Biotechnology Research and Innovation (2020) 3, 42–58

Biotechnology Research & Innovation

http://www.journals.elsevier.com/biotechnology-research-and-innovation/

RESEARCH PAPER

Evaluation of *Setaria viridis* physiological and gene expression responses to distinct water-deficit conditions

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Received 31 October 2019; accepted 23 March 2020
Available online 11 April 2020

KEYWORDS
Green foxtail;
Drought;
Water-deficit stress

Abstract *Setaria viridis*, a C_4 monocot, was proposed as a model plant for studies on the response to stress conditions. Water-deficit ranks among the top three most devastating stresses and its importance will likely increase in the scenario of climate change. The aim of this work was to evaluate physiological and molecular water-deficit responses of *S. viridis* subjected to different conditions. Principal component analysis highlighted the physiological differences between vegetative and reproductive stages of *S. viridis*, as well as the differences between two methods of water-deficit induction: polyethylene glycol and air-drying. Network interactions were observed in distinct developmental stages and water-deficit induction methods tested, allowing classification of root and shoot fresh weight and non-photochemical quenching as the best physiological parameters to group the networks. Variations in the gene expression patterns of delta 1-pyrroline-5-carboxylate synthase 2 (*SvP5CS2*), Dehydrin 1 (*SvDHN1*) and the transcription factors WRKY DNA-binding domain 1 (*SvWRKY1*), dehydration-responsive element-binding protein 1 class C (*SvDREB1C*) and NAC protein 6 (*SvNAC6*) were observed. Among these genes, it was observed two expression patterns predominant during water-deficit: inducible (*SvDHN1* and *SvNAC6*) and repressed (*SvP5CS2*, *SvWRKY1* and *SvDREB1C*) genes. *SvDHN1* showed the highest expression level in all the conditions tested. PEG treatment during the reproductive stage promoted the upregulation of the five marker genes in roots. The discriminative analysis suggested that the physiological and molecular responses in *S. viridis* adjusted according to the evaluated water-deficit conditions, especially after PEG treatments, indicating that the PEG method of water-deficit may better replicate field conditions.

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https://doi.org/10.1016/j.biori.2020.03.001

Introduction

Monocot C_4 crops such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*), pearl millet (*Pennisetum glaucum*), switchgrass (*Panicum virgatum*) and others are major sources of food, feedstock and biofuel (Ehleringer, Cerling, & Helliker, 1997). The majority of C_4 plants are well adapted to high light intensities, elevated temperatures and relatively poor soils, which makes them targets of intense research (Ehleringer et al., 1997; Sage & Zhu, 2011). However, studies with most of C_4 crops are difficult, because of their size, large and/or polyploid genome and long life span (Saha & Blumwald, 2016). In 2010, the monocot C_4 plant *Setaria viridis*, popularly known as green foxtail was proposed as a model plant for C_4 metabolism studies (Brutnell et al., 2010). The small size (10–15 cm), short life cycle (6–9 weeks), high seed production (~13,000 per plant) and relatively small genome (approximately 515 Mb) make *S. viridis* an ideal model for research of C4 species, including physiological and molecular responses to water-deficit (Martins et al., 2016; Saha et al., 2016).

Stress conditions are a direct cause of disturbance of essential physiological functions and photosynthetic processes, causing increasing of oxidative stress and declining in water content, which affect the plant development (Zivcak et al., 2013). Water-deficit can reduce substantially the growth and modify root architecture, compromising development and functionality of the aerial parts (Novák & Lipiec, 2012). Root length, along with a balanced exposed leaf area ratio are directly associated to soil water content and nutrient acquisition (Diaz-Espejo et al., 2012).

In *S. viridis*, water-deficit conditions lead to considerable reduction of the length and volume of the main root system and the growth inhibition of crown roots (Sebastian et al., 2016). In addition, a decrease in biomass dry weight was also observed in this species (Saha et al., 2016; Sebastian et al., 2016). Studies performed with different accessions of *S. viridis* demonstrated the physiological response patterns during water-deficit. In general, maximum and minimum rates of photosynthesis, transpiration and stomatal conductance are significantly reduced in comparison to well-watered groups (Saha et al., 2016). In the comparison of different accessions, Zha-1, A10.1 and Ula-1 presented significantly higher leaf water potential, photosynthesis, transpiration and stomatal conductance rates, compared to Ast-1, Aba-1 and Sha-1 when grown under water-deficit conditions. Together, physiological, biochemical and gene expression analysis showed that A10.1 is a tolerant accession in response to water-deficit and heat stresses (Saha et al., 2016).

Plant responses to water-deficit stress depend on the selective activation of molecular mechanisms, specifically, those that alter the expression levels of particular genes that may lead to tolerance (Qi, Xie, Liu, Yi, & Yu, 2013). During water-deficit, transcription factors (TFs) participate in the gene network regulation through the interaction of their DNA-binding domain with different gene promoters, mediated by abscisic acid (ABA) dependent or independent signal transduction pathways (Yang, Sornaraj, Borisjuk, Kovalchuk, & Haefele, 2016). TF families, such as dehydration-responsive element-binding protein (DREB), NAC (from NAM, ATF1 and -2 and CUC2 TFs) and WRKY (WRKY binding domain) play an important role in abiotic stress responses in crops such as *Oryza sativa*, *Triticum aestivum* and *Z. mays*, which are important in agriculture (Banerjee & Roychoudhury, 2015; Liu et al., 2014; Mao, Chen, Li, Zhai, & Jing, 2014; Nakashima, Yamaguchi-Shinozaki, & Shinozaki, 2014; Qiao, Li, & Zhang, 2016; Shan et al., 2013). In addition, targeted genes of these TFs, such as dehydrin (DHN) and P5CS2 (delta 1-pyrroline-5-carboxylate synthase 2 enzyme, responsible for proline biosynthesis) are involved in osmotic regulation, being important mechanisms of water-deficit response (Fang & Xiong, 2015). Together, TFs and target genes can be important drought markers as shown in recent reports that evaluated the water-deficit response (Amini, Ghobadi, & Yamchi, 2015; Graether & Boddington, 2014; Singh & Laxmi, 2015).

In general, monocots are more resistant to desiccation in comparison to dicots (Alpert, 2000), therefore the development of water-deficit protocols for the study of molecular responses should be carefully evaluated. For this, we employed a combination of physiological and molecular measurements of *S. viridis* in shoots and roots during water-deficit triggered by two different methods: acute water deprivation and polyethylene glycol (PEG) induced water-deficit. Both methods are widely used to study gene expression response to water-deficit and are indicated for when the target tissue is the roots, since it can be rapidly and easily collected (Bencke-Malato et al., 2019; Conforte, Guimarães-dias, Neves-borges, Felix-whipps, & Alves-Ferreira, 2017; Guimarães-Dias et al., 2016; Li et al., 2016; Minh-Thu, Hwang, Jeon, Nahm, & Kim, 2013; Trachsel, Stamp, & Hund, 2010; Yoo et al., 2014). Physiological parameters, such as biomass fresh weight, electrolyte leakage and chlorophyll fluorescence were evaluated in combination with gene expression analysis of the transcription factors (*SvWRKY1*, *SvDREB1* and *SvNAC6*) and osmorregulators (*SvDHN1* and *SvP5CS2*), which are candidate gene markers for water-deficit.

Materials and methods

Seed germination and plant growth conditions

S. viridis seeds (A10.1 accession) were germinated and grown in a plant chamber with a photoperiod of 16/8 h (light/dark cycle). Temperature and humidity were controlled and monitored by a HOBO (UX100 Temperature Relative Humidity Data Logger) and maintained during the day at $31 \pm 2\,^{\circ}C$/40–60% and at night at $22 \pm 2\,^{\circ}C$/60–80%. Light intensity and vapor pressure deficit were maintained at 900 $\mu mol\, m^{-2}\, s^{-1}$ and 2.42 kPa, respectively.

The dormancy break of seeds was performed with gibberellic acid (GA_3) and potassium nitrate (KNO_3). GA_3 (Sigma) was dissolved in 95% ethanol to obtain a stock solution at 200 mM, then stored at $-20\,^{\circ}C$. The working solution concentration (2.89 mM) was achieved by dissolving GA_3 in distilled water. Separately, KNO_3 stock solution at 500 mM was dissolved in distilled water to a 30 mM concentration. GA_3 and KNO_3 working solutions were mixed to a 1:1 proportion. Seeds were separated in sterilized 2 mL Eppendorf

tubes (about 50 seeds per tube). 1.5 mL of GA_3/KNO_3 solution was added to each tube. After 24 h, seeds were washed three times with distilled water and placed on paper filter for germination (Sebastian, Wong, Tang, & Dinneny, 2014). The cotyledon disruption and leaf primordium emergence were observed within 5–6 days. The average germination rate was 78.5%.

Seedlings were transferred to a hydroponic system containing 25% Hoagland's solution no. 2 (Caisson labs), with a concentration of 0.4075 g/L and pH 5.5–6.0, and 7.5 mg/L of Daconil (3.0 g/L) for fungal growth control. Hydroponic cultivation was chosen to maintain the nutritional homogeneity of plants and root integrity. Plants were grown for twenty days after germination (20 DAG; Fig. S1A), to vegetative stage when the third leaves were totally expanded, or until reaching reproductive stage (grain-filling period), at which point immature grains were observed (45 DAG; Fig. S1B). The hydroponic solution was changed weekly.

