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ORIGINAL ARTICLE

Integrated proteomics and metabolomics to unlock global and clonal responses of *Eucalyptus globulus* recovery from water deficit

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Abstract
Background and aims Water availability is well known for impacting productivity of *Eucalyptus* but comprehensive knowledge on cellular pathways involved in recovery and tolerance is scarce. In this context, we aimed to unveil putative mechanisms that account for drought recovery of *E. globulus*, and to identify specific strategies that make a clone more adapted to water deficit.
Methods We resorted to comparative proteome (using difference gel electrophoresis) and metabolome [using Gas chromatography–mass spectrometry (GC–MS)] analyses in two *E. globulus* clones that exhibit physiological differences in their capacity to tolerate water shortage and restoration; also, interpretable networks were constructed coupled with previously assessed physiological matrices in order to interrogate the large datasets generated and develop a clear and integrative analysis.
Results Our study enabled the separation and isolation of 2031 peptide spots, 217 of which were identified. GC–MS yielded the detection of 121 polar metabolites. Water shortage negatively affected photosynthesis, gene regulation, cell growth and secondary metabolites; enhanced photo protection, osmoprotection, and other defence-related pathways; and caused a shift from chloroplastic to mitochondrial energy generation. Recovery was characterised by upregulation of all previously described pathways. The analysis of the resilient clone AL-18, which presented a network very distinct from the responsive clone AL-10, reinforced the role of specific photosynthetic and defence-related proteins as key players in mediating drought tolerance and revealed new players: glutamine synthetase, malate dehydrogenase and isoflavone reductase-like protein.
Conclusion This study provides a set of novel proteins and pathways involved in drought stress that represent potential drought tolerance markers for early selection of *Eucalyptus*.

Keywords DIGE · Forest tree · GC–MS · Plant · Stress

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1 Introduction

Eucalypts represent one of the most significant pulpwood planted genera in the world (Freeman et al. 2013). *Eucalyptus globulus* Labill. is successfully cultivated in the Mediterranean region, but experiencing environmental stress is unavoidable for this plant species particularly in light of a rapidly changing environment where drought is one of the major abiotic factors limiting plant growth (Galmés et al. 2007). Water deficit can disrupt cell structures and impair biological functions leading to inhibition

of photosynthesis, metabolic dysfunction, and damage to membranes and proteins (Krasensky and Jonak 2012).

Due to its global significance, the impact of drought stress on *Eucalyptus* has received much scientific interest and most of the morphological and physiological responses that occur during water deficit are already documented (Costa e Silva et al. 2004; Shvaleva et al. 2006; Coopman et al. 2008; Granda et al. 2011; Correia et al. 2014a, b). Earlier studies from our group indicate that *E. globulus* responds to drought mainly by reducing stomatal conductance, gas exchange, height, biomass and water potential; whilst pigments (chlorophylls and carotenoids), malondialdehyde (MDA) and some hormones, such as abscisic acid (ABA) increase (Correia et al. 2014a, b). However, studies that explore the capacity for drought recovery of this species are less abundant (Correia et al. 2014a, b; McKiernan et al. 2015) and little is known about the cellular pathways regulating recovery from stressful conditions in *Eucalyptus*.

As previously stressed by other authors, proteomic and metabolomic variations are far less researched in forest species than in herbaceous plant species (Warren et al. 2011a; Valdés et al. 2013). Valdés et al. (2013) analysed two *E. globulus* provenances with contrasting drought tolerance and found differences in morphology and accumulation of endogenous contents of ABA and proteins involved in abiotic stress processes in the tolerant provenance. Warren et al. (2011a) used gas chromatography–mass spectrometry (GC–MS) to examine the response of leaf metabolites to a 2 month water stress in two species of *Eucalyptus* (*E. pauciflora* and *E. dumosa*) reporting that metabolites were differentially affected during both water stress and rewatering with species-related variations. McKiernan et al. (2014) investigated the influence of water availability on a range of foliar secondary metabolites in juvenile *E. globulus* and *E. viminalis*. These authors concluded that significant species variation occurred but with a minimal treatment effect. These studies highlight the need to incorporate multidisciplinary approaches that investigate plants' ability to recover and unveil the cellular pathways involved. In order to reduce this gap in our knowledge of the cellular processes taking place in *E. globulus* after drought imposition and rewatering, we performed comparative proteome and metabolome analyses using two clones that exhibit differences in their capacity to tolerate water shortage and restoration. In addition, considering the large amount of experimental data generated and the need for a clear, integrated and meaningful view of the investigated biological events, we resorted to the construction of interpretable networks coupled with previously assessed physiological matrices. This approach has led to novel insights regarding the processes involved in drought tolerance and recovery in *E. globulus*. Our main goals were: (i) to unveil putative mechanisms that account for the extraordinary ability of *E. globulus* to recover from water deficit, and (ii) to identify specific strategies that make a clone more adapted to water deficit conditions.

2 Materials and methods

2.1 Plant material and experimental design

Experiments were undertaken in two clones of *E. globulus* Labill. (AL-18 and AL-10) obtained from Altri Florestal, SA (Portugal), which present differential physiological and biochemical responses to water shortage and restoration (Correia et al. 2014a, b). Clone AL-18 was identified as a resilient clone, less reactive to water availability, and AL-10 is considered as more sensitive and responsive in responding to drought stress and rehydration.

