# The Accumulation of miRNAs Differentially Modulated by Drought Stress Is Affected by Grafting in Grapevine[1][OPEN]

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Grapevine (*Vitis vinifera*) is routinely grafted, and rootstocks inducing drought tolerance represent a source for adapting vineyards to climate change in temperate areas. Our goal was to investigate drought stress effects on microRNA (miRNA) abundance in a drought-resistant grapevine rootstock, M4 (*Vitis vinifera* × *Vitis berlandieri*), compared with a commercial cultivar, Cabernet Sauvignon, using their autografts and reciprocal grafts. RNA extracted from roots and leaves of droughted and irrigated plants of different graft combinations was used to prepare cDNA libraries for small RNA sequencing and to analyze miRNAs by quantitative real-time polymerase chain reaction (RT-qPCR). Measurements of leaf water potential, leaf gas exchange, and root hydraulic conductance attested that, under irrigation, M4 reduced water loss in comparison with cultivar Cabernet Sauvignon mostly through nonhydraulic, root-specific mechanisms. Under drought, stomatal conductance decreased at similar levels in the two genotypes. Small RNA sequencing allowed the identification of 70 conserved miRNAs and the prediction of 28 novel miRNAs. Different accumulation trends of miRNAs, observed upon drought and in different genotypes and organs, were confirmed by RT-qPCR. Corresponding target transcripts, predicted in silico and validated by RT-qPCR, often showed opposite expression profiles than the related miRNAs. Drought effects on miRNA abundance differed between the two genotypes. Furthermore, the concentration of drought-responsive miRNAs in each genotype was affected by reciprocal grafting, suggesting either the movement of signals inducing miRNA expression in the graft partner or, possibly, miRNA transport between scion and rootstock. These results open new perspectives in the selection of rootstocks for improving grapevine adaptation to drought.

The silencing phenomena mediated by small noncoding RNAs finely tune physiological and biochemical processes that tightly regulate plant development (Li and Zhang, 2016) and adaptation to the surrounding environment (Kruszka et al., 2012; Sunkar et al., 2012). Among small noncoding RNAs, microRNAs (miRNAs) constitute the most studied group of endogenous posttranscriptional silencing effectors, which serve as guides for either the cleavage or translational inhibition of complementary target transcripts (Voinnet, 2009; Rogers and Chen, 2013). Plant miRNAs are single-stranded RNA molecules of approximately 21 nucleotides in length generated by DICER-LIKE1 (DCL1) enzymes from stem-loop precursors encoded by endogenous *MIR* genes (Nozawa et al., 2012). In recent years, increasing experimental evidence has suggested a role for plant miRNAs as crucial mediators in the regulation of molecular signaling cascades upon exposure to abiotic stresses, particularly drought and salinity (for review, see Ding et al., 2013; Ferdous et al., 2015; Zhang, 2015). Indeed, in many herbaceous plants, such as Arabidopsis (*Arabidopsis thaliana*; Liu et al., 2008; Song et al., 2013; Xu et al., 2014), tobacco (*Nicotiana tabacum*; Frazier et al., 2011), tomato (*Lycopersicon esculentum*; Candar-Cakir et al., 2016), *Medicago truncatula* (Trindade et al., 2010; Wang et al., 2011), cotton (*Gossypium hirsutum*; Wang et al., 2013), soybean (*Glycine max*; Kulcheski et al., 2011), and various cereals (for review, see Budak et al., 2015), as well as in woody species, such as poplar (*Populus* spp.; Ren et al., 2012;

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C.P. performed most of the experiments, analyzed and compared all data, and wrote the article; M.V. carried out the physiological measurements and analyzed the corresponding data; M.F. collaborated on the molecular analyses and analyzed the data; N.V. performed the sequencing analysis and bioinformatic elaboration of data; M.I. collaborated on the molecular analyses and complemented the writing; C.L. supervised the physiological experiments, analyzed data, and complemented the writing; G.V. coordinated the sequencing analyses; A.S. conceived the project, supervised all the experiments, and critically revised the article; all authors read and approved the article.

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Li et al., 2013a; Shuai et al., 2013) and fruit trees (Solofoharivelo et al., 2014), conserved and novel miRNA families have been shown to dynamically respond to abiotic stress. Although these findings contributed greatly to identify a large source of stress-related miRNAs, further efforts are needed to advance current understanding of the interrelated molecular pathways controlling miRNA biogenesis and action (Borges and Martienssen, 2015; Reis et al., 2015). Progress in this direction was made recently by the identification of stress-responsive regulatory sequences located within the *MIR* gene promoters (Devi et al., 2013) and by the discovery of salt stress-responsive miR-SSR markers (Mondal and Ganie, 2014).

Grapevine (*Vitis vinifera*) is a major fruit crop worldwide, and it features a relatively high tolerance to drought, determined by a still poorly explored array of morphological, biochemical, and molecular mechanisms (Cramer et al., 2007, 2013; Perrone et al., 2012; Ferrandino and Lovisolo, 2014; Corso et al., 2015). Previous studies identified conserved and novel miRNAs in grapevine (Carra et al., 2009; Mica et al., 2010; Pantaleo et al., 2010, 2016; Wang et al., 2012), and a grapevine miRNA expression atlas was published recently (Belli Kullan et al., 2015). However, no studies specifically addressing the identification of drought stress (DS)-responsive miRNAs have been reported in *Vitis* spp. Further investigation of such miRNAs could indeed offer important clues to their specific function within the context of regulatory networks promoting grapevine adaptation to stressful environments.

Grafting is an ancient agronomical technique widely used in horticulture for diverse purposes, such as vegetative propagation, reduction of juvenility, and tolerance to soil pathogens. European grapevines are routinely grafted on interspecific hybrid rootstocks in order to control infestation by phylloxera (*Daktulosphaira vitifoliae*; Mudge et al., 2009). In parallel, rootstocks affect scion growth vigor and resistance to abiotic stresses (Berdeja et al., 2015; Lovisolo et al., 2016; Lavoie-Lamoureux et al., 2017). Specific rootstocks induce tolerance to DS (Carbonneau, 1985; Soar et al., 2006; Koundouras et al., 2008; Tramontini et al., 2013), and it was shown that particular genetic regions in their genome are linked to the induction of stress tolerance (Marguerit et al., 2012). Thus, rootstock management is considered a promising tool to enhance the resilience of grapevine to water scarcity (Berdeja et al., 2015). A recent transcriptomic study demonstrated that grafting can determine stock-specific transcript concentration changes in the grapevine scion (Corso et al., 2015), and this could depend at least in part on modifications in the concentration of miRNAs, as shown recently in grafts of cucumber (*Cucumis sativus*) and pumpkin (*Cucurbita moschata*; Li et al., 2014).

In this study, we provide new insights on the regulatory effect exerted by DS on the abundance of grapevine miRNAs. In particular, we identified, by the use of high-throughput next-generation sequencing technology and in silico analysis, novel putative miRNAs in roots and leaves of two grapevine genotypes, cv Cabernet Sauvignon (CS), a widely known commercial variety, and the recently selected, water stress-tolerant *Vitis* hybrid M4. We further quantified miRNA expression levels under DS by applying a well-established stem-looped quantitative real-time PCR (RT-qPCR) approach and focusing on a group of selected conserved and novel miRNAs. These analyses were performed in autografted as well as in heterografted plants in order to unravel changes in miRNA accumulation induced by grafting. We show that several conserved and novel miRNAs, potentially influencing biological processes that affect the whole-plant capacity to cope with DS, are modulated by the imposed treatment. Moreover, we report that heterografting alters miRNA abundance in the graft partners, possibly due to the movement of stress signals between them.

## RESULTS

### Water Status and Gas Exchange in Autografted and Heterografted Grapevines

In order to outline the physiological behavior of autografted CS and M4 plants under DS, we withdrew irrigation and followed dynamic changes in leaf water potential ($\Psi_{leaf}$) and stomatal conductance ($g_s$; Fig. 1). Upon irrigation, $g_s$ rates were significantly lower in M4/M4 vines than in CS/CS vines (Fig. 1A). Nevertheless, when severe DS occurred (about 10 d after the treatment imposition), $g_s$ decreased strongly up to 1 order of magnitude in both the autografts (Fig. 1C). Accordingly, $\Psi_{leaf}$ was significantly lower in droughted plants than in irrigated plants (Fig. 1, B and D).

Physiological responses to drought also were investigated on the reciprocal heterografts of the two genotypes (CS/M4 and M4/CS), with the final goal to obtain more information on the relative role of either root or shoot in reducing $g_s$ of M4 autografted plants. Irrigated M4/CS and CS/M4 vines showed comparable $g_s$ values, slightly lower than CS/CS vines but significantly higher than those measured in M4/M4 plants (Fig. 1A). Under irrigation, $\Psi_{leaf}$ was significantly more negative in heterografts than in autografts, and in particular, the lowest values were observed for the CS/M4 combination (Fig. 1B). In heterografted plants, drought treatment strongly reduced $g_s$ (Fig. 1C) together with an evident decrease in $\Psi_{leaf}$ values, which were close to −1.4 MPa on average (Fig. 1D). The observed trends in $g_s$ were well mirrored by leaf transpiration rates measured on the tested plants (data not shown).