Water-deficit treatments

20 DAG and 45 DAG plants were air-dried (AD) for 25, 50, 150 and 250 min at 28 °C on paper towel layers, or had their roots submerged in 20% polyethylene glycol 8000 (PEG −0.55 MPa; Sigma-Aldrich) for 1, 3, 5 and 7 h, according to previous study performed with *Setaria italica* (Qi et al., 2013). AD is considered an acute water-deficit method because of the fast water loss (Guimarães-Dias et al., 2016; Minh-Thu et al., 2013), while PEG is a moderate water-deficit method for it promotes osmotic stress through slow water loss (O'Donnell et al., 2013). Shoots and roots were used in physiological analysis. For molecular analysis, roots and the third leaves (the first leaf that presents C_4 metabolism) of water-stressed and non-stressed (control groups) plants were harvested separately and placed immediately in liquid nitrogen. A pool of five roots/leaves composed one biological replicate. Triplicates for RNA extraction were stored in a freezer at -80 °C.

Physiological measurements

To measure biomass fresh weight (FW) and electrolyte leakage (EL), total root and shoot samples of plants 20 DAG and 45 DAG ($n = 10$) were collected after water-deficit stress. To access the level of water loss during stress treatments, the FW of plants was determined on an analytical balance (S1502 model, BEL Engineering). EL was measured according to the method of Liu et al. (2015). 0.4 g of tissue was placed in a 50 mL tube containing 40 mL deionized water. After 24 h, the solution conductivity ($C1$) was measured with a conductivity meter (3540 model, Jenway). The samples were then autoclaved at 120 °C for 30 min, and when the tubes cooled to room temperature, the conductivity of the solution containing the dead tissue was measured ($C2$). The relative EL was calculated using the formula: $EL\ (\%) = (C1/C2) \times 100$ (Liu et al., 2015b).

S. viridis at 20 DAG and 45 DAG were individually placed in a closed fluorescence chamber (Fluorcam 800 MF, Photon Systems Instruments) to estimate the chlorophyll fluorescence of water-deficit treated plants and control groups ($n = 10$). After calibration of the equipment, the quenching analysis protocol, employing the actinic light 2 (wavelength from royal-blue), was selected for the evaluation of indicators. The third leaf of 20 DAG plants and the youngest leaf on the main axis of 45 DAG plants were chosen for analysis. Evaluated chlorophyll fluorescence parameters comprised exposed leaf area (mm^2), photosynthetic quenching (qP), non-photochemical quenching (NPQ), maximum PSII quantum yield (F_v/F_m) and fluorescence decrease ratio (Rfd). These parameters are described in previous studies as indicators of water stress response (Gorbe & Calatayud, 2012; Jiang, Yao, & Wang, 2012; Salvatori et al., 2014; Yoo et al., 2014).

Identification and selection of candidate reference genes

Thirteen candidate reference genes were selected using as criterion reference genes previously reported as suitable for transcript normalization in *O. sativa* (Maksup, Supaibulwatana, & Selvaraj, 2013), *Z. mays* (Manoli, Sturaro, Trevisan, Quaggiotti, & Nonis, 2012) and *S. viridis* (Lambret-Frotté et al., 2015). Initially, the *S. italica* genome database available in Phytozome 12.1v. (http://phytozome.jgi.doe.gov/) was used as a reference to retrieve orthologue gene sequences. Following the release of the *S. viridis* genome sequence at Phytozome database, the identity and specificity of all primer sequences used in this present study was confirmed. Following *S. italica* orthologue identification, primer design for the 13 candidate genes was performed based on these sequences. BLASTP analysis in Phytozome v12.1 was conducted to determine the identity in *S. viridis*. With the exception of the glycine-rich RNA binding protein (*Si025395*), all the *S. viridis* identities were obtained, with the poorest alignment result observed with a 94.5% identity to ubiquitin carboxyl terminal hydrolase (*Si021145*). Phytozome ID, *S. viridis* identity, biological function annotation, primer sequences and amplicon length information are summarized in Table S1.

Primer design

Primer3 Plus (http://bioinformatics.nl/cgi-bin/primer3plus/) was employed for primers design, with the following parameters: Tm around 60 °C, GC content between 35 and 65% and amplicon length of 20–22 bp. The amplicons varied from 80 to 180 bp in length and were designed to span intronic regions whenever possible (Table S1). The specificity of the primers was validated both *in silico* and by RT-PCR.

RNA isolation and cDNA synthesis

Approximately 100 mg of plant tissue was used for RNA isolation. Total RNA from roots and leaves was isolated using the SV Total RNA Isolation System (Promega), according to the manufacturer's protocol. Total RNA quantity and purity was measured with a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Samples with 260/280 and 260/230 ratios between 1.6 and 2.2 were selected for cDNA synthesis. RNA integrity was verified in 1% agarose gel electrophoresis.

cDNA synthesis was performed using SuperScript™ III Reverse Transcriptase (Invitrogen). 50 μM oligo-dT and 10 mM of each dNTP was added to 1 μg total RNA, completing the volume to 11 μL with milli-Q water. After incubation at 65 °C for 5 min on a thermocycler, the reaction was cooled on ice for 5 min, followed by addition of 4 μL First-Strand 5X buffer, 2 μL DTT (0.1 M) and 200 U SuperScript™ III RT (total volume of reaction: 20 μL). The reaction was incubated at 50 °C for 1 h followed by enzyme inactivation at 70 °C for 15 min. PCRs with specific primers were performed and bands observed in 1% agarose gel electrophoresis to identify gDNA contamination. cDNA samples were diluted (1:50) prior to use in RT-qPCR assays, where dissociation curves were analyzed.

Quantitative real-time PCR conditions

Reactions were performed on the 7500 Fast Real-Time PCR System (Applied Biosystems) and viewed using the 7500 software v.2.0.5. cDNA amplification reactions contained: 2 μL of the SYBR Green detector (1×) (Molecular Probes); 0.4 μL of ROX reference dye; 10 μL of 1:50 diluted cDNA; 0.8 μL of primer pair (10 μM of each); 0.05 μL of each dNTP (10 mM); 2 μL of PCR buffer (10×); 1.2 μL of 50 mM magnesium chloride; 0.25 units of Platinum® Taq DNA Polymerase (Invitrogen) and 3.5 μL of water, totaling a final volume of 20 μL. In the control reaction, the cDNA was replaced by ultrapure and sterile water.

Reaction parameters included initial denaturation at 94 °C for 5 min, followed by 40 cycles at 94 °C for 15 s, annealing of primers at 60 °C for 10 s, extension by the enzyme at 72 °C for 15 s and fluorescence reading at 60 °C for 35 s. The dissociation curve cycle was at 95 °C for 15 s, followed by 60 °C for 1 min, 95 °C for 30 s and finally, 60 °C for 15 s. After 40 cycles, the specificity of the amplifications was analyzed through the dissociation curve profiles. All assays were performed using three biological replicates with three technical replicates each and a non-template control.

In order to obtain the C*q* values and primers efficiency, the raw fluorescence data was submitted to the Real-Time PCR Miner online software (Zhao & Fernald, 2005). The qBase v1.3.5 software was used to calculate the non-normalized expression values, using the formula $Q = E^{\Delta Cq}$, in which E represents the efficiency of gene amplification and Δ^{Cq} is the difference between the sample with the lowest expression in the dataset minus the C*q* value of the sample analyzed (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007).

Measuring the expression stability of candidate reference genes

Results obtained from qBase were imported into two different softwares that classify the best constitutive genes, namely geNORM (Vandesompele et al., 2002) and NormFinder (Andersen, Jensen, & Orntoft, 2004). These programs are based on different algorithms that use as information the analysis of the CT of the genes in the different analyzed tissues, their biological replicates and the efficiency of the primer in each sample. With this approach, we aim to identify the genes that present the smallest variation in the number of transcripts from each tissue and to identify the best reference genes for the normalization of RT-qPCR data of *S. viridis* under water-deficit conditions. Global analysis was performed using all datasets. Subsequently, each experimental set was assessed to define specific reference genes for proper normalization.

To test expression stability in a set of samples, which included control and water stressed plants, RT-qPCR assays were performed. Analysis of melting curves revealed a single dominant peak of the specific amplicon with no detectable amplifications in the no-template controls (NTCs), suggesting the absence of any primer dimers and specific amplification from the respective primer pair (data not shown). The C*q* curve medium values and PCR efficiency varied between 16.8 ± 0.70 and 25.4 ± 1.36 and 74.9–92.3%, respectively (Table S1).

Water-deficit marker gene identification and selection

Selection of water-deficit marker gene candidates was based on the analysis of drought responsive genes described in previous studies with *A. thaliana*, *Z. mays* and *O. sativa* (Fang, You, Xie, Xie, & Xiong, 2008; Kumar et al., 2015; Liu et al., 2015a; Qiao et al., 2016; Wang et al., 2014; Wei, Chen, Chen, Wu, & Xie, 2012). Genes encoding delta 1-pyrroline-5-carboxylate synthase 2 (*P5CS2*), Dehydrin 1 (*DHN1*) and the transcription factors WRKY DNA-binding domain 1 (*WRKY1*), dehydration-responsive element-binding protein 1 class C (*DREB1C*) and NAC domain-containing protein 6 (*NAC6*) were all selected as potential water-deficit markers.