Six month old rooted cuttings of each clone were transferred to a greenhouse in 2 L plastic pots for a one-month acclimation and plants were automatically well watered with nutritive solution. After this period, AL-18 and AL-10 cuttings were divided into groups assigned to either a well-watered regime (WW: water supplied daily until soil water content reached around 80 % field capacity, FC) or a water stress regime (WS: water supplied daily until soil water content reached around 25 % FC) for 7 days. After this period, the stress in the WS group was intensified (water supplied daily until soil water content reached around 18 % FC). This lasted for 14 days prior to the initial sampling (stress point, WW and WS plants sampled). After this period, the WS cuttings were re-watered to the well-watered regime and recovery was monitored 1 day (1dR) and 7 days (7dR) after rehydration. Detailed methods can be found in Supplementary Material 1.

2.2 Physiological analysis

Previously published physiological data (Correia et al. 2014b) were introduced into our data analysis in order to obtain an integrated and meaningful interpretation of the proteome and metabolome datasets. Data are summarised in Supplementary Table S1 and full experimental details are published elsewhere (Correia et al. 2014b) and briefly described in Supplementary Material 1.

2.3 Proteomics analysis

Proteins were extracted and prepared from 200 mg of foliar tissue as described by Vítámvás et al. (2012). Protein labelling, 30 μg for each sample, and migration, gel scanning and analysis, as well as spot picking, digestion

and MS analysis followed the procedure reported by Printz et al. (2013). Detailed methods can be found in Supplementary Material 1.

2.4 Metabolomics analysis

Metabolites were extracted according to an adaptation of the procedure previously reported by Weckwerth et al. (2004). Samples were derivatised as described in Furuhashi et al. (2012) and GC–MS measurements were carried out following the protocol of Valledor et al. (2013). Metabolites were identified based on their mass spectral characteristics and GC retention times, by comparison with retention times of reference compounds from an in-house reference library. Peak areas corresponding to each metabolite were normalised to the total peak area in the sample. Detailed methods can be found in Supplementary Material 1.

2.5 Statistical and multivariate analysis

All statistical procedures described were performed using the software R v3.1.2 (R Core Team 2014) core functions plus the package mixOmics v.4.0.2 (González et al. 2011) following the recommendations of Valledor and Jorrin (2011). Detailed methods can be found in Supplementary Material 1.

3 Results

3.1 Proteome and metabolome profiling

The proteome profile of the two *E. globulus* clones after drought and during rewatering was established using proteins extracted from leaves of 32 individuals (4 biological replicates of WW, WS, 1dR and 7dR from clones AL-18 and AL-10). DIGE enabled the separation and isolation of 2031 spots (Supplementary Table S2), 217 of which could be identified (Supplementary Tables S3, S4). Considering the identified proteins, the abundance of 124 was significantly altered because of the watering dynamics and 68 revealed significant differences between the genotypes. The abundance of 32 proteins was significantly affected by interaction between watering dynamics and tree clone.

The foliar metabolic profiles of the two genotypes subjected to drought and rewatering were compiled using GC–MS. The analysis of 24 samples (4 biological replicates of WW, WS and 7dR from clones AL-18 and AL-10) yielded the detection of 121 polar metabolites (Supplementary Table S5). Of these, exact metabolite identities could be assigned to 62, 44 were characterised according to their molecular composition and 15 remained as unknown compounds. From the identified or characterised metabolites, 35 showed significant changes due to the watering dynamics and 15 metabolites were differently abundant between genotypes. Only two metabolites exhibited a clone × watering interaction in their abundance.

3.2 Drought-induced changes in primary metabolism reflect decreased photosynthesis that is rapidly restored after rewatering, and enhanced photo and osmoprotection

At the photosynthetic electron transfer chain level, photosystem II (PSII) reaction centre PSB28, plastocyanin 1 and cytochrome b559 increased in WS, a trend that was maintained during rehydration (Table 1). Ferredoxin–thioredoxin reductase also increased during WS and recovery. Taken together, these results indicate the induction of cyclic electron transfer through PSII, relieving over-reduction of the electron transport chain and thereby suppressing the generation of ^{3}P680* and $^{1}O_2$. The latter occurs under conditions of excess excitation energy caused by a reduced availability of CO_2, lower CO_2 assimilation rates and a lower demand for reducing power (Asada 2006).

Several proteins involved in the Calvin cycle were significantly altered in abundance in response to drought stress (Table 1). The RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) large subunit, plastidic aldolase, transketolase, and phosphoribulokinase were all less abundant under WS conditions, however the RuBisCO small subunit increased in abundance at this sampling point. This reduction in Calvin cycle enzyme abundance was concomitant with the parallel accumulation of ribulose-1,5-bisphosphate (ribulose-1,5-BP). Similarly, fructose, galactose, xylose, arabinose, myo-inositol, ribose, pentose, hexose, and pentitol accumulated during stress (Table 1). The decrease in abundance of photosynthesis-related proteins is a common stress response (Wade et al. 2002; Kottapalli et al. 2009; Sergeant et al. 2011). Beyond that, it is suggested that an overall reduction of photosynthesis-related proteins during water stress may occur in tolerant genotypes that correlated with a rapid decrease in transpiration and photosynthetic rates (Kottapalli et al. 2009). These results in *E. globulus* are in line with results of other *Eucalyptus* described by Warren et al. (2011b).

