficit irrigation might be claimed as having a better fitness and, hence, higher drought tolerance. In this concern, R32 displayed the highest ShLA throughout seasons and cultivars and thus performed as the best rootstock under water deficit conditions. Although some of the other rootstocks were not significantly lower in ShLA than R32 in some of the seasons by cultivar combinations (R50 and R70 in CS season2; R65 and RF in Sy season1; and R56 and 140Ru in Sy season1), R32 was the only one to consistently enable the highest ShLA during both seasons and in both cultivars.

Deficit irrigation (T30) reduced midday stem water potential (Ψx) in both cultivars for both season but to a higher extent during the

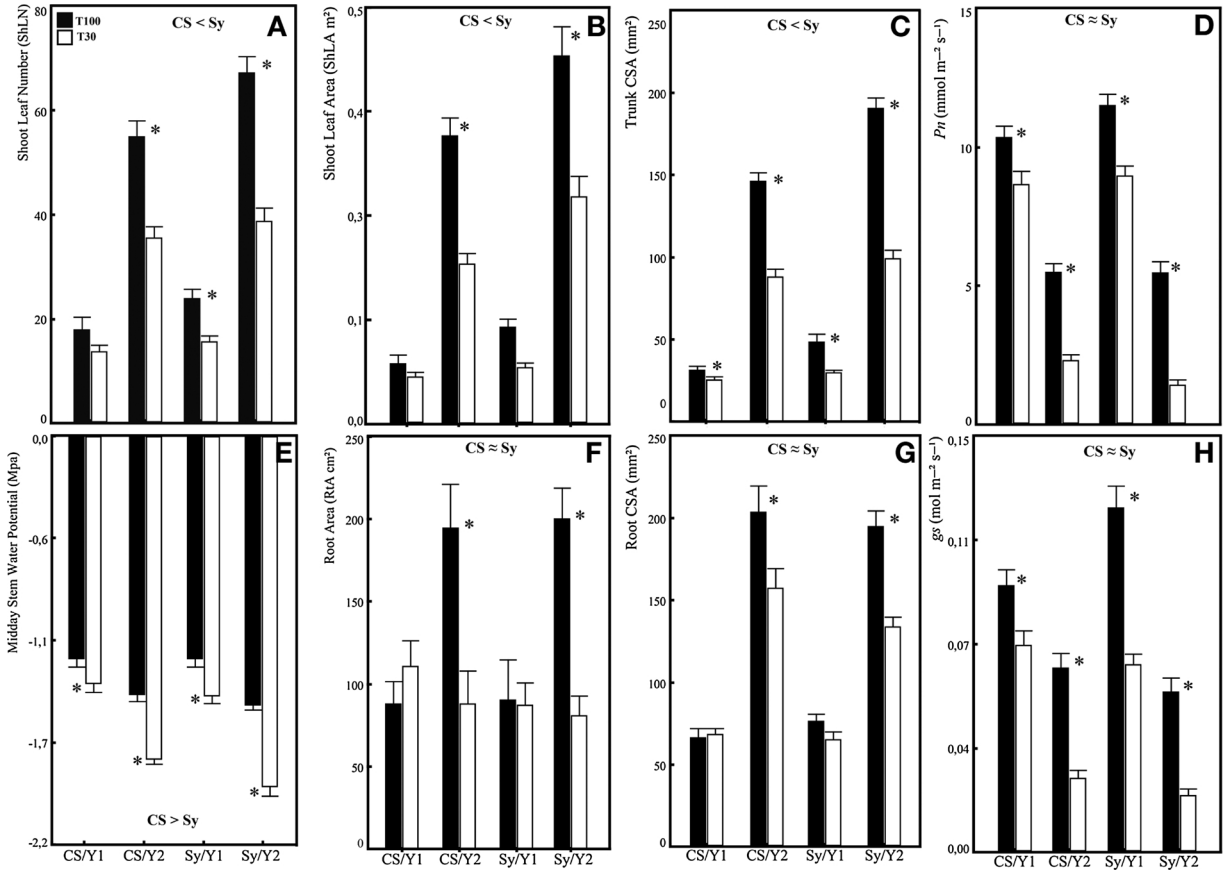


Fig. 1. Response to full (T100, black bars) and 30 % irrigation (T30, white bars) of cultivars (CS: Cabernet Sauvignon, Sy: Syrah) and seasons (Y1 and Y2) on shoot leaf number (ShLN; A) shoot leaf area (ShLA; B) and root area (RtA; F), trunk (TkCSA; C) and rootstock cross sectional area (RsCSA; G), net photosynthesis (P_n; D) stem water potential (Ψ_x, E), and stomatal conductance (g_s; H). *: statistical differences between treatments; Differences between cultivars: significantly higher > ; significantly lower < ; ≈ not different; Tukey ($\alpha = 0.05$).

second season (Fig. 1E). This indicates a mild water stress during the first season and a severe one during the second season. In both seasons Ψ_x was lower in Sy than in CS, which is consistent with the classification of Sy as a near-anisohydric cultivar and CS as a near-isohydric one (Hochberg et al., 2013). It was noteworthy that 140Ru exhibited low ShLA in CS as compared when using R32 (Fig. 2), since 140Ru have shown to increase yield and water use under reduced irrigation for Syrah (Stevens et al., 2010) and Cabernet Sauvignon (Williams, 2010), but also achieved high ShLA in the present study in Syrah. This contrasting effect between cultivars, which was also found for other rootstocks in this study (such as R57 and R70) that exhibited high ShLA in CS but not in Sy, is in line with the results of Keller et al. (2012) who found contrasting effects of rootstocks on shoot growth and yield of three different grapevine cultivars submitted to deficit irrigation. Thus, ShLA trait can be a good proxy for analysing the performance of both vines and corresponding rootstocks under water deficit as it's directly linked to yield potential (Petrie et al., 2000).

