A cold change: how short low temperature exposure affects primary metabolism in leaves and stems of two eucalyptus species

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Abstract Plants often modify their metabolism in order to regain homeostasis and maintain survival in the face of stressful conditions. Here, two species of eucalyptus, *E. globulus* and *E. grandis* (adapted and non-adapted to low temperature, respectively), were exposed to either 10 °C or 25 °C over 24 h, and changes in gene expression and metabolite levels were analyzed. The aim of this experiment was to investigate the dynamic of short period changes in the energy metabolism of source (leaves) and sink (stem) tissues

in these contrasting species regarding low temperature. We expected to observe a distinct pattern on carbon metabolism and source-to-sink relationship between both species which would be related to their different vegetative responses when facing low temperatures. In that way, *E. globulus* plants showed a differential expression in leaves and stems of SnRK1 genes system (responsible for energy availability control in plants), that was strongly associated to the changes in carbon metabolism and the main difference between the response when both species face cold. Taken together, the results suggest that low temperatures (10 °C) are able to increase the sink strength of stem tissues and the carbon assimilation in leaves of *E.*

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globulus, supporting a higher vegetative growth rate. In *E. grandis*, on the other hand, exposure to 10 °C promoted a higher consumption of carbon skeletons without better growth rate as a counterpart, suggesting that under cold conditions, these two eucalyptus species differ in the way they coordinate the interaction between the activation of SnRK1 system and primary metabolism in source and sink tissues.

Keywords Eucalyptus · SnRK1 system · Primary metabolism · Cold stress

Abbreviations

BCAAs	Branched-chain amino acids
CDD	Conserved domains database
CTAB	Cetyl trimethylammonium bromide
EUCAGEN	Eucalyptus Genome Network
GC–MS	Gas chromatography–mass spectrometry
GMD	Golm metabolome database
IDH	Isocitrate dehydrogenase
KIN10	Kinase protein 10
KIN11	Kinase protein 11
PC	Principal component
PCA	Principal components analysis
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
SnRK1	SNF-related kinase 1
TCA	Tricarboxylic acid cycle
TOR-1	Target of rapamycin kinase-1

1 Introduction

Changes of irradiance and temperature imposed by movements of the Earth are two of the main external events capable of altering plant development (Kooke and Keurentjes 2011; Shin et al. 2017; Tarkowski and Van den Ende 2015). In this sense, although there are differences between later physiological and metabolic responses when plants face abrupt or gradually decreasing temperatures, the molecular pathways firstly activated in response to cold stress are common to both cases, being more sensitive to the range of the minimum temperature achieved (Zarka et al. 2003; Kidokoro et al. 2017; Hao et al. 2018). Cold responses strongly differ if the temperature drops down below ice formation, promoting a freezing stress condition,

or if it ranges between 0 and 15 °C, characterized as chilling events (Fenollosa et al. 2018; Hao et al. 2018). Under natural conditions, plants growing in tropical and subtropical zones are more likely to be exposed to chilling events, affecting how they respond to those seasonally events and grow during the year (Fenollosa et al. 2018). Therefore, during autumn and winter seasons, massive changes in gene expression patterns, enzymatic activities, and specific metabolite concentrations tend to slow down plant growth rates, allowing them to decrease energy consumption and survive these harsh periods of the year (O’Brien et al. 2014; Budzinski et al. 2016; Hoermiller Imke et al. 2017). A well-known characteristic of cold-tolerant plants is their ability to continuously provide sugars and other energetic substrates to non-photosynthetic tissues in a minimally altered manner, allowing relatively minor changes in vegetative development during winter (Heidarvand et al. 2017; Hoermiller Imke et al. 2017; O’Brien et al. 2014).

Several other physiological events are regulated by sugar signaling such as those downstream to the *Target of rapamycin kinase-1/SNF-related kinase 1* (TOR-1/SnRK1) system. The TOR-1/SnRK1 system is responsible for controlling the availability of nutrients, integrating a wide variety of environmental and endogenous signals and regulating anabolic and catabolic processes in order to maintain energy balance and sustain an optimal growth rate (Caldana et al. 2013; Tomé et al. 2014; Williams et al. 2014; Wurzing et al. 2018). While the TOR-1 portion of the complex shows high activity in meristematic tissues, the SnRK1 portion is active in both source and sink tissues of plants (Tomé et al. 2014; Williams et al. 2014; Wurzing et al. 2018). The SnRK1 portion, whose catalytic subunit is composed of the kinase proteins KIN10 and KIN11, is associated with the activation of metabolic processes that increase the amount of available energy, such as higher photosynthetic activity and catabolism, especially in situations of elevated metabolic demand (Baena-Gonzalez et al. 2007; Schluepmann et al. 2011). Additionally, the activity of SnRK1 also depends on the tissue type analyzed. At first, the catabolic behavior promoted by SnRK1 complex may appear to be counter-intuitive in heterotrophic tissues, which require a plenty of substrates available while promote their growth (Schluepmann et al. 2011; Wu and Birch 2010; Wurzing et al. 2018). Nonetheless, source-sink dynamics must