The RuBisCO large subunit and phosphoribulokinase increased, and RuBisCO activase showed a maximum peak following 1 day of rewatering (1dR, Table 1). Most of the carbohydrates that increased during water deficit diminished after rewatering, whereas pentitol and other unknown

Table 1 Impact of drought and rewatering on proteins associated with photosynthetic electron transport and carbon fixation, and primary carbohydrates

	Relative abundance (WW-WS-1dR-7dR)		Two-way ANOVA		
Protein	AL-18	AL-10	Clone	Water	C x W
Photosystem II reaction centre PSB28			0.169	0.043	0.834
Plastocyanin 1			0.482	0.000	0.587
Cytochrome b559			0.603	0.004	0.743
RuBisCO large subunit			0.379	0.036	0.037
RuBisCO small subunit			0.000	0.000	0.020
Plastidic aldolase			0.909	0.021	0.696
Transketolase			0.003	0.000	0.004
Phosphoribulokinase			0.625	0.000	0.608
RuBisCO activase			0.224	0.043	0.169
Ferredoxin–thioredoxin reductase			0.111	0.001	0.015
	Relative abundance (WW-WS-7dR)		Two-way ANOVA		
Metabolite	AL-18	AL-10	Clone	Water	C x W
Ribulose-1,5-BP			0.882	0.018	0.514
Fructose			0.916	0.000	0.035
Galactose			0.607	0.000	0.129
Xylose			0.967	0.001	0.601
Arabinose			0.483	0.017	0.369
Myo-inositol			0.009	0.032	0.507
Ribose			0.822	0.004	0.111
Pentose			0.880	0.009	0.561
Hexose			0.327	0.000	0.410
Pentitol			0.822	0.002	0.862

Abundance data is presented on a scale relative to the lowest value among sampling points and *p*-values for significance differences dependent on clone, watering regime or the interaction between clone and watering regime (C × W) as estimated by two-way ANOVA are indicated

sugars maintained a high content during rehydration (Table 1). The increased abundance in some spots containing RuBisCO small subunit may indicate either degradation of RuBisCO during exposure to abiotic stress, as reported in other studies (Feller et al. 2008; Sergeant et al. 2011) or it can be related to the different cellular generation between large and small RuBisCO subunits. The maintenance of pentitol and other unspecified sugars after water deficit relief may suggest a strategy to maintain higher osmotic potential to permit stomatal opening and gas exchange under future water stress (Warren et al. 2011a).

3.3 Gene regulation and cell growth were affected by water availability

The impact of drought stress on cell growth was confirmed with a reduction in the relative abundance of actin, which was recovered after prolonged rehydration (7dR, Table 2). Proteome remodelling, and its related metabolism and

growth pattern reprogramming, is the consequence of a complex regulatory process involving signal transduction, gene regulation, and protein biosynthesis and degradation. Regarding signal transduction mechanisms, different proteins were responsive to water availability. Calmodulin, plastid fibrillin 2, and 20 kDa chaperonin increased with water stress (Table 2), representing potential indicators of enhanced cellular oxidation as a result of the environmental stress. Furthermore, transcriptional regulation was also affected: water deficit caused a decline in mRNA binding protein but rewatering led to an increment, also shown by a maximum peak of RNA-binding family protein isoform 2 at 1dR (Table 2).

We were unable to detect changes at the protein translation level as we could not identify ribosomes or eukaryotic initiation factors. On the other hand, we did observe an increase in a 20 kDa chaperonin following water deficit and recovery, indicative of an increased

Table 2 Impact of drought and rewatering on proteins associated with growth, gene regulation and plastid metabolism, and related metabolites

	Relative abundance (WW-WS-1dR-7dR)		Two-way ANOVA		
Protein	AL-18	AL-10	Clone	Water	C x W
Actin			0.184	0.001	0.383
Calmodulin			0.023	0.011	0.009
Plastid fibrillin 2			0.187	0.003	0.415
20/kDa chaperonin, chloroplastic			0.404	0.000	0.977
mRNA-binding protein			0.711	0.015	0.211
RNA-binding family protein isoform 2			0.655	0.000	0.272
Polyubiquitin 4-like protein			0.074	0.001	0.184
Soluble inorganic pyrophosphatase			0.612	0.000	0.579
Malate dehydrogenase, glyoxysomal-like			0.030	0.000	0.102
ATP-dependent Clp protease ATP-binding subunit ClpC			0.157	0.007	0.810
ATP synthase CF1 alpha subunit			0.355	0.012	0.262
ATP synthase D chain, mitochondrial			0.982	0.000	0.686
Glutamine synthetase precursor			0.016	0.006	0.131
	Relative abundance (WW-WS-7dR)		Two-way ANOVA		
Metabolite	AL-18	AL-10	Clone	Water	C x W
Glycine			0.948	0.030	0.601
Serine			0.180	0.132	0.275
Valine			0.303	0.005	0.289
Proline			0.884	0.033	0.068
Alanine			0.191	0.267	0.361
Glyceric acid			0.271	0.027	0.310

Abundance data is presented on a scale relative to the lowest value among sampling points and *p*-values for significance differences dependent on clone, watering regime or the interaction between clone and watering regime (C × W) as estimated by two-way ANOVA are indicated

requirement for protein refolding. Similarly, an increase in the abundance of a polyubiquitin 4-like protein after prolonged recovery (7dR) indicated a potential requirement for protein degradation and turnover during recovery (Table 2).