Furthermore, Principal Component Analysis (PCA) confirmed that R32 was closely associated to ShLA irrespective of season and cultivar and even opposed to P_n, g_s and Ψ_x during the second season during which water stress was severe (Fig. 3). Actually, higher ShLA, for both seasons, was closely related to increased root growth and cross sectional area of rootstock and trunk, traits which were also closely related to R32 for both seasons and cultivars (Fig. 3). This positive correlation of root growth traits and cross sectional areas with ShLA might indicate that increased water interception in the soil, and transport along the stems, respectively, would enable a higher shoot development under deficit irrigation. Moreover, intrinsic water use efficiency, which has been often proposed to increase performance under water stress (Tomás et al., 2014), was unrelated to higher ShLA, which rules out this trait as a target for increasing drought tolerance.

3.2. Differentially Expressed genes (DEG) assessment

Based upon previous results, we selected both cultivars grafted onto R32 rootstock for assessing leaf and root tissues in expression analysis through RNA sequencing approaches in both T100 and T30 conditions. Information retrieved from RNA-Seq included 100 base fragments accounting for a total of 94.511.799.390 sequenced base pairs (bp). On average, each library sample yielded 3.937.991.641 bp, with a total number of Illumina paired-end sequencing (read1 plus read2) reads of 935.760.390, with an average of 38.990.016 reads per sample (Supplemental Table 1) and an average of mapped reads of 93.8 % and 94.9 % for Cabernet Sauvignon and Syrah respectively. Mapping parameters of CS and Sy libraries against reference genome were also determined (Supplemental Tables 2 and 3).

DEG analysis was performed by using filter parameters of false discovery rate (FDR) < 0,05 and > 2—fold change, comparing differential expressed genes between both irrigation treatments and combinations of tissues in both cultivar shoots grafted over R32 rootstock (and corresponding roots). DEG totals have shown major differences between both cultivars: CS displayed a number of 1249 DEG at shoot level, whereas Sy displayed 2-fold those genes (2649 DEG) that were both up- and down-regulated by T30 deficit irrigation. Regarding R32 rootstock tissues, a similar pattern was detected: CS-scion DEG displayed roughly half of Sy-scion DEG in response to drought stress, accounting for 2409 and 4784 DEG respectively (Fig. 4 upper panel). Moreover, number of annotated genes reached an average of 90 % in all

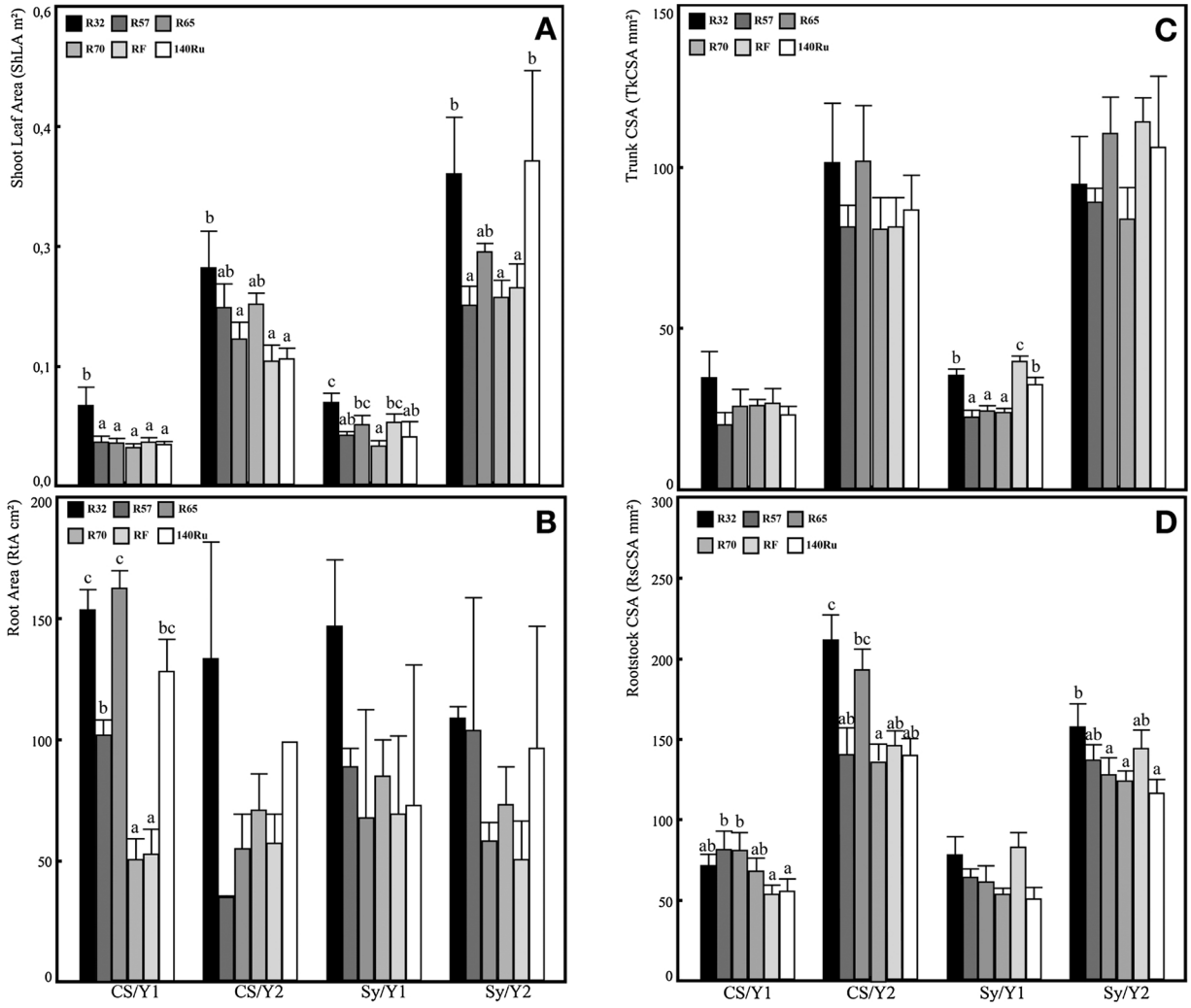


Fig. 2. Rootstocks effects over responses to 30 % irrigation on Shoot leaf area (ShLA; A), Roots area (RtA; B); trunk (TkCSA; C) and rootstock cross sectional area (RsCSA; D). Each bar represents average of both Rootstock genotype (R32, R57, R65, R70) and controls (RF: self-grafted; 140Ru 140 Ruggeri), cultivar scions (CS: Cabernet Sauvignon, Sy: Syrah) over two season (Y1 and Y2). Different letters indicate significant differences; Tukey ($\alpha = 0.05$).