be taken into account, in those tissues: when the amount of available sucrose is sufficiently high, SnRK1 activity is inhibited, activating anabolic pathways required for growth. However, as sucrose is used as a substrate for growth, its content falls and thus the activity of SnRK1 increases, facilitating remobilization of stored carbon skeletons to enable the normal growth of these tissues (Bechtold and Field 2018; Halford and Hey 2009; Schluepmann et al. 2011; Wu and Birch 2010). In leaves, due to their high photosynthetic rate, the amount of sucrose tends to be high during the day, inhibiting the activity of SnRK1. However, if a plant is subject to conditions that limit its photosynthesis such as saline and thermal stresses, activation of SnRK1 system and the promotion of pathways favoring a greater carbon fixation are observed (Baena-Gonzalez et al. 2007; Bechtold and Field 2018; Schluepmann et al. 2011).

Cell wall formation represents an important physiological event in plants and carries a high energy burden (Allahverdiyeva et al. 2015; Cerasuolo et al. 2016; Osakabe et al. 2013). In woody plants, cell wall may account for up to 50% of the total dry mass, mostly represented by the high investment in stem growth (Arias et al. 2014; Campoe et al. 2013; Cerasuolo et al. 2016; Osakabe et al. 2011). Currently, species and hybrids of eucalyptus (genus *Eucalyptus*, family Myrtaceae) are the main forest crop supplying wood for the cellulose pulp and paper industry (Araújo et al. 2014; Budzinski et al. 2016; Campoe et al. 2013; Turnbull and Booth 2002). *E. grandis* is amongst the most globally cultivated eucalyptus species and it is valued for its rapid growth, whereas *E. globulus* is characterized by a better wood quality (related to cellulose yield) and higher productivity compared to *E. grandis* when both species grow under low temperature conditions (Budzinski et al. 2016; Fernández et al. 2010; Ruedell et al. 2013).

The objective of this work was to evaluate the main changes in energy metabolism of *E. globulus* and *E. grandis* exposed for 24 h to 10 °C and identify differential responses in primary metabolism of leaf and stem tissues, representing source and sink photoassimilates tissues, respectively. We expected to identify patterns associated to each tissue and how they change under low temperature, and the impact on the energy availability according to catabolic and anabolic regulations. Therefore, we could observe that the differential stem growth of *E. globulus* compared

to *E. grandis* when both species grow under cold environments (Domingues-Junior et al., MS in preparation; Costa e Silva et al. 2009; Fernández et al. 2010; Goulao et al. 2011; Kirst et al. 2004; Mora and Serra 2014; Ruedell et al. 2013) is related to a distinct carbon metabolism in leaves and stems, which differs from the source-to-sink relationship observed in *E. grandis* at the same condition.

2 Material and methods

2.1 Plant material and low temperature treatment

Seeds of *E. globulus* and *E. grandis*, obtained from clonal gardens (respectively GloPirAPSF1 and GraPirAPSF2, Caiçara Sementes—<http://www.sementescaicara.com>, Araújo et al. 2018) were germinated and cultivated in the greenhouse for 34 weeks. Eight weeks before the beginning of the treatment, plants were transferred to a growth chamber with 12:12 light/dark photoperiod, 500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ and average temperature of 25 °C. After this acclimation period, plants with similar visual height and stem diameter were randomly divided into two groups: (a) control, kept in the same conditions of the acclimation period; and (b) cold treatment, where plants were kept under the same photoperiod and light condition, but the chamber temperature was set to 10 °C, starting with the light period. Leaf and stem samples were harvested at regular intervals: (I) 0.5 h; (II) 6 h; (III) 12 h; (IV) 18 h and (V) 24 h after the change in temperature and immediately frozen in liquid nitrogen. Throughout the text we referred to the light period as day and to the dark period as night. Stem samples, counted from the apex to the leaf at height of the fifth node, were collected, as well as all the leaves of this portion. Frozen plant material was stored at – 80 °C until further analysis. For all analyses, five biological replicates were used, each representing material pooled from five independent plants.

