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Drought stress priming induces morphological adaptation and methylome changes in apple seedlings

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Abstract

Developing multiple stress-tolerant crops is the goal of many breeders in the current context of seasonal variability precipitated by the ongoing climate change. Among the most pressing stress, drought is becoming a major concern in many regions of the world. Plant responses to drought have been studied in many species, including perennial plants such as apple trees (*Malus domestica*); however, the effects of recurrent stress and the potential for stress memory, or priming, remain poorly described. Priming reflects the capacity of plants to "memorize" environmental stress experiences and potentially improve their responses to recurring stress. Such information may be retained through epigenetic modifications of DNA and associated histone proteins. This ability could enable training of plants to face future challenges. In this study, we explored the effects of recurrent drought stress on apple seedling morphology and investigated the epigenetic changes in plants subjected to this treatment. Our results indicate that naïve apple seedlings can be primed upon first exposure to drought stress, and that primed plants respond differently to recurrent stress later in the season. Analyses of differentially methylated regions further indicate that primed plants' molecular responses differ significantly from plants exposed to drought stress for the first time. Finally, our results suggest that following a winter period, the previously induced priming effect may be partially lost. Overall, our study demonstrates the importance of studying recurrent stress to better understand the memory-associated mechanisms leading to morphological and DNA methylation modifications in perennial plants.

Keywords Recurrent drought stress, Apple seedlings, DNA methylation, Stress memory

Background

Plants in natural environments are often faced with multiple and recurrent stresses. To survive, perennial species have evolved strategies to withstand these conditions. Recurrent exposure to non-lethal stress can improve plant performance, a phenomenon known as priming [1]. Primed plants respond differently to stress compared to naïve ones [2], suggesting the existence of persistent biological 'memory' that influences future responses.

Water availability is a major factor affecting plant growth and crop yield in many regions of the world. Drought stress, combined with extreme temperatures and high salinity, are among the most important limiting factors in apple production, particularly under the ongoing climate change scenario [3]. Water shortage leads to morphological and physiological changes that minimize water loss, maximize water uptake, and prevent cellular damage caused by decreasing tissue water content

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[4–6]. Drought avoidance is accompanied by a reduction in overall photosynthetic performance which, combined with hydraulic properties, determines growth performance [4].

Additionally, drought stress in sessile organisms trigger changes in gene expression, affecting plant growth and metabolism regulation. In apple, transcriptome analyses, enabled by the complete genome sequence [7], permitted the identification of differentially expressed genes (DEGs) linked to growth, fruit development and stress regulation. Li et al. [8] investigated transcriptome dynamics under drought, cold and high salinity stress, highlighting the cross-regulation mechanisms involved in the various stress responses. RNA-seq analyses performed on apple trees treated with exogenous dopamine revealed the involvement of WRKY, ERF and NAC transcription factors in drought tolerance [9]. WRKY genes were also identified as differentially expressed in response to both waterlogging and drought stress, pointing to a potential implication of this gene family in apple's response to stress [10]. More recently, Liu et al. [11] found that the over-expression of *MdPL9* (an ABA receptor), increased the drought resistance of apples under stress by reducing the downregulation of genes encoding CHS (chalcone synthase). Recent studies have also demonstrated the role of epigenetics in plant response to drought. Wang et al. [12] combined ATAC-seq and RNA-seq analyses to investigate the effect of variation in chromatin accessibility on apple plants under drought stress. Cytosine methylation level variation, an essential feature of epigenetic regulation, was also investigated. Whole Genome Bisulfite (WGB) analyses, combined with RNA-seq data from drought-sensitive and drought-tolerant apple cultivars grown under water deficit conditions, revealed that a wide array of genes and transcription factors were differentially expressed between the cultivars [13]. Moreover, the water deficit was associated with changes in methylation level of these genes, particularly in their promoter regions. Comparison of the transcriptomic and epigenetic dynamics of 'Golden Delicious' and the wild species *Malus prunifolia* exposed to drought stress revealed an overexpression of the *MdDREB2A* gene, which homolog is involved in drought tolerance in Arabidopsis [14], together with a significant decrease in the methylation level of that gene's promoter [15]. More recently, the methylation of a miniature inverted-repeat transposable element (MITE) inserted in the promoter region of the *MdRFNR1-1* (root-type ferredoxin-NADP+ oxidoreductase) gene was found to be involved in the overrepression of that gene and positively associated with an increased tolerance to drought stress in apple [16].

Apple production relies primarily on improved cultivation practices and breeding for stress tolerance. While these approaches are essential, they could be further complemented by plant priming strategies. However, few studies have investigated the molecular and physiological responses triggered by recurrent drought cycles. In this study, we report the effects of several cycles of drought stress on apple seedlings and their capacity to modulate adaptive responses through a phenomenon known as priming. We investigated the morphological changes induced by three consecutive drought periods interspersed with recovery stages and investigated the potential priming effect of such recurrent stress cycles by monitoring seedlings' growth reduction and recovery rates. In parallel, we conducted methylome analyses of leaves and roots to investigate the regulatory mechanisms involved in their adaptation. To investigate a potential long term priming effect, groups of control (naïve) and drought-primed plants were submitted to a single drought stress the following year and their morphological and molecular responses were investigated. This study provides insights into the physiological and epigenetic shifts driving apple seedlings' adaptation to recurrent drought and recovery cycles.

Materials and methods

Plant material

Plants were grown in the greenhouses of France's National Research Institute for Agriculture, Food and Environment (INRAE) in Angers, France (47° 28' 36.804" N 0° 36' 41.4" W). The apple seedlings were obtained from seeds of 'Golden Delicious' double haploid 18 (GDDH18) [17]. The seeds were produced at INRAE from self-pollination of the GDDH18 tree, then stratified for 3 months in a 1:1 mixture of sand and vermiculite (humidity of 70%) and kept at 3°C for 90 days. The seeds were then sown in a soil mixture containing 50% peat moss, 40% perlite, 10% coco fibers, 7.5kg/ea carbonates and N-P-K mix 14-16-18. Sowing was performed in three blocks, interspersed by one week each, representing three biological replicates. The germination was carried out in a greenhouse (ambient light; 21°C ± 5°C; relative humidity 60% ± 20; the greenhouse minimum/maximum temperature and humidity values were monitored and documented during the whole experiment (Table S1). Four weeks after germination, seedlings were transplanted into 0.4 L plastic pots using the same soil mixture. At eight weeks of age, the seedlings were transferred to 1 L pots and grown for an additional four weeks before the initiation of drought stress (DS) cycles. The DS assays were conducted on a total of 163 seedlings, distributed across three biological replicates in greenhouse conditions.

Drought stress cycles

Irrigation management was conducted by monitoring the soil water content (SWC) of all plants every two days by

pot weighing. During well-watered (WW) periods, plants were maintained near field capacity (FC; 80% SWC), whereas during DS periods, irrigation was adjusted to maintain SWC at 45% of FC. The SWC corresponding to 100% FC was determined for the soil mix by saturating pots with water and allowing free drainage for 24 h. Three distinct irrigation regimes were established (Fig. S1):

(I) Control group (64 plants): maintained under WW conditions throughout the experiment.
(II) Multiple-DS group (61 plants): subjected to three consecutive drought cycles, each consisting of three weeks under DS conditions followed by three weeks of recovery (WW condition).
(III) Unique-DS group (38 plants): exposed to a single three-week DS period at the beginning of the experiment, followed by continuous WW condition until the end of the study. This treatment only was performed with two biological replicates due to the limited availability of GDDH18 seedlings.

To investigate long-term responses, plants were transplanted into 3 L pots at the end of the 19-week experimental period and transferred from the greenhouse to outdoor shelters, where they were exposed to natural environmental conditions, allowing them to complete their phenological cycle. In the subsequent spring, 22 and 23 plants from the previous Control and Multiple-DS groups, respectively, were reintroduced in the same controlled greenhouse environment to resume vegetative growth. A new drought stress cycle of three weeks at 45% FC followed by three weeks of WW (80% FC), was then applied (Fig. S1).

