UNIVERSIDAD DE CONCEPCIÓN
Dirección De Postgrado
Facultad de Ciencias Forestales
Programa de Doctorado en Ciencias Forestales

NAC: una superfamilia de factores transcripcionales y su rol en la tolerancia al estrés abiótico en *Eucalyptus globulus*

Tesis presentada para optar al grado académico de Doctor en Ciencias Forestales

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CONCEPCIÓN- CHILE
Julio 2024

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DEDICATORIA

A mi Emilia paz, eres importante, eres amada, eres valiente y empoderada, y mama te ama incondicional.

AGRADECIMIENTOS

A mi profesora guía la Dra. Sofía Valenzuela, por su apoyo, motivación y absoluta confianza en mi trabajo, a tal punto que me dejo durante dos años y volvió en la recta final. su guía además de enfrentarme a desafíos me ha permitido crecer, ya son 17 años desde que iniciamos esta carrera juntas y seguimos!!

Agradecer al Dr. Claudio Balocchi por aceptar ser mi co-guía de tesis gracias por su completa disposición y por confiar en mis actitudes no solo de alumna de doctorado si no también de coordinadora en el Curso Internacional de Genética y Biotecnología Forestal.

Al Centro de Biotecnología (CB-UdeC) de la Universidad de Concepción donde realicé la mayor parte de mi programa de doctorado.

Al Laboratorio de Genómica Forestal donde realicé la parte experimental de este proyecto.

A mis queridos tesis, Macarena, Nicolás y Daniel, que fueron un apoyo fundamental para poder sacar este proyecto adelante.

A mi pareja, por su apoyo y disposición siendo parte del proceso montando ensayos y regando plantas.

A mi mamá, sin tu apoyo y cuidados a nuestra Emi todo hubiera sido más difícil.

A mi Vicky por que el sufrimiento del doctorado lo vivimos a distancia pero juntas.

Finalmente agradecer a las fuentes de financiamiento de esta tesis:

Proyecto Fondecyt 1161063

Beca de doctorado Nacional – ANID folio 21211524

Bioforest-Arauco S.A.

Genómica Forestal S.A

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RESUMEN

El estrés abiótico en plantas conduce a pérdidas económicas significativas al reducir el rendimiento, efectos agravados por el cambio climático actual. Debido a su rápido crecimiento, adaptabilidad y propiedades de la madera, *Eucalyptus globulus* se ha convertido en una especie fundamental en la industria forestal. Sin embargo, es vulnerable al estrés hídrico y sensible a las bajas temperaturas, lo que se traduce en una disminución de las tasas de crecimiento y un aumento en la mortalidad de las plantaciones. Para contrarrestar estos efectos las plantas generan cambios moleculares, donde la expresión diferencial de determinados genes genera mecanismos de tolerancia. Los factores de transcripción NAC son una extensa familia de reguladores genéticos implicados en múltiples procesos incluyendo la respuesta al estrés abiótico. Se identificaron 101 FT NAC en un transcriptoma de *E. globulus* sometido a tratamiento de estrés hídrico y frio. El análisis *in silico* reveló que 13 de ellos mostraban una mayor abundancia de transcritos en respuesta a los tratamientos de estrés abiótico. Estos resultados proporcionan la base para futuros estudios y análisis de los genes NAC y su rol en la respuesta al estrés abiótico en *E. globulus*.

ABSTRACT

Abiotic stress in plants leads to significant economic losses by reducing yield, effects enhanced by current climate change. Due to its rapid growth, adaptability, and wood properties, *Eucalyptus globulus* has become a key species in the forestry industry. However, it is vulnerable to drought stress and sensitive to low temperatures, resulting in decreased growth rates and increased plant mortality. To face these effects, plants generate molecular changes, where the differential expression of certain genes generates tolerance mechanisms. NAC transcription factors are an extensive family of genetic regulators involved in multiple processes, including response to abiotic stress. A total of 101 NAC TFs were identified in a transcriptome of *E. globulus* subjected to water and cold stress treatments. *In silico* analysis revealed that 13 of these genes showed higher transcript abundance in response to both stress treatments. These results provide important information for future studies and analysis of NAC genes and their role in abiotic stress response in *E. globulus.*