3.4 Water stressed *E. globulus* exhibit a shift from chloroplastic to mitochondrial energy generation

Our dataset suggested a reduction in cellular metabolism, particularly at the chloroplast level. We observed a decrease in the relative abundances of inorganic pyrophosphatase, malate dehydrogenase, ATP-dependent Clp protease ATP binding subunit ClpC and ATP synthase CF1 following water stress (Table 2). The reduction in the chloroplastic ATP synthase CF1 was accompanied by a rise in the mitochondrial ATP synthase D (Table 2), which may indicate an alteration in the primary site of ATP production. After rewatering, most of these proteins increased to WW or kept their high abundance (Table 2).

3.5 Multiple stress and defence related pathways are involved in alleviating drought stress effects

Several proteins implicated in stress and defence showed an enhanced accumulation during drought and rewatering (Table 3): 2-*cys*-peroxiredoxin, peroxiredoxin, thioredoxin superfamily protein, oxidoreductase, zinc-binding dehydrogenase family protein isoform 1, putative quinone reductase, phi class glutathione *S*-transferase protein and a putative translationally-controlled tumor protein. Thioredoxin and thioredoxin–peroxiredoxin systems are best characterised in plant chloroplasts, where they have been demonstrated to function in the antioxidant water–water cycle as an alternative in place of APX (Asada 2006). Moreover, the maintenance of these proteins during recovery may also be involved in RuBisCO activase modulation in response to rehydration, as was observed for light regulation of RuBisCO (Zhang and Portis 1999). Epoxide hydrolase 2-like, probable protein Pop3 and heat shock protein 70 (HSP 70) declined in WS and increased after rehydration (Table 3).

3.6 Secondary metabolites were less abundant under water stress

Secondary metabolism was negatively affected by the imposed water deficit. Key enzymes required for isoflavonoid (isoflavone reductase) and terpenoid (linalool synthase) biosynthesis were reduced in abundance during water stress (Table 3). Similarly, the abundance of phenolic compounds and their precursors, including shikimic acid, a key precursor for the biosynthesis of aromatic amino acids that form the entry precursor to phenylpropanoid biosynthesis, the hydroxycinnamate 3-*trans*-caffeoylquinic acid, the flavonoid quercetin and the stilbenoid piceatannol were diminished after water stress (Table 3). The majority of the metabolites remained at low concentration following rewatering, although levels of piceatannol increased after the reapplication of water.

3.7 Integrated proteomic, metabolomic and physiological analysis reveal topological differences in correlation networks between the responsive and resilient clone

Drought stress induced clone specific proteome and metabolome profiles in parallel with the previously reported physiological behaviour (Correia et al. 2014b). Clone AL-18 exhibited a greater abundance in many proteins and metabolites, particularly those associated with the Calvin cycle and elements of the photosynthetic electron transfer chain, irrespective of water stress or abundance. A number of stress and defence-related proteins/metabolites also highlighted clonal differences.

To integrate the different *omics* datasets and physiological responses, we applied multivariate analysis integrating previously described physiological measurements (Correia et al. 2014b). This analysis provided us a comprehensive overview of plant stress responses, identifying clone-dependent interaction networks.

Initial comparison based on principal component analysis (PCA) exhibited a clear separation between well-watered and water stressed samples in both *E. globulus* clones (Fig. 1). Further examination of PCA plots indicated that variability assigned to principal component (PC 1) was primarily related to biological variability between individuals. On the other hand, PC2 describes variability due to watering regime where well-watered plants were clearly separated from water-stressed and recovered plants.

The construction of correlation networks based on sparse partial least squares (sPLS) allowed the determination of the specific components behind the observed phenotypical changes considering proteomic, metabolomic and physiological levels. These networks (Fig. 2 a, b) showed clone-specific topologies and provided a comprehensive visualization of the differential responses to stress between the analysed clones. Clone AL-18 (Fig. 2a) showed an inverse correlation of height, biomass, and water potential to the studied pigments, MDA, pentitol and sugars. This connection was mediated by specific aldolases, glutamine synthetase, malate dehydrogenase and phosphoribulokinase. Isoflavone reductase was highly correlated with water potential, having a negative impact on chlorophyll contents. RuBisCO large subunit was positively correlated to

Table 3 Impact of drought and rewatering on proteins associated with stress and defence, and secondary metabolism, and related metabolites