2.2 Sequence analysis in Forest database and primer design

The databases *Forest*, *Eucalyptus Genome Network* (EUCAGEN, <http://www.eucagen.org>) (Mizrachi et al. 2010) and *Phytozome* (<http://www.phytozome.net/>) were used to search for homologues of *AtKIN10*

genes in eucalyptus. The search for sequences was done using conserved regions of the same genes, obtained through the analysis of sequences of *Populus* and *Arabidopsis*, found in NCBI database (<http://www.ncbi.nlm.nih.gov/>). The amino acid sequences for KIN10 homologous of *Arabidopsis thaliana* (AEE73609.1; AEE77544.1), *Mesembryanthemum crystallinum* (AFN89137.1), *Malus hupehensis* (ABV49061.1), *Nicotiana benthamiana* (AAX12442.1), *Pisum sativum* (CAI96819.1), *Populus trichocarpa* (XP_002306053.1; XP_002319341.1), *Solanum berthaultii* (AGF39571.1), *Solanum lycopersicum* (NP_001233965.1), *Vicia faba* (CAI96818.1) and *Zea mays* (ACG33875.1; NP_001105673.1) were aligned with the eucalyptus sequences identified here, using the ClustalW included in MEGA 5 software to perform this alignment (Tamura et al. 2011). Next, this alignment was used to construct a phylogenetic tree according to the neighbor-joining algorithm (Saitou and Nei 1987). Bootstrap values were obtained after 5000 replications. Additionally, the online tools *Blastx* (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and *Conserved Domains Database—CDD* (<http://www.ncbi.nlm.nih.gov/cdd/>) were also used to search for KIN10 domains of *Arabidopsis thaliana* in sequences obtained from Eucalyptus.

The primers for eucalyptus *KIN10s* genes were designed using CAPE algorithm (BioEdit software) (Hall 1999) and CLUSTALX software (Thompson et al. 1994). Table S1 shows the primer sequences of the genes analyzed in the present study.

2.3 Gene expression analysis

The cetyl trimethylammonium bromide (CTAB) method (Quang et al. 2012) was used to extract total RNA, which was further treated with rDNase I (RNase-Free, Bioagency) and quantified in a Qubit fluorometer (Invitrogen). The quality of RNA was inspected in 1% agarose gel electrophoresis with ethidium bromide and visualization under UV light. cDNA was obtained using SuperScript^R VILOTM cDNA synthesis (Invitrogen).

Analyzes by qRT-PCR were made in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) using SYBR green PCR Master Mix (Applied Biosystems). Each sample was analyzed with three technical replicates in a reaction mix containing 20 µL

of reverse-transcribed cDNA from 5 ng total RNA. Isocitrate dehydrogenase (IDH) gene was used as constitutive gene as recommended by Moura et al. (2012) (Table S1) and the relative expression was presented using the method $2^{-\Delta\Delta C_t}$ (Figs. 1, 2) and $2^{-\Delta C_t}$ (Figures S4 and S5), according to Livak and Schmittgen (2001).

2.4 Extraction and identification of major polar metabolites

Primary metabolites extraction and derivatization were carried out in the same samples used for molecular analysis, following the protocol described by Lisec et al. (2006). Briefly, 50 mg of frozen plant material (leaves and stems) were used for each biological sample. Extraction in 700 µl of methanol per sample, with shaking at 70 °C, was performed for 1 h. Ribitol was used as internal standard. After centrifugation at 15,300×g, the supernatant was taken, following by the separation of polar and apolar phases by adding chloroform (300 µl) and water (300 µL) to the supernatant. After a new centrifugation, 1 mL of the polar phase was taken and reduced by dryness. Derivatization and analysis by gas chromatography coupled to mass spectrometry (GC–MS) were performed according to the same work (Lisec et al. 2006). Data were normalized and transformed as described by Lisec et al. (2006) and Caldana et al. (2013). Total protein (Bradford 1976) and starch content (Critchley et al. 2001) were analyzed according to standard protocols described in the literature.