Plant growth monitoring

Stem height, number of nodes and internode length were evaluated in control ($n = 5 \times 3$), Multiple-DS ($n = 5 \times 3$), and Unique-DS ($n = 5 \times 2$) groups. Data collection was performed using the MicroScribe® digitizer, a portable coordinate measurement machine, every week during the experiment. The input XYZ coordinates from MicroScribe® were processed by an *in-house* R script using RStudio®. For stem diameter measurement, a digital caliper was used. The measures were performed at t_6 (see Fig. S1) on all plants. The overall stability (Ost) of plant growth was calculated based on Ribeiro et al. [18]. Here, the recovery rate (RR) was estimated by the slope of the linear regression of plant stem growth during the 3 weeks of recovery. The disturbance rate (DR) was obtained by the slope of the linear regression of plant growth during the 3 weeks of water deficit. We estimated the Ost by the RR/DR ratio for each of the 3 cycles. At the end of the experiment (t_6 at Year n and after DS + WW at Year $n+1$; Fig. S1), six plants of each group were collected to determine the biomass (root and stem fresh and dry weight). The dry weight was obtained after 48 h of 60 °C oven-drying.

Leaf anatomical studies

Leaf anatomical analyses were performed using three fully expanded leaves randomly collected from different seedlings for condition and biological repetitions (Control: $n = 3 \times 3$; Multiple-DS: $n = 3 \times 3$; Unique-DS: $n = 3 \times 2$). The selected leaves had developed during the drought stress (DS) periods t_0–t_1 (DS1) t_4–t_5 (DS3) and in the year $n+1$ before DS and after DS (Fig. S1). From each leaf, three 0.25 cm^2 sections were excised from the central region of the blade and frozen in liquid nitrogen. The samples were embedded in Cryo-Gel tissue freezing medium (Leica Biosystems, Germany), and 60 µm transverse sections were obtained using a Cryostat CM3050 S (Leica Biosystems, Germany) at -20 °C. The sections were observed under a Leica DM1000 LED optical microscope (Leica Biosystems, Germany) equipped with a digital camera. For each 0.25 cm^2 section, two images were acquired, and two distinct regions per image were measured, resulting in a total of 54 images and 108 measurements per condition for both the Control and Multiple-DS. At t_1, plants from the Multiple-DS group were used to analyze the first cycle of water deficit (DS1) as they were under the same conditions as the Unique-DS group. This approach also allowed the same plants to be used for analysis at the end of the third cycle of water deficit (DS3) at t_5. For Unique-DS at t_5, 36 images and 72 measurements were obtained. Image analysis was carried out using ImageJ software [19]. Leaf blade thickness was measured between the upper and lower epidermis (Fig. S3). The two mesophyll layers (palisade and spongy) were measured separately (Fig. S3). Total mesophyll thickness was obtained by summing the palisade and spongy parenchyma measurements, while epidermis thickness was calculated by subtracting the mesophyll thickness from the total leaf blade thickness (Table S2). Mean values across treatments and time points were compared using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$) in GraphPad Prism.

Sampling of leaves and roots for nucleic acid extraction

For the Control and Multiple-DS treatments, fully expanded young leaves were collected at t_1 and t_5, corresponding to the end of each DS cycle (Fig. S1). For each biological replicate, a pool of six leaves ($n = 6 \times 3$) was used for DNA/RNA extraction. Leaf pools were frozen in liquid nitrogen after sampling. For roots, approximately 200 mg of white root tips per biological replicate were collected from randomly selected plants at t_1 and t_5 for Control and Multiple-DS groups ($n = 6 \times 3$; Fig. S1). Prior

to liquid nitrogen freezing, roots were washed with water and dried with paper towels. All leaf and root samples were ground into a powder in liquid nitrogen and stored at -80 °C until nucleic acid extraction.

Transcriptomic analysis

RNA-seq analyses were conducted on leaf and root samples collected after 3 weeks of water deficit in the cycle 1 (t_1; Control_t_1, Multiple-DS_t_1: Fig S1). Plants from the Multiple-DS group were used to analyze the first cycle of water deficit (DS1) because, at this time point, they were under the same conditions as the Unique-DS group. In addition, three biological replicates were available for the Multiple-DS group, which was not the case for the Unique-DS group. For each tissue and condition, approximately 50 mg of fresh weight was used for total RNA extraction with the NucleoSpin® RNA Plus kit (Macherey-Nagel, Germany). RNA quality was verified by 1% agarose gel electrophoresis, and RNA Integrity Number (RIN) was assessed using a Bioanalyzer (Agilent Technologies, USA). Total RNA (0.5 µg) was sent to the Genome Quebec platform (Quebec, Canada), where libraries were prepared using the NEB® mRNA Stranded Library Prep Kit (New England Biolabs, USA). Sequencing was performed on an Illumina NovaSeq S4 platform in paired-end mode (2 × 100-bp), generating a minimum of 25 million reads per sample. Raw sequencing quality was assessed using FastQC [20]. Transcript-level quantification was performed using Salmon [21] with default parameters, using the *M. domestica* GDDH13 v1.1 reference genome [17]. Differential gene expression analysis was carried out using the DESeq package in R [22]. DEGs (Differentially Expressed Genes) are defined as genes with logFC ≥ 1 or logFC ≤ − 1 and an adjusted p-value of < 0.1.

Whole Genome Bisulfite Sequencing (WGBS)

Genomic DNA (gDNA) was purified from 50 mg of tissue powder, collected at the same time points as the RNA-seq samples, using the NucleoSpin™ Plant II kit (Macherey-Nagel, Germany). DNA quality and concentration were assessed by 1% agarose gel electrophoresis and Nanodrop® spectrophotometry (Thermo Scientific, USA). Samples of gDNA (0.7 µg) were sent to the Genome Quebec Sequencing Platform (Quebec, Canada). Libraries were prepared using the NEBNext® Enzymatic Methyl-seq Library Prep Kit (New England Biolabs, USA). Paired-end sequencing (2 × 150 bp) was performed on the Illumina NovaSeq 6000 system with a minimum of 35 million reads per sample. Adapter trimming and removal of low-quality reads were carried out using Trimmomatic [23]. Sequencing quality was assessed using MultiQC [24], and alignments were performed with Bismark [25] using the *M. domestica* GDDH13 v1.1 genome. Differentially methylated regions (DMRs) were analyzed using a sliding-window approach implemented in the methylKit R package [26], with a window size of 1000 bp and a step size of 100 bp. Only CpG sites with a minimum coverage of 10× were included. A minimum 25% difference in methylation levels and a q-value < 0.01 were used as DMRs threshold. All three methylation contexts (CG, CHG, and CHH) were examined. Genes located within 2 kb of each DMR were identified. This process was resumed at the 'BISEPS2' pipeline [27].

Gene ontology enrichment

Gene ontology (GO) enrichment analysis was performed using the agriGO v2.0 platform [28]. The Singular Enrichment Analysis method was applied using *M. domestica* as a reference background. The Hochberg false discovery rate (FDR) correction was used for multiple testing, with a minimum significance level of p-value < 0.05. A minimum of five mapping entries was required, and a full GO run was conducted.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 10.01 (GraphPad Software, La Jolla, California, USA). One-way ANOVA followed by multiple comparison tests (Tukey or Dunnett, depending on the dataset and described in the figure legends) were used to evaluate differences among groups. Statistical significance was set at $p < 0.05$. Results are expressed as means ± standard error (SE).

Results

Drought-primed apple seedlings show a distinct growth response when exposed to a new cycle of water deficit

The methodology employed to monitor plant irrigation in this study indicates that within one week of the drought stress (DS) induction, Unique-DS and Multiple-DS plants were subjected to a 20% reduction in SWC compared to Control plants (Fig. 1A). In the same week, the absolute growth rate (AGR) of the apple seedlings, which represents the increment in plant stem size (mm) per unit of time (day) was not yet impacted (Fig. 1B).