For the identification of candidate genes of water-deficit stress in *S. viridis*, protein sequences of *A. thaliana*, *O. sativa*, *Z. mays*, *S. bicolor* and *S. italica* genes and their putative orthologues were used to construct dendrograms. P5CS (also known as glutamate-5-semialdehyde dehydrogenase, GPR) and DHN amino acid sequences were obtained from the Ensembl Genomes database (http://ensemblgenomes.org/), while amino acid sequences of WRKY, DREB and NAC TF families were obtained from the Plant Transcription Factor Database platform v.3.0 (http://planttfdb.cbi.pku.edu.cn/). Before construction of dendrograms, BLASTP of the amino acid sequences identified in the literature was performed against own species in the databases (Ensembl Genomes or Plant TFDB) to confirm the accession ID.

A multiple alignment of the amino acid sequences for each selected gene was conducted using Muscle in Molecular Evolutionary Genetics Analysis (MEGA) 7.0 v. (Kumar, Stecher, & Tamura, 2016). Maximum likelihood (ML) was performed in MEGA to identify the best model for each water-deficit marker candidate using the neighbor-joining algorithm as statistical method and Bootstrap test of phylogeny (1000 replications) (Simonsen, Mailund, & Pedersen, 2008). Dayhoff + G (gamma distribution) was the ML amino acid substitution model recommended for the phylogeny of DHN and WRKY; while the JTT (Jones–Thornton–Taylor) + G model was recommended for P5CS, DREB and NAC.

The *S. italica* sequences that presented the highest similarity in the dendogram to the gene ID identified previously in bibliographic survey carried out in *A. thaliana*,

O. sativa or *Z. mays*, were considered as putative homologues. For the sake of simplicity, we named these genes as homologues. BLASTP using the ID recognized *S. italica* genes was performed against the genome of *S. viridis*, with the criterion of *e*-value $\leq$0.0 in Phytozome 12.1 (http://phytozome.jgi.doe.gov/). The *S. viridis* identities in comparison to *S. italica* of water-deficit marker gene candidates were 100% (Table S1), confirming the phylogenetic proximity of these two species. A *S. viridis* sequence identified as a putative homolog for each water-deficit marker gene candidate was included in the analysis for dendrogram construction, using the same parameters in MEGA program, as previously mentioned.

After homologue identification, primer design was performed and quantitative gene expression was evaluated by RT-qPCR. Presence of primer dimers and unspecific amplification was evaluated according to melting curves (data not shown). The medium values of CT curves varied between 23.1 $\pm$ 3.40 and 32.9 $\pm$ 6.01 and PCR efficiency varied between 83.8 and 90.0% (Table S1).

Statistical analysis

For physiological analysis between control and treated-groups, normal distribution of the data and the homogeneity of variances were verified with the Kolmogorov–Smirnov and Bartlett's tests, respectively. Mostly, the data evaluated did not present a normal distribution therefore a nonparametric statistic was applied. To test the effect of the water-deficit treatment, a Kruskal–Wallis test was performed, followed by a Dunn's test, for multiple comparison between the control group and water-deficit treatments, using the GraphPad Prism 6 program. Results were expressed as mean $\pm$ SD (standard deviation). Values with $p \leq 0.05$ were considered significant.

Physiological parameters were used to perform a discriminant analysis (DA). DA is a statistical procedure that extracts the most important information from the data table to discriminate the treatments and identifies the linear discriminant (LD) components compressing the size of the data set (Abdi & Williams, 2010). Those LD can be associated to variables to capture the most important in discriminate the treatments. Firstly, data normality was determined using the Shappiro–Wilk test (Shapiro & Wilk, 1965). The analyses were carried out using R version 3.2.2 (''R Development Core Team,'' 2011). DA was performed using the MASS R package (Venables & Ripley, 2002). The DA were performed to discriminate the developmental stages, induction methods and time, used as sample categories in DA. We considered roots and shoots of control and treated-groups.

To analyze the relationship among the physiological parameters evaluated, we also performed the linear Pearson's correlation (*r*) analysis considering only shoots. To understand the relationship among variables in each treatment and to verify whether their pattern changes among treatments we performed network analysis. The nodes of networks represent the physiological measurements and the edges are statistical significance between the variance. To evaluate the importance of a measurement in the system studied we calculated Eigenvector centrality. This network centrality measure indicates if a node has a great number of edges and if its neighbor (node connected with it) is also important. We build networks for the two drought-induction methods (air-drying and PEG), for each developmental stage using the samples of measurements times to calculate the correlation coefficients among variables. Spearman's correlations were calculated in the R Hmisc package (Husson, Josse, Le, & Mazet, 2007) and networks were built and drawn using the R Igraph package (Csardi & Nepusz, 2006). The links between the nodes (*i.e.* physiological measures) were created only when the correlation coefficient was statistically significant (p-value ≤ 0.05).

Relative expression level of water-deficit marker gene candidates was calculated using the relative expression software tool (REST), which compares treatments *versus* control-group and calculates the relative expression ratio between them. It presents coupled statistical analysis. The algorithm is based on PCR efficiencies and the mean CT deviation of target genes between the sample and the control group, normalized by the mean CT deviation of the reference genes. REST presents a statistical analysis test that analyzed CT values by a pair-wise fixed reallocation randomization test (Pfaffl, 2001). A heat map was constructed to simplify the expression level visualization among the treated-groups. Candidate genes with relative expression levels (2-log) $\geq$1.0 were considered as good water-deficit markers under the specific conditions tested.

Results

Physiological response of *S. viridis* under water stress

S. viridis plants were evaluated in the vegetative (20 DAG) and the reproductive (45 DAG) stages when subjected to air-drying or PEG $-$0.55 MPa water-deficit conditions at different sampling times. Water stress responses in both well-watered and water-deficit plants were measured using seven physiological parameters in shoots (fresh weight, FW; electrolyte leakage, EL; exposed leaf area; photochemical quenching, qP; maximum PSII quantum yield, F_v/F_m; non-photochemical quenching, NPQ; and fluorescence decrease ratio, Rfd) and two parameters in roots (FW and EL). Fresh weight (FW) and electrolyte leakage (EL) were important parameters evaluated in water-deficit experiments (Liu et al., 2015b). FW indicates the quantity of water loss and EL indicates oxidative damage caused by accumulation of reactive oxygen species, which often results in programmed cell death (Demidchik et al., 2014).

All results from the physiological parameters are presented in Table S2. However, for data integration and to better evaluate the contribution of each parameter in discriminating control from water-deficit treated plants, DA was performed using all the physiological parameters considering the developmental stages, water-deficit induction methods and exposure times. Through vector analysis, clear separation was observed between the vegetative (VS) and reproductive (RS) stages. The samples of each stage are projected in opposite sides of the first axis (LD1) in the plot (Fig. 1A). The two main variables explaining differences between stages (the first dimension) are F_v/F_m and Rfd (Fig. 1A and Table S2). The differences between

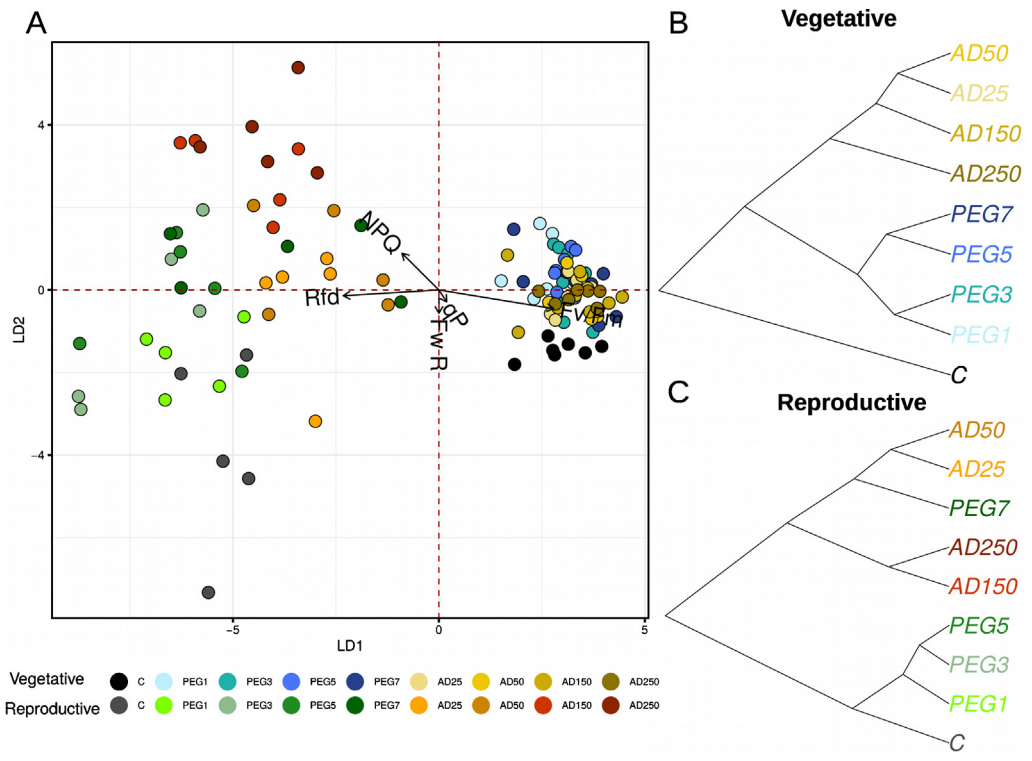

Figure 1 Discriminant analysis (DA) of *S. viridis* physiological parameters in vegetative and reproductive stages under water-deficit conditions (A). The arrows represent the variables with the greatest weight in the separation among the groups. Hierarchical clustering of vegetative (B) and reproductive (C) stages showing the grouping according to water-deficit treatment and time of exposition. C, control-group; FW R, fresh weight of roots; qP, photochemical quenching; NPQ, no-photochemical quenching; F_v/F_m, maximum PSII quantum yield; Rfd, fluorescence decrease ratio.

control and treated groups are related to the second axis (LD2) in Fig. 1A. The variable with the higher weight in discriminating control from treatments is the fresh weight in roots (FW R). Together, dimensions 1 and 2 can discriminate 94.7% of the samples in response to water-deficit treatment of all data. Analyzing each stage and comparing the treatments, F_v/F_m and FW in shoots were the most important variable to discriminate PEG from AD in reproductive stage (Fig. S2B). In vegetative stage, F_v/F_m also discriminates the treatments together with NPQ (Fig. S2A).