2.5 Statistical analyses

Results were expressed as mean ± standard error and were compared by the Tukey test at 5% probability. The relationship between gene expression and identified metabolites were evaluated through Pearson correlation test at 5% probability (Ruprecht and Persson 2012). For both analyzes, we used GraphPad Prism software, version 8.00 for Windows (Microsoft), GraphPad Software, San Diego, California, USA. Principal components analysis (PCA) was carried out using R software version 2.12.2. (<http://www.r-project.org>), with criteria established by Ruprecht et al. (2011).

Fig. 1 Relative expression of the genes (a), (c) *KIN10B* and (b), (d) *KIN10C* in (a), (b) leaves and (c), (d) stems of *E. globulus* and *E. grandis* harvested over 24 h of exposure at 10 °C. Letters indicate statistical differences between species within the same harvesting time and numerals indicate

differences between harvesting times within the same species and treatment. Experiment performed with triplicates. Differences among experimental groups evaluated by One-way ANOVA with Tukey test at 5% probability. Error bars: standard error

Fig. 2 Content of (a) starch and (b) total proteins in leaves of *E. globulus* and *E. grandis* maintained at 10 °C and 25 °C over 24 h. Capital letters indicate statistical differences between species within the same harvesting time and treatment; lower case letters indicate differences between treatments for the same harvesting timepoints and species; numerals indicate

differences between harvesting timepoints within the same species and treatment. Experiment performed with five biological samples. Differences among experimental groups evaluated by One-way ANOVA with Tukey test at 5% probability. Error bars: standard error

3 Results

3.1 Identification of Eucalyptus genes homologous to *AtKIN10*

Two complete cDNA sequences of *Eucalyptus grandis* genes homologous to *AtKIN10* (*EgKIN10B* and *EgKIN10C*) were recovered from search of public databases. Using the online tools *Blastx* (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and *Conserved Domains Database—CDD* (<http://www.ncbi.nlm.nih.gov/cdd/>) it was possible to identify *Arabidopsis thaliana* domains in sequences obtained from Eucalyptus (Figures S1 and S2), suggesting their homology to *AtKIN10*. Additionally, comparisons of the deduced protein sequence with sequences already characterized in *A. thaliana*, *Mesembryanthemum crystallinum*, *Malus hupehensis*, *Nicotiana benthamiana*, *Pisum sativum*, *Populus trichocarpa*, *Solanum berthaultii*, *Solanum lycopersicum* and *Zea mays* emphasized this fact (Figures S2 and S3).

3.2 Expression of *KIN* genes

KIN10B showed higher expression in leaves of *E. globulus* than in *E. grandis* in response to cold (Fig. 1a and Fig. S4A). Moreover, leaves of *E. globulus* showed a considerable daily variation in *KIN10B* expression with a maximum soon after the onset of exposure to cold, a subsequent fall throughout the day and a new increase just before dawn of the next day. *KIN10C* (Fig. 1b and Fig. S4B) had a more modest relative expression and few changes throughout the day in leaves of *E. globulus* when exposed to cold; the same occurs in *E. grandis* during the light period, with an increased expression during the first 6 h of dark and subsequent decline until the end of cold treatment.

The expression profiles of *KIN10B* and *KIN10C* genes were similar in stems of *E. grandis* plants (Fig. 1c, d and Figs. S5A and S5B, respectively); a maximum relative expression was observed after 18 h of exposure to cold and few differences were observed at other harvest times. Both genes also showed a similar pattern in *E. globulus* stems exposed to cold with: higher values during the daytime period, and a reduction of the relative expression in transition to night, with a second increase at the end of treatment.

3.3 Primary metabolism

3.3.1 Macromolecules quantification: starch and proteins

In general, irrespective of the thermal treatment, *E. globulus* had less starch in leaves when compared to *E. grandis* during the day, with the inverse behavior observed during the night (Fig. 2a). Moreover, *E. globulus* leaves exhibited lower oscillation in starch content over 24 h compared to *E. grandis*. By contrast, leaves of *E. grandis* exposed to 10 °C showed a marked decrease in the starch content, with the lowest level observed after 24 h of treatment.

While the amount of foliar protein in *E. globulus* did not dramatically change over 24 h in the control (25 °C) and treated (10 °C) plants (Fig. 2b), leaves of *E. grandis* generally exhibited higher leaf protein content than *E. globulus* at both temperatures.

In stems, starch (Fig. 3a) and total protein (Fig. 3b) contents were higher in *E. grandis* samples when compared to *E. globulus*, regardless of harvest time-point or thermal treatment. *E. grandis* had the highest accumulation of starch 6 h after beginning of cold treatment, and later falling during the night period. *E. globulus* also showed maximum accumulation at 6 h of cold exposure.