However, from week 2 of DS1 we observed a reduction (around 50%) in plant growth rate in Multiple-DS and Unique-DS groups compared to the Control group (4.2 ± 0.5, 4.1 ± 0.5, 9.2 ± 0.8 mm.day^{-1}, respectively; Fig. 1B). From week 3, the reduction was accentuated (around 70%) with an AGR of 3.3 ± 0.4 mm.day^{-1} for Multiple-DS, 3.0 ± 0.8 mm.day^{-1} for Unique-DS, and 11.1 ± 0.7 mm.day^{-1} for Control group, even if the irrigation was kept constant (Fig. 1A). During the first recovery period (WW1) after 3 weeks of drought stress, our data indicated that toward the end of WW1 in both Multiple-DS and Unique-DS groups, the AGR was stronger than

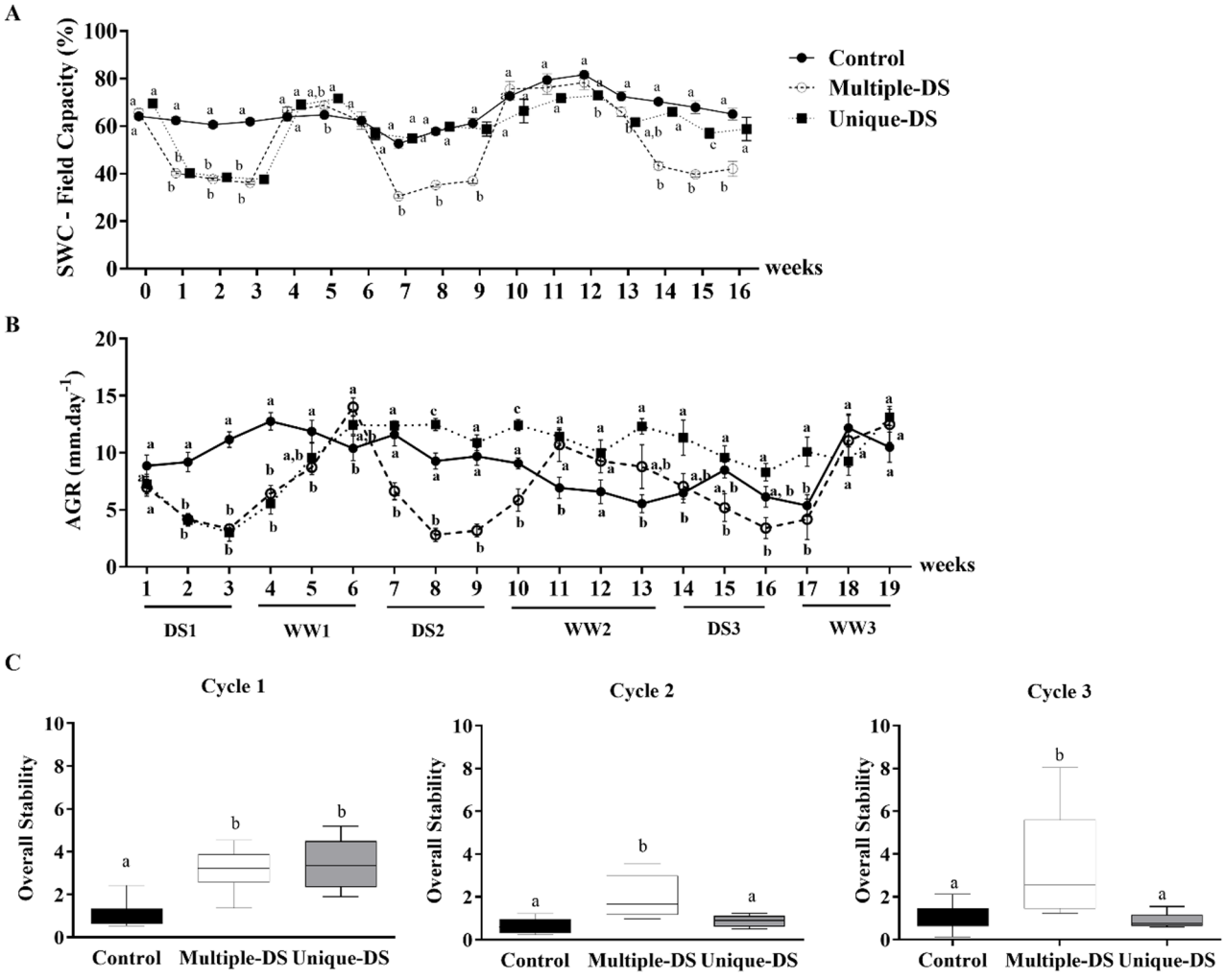

Fig. 1 Monitoring of soil moisture and plant growth dynamics during drought and recovery periods. **A** Soil water content (SWC) was monitored and irrigation was adjusted according to the drought stress (DS) treatment. The measurements were performed three times per week by pot weighing and expressed as a percentage of field capacity. **B** Absolute growth rate (AGR), defined as the weekly increment of stem length in millimeters per day ($mm{\cdot}day^{-1}$), was measured from the first until the third drought stress (DS) and well-watered (WW) periods. **C** Overall stability of plant growth was calculated for each cycle as the ratio between growth during recovery and drought conditions (see Materials and Methods for details). Data were compared among treatments at the same time-point or cycle using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).

that observed by the Control group, indicating the presence of a potential growth compensation mechanism in previously stressed plants.

During DS2 cycle, Multiple-DS plants demonstrated a faster response to drought stress (compared to DS1), with a 2x reduction of growth speed in week 7, followed by 3.5x reduction in weeks 8 and 9, under the same irrigation conditions of DS1 (Fig. 1A and B). These observations indicate that the second stress affected the plants differently from the first and suggest a potential adaptation of Multiple-DS seedlings, as they were able to perceive and respond to the water deficit more rapidly than during the initial exposure. This hypothesis is confirmed by the fact that plant recovery was also more dynamic at WW2 compared to WW1, where it took only one week to reach the same growth speed as Unique-DS group that were under constant irrigation (Fig. 1B). In the DS3 cycle, no significant difference in AGR was observed between the Multiple-DS and Control groups, even if the irrigation management via SWC was significantly different between groups (Fig. 1A and B). Temperature and humidity control issues registered in the greenhouse during that period may have affected Control plant growth. However, even under these conditions, Unique-DS plants exhibited consistent growth and appeared to be less affected by environmental constraints than plants that had never been exposed to any water deficit (Fig. 1B).

To confirm the physiological stress status of the plants, we estimated the index of plant overall stability (OSt) described by Ribeiro et al. 2021, which is an indicator of intra-group growth variability (Fig. 1C). Groups of plants growing at a similar rate are expected to exhibit an OSt

value of one [18]. In cycle 1, the Unique-DS and Multiple-DS groups displayed OSt values between three and four, indicating stress, whereas the Control group maintained an OSt value close to one (Fig. 1C). Furthermore, during drought cycles 2 and 3, only the Multiple-DS group which was confronted with water deficit exhibited an OSt value above one, and significantly different from the values estimated from the Control and Unique-DS groups (Fig. 1C). These parameters demonstrate that the various groups of plants were indeed drought-stressed during the intended periods.

Regarding plant morphological traits, Multiple-DS apple seedlings exhibited a reduction of 20% of plant height (1136.8 ± 41.4 mm) compared to Control plants (1375.6 ± 40.8 mm; Fig. 2A) at the end of DS3. Interestingly, Unique-DS plants were 11% taller than the Control group (1526.7 ± 26.1 mm; Fig. 2A, Fig. S2A) in the end of WW3.

At the end of the 3 cycles of water deficit and recovery periods (WW3), no significant difference in the number of nodes was observed between Control and Multiple-DS or Control and Unique-DS plants (Fig. 2B), indicating that the difference observed between the two groups may be due to different internode lengths. Confirming this hypothesis, the increment in stem length was affected during all DS periods for plants facing a water deficit (Fig. 2C). The primary stress response (DS1) reduced the stem length growth of DS-stressed plants to about 30% of that observed in the Control group. (Multiple-DS: 39.23 ± 6.5 mm; Unique-DS: 45.2 ± 6.2 mm, Control: 127.6 ± 12.9 mm; Fig. 2C).