In RS, vectors representing 25 min AD and 1 h PEG were closer to the control, and the other vectors related to AD treatments were grouped apart (Fig. 1B). The 7 h PEG treatment samples were closer to AD treatments in comparison to other PEG treatments (Fig. 1B). Vectors representing PEG at 1, 3 and 5 h show extensive overlap and proximity in the hierarchical clustering analysis (Fig. 1A and C). When VS was analyzed separately, the main component that distinguishes the control and stressed-groups was FW R (LD1 and LD2 was −13, 39 and −60.4, respectively), although FW in shoots and F_v/F_m are the main variables that explain the differences between PEG and AD treatments (Fig. S2A). Root FW decreased significantly in AD treatment after 50, 150 and 250 min of water-deficit exposition ($p < 0.05$; Table S2). In general, water-deficit treatments presented a great overlap (Fig. 1), but the samples representing 1, 3, and 5 h PEG water-deficit induction exhibited a greater distance of samples that represent 7 h PEG treatment, which tended to be close to 50 and 150 min air-drying water stress group (AD) vectors (Fig. 1A). The vector representing the AD group treated by 250 min was the furthest from the other vectors, especially in relation to the control and PEG treatments. A clear separation was observed between control and treated groups in VS (Fig. S2A).

To clarify the differences and similarities between each physiological parameter evaluated, a linear correlation coefficient calculation (r) was performed. Analysis showed that, under water-deficit, there was significant correlation between all the variables analyzed in shoots, in both VS and RS ($p < 0.01$; Table 1). The correlations of NPQ with EL, FW and exposed leaf area were the largest, with r of 0.76, 0.75 and 0.75, respectively ($p < 0.0001$). Correlation between Rfd and exposed leaf area also showed statistical significance (0.73; $p < 0.0001$). All these parameters with high r were valid to substantiate the VS and RS separation in PCA (Fig. 1).

The network connections between the physiological parameters in developmental stages and distinct water-deficit induction methods were evaluated to verify the relationship between the variables measured and if this relationship changes between treatments. In the RS (PEG treatment) and VS (in both PEG and AD treatments), it was observed that shoot FW presented a higher number of connections, with a high centrality degree and low number of connections in RS air-drying. NPQ and roots FW were found with lower centrality degrees, but with number of connections statistically significant ($p \leq 0.05$; Fig. 2A–D).

Table 1 The correlation coefficient *r* between physiological measurements in shoots of *Setaria viridis* submitted to distinct water-deficit treatments.

Parameters	Shoots						
	FW (g)	EL (%)	Leaf area (mm^2)	qP	F_v/F_m	NPQ	Rfd
FW (g)	1	−0.75****	0.66****	0.35****	0.46*,***	−0.75****	0.67****
EL (%)		1	−0.72****	−0.38****	−0.55****	0.76****	−0.63****
Leaf area (mm^2)			1	0.45***	0.46****	−0.75****	0.73****
qP				1	0.27**	−0.37****	0.41****
F_v/F_m					1	−0.56****	0.45****
NPQ						1	−0.74****
Rfd							1

FW, fresh weight; EL, electrolyte leakage; qP, photosynthetic quenching; NPQ, no-photosynthetic quenching; F_v/F_m, maximum PSII quantum yield; Rfd, fluorescence decrease ratio. Statistical significance: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; **** $p \leq 0.0001$ ($n = 10$).

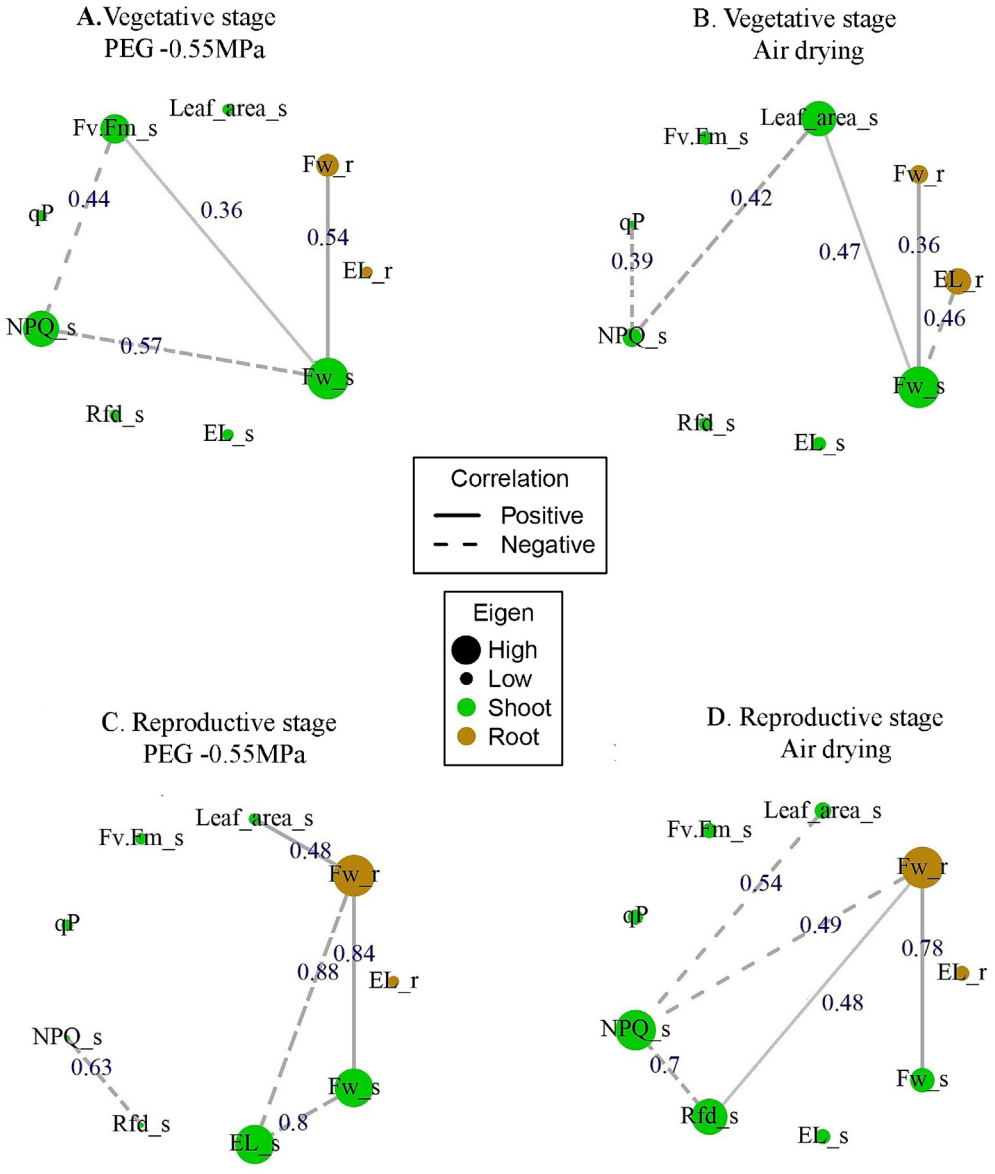

Figure 2 Interaction networks of physiological parameters. (A) PEG −0.55 MPa and (B) air-drying treatments in vegetative stage; (C) PEG −0.55 MPa and (D) air-drying treatments in reproductive stage. Circle sizes represent the degree centrality. Values represent the significant correlation coefficients between the variables ($p \leq 0.05$, using Spearman correlation). Filled and dashed lines represent positive and negative correlations, respectively. FW, fresh weight; EL, electrolyte leakage; qP, photosynthetic quenching; NPQ, no-photosynthetic quenching; F_v/F_m, maximum PSII quantum yield; Rfd, fluorescence decrease ratio.

Comparing the water-deficit induced methods (PEG × AD), the physiological parameters FW (roots and shoots) and NPQ showed network connections in the VS (Fig. 2A and B), while for FW (roots and shoots), NPQ and Rfd connections were statistically significant in RS (Fig. 2C and D). In PEG treatments, there was FW (roots and shoots) and NPQ network links in common among VS and RS (Fig. 2A and C). FW (roots and shoots), exposed leaf area and NPQ appeared with connections in AD treatments, in both VS and RS (Fig. 2B and D). In VS, root EL, exposed leaf area, F_v/F_m and qP were the parameters which differentiate PEG and AD treatments (Fig. 2A and B). Shoot EL was the main parameter that distinguished AD and PEG in RS (Fig. 2C and D).