INTRODUCCIÓN GENERAL

El estrés en las plantas se define como cualquier interferencia negativa causada por factores externos que impactan en el crecimiento o desarrollo de estas (Lichtenthaler, 1998). Esta interferencia comúnmente desencadena respuestas a nivel molecular, bioquímico o fisiológico, que tienen como finalidad el preservar las funciones esenciales del organismo y con ello su supervivencia (Taiz y Zeiger, 2006). Dependiendo de la naturaleza de la interferencia, el estrés puede clasificarse en estrés biótico, cuando es causado por organismos vivos, y estrés abiótico, cuando es provocado por factores ambientales adversos (Nawaz et al., 2023).

El estrés abiótico conlleva significativas pérdidas económicas al reducir el rendimiento y calidad de especies agrícolas y forestales. El cambio climático agrava los efectos adversos causados por el estrés abiótico provocando un aumento en las tasas de perdida para las industrias que utilizan como materia prima especies vegetales. Según las proyecciones climáticas globales, el cambio climático persistirá, dando lugar a eventos de estrés abiótico, tales como cambios extremos en la temperatura, salinidad, inundaciones, sequías y otros, con una mayor frecuencia y severidad (Teshome et al, 2020).

Actualmente el cambio climático se ha manifestado a través de un aumento apresurado en las temperaturas y alteraciones en las precipitaciones, lo que resulta en periodos de lluvia intensa intercalados con periodos prolongados de sequía. El impacto del estrés por sequia puede ser especialmente perjudicial para plantaciones forestales, principalmente debido al tamaño de los especies utilizadas, que requiere un sistema vascular complejo para transportar agua desde el suelo hasta el dosel, y su largo tiempo de generación, lo que resulta en una adaptación ambiental lenta (Rosso et al, 2023).

El estrés hídrico, tiene repercusiones negativas en las plantas de varias maneras. El cierre estomático, una respuesta frecuente ante la sequía, causa desequilibrios en la absorción y fijación del carbono, así como una disminución de las reservas de carbohidratos. Además, este cierre reduce la transpiración, lo que limita el enfriamiento por evaporación de las hojas y aumenta el estrés térmico en los procesos metabólicos, especialmente en la fotosíntesis. La acumulación de especies reactivas de oxígeno (ROS), otro efecto común de la sequía, daña tanto proteínas como membranas celulares. Por otro lado, la disminución del potencial hídrico del suelo expone el sistema de transporte del xilema a riesgos de cavitación y formación de embolias, lo que puede resultar en fallas hidráulicas y la muerte de ramas o incluso de toda la planta (Ilyas et al, 2021; Li et al, 2023).