As the results showed that autografted M4 plants lost less water than CS autografts under irrigated conditions, we compared the specific root hydraulic conductance (root $k_s$) of M4 with that of CS upon well-watered conditions. The results indicated that root $k_s$ of M4 was higher, although not significantly, than in CS (Fig. 2), suggesting that factors other than hydraulic signals should contribute to control $g_s$, and hence transpiration, of this genotype.

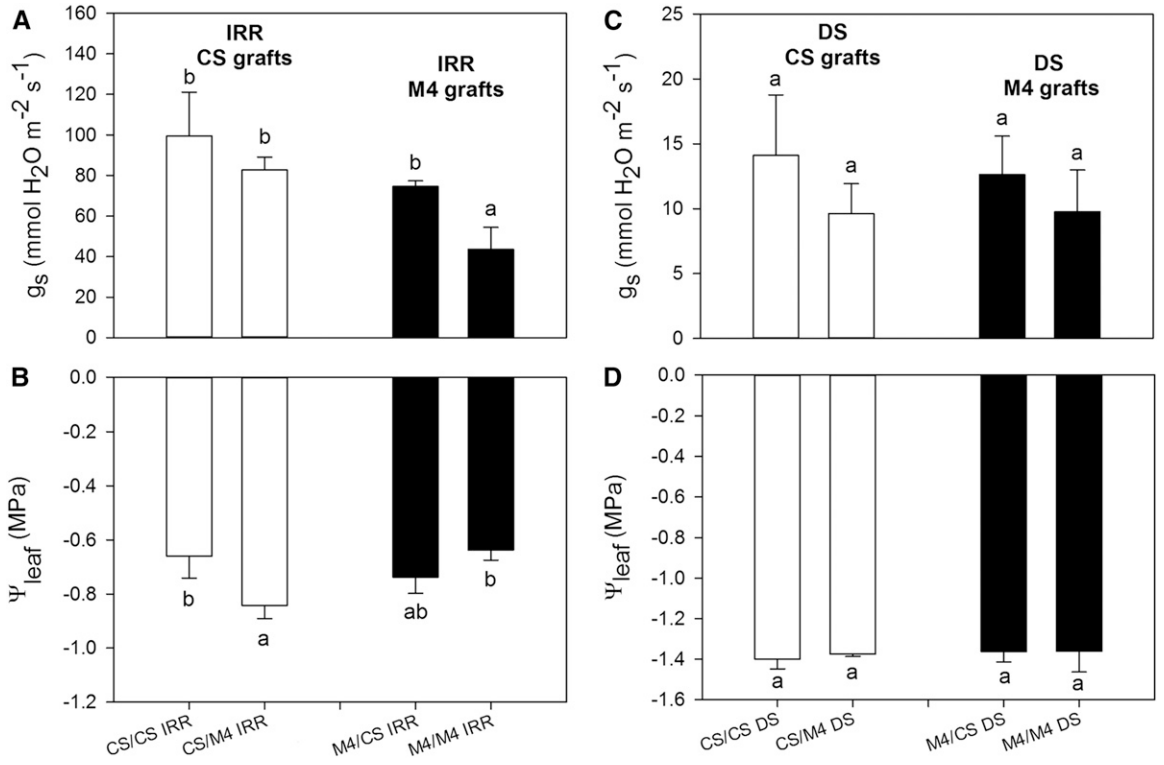


**Figure 1.** Measurements of $g_s$ (A and C) and $\Psi_{leaf}$ (B and D) in autografted and heterografted plants of CS and M4 genotypes in irrigated conditions (IRR; A and B) and after 10 d of DS (C and D). Lowercase letters above and below the bars denote significant differences ($P < 0.05$) attested using Tukey's honestly significant difference (HSD) test. Error bars represent SE ($n = 10$).

### miRNA Sequencing and Identification

In order to identify novel candidate miRNAs affected by DS, we prepared eight small RNA libraries from pooled low molecular weight (LMW) RNA samples extracted from roots and leaves of CS and M4 autografted plants, either irrigated or exposed to DS treatment. When subjected to SOLiD deep sequencing, the libraries yielded more than 180 million raw reads in total. After removing low-quality sequences, adapters, small sequences (fewer than 17 nucleotides), noncoding RNAs, and other contaminants, an average of more than 7 million nonredundant reads, with length ranging from 17 to 25 nucleotides, was obtained for each library and aligned to the *Vitis* genome (Vitis 12X database version V1 [12X V1]; http://genomes.cribi.unipd.it/grape/). The sequences perfectly matching at least one genome locus were then analyzed to either retrieve conserved/known miRNAs, by further mapping these sequences on miRBase (version 21; http://www.mirbase.org/), or identify novel candidate miRNAs using two dedicated computational softwares (miRCat and miRDeep-P; for details, see "Materials and Methods"). A summary of small RNA sequencing analysis is reported for each library in Table I.

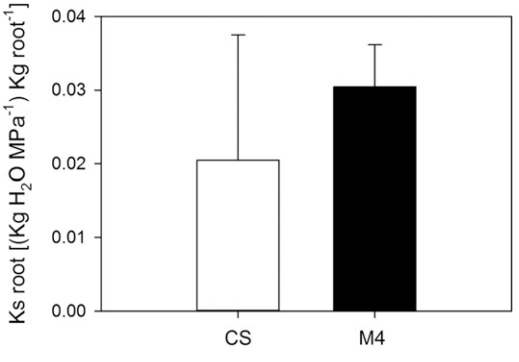


**Figure 2.** Specific hydraulic conductance (i.e. root $k_h$), with reference to root dry weight ($K_s$), in M4 and CS roots under irrigated control conditions. Error bars represent SE ($n = 10$). No significant differences were observed in the pairwise comparison when performing Student's *t* test ($P < 0.05$).

Sequencing elaboration allowed the identification of 635 miRNAs, and filtering above a threshold value (100 or more unique normalized reads) reduced the number to 98. Among these, 70 were classified as conserved/known miRNAs belonging to 42 families (miRBase annotation version 21; Supplemental Table S3), whereas 28 were predicted as putative novel miRNAs (Supplemental Table S4). The majority (70%) of conserved miRNA sequences fell within the 21-nucleotide category (Fig. 3A), one of the most abundant size classes for known grapevine DCL1-derived products

**Table I.** *Statistics of small RNA sequencing data analysis*

Small RNA libraries were obtained from leaf (L) and root (R) samples of CS and M4 autografted vines upon DS or irrigated (IRR) conditions.

| Library Name | Total Raw Reads | Adapter Trimming[a] | Contaminant Filtering[a] | Noncoding RNA Filtering[b] | Unique Reads Matching the Grape Genome[c] |
|---|---|---|---|---|---|
| CS IRR L | 20,579,159 | 16,922,667 | 16,053,719 | 9,680,892 | 1,365,464 |
| CS DS L | 23,614,667 | 15,125,262 | 13,881,849 | 6,290,048 | 679,650 |
| CS IRR R | 16,015,312 | 13,289,525 | 11,884,302 | 6,197,784 | 247,795 |
| CS DS R | 13,677,360 | 8,040,667 | 7,188,734 | 2,985,063 | 67,588 |
| M4 IRR L | 27,596,925 | 21,264,151 | 19,223,470 | 7,904,996 | 759,658 |
| M4 DS L | 26,706,483 | 22,914,129 | 20,900,171 | 8,748,394 | 179,474 |
| M4 IRR R | 27,028,350 | 21,354,251 | 17,907,958 | 9,592,832 | 682,325 |
| M4 DS R | 29,588,911 | 21,875,311 | 18,629,316 | 11,112,533 | 1,518,832 |

[a]Sequences obtained after quality, adapter, and size (read lengths of less than 17 nucleotides) filtering. [b]Sequences obtained after filtering noncoding RNAs (e.g. tRNA, rRNA, snoRNA, and snRNA). [c]Total of unique sequences from conserved and novel miRNAs matching the grape genome (12X V1 GGDB CRIBI) and/or miRBase (version 21).

(Pantaleo et al., 2010; Wang et al., 2012; Belli Kullan et al., 2015). Among novel candidate miRNAs, two major (each above 30%) size categories were observed at 21 and 22 nucleotides (Fig. 3B).