Total protein content of both species oscillated less in stem samples when compared to leaves. For *E. globulus*, the main difference between thermal treatments was the moment when protein levels were at their lowest: plants exposed to 10 °C showed a reduction of total protein content in transition from day to night (12 h after the start of treatment), while the same occurred 6 h earlier in plants exposed to 25 °C.

3.3.2 Primary metabolome analyses

Using these same samples, characterization of several primary metabolites in polar extracts was carried out by GC–MS, identifying 44 compounds (Figs. 4, 5, Figures S4 to S7 and Dataset S1): 12 amino acids, 12 carbohydrates, eight organic acids, six phenolic compounds, four polyols and two miscellaneous compounds (putrescine and phosphoric acid).

In general, 10 °C exposure mostly affected leaf amino acids and carbohydrates, with a large reduction in isoleucine, leucine and valine levels occurring in

Fig. 3 (a) Starch and (b) total proteins contents in stems of *E. globulus* and *E. grandis* maintained at 10 °C and 25 °C over 24 h. Capital letters indicate statistical differences between species within the same harvesting time and treatment; lower case letters indicate differences between treatments for the same harvesting timepoints and species; numerals indicate

differences between harvesting timepoints within the same species and treatment. Experiment performed with five biological samples. Differences among experimental groups evaluated by One-way ANOVA with Tukey test at 5% probability Error bars: standard error

leaves of *E. globulus* at the end of the 24 h of exposure to cold (Fig. 4). While leaf sucrose levels were similar irrespective of species, harvest timepoints or thermal treatment (Fig. 6b), glucose content differed between the two species. *E. globulus* displayed increase of glucose levels shortly after 6 h of treatment, with subsequent stabilization in plants exposed to 10 °C (Fig. 6a) and subsequent decrease in plants exposed at 25 °C. *E. grandis* displayed the reverse response, while plants kept at 10 °C showed an increase in glucose content only at the end of the night, the same compound accumulated at higher levels at 25 °C during the first harvest timepoint, with a subsequent fall and stabilization (Figs. 4, 5, 6).

Less intense changes throughout the day were observed for organic acid profiles in leaves (Dataset S1). However, a reduction of compounds of the tricarboxylic acid (TCA) cycle, i.e., citrate, malate and, in a lesser extent, succinate, is notable in *E. globulus* samples harvested in the late evening (Fig. 4 and S4). When both species were maintained at 10 °C, larger amounts of citrate and malate were observed at the end of the night period.

In stems, many amino acids and carbohydrates were present in larger amounts early in the morning with a reduction in content occurring throughout the day, with this pattern being more intense for amino acids of *E. globulus* and for carbohydrates of *E. grandis* when both species were kept at 25 °C (Fig. 5, S6 and S7 and Dataset S1). Additionally, less severe oscillations than those presented in leaves also occurred among organic acids, phenolic compounds, polyols and other compounds identified in stems samples, regardless of species and temperature treatment (Figures S6 and S7).

The levels of the major sugars present in stems of both species (Fig. 7) oscillated more intensely than in leaves, responding to the variations of the day and the environmental temperature to which plants were exposed. Glucose content (Fig. 7a) in *E. globulus* stems was reduced during night for both temperature treatments, with more pronounced reduction in plants exposed to 25 °C. Throughout the day, accumulation of this hexose was less intense in stems exposed to 10 °C. Samples of *E. grandis* displayed opposite pattern of change, with plants kept at 10 °C showing

Significant negative relationships ($-0.6 < R < -0.3$) between starch and sucrose levels were identified in leaves of *E. globulus* exposed to 10 °C, while its relations with glucose and fructose was strongly positive ($+0.6 < R < +1.0$) at the same temperature condition for this specie at 10 °C. For *E. grandis*, starch only correlated positively (+

Fig. 5 Main metabolites identified in stem samples of *E. globulus* (upper squares) and *E. grandis* (lower squares), according to the harvest time. Values relative to control treatment (25 °C). Shades of blue indicate higher relative values observed in samples exposed to cold nights; red shades indicate

lower relative values observed in samples exposed to cold nights. Compounds in gray color were not identified. Abbreviations of the compounds are identified in Table S1. (Color figure online)

0.6 < R < + 1.0) with *KIN* gene expression at the same thermal treatment.