Identification and selection of suitable reference genes in *S. viridis* for gene expression analysis under water-deficit conditions

The identification of reliable genes is essential for accurate normalization of gene expression data in qPCR assays. Thirteen candidate genes (Table S1), selected from previous studies that investigated transcript normalization in *O. sativa* (Maksup et al., 2013), *Z. mays* (Manoli et al., 2012) and *S. viridis* (Lambret-Frotté et al., 2015) in well-watered conditions, were selected to analyze expression under water-stress. Performance of the candidates as potential reference genes for *S. viridis* was assessed in vegetative (VS) and reproductive stages (RS), in roots and leaves of water-stressed plants (PEG) or following air-drying (AD) in four sampling times. Using geNORM, two parameters were estimated to evaluate the expression stability of these genes: the average expression stability value (*M* value) and the pairwise variation (Vn/*n* + 1). The first is based on the pairwise variation between a specific gene compared to all others and the second determines the required number of genes to result in a more accurate normalization (Vandesompele et al., 2002). When considering the entire dataset, *Si002651/Si017354* (*M* = 0.60) was the best pair to normalize all samples (Fig. S3A). Taking into account 0.15 as a pairwise variation cut-off, the optimal number of reference genes recommended for normalization was seven (V7/8; Fig. S4). In the analysis with NormFinder, the best pair genes for reliable normalization was *Si018608/Si021373*, with a stability value of 0.382, while *Si025395* was the least stable gene among the samples observed with both geNORM and NormFinder, with expression stability values of 1.61 and 2.084, respectively (Table S3).

Due to the heterogeneity of samples and conditions, experimental conditions were analyzed by different combinations of the data set, grouping developmental stages (vegetative and reproductive), tissues (roots and leaves) and water-deficit induction methods (PEG −0.55 MPa and air-drying) separately (Figs. S3, S4 and Table S4). The rankings in both geNORM and NormFinder algorithms showed that it is necessary to analyze vegetative (VS) and reproductive (RS) stages separately for ensure an accurate normalization for this study. GeNORM and NormFinder rankings for the distinct developmental stages are summarized in Table 2. While GeNORM analysis showed *Si002651/Si014034* (*Triosephosphate isomerase/GAPDH*) as the best gene pair ranked for VS, NormFinder ranked *Si002651/Si018608* (*Triosephosphate isomerase/Ubiquitin-conjugating enzyme E2*). In RS, the most stable genes were *Si017354/Si021373* (*Serine incorporator/Cullin*) in geNORM and *Si018608/Si021373* (*Ubiquitin-conjugating enzyme E2/Cullin*) in NormFinder analysis.

Identification and analysis of candidate genes as water-deficit molecular markers in *S. viridis*

With the aim of identifying water-deficit responsive genes in *S. viridis*, we first selected genes that were previously characterized as responsive to drought in *A. thaliana*, *O. sativa* and *Z. mays* using information available in the literature (Fang et al., 2008; Kumar et al., 2015; Liu et al., 2015a; Qiao et al., 2016; Wang et al., 2014; Wei et al., 2012b). For each gene selected as a putative water-deficit responsive gene, the complete amino acid sequence from *A. thaliana*, *O. sativa*, *Z. mays*, *S. bicolor* and *S. italica* and their putative paralogs were obtained from databases previously cited. We focused our selection on genes that have been well characterized by previous studies and are considered reliable molecular makers for water-deficit stress (described in ''Materials and methods'' section).

The construction of dendrograms was performed with the putative homologues of delta 1-pyrroline-5-carboxylate synthase (P5CS), dehydrin (DHN), WRKY DNA-binding domain (WRKY), dehydration-responsive element-binding protein (DREB) and NAC domain-containing protein (NAC) amino acid sequences from *A. thaliana*, *O. sativa*, *Z. mays*, *S. bicolor* and *S. italica* using the best maximum likelihood model (MEGA 7.0) and neighbor-joining analysis. A BLASTP search in the Phytozome database with the putative water-deficit homologue genes of *S. italica* against the *S. viridis* genome allowed us to identify homologues to *P5CS2*, *DHN1*, *WRKY1*, *DREB1C* and *NAC6*, and construct the dendrograms including the identified *S. viridis* homologues (Table S2 and Fig. 3).

Analysis of the putative water-deficit marker genes in *S. viridis* under air-dry and PEG treatments

The *SvP5CS2*, *SvDHN1*, *SvWRKY1*, *SvDREB1C* and *SvNAC6* genes were evaluated by RT-qPCR. Expression analysis included samples of PEG and air-drying water-deficit treatments in distinct developmental stages, tissues and time points after stress initiation (Figs. 4 and 5). In PEG treated-groups, *SvWRKY1*, *SvDREB1C* and *SvNAC6* were exclusively up-regulated in roots of reproductive stage (RS), while the expression of *SvDHN1* was induced in both organs and all stages of development (Fig. 4). In leaves, *SvDHN1* was up-regulated in both VS and RS in all sampling times tested (Fig. 4B and F). In VS, induction of expression in *SvP5CS2* was observed at 3, 5 and 7 h, whereas *SvDREB1C* only at 7 h of PEG treatment (Fig. 4A, D and F). *SvP5CS2* was up-regulated at 7 h of PEG treatment in RS (Fig. 4A and F), while *SvWRKY1* and *SvDREB1C* were down-regulated in all the times (Fig. 4C, D and F).

Response of the putative water-deficit marker genes in plants submitted to air-drying showed a distinct profile when compared to PEG treatments (Fig. 5). *SvP5CS2*, *SvWRKY1* and *SvDREB1C* presented a similar expression profile, with

Table 2 geNORM and NormFinder rankings of the thirteen reference genes candidates to suitable normalization by RT-qPCR.

Ranking	Vegetative stage		Reproductive stage	
	geNORM	NormFinder	geNORM	NormFinder
1	Si002651	Si018608	Si017354	Si018608
2	Si014034	Si002651	Si021373	Si021373
3	Si018608	Si021373	Si002651	Si014034
4	Si021373	Si034613	Si014034	Si035045
5	Si017354	Si017354	Si021145	Si003209
6	Si034613	Si014034	Si018608	Si034613
7	Si018607	Si003209	Si022372	Si021145
8	Si025395	Si025395	Si034613	Si017354
9	Si035045	Si000245	Si003209	Si002651
10	Si003209	Si021145	Si035045	Si022372
11	Si000245	Si018607	Si000245	Si000245
12	Si021145	Si035045	Si018607	Si018607
13	Si022372	Si022372	Si025395	Si025395
Best pair	Si002651/Si014034	Si002651/Si018608	Si017354/Si021373	Si018608/Si021373

The IDs represent *Setaria viridis* homologues in *Setaria italica*.

repression in leaves of RS and mainly induction in roots of VS and RS (Fig. 5A, B and F). Higher induction levels of *SvWRKY1* and SvDREB1C genes were observed in the roots of RS. However, the highest level of expression was observed for *SvDHN1* in leaves of VS after 150 and 250 min of air-drying (Fig. 5B and F).

In both PEG and air-drying water-deficit treatments it was observed that *SvDHN1* was highly responsive in leaves and roots, with relative expression level (2-log) $\geq$10 (Figs. 4B and F and 5B and F), when compared with the relative expression level of the other putative water-deficit markers tested. The highest induction levels of *SvDHN1* were found in roots and leaves treated with the PEG water-deficit method (Fig. 4B and F).

Discussion

Distinct water-deficit conditions promote variation in physiological responses of *S. viridis*

Physiological measurements indicate that water-deficit responses in *S. viridis* differ according to the developmental stage and stress induction methods (Table S2). The significant linear correlations between the parameters and the high number of interactions in the network analysis showed that all the variables evaluated help to explain the physiological responses of *S. viridis* to water-deficit (Table 1 and Fig. 2). During the reproductive stage, initial times in PEG solution promote few alterations in the physiological parameters evaluated when comparing to air-drying (Fig. 1). Treatments with PEG for 1, 3 and 5 h showed only small differences between the physiological responses in these water stress induction times. However, our results suggest that 7 h of PEG water-deficit can be as acute as the air-drying method (Fig. 1).

Discriminant analysis (DA) indicated a key divergence between vegetative (VS) and reproductive (RS) stages in both water-deficit methods used (Fig. 1). The morphological variations observed between VS and RS – such as the quantity of secondary roots, shoot ramifications and grain-filled inflorescences – have a direct relationship with the physiological differences found in both water stress treatments tested (see Fig. S1). The network analysis revealed that FW (roots and shoots) and NPQ are the most representative physiological parameters to evaluate all the data set. Rfd is the main parameter that distinguishes the developmental stage response to water-deficit in the network analysis, presenting connections only in RS (Fig. 2).

The physiological measurements in *S. viridis* revealed that the reproductive stage was more sensitive to water-deficit than the vegetative stage. This result is expected since crops and most plant species are more sensitive to water-deficit during flowering and the initial stage of grain-filling, and less in the vegetative or the maturation phase of the harvested organ (Pushpavalli et al., 2015). Higher plant sensitivity during RS can be mainly explained by the increase in photosynthetic parameters (here explained by F_v/F_m NPQ and Rfd alterations), starch accumulation and, consequently, higher demand for water supply during grain formation (Yang & Zhang, 2006). Therefore, *S. viridis* presents a similar behavior in water-deficit to other C_4 monocot crops, supporting its use as model plant in water-deficit studies (Yoo et al., 2014; Zinta et al., 2014).