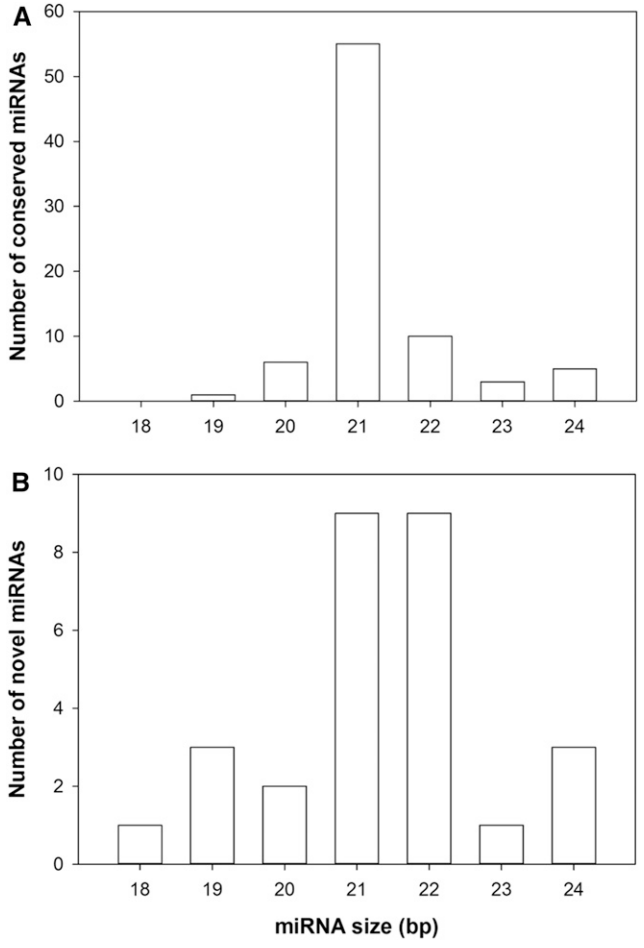


**Figure 3.** Size distribution of conserved (A) and novel (B) miRNAs identified by SOLiD sequencing analysis.

### Drought-Induced Changes of Conserved miRNA Accumulation in Autografted Plants

Based on results from the sequencing experiment (Supplemental Table S3), the concentration of a group of conserved or grape-specific known miRNAs was analyzed by RT-qPCR in autografted plants. The conserved *miR156*, *miR159*, *miR393*, and *miR396* and the grape-specific *miR3624* (Carra et al., 2009) were chosen because they were highly expressed in our sequencing data set (*miR159*, *miR396*, and *miR3624*; Supplemental Table S3) and/or were reported previously to play a key role in the regulation of abiotic stress responses in plants (*miR156*, *miR159*, *miR393*, and *miR396*; Covarrubias and Reyes, 2010; Ferdous et al., 2015, and refs. therein; Zhang, 2015).

In control plants, these miRNAs were detected at comparable concentrations in both genotypes, with the exception of *miR159*, which was expressed at a lower level in CS compared with M4, independently of tissue type (Fig. 4B). DS induced significant expression changes in these miRNAs, and in some cases, accumulation trends were common for the two genotypes (Fig. 3). For instance, *miR156* abundance was lowered significantly by drought in the leaves of both genotypes, whereas in roots, it was not affected significantly (Fig. 4A). Conversely, *miR393* increased in DS root samples of both CS and M4, while in DS leaves, it was slightly less abundant (Fig. 4C). A negative effect of DS also was evident for *miR3624*, particularly in roots, where this miRNA was either much less accumulated (M4) or almost absent (CS; Fig. 4E).

On the contrary, two miRNAs showed distinct expression patterns in CS and M4 genotypes. *miR159* abundance did not change in CS samples independently of the treatment and tissue type, whereas in M4 plants, this miRNA was much less abundant in DS tissues (Fig. 4B). *miR396* was accumulated upon drought in M4 roots, while in M4 leaves and in CS vines, the treatment significantly lowered its levels (Fig. 4D).

### Quantification of Target Transcripts of Conserved miRNAs

In order to characterize the downstream effects of transcriptional changes occurring in conserved miRNA

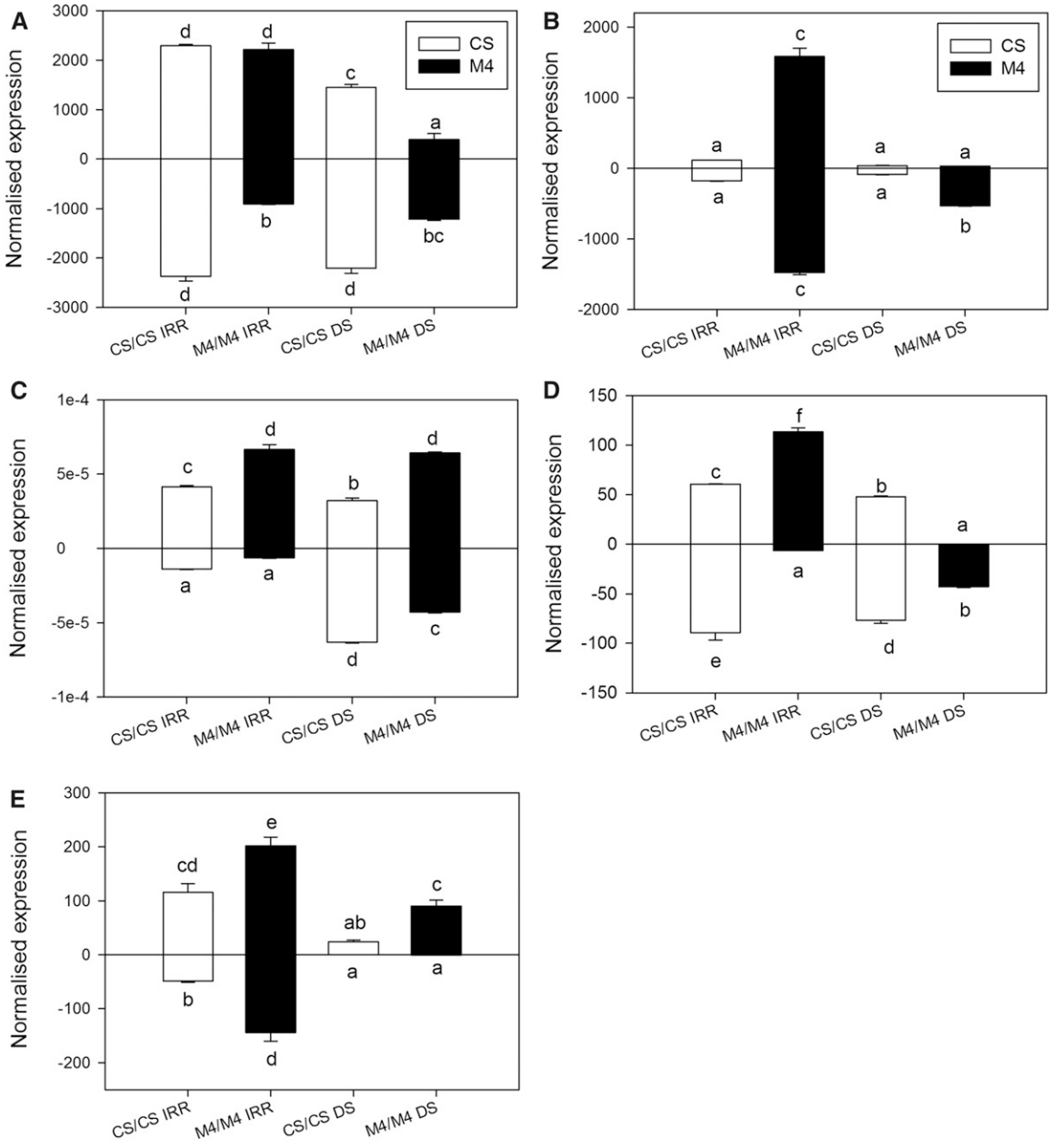


**Figure 4.** Expression changes occurring in a group of conserved miRNAs. RT-qPCR analyses are shown for *miR156* (A), *miR159* (B), *miR393* (C), *miR396* (D), and *miR3624* (E) on leaf (top bars) and root (bottom bars) samples obtained from CS and M4 autografted vines upon irrigated (IRR) and DS conditions. The small nuclear RNA U6 (*VvsnRU6*) was used as the endogenous control gene for the normalization procedure. Lowercase letters above and below the bars denote significant differences ($P < 0.05$) attested using Tukey's HSD test. Error bars represent SE ($n = 3$).

accumulation upon stress, we quantified selected targets by RT-qPCR (for details, see Supplemental Table S6). In many cases, as expected, the expression of the miRNA and its target gene followed opposite trends, although this was not always the rule. In all conditions, the abundance of *miR156* was inversely correlated to the zinc finger protein *CONSTANS5-like* target mRNA (*VIT_04s0008g07340*; Supplemental Fig. S1A). In the case of *miR159*, accumulation of the target transcripts *VIT_19s0090g00590*, encoding the putative grape ortholog of Arabidopsis MYB101, *VIT_09s0002g08370*, encoding TOPLESS-RELATED PROTEIN4-like, and *VIT_00s0287g00040*, encoding GATA TRANSCRIPTION FACTOR26-like, also followed divergent patterns from the miRNA (Supplemental Fig. S1, B–D). The *miR393* target mRNA, encoding the auxin-responsive factor TIR1-like (*VIT_14s0068g01330*), exhibited a divergent pattern of expression in leaves (Supplemental Fig. S1E),

while the analysis of *miR396* target transcript, encoding a growth-regulating factor (*VIT_00s0494g00010*), displayed an expression profile opposite to that of *miR396* in M4 but not in CS (Supplemental Fig. S1F). Besides the genes targeted by these four conserved miRNAs, the target transcript of the grape-specific *miR3624* (Carra et al., 2009) was analyzed. The expression profile of this gene, encoding a metal ion-binding protein (*VIT_00s0194g00340*), followed an opposite trend when compared with the cognate miRNA (Supplemental Fig. S1G).