A pesar de las proyecciones de calentamiento global para los próximos años, los modelos climáticos indican que los eventos de frío extremo persistirán y serán más intensos que en el pasado reciente (Collins et al., 2013; Li et al., 2023). El frío, en referencia a temperaturas no congelantes (entre 15 y 0°C), conlleva síntomas como la reducción en la expansión del área foliar, marchitamiento, clorosis e incluso necrosis de los tejidos expuestos (Wang 1990). Por otro lado, bajo temperaturas de congelamiento (<0°C), se produce la muerte de los tejidos, principalmente como respuesta al daño provocado en las membranas celulares, lo que resulta en la pérdida de electrolitos en el apoplasto y un desequilibrio en las propiedades de transporte de las estas (Kazemi-Shahandashti y Maali-Amiri 2018). Además, formación de cristales de hielo a nivel celular conlleva a una deshidratación, que comienza en el apoplasto, donde el fluido extracelular tiene un mayor punto de congelación que el intracelular debido a la menor concentración de solutos. Esto produce una pérdida de agua desde el interior de la célula hacia el apoplasto, lo que contribuye al crecimiento de los cristales de hielo los que ejercen una tensión mecánica entre la pared celular y la membrana citoplasmática, resultando en una ruptura celular (Thomashow 1999; Kaminska-Rozek y Pukacki 2005). La sensibilidad y daños en las plantaciones provocadas por estas alteraciones ambientales va a depender de la velocidad, el tiempo de exposición, la edad, la presencia de otros tipos de estrés y la resistencia de la especie (Nievola et al. 2017). En plantas, los mecanismos de resistencia al estrés abiótico se dividen en dos estrategias adaptativas: evitación y tolerancia, donde esta última se refiere a la capacidad de resistir las alteraciones causadas por el estrés e implica cambios en los mecanismos fisiológicos, expresión génica y diversas actividades de desarrollo para tolerar el efecto de estreses específicos (Rihan et al. 2017; Nievola et al. 2017). En ausencia de una estrategia para evitar las alteraciones ambientales, la supervivencia de algunas especies va a depender solo de su capacidad de aclimatación, una estrategia de tolerancia en respuesta a los cambios ambientales que pueden ocurrir en días o semanas y en presencia de estrés continuo o puede ser transmitida por la experiencia pasada de una alteración ambiental (Kozlowki, 2002; Gessler et al. 2020). El proceso de aclimatación al estrés abiótico implica una reprogramación de la expresión génica como resultado de una cascada de señalización producto de la detección del estrés, capaz de activar mecanismos de protección tales como osmorregulación, desintoxicación, activación de proteínas chaperonas, entre otras, con el fin de ajustar el metabolismo de las plantas para hacer frente a los daños que el estrés pueda causar (Oberschelp et al., 2020). Es así como, desde la década de los 90´, la identificación y caracterización de genes en respuesta al estrés abiótico ha sido de gran interés, lo que se ve reflejado en el aumento exponencial de las publicaciones asociadas a este tópico en los últimos años (Fig. 1) que van de la mano, con el desarrollo de las tecnologías de secuenciación de nueva generación (NGS), tales como RNA-sequencing o RNA-seq (Lu et al., 2023).

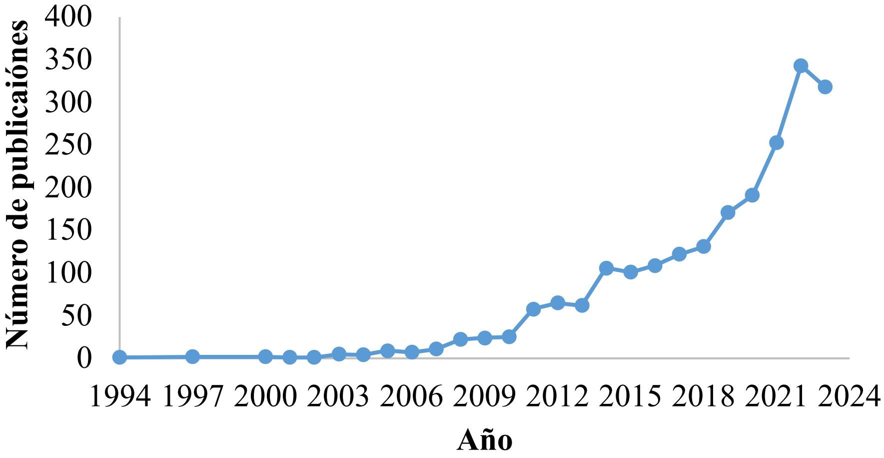

Fig. 1 Número de publicaciones científicas asociada a la identificación de genes en respuesta al estrés abiótico árboles, fuente https://www.webofscience.com/wos/woscc/basic-search.

RNA-seq, es una herramienta crucial en los análisis genéticos de un determinado tejido en un momento especifico, en la actualidad, una gran variedad de trabajo ha utilizado esta herramienta para la identificación de genes en respuesta al estrés abiótico en plantas, ya que permite la identificación de variantes, la expresión específica de genes, la caracterización transcriptómica de todo el genoma y análisis diferencial de la expresión génica (Lowe et al, 2017; Shinozaki et al, 2022).