The differences between acute (air-drying, AD) and moderate (PEG) water-deficit treatments were also observed in the physiological responses, showing that AD is a more severe method in comparison to PEG, especially at later time points in both VS and RS (Fig. 1). The AD treatment triggers a rapid and severe water-deficit stress and has been successfully used to identify genes involved in early water stress responses in rice, soybean and other crops (Guimarães-Dias et al., 2016; Minh-Thu et al., 2013; Oh et al., 2005; Zhou et al., 2007). In contrast, moderate water-deficit with PEG induces water loss more slowly by osmotic imbalance, which may potentially reproduce better field conditions (O'Donnell et al., 2013). The osmotic imbalance caused by PEG treatments promoted oxidative

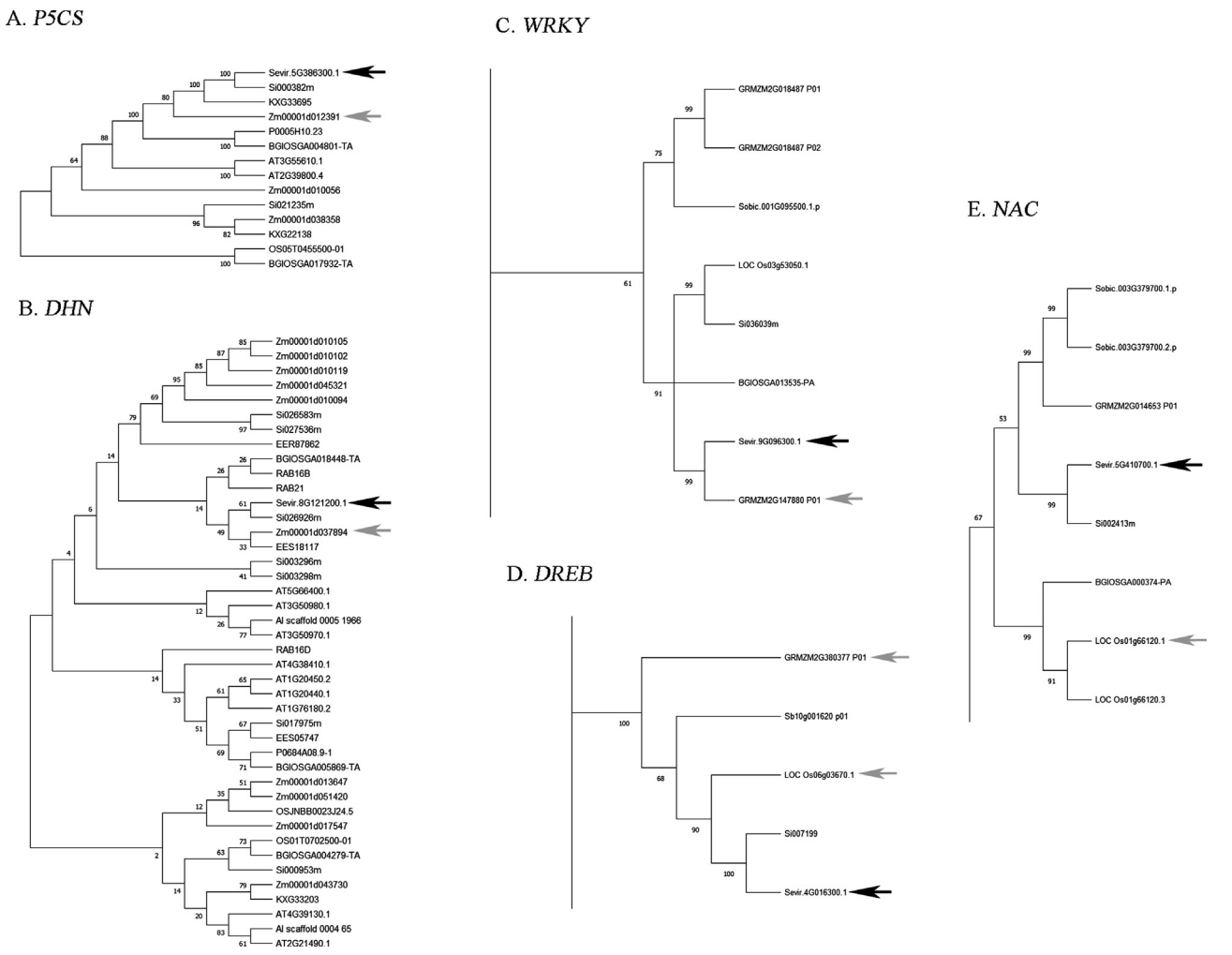

Figure 3 Dendrograms from *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Setaria italica* and *Setaria viridis* based on the amino acid sequences. (A) Delta 1-pyrroline-5-carboxylate synthase, *P5CS*; (B) Dehydrin, *DHN*; (C) WRKY DNA-binding domain, *WRKY*; (D) dehydration-responsive element-binding protein, *DREB*; (E) NAC domain-containing protein, *NAC*. The multiple alignment was made using Muscle, and the dendrograms were built using the MEGA 7.0 software using the neighbor-joining with a consensus of 1000 bootstrap replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The gray arrows indicate the *O. sativa* or *Z. mays* reference key genes and the respective homolog *S. viridis* genes was indicated with black arrows. The complete dendrograms of *P5CS* and *DHN* were shown in this figure. *WRKY*, *DREB* and *NAC* complete dendrograms can be found in Fig. S5A–C.

stress in the photosynthetic apparatus of *S. viridis* according to chlorophyll fluorescence data, which has been previously reported in sorghum cultivated in hydroponic medium (O'Donnell et al., 2013) and confirmed in our study. The damage got worse when there was an increase in the time of exposure of the plants to the drought induction agent (Fig. S2A). Several studies have applied PEG to induce water-deficit stress conditions using hydroponic systems, showing it to be an appropriate method to apply water-deficit in controlled systems, especially because it does not impact root integrity (Ashraf, Ali, & Ashraf, 2013; Kocheva, Petrov, & Georgiev, 2013; Leung, 2015; Li et al., 2015, 2016; O'Donnell et al., 2013; Silveira et al., 2016). All these findings reveal that the physiological indicators tested are important parameters for assessing water-deficit responses in *S. viridis*, enabling robust characterization of water-deficit.

Selection of suitable reference genes for accurate normalization of RT-qPCR data

Identification and selection of reference genes was conducted for accurate evaluation of gene expression during water-deficit. A set of thirteen candidate reference genes described in previous studies as robust reference genes were selected for analysis (Lambret-Frotté et al., 2015; Maksup et al., 2013; Manoli et al., 2012). We opted to use the best ranked reference genes indicated by NormFinder for subsequent gene expression analysis, as this algorithm also evaluates intragroup stability (González-Aguilera, Saad, Chávez Montes, Alves-Ferreira, & de Folter, 2016; Lambret-Frotté et al., 2015; Nardeli et al., 2017). Based on the DA data that indicated a sharp separation between vegetative and reproductive stages, we identified the best references genes for each developmental stage in the conditions tested