### Changes of Novel miRNA Abundance in Autografted Plants

We selected a group of novel candidate miRNAs showing important changes in the number of sequencing reads (Supplemental Table S4) upon water stress application (*n_22*, *n_47*, *n_55*, *n_191*, *n_231*, *n_312*, *n_327*, *n_337*, *n_346*, *n_479*, *n_520*, and *n_622*), and we analyzed their expression in autografted plants by RT-qPCR. The relative abundance of these putative miRNAs was variable: *n_55*, *n_327*, and *n_479* were the most abundant (more than $10^2$ times higher than the small nuclear RNA *U6*), followed by *n_47*, *n_231*, *n_337*, and *n_622*, while *n_22*, *n_191*, *n_312*, *n_346*, and *n_520* were the least concentrated (Fig. 5).

In control conditions, the relative concentration of the analyzed miRNAs was modulated by genotype and tissue type. Expression of *n_191* was significantly greater in CS than in the corresponding M4 tissues (Fig. 5D). Tissue specificity independent of genotype was observed in *n_22*, which was significantly more abundant in roots (Fig. 5A). A group of putative miRNAs showed tissue specificity in one genotype only. In CS, expression in roots was lower for *n_55* and *n_191* (Fig. 5, C and D) and higher for *n_622* (Fig. 4I). In M4, *n_337* and *n_520* were less abundant in roots (Fig. 5, G, H, and K), whereas *n_47*, *n_231*, and *n_479* were more concentrated (Fig. 5, B, E, and L).

In a few cases, water stress activated the expression of putative miRNAs: *n_231* was positively induced by drought, with the exception of M4 root, where it was significantly repressed (Fig. 5E). Upon stress, three miRNAs underwent significant overexpression in CS/CS plants: *n_312* in leaves and roots (Fig. 5F), *n_622* in leaves (Fig. 5L), and *n_346* in roots (Fig. 5I).

The abundance of a larger group of putative miRNAs decreased upon DS. The concentration of the root-specific *n_22* candidate miRNA decreased to very low levels upon drought in CS/CS and M4/M4 (Fig. 5A). On the contrary, *n_622* showed a genotype-dependent stress response in leaves, and it increased significantly in CS, whereas it was much less accumulated in M4

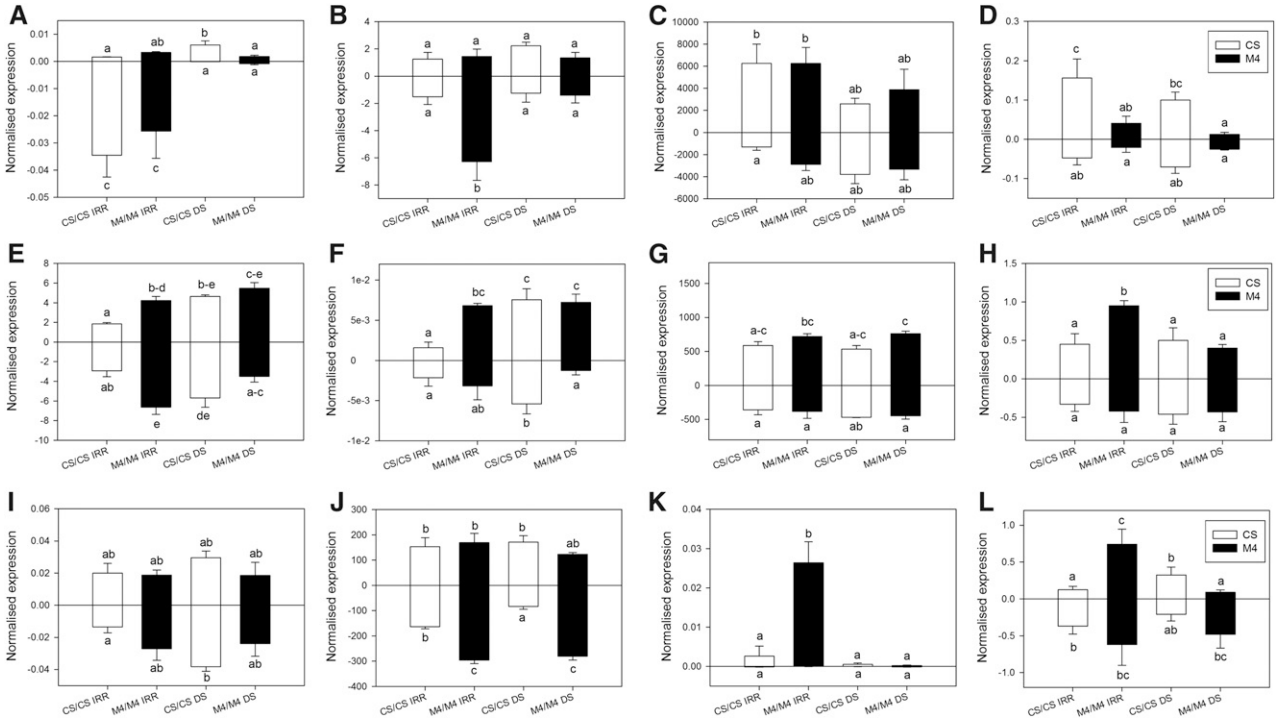


**Figure 5.** Expression changes occurring in a group of selected novel candidate miRNAs. RT-qPCR analyses are shown for *n_22* (A), *n_47* (B), *n_55* (C), *n_191* (D), *n_231* (E), *n_312* (F), *n_327* (G), *n_337* (H), *n_346* (I), *n_479* (J), *n_520* (K), and *n_622* (L) on leaf (top bars) and root (bottom bars) samples collected from CS and M4 autografted vines upon irrigated (IRR) and DS conditions. *VvsnRU6* was used as the endogenous control gene for the normalization procedure. Lowercase letters above and below the bars denote significant differences ($P < 0.05$) attested using Tukey's HSD test. Error bars represent SE ($n = 3$).

(Fig. 5L). The stress treatment significantly decreased the abundance of *n_479* in CS roots (Fig. 5J), while the abundance of *n_337* and *n_520* was significantly lower upon stress in M4 leaves (Fig. 5, H and K).

### Changes of Novel miRNA Abundance in Heterografted Plants

The expression profiles of seven candidate miRNAs (*n_55*, *n_191*, *n_312*, *n_327*, *n_337*, *n_346*, and *n_520*) were investigated further in the reciprocal scion/stock combinations CS/M4 and M4/CS (Fig. 6).

In control conditions, the CS-specific (in autografted plants) *n_191* was either equally distributed (M4/CS) or more abundant in M4 tissues (M4/CS; Fig. 6B). Also, the tissue specificity observed for *n_55*, *n_191*, *n_337*, and *n_520* in autografted plants was lost in both heterografted combinations (Fig. 6, A, B, E, and G). On the contrary *n_312*, equally present in both genotypes and tissues in the autografted combinations, was nearly absent in M4 tissues in the reciprocal grafts (Fig. 6C).

In a few cases, the effects of drought on miRNA concentration observed in autografts were detected in the corresponding genotypes and tissues of heterografts, as in the case of the up-regulation of *n_346* in CS roots in both autografts and heterografts (Fig. 6F). In two cases (*n_312* in CS roots and *n_520* in M4 leaves), the effects of drought observed in autografted plants were reversed in the same tissues of heterografts (Fig. 6, C and G). Nevertheless, in most cases, significant up- or down-regulation trends in heterografted plants were observed for putative miRNAs whose expression was not affected by drought in autografted plants. As an example, *n_337* was induced by drought treatment in leaves and roots of CS/M4 heterografts (Fig. 6E), an effect not observed previously in autografts (Fig. 5H). Another interesting case was *n_346*, for which a significant stress-induced up-regulation was observed in almost all tissues collected from heterografts (Fig. 6F), different from what was observed for autografted vines (Fig. 5K). Conversely, drought significantly decreased the expression of *n_55* in CS roots and in M4 roots and leaves collected from heterografts (Fig. 6A), but not in the corresponding tissues of autografts (Fig. 5C). Significant effects of drought also were noticed for *n_191* in both leaves and roots of CS/M4 grafts (Fig. 6B) and for *n_312* exclusively in the root (CS) of the opposite graft combination (Fig. 6C), but not in the corresponding tissues of autografted vines (Fig. 5, D and F). Unlike what was described in autografted plants (Fig. 5G), drought negatively affected the expression of *n_327* in heterografts, with particular evidence in the roots of both graft combinations (Fig. 6D).

### In Silico Prediction of Novel miRNA Target Genes and Quantification of Related Expression Changes

Target gene sequences of the putative novel miRNAs analyzed in this study were predicted in silico using two computational algorithms in parallel, psRNA-Target and Target-align. Some of the miRNAs targeted more than one transcript (i.e. *n_622*, *n_479*, and *n_327*). For *n_312* and *n_337*, no targets could be predicted. The Gene Ontology annotation showed that target transcripts encoded proteins, enzymes, or transcription factors involved in a broad range of biological processes, among which stress defense and stress signaling response mechanisms were the most common hits (Supplemental Table S5).

The transcriptional changes of six of these predicted target genes, chosen among the most interesting target genes in terms of possible role in the context of stress response regulation (*n_22*, *n_47*, *n_191*, *n_231*, *n_312*, *n_479*, *n_520*, and *n_622*), were assessed by RT-qPCR analysis (Fig. 7). Overall, target transcripts showed opposite expression trends when compared with those of the related novel miRNAs, supporting negative posttranscriptional regulation driven by the miRNA. This was the case in CS samples: for *VIT_ 12s0035g0026* (Fig. 7A), encoding a calmodulin-binding protein (MLO16), target of *n_22*; for *VIT_09s0096g00830*, target of *n_47*, encoding a NBS-LRR defense protein (Fig. 7B); for transcripts of a polyphenol oxidase (*VIT_00s0480g00100*), target gene of *n_231* (Fig. 7D); and for a squamosa promoter binding protein-like transcription factor gene (*SPL6*; *VIT_01s0011g00130*), targeted by *n_327* (Fig. 7E). The same pattern was observed in M4 samples for the CBL interacting binding protein kinase gene *CIPK13* (*VIT_13s0067g0248*), target of *n_191* (Fig. 7C); or in both genotypes, as in the case of a zinc finger transcription factor gene (*VIT_06s0004g06440*), targeted by *n_520* (Fig. 7G). Transcriptional regulation of target genes occurred only in specific tissues. This happened for *VIT_09s0002g04950* (Fig. 7F), target gene of *n_479*, which encodes an RPS5-like defense protein belonging to the NBS-LRR superfamily: in leaves, its expression trend followed that of its putative cognate miRNA, whereas in the root of both genotypes, expression changes were opposite (Fig. 7F). A similar result was observed in CS root upon both control and stress conditions for transcripts of the hexose transporter HT5 (*VIT_05s0020g03140*), targeted by *n_622* (Fig. 7H).

## DISCUSSION

### Conserved and Novel Grapevine miRNAs Respond to DS in the Two Genotypes

In order to separate the shoot-controlled physiological processes from those regulated at the root level, we investigated the effects of stress on autografts and heterografts of two grapevine genotypes, CS and M4. Under irrigated conditions, $g_s$ was significantly lower in M4 than in CS, and both genotypes significantly decreased $g_s$ with DS. Heterografted plants also showed significant decreases in $g_s$ with DS, and there was no significant difference in the response with rootstock.

The root $k_s$ in M4 was higher but not significantly different from that in CS, suggesting that the lower $g_s$ induced

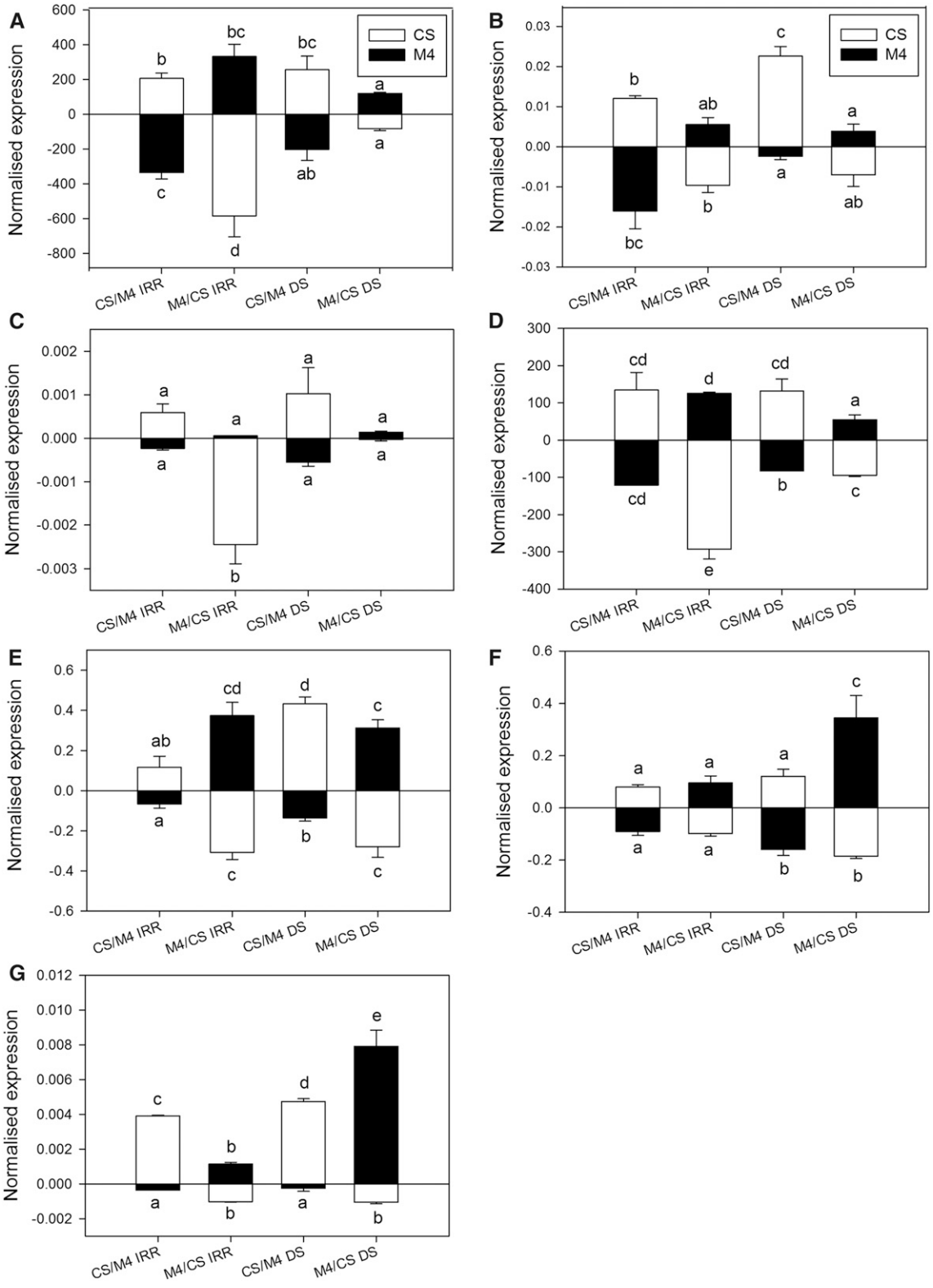


**Figure 6.** Expression changes of novel candidate miRNAs in heterografted plants. Expression profiles are shown for *n_55* (A), *n_191* (B), *n_312* (C), *n_327* (D), *n_337* (E), *n_346* (F), and *n_520* (G) in leaf (top bars) and root (bottom bars) samples collected from CS and M4 heterografted vines (CS/M4 and M4/CS) upon irrigated (IRR) and DS conditions. *VvsnRU6* was used as the endogenous control gene for the normalization procedure. Lowercase letters above and below the bars denote significant differences ($P < 0.05$) attested using Tukey's HSD test. Error bars represent SE ($n = 3$).

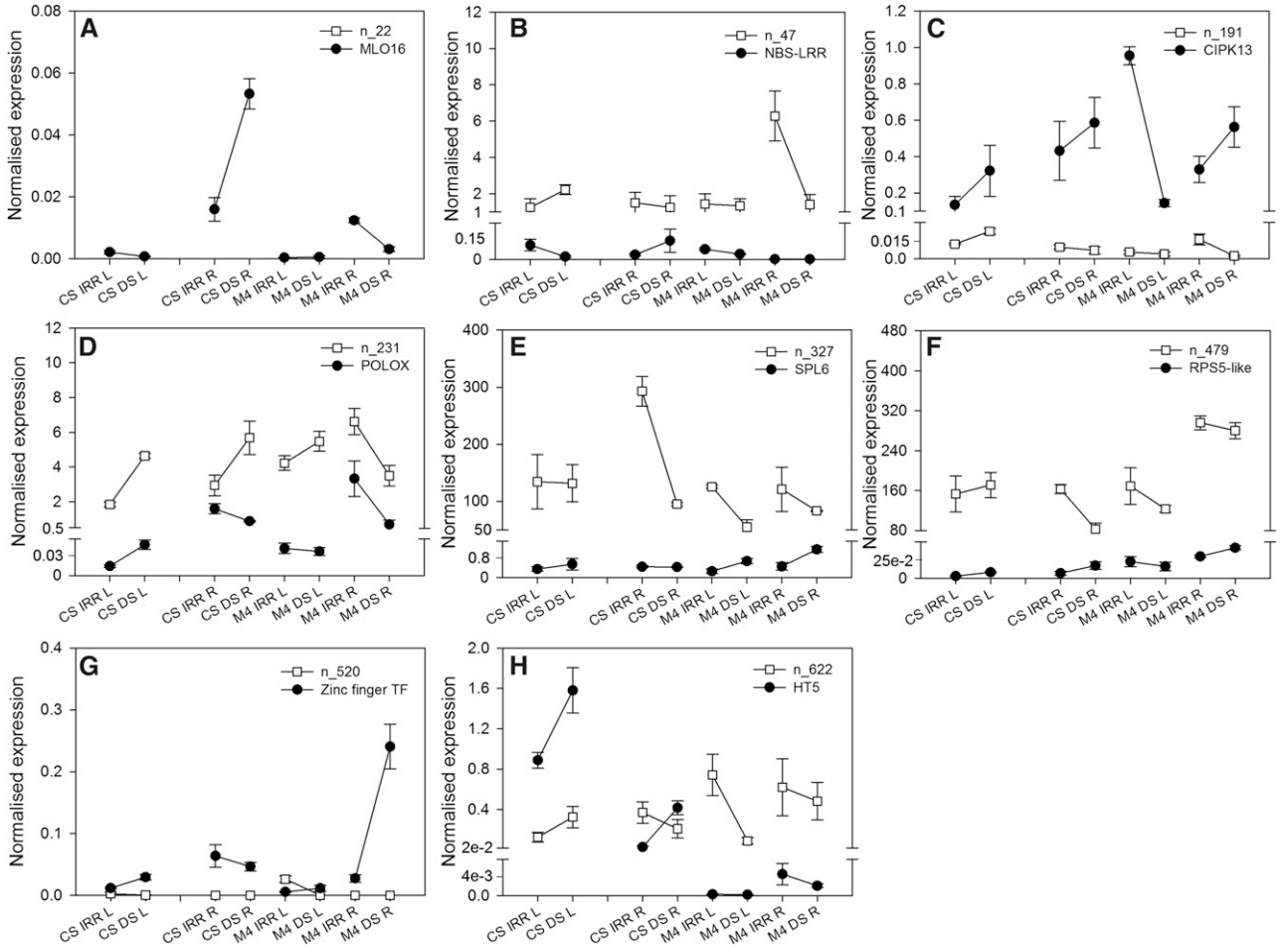


**Figure 7.** Expression patterns of novel miRNA target transcripts. RT-qPCR profiles are shown for *n_22* (A), *n_47* (B), *n_231* (C), *n_327* (D), *n_191* (E), *n_520* (F), *n_479* (G), and *n_622* (H) target genes in correlation with each related novel miRNA, measured in leaf (L) and root (R) samples collected from either CS and M4 autografted (A–C and F–H) or heterografted (D and E) vines upon irrigated (IRR) and DS conditions. The ubiquitin (*VvUBI*) and actin (*VvACT1*) genes were used as endogenous controls for the normalization procedure of target transcripts, while *VvsnRU6* was the endogenous control for miRNA expression analysis. Error bars represent SE ($n = 3$).

by M4 may not rely on hydraulic signals but rather on other factors, such as signaling molecules. We reported previously that grapevine rootstocks control $g_s$ when water potential decreases (Tramontini et al., 2013) and that root-originated abscisic acid (ABA) limits $g_s$ during water stress and upon rehydration after drought events (Lovisolo et al., 2008; Tramontini et al., 2014).

During the last few years, an emerging number of drought-related miRNAs have been reported in several crop plants, such as barley (*Hordeum vulgare*; Hackenberg et al., 2015), rice (*Oryza sativa*; Zhou et al., 2010; Barrera-Figueroa et al., 2012), cotton (Xie et al., 2015), potato (*Solanum tuberosum*; Zhang et al., 2014), maize (*Zea mays*; Li et al., 2013b), tomato (Candar-Cakir et al., 2016), cowpea (*Vigna unguiculata*; Barrera-Figueroa et al., 2011), peach (*Prunus persica*; Eldem et al., 2012), and poplar (Li et al., 2011; Ren et al., 2012). Recently, Sun et al. (2015) described changes in grape miRNA abundance induced by cold stress and Pantaleo et al. (2016) characterized novel miRNAs in virus-infected grapevine contemporaneously subjected to water stress. However, to the best of our knowledge, this is the first time that the effects of drought alone were analyzed on grape miRNA populations.

As the accumulation of miRNAs can vary significantly among diverse organs or tissues (Ferdous et al., 2015), we investigated miRNA expression changes in roots and leaves of two genotypes. Our analysis of conserved miRNAs confirms their response to osmotic stress and variation among tissue types. The grape-specific *miR3624* was inhibited by drought, while its target gene, encoding a metal ion-binding protein, increased greatly, in accordance with the known up-regulation of metallothionein-induced abiotic stress (Kim et al., 2014). *miR393* positively responded to drought exclusively in the roots of the tested genotypes.

This is consistent with previous work showing the root-specific up-regulation of this miRNA (Gao et al., 2016) and revealing its essential role in the adaptation of lateral root growth to DS (Chen et al., 2012). Additional evidence supporting the stress-mediated regulation of *miR393* is the presence of specific drought-induced cis-elements in the promoter region of the related *MIR* gene (Devi et al., 2013). In Arabidopsis, *miR393* is a key component of the auxin signaling cascade and tunes the expression of *TIR1/AFB2* genes (Si-Ammour et al., 2011); this function also was confirmed in other plant species (Ferreira et al., 2012; Xia et al., 2012). In our study, however, the expression of the homologous grape target transcript, encoding the auxin-responsive factor TIR1-like, was inversely correlated to the abundance of *miR393* only in leaves, implying that, in root, other stress-induced regulatory mechanisms could interfere with the miRNA inhibitory activity (Rogers and Chen, 2013).

*miR156*, *miR159*, and *miR396* are abundant in Arabidopsis plants exposed to osmotic stress (Covarrubias and Reyes, 2010), while in our experiments, they are mostly down-regulated by drought. Data on the regulation of conserved miRNAs divergent from that observed in Arabidopsis have been reported before: upon water stress, a lower accumulation of *miR156* and *miR393* was observed in cotton (Xie et al., 2015) and in cowpea (Barrera-Figueroa et al., 2011), while the expression of *miR396*, another drought-induced miRNA in Arabidopsis (Liu et al., 2008), was inhibited in rice (Zhou et al., 2010). *miR156* is one of the most highly conserved miRNAs in plants and is involved primarily in the control of floral transition phases by targeting members of the SPL transcription factor family, as demonstrated experimentally in Arabidopsis (Wu and Poethig, 2006) and tomato (Ferreira e Silva et al., 2014). Besides SPL genes, other species-specific targets were predicted in grapevine by degradome analysis (Pantaleo et al., 2010). Here, we showed that one of these predicted genes, encoding a zinc finger CONSTANS5-like protein, is induced by stress while *miR156* decreases, supporting its status as a target of this miRNA. In our analysis, *miR396* levels increased upon drought exclusively in M4 root, while *GRL1-like1*, a member of the growth-regulating factor family, already shown to be targeted by *miR396* in Arabidopsis (Liu et al., 2009) and grapevine (Pantaleo et al., 2016), followed an opposite trend in the same genotype. The diverse expression profiles observed for *miR396* and its target in M4 and CS tissues are in accordance with other reports on genotypes with different sensitivity to DS (Kulcheski et al., 2011; Candar-Cakir et al., 2016). Grape targets of *miR159* (Pantaleo et al., 2010) include the GAMYB transcription factors MYB33, MYB65, and MYB101 (Achard et al., 2004), which take part in the signaling cascades induced by ABA accumulation in the presence of stress (Reyes and Chua, 2007). In our study, three putative *miR159* targets, encoding a TOPLESS4-like protein, a GATA transcription factor, and the putative *Vitis* ortholog of AtMYB101, showed an opposite expression profile than the cognate miRNA, supporting the hypothesis that, in grapevine, *miR159* may control additional molecular pathways than in Arabidopsis.

Thus, conserved miRNAs affected by stress in Arabidopsis may be involved in stress defense mechanisms in grapevine, but their modes of response and action seem to be different from those in the model plant. Our findings also confirm that members of a miRNA family can oppositely respond to stress in different plants and that their abundance can vary greatly in different organs or tissues (e.g. *miR393*) under water deficit (Zhang, 2015). Nevertheless, it should be mentioned that these variations among species could depend on experimental conditions, such as the timing and severity of DS (Frazier et al., 2011; Budak et al., 2015).

Besides conserved miRNAs, we identified 12 novel putative miRNAs whose abundance was affected significantly by DS. These novel miRNAs add to the emerging number of miRNAs identified in *Vitis* spp. (Carra et al., 2009; Mica et al., 2010; Pantaleo et al., 2010, 2016; Wang et al., 2012). The relative concentrations of these novel putative miRNAs were mostly lower than those of conserved miRNAs measured in the same samples. A similar situation was observed previously for novel miRNAs isolated in this and other plant species (Mica et al., 2010; Li et al., 2011; Ren et al., 2012; Ge et al., 2013). Moreover, since miRNAs belonging to low-abundance categories have emerging big roles in plant developmental processes (Wang and Guo, 2015), we hypothesize that the analyzed novel miRNAs that are less abundant than conserved miRNAs could function to modulate the cross talk between plant development and the abiotic stress response. For instance, the novel candidate miRNA *n_22* was predicted to target an *MLO* calmodulin-binding protein (MLO16)-encoding transcript belonging to a plant gene family involved in pathogen defense and abiotic stress response processes (Piffanelli et al., 2002). Upon drought, *MLO16* transcriptional rates increased in CS root, while *n_22* decreased accordingly. Grape-specific miRNAs have already been shown to target members of the NBS-LRR superfamily (Carra et al., 2009). Since miRNA-regulated NBS-LRR genes are involved in immune responses (Li et al., 2012), a role for these proteins in the response to abiotic stress also is conceivable. Here, NBS-LRR transcripts were predicted to be targets of *n_47* and *n_479* and showed transcriptional trends opposite to the expression profiles of these miRNAs. NBS-LRR gene biological functions often overlap (Meyers et al., 2003), and interestingly, it was reported that miRNAs can effectively control members of this protein family by triggering the biogenesis of phased secondary small interfering RNAs that, in turn, target NBS-LRR genes (Zhai et al., 2011). This strengthens the power of miRNAs as mediators of the integrated network of stress-induced molecular signals (Atkinson and Urwin, 2012).