Measurement of stem diameter at the end of cycle 3 revealed higher values for Unique-DS plants (7.1 ± 0.1 mm) and lower values for Multiple-DS plants (5.3 ± 0.1 mm; Fig. 2D). This is consistent with the increased plant height observed in Unique-DS plants, likely related to the peak in stem length increment that occurred during DS2 (Fig. 2A and C). These results indicate that Unique-DS plants recovered completely from their first stress and even outgrew the Control plants, suggesting a potential growth compensation mechanism. Moreover, they seem to respond differently from Multiple-DS apple seedlings. Regarding root system morphology, no significant differences among treatments were

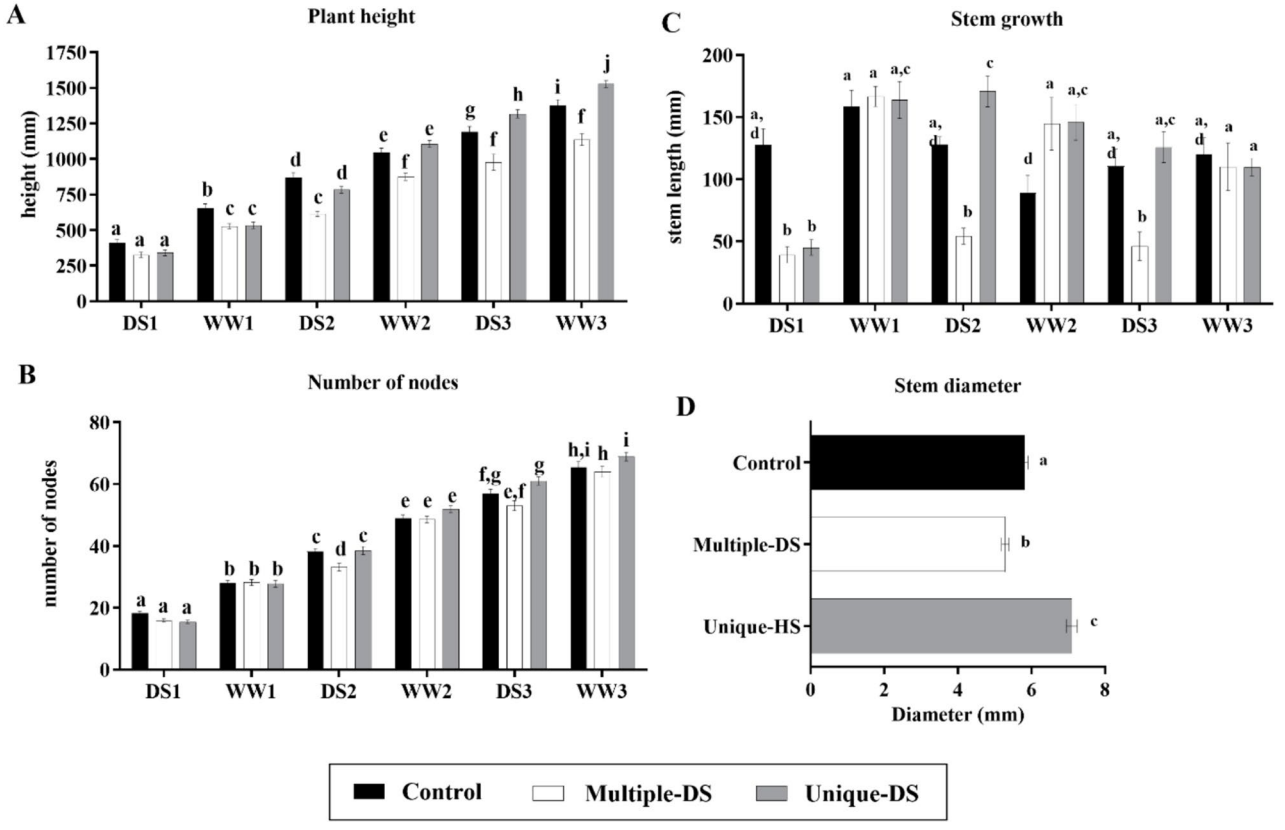

Fig. 2 Morphological traits of plants in response to multiple water irrigation treatments. **A** Plant height (mm) (**B**) number of nodes and **C** stem growth increment (mm) was measured weekly using a MicroScribe® digitizer. Values shown correspond to the mean ± SE of all evaluated plants in the final week of each treatment period (DS or WW). Different letters indicate statistically significant differences between treatments and periods, as determined by two-way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). **D** Stem diameter was measured at the end of the experiment, after WW3 period (t_6) using a digital caliper. Statistical differences between stem diameters measurements were evaluated using one-way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$)

Table 1 Total biomass and biomass allocation of Non- and DS-primed plants after overcoming dormancy and a new cycle of drought stress (year n+1)

	Before drought stress		After drought stress	
Organ	Non-primed	DS-primed	Non-primed	DS-primed
Stem	18.22 ± 0.79 a	13.68 ± 1.17 b	20.52 ± 2.25 a	23.69 ± 2.96 a
Branches	6.58 ± 0.45 a	4.58 ± 0.45 b	6.31 ± 0.71 a	10.86 ± 1.67 b
Leaves	19.14 ± 1.18 a	14.27 ± 1.12 b	17.22 ± 2.19 a	12.72 ± 1.78 a
Roots	7.58 ± 1.08 a	7.37 ± 0.61 a	8.24 ± 0.97 a	9.67 ± 0.86 a
Total DM	51.52 ± 2.69 a	39.83 ± 2.74 b	52.30 ± 5.37 a	56.93 ± 4.15 a
	Biomass allocation ($g.g^{-1}$) after winter			
	Before drought stress		After drought stress	
Variable	Non-primed	DS-primed	Non-primed	DS-primed
SMF	0.35 ± 0.01 a	0.34 ± 0.01 a	0.39 ± 0.01 a	0.40 ± 0.03 a
BMF	0.13 ± 0.01 a	0.11 ± 0.01 a	0.12 ± 0.01 a	0.18 ± 0.02 b
LMF	0.37 ± 0.01 a	0.36 ± 0.01 a	0.32 ± 0.01 a	0.24 ± 0.04 a
RMF	0.15 ± 0.02 a	0.19 ± 0.02 a	0.16 ± 0.01 a	0.17 ± 0.01 a
S/R ratio	6.31 ± 1.00 a	4.55 ± 0.50 a	7.27 ± 0.81 a	5.50 ± 0.69 a

Biomass is expressed as the dry mass (g) of each plant organ measured. For details on biomass allocation calculations, see the Materials and Methods section. Statistical differences between non- and DS-primed plants,for each organ or variable, are indicated by different letters ($p \leq 0.05$). Unpaired t-tests were used, and values are presented as mean ± standard error (SE)

DM dry mass, *DS* drought stress, *SMF* stem mass fraction, *BMF* branches mass fraction, *LMF* leaf mass fraction, *RMF* root mass fraction, *S/R* ratio shoot to root ratio

observed (Fig. S2B). Additionally, there was no difference in the ratio of fresh weight from the ratio stem: roots (Fig. S2C).

To investigate the long-term impact of recurrent drought stress, in the year following the first drought stress cycles (year $n+1$), after the plants had completed one winter cycle and resumed vegetative growth, we continued phenotyping plants from the Control and Multiple-DS groups. Morphological traits and biomass were measured before and after a new drought stress cycle.

Prior to drought stress, analysis showed that Multiple-DS primed plants in the spring of year $n+1$ were shorter in height compared to Control plants (108.5 ± 3.7 vs. 135.2 ± 3.6 cm on average, respectively; Table S3). No difference was observed in the number of nodes or stem diameter. However, there was a tendency for a higher ratio of viable to non-viable buds in Multiple-DS primed plants (Table S3). DS-primed plants also exhibited significantly lower dry mass in stems, branches, and leaves, resulting in reduced total biomass compared with non-primed plants, whereas root biomass did not differ between treatments. Moreover, no difference in biomass allocation preferences was observed between Non- and DS-primed apple seedlings (Table 1).

Interestingly, the evaluation of the biomass of DS-primed plants after a new three-weeks drought stress cycle revealed no significant difference in stem, leaf, or root biomass, indicating that growth was less affected by water deficit than during the initial stress exposure (Table 1). Notably, DS-primed plants allocated significantly more biomass to branches.

Drought-primed plants display incresead leaf thickness even under well water conditons

To further study the effects of recurrent drought stress on apple seedlings, we investigated leaf thickness evolution. Overall, the measurements indicated that leaf thickness after one cycle of water deficit (t_1) in the Multiple-DS plant group (exposed to a single drought stress cycle) was significantly higher than that observed in the Control group (221.8 ± 2.7 µm and 189.6 ± 2.6 µm, respectively; Table 2). The increase was driven by the mesophyll via the spongy parenchyma layer and leaf epidermis (Table 2; Fig. S3A and B). As expected, these results indicate that plants adjust their leaf anatomy by increasing mesophyll thickness in leaves developed during the stress period, helping to enhance water-use efficiency.

After recurrent water deficit cycles (t_5), we observed a thicker leaf blade for Multiple-DS (230.9 ± 4.8 µm) and Unique-DS (230.0 ± 4.5 µm) groups compared to Control

Table 2 Epidermis and mesophyll layers thickness of apple leaves under well-watered and drought conditions measured after the first (t1) and third (t5) water stress cycles

	Leaf blade (µm)		**EP (µm)**		**Meso (µm)**	
	t_1	**t_5**	**t_1**	**t_5**	**t_1**	**t_5**
Control	189.6 ± 2.6 a	207.7 ± 2.6 c	25.94 ± 0.7 a	28.0 ± 0.6 a	164.3 ± 2.3 a	180.5 ± 2.6 b
Multiple-DS	221.8 ± 2.7 b	230.9 ± 4.8 b	32.9 ± 0.8 b	27.8 ± 0.7 a	188.9 ± 2.3 b,c	203.7 ± 4.3 c
Unique-DS	-	230.0 ± 4.5 b	-	26.6 ± 1.0 a	-	197.4 ± 4.5 c
	PP (µm)		**SP (µm)**			
	t_1	**t_5**	**t_1**	**t_5**		
Control	43.2 ± 0.9 a	43.5 ± 0.9 a	121.1 ± 2.0 a	137.0 ± 2.0 b		
Multiple-DS	47.5 ± 0.8 a	61.3 ± 1.9 b	141.4 ± 2.1 b	142.4 ± 2.8 b,c		
Unique-DS	-	55.49 ±2.1 c	-	148.0 ± 3.0 c		

Significant differences are indicated by different letters accompanyingmean ± SE values, based on one-way ANOVA followed by Tukey's multiple comparisons test ($p \leq 0.05$; GraphPad Prism)

EP leaf epidermis, Meso leaf mesophyll, PP palisade parenchyma, SP spongy parenchyma

(207.7 ± 2.6 µm; Table 2; Fig. S3C–E), due to an increase of the mesophyll layer (Table 2).

Interestingly, Unique-DS plants which had been under well-watered conditions for the last 12 weeks following the DS1 presented a leaf thickness similar to that observed for Multiple-DS that were under recurrent water deficit (Table 2; Fig. S3D-E). Since the leaf thickness measures were performed on leaves that had been formed after DS1, these results indicate that plants from the Unique-DS group maintained a thicker leaf blade anatomy since the end of DS1, suggesting that the "drought-stress" memory, or priming, may have been retained in these plants throughout the growing season.

To investigate the long-term effects of recurrent drought stress, we measured leaf thickness of Control and Multiple-DS plants in the following year (year $n+1$; Fig. S1). Prior to the application of a new drought cycle, leaves from both groups showed comparable thickness (155.1 ± 1.6 µm in Control plants and 151.8 ± 2.0 µm in Multiple-DS plants) with no significant differences observed in tissue layers (Fig. 3).

After a new drought stress cycle was applied, a significant difference was identified between the Control and Multiple-DS plants. The average leaf thickness of leaf blade from Control plants was measured at 189.3 ± 1.9 µm, while the average leaf thickness of Multiple-DS plants was measured at 182.3 ± 2.0 µm. This difference was associated with changes in both the epidermis and mesophyll layers, including the palisade and spongy parenchyma (Fig. 3). Altogether, these data indicate that during winter, the information captured by plants submitted to recurrent drought stress may have been partially lost since leaves thickness was similar between the two groups before the stress. However, the ability of plants submitted to drought stress to increase their leaf thickness was maintained in both Control and Multiple-DS plants. The lower increase in leaf thickness observed in Multiple-DS plants compared to Control plants after drought-stress application suggests that Multiple-DS plants may have developed alternative mechanisms to cope with that stress.

Molecular signatures underlying physiological responses to water deficit in apple seedlings

Gene expression changes in leaves were analyzed after a single cycle of drought stress (DS1) to validate the physiological impact of water deficit on apple seedlings. This analysis aimed to determine whether the imposed drought treatment elicited a transcriptional response consistent with drought stress perception and signaling. Differential gene expression analyses were therefore performed on samples collected after DS1. In leaf tissues, a total of 3,202 differentially expressed genes (DEGs) were identified, of which 1,505 were upregulated and 1,697 were downregulated (Fig. 4A).

Several well-characterized regulators of dehydration and water-deficit stress were identified among the DEGs. The gene MD17G1089700 is annotated as a dehydration-responsive element-binding protein 2 A, homologous to *Arabidopsis thaliana* AT2G40340.1, while MD12G1067600 corresponds to dehydration-responsive element-binding protein 2 F (AT3G57600.1). Both genes encode transcription factors involved in drought-responsive gene regulation. Additional genes associated with early dehydration responses included MD10G1325800, homologous to AT4G22120.1 and annotated as an early-responsive to dehydration stress (ERD) family protein, as well as MD06G1023400 and MD08G1076400, homologous to AT1G32090.1 and AT1G10090.1 respectively, both annotated as early responsive to dehydration stress protein ERD4. The drought-responsive gene set also included MD02G1011700, homologous to AT1G80710.1 and annotated as DROUGHT SENSITIVE 1, further supporting the involvement of conserved molecular

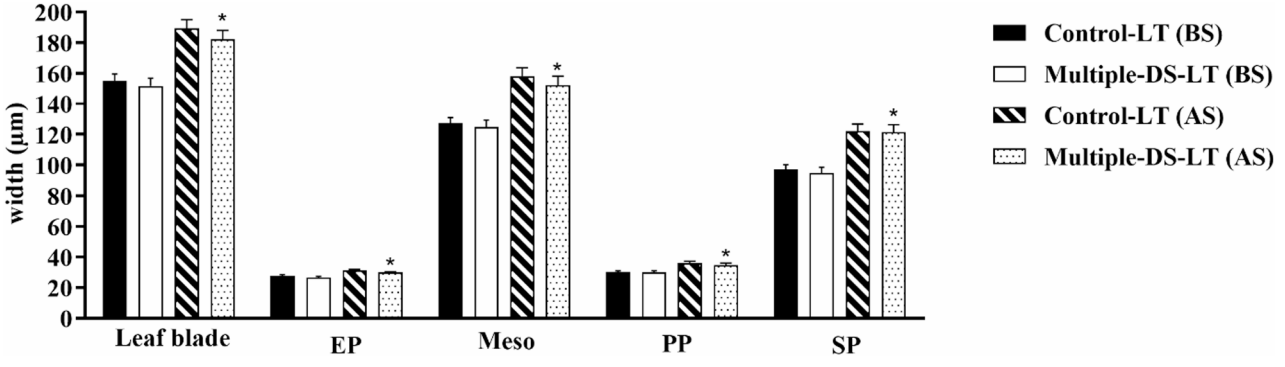

Fig. 3 Anatomical measurements of Control and Multiple-DS plants in the growing season following drought stress cycles (LT). Leaf blades were sectioned using a Cryostat® (Leica Biosystems, Germany), and 60 µm transverse sections were analyzed under optical microscopy. Anatomical structures assessed included the upper and lower epidermis (EP), as well as the spongy (SP) and palisade (PP) parenchyma of the leaf mesophyll (Meso). Measurements were taken before (BS) and after (AS) a new cycle of drought stress of year + n. Statistical comparisons between treatments (Control vs. Multiple-DS) under the same time-point conditions (BS or AS) were performed using t-tests (p-value ≤ 0.05)

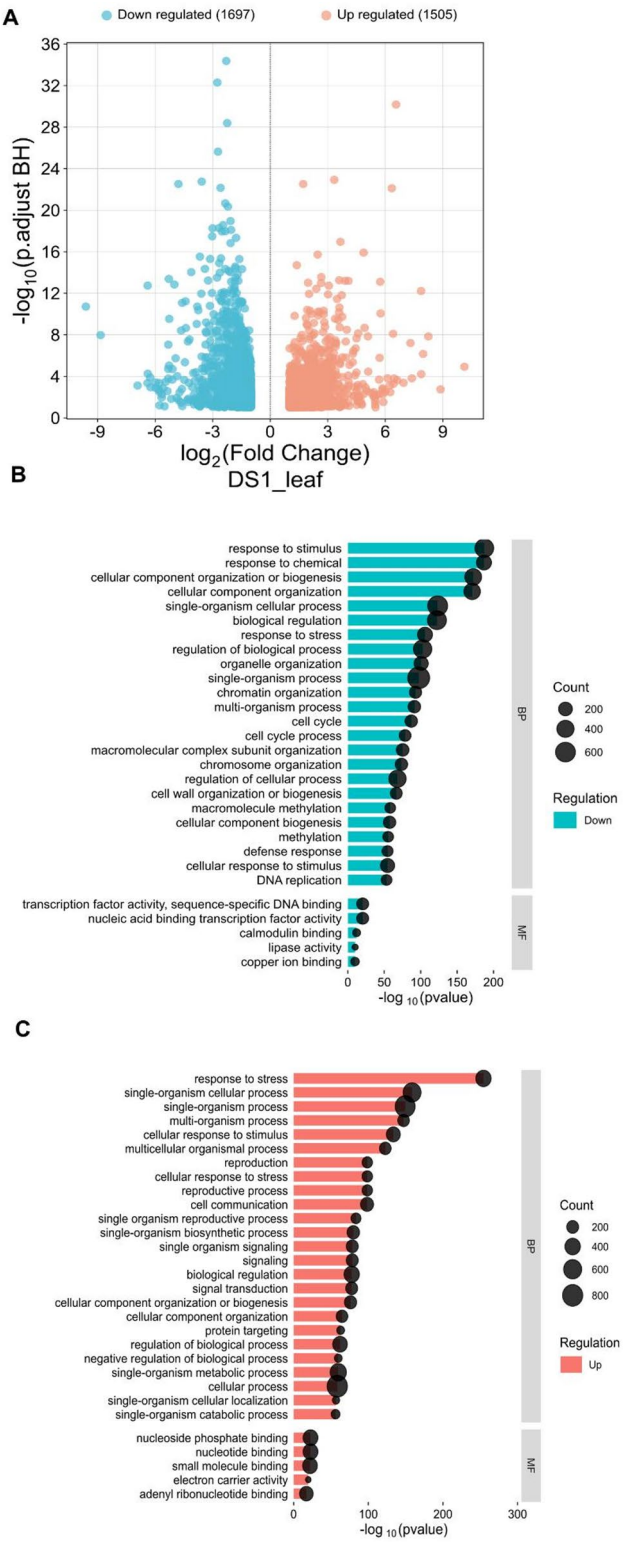

Fig. 4 Gene expression of apple trees under DS1 in leaves. **A** Volcano plot showing the DEGs found in leaves. Gene ontology (GO) enrichment of the DEGs found in RNAseq analysis for down (**B**) and up (**C**) DEGs. The search was performed with agriGO v2.0 tool for both ontology terms: biological process (BP) and molecular function (MF), using *Malus x domestica* GO terms as background. The circles represent the number of DEGs associated with each respective BP or MF. The x-axis shows the $-\log_{10}$ of the adjusted *p*-value used in the analysis

pathways underlying drought perception and response. Moreover, several upregulated genes were homologous to *A. thaliana* genes known to be involved in drought tolerance and dehydration responses. These included MD15G1374300, homologous to BETA GLUCOSIDASE 15 (BGLU15; AT2G44450), which is involved in drought tolerance [29], and MD00G1127700, homologous to DROUGHT-INDUCED 21 (DI21; AT4G15910), a gene responsive to drought stress [30].

To further characterize the biological processes affected by drought stress after DS1, Gene Ontology (GO) enrichment analyses were performed separately for downregulated (Fig. 4B) and upregulated (Fig. 4C) genes in leaf tissue.

GO enrichment analysis of downregulated genes revealed a strong overrepresentation of terms related to cellular organization and growth-associated processes (Fig. 4B). Enriched biological processes included cellular component organization, cellular component biogenesis, chromosome organization, cell cycle, and DNA replication, indicating a general repression of cell division and structural maintenance under drought conditions. In addition, terms associated with response to stimulus and response to chemical were also significantly enriched among downregulated genes, suggesting an attenuation of certain stimulus-responsive pathways. Molecular function categories were dominated by nucleic acid binding, transcription factor activity, and DNA-binding functions, further supporting a drought-induced suppression of transcriptional and cell-cycle–related regulatory activities.

In contrast, GO enrichment analysis of upregulated genes showed a clear enrichment of stress- and signaling-related biological processes (Fig. 4C). Highly represented terms included response to stress, cellular response to stimulus, signal transduction, and biological regulation, reflecting activation of drought-responsive signaling networks. Additional enriched categories such as protein targeting, cell communication, and regulation of biological processes indicate coordinated cellular adjustments to

water deficit. At the molecular function level, enriched terms included nucleotide binding, nucleoside phosphate binding, and small molecule binding, consistent with enhanced signaling and metabolic regulation during drought stress. Altogether, these results indicate that our drought-inducing protocol was perceived by the apple seedlings as a stress and induced a water deficit related transcriptional response in leaves.

The number of DMR increases with the recurrence of drought stress in apple leaves

To assess the potential impact of recurrent water deficit on DNA methylation in apple leaves and roots, we performed WGBS. The sequencing achieved an average depth of 20x–30x across the ~700 Mb apple genome. In total, when comparing Control and drought-stress leaves after DS1, we identified 340 DMRs, including 39 DMRs in the CG context, 8 DMRs in the CHG context and 293 DMRs in the CHH context. The total number of DMRs identified between the Control and Multiple-DS groups after DS3 increased to 3005 DMRs, including 2170 in the CG context, 669 in the CHG context, and 166 in the CHH context (Table 3). In leaves, DS3 exhibited a relatively balanced pattern of hypo- (1026) and hypermethylation (1144) in the CG context, while CHG changes were also distributed between hypo- (358) and hypermethylation (311); in contrast, CHH sites were predominantly hypomethylated (144 vs. 22 hyper).

Whether for the DS1 or DS3 analyses, about 50% of DMRs in all three contexts were localized close to putative coding genes (Fig. 5). Of the 340 DMRs identified after DS1, 180 were localized within 2 kb of coding genes. Similarly, after DS3, of the 3005 DMRs, 1461 were localized within 2 kb of coding genes.

Two DMRs in the CG context were identified in common between DS1 and DS3 leaves (Table 3). Both DMRs are located within the coding region of MD15G1070300, annotated as a galactose oxidase. The increase in the total number of DMRs identified between the Control and Multiple-DS groups across successive drought stress periods, together with the observation that only two DMRs were shared after recurrent stress, indicates that the plant methylome dynamically evolves in response to stress perception.

To assess the long-term impact of recurrent drought stress, a DMR analysis was performed on leaf tissues collected from Control and Multiple-DS plants in the following year (year $n+1$; Fig. S1). This group is hereafter referred as LTM. This analysis yielded a total of 2187 DMRs between the two groups of plants, including 1380 DMRs in the CG context, 510 in the CHG context, and 297 in the CHH context (Table 3). LTM in leaves showed a predominance of hypermethylation in CG (877 vs. 503 hypo), whereas CHH DMRs were mainly hypomethylated (248 vs. 49 hyper), indicating context-specific methylation shifts (Table 3). Of these, 19 DMRs were in common with those identified after DS3 and included 15 DMRs in the CG context and 4 DMRs in the CHG context (Table 3; Table S4).

Of these 19 overlapping DMRs, 16 were located within 2 kb of annotated coding genes. In the CG context, the common DMRs were associated with genes encoding proteins involved in stress response and metabolism, including Leucine-rich repeat (LRR) family proteins (MD07G1006700 MD17G1130800 homologous to AT3G12610 and AT3G17640, respectively), 3′-5′-exoribonuclease family protein (MD17G1115700 – homologous to AT3G07750), and a P-loop containing nucleoside triphosphate hydrolase (MD14G1096800 - homologous to AT3G53110; Table S4). Additionally, DMRs were found in the ATP-binding cassette subfamily B19 gene (MD09G1177100 - homologous to AT3G28860), which showed consistent hypermethylation in both LTM and DS3 plants. Overlaps were also detected in the plastidic glucose translocator (MD09G1254500 – homologous to AT5G16150) and the gamma subunit of

Table 3 DMR overview

Tissue	Group	DMR context								
		CG			CHG			CHH		
		Total	Hypo	Hyper	Total	Hypo	Hyper	Total	Hypo	Hyper
Leaf	DS1	39	27	12	8	2	6	293	275	18
	DS3	2170	1026	1144	669	358	311	166	144	22
	LTM	1380	503	877	510	250	260	297	248	49
	DS3 vs. DS1	2	na		0	na		zero	na	
	LTM vs. DS3	15	na		4	na		zero	na	
Roots	DS1	zero	na		zero	na		zero	na	
	DS3	zero	na		1	1	0	4	3	1
	LTM	50	26	24	9	5	4	243	164	79
	DS3 vs. DS1	zero	na		zero	na		zero	na	
	LTM vs. DS3	zero	na		zero	na		zero	na	

T treatment, *C* common, *DS* Drought stress (DS1 or DS3 treatment), *LTM* Long-term memory (year n+) treatment, *na* not applicable

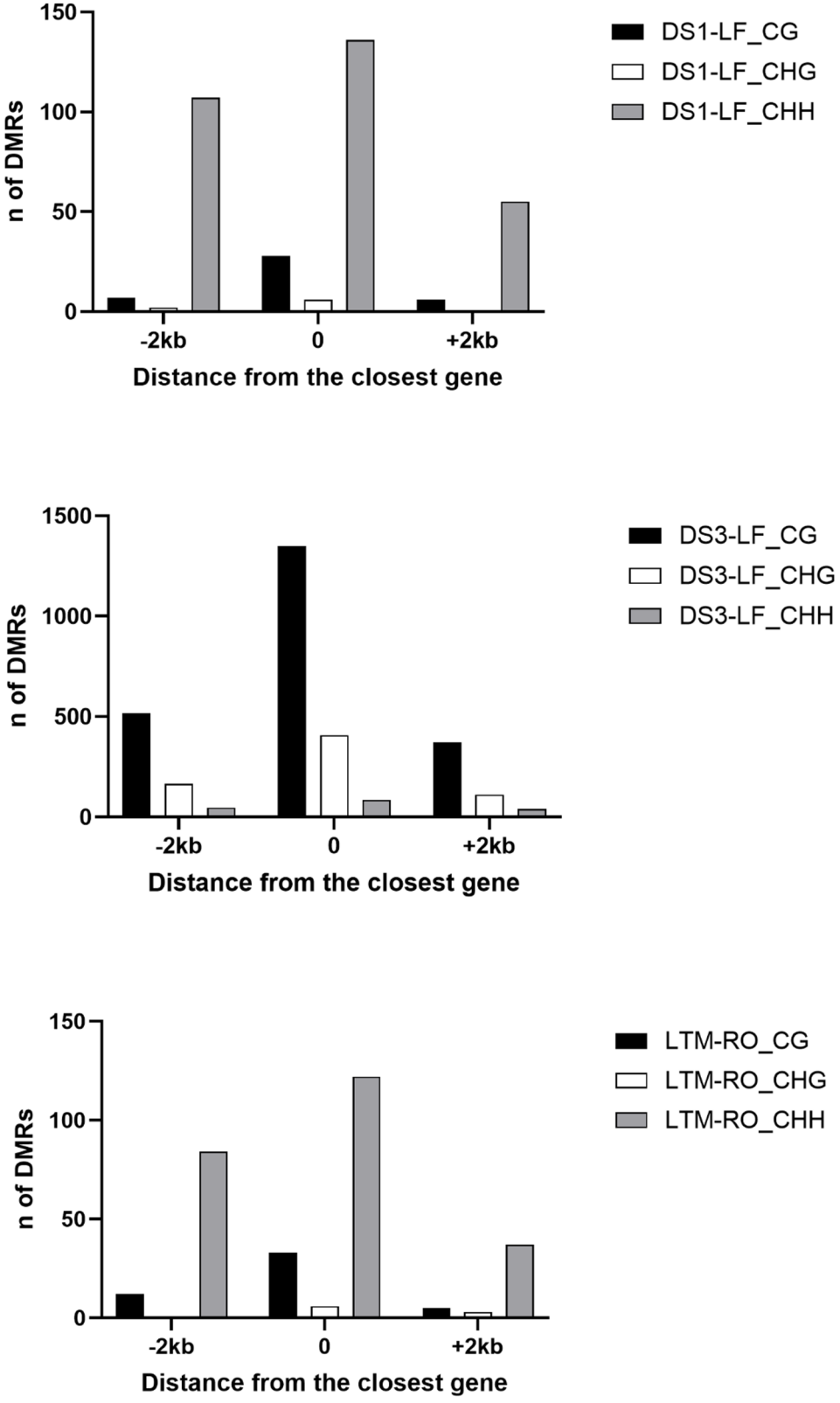

Fig. 5 Distribution of DMRs relative to the nearest gene in apple seedlings under DS1, DS3 and LTM. RO: roots, LF: leaf; n: number; LTM: Long-term memory (year n+) treatment. DS: Drought stress (DS1 or DS3 treatment)

mitochondrial ATP synthase (MD06G1202600 – homologous to AT2G33040).

In the CHG context, common DMRs were identified in genes encoding tetratricopeptide repeat (TPR)-like superfamily proteins (MD10G1008400, MD17G1024500) and aspartic proteinase A1 (MD17G1098900). These loci consistently displayed hypomethylation across both LTM and DS3 plants (Table S4).

Recurrent drought stress has limited impact on the root system methylome

To investigate the effects of drought stress on apple seedling roots, WGBS was also performed after the first (DS1) and third (DS3) stress cycles. No DMRs were detected following DS1, indicating an absence of early methylation changes in roots. After DS3, only five DMRs were identified, one in the CHG context and four in the CHH context (Table 3), demonstrating a very limited epigenetic response even after repeated stress. Of these, just one DMR was located within 2 kb of a coding gene (MD02G1068200; unknown protein).

In contrast, LTM plants exhibited 302 DMRs in roots, comprising 50 in the CG context, 9 in CHG, and 243 in CHH (Table 3). In the CHH context, both hypo- (164) and hypermethylated (79) regions were observed, with a moderate predominance of hypomethylation. Most LTM-associated DMRs were located within coding regions (Fig. 5). Notably, no overlap was found between DMRs detected in LTM and those identified after DS3.

Together, these results indicate that, unlike leaves, the root methylome shows minimal and largely non-overlapping changes in response to recurrent drought stress, suggesting a distinct, tissue-specific epigenetic response.

Discussion

While the impact of drought stress on apple tree growth and fruit quality has been the subject of numerous investigations [31–34], the impact of recurrent drought stress on apple seedling growth has rarely been investigated. In our study, plants submitted to recurrent drought stress followed by re-watering were found to be about 30% smaller than Control plants. Surprisingly, plants submitted to a unique drought stress early in their development ended up 11% taller than Control plants, driven by longer internodes length, indicating a potential growth compensation mechanism in the seedlings. Investigating this mechanism in seedlings with different genetic backgrounds or in scion varieties may prove useful for breeders and tree nurseries to increase young plants' growth.

Our data also indicated that plants' responses to drought stress evolved with the recurrence of that stress. We showed that plants confronted with 2 or 3 consecutive water deprivation cycles responded faster in terms of growth speed reduction during the first days of stress. This faster response early in the stress period was mirrored by a more dynamic growth recovery during the re-watering period, suggesting a potential evolution in the adaptation of seedlings to recurrent stress, and possibly a priming effect.

Previous studies have shown that overcompensation and increased growth rates can occur in several species following drought events [35]. This recovery response may be associated with the accumulation of non-structural carbohydrates during drought, enabling rapid carbohydrate availability to support growth upon rewatering. Consistent with this hypothesis, our results identified genes upregulated in DS1 that are involved in starch synthesis and carbohydrate metabolism. These include MD07G1169000, homologous to *AT4G30310.2* and associated with the FGGY family of carbohydrate kinases; MD10G1321600 and MD10G1321800, both homologous to *AT1G11720.2* and encoding soluble starch synthase 3; and MD06G1122100, homologous to *AT1G32900.1*, which encodes granule-bound starch synthase 1.

Additionally, leaf thickness was used in this study as an indicator of drought stress, as it has been shown to decrease during dehydration and increase upon rehydration [36–39]. In our experiment, leaves that developed after a drought stress event were significantly thicker than those formed prior to the stress. In apple, leaf thickness has previously been associated with enhanced drought tolerance [40]. In that study, among the eight rootstock varieties evaluated, the most drought-tolerant genotype exhibited the greatest leaf thickness. Mechanistically, increased leaf thickness may limit water loss by reducing evaporation, as thicker epidermal and palisade tissues can effectively decrease transpiration rates. However, the differences in leaf thickness reported by Sun et al. [40] were genotype-dependent rather than stress-induced. Similarly, studies conducted on sugarcane showed that, under arid conditions, lamina thickness increased in drought-tolerant genotypes [41]. In contrast to these genotype-based differences, our results indicate that drought stress itself induced an increase in leaf thickness, which was maintained in newly formed leaves for several weeks. This sustained response suggests a potential priming effect triggered by the initial drought event, which may partially explain the improved adaptation of apple seedlings to subsequent stress. However, this priming effect was not persistent across seasons. In the following growing season, previously stressed and control plants exhibited comparable leaf thickness and similar physiological responses to renewed drought, indicating that overwintering likely reset the plants to a naïve state.

Although no persistent changes in leaf thickness were observed under long-term stress memory, the increased

biomass accumulation in the branches of primed plants (detected only after a renewed period of water deficit in year $n+1$) suggests that previous drought exposure may confer a growth advantage during subsequent stress. This response likely reflects the establishment of a physiological memory that is not fully reset during overwintering. Shoot branching is strongly regulated by phytohormones, particularly cytokinins and strigolactones [42]. Therefore, further studies investigating the dynamics of these hormones under recurrent drought conditions could provide valuable insights into the mechanisms underlying stress priming and adaptive growth responses. In contrast to the responses observed in leaves, root morphological traits appeared to be less affected by drought stress than the aerial system or required longer stress periods to exhibit significant phenotypic changes. Alternatively, the indicators we used in this study may not have been sensitive enough to capture the effects of stress on roots.

Besides morphological changes, our transcriptomic analysis revealed a broad set of differentially regulated genes in response to DS1, with an overrepresentation of gene categories associated with cellular processes, cellular component organization or biogenesis, biological regulation, and cellular responses to stimuli. These patterns are consistent with the findings of Liu et al. [11], who reported similar functional enrichments in apple trees subjected to a single drought stress event. To further our understanding of the molecular modifications induced by recurrent stress, we surveyed the evolution of the methylome. Surprisingly, a limited number of DMRs were identified between the two groups after DS1. This result is in contrast with the study conducted by Xu et al. 2018 [13] on the apple cultivars 'Qinguan' and 'Honeycrisp', who identified up to 60,000 DMRs between the control and stress conditions for both cultivars. Importantly, our study differed from Xu and colleagues since their analysis was conducted on adult grafted trees, using different cultivars and drought stress conditions.

In contrast to DS1, the number of DMRs increased substantially after recurrent drought stress (DS3). Despite the absence of a large number of common DMRs between the two analyses (DS1 vs. DS3), the observed increase may reflect an enhanced priming effect. Finally, 2,187 DMRs were identified between Control and Multiple-DS groups in the following year (LTM), and 19 DMRs were found in common in leaf samples within DS3 plants. Overall, the common DMRs between LTM and DS3 were predominantly located in genes related to stress signaling (LRR, TPR-like proteins), energy metabolism (ATP synthase, glucose translocator), and transport (ABC transporters), suggesting potential involvement in long-term gene regulation associated with stress memory (Table S4).

While our results provide evidence for methylome remodeling associated with recurrent drought stress, some limitations should be considered. First, DNA methylation profiling was performed on bulk tissue, which integrates signals from multiple cell types and developmental stages. Consequently, cell-type-specific or spatially restricted methylation changes may be masked, potentially underestimating epigenetic heterogeneity associated with stress priming. This limitation has been previously demonstrated in studies analyzing multiple cell populations in plant tissues. For example, analysis of six major cell types in the Arabidopsis root meristem revealed widespread cell-type-specific DNA methylation patterns, particularly in the CHH sequence context [43]. In addition, the functional interpretation of DMRs is complicated by the context- and position-dependent nature of DNA methylation in plants. Cytosine methylation occurs in CG, CHG, and CHH sequence contexts, which are maintained by distinct molecular pathways and may exert different regulatory effects. Generally, CG methylation is frequently found in gene bodies and promoter regions, whereas non-CG methylation is more commonly enriched in transposable elements (reviewed by Zhang et al. [44]).

Moreover, the regulatory impact of methylation strongly depends on its genomic location. Promoter methylation is commonly associated with transcriptional repression, although exceptions exist. For instance, promoter methylation can also promote transcription in specific genes, such as the ROS1 gene in A. thaliana and several genes involved in fruit ripening regulation in tomato (reviewed by Zhang et al. [44]). In contrast, gene body methylation is often associated with constitutively expressed genes and may contribute to transcriptional stability, while methylation of transposable elements contributes primarily to genome integrity, transposon silencing, and regulation of nearby genes. Consequently, the presence of DMRs near to annotated genes does not necessarily imply predictable transcriptional outcomes. Future studies integrating cell-type-resolved epigenomics, chromatin accessibility profiling, and functional validation approaches will help to clarify the precise contribution of methylation dynamics to drought stress memory in apple seedlings.

The primary objective of this study was to determine whether apple seedlings can be primed against drought stress. Our results clearly indicate that prior drought exposure enhances the response of seedlings to subsequent stress, supporting the existence of a priming effect in this perennial species. We further show that this physiological priming is accompanied by measurable remodeling of the DNA methylation landscape, as evidenced by the accumulation of DMRs in primed plants compared

to non-primed controls. Notably, approximately 50% of these DMRs are located within 2 kb of annotated coding genes, suggesting preferential methylation changes in gene-proximal regions with potential regulatory relevance.

Although we did not detect a strong genome-wide correlation between DMRs and differential gene expression, accumulating evidence indicates that the relationship between stress-induced methylation and transcription is highly context-dependent. While locus-specific associations have been reported in some systems [45] more recent syntheses highlight that many stress-associated methylation changes do not systematically translate into detectable transcriptional variation and some causal relationships remain difficult to establish [46]. Therefore, the absence of a global correlation in our dataset does not preclude biologically meaningful effects. Instead, it suggests that methylation remodeling associated with priming may operate through locus-specific regulation, chromatin configuration changes, stress memory mechanisms, or genome stabilization processes rather than immediate steady-state transcriptional modulation. Together, our findings support the hypothesis that drought priming in apple involves targeted epigenomic adjustments, while underscoring that their precise functional contribution to transcriptional regulation requires further experimental validation.

Conclusion

The observed differences in plants physiological response and DMRs in leaves between DS1 and DS3 may result from a priming effect. Priming is defined as the capacity of plants to memorize environmental stress experience which may improve their response to recurring stress [2]. Here, we showed that a naïve seedling may be primed upon first exposure to a stress. Lämke and Bäurle [47] proposed that primed plants may respond in a sensitized fashion so that the response is triggered at a lower threshold, and that primed plants may modify their response pattern and regulate a network of genes that differs from that involved in a naïve plant. Our analyses of DMRs in leaves indicate that apple seedlings may respond in such a way to recurrent drought stress, which may explain the observed increase in the number of DMRs between the time points and the relative lack of correspondence between the methylome analyses. Finally, our analysis of plants' phenotype and methylome indicated that following a winter period, previously primed-plants respond to a stress similarly to naïve plants, except for the measure of lateral branch biomass production during the stress period, indicating that priming may have been partially lost during the winter period.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-026-08631-6.

Supplementary Material 1: Fig. S1. Schematic representation of the experimental design of the recurrent drought cycles. Fig. S2. Shoot and root responses of GDDH18 plants under Multiple and Unique drought stress cycles. Fig. S3. Leaf anatomy of apple seedlings subjected to unique and multiple drought cycles.

Supplementary Material 2: Table S1. The min-max temperature and humidity registered throughout the weeks in the experimental greenhouse. Table S2. Leaf thickness measurements during the DS1, DS3, and LTM periods. Table S3. Morphological traits measures of Control (non-drought-primed) and Multiple-DS (drought-primed) plants the following year (n+1) before and after one cycle of drought stress. Table S4. Common DMRs found between LTM and DS3 treatments in leaves.

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Authors' contributions

AMC performed the experiments, analyzed the data, and interpreted the results. JMC conceived the study and interpreted the results. PM conducted leaf thickness analyses and plant growth monitoring. SH monitored apple seedling growth in the greenhouse. BB and MC contributed to nucleic acid extraction and quality assessment. ALF edited the manuscript. SK and SB contributed to RNA-seq and BS-seq analyses. AMC, JMC, and SB wrote the original draft. All authors contributed to reviewing and editing the manuscript.

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Data availability

The RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE306229. The BS-seq data are available in the same repository under accession number GSE307875.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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