	Relative abundance (WW-WS-1dR-7dR)		Two-way ANOVA		
Protein	AL-18	AL-10	Clone	Water	C x W
2-*cys*-peroxiredoxin, partial			0.031	0.001	0.162
Peroxiredoxin			0.014	0.011	0.119
Thioredoxin superfamily protein			0.006	0.023	0.704
Oxidoreductase, zinc-binding dehydrogenase family protein			0.439	0.003	0.782
Putative quinone reductase, partial			0.210	0.050	0.410
Phi class glutathione *S*-transferase protein			0.957	0.031	0.421
Putative translationally-controlled tumor protein			0.041	0.044	0.479
Epoxide hydrolase 2-like			0.007	0.000	0.199
Heat shock protein 70			0.048	0.001	0.329
Isoflavone reductase homolog Bet v 6.0101			0.013	0.000	0.242
Linalool synthase			0.377	0.025	0.042
	Relative abundance (WW-WS-7dR)		Two-way ANOVA		
Metabolite	AL-18	AL-10	Clone	Water	C x W
Shikimic acid			0.344	0.001	0.699
3-*trans*-caffeoylquinic acid			0.479	0.007	0.733
Quercetin			0.089	0.000	0.490
Piceatannol			0.090	0.020	0.047

Abundance data is presented on a scale relative to the lowest value among sampling points and *p*-values for significance differences dependent on clone, watering regime or the interaction between clone and watering regime (C × W) as estimated by two-way ANOVA are indicated

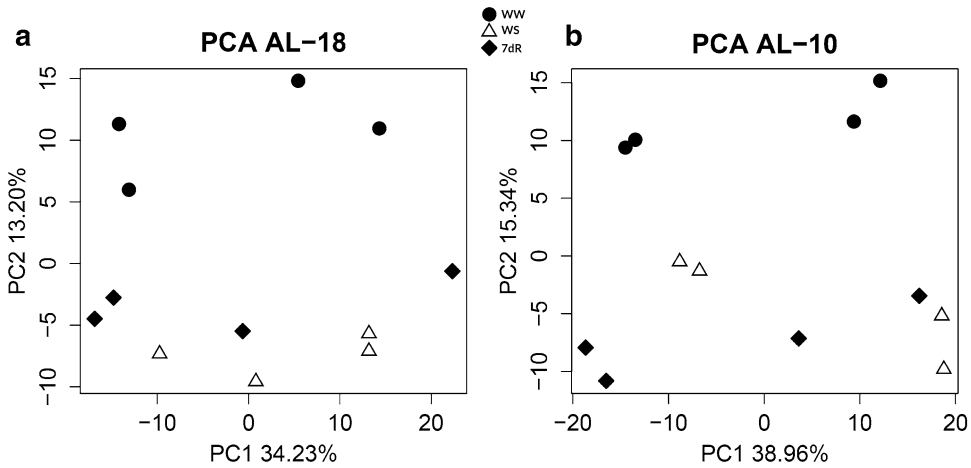
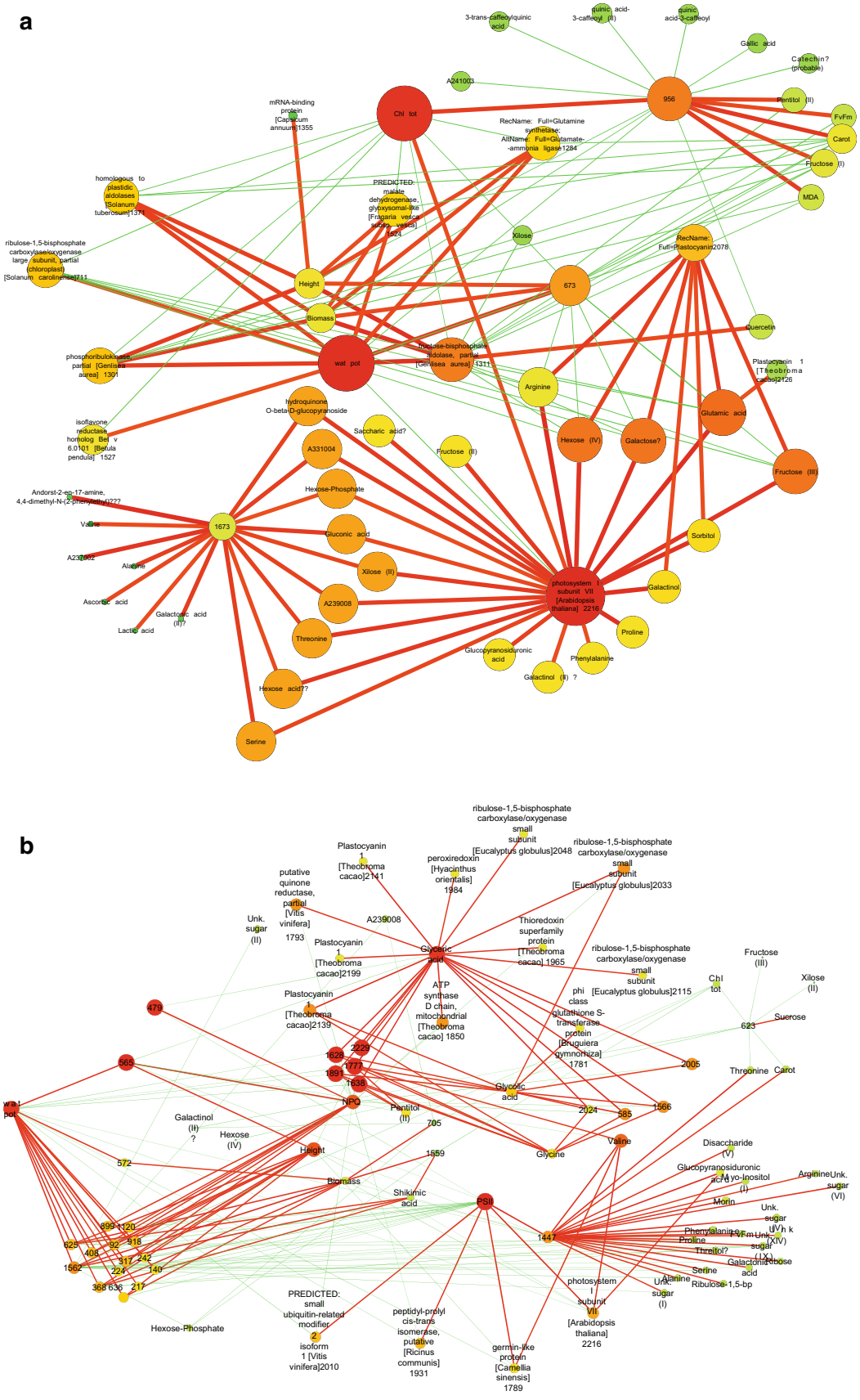


Fig. 1 Principal Component Analysis of the AL-18 (**a**) and AL-10 (**b**) datasets. First two components are plotted in the main graph. The proportion of variance explained by each component is indicated on *axes* labels

water potential and negatively correlated to sugars and glutamic acid. Glutamic acid, arginine, galactose, hexose and fructose were positively correlated with plastocyanin and photosystem I subunit VII.

The interaction network of clone AL-10 (Fig. 2b) showed a structure different from AL-18, with glyceric acid, operating quantum yield of PSII (Φ_{PSII}), and water potential amongst the nodes showing higher centralities.


a
3-trans-caffeoylquinic acid
quinic acid-3-caffeoyl (II)
quinic acid-3-caffeoyl
Gallic acid
Catechin? (probable)
A241003
956
Pentitol (II)
FvFm
Carot
Fructose (I)
MDA
mRNA-binding protein [Capsicum annuum]1355
Chl tot
RecName: Full=Glutamine synthetase; AltName: Full=Glutamate--ammonia ligase1284
homologous to plastidic aldolases [Solanum tuberosum]1371
PREDICTED: malate dehydrogenase, glyoxysomal-like [Fragaria vesca subsp. vesca]1624
Xilose
RecName: Full=Plastocyanin2078
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Solanum carolinense]711
Height
673
Biomass
Quercetin
phosphoribulokinase, partial [Gentiana lutea] 1391
wat pot
ribulose-bisphosphate aldolase, partial [Gentiana lutea] 1311
Plastocyanin 1 [Theobroma cacao]2126
Arginine
hydroquinone O-beta-D-glucopyranoside
Glutamic acid
isoflavone reductase homolog Bet v 6.0101 [Betula pendula] 1527
Saccharic acid?
Hexose (IV)
Galactose?
A331004
Fructose (II)
Fructose (III)
Andorst-2-en-17-amine, 4,4-dimethyl-N-(2-phenylethyl)?22?
Hexose-Phosphate
Valine
1673
Gluconic acid
Sorbitol
A230002
Alanine
Xilose (II)
photosystem I subunit VII [Arabidopsis thaliana] 2216
Galactinol
Ascorbic acid
A239008
Galactonic acid (II)?
Lactic acid
Threonine
Proline
Glucopyranosiduronic acid
Phenylalanine
Galactinol (II) ?
Hexose acid??
Serine
b
ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [Eucalyptus globulus]2048
Plastocyanin 1 [Theobroma cacao]2141
peroxiredoxin [Hyacinthus orientalis] 1984
ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [Eucalyptus globulus]2033
putative quinone reductase, partial [Vitis vinifera]
Unk. sugar (II)
1793
A239008
Thioredoxin superfamily protein [Theobroma cacao] 1965
Plastocyanin 1 [Theobroma cacao]2199
Glycolic acid
ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [Eucalyptus globulus]2115
Fructose (III)
Chl tot
Xilose (II)
Plastocyanin 1 [Theobroma cacao]2139
ATP synthase D chain, mitochondrial [Theobroma cacao] 1850
phi class glutathione S-transferase protein [Bruguiera gymnorhiza] 1781
Suerose
623
478
2229
565
1626
1773
2005
Threonine
Carot
1891
1638
wat pot
NPQ
Glycolic acid
Pentitol (II)
2024
1566
585
705
Galactinol (II) ?
Hexose (IV)
Valine
Disaccharide (V)
Height
1659
Glycine
572
Glucopyranosiduronic acid
Arginine
Unk. sugar (VI)
Biomass
Shikimic acid
PSII
Myo-Inositol (I)
Morin
Unk. sugar (XIV)
699
1120
1447
Phenylalanine
FvFm
Unk. sugar (IX)
629
92
918
Proline
Threitol?
408
317
242
Galactonic acid
Ribose
1562
224
140
Serine
Ribulose-1,5-bp
Alanine
Unk. sugar (I)
368
636
217
photosystem I subunit VII [Arabidopsis thaliana] 2216
PREDICTED: small ubiquitin-related modifier 2 isoform 1 [Vitis vinifera]2010
peptidyl-prolyl cis-trans isomerase, putative [Ricinus communis] 1931
germin-like protein [Camellia sinensis] 1789
Hexose-Phosphate

◀**Fig. 2** sPLS-based interaction network of water deficit and rehydration in clones AL-18 (**a**) and AL-10 (**b**). Network is presented using the Force Directed layout, which is based on the "force-directed" paradigm. *Colour and size* of nodes illustrate radiality on a *small* to *large* and *green to red scale* (radiality of a node is calculated by computing the shortest path between the node and all other nodes in the graph); *colour and size* of edges reflect weight with *red and green edges* illustrating positive and negative correlations, respectively

Among correlations, RuBisCO small subunit, plastocyanin 1, phi (II) class glutathione *S*-transferase and other unidentified proteins positively correlated with glycolic acid. Many of the aforementioned unidentified proteins and plastocyanin I also correlated with glyceric acid. Small ubiquitin-related modifier 2 isoform 1 and peptidyl-prolyl *cis-trans* isomerase are positively correlated with Φ_{PSII} and negatively correlated with water potential and NPQ.

The potential biological implications of the detected relationships are explored in the discussion section.

4 Discussion

In this work, we combined an analysis of the proteome and metabolome with previously obtained physiological parameters in two *E. globulus* clones under drought and rehydration. The physiological characterisation of the response of the studied clones to drought and recovery demonstrated different degrees of tolerance (Correia et al. 2014b). We identified clone AL-18 as resilient and less reactive to water availability, and AL-10 as more sensitive and responsive in responding to water shortage and restoration. These differences were mainly related to growth, carotenoids, effective quantum yield of PSII, non-photochemical quenching and photosynthetic rate, which rapidly varied in response to water availability in clone AL-10, while clone AL-18 took more time to respond (Correia et al. 2014b).

Through a comprehensive analysis of the proteomic and metabolomic datasets, we can now identify mechanisms behind these altered physiological responses, which confirmed previous knowledge but, most importantly, revealed novel responsive mechanisms.

First, we have confirmed several reported results that include decreased photosynthesis, disrupted cell growth, and alterations in gene regulation, in parallel with enhanced photo and osmoprotection, and the activation of other stress and defence related pathways (Wade et al. 2002; Chaves et al. 2003; Kottapalli et al. 2009; Brossa et al. 2015; Kattam et al. 2016). However, our results also indicate novel responses that have not been reported previously. These include a shift of energy metabolism away from chloroplasts and towards mitochondria as evidenced by changes in the abundance of subunits of chloroplastic or mitochondrial ATP synthases. Similarly, the observation that secondary metabolism and particularly phenylpropanoid metabolism was downregulated by drought stress is not commonly reported. Phenylpropanoids are implicated in defence against biotic stresses (Cheynier et al. 2013) and hence this result has significance in terms of the impact of abiotic stress on biotic interactions. For example, it has previously been reported that water stress can negatively influence defence against aphid infestation (Foyer et al. 2016).

Second, we verified that rehydration is mainly characterised by a reversion of the altered parameters, well-illustrated by our PCA analysis and once again confirming our physiological profiles: scores from recovered (4R) plants tend to migrate to WW scores, with AL-10 scores keeping farther from the WW plants relative to AL-18.

Finally, we were able to unveil interesting *E. globulus* responses described before (Correia et al. 2014b) such as overcompensation of CO_2 assimilation rate after rehydration, and the different response profiles displayed by the clones, more easily understood after integration with *omics* datasets and network analysis.

Considering CO_2 assimilation rate, we hypothesise that it can be explained by a tightly controlled mechanism involving specific proteins of the Calvin cycle and photosynthetic electron transfer chain, which together with several defence-related proteins act to prevent oxidative damage to the photosynthetic machinery, reducing photosynthesis as the water deficit occurs, and later to reinforce photosynthetic rates. Our hypothesis is based on our results that show a specific relation among RuBisCO, RuBisCO activase, phosphoribulokinase and proteins of the ferredoxin–thioredoxin systems.

A decrease in photosynthetic enzymes in conjunction with an increase in thiol-mediated defence systems prevents oxidative damage during stress. The positive induction of both photosynthetic and redox defence systems during recovery is probably responsible for the re-activation of photosynthesis at several levels: photosynthesis is already known to be regulated through phosphoribulokinase/ferredoxin/thioredoxin system (Wolosiuk and Buchanan 1978) or to respond to light modulation by RuBisCO activase/thioredoxin (Zhang and Portis 1999), processes possibly occurring after rehydration and leading to an overcompensation of CO_2 assimilation. Interestingly, although both clones exhibited a reduced glycine to serine ratio, indicative of reduced photorespiration, following drought stress and recovery, this value was higher for the resilient clone AL-18, suggesting higher photorespiration under conditions of abiotic stress, possibly as protective sink against excess oxidative stress.