The novel candidate miRNA *n_231* was predicted to target a chloroplastic polyphenoloxidase (PPO)-encoding gene, repressed upon drought, whereas the related

miRNA was strongly activated. PPO enzymes play a role in the control of reactive oxygen species (Sharma et al., 2012), and inhibition of the *PPO* gene, putatively mediated by *n_231*, could counteract drought-induced oxidative stress damage. The candidate miRNA *n_622* has three putative targets, including the hexose transporter *HT5*, which was functionally characterized in grapevine as a homolog of the Arabidopsis sugar transporter *STP13* (Hayes et al., 2007, 2010). This could support an induction of sugar signaling pathways, which, in woody species, can facilitate adaptation and/or recovery in response to dehydration (Secchi et al., 2016).

A gene encoding CIPK13 and a transcription factor, SPL6, belonging to the squamosa promoter-binding protein-like superfamily were predicted and analyzed as target sequences of *n_191* and *n_327*, respectively. In higher plants, CIPKs form an interacting network with CBL calcium sensors, which control calcium-dependent molecular cascades involved in environmental stress adaptation (Weinl and Kudla, 2009) and were reported as target transcripts of species-specific miRNAs tied to abiotic stress tolerance (Jeong and Green, 2013). Accordingly, in our experiments, the *CIPK13* gene was, in most cases, activated significantly upon stress and showed an expression profile opposite to that of *n_191*. Overall, expression levels of *SPL6* increased in drought-stressed plants and were divergent from those of *n_327*. The predicted target gene of *n_520* encodes a zinc-finger domain protein, which belongs to a wide group of transcription factors directing unique biological processes in plants, such as flower development, light-regulated morphogenesis, and pathogen responses (Takatsuji, 1998). The mentioned target exhibits an increasing expression in M4 roots and leaves upon drought occurrence, where contemporaneously low levels of *n_520* were observed.

The occurrence of opposite RT-qPCR trends between target transcripts and related miRNAs suggests their interaction in planta, but for some targets (*RPS5-like*, *HT5*, and *CIPK13*), this was not observed, especially upon stress conditions. However, the quantification of target mRNAs does not provide information on the possible effects of miRNAs on protein synthesis through translational repression, a widespread mechanism in Arabidopsis (Brodersen et al., 2008) and recently reported in woody species (Li et al., 2013a), which could be effective in grape as well. Furthermore, it must be taken into account that stress could affect the expression of target genes through regulation mechanisms independent of miRNA-mediated silencing (Hirayama and Shinozaki, 2010).

Most of the predicted target genes, especially those of conserved miRNAs, encoded proteins involved in embryogenesis and cell development (e.g. *SPL* genes), apparently not linked to stress defense. These data are not surprising, since plants coping with a changing environment must regulate the transcription of genes tied to cell proliferation, development, and control of phenological phases (Sunkar et al., 2012). This is supported both by recent work on *Brachypodium distachyon* (Bertolini et al., 2013), where the role of miRNAs upon drought was mostly correlated to the regulation of leaf cell growth reprogramming and development, and by more specific studies unraveling the induction of stress tolerance strategies through the miRNA-mediated regulation of key transcription factors tied to plant development (Stief et al., 2014).

### The Accumulation of Some Novel miRNAs Is Differently Modulated in Heterografted Plants

Grafting is performed routinely in grapevine management to obtain resistance against phylloxera, but grafting on specific rootstocks also can optimize plant growth rate, fruit quality, and tolerance to abiotic stresses. In pumpkin and cucumber, it has been shown that reciprocal grafting modifies the abundance of several miRNAs and predicted target genes (Li et al., 2014). As grapevine miRNAs are responsive to DS and are differently expressed in specific genotypes, reciprocal grafting of these genotypes could modify the scion or stock concentration of these miRNAs. To test this hypothesis, we assessed the concentrations of a group of novel putative miRNAs in reciprocal grafts of the stress-tolerant M4 with the stress-sensitive CS.

Grafting affected miRNA abundance, particularly for some of the analyzed novel candidates. This is the case for *n_191*, which in autografted vines was more abundant in CS, while in heterografted plants, significant differences in the accumulation of this miRNA between stock and scion were evident upon stress when M4 was used as stock. Another interesting case is *n_520*, which, in autografted vines, was present nearly exclusively in the leaves of M4 and only under irrigated conditions, whereas in heterografted plants, a relatively high abundance of this miRNA was present in CS root and leaves. However, the down-regulation of this putative miRNA observed upon drought in M4 was not noticed in CS tissues of heterografted plants. Thus, upon grafting, *n_520* was present in heterografted CS, but it was not affected by drought.

Some degree of immune response to grafting is experienced even in these compatible genotypes, and a recent report demonstrated the up-regulation of stress responses in heterografting with nonself rootstocks (Cookson et al., 2014). This general response also could be the basis of the changes in expression that we observed for some candidate miRNAs. The abundance of *n_55*, *n_191*, and *n_312* was 1 order of magnitude lower in heterografted plants than in autografted plants. Thus, a nonself response could affect the accumulation of specific miRNAs. The movement of hormones between stock and scion has been demonstrated previously (Foo et al., 2007), and many signals, such as ABA, commonly move across the graft junction in grapevine (Ferrandino and Lovisolo, 2014). This implies that altered signal fluxes induced by grafting could affect the regulation of miRNA expression in either one or both of the graft partners. Accordingly, several research studies

have elucidated that miRNAs are key players in hormone (e.g. ABA and auxin) signaling cascades driving the response to DS (Ding et al., 2013; Zhang, 2015).

Finally, results obtained from the analysis of phloem sap have supported the hypothesis that, under stress, miRNAs can accumulate in phloem vessels, thus potentially serving as long-distance signaling molecules (Pant et al., 2008; Marín-González and Suárez-López, 2012). Testing of this possibility is not straightforward in grapevine and most other plants where the application of genetic approaches, such as the generation of specific mutant lines used as rootstock in grafting experiments, is still limited due to the absence of effective transformation protocols and long reproductive cycles (Gambino and Gribaudo, 2012). It is worth noting that, until now, small RNAs have been detected in the phloem and not in the xylem sap (Pant et al., 2008); thus, direct movement should explain the presence of scion-specific miRNAs in stocks but not the reverse. In this context, an interesting result concerns miRNA *n_312*, whose abundance was similar in CS and M4 autografted plants, but it accumulated to different extents in heterografts, with higher concentration in M4 (either leaf or root) heterografted plants, particularly upon stress.

Taken together, our findings highlight changes in the accumulation patterns of conserved and novel miRNAs dependent on DS, tissue type, and grapevine genotype. Moreover, the analyses of diverse grafting combinations reveal the existence of even more complex genotype-dependent molecular networks that tune stress-related miRNA abundance and mode of action. These effects could be triggered in a grafted genotype through the interaction of signaling molecules (e.g. ABA) produced by the other graft component.

## MATERIALS AND METHODS

### Plant Material and Experimental Setup

Two-year-old autografted grapevines of *Vitis vinifera* 'Cabernet Sauvignon', M4 [a new hybrid genotype selected at the University of Milan by crossing (*V. vinifera* × *Vitis berlandieri*) × *V. berlandieri* 'Resseguier n.1' (Meggio et al., 2014)], and reciprocal grafting combinations (heterografts: CS shoot/M4 root and M4 shoot/CS root) were grown in a greenhouse under partially controlled climate conditions (20 plants per graft combination). The temperature in the greenhouse was maintained in the 26°C to 35°C range, and natural light/dark cycles were followed. Maximum photosynthetic photon flux density ranged between 1,330 and 1,580 $\mu$mol $m^{-2}$ $s^{-1}$. Grapevine genotypes often show a capacity to explore diverse volumes of soils, and for this reason, we used a limited substrate volume in all the grafting conditions tested. Each plant grew in a 5-L pot filled with a substrate composed of a sandy loam soil (pH 7; available phosphorus, 7.9 mg $kg^{-1}$; organic matter, 1.37%; cation-exchange capacity, 4.58 mE 100 $g^{-1}$):expanded clay:peat mixture (2:1:1, w/w). From bud break (February 15) to the beginning of the experimental period (July 24), plants were irrigated twice per week to maintain water container capacity. A DS treatment was applied during a period of high atmospheric evaporative water demand (vapor pressure deficit averaging 25 mbar $bar^{-1}$). Half of the plants of each graft combination were maintained at container capacity (Lovisolo and Schubert, 1998) and used as irrigated controls, whereas the other 40 plants were subjected to a DS treatment by withholding irrigation for a period of about 10 d. During the treatment, $\Psi_{leaf}$ and $g_s$ were measured daily. Droughted plants were stressed until the measured $\Psi_{leaf}$ and $g_s$ values were lower than −1.2 MPa and 0.05 mmol water $m^{-2}$ $s^{-1}$, respectively, which can be considered levels of severe water stress according to grapevine adaptations to water stress (Lovisolo et al., 2010).