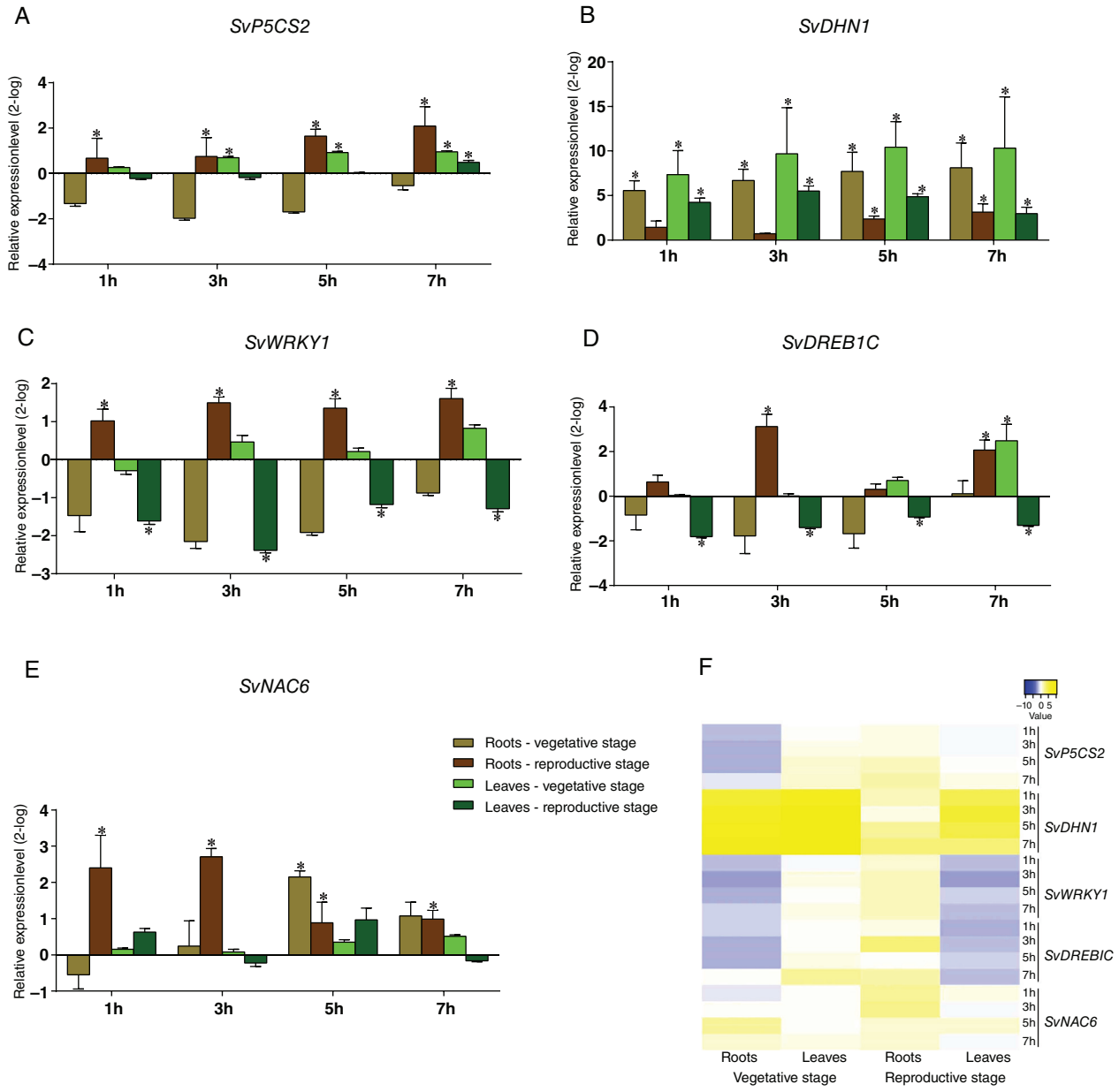

Figure 4 Relative expression level of water-deficit marker genes candidates measured by RT-PCR in plants submitted to PEG −0.55 MPa water-deficit induction method. (A) Delta 1-pyrroline-5-carboxylate synthase 2, *SvP5CS2*; (B) Dehydrin 1, *SvDHN1*; (C) WRKY DNA-binding domain 1, *SvWRKY1*; (D) dehydration-responsive element-binding protein 1 class C, Sv*DREB1C*; (E) NAC domain-containing protein 6, *SvNAC6*. (F) Heat map representing the relative expression level (2-log) of water-deficit marker genes evaluated. Expression level was calculated by relative expression software tool (REST) program ($p < 0.05$). Error bars represent standard error of the mean ($n = 3$).

in this study. *Triosephosphate isomerase* and *Ubiquitin-conjugating enzyme E2* were the highest ranked gene pair for VS, with *Ubiquitin-conjugating enzyme E2* and *Cullin* the most appropriate for RS (Table 2). Martins et al. (2016) also identified reference genes for *S. viridis* at the reproductive stage under water-deficit conditions. However, in this previous study a field capacity reduction method was applied as the water-deficit method. Among the 13 genes tested in our study, coincidently the two best ranked genes in the analysis of Martins and collaborators, *Si021373* (Cullin) and *Si003209* (Ribosomal protein), were also included in our evaluation of candidate normalization genes. The Cullin gene was considered one of the best reference genes based on NormFinder analysis in both studies.

Distinct water-deficit conditions promote variation in the molecular response of marker genes in *S. viridis*

The genes evaluated as water-deficit markers belong to two general categories, namely transcription factors (*SvWRKY1*,

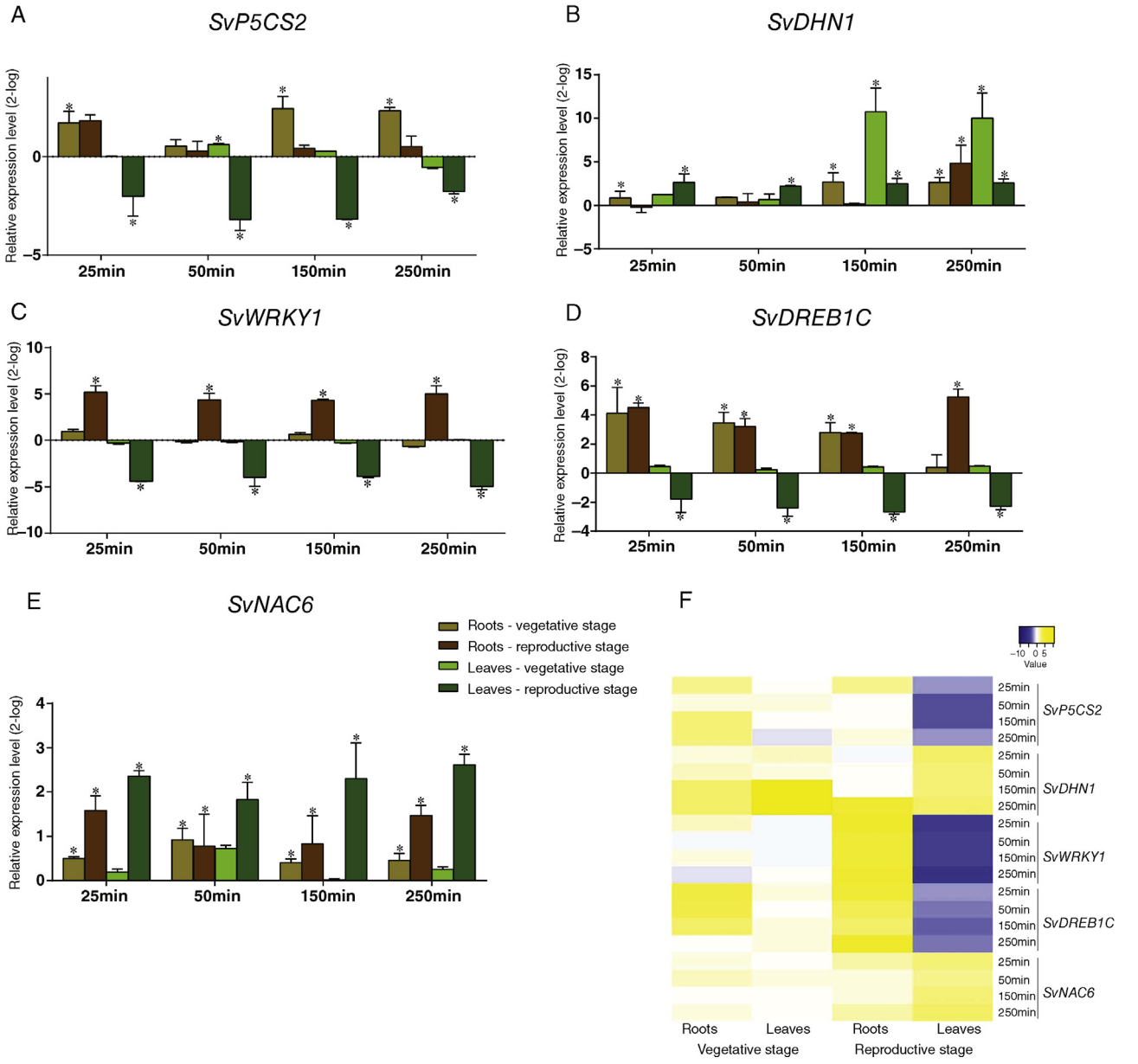

Figure 5 Relative expression level of water-deficit marker genes candidates measured by RT-PCR in plants submitted to air-drying water-deficit induction method. (A) Delta 1-pyrroline-5-carboxylate synthase 2, *SvP5CS2*; (B) Dehydrin 1, *SvDHN1*; (C) WRKY DNA-binding domain 1, *SvWRKY1*; (D) Dehydration-responsive element-binding protein 1 class C, Sv*DREB1C*; (E) NAC domain-containing protein 6, *SvNAC6*. (F) Heat map representing the relative expression level (2-log) of water-deficit marker genes evaluated. Expression level was calculated by relative expression software tool (REST) program ($p < 0.05$). Error bars represent standard error of the mean ($n = 3$).

SvDREB1C, *SvNAC6*) and target genes (*SvP5CS2* and *SvDHN1*). These genes have been described as responsive to water-deficit in other species such as *A. thaliana*, *O. sativa*, *Z. mays* and *S. italica* (Fang et al., 2008; Kumar et al., 2015; Liu et al., 2015a; Qiao et al., 2016; Wang et al., 2014; Wei et al., 2012b). The expression pattern, for most of the genes selected in this work was assessed in distinct organs or across time courses. This information is mostly missing for these genes in the species previously evaluated. Nevertheless, our study corroborates previous results, that *WRKY1*, *DREB1C*, *NAC6*, *P5CS2* and *DHN1* are water-deficit marker genes (Fang et al., 2008; Kumar et al., 2015; Liu et al., 2015a; Qiao et al., 2016; Wang et al., 2014; Wei et al., 2012b). It is important to mention that further experiments are necessary to confirm the *S. viridis* genes as functional orthologues of the genes previously characterized (Fig. 3). In addition, we applied these maker genes to characterize distinct water-deficit conditions in *S. viridis*. According to relative expression levels calculated, we were able to identify patterns of responses of the water-deficit markers considering the different developmental stages, tissue specificity and water-deficit induction methods (Fig. 6).

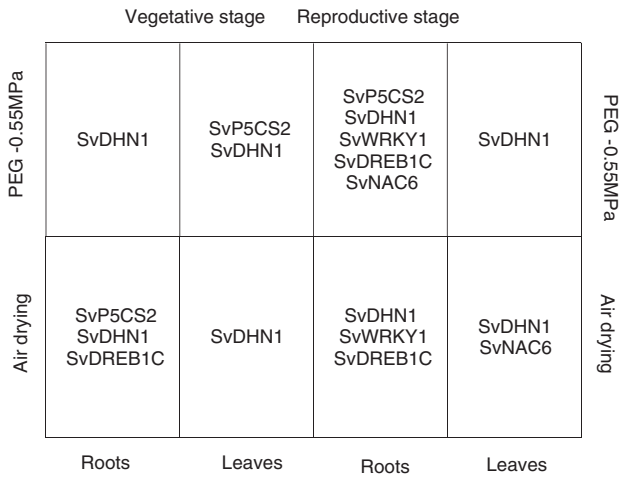

Figure 6 Diagram representing a summary of the best water-deficit marker genes according to conditions tested. We applied a threshold of (2-log) > 1.0 to the relative expression level in order to include the genes as a marker of drought stress.