These global responses are differently triggered in each clone and lead to differences in tolerance to drought stress

as reflected in the interaction networks. These plots illustrated the different physiological and biochemical rearrangements induced by drought in the two studied clones, confirming previous results and providing additional information concerning the underlying molecular causes of observed physiological differences.

Focusing our attention in the network relative to clone AL-18, we found the involvement of key proteins, namely specific aldolases, glutamine synthetase, phosphoribulokinase, isoflavone reductase, malate dehydrogenase and RuBisCO large subunit, mediating a close inverse correlation among height, biomass and water potential, on one hand, and pigments, MDA, pentitol and sugars on the other hand. These results strengthen our conclusions that specific photosynthetic and defence-related proteins are key players in mediating drought tolerance in *E. globulus* and reveal new players. Recently, Kaminski et al. (2015) suggested that cytosolic glutamine synthetase genes are important for limiting nitrogen loss due to photorespiration in potato under well-watered conditions possibly by improving photosynthetic and water use efficiency. The involvement of glutamine synthetase was also reported by Bernard and Habash (2009), which found that cytosolic glutamine synthetase overexpression enhances water stress resistance by promoting photorespiratory activity, and providing a protective sink for electrons from photosynthetic reaction centres. Confirming this, malate dehydrogenase is also described as significant regulator of respiratory rate in plants (Tomaz et al. 2010) and appears with a role in adaptation to drought stress (Pastore et al. 2007). Finally, the down-regulation of isoflavone reductase-like protein was also described as a novel drought-responsive mechanism (Wade et al. 2002).

Interestingly, regarding the network relative to clone AL-10, we found a completely different scenario: not only none of these "tolerance players" is present, not reflecting any pathways described above, as the correlations found are weaker and poorly supported by physiological markers. The lack of activation of stress-responsive pathways in AL-10 may explain the increased sensitivity to drought compared to AL-18. Moreover, the observed correlations among glyceric acid and several plastocyanins and antioxidant-related proteins, coupled with the involvement of several key amino acids (valine, proline and alanine) appears to be more related to increased salinity tolerance rather than drought (Sanchez et al. 2008).

5 Conclusion

We reported here a forward-looking approach that enabled us to go further than a classic descriptive analysis of *omics* results and look for an integrative and meaningful view of the biological events that take place not only during water deficit but also after rewatering. This approach represents a powerful tool for monitoring changes in response to environmental disturbances (Kottapalli et al. 2009; Liu et al. 2013; Scalabrin et al. 2015), and in our hands allowed not only to survey of global biological alterations that characterise *E. globulus* response to drought and rewatering (i), but also to successfully identify specific strategies that make a clone more adapted to water deficit conditions (ii) (Fig. 3).

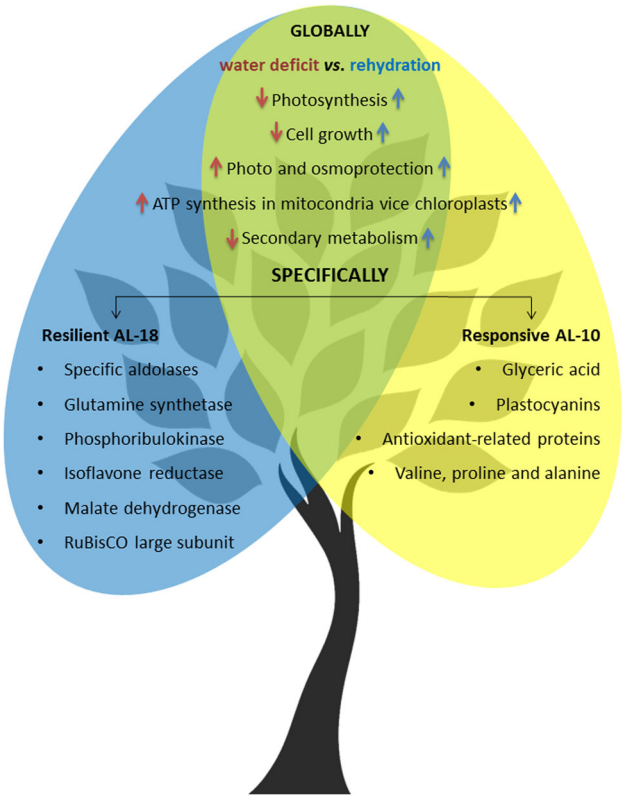


Fig. 3 Schematic illustration of the main conclusions of the work. Global pathways altered by water deficit and rehydration responses in *E. globulus* are indicated by *red arrow and blue arrows*, respectively. The direction of the *arrows* indicates whether pathways are up- or down-regulated. Also indicated are elements of the specific responses of clones AL-18 and AL-10 achieved following network analysis and highlighting key players that mediate each response profile

This work increases our understanding of drought tolerance in *E. globulus* and it may lead to applications in breeding for enhanced drought tolerance in this and other species. Specifically, this study provides a set of new relevant proteins and pathways involved in drought stress that can be the subject of further research in order to check their relevance as tolerance markers for early selection.

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Compliance with ethical standards

Conflict of interest The authors declare that no competing interests exist.

Ethical approval This article does not contain any studies with human or animal subjects.

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