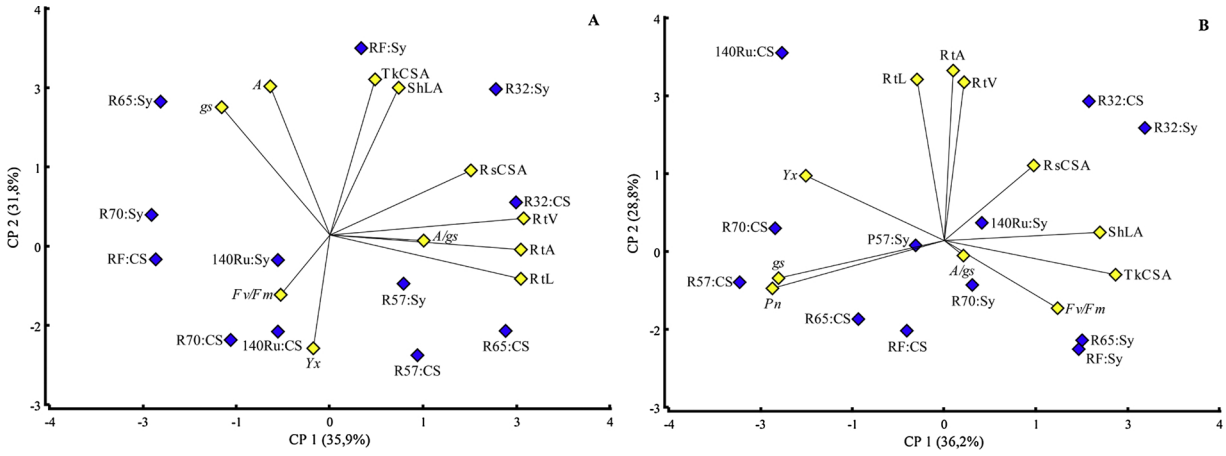


Fig. 3. Principal components (PC) for seasons 1 (A) and 2 (B) for treated grapevines. Yellow symbols represent traits: roots area (RtA), volume (RtV) and length (RtL); shoot leaf area (ShLA), cross sectional area of rootstock (RsCSA) and trunk (TkCSA), net photosynthesis (*Pn*), stomatal conductance (*gs*), intrinsic water use efficiency (*A/gs*), maximum quantum use efficiency (*Fv/Fm*) and stem water potential (*Yx*). Blue symbols indicate rootstock:cultivar combination (CS: Cabernet Sauvignon; Sy: Syrah). The relative contribution of each PC is indicated between brackets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

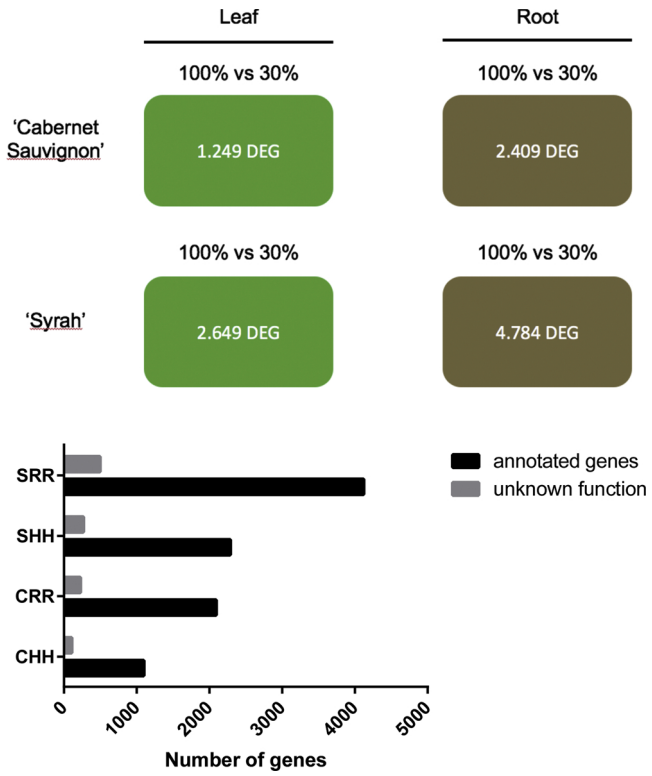


Fig. 4. Number of Differentially Expressed Genes (DEG) for analysed tissues considering control versus water deficiency in grafted vines over R32 Roostock. Number of annotated genes vs unknown function from DEG are depicted in lower panel.

tissues/cultivar combinations grafted onto R32 rootstock. Specific quantities of unknown function transcripts were 11 % in CS leaf tissues and 10 % in CS/R32 rootstock tissues, whereas in Sy leaf tissues 10 % transcripts were of unknown function and 9 % in Sy/R32 rootstock tissues (Fig. 4 lower panel).

When considering tissue specificities and commonalities between combinations (Fig. 5), number of shared DEG at shoot level between CS and Sy accounted for 412 genes, whereas DEG exclusive for CS/R32 were roughly half than Sy/R32 (535 DEG versus 1214 respectively). At R32 rootstock level, there was a higher subgroup of shared DEG (1474 transcripts), meanwhile in CS/R32 number of exclusive DEG was less than 3-fold that Sy/R32 (633 vs 2287). This pattern was further compared considering cultivar by tissue in each combination. Regarding specificities and commonalities between both combinations (Fig. 5), number of share DEG between tissues in CS was 302 transcripts, whereas major amount of DEG was achieved in R32 rootstock in comparison to leaf tissues (2107 vs 947 respectively), suggesting that responses were highly tissue-specific when considering the common DEG (3-fold leaf and 7-fold root). A similar pattern was determined in Sy, common DE genes were 1,023, but DE genes at leaf tissues were 1626 and at R32 rootstock tissues were 3761 although with different magnitude despite bigger amounts (1,5-fold leaf tissues and 3,5-fold root tissues).

These results denote that major changes did occur at root transcriptional level, which might suggest that an active cross-talk system was induced at root level in response to water deficiency treatment. Another main difference between cultivars suggested that a far more stable transcriptional landscape in cv CS might be the near-isohydric strategy operating at shoot level, whereas a far more unstable transcriptional behaviour was reached in cv Sy related to its near-anisohydric strategy.

3.3. DEG and Gene Ontology (GO) Analysis

From filtered DEG results, we constructed a Heatmap in order to display the global expression profiles of differentially expressed genes between treatments (Supplemental Fig. 2). Several processes were modified by irrigation deficit, and these were further grouped by Biological process, indicating targets for both up-regulated and down-regulated processes. Specific redundancy in GO terminology for individual DEG was resolved by means of REVIGO tool of gene groups

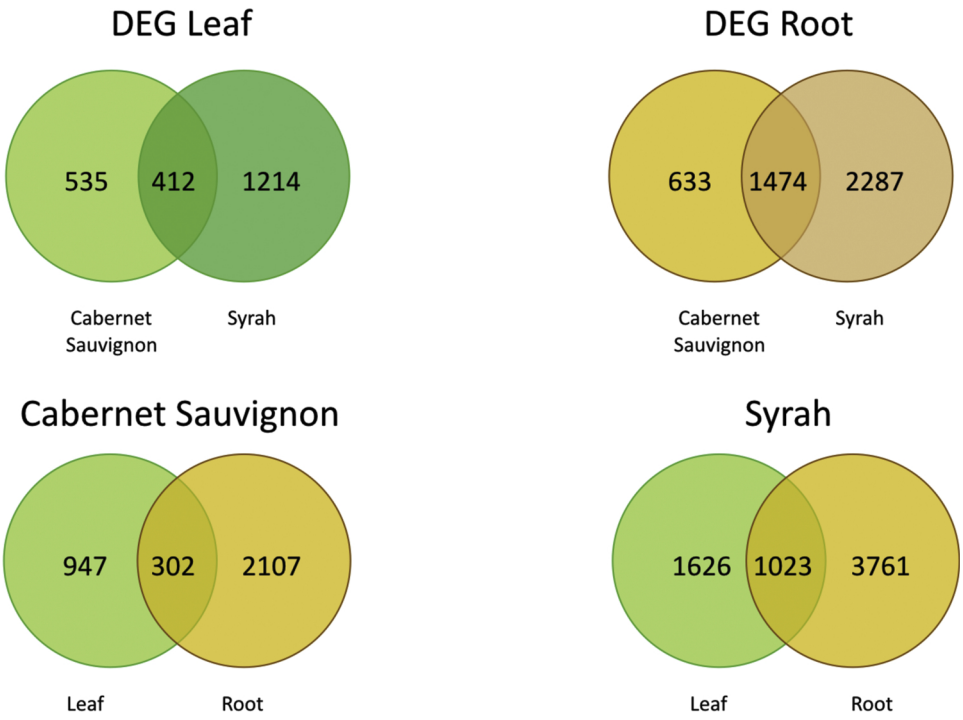


Fig. 5. Number of Differentially Expressed Genes (DEG). A) DEG grouped by tissues, considering control versus water deficiency DEG in grafted vines over R32 Rootstock. **B)** DEG grouped by cultivars, considering control versus water deficiency DEG in grafted vines over R32 Rootstock.

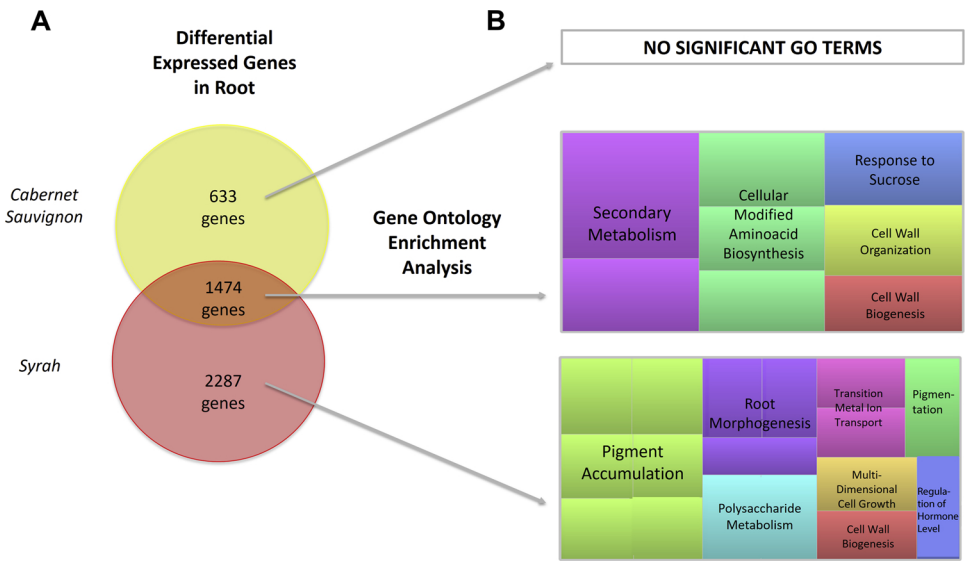


Fig. 6. Differential expression analysis in R32 rootstock tissues. A, distribution of differentially expressed genes between the conditions of normal irrigation (T100) and the water stress condition (T30) in R32 rootstock tissues for each cultivar. B, gene ontology enrichment analysis of each sub-group of genes from the previous analysis. The analysis shows the results using TreeMap view of REVIGO tool. Size of each rectangle reflects its relative importance according to the level of significance associated with the *p-value*.

generated from the Venn diagram of Fig. 5. Firstly, we analysed exclusive and shared gene clusters in R32 rootstock tissues between cvs CS and Sy. This group was identified as the most interesting and possibly associated with an isohydric/anisohydric–like driven regulation. From such analysis, a group of genes shared between both cultivars (1474 genes) was significantly associated with 21 GO terms (Fig. 6A). These processes were shared between both grafted cultivars and thus might be related to a strict water stress response by R32 rootstock. REVIGO analysis grouped these processes within “secondary metabolism”, “amino acid synthesis”, “sucrose response” and “cell wall biogenesis” (Fig. 6B). Interestingly, a group of genes that were exclusively expressed in R32 rootstock tissues grafted to CS (633 genes) were not significantly associated with any particular process. In contrast, genes expressed exclusively in R32 rootstock tissues grafted to Sy (2287 genes) were significantly associated with 28 GO terms (Fig. 6B). The most representative terms (REVIGO) were “pigment accumulation”, “root morphogenesis”, “polysaccharide metabolism”, “ion transport”, “multidimensional cell growth”, “cell wall biogenesis” and “regulation of hormone levels”. Another important group of genes were over-expressed exclusively in Sy leaf tissues (78 genes), significantly associated to "polysaccharide metabolic process", "cell wall organization or biogenesis" and "multidimensional cell growth", again associated to cell wall metabolism. From these genes, most were differentially expressed to a greater extent at full irrigation (T100). One of the genes expressed almost three-fold in full irrigation than in deficit irrigation in R32 rootstock tissues with Sy, was *glycosyl hydrolase 9A1*, also identified as *KOR1*, which corresponds to membrane-bound endo-1,4-beta-D-glucanase involved in cellulose biosynthesis. Catalytic function of cellulose cleavage is associated with a finely controlled remodelling activity of cell wall to allow expansion in growth stages (Ueda, 2014).

Only 22 genes were differentially up-regulated in water deficit treatment (T30), from which a *cellulose synthase-like G2* exceled. Accompanied by this gene, other members of cellulose synthase and glycosyltransferases families were observed, suggesting a synthesis increase of cell wall material. Another interesting group of 93 genes was significantly associated to "response to external stimulus". A class III *chitinase A* gene was highlighted, which is expressed exclusively under environmental stress conditions (Takenaka et al., 2009). Members of class III *chitinase* were only expressed when plants were exposed to environmental stresses, especially to salt and wound stresses. Another gene in this group codes for *peroxisomal 3-ketoacyl-CoA thiolase 3*, which is involved in fatty acid b-oxidation. The evidence suggests that this gene positively regulates ABA signalling in all of the major ABA responses, including stomatal closure and stomatal opening inhibition (Jiang et al., 2011).

3.4. Differential Expression Analysis considering irrigation treatments

Differentially expressed genes were identified (DEG, FDR < 0.05 and $\log_2$ FC > 2; < -2) for each cv and tissue analysed between T100 and T30, and were further classified into two groups: those with a significant up-regulation in transcript levels at T100 condition with respect to T30, and those with a significant up-regulation in transcript levels at T30 condition with respect to T100. Gene groups were analysed by gene ontology (AgriGO) and subsequent application of the REVIGO tool to limit and eliminate their redundancy. Next, each group of genes overexpressed in each treatment was analysed for each of comparisons. Table 1 shows most significant GO terms associated with genes with significantly higher expression levels in T100 compared to T30. Interestingly, greater number of up-regulated genes were observed in R32 rootstock tissues than in leaf tissues for both cultivars, being higher in CS (2377 genes grouped in 96 significant GO terms) compared to Sy (1503 genes grouped in 71 significant GO terms). Among common processes, cell wall remodelling and biogenesis processes (GO: 0,042,546) stand out. Thus, up-regulation of gene encoding members of cellulose synthase family, glycosyl hydrolase, galacturonosyl transferase, nucleotide-diphospho-sugar transferases, O-acetyltransferase, amid others were included. Term "xylem development" (GO: 0010089) was also identified as common process, grouping genes involved in secondary cell wall biosynthesis as cellulases synthases, members of COBRA family, phytochelatin synthetase, and specific NAC transcription factor (*NAC58*). Secondary metabolism was also represented in shared genes, grouping those involved in phenylpropanoid metabolism, flavonoids biosynthesis, carbohydrates and some amino acids.

When considering specificities between both cultivars at T100, overexpression in Sy was significantly induced with "ethylene biosynthesis" (GO: 0009693), "anion transport" (GO: 0006820) and "respiratory burst involved in defence response" (GO: 0002679). Conversely, greatest number of significant terms were associated with CS, pointing towards a major difference of cell proliferation processes. Hence, it was observed a significant association with "DNA replication" (GO: 0006260), "cell cycle" (GO: 0007049), "M phase" (GO: 0000279), "anaphase" (GO: 0051322), and "microtubule cytoskeleton organization" (GO: 0000226). Moreover, several genes were related to processes that suggest epigenetic control, i. e. “histone methylations” and “chromatin silencing”. There were remarkable differences in root expansion mechanisms, associated with cell proliferation and elongation processes in CS, whereas a predominance of cell elongation over

Table 1
Gene Ontology significant terms associated to up-regulated genes in condition of normal irrigation (T100) compared to drought stress condition (T30).

Variety	Organ	GO Term	Description	FDR
Cabernet	Leaf	GO:0010200	Response to chitin	9.60E-07
Sauvignon	*28 GO terms**	GO:0050896	Response to stimulus	1.10E-02
	*234 genes***	GO:0050794	Regulation of cellular process	1.40E-02
		GO:0023052	Signaling	2.70E-02
		GO:0002376	Immune system process	4.60E-02
	Root	GO:0042546	Cell wall biogenesis	2.20E-07
	96 GO terms	GO:0051322	Anaphase	3.20E-06
	2377 *genes*	GO:0007017	Microtubule-based process	1.40E-05
		GO:0048646	Anatomical structure formation involved in morphogenesis	4.40E-05
		GO:0006260	DNA replication	7.00E-04
		GO:0008283	Cell proliferation	3.80E-03
		GO:0009914	Hormone transport	1.00E-02
		GO:0009813	Flavonoid biosynthetic process	1.40E-02
Syrah	Leaf	GO:0009266	Response to temperature stimulus	1.90E-09
	26GO terms	GO:0006457	Protein folding	5.20E-08
	443 genes	GO:0000302	Response to reactive oxygen species	2.20E-07
		GO:0009404	Toxin metabolic process	1.00E-04
		GO:0009414	Response to water deprivation	2.20E-02
	Root	GO:0042546	Cell wall biogenesis	4.90E-10
	71 GO terms	GO:0042398	Cellular amino acid derivative biosynthetic process	3.10E-06
	1503 *genes*	GO:0009698	Phenylpropanoid metabolic process	1.50E-05
		GO:0015698	Inorganic anion transport	2.20E-03
		GO:0009611	Response to wounding	4.20E-02

* Total number of significantly associated GO terms.
** Total number of significantly associated genes.

proliferation in Sy. Scion-driven control of these processes over R32 rootstock tissues suggested a machinery that needs further understanding to unravel molecular machinery involved in this response.

Considering genes that up-regulated expression in leaf tissues, differences between both cultivars were also detected: CS displayed association with plant defence response activation, associated to biotic stress. Connection of "response to chitin" (GO: 0010200) was significantly determined, highlighting regulatory genes that code for *polynucleotidyl transferase*, *integrase-type DNA-binding*, transcription factor *NAC2* and *zinc-finger protein 3*. However, these transcription factors also increased their expression in Sy, which suggests that although GO terms are related to different described responses in literature, they are likely to share similar mechanisms and crosstalk response to stress.

Conversely, biological processes associated to significant up-regulated genes by water stress compared to control are shown in Table 2. A similar trend was determined in leaf tissues: DEG number in Sy doubled those in CS (as shown in Table 1). Common processes exhibited by both cultivars included those related to plant growth such as "meristem growth" (GO: 0035266), and "syncytium formation" (GO: 0006949). Accompanying these processes were also identified "growth" (GO: 0040007), "cell wall biogenesis" (GO: 0071554) and "lipid transport" (GO: 0006869), suggesting new tissue formation under water stress conditions. There were important differences at shoot level: few

Table 2
Gene Ontology significant terms associated to up-regulated genes in condition of drought stress (T30) compared to normal irrigation condition (T100).

Variety	Organ	GO Term	Description	FDR
Cabernet	Leaf	GO:0035266	Meristem growth	2.30E-04
Sauvignon	*37 GO terms**	GO:0071554	Cell wall organization or biogenesis	4.40E-04
	*696 genes***	GO:0040007	Growth	8.80E-03
		GO:0007167	Enzyme linked receptor protein signaling pathway	1.60E-02
		GO:0051322	Anaphase	2.40E-02
	Root	GO:0006979	Response to oxidative stress	1.80E-05
	17 GO terms	GO:0006457	Protein folding	1.30E-03
	458 genes	GO:0006950	Response to stress	2.30E-03
		GO:0019748	Secondary metabolic process	5.70E-03
		GO:0015698	Inorganic anion transport	1.90E-02
Syrah	Leaf	GO:0071554	Cell wall organization or biogenesis	2.40E-04
	59 GO terms	GO:0040007	Growth	7.80E-04
	1495 genes	GO:0010075	Regulation of meristem growth	2.00E-03
		GO:0008283	Cell proliferation	6.00E-03
		GO:0009725	Response to hormone stimulus	9.50E-03
		GO:0051322	Anaphase	9.50E-03
	Root	GO:0042546	Cell wall biogenesis	4.90E-10
	71 GO terms	GO:0042398	Cellular amino acid derivative biosynthetic process	3.10E-06
	1503 *genes*	GO:0009698	Phenylpropanoid metabolic process	1.50E-05
		GO:0015698	Inorganic anion transport	2.20E-03
		GO:0009611	Response to wounding	4.20E-02

* Total number of significantly associated GO terms.
** Total number of significantly associated genes.

biological processes were found to be expressed exclusively in CS grouping 47 genes. Only "extracellular region" process (GO: 0005576) should be underlined, whereas greatest differences were determined in up-regulation genes for enzymes with glycosyl hydrolase, peptidase and peroxidase activity. Conversely, 609 genes were up-regulated exclusively in Sy leaf tissues under water stress. These genes were significantly associated with a total of 35 biological processes: "regulation of cell cycle" (GO: 0051726), "cell proliferation" (GO: 0008283), and "cell wall biogenesis" (GO: 0070882), with significant association to processes such as "response to hormone" (GO: 0009725), "biological regulation" (GO: 0065007), and "histone H3-K9 methylation" (GO: 0051567). These clusters might point to activation to adaptive regulatory mechanisms to water stress not observed in CS, and suggests that Sy leaf tissues have undergone greater changes reflecting greater metabolic adjustments of its anisohydric-like strategy for coping with water stress. One example of up-regulated genes was a key enzyme in the synthesis of Raffinose family oligosaccharides, which function as osmoprotectants in plant cells (Peters et al., 2007).

Regarding genes that increased their expression in R32 rootstock tissues under water stress, a similar trend was observed in activation of biological processes between CS and Sy. A common process activated in these tissues was "response to oxidative stress" (GO: 000697). Additionally, various heat shock proteins, proteases and chaperones were induced, consistent with the onset of biological mechanisms in response to water stress. Specific up-regulated 156 genes triggered in CS/R32 rootstock tissues were not significantly associated with any particular biological process. However, Sy/R32 rootstock tissues increased significantly the expression of 298 genes associated with 25 biological processes under water stress. Most significant processes were related to "ion transport" (GO: 0006811), "response to starvation" (GO: 0042594), "cell communication" (GO: 0007154), "localization" (GO: 0051179) and "cellular homeostasis "(GO: 0019725). Despite same trend between cultivars, expression level was always greater in Sy than in CS, suggesting particular requirements for metabolic adjustments for adaptation in Syrah.

3.5. DEG transcriptional activities display contrasted tissue-specific behaviour

A subset of DEG obtained by RNA-Seq were further assessed by means of qPCR in both cv leaf and R32 rootstock tissues, for verifying transcriptional behaviour of each specific gene in response to water deficit. A correlation matrix analysis was performed in order to cluster genes with high similarity expression (Pearson's $r > 0.980$) in RNA-Seq experiments that are presented in Fig. 7. This subset was composed by UDP-Glycosyltransferase superfamily protein (*VvUGT*), Nitrate transporter 1.7 (*VvNRT1.7*), Osmotin 34 (*VvOSM34*), Expansin A17 (*VvEXPA17*), Root hair specific 19 (*VvRHS19*), High-affinity K^+ transporter 1 (*VvHKT1*) and Lipid transfer protein 3 (*VvLTP3*). These transcripts were indeed significantly induced by water deficit both in CS and Sy leaf tissues, but the induction magnitude was higher in Sy, in line with its hydric behaviour. Remarkably, major differences were determined in R32 rootstock tissues from both cultivars, where most of genes were already significantly induced in Sy/R32 rootstock tissues at control conditions (Log_2 FC > 2), suggesting that scion influence was determinant in regulation of this response in R32 rootstock tissues. Moreover, expression magnitude was lower than registered in stressed CS/R32 rootstock tissues, suggesting an induced steady-state that was even present in Sy/R32 control leaves. Indeed, noteworthy up-regulation of *VvEXPA17* and *VvLTP3* in Sy control leaves might indicate that some of stress adaptive mechanisms were active despite non-stressful conditions, in particular since *VvLTP3* has been previously described as mediating drought responses (Guo et al., 2013), and induction of *VvEXPA17* in Sy/R32 control leaf tissues might be linked to putative peroxidase activities as well and since growth requires intensive cell-wall modification. This latter might be linked to isohydric-like (CS) and anisohydric-like (Sy) contrasting behaviour, since differential up-regulation of *VvNRT1.7* also suggested onset of remobilization process, in line with role for nitrate phloem loading in source leaves to allow transport out of older into younger leaves, according to previous determinations that source-to-sink remobilization of nitrate is mediated by phloem (Fan et al., 2009). Likewise, *VvOSM34* belongs to the PR-5 family of Pathogenesis-related (PR) proteins induced in response to diseases caused by various biotic, and abiotic stresses related to osmotic responses. *Osmotin* was also involved in apoptosis initiation and programmed cell death, whereas its overexpression causes accumulation of proline (Hakim Ullah et al., 2017). Glycosyltransferases (GTs) family modify activities of structural and regulatory metabolites, UDP-glycosyltransferase (*PsUGT1*) is essential for plant development, and a regulation role of cell division has been suggested. Indeed, subfamily members' alterations resulted in changes in life cycle, leaf morphology, auxin response, and root development. Plant phenotypes suppressed by RNAi mutagenesis were very similar to those occurring in plants with altered expression of *PsUGT1* (Woo et al., 2007).

To our knowledge, one report on root hair specific 19 (*RHS19*) described probable role as peroxidase during root hair elongation. RHS19 is a class III peroxidases that have peroxidative and hydroxylic activities acting in the cell wall, thus modulating both cell wall loosening and stiffening (Passardi et al., 2004; Won et al., 2009). RHS19 is also described to function in peroxidase activity, metal ion binding and heme binding, involved in oxidation reduction and response to oxidative stress, according to TAIR resources (The Arabidopsis Information Resource, 2019). Moreover, high expression level of *VvEXPA17* in stressed CS/R32 rootstock and both control and stressed Sy/R32 rootstock tissues might indicate the active process of this expansin cell wall-loosening protein, which have been also reported as required for root hair elongation in rice (*Oryza sativa* L.) and suppression of *OsEXPA17* by RNA interference further confirmed the requirement for the gene in root hair elongation process (ZhiMing et al., 2011).

Moreover, plant high-affinity K^+ transport (HKT) proteins mediate high-affinity K^+ uptake and are K^+-selective uniporters or Na^+-K^+ symporters, but HKT proteins also functions as a Ca^{2+}-permeable cation channel that conducts current carried by a wide range of monovalent and divalent cations. The HKT is expressed in several cell types, including root hairs and vascular parenchyma cells, and is localized to the plasma membrane, thereby providing a mechanism for cation uptake and extrusion, which further extends the function of HKT proteins to Ca^{2+}-linked processes and, in so doing, defines a previously undescribed type of Ca^{2+}-permeable cation channels in plants (Lan et al., 2010). Additionally, an up-regulation of *VvLTP3* might support onset stress adaptive mechanisms in stress, because *LTP3* has been previously described as mediating drought responses (Guo et al., 2013). Primary plant cell walls consist of cellulose fibrils interconnected by hemicellulose tethers, such as xyloglucan and arabinoxylan, and embedded in a pectin gel (Tenhaken, 2015), which also contains phenolics, *peroxidases*, pectin esterases, and other extensins, expansins. Since drought and other osmotic stress can cause ROS accumulation and changes in cell wall (Zhu, 2016), induction of this peroxidase superfamily protein is a first experimental evidence of its up-regulation. Indeed, transcriptional responses in grapevine cv Sangiovese to water deficit were influenced by anisohydric-like strategy, where genes involved in ROS scavenging during oxidative burst and in oxidative stress-induced protein damage repair pathway were intensely induced (Dal Santo et al., 2016). In parallel to activation of 'primary mechanisms' of ROS scavenging, a drought-tolerant M4 rootstock genotype may also induce 'secondary mechanisms' leading to biosynthesis of secondary compounds in roots and leaves (Corso et al., 2015). Very recently, Migicovsky et al. (2019) reported rootstock-specific patterns of gene

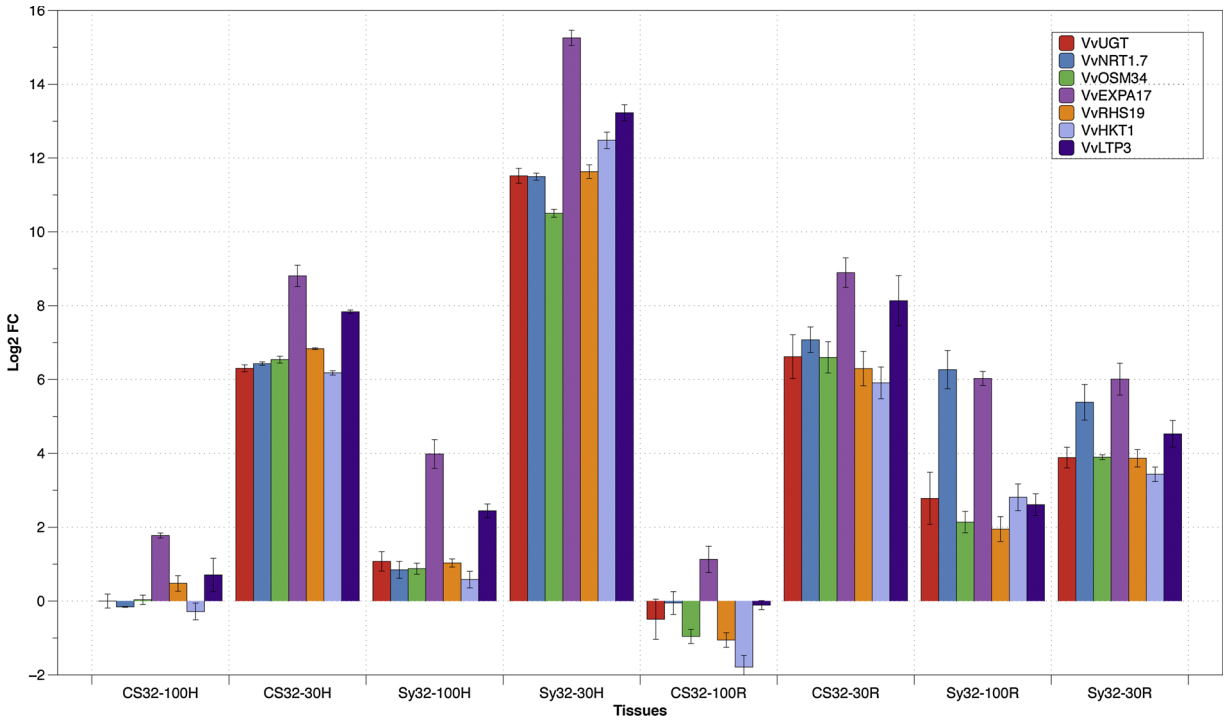


Fig. 7. Expression level of target genes by RT-qPCR. Differentially expressed genes by RNA-Seq of leaf (H) and root (R) tissues of cvs Cabernet Sauvignon (CS) and Syrah (Sy) vines grafted onto R32 rootstock, in response to control (100 %) and deficit irrigation (30 %) treatments. Colour bars denote average expression (Log2 Fold Change) and SEM of biological triplicates. *VvUbiquitin* was used as normalizer.

expression in grafted plants when compared to ungrafted vines, revealing subtle and complex effects of grafting on leaf morphology, ion uptake, and gene expression in cv 'Chambourcin', which might indicate the deep coordination processes occurring in grafted plants to adapt to abiotic stressors. Furthermore, scion has the potential to give a useful trait to the stock by changing its epigenetic state, and vice versa (Tsutsui and Notaguchi, 2017).

4. Conclusions

Naturalized R32 rootstock increased performance of both CS (near-isohydric) and Sy (near-anisohydric) under reduced irrigation during two seasons as compared to other naturalized rootstocks, self-grafted and scion-grafted 140Ru. R32 rootstock performance was related to enhanced root growth, and cross-sectional area (scion and rootstock). Functional grapevine performance brought about by rootstocks might be related to increased water interception by roots and transport through stems and not to increased physiological performance. Transcriptomic changes were significantly up-regulated by water deficit, but magnitude of induction was higher in Sy, with several genes induced at control conditions (Log_2 FC > 2). Amusingly, major transcriptional differences were observed in R32 rootstock, where water deficit triggered a significant magnitude of DEG in Sy/R32 rootstock tissues (ca. two-fold than CS and leaf), revealing that major changes at transcriptional level did occur at root level, thus suggesting a scion-driven induced system. Another major difference between cultivars registered a far more stable transcriptional landscape in CS than in Sy, which might rest upon hydric strategy at shoot level, with bigger inducible transcriptional changes observed in Syrah's (near-anisohydric). Finally, R32 rootstock can be considered a drought tolerant rootstock promising for enhancing grapevine performance for both near iso and anisohydric cv and might serve as adaptive strategy in face of expected climate constrains.

Declaration of Competing Interest

No conflicts of interest declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2019.109031.

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