In stems of *E. globulus* plants exposed to 10 °C, the total protein content positively correlated (+ 0.6 < R < + 1.0) with the amount of different amino acids (i.e., BCAAs, asparagine, phenylalanine and tryptophan), as well as some carbohydrates (i.e., glucose, maltose and mannose). In relation to starch content, there were practically no significant

relationships with any other metabolic parameters for *E. globulus* in both treatments.

A principal component analysis (PCA) was performed with metabolome dataset obtained (leaves represented in Figures S8 and stems in Figure S9). A clear distinction of each experimental group was observed for any combination of the three main principal components (PCs). For leaves, the differences between the thermal treatments predominate (i.e., factor best explained by PC1, Figure S8), while

Fig. 6 Relative content of (a) glucose and (b) sucrose in leaves of *E. globulus* and *E. grandis* maintained at 10 °C and 25 °C over 24 h. Capital letters indicate statistical differences between species within the same harvesting time and treatment; lower case letters indicate differences between treatments for the same harvesting timepoints and species; numerals indicate

differences between harvesting timepoints within the same species and treatment. Experiment performed with five biological samples. Differences among experimental groups evaluated by One-way ANOVA with Tukey test at 5% probability Error bars: standard error

Fig. 7 Relative content of (a) glucose and (b) sucrose in stems of *E. globulus* and *E. grandis* maintained at 10 °C and 25 °C over 24 h. Capital letters indicate statistical differences between species within the same harvesting time and treatment; lower case letters indicate differences between treatments for the same harvesting timepoints and species; numerals indicate

differences between harvesting timepoints within the same species and treatment. Experiment performed with five biological samples. Differences among experimental groups evaluated by One-way ANOVA with Tukey test at 5% probability Error bars: standard error

for stems (Figure S9), interspecific differences provide the best separation in PC1. Moreover, it should be noted that, for stem samples thermal, differences were well explained by the components present in PC2.

4 Discussion

4.1 Relationship between sugar profile and expression of *KIN* genes

Although the main carbohydrates profile in leaves and stems (Figs. 2a, 3a, 6, 7) of *Eucalyptus* respond similarly to cold exposure, the manner how they do so is tissue-dependent. Such behavior was already expected, given the functional differences of each organ (Bauerfeind et al. 2015). Furthermore, this behavior is consistent with expression pattern of *KIN10* genes (Fig. 1), a major component of catalytic complex of SnRK1 system (Tomé et al. 2014; Williams et al. 2014).

In that way, it is important to highlight that in situations of low energy availability, SnRK1 is responsible for activating and regulating genes related to numerous catabolic pathways, accessing alternative sources of energy and substrates and suppressing several pathways with a high energy demand (Tomé et al. 2014; Wurzinger et al. 2018). Nonetheless, the activity of the SnRK1 system is a highly complex process, controlling, and in turn being controlled by, a myriad of regulatory proteins and signaling metabolites such as soluble sugars and also being dependent on the tissue nature investigated (Halford and Hey 2009; Lunn et al. 2014; Smeekens et al. 2010).

Therefore, the greater expression of leaf *KIN* genes during the day (Fig. 1a, b) may be especially important in *E. globulus* which displayed no significant increase in amount of the major carbohydrates (i.e., glucose and sucrose) in their leaves during the daytime under cold conditions, even though this species displays a higher rate of carbon assimilation when exposed to low temperatures (Domingues-Junior et al., MS in preparation). One possible explanation for this phenomena would be a greater sink strength of the stem, stimulated by cold signaling and starting to consume high quantities of photoassimilates (Arias et al. 2014; Campoe et al. 2013; Çetinkol et al. 2012). In this sense, it is worth mentioning that *E. globulus* shows a faster growth rate compared to *E. grandis*

when both species are exposed to low temperatures (Costa e Silva et al. 2009; Fernández et al. 2010; Goulao et al. 2011; Kirst et al. 2004; Mora and Serra 2014; Moura et al. 2010; Ruedell et al. 2013). Moreover, this hypothesis is consistent with observations described in Fig. 1c, d, wherein stems of this species also displayed greater expression of *KIN10* genes during day, suggesting a higher demand for energy resources.

In *E. grandis*, leaves and stems kept at 10 °C showed higher expression of *KIN10* during the night. Associating this with the stressful impact that cold exerts on the development of this species (Budzinski et al. 2016) and the small oscillation observed in carbohydrate levels at night (Figs. 6 and 7), we postulate that the greater expression of *KIN10* genes favors the increase of available carbon skeletons which might be used as an energy supply and/or as well as substrate to the biosynthesis of defense metabolites.

We, therefore, suggest that the two species differ in the way they coordinate the activation of the SnRK1 system with sugar metabolism and vegetative growth when exposed to low temperatures. For *E. globulus*, this system is directed to a higher carbon assimilation and greater stem growth rate during the day, while in *E. grandis*, it would appear to be associated to providing substrates for responses against cold (Hörmiller Imke et al. 2017; Tomé et al. 2014). However, future studies seeking the association of this pattern with the response of genes related to the cell cycle, the activity of metabolic protection systems (such as antioxidant enzymes), as well as a prolonged low temperature exposure will allow the confirmation or refutation of the hypothesis raised here.

4.2 Cold provokes different changes in primary metabolism of *E. globulus* and *E. grandis*

The development and widespread adoption of gas chromatography mass spectrometry (GC-MS) has facilitated numerous studies characterizing the metabolic profiles of different plant species in response to endogenous and external alterations (Bauerfeind et al. 2015; Dhuli et al. 2014; Mokochinski et al. 2018; Tarkowski and Van den Ende 2015). However, the majority of the studies to date have focused on herbaceous plants, with only a few being related to tree species (Correia et al. 2018; Dhuli et al. 2014; Warren

Fig. 8 Relations between source and sink tissues of (a) *E. globulus* and (b) *E. grandis* when both species are exposed to 10 °C. This schematic representation combines molecular and metabolic data of the present work and observations discussed in other work of our group (Domingues-Junior et al., MS in preparation). Small arrows indicate changes in parameters

et al. 2012; Warren 2011). To our knowledge, the present study is the first to analyze daily profile of the main central metabolites in leaves and stems of Eucalyptus exposed to low temperatures.

The compounds presented herein (Figs. 4, 5 and Dataset S1) comprise several metabolic classes, including non-structural carbohydrates, amino acids, organic acids, polyols and two compounds classified as “miscellaneous” (putrescine and phosphate). In general, the division between periods of light and dark significantly impacted levels of the different metabolites described in this study.

When analyzing each metabolite in the context of the biochemical class to which it belongs (Figures S4 to S7), a similar pattern was observed between *E. globulus* and *E. grandis*. The metabolite oscillations were smaller in stems when compared to leaves and plants exposed to 10 °C displayed higher levels of free amino acids, especially of the branched-chain amino acids (BCAAs, i.e. leucine, isoleucine and valine) (Figs. 4, 5). According to Avin-Wittenberg et al. (2012) and Tomé et al. (2014), stress conditions are responsible for increasing the content of free amino acids, as a result of either enhanced degradation and/or reduced synthesis of proteins. Moreover, free BCAAs can be catabolized in order to maintain the levels of the TCA cycle intermediates and to provide electrons to mitochondrial electron carrier chain (Arias et al. 2014; Avin-Wittenberg et al. 2012; Budzinski et al. 2016). In this sense, the increase of citrate, malate and succinate

values in plants exposed to cold (10 °C) compared to plants of control group (25 °C): (upward arrow) indicates increased values of those parameters and (downward arrow) indicates decreased values of those parameters. Dashed line indicates reduction of carbon substrates available to exportation from source to sink tissues

at the end of the night period is consistent with the decline observed in BCAA content in the leaves of both eucalyptus species following exposure to cold.

Similar changes in profile of BCAAs and TCA cycle components were described in plants of pea submitted to conditions of low energy availability, being regulated by the SnRK1 system (Radchuk et al. 2010). Thus, changes in levels of amino and organic acids observed in leaves and stems of *E. globulus* and *E. grandis* exposed to 10 °C would be anticipated, given the changes observed here in *KIN10* gene expression (already discussed above). However, no other relationships between the content of these metabolites and the expression of the *KIN10* genes were observed. We, therefore, postulate the existence of additional factors, not analyzed in the present work, including the possibility of increased activity of degradative enzymes activity or post-translational regulatory events of this process, as links between the initial signaling via SnRK1 and the catabolic response. Future studies should encompass analysis at multiple different levels namely transcriptomic, proteomic and metabolomic levels in order to gain a more detailed insight into the underlying mechanisms.

The principal component analysis (PCA) of the metabolic profile data revealed a clear distinction between species and thermal environments (Figures S8 and S9), indicating that, even for a short period, cold exposure was able to evoke distinct responses in *E. globulus* and *E. grandis* plants. When analyzing

variables according to the tissues, we can observe a greater susceptibility of photoassimilates sources organs (leaves) to thermal treatments. In this manner, TCA cycle components, BCAAs and glucose were the main components responsible for this response (Figure S8 and Table S3). Such behavior is perfectly plausible given the high sensitivity of both carbon assimilatory and respiratory processes in leaves exposed to low temperatures (Navarrete-Campos et al. 2013; Tarkowski and Van den Ende 2015). Also, leaf, working as the main organ for perception of external environment and converging thermal, nutritional and luminous signals must respond immediately to any change in its environment, in order to generate both rapid and long term responses (Tomé et al. 2014).

Moreover, the lack of separation of temperature treatments according to PC1 for stem metabolites (Figure S9 and Table S4) and the lower number of cold induced changes observed on those variables suggest a lower susceptibility of stems to the thermal environment (at least, to the conditions presented in this work). Moreover, the responses observed for stem samples indicate that intrinsic differences found in *E. globulus* and *E. grandis* are larger and more significant than changes evoked by the thermal environment imposed here. It is probably due the fact that stem is mainly composed of vascular tissues, where cell walls are important sink of carbon (Allahverdiyeva et al. 2015; Epron et al. 2012); therefore, the biosynthesis of their component affects entire development, once it exerts great influence on the plant energy balance, and must be under strict metabolic and genetic control to allow the best performance according to the environmental conditions.

Therefore, we could observe in this work that the expression of *KIN10* genes in leaves and stems of *E. grandis* is consistent with their basal function (being active in situations of energy deficit) (Tomé et al. 2014; Williams et al. 2014; Wurzinger et al. 2018); however, in *E. globulus*, the expression of those genes during the day and under cold, a condition considered not harmful for its vegetative growth, suggests two possible scenarios: (I) there is little energy available from photosynthesis; or (II) the converted energy is rapidly consumed. Moreover, the soluble sugar contents suggest that the increase of glucose in *E. grandis* stems at cold night period is consistent with the higher expression of *KIN10* genes soon after the transition to the dark period, meaning that, with *KIN10* genes

signaling, catabolic pathways would guarantee glucose supply for the nocturnal period (Tomé et al. 2014; Williams et al. 2014; Wurzinger et al. 2018) in stems of this species. By other way, in *E. globulus* plants exposed to cold, the small increase of leaf glucose, associated with the absence of oscillation in sucrose levels and starch content, suggests the existence of a strong photoassimilate sink (Wu and Birch 2010). Moreover, our group has observed a high photosynthetic rate when *E. globulus* is maintained at 10 °C (Domingues-Junior et al., MS in preparation), thus higher rate of photoassimilate synthesis is expected under this condition. In this manner, the small decrease in sucrose content summed to the absence of oscillation of glucose levels in *E. globulus* stems suggests an increased sink strength (Wu and Birch 2010), possibly by investing in a higher cell wall deposition and growth (Cerasuolo et al. 2016; Osakabe et al. 2013), behavior possibly triggered by low temperature signaling.

In that way, we could observe that cold (10 °C) was able to promote a higher *KIN10* gene expression in leaves of *E. globulus*, stimulating photosynthesis and promoting photoassimilates production. Another work from our group and carried out parallel to the present study (Domingues-Junior et al., MS in preparation) has observed that the same low temperature conditions applied here were able to promote a higher stem growth rate in *E. globulus* when this species were exposed to cold for 30 days. Therefore, this higher stem growth rate triggered by low temperature signaling probably increases the sink strength of this tissue, consuming the photoassimilate surplus and thereby maintaining a higher growth rate. If this scenario is maintained over time, such dynamics would guarantee the higher vegetative development of *E. globulus* when plants of the species are subjected to low temperatures. A schematic representation of this hypothesis is presented in Fig. 8.

In conclusion, the present work is the first to identify changes on primary metabolic profile of source and sink tissues of two Eucalyptus species when exposed to 10 °C for 1 day cycle. The fact that it was possible to distinguish species according to the metabolic profile and the content of important carbon sinks (starch and total protein content) suggests starting points for future studies, in which such interactions between primary metabolism and cell wall biosynthesis should be carefully analyzed in

order to select desirable parameters for higher production of cellulose and biofuels from *Eucalyptus*.

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Author contributions APDJ, PM and ARF designed the experiments; APD-J, DDM, MM, LR-S and LPS carried out the experiments; APD-J, ARF and PM wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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