### Leaf Gas-Exchange and $\Psi_{leaf}$ Measurements

The $g_s$ was measured on adult, nonsenescing leaves well exposed to direct sunlight (photosynthetic photon flux density of 400–700 nm) using a portable gas-exchange fluorescence system (GFS-3000; Heinz Walz). The measurements were performed using an 8-$cm^2$ leaf chamber with artificial irradiation (1,200 $\mu$mol photons $m^{-2}$ $s^{-1}$), setting the chamber temperature at 26°C to avoid overheating of the leaf. $CO_2$ and relative humidity were maintained at greenhouse conditions (420 $\mu$L $L^{-1}$ and 50%, respectively). Measurements were taken on two leaves per plant between 9 AM and 12 noon on each experimental day. After measuring $g_s$, the same leaves were excised and used to determine $\Psi_{leaf}$ using a Scholander-type pressure chamber (Soil Moisture Equipment).

### Measurements of Root $k_h$

Root $k_h$ was measured on well-watered rootstocks of CS/M4 and M4/CS plants under laboratory conditions by means of an HPFM-XP Hydraulic Conductance Flowmeter (Dynamax; http//www.dynamax.com/) following the protocol described by Lovisolo and Tramontini (2010). Five transient measurements were run by increasing the pressure up to 0.4 MPa at a rate of ~5 KPa $s^{-1}$ and by measuring the instantaneous flow. The root $k_h$ corresponds to the slope of the linear regression obtained by plotting flow data versus pressure data. Root $k_s$ was calculated by normalizing the root $k_h$ on the root dry weight.

### Small RNA Library Preparation and Sequencing

Leaf and root samples were collected from DS and irrigated autografted plants of both genotypes (CS/CS and M4/M4), weighed (about 1 g each), immediately frozen in liquid nitrogen, and stored at −80°C. In detail, leaf samples were always taken from adult, nonsenescing leaves, whereas roots were sampled by collecting the whole root system. Root and leaf samples obtained from all the plants ($n$ = 10) of each condition were pooled together, and LMW RNA was extracted following the protocol by Carra et al. (2007). The integrity and quantity of LMW RNA were checked with a 2100 Bioanalyzer (Agilent). Only LMW RNA samples obtained from autografted plants, representing eight tested conditions (2 genotypes × 2 organs × 2 treatments), were processed further for library preparation; only one replicate per condition was considered for next-generation sequencing analysis. LMW RNA samples were processed according to the protocol for the SOLiD Total RNA-Seq Kit (Applied Biosystems) with minor modifications: the first step was the ligation of adaptors that ensure the directionality of sequencing; after the reverse transcription step, a size selection was done on a 10% acrylamide denaturing gel. At the end of the process, the obtained cDNA libraries were amplified by emulsion PCR, barcoded, and sequenced using the SOLiD platform (Applied Biosystems). At the end of the sequencing process, raw sequence data were trimmed to remove adaptor sequences, filtered for quality, and aligned against the Rfam 11 database (http://rfam.xfam.org/; Gardner et al., 2011) to exclude from the genomic mapping contaminants and small noncoding RNAs not belonging to the miRNA clade (i.e. tRNAs, rRNAs, scRNAs, snoRNAs, snRNAs, and RDPs). The unique number of reads was obtained by mapping the filtered sequences on the grapevine reference genome (12X V1; http://genomes.cribi.unipd.it/grape/) and, in parallel, on miRBase (http://www.mirbase.org/; version 21) to allow the detection of already annotated miRNA sequences among the predicted novel ones. Finally, to compare the number of reads obtained for each predicted miRNA among the different libraries, a normalization step was performed, and the relative number of miRNA reads was calculated for each miRNA, as follows:

$$\textit{Relative number of miRX reads} = \textit{number of miRX reads in libraryX} / \textit{total number of libraryX reads} * 1,000,000$$

in which miRX is a predicted known or novel miRNA, libraryX is one of the sequenced library samples, and 1,000,000 is a constant value.

Novel miRNAs and their precursors were identified on the reference grapevine genome by applying two dedicated prediction softwares, mirCat (Moxon et al., 2008) and miRDeep-P (Yang and Li, 2011). Precursor sequences of the predicted novel miRNAs were further processed using MFOLD version 2.3 (http://unafold.rna.albany.edu/?q=mfold; Zuker, 2003) to analyze the folding of secondary hairpin structures. Conserved and known miRNAs were

identified through alignment on miRBase (Kozomara and Griffiths-Jones, 2014) by setting the PASS algorithm in order to consider only the alignments showing no mismatches with the miRBase entries and with a minimum coverage of 17 bp, because in miRBase the size range of annotated sequences is between 17 and 26 nucleotides.

## Prediction of Novel miRNA Target Genes

Putative target genes of candidate miRNAs were predicted in silico by applying two computational approaches: psRNATarget (http://plantgrn.noble.org/psRNATarget/; Dai and Zhao, 2011) and Target-align (http://www.leonxie.com/targetAlign.php; Xie and Zhang, 2010).

psRNATarget is a recent evolution of a previous software, miRU (Zhang, 2005), which calculates the score of complementarity between the small RNA and its target gene and the target site accessibility, which is assessed by calculating the energy required to unpair the secondary structure around a small RNA target site on the mRNA. Target gene sequences were retrieved by alignment with 12X V1 using the default setting parameters of psRNATarget, with the only exception that of the maximum expectation value, for which a cutoff threshold of 2 was applied. The Target-align algorithm, which was also developed as an implementation of the Smith-Waterman algorithm, as well as psRNAtarget, scores for a specific miRNA and its potential targets considering all potential factors that can affect the identification of miRNA targets (number of consecutive mismatches, base site restrictions, number of G:U wobbles, and number of gaps), according to the indications reported by Jones-Rhoades and Bartel (2004).

Predicted target sequences were then aligned to the M4 resequenced genome (http://genomes.cribi.unipd.it/grape/serres/) in order to check for nucleotide mismatches in the region of miRNA complementarity, M4 being a hybrid *Vitis* genotype.

Target genes were also annotated functionally using the Blast2GO software (version 2.7.1; http://blast2go.com/b2ghome) with default parameters.

## Real-Time PCR Analysis

Expression changes of conserved and novel miRNAs and of target transcripts were determined on leaf and root samples collected from both autografts and heterografts by stem-looped RT-qPCR assay.

Total RNA was extracted in triplicate from autografted and heterografted plants of the two genotypes, following the protocol by Chang et al. (1993) with only slight modifications. RNA samples were quantified and qualified with the 2100 Bioanalyser and treated with DNase I, RNase-free (50 units $\mu L^{-1}$; Fermentas) to avoid any risk of DNA contamination. First-strand cDNA was synthesized starting from 10 $\mu g$ of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's instructions. Only in the case of miRNA-specific cDNA was the retrotranscription protocol modified using a stem-loop primer (rather than random primers) specific for each of the target miRNAs. The use of stem-loop primers in RT-qPCR experiments is an effective and well-developed strategy for the detection of miRNAs, first reported by Chen et al. (2005) and Varkonyi-Gasic et al. (2007) and applied successfully in many research experiments addressing the study of abiotic stress-responsive miRNAs (Shen et al., 2010; Eldem et al., 2012; Xie et al., 2014; Candar-Cakir et al., 2016; Gao et al., 2016; Yadav et al., 2016). A list of the stem-loop primers used in this study is given in Supplemental Table S1.

Real-time PCR was carried out in the StepOnePlus RT-qPCR System (Applied Biosystems) using the SYBR Green (Applied Biosystems) method for quantifying amplification results. Thermal cycling conditions were as follows: an initial denaturation phase at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min (only in the case of miRNA expression analysis, a step at 58°C for 15 s was added to the cycling stage). Specific annealing of primers was checked on dissociation kinetics performed at the end of each RT-qPCR run.

Expression levels of miRNAs and of the related target transcripts were quantified after normalization to either *VvsnRU6* or *VvUBI* and *VvACT1* genes used as internal controls, respectively. Gene-specific primers used in real-time PCR experiments are listed in Supplemental Table S2. Three biological replicates were run for each RT-qPCR experiment.

In the case of novel miRNA expression analysis, RT-qPCR products were purified, cloned into the pGEM-T vector (Promega), and sequenced using T7 and SP6 forward and reverse primers in order to further ascertain the accuracy of the amplification results.

## Statistical Analyses

Significant differences among treatments were statistically analyzed by applying a one-way ANOVA test, using Tukey's HSD posthoc test to separate means when ANOVA results were significant ($P < 0.05$). Significant differences of pairwise comparisons were assessed by Student's $t$ test.

The SPSS statistical software package (SPSS; version 22) and Sigma Plot software (Systat Software) were used to run the statistical analyses reported above and to elaborate figure charts, respectively.

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Expression patterns of conserved miRNA target transcripts.

**Supplemental Table S1.** Stem-loop primers used in this study for miRNA-specific RT-qPCR assay.

**Supplemental Table S2.** Oligonucleotides used in this study for RT-qPCR analysis of miRNAs and related target transcripts.

**Supplemental Table S3.** Conserved and known miRNAs identified by high-throughput SOLiD sequencing in CS and M4 root and leaf samples upon irrigated control and DS conditions.

**Supplemental Table S4.** Putative novel miRNAs identified by high-throughput SOLiD sequencing in CS and M4 root and leaf samples upon irrigated control and DS conditions.

**Supplemental Table S5.** Target transcripts of the novel candidate miRNAs analyzed in this study.

**Supplemental Table S6.** Target transcripts of the conserved/known miRNAs analyzed in this study.

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