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Gene expression in two contrasting hybrid clones of *Eucalyptus camaldulensis* x *Eucalyptus urophylla* grown under water deficit conditions

Guilherme Silva Martins*, Natália Chagas Freitas, Wesley Pires Flausino Máximo, Luciano Vilela Paiva

Laboratório Central de Biologia Molecular, Departamento de Química, Universidade Federal de Lavras, 37200-000, Lavras, MG, Brazil

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ABSTRACT

The physiological and molecular responses to water stress are mediated by a range of mechanisms, many of which involve abscisic acid (ABA)-dependent signaling pathways. In addition, plants contain drought response genes that can be induced by ABA-independent routes, mediated by secondary messengers such as Ca^{2+}, or regulated by epigenetic modifications. The complex processes involved in the response to water stress can be investigated using molecular techniques to evaluate the expression patterns of genes of interest and to infer the behavior of different genotypes and species. In the present study, we first analyzed the stability of a set of reference genes for normalization of the gene expression with real-time quantitative polymerase chain reaction (RT-qPCR), since there were no results related to the genotype used in this study. We verified that although there were some variations between algorithms used, the three most stable reference genes were SAND, PP2A-3 and EF-1α. The expressions of genes encoding for proteins associated with drought-tolerance responses, namely 9-*cis*-epoxycarotenoid dioxygenase 3 (EgrNCED3), pyrabactin resistance 1 (EgrPYR1), dehydration-responsive element-binding 2.5 (EgrDREB2.5) transcription factors, calcium-dependent protein kinase 26 (EgrCDPK26), methyl transferase 1 (EgrMET1) and deficient in DNA methylation 1 (EgrDDM1) protein, were determined by RT-qPCR in leaf samples from drought sensitive (VM05) and drought tolerant (VM01) clones of the hybrid *Eucalyptus camaldulensis* x *Eucalyptus urophylla* grown under water stress and irrigation conditions. When the two clones were maintained under conditions of water deficiency, VM01 exhibited higher expression levels of *EgrNCED3* and *EgrPYR1* genes than VM05 at all sampling times, implying that ABA biosynthesis and subsequent induction of the ABA-dependent cascade mediated by the PYR1-ABA receptor complex were enhanced in the tolerant clone. Under water-stress conditions, this clone also presented increased expression of the *EgrDREB2.5* gene, representative of an ABA-independent cascade, and of the *EgrCPK26* gene, related to stomatal opening and closure. On the other hand, the expression levels of *EgrMET1* and *EgrDDM1* genes in the sensitive clone were higher than in the tolerant clone under all conditions, showing a putative impact of epigenetic modifications on tolerance to water deficiency. The results obtained indicate that the superior ability of the VM01-tolerant clone to perceive water deficiency and activate drought-resistance genes is associated with the high expression levels of *EgrNCED3, EgrPYR1* and *EgrDREB2.5* under water-stress conditions. These findings will facilitate future research on the functional characterization of stress-related response genes, the identification of molecular markers, the evaluation of drought tolerance and genetic transformation in tree species.

Abbreviations: ABA, abscisic acid; BLAST, Basic Local Alignment Search Tool; CDPK, calcium-dependent protein kinase; CTAB, cetyltrimethylammonium bromide; DDM1, deficient in DNA methylation 1; DREB, dehydration-responsive element-binding; EF1α, elongation factor 1α; ERE, ethylene responsive element; IDH, isocitrate dehydrogenase; LEA, late embryogenesis abundant; MET1, methyl transferase 1; NCED3, 9-*cis*-epoxycarotenoiddioxygenase 3; PP2A, protein phosphatase 2A; PP2C, protein phosphatase 2C; PYRI/PYL/RCAR, pyrabactin resistance 1/pyrabactin resistance 1-like/regulatory components of ABA receptors; RT-qPCR, real-time quantitative polymerase chain reaction; SAND, SAND domain-containing proteins; SLAC1, slow anion channel 1; SNF1, sucrose non-fermenting 1; SnRK2, SNF1-related protein kinase 2; TUB, β-tubulin; UBQ, ubiquitin

* Corresponding author.
E-mail addresses: guilhermesm91@hotmail.com (G.S. Martins), natalia_biotec@hotmail.com (N.C. Freitas), wesleypfm@hotmail.com (W.P.F. Máximo), luciano@dqi.ufla.br (L.V. Paiva).

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1. Introduction

Water deficit is one of the conditions that most threatens production in *Eucalyptus* plantations because it affects vital processes such as photosynthesis, respiration, carbohydrate metabolism and ion absorption. In order to reduce or even avoid the impact of water deficit, plants frequently undergo biochemical and morphological changes that enable them to acclimatize to stressful conditions (Chaves et al., 2009). Therefore, understand the complex mechanisms underlying physiological and molecular responses to water stress is crucial for the generation and selection of tolerant genotypes (Ahuja et al., 2010). In South America, one of the hybrids with the greater economic importance is *Eucalytpus camaldulensis vs Eucalyptus urophylla* mainly because of its drought tolerance, wood quality, disease resistance and shoot development. However, regardless its better capacity to tolerate drought conditions compared to its counterparts, the productivity is still affected by this abiotic stress.

A diverse array of strategies may be involved in the adaptation of a plant to water stress, including stomatal closure, induction of stress responsive proteins and accumulation of cell protective metabolites mediated by the phytohormone abscisic acid (ABA) (Umezawa et al., 2010). The enzyme that catalyzes the rate-limiting step in the ABA biosynthesis is 9-*cis*-epoxycarotenoid dioxygenase (NCED3), and many reports have confirmed that increases in *NCED3* gene expression are associated with higher production of ABA and ABA-induced responses to water stress (Iuchi et al., 2001; Daszkowska-Gollec and Szarejko, 2013) in some species as, for example, *Arabidopsis thaliana* (Woo et al., 2011), *Zea mays* (Su et al., 2011) and *Nicotiana tabacum* (Zhang et al., 2009).

Findings from studies involving *A. thaliana* led to the concept of an ABA perception/signaling model that elicits drought tolerance and involves three main components, namely the Pyrabactin resistance 1/Pyrabactin resistance 1-like/Regulatory components of ABA receptors (PYR1/PYL/RCAR) and two phosphatase/kinase enzyme pairs with opposite functions, i.e. protein phosphatase 2C (PP2C) and sucrose non-fermenting (SNF1)-related protein kinase 2 (SnRK2) (Mehrotra et al., 2014). The conformational changes that occur when intracellular ABA binds to the PYR1/PYL/RCAR ABA receptor induce the inactivation of PP2C proteins and the activation of SnRK2 proteins culminating in the expression of genes and ABA-dependent transcription factors and, ultimately the triggering of the downstream pathway (Gonzalez-Guzman et al., 2012; Santiago et al., 2009; Yoshida et al., 2015).

Drought-tolerance responses can also be induced by ABA-independent routes (Kuromori et al., 2014) involving, for example, the attachment of Dehydration-responsive element-binding (DREB) transcription factors to drought-responsive genes through recognition of A/GCCGAC sequences in promoter regions (Agarwal and Jha, 2010; Matsukura et al., 2010). Genes encoding DREB transcription factors have been described and evaluated in *A. thaliana* (Liu et al., 1998, 2000), *Glycine max* (Chen et al., 2007; Mizoi et al., 2012), *Oryza sativa* (Matsukura et al., 2010), *Triticum aestivum* (Shen et al., 2003) and *Z. mays* (Qin et al., 2007).

Additionally, drought-resistance responses can be mediated by secondary messengers such as Ca^{2+}, the mobilization of which from intracellular compartments generates increased concentration in the cytoplasm resulting in conformational changes and activation of calcium-dependent protein kinases (CDPKs) (Boudsocq and Sheen, 2013). Members of the *CDPK* multigene family participate in the activation and inhibition of a range of enzymes, ion channels and transcription factors. For example, the *AtCPK6* gene of *Arabidopsis* plays a role in stomatal closure through activation of guard-cell slow anion channel 1 (SLAC1) and calcium channels and the scavenging of reactive oxygen species (Geiger et al., 2010), while some studies have demonstrated that the over-expression of *AtCPK6* can induce drought tolerance in various species by regulating the production of osmolytes such as proline (Xu et al., 2010; Schulz et al., 2013).

Furthermore, it is known that the epigenome strongly influences the expression or repression of genes at times of stress, and that epigenetic regulatory mechanisms enable plants to survive and reproduce successfully in unfavorable environments. The epigenetic changes in response to water stress that have received most research attention are DNA methylation, histone modifications and chromatin remodeling (Han and Wagner, 2013). Although the mechanisms have not been fully elucidated, it has been shown that DNA methylation and decomposition of chromatin in plants grown under drought conditions are mediated by DNA methyltransferases such as MET1 and chromatin remodeling factors such as deficient in DNA methylation 1 (DDM1) protein (Karan et al., 2012; Kim et al., 2014).

Gene expression analysis is commonly employed in plant research for identifying molecular markers, performing genetic manipulation and evaluating the tolerance to biotic and abiotic stresses. Alterations in the expression of specific target genes in relation to endogenous control genes (reference genes) can be detected using real-time quantitative polymerase chain reaction (RT-qPCR), the advantages of which include reliability, swiftness, sensitivity and specificity (Pfaffl et al., 2004). With the aim of identifying different strategies for drought-resistance in *Eucalyptus* spp. we have: (i) evaluated the expression of six genes encoding for proteins associated with drought-tolerance responses, namely *EgrNCED3, EgrPYR1, EgrDREB2.5, EgrCDPK26, EgrMET1* and *EgrDDM1,* in leaf samples from drought sensitive (VM05) and drought tolerant (VM01) clones of the hybrid *Eucalyptus camaldulensis* x *Eucalyptus urophylla* using RT-qPCR, and (ii) correlated the levels of expression with the physiological and morphological characteristics of these genotypes grown under water stress and irrigation conditions.

2. Materials and methods

2.1. Plant material, growth conditions and sampling

3 months-old seedlings (~40 cm length) of two clones (VM01-tolerant and VM05-sensitive) of the hybrid *E. camaldulensis* Dehnh. x *E. urophylla* S.T.Blake were used in this study. They were planted in pots containing 1500 mL of CSC® commercial substrate (Carolina Soil do Brasil, Santa Cruz do Sul, RS, Brazil) composed of sphagnum moss, expanded vermiculite, dolomitic limestone, agricultural gypsum and traces of NPK. Prior to the experiment, seedlings of both clones were subjected to constant irrigation for 10 days in a greenhouse with controlled temperature and humidity, and subsequently submitted to either water stress (no irrigation) or constant irrigation conditions. The experiment was carried out in biological triplicate for each sampling time (5, 10 and 15 days of water stress or irrigation conditions), such that a total of 18 seedlings were assessed for each clone. For each plant, 4 leaves per seedling, localized in the middle node of the plant were harvested for the subsequent experiments.

2.2. Analysis of water potential

A Scholander pressure pump was used to measure the internal water potential in plants before dawn (typically between 2 and 4 h a.m) on the sampling day. The tests were performed on totally expanded mature leaves located at the middle third portion of each seedling.

2.3. Extraction of total RNA and cDNA synthesis

Total RNA was extracted from leaf tissues using the method described by Chang et al. (1993) with some modifications. Briefly, cetyltrimethylammonium bromide (CTAB) extraction buffer (containing 2% w/v CTAB, 2% w/v polyvinylpyrrolidone-40, 2 M NaCl, 100 mM Tris-HCl pH 8.0, 25 mM ethylenediaminetetraacetic acid pH 8.0 and 0.5 $g\,L^{-1}$ spermidine) was preheated to 65 °C and added, along with β-mercaptoethanol (0.2%). The mixture was homogenized for 2 min and extracted twice with chloroform : isoamyl alcohol (24:1; v/v). Each