Between the two target genes evaluated *SvP5CS2* and *SvDHN1*, the former is mostly induced in the conditions tested in this work, but it is repressed in roots of plants during vegetative stage at PEG water-deficit and in leaves of plants during reproductive stage at AD water-deficit (Figs. 4A–B and 5A–B). P5CS2 enzyme is involved in proline biosynthesis, which is directly related in the adaptive response of plant tissues to osmotic imbalances that may occur in cells, including response to various abiotic stress conditions (Amini et al., 2015). Even though we did not perform proline quantification, there are studies showing the positive relationship between *P5CS* expression level and proline accumulation during drought stress in C3 and C4 monocots (Iskandar, Widyaningrum, & Suhandono, 2014; Yooyongwech, Cha-um, & Supaibulwatana, 2012). Therefore, proline biosynthesis might be relevant to maintaining homeostasis conditions, stabilizing cell structure and preventing protein degradation in *S. viridis* during drought stress (Taiz & Zeiger, 2010). When compared to *SvP5CS2* and the other three molecular markers evaluated (*SvWRKY1*, Sv*DREB1C*, *SvNAC6*), *SvDHN1* gene showed the highest expression levels in the conditions tested (Figs. 4B and 5B). Previous studies showed that dehydrin genes are induced by water-deficit in *S. italica* (Qi et al., 2013). Moreover, *DHN* has been described as an excellent drought stress marker gene in other plant species (Liu et al., 2018; Shakirova et al., 2016). The high levels of electrolyte leakage (EL) and non-photochemical quenching (NPQ) in roots caused by water-deficit in PEG and AD treatments might explain not only the high expression of *DHN1* but also the induction of *P5CS2* expression. Both proteins encoded by these genes have be implicated in protective roles of oxidative damage of cell wall and photosynthetic apparatus during salt and drought stress in Arabidopsis (Saibi, Feki, Ben Mahmoud, & Brini, 2015).

Here, we identified three *S. viridis* putative homologues of the TFs: *SvDREB1C*, *SvWRKY1* and *SvNAC6* (Fig. 3). These TFs have been extensively characterized in other species (Chen et al., 2018; Dong et al., 2017; He et al., 2016; Hong et al., 2016; Hu et al., 2019; Mao et al., 2016; Sahebi et al., 2018; Souza et al., 2019; Wang et al., 2018). *SvDREB1C* and *SvWRKY1* presented a similar expression pattern in both water-deficit methods used in the work, being mainly down-regulated in leaves of reproductive stage and up-regulated in roots of reproductive stage (Figs. 4C–D and 5C–D).

SvWRKY1 belongs to a family of DNA-binding proteins (WRKY) that are involved in responses to abiotic stress, but also plant disease resistance, nutrient deprivation, senescence, seed and trichome development, embryogenesis, as well as additional developmental and hormone-controlled processes (Bakshi & Oelmüller, 2014). *S. viridis* plants in reproductive stage showed high expression of *SvWRKY1* in roots and a dramatic down-regulation in leaves (Fig. 4C and 5C). Interestingly, Qiao and collaborators (2016) reported that *wrky1* knockout mutant of *Arabidopsis* was more tolerant to drought stress than the wild type. The authors suggested that the loss-of-function T-DNA insertion mutant *wrky1* was more sensitive to ABA, in especial for stomatal movement (Qiao et al., 2016). Stomatal closure is one of the most important physiological responses described in the literature as a mechanism of resistance to dehydration (Osakabe, Osakabe, Shinozaki, & Tran, 2014). The repression of *SvWRKY1* observed in leaves during water-deficit in reproductive stage, added to considerable reduction of leaf area and FW in plants may be a strategy of *S. viridis* to relieve the water-deficit effects and acquirement of drought tolerance.

SvDREB1C presents a similar expression pattern to *SvWRKY1* during reproductive stage as mentioned above. DREB is a subfamily of the large AP2/ERF family of TFs that is characterized on the basis of the number of repetitions and the sequence of the AP2 domain (Sakuma et al., 2002). Members of DREB subfamily have been extensively studied and most of them are induced after drought stress (Dong et al., 2017; Hu et al., 2019; Souza et al., 2019). *SvDREB1C* is intensely induced in roots in the AD water-deficit method (Figs. 4D and 5D). However, the expression observed in roots of plants induced with PEG (RS) was less intense (Figs. 4D and 5D). Interestingly, *SvDREB1C* is repressed in leaves of RS in both water-deficit methods, the same expression pattern of *SvWRKY1* (Figs. 4D and 5D). On the other hand, the expression pattern of *SvDREB1C* and *SvWRKY1* in roots during VS, when the water-deficit methods are compared, were opposing (Figs. 4D and 5D). This result highlights the differences between the two methods of water-deficit induction, air-dry and PEG8000. Remarkably, the target gene *SvP5CS2* also presents an expression pattern very similar to *SvDREB1C* and *SvWRKY1*, which suggests that it may be regulated by these TFs. Actually, some studies have suggested that members of the WRKY family control proline metabolism (Jiang et al., 2016; Xu et al., 2015). The differences in the expression patterns among the tested genes when comparing the two water-deficit methods were expected based on our physiological data. When PEG is used, the water loss is slower, allowing the plant to acclimate to the unfavorable environmental condition. On the other hand, when the AD is applied, the water loss is fast, not allowing the plant to acclimate to the stress condition. When vegetative and reproductive are compared, *SvDREB1C*, *SvWRKY1* and *SvNAC6* presented more intense responses during reproductive stage in both treatments tested (Figs. 4D–E and 5D–E). These results are in

agreement with the physiological data, plants at reproductive stage have a considerable reduction on fresh weight (FW) and increase in EL in roots compared to vegetative stage. As mentioned above, it has been observed a higher sensibility to water-deficit stress during reproductive stage, especially during grain-filling, in several plant species. In sorghum, physiological and morphological traits like stay green, limited transpiration under high vapor pressure deficit, canopy temperature depression, and root architecture were used to identify drought stress in different developmental stages, which was proved the sensibility during grain filling-period (Prasad et al., 2018).

Contrary to *SvDREB1C* and *SvWRKY1* TFs, *SvNAC6* showed a high expression in most of conditions tested in this work (Figs. 4E and 5E). NAC transcription factors are one of the largest families of transcriptional regulators in plants, and members of the NAC gene family are involved in pivotal roles of transcriptional reprogramming associated with plant stress responses (Shao et al., 2015). Stress-related NAC transcription factor members had the transcription levels increased in water-stressed conditions, showing a potential role on tolerance regulation (Lu, Sun, Zhang, Wang, & Pan, 2015). For instance, transgenic plants overexpressing *OsNAC6* showed an increased tolerance to water-deficit (Rachmat, Nugroho, Sukma, Aswidinnoor, & Sudarsono, 2014). Therefore, *SvNAC6* should participate in water-deficit responses in roots and leaves of *S. viridis* and also it can be a good target for further studies to explore drought tolerance in monocots.

Conclusions

Our analysis revealed that FW (roots and shoots) and NPQ are the most representative physiological parameters to evaluate water-deficit in *S. viridis*. In addition, we showed that the *S. viridis* putative homologues to *SvDHN1*, *SvP5CS*2, Sv*DREB1C*, *SvWRKY1* and *SvNAC6* are suitable water-deficit markers for *S. viridis*. However, the expression level of *SvDHN1* allows us to rank it as the best water-deficit marker gene for all the conditions tested in comparison to the other markers. Although gene expression analysis of *SvDHN1*, *SvP5CS*2, Sv*DREB1C*, *SvWRKY1* and *SvNAC6* strongly suggest their involvement in water-deficit responses in *S. viridis* and further experiments are required to qualify them as functional orthologues. Analysis of physiological parameters and gene expression indicate that *S. viridis* is more susceptible to stress at the reproductive stage. Our results also indicate that PEG treatment is the most adequate method to induce water-deficit in plants of *S. viridis* when the main objective is to characterize early gene expression responses. PEG 8000 can simulate water-deficit and, in combination with hydroponic system, allows for a feasible way to sample roots.

Conflicts of interest

The author declares no conflicts of interest.

Acknowledgments

This research was supported by the CNPq (National Council for Scientific and Technological Development), CAPES (Coordination for the Improvement of Higher Education Personnel/Ministry of Education), FAPERJ (Carlos Chagas Filho Fundation of Support for Research in the State of Rio de Janeiro) and INCT (National Institute of Science and Technology) for financial support for the development of this study. This work is part of the doctoral thesis of T.S.R. in Plant Biotechnology (Programa de Pós-Graduação em Biotecnologia e Bioprocessos, Universidade Federal do Rio de Janeiro, Brazil), supported by a scholarship from CNPq (162676/2015-8). The author's research was supported by Conselho Nacional de desenvolvimento Científico e Tecnológico (CNPq) (308832/2017-5) and Instituto Nacional de Ciência e Tecnologia (INCT Biotec Seca-Pragas; 465480/2014-4 and INCT-Bioethanol; FAPESP 2008/57908-6 and CNPq 574002/2008-1) and Fundação de Amparo à Pesquisa do Rio de Janeiro (FAPERJ CNE; E-26/202.631/2019).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biori.2020.03.001.

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