La expresión génica está determinada en gran medida por los factores de transcripción (FT). Los factores de transcripción, son proteínas que poseen dominios de unión al ADN, reconociendo específicamente los elementos en cis dispuestos en las regiones promotoras de los genes diana, estos pueden ser clasificados en FT generales o basales, que participan en la formación del complejo de iniciación de la transcripción de genes encontrándose prácticamente en todas las células de un organismo y en FT específicos cuya función radica en activar o inhibir la expresión de genes específicos en respuesta a diferentes estímulos (Agarwal et al., 2010; Clark et al 2019).

En los genomas de plantas, aproximadamente el 7% de las secuencias codificantes son asignados como FT, en *Arabidopsis thaliana* la planta modelo para varios estudios de respuesta a estrés se ha registrado que entre un 6 a 10% de los genes que constituyen el genoma de la especie codifican para FT (Lindemose et al. 2013; Wisniewski et al. 2018).

Se ha demostrado que diferentes familias de factores de transcripción están involucradas en la respuesta de las plantas al estrés abiótico, ya sea en vías dependientes o independientes del ácido abscísico (ABA). Estos incluyen, factores de unión a elementos de respuesta APETALA2/etileno (AP2/ERF), cremallera básica de leucina (bZIP), WRKY, MYB y tres grupos de factores de transcripción, sin meristema apical (NAM), ATAF1-2 y cotiledón en forma de copa (CUC2) que constituyen una gran familia de factores de transcripción llamados NAC (Wang et al. 2016; Trono et al 2023).

Las proteínas NAC constituyen una de las familias de factores de transcripción más extensa y específica de plantas distribuyéndose en una amplia variedad de especies (Olsen et al. 2005). Inicialmente caracterizadas en petunia (**N**AM) y Arabidopsis (**A**TAF1/2 y **C**UC2), estas proteínas exhiben un dominio altamente conservado localizado en la región N-terminal compuesto por aproximadamente entre 150-160 aminoácidos y dividido en cinco subdominios (A a E), cuya conservación se clasifica en A > C > D > B > E (Han et al. 2023). La función de cada subdominio ha sido identificada: el subdominio A participa en la formación de dímeros funcionales, como homodímeros o heterodímeros. Los subdominios C y D, contienen señales de localización nuclear y están estrechamente relacionados con la unión a elementos específicos del promotor. Además, la subunidad D de varias proteínas NAC contiene un dominio regulador negativo altamente hidrofóbico. Estudios han revelado que el dominio de represión NAC (NARD) puede inhibir la actividad transcripcional de otros FT como WRKY y AP2/DRE (Hao et al., 2010). La conservación de los subdominios B y E es relativamente baja y contribuye a la diversidad funcional de los factores de transcripción NAC (Ernst et al., 2004). Por otro lado, en la región C-terminal se encuentra el dominio regulador de la transcripción, el cual puede actuar como activador o represor transcripcional. Esta región se caracteriza por su alta variabilidad entre especies, tanto en longitud como en secuencia, lo que contribuye a la diversidad funcional de los miembros de la familia de FT NAC (Ooka et al., 2003; Shao et al., 2015).

En el transcurso de los años esta familia de FT ha sido implicada en la regulación de una amplia variedad de procesos tales como, el desarrollo de semillas y embriones, la formación de meristemas apicales de brotes, el desarrollo de fibras, la senescencia de las hojas, la división celular y la respuesta al estrés biótico y abiótico (Nakashima et al. 2012; Singh et al.

2021). Los miembros de la familia de FT NAC han sido ampliamente estudiados en respuesta a diversas condiciones de estrés, incluyendo calor, anegación, salinidad, frío y sequía (Han et al., 2023). El rol exacto de cada miembro en respuesta a señales ambientales sigue siendo objeto de investigación y con un número limitado de estudios realizados en especies leñosas de importancia forestal. En *E. grandis*, Hussey et al. (2015) caracterizó 189 EgrNAC, dentro de los cuales se identificaron algunos candidatos involucrados en la regulación del desarrollo de la madera opuesta y de tensión y en respuestas a frio. Hu et al. (2019) mostraron que *BpNAC012*, un gen NAC caracterizado en *Betula platyphylla,* es regulado positivamente en respuesta al estrés osmótico. Yao et al. (2020) evaluaron los patrones de expresión de la familia PtNAC presente en *Populus simonii*×*Populus nigra,* bajo estrés salino, donde 20 genes NAC fueron involucrados a esta respuesta. Recientemente, Chen et al. (2022) evaluó la sobreexpresión de líneas transformadas de *P. tomentosa* con el gen *PeNAC122,* un miembro de la familia de FT NAC altamente expresado en el xilema de *P. euphratica*, los resultados mostraron una mayor tolerancia al estrés osmótico por parte de las líneas transformadas acompañadas de un mejor rendimiento fotosintético, mayor actividad antioxidante y menor acumulación de especies reactivas de oxígeno (ROS).

Para Chile, la industria forestal es un pilar fundamental en su crecimiento económico, con un patrimonio de 2,3 millones de hectáreas (ha) constituidas principalmente por las especies de pinus y eucaliptus, con una contribución por año en promedio del 2% al PIB y siendo el primer sector exportador de recursos naturales renovables, esta industria ha consolidado su posición en el desarrollo del país con éxito (Corporación Nacional Forestal 2013, INFOR 2023). Al año 2021, el 38% de la superficie total de plantaciones forestales corresponden al genero Eucalyptus, siendo *Eucalyptus globulus* el líder con 483.000 ha (INFOR, 2023).

Debido a su rápido crecimiento, madera de alta calidad para la industria y cortos periodos de rotación, *E. globulus* es la principal especie para la producción de pulpa de celulosa (Carrillo et al, 2018). Sin embargo, a pesar de su capacidad de adaptación a una amplia gama de sitios, la disponibilidad de agua y las temperaturas tendrán un rol clave en la distribución de las plantaciones de esta especie en el futuro. Si bien bajo condiciones de estrés hídrico moderado esta especie no se ve significativamente afectada, frente a un estrés prolongado la vulnerabilidad de la especie aumenta disminuyendo las tasas de crecimiento y la mortalidad de las plantaciones (Battaglia y Bruce 2017). Por otro lado, una de las principales restricciones en la distribución de *E. globulus* reside en su sensibilidad a las bajas temperaturas, su baja resistencia a las heladas ha resultado ser un obstáculo para la expansión de las plantaciones, donde plantas juveniles presentan una menor resistencia a las temperaturas de congelación que las adultas, dificultando el establecimiento de las plantaciones (Davidson et al. 2004). Desde principios del siglo XX, se han implementado programas intensivos de mejoramiento genético en especies forestales como el pino, álamo y eucalipto, con énfasis en el aumento del rendimiento, la resistencia a plagas y patógenos, y la mejora de la calidad de la madera (Rosso et al., 2023). Sin embargo, considerando las proyecciones relacionadas con el cambio climático, es necesario ajustar estos objetivos para priorizar la mejora en la tolerancia al estrés abiótico. Las herramientas genómicas empleadas para entender las respuestas moleculares de los árboles al estrés abiótico, identificando genes clave y vías de señalización involucradas en la adaptación, son fundamentales (Ma et al., 2012). Estos estudios nos ofrecen la oportunidad de comprender cómo los árboles responden a los desafíos ambientales, facilitando el camino para el desarrollo de estrategias efectivas para el manejo forestal sostenible y programas de mejoramiento. Los factores de transcripción son reguladores cruciales de numerosos genes relacionados con la respuesta a diversos estreses, convirtiéndolos en excelentes candidatos para futuros programas de mejoramiento genético (Joshi et al., 2016). El estricto control y ajuste de los genes NAC durante las respuestas al estrés en las plantas contribuyen a la formación de complejas redes de señalización, destacando su importancia para conferir tolerancia al estrés abiótico (Han et al., 2023). Es por ello, que en el presente estudio se plantea identificar y caracterizar los FT NAC, cuya expresión es regulada frente a condiciones de estrés hídrico y frio en *E. globulus*. Para ello, se realizó una búsqueda de miembros de dicha familia en los transcriptomas de plantas de *E. globulus* previamente sometido a tratamientos de estrés, generados mediate RNA-Seq en el marco del proyecto FONDECYT 1161063, información disponible en la base de datos NCBI BioProject (PRJNA486291). Posteriormente, se realizó un análisis de expresión diferencial de genes (DEG) donde se identificaron posibles genes candidatos dentro de la familia NAC con respuesta significativa a los tratamientos estresantes. Adicionalmente, se analizó la expresión relativa de estos genes candidatos en genotipos contrastantes de *E. globulus* para validar que la expresión de estos no corresponde a un evento propio del genotipo secuenciado si no a una respuesta general ante los tratamientos expuestos. Finalmente, el FT NAC más relevante para ambos tratamientos (hídrico y frio) fue seleccionado para un análisis de red de interacción de proteína-proteína utilizando la proteína NAC descrita para *Arabidopsis* con mayor porcentaje de identidad a la identificada en *E. globulus*, aquellos genes identificados en esta red, descritos con alguna respuesta a estrés abiótico fueron seleccionados para análisis de patrones de expresión mediante qRT-PCR en genotipos contrastantes de *E. globulus*.

HIPÓTESIS

Diferentes factores transcripcionales NAC están involucrados en la respuesta de *Eucalyptus globulus* al estrés por frío e hídrico y su nivel de expresión está asociado con la susceptibilidad o tolerancia de esta especie a estos estreses.

OBJETIVO GENERAL

Evaluar la abundancia de transcritos de los factores de transcripción NAC presentes en el transcriptoma de un genotipo tolerante al estrés por frio e hídrico de *Eucalyptus globulus* y comparar los niveles de expresión relativa de los miembros con mayor abundancia de transcritos en otros genotipos.

OBJETIVOS ESPECIFICOS

- Establecer la identidad de secuencias en el transcriptoma de *E. globulus* con los factores de transcripción NAC reportados en otras especies.
- Caracterizar estructural y filogenéticamente los factores transcripcionales NAC identificados en *E. globulus*.

- Evaluar la expresión diferencial *in silico* de los factores de transcripción NAC de *E. globulus* bajo condiciones de estrés por frio e hídrico.
- Evaluar la expresión relativa de los genes con mayor abundancia de transcrito mediante qRT-PCR bajo condiciones de estrés por frio e hídrico.

CAPITULO I: Transcriptome-wide identification of WRKY family genes and their expression under cold acclimation in *Eucalyptus globulus*[1]

Paula Aguayo, Catalina Lagos, Daniel Conejera, Diego Medina, Marta Fernández, Sofía Valenzuela

1.1 Abstract

The WRKY transcription factor family is involved in multiple biological processes, especially in the transcriptional regulation associated with plant abiotic stress response. This gene family has not been previously characterized in *Eucalyptus globulus*, a sensitive specie to freezing temperatures; able to develop a certain degree of frost tolerance when exposed to low but non-freezing temperatures, phenomenon known as cold acclimation. In this study, based on the RNA-seq data from cold acclimated *E. globulus* plants, a total of 51 *Egl*WRKY genes were identified. Phylogenetic analysis allowed to classify these genes in three main groups

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[1] Trabajo publicado 4 de mayo 2019 en revista Trees https://doi.org/10.1007/s00468-019-01860-3

and five subgroups, according to their putative orthologous genes in *Arabidopsis*. The expression patterns of 51 *EglWRKY* genes under cold stress were determined by *in silico* DEG analysis in leaf and root. Transcriptome profiling indicated that the expression of multiple *EglWRKY* genes is activated under chilling (4 ºC) and freezing temperatures (-6 ºC). Expression patterns derived from qRT-PCR, showed that 11 *EglWRKY* genes are regulated during cold acclimation in leaf tissue. These results provide the basis for further studies and analysis of *WRKY* genes to identify their function and molecular mechanisms involved in plant abiotic stress responses in *E. globulus*.

Keywords: RNA-Seq, abiotic stress, cold stress, transcription factors.

1.2 Introduction

Plants are capable to manage various stresses during their life cycle, developing several mechanisms to adapt to biotic and abiotic stress conditions. Families of transcription factors (TFs) play an important role in stress responses by regulating the expression of target genes, interacting with specific *cis*-acting elements present in their promoter regions, and acting as activators or repressors in plant stress responses (Golldack et al., 2011; Kiranmai et al., 2016; Zhang et al., 2018). Several studies have demonstrated that WRKY TFs play a critical role in plant growth and development, as well as the response to biotic and abiotic stresses (Wang et al., 2014); they are involved in processes such as morphogenesis of embryos, senescence, dormancy, metabolomic pathways; as well as in flavonoid and lignin biosynthesis (Rushton et al., 2010; Grunewald et al., 2012). *WRKY* genes, also respond to wounding, drought, heat, cold or heat pre-treated chilling (Zhang et al., 2017). The WRKY TF family is the largest family within plants, with approximately 60 conserved amino acid residues; containing the heptapeptide WRKYGQK that was identified in the N-terminal, together with zinc-binding motif at the C-terminus (Eulgem et al., 2010). The zinc-finger structure is either $Cx_{4-5}Cx_{22-23}HxH$ or $Cx_7Cx_{23}HxC$, which could bind with Zn^{+2} to facilitate the DNA-binding process (Rushton et al., 2010). WRKY TFs are classified into three groups according to variations present in the WRKY domain and zinc finger motif in the C-terminus; WRKY proteins having two WRKY domains and a C2H2 zinc-finger motif belong to group I, whilst proteins of groups II and III contain a single WRKY domain. Group II has been subdivided into five subgroups (IIa, IIb, IIc, IId and IIe), while group III proteins contain a variant zinc-finger, which ends with HXC. Recently, based on an accurate phylogenetic analysis, a fourth WRKY group for *Vitis vinifera* was described (Wang et al., 2014); corresponding to WRKYs that contain the WRKY domain, but lack a complete zinc-finger structure in the C-terminus. WRKY TFs regulate downstream gene expression interacting with a binding site known as the W-box (TTGACT/C) found within the promoter region of a large number of plant target genes; although alternative binding sites have been identified (Cheng et al., 2012; He et al., 2012). Analyses of *cis*-acting elements present within the promoter regions have helped to understand the role of *WRKY* genes in many species (Chen et al., 2015).

The WRKY genes have been characterized in multiple plant species, in *Arabidopsis*, the WRKY family comprises 74 members (*AtWRKY*) (Eulgem et al., 2000; Rushton et al., 2010; Li et al., 2016). Recent work in peach (*Prunus persica)* have reported 58 *WRKY* and 119 candidate *WRKY* genes (*MdWRKY*) were identified in apple (*Malus domestica*) (Cheng et al., 2012), from which 63 *MdWRKY* were differentially expressed in response to fungal pathogen and hormone treatments (Lui et al., 2017). In the poplar genome (*Populus trichocarpa*), 104 *WRKY* genes (*PtWRKY*) were identified (He et al., 2012), while in the genome of *Eucalyptus grandis* 79 *WRKY* genes (*EgrWRKY*) have been identified (Myburg et al., 2014; Fan et al., 2018).

Many studies have demonstrated that members of the WRKY family play an important regulatory role in cold stress response; in *A. thaliana* the expression of some *AtWRKY* genes were induced by cold stress (Zou et al., 2010). In tomato (*Solanum lycopersicum*) ten *SlWRKY* were induced during cold stress and 12 *SlWRKY* genes were significantly down regulated (Cheng et al., 2012). In *P. trichocarpa,* the RT-PCR analysis of group III *PtWRKY* genes showed that they were differentially expressed when seedlings were exposed under different stresses, being six genes upregulated by cold stress conditions (He et al., 2012). Wang *et al*. (2014) identified 59 *WRKY* genes in *V. vinifera* genome, of which 15 *VvWRKY* genes showed stress-induced expression patterns in response to cold. In tea (*Camellia sinensis*) 50 putative

WRKY genes (*CsWRKY*) have been identified; subgroup IId members *CsWRKY*33, *CsWRKY*34, *CsWRKY*37, *CsWRKY*38 and *CsWRKY*39 genes were up-regulated under cold exposure (Wu et al., 2016). Londo *et al* (2018) examined whole-transcriptome gene expression patterns in *V. vinifera*, exposed to chill and freeze shock, among the genes most commonly observed to be differentially regulated was the WRKY family.

Eucalyptus globulus Labill has an exceptional wood quality combined with fast growth in Mediterranean areas. However, low temperature stress limits their productivity and geographical distribution. To survive temperate climates, *E. globulus* has developed an acquired cold tolerance known as cold acclimation (CA), which, takes place after exposure to low nonfreezing temperatures, activating multiple mechanisms which contribute to enhance cold tolerance (Keller et al., 2013). Most of these changes, involve membrane and protein protection, and are regulated by transcriptional regulatory networks (Navarro et al., 2009).

Despite of its important role described in different species under abiotic stress, there is no information on *WRKY*s in *E. globulus*. Fernandez *et al.,* (2015) reported an increase in WRKY transcript levels in cold tolerant genotype of *E. globulus* plants under cold acclimatation; but the organization and exact function of these proteins especially in response to cold stress in this species are still unknown. This work provides the first identification and composition of the *WRKY* gene family in *E. globulus* (*EglWRKY*). Based on RNA-Seq data from *E. globulus* plants subjected to a cold acclimation profile, *EglWRKY* genes were identified on leaves and roots transcriptomes. The sequences obtained for *EglWRKY* gene family were aligned and analyzed in light of homology assessment in a phylogenetic tree; transcript sequences were executed by functional annotation and comparative analysis with other species to determine their functional and evolutionary aspects. The *EglWRKY* genes were classified in three groups according to comparative analysis and their predicted WRKY domains. Based on the *in silico* differential expressed gene (DEG) results, *EglWRKY* genes were selected for a comparative quantitative real-time polymerase chain reaction (qRT-PCR) analysis using leaf and root tissues of cold acclimated *E. globulus* plants for validation.

1.3 Materials and methods

1.3.1 Plant material and cold treatment

Five genotypes of *E. globulus* provided by Bioforest S.A., Chile were employed in this study. Genotypes had been characterized as having contrasting freezing resistance under field condition; three cold resistant genotypes (T1, T2 and T3), and two cold susceptible genotypes (S1 and S2). Eighty-eight ramets of each genotype were placed in a cold chamber with controlled temperature and photoperiod. Plants were kept in aislapol boxes with substrate covered with vermiculite to maintain substrate humidity under control conditions; 14 h day at temperatures of 20/12 ºC (day/night). Cold acclimation (CA) profile was performed following a modified procedure previously described (Fernandez et al., 2010). At day 30, non-acclimated treatment (NA), roots and leaves from five ramets of each genotype were collected, frozen in liquid nitrogen and stored at -80 ºC until use. The second treatment, cold-acclimated before frost (CABF), genotypes were maintained on 14 h day at day/night temperatures of 8/4 ºC; samples of leaves and roots were collected after one week. For the last treatment, cold-acclimated after frost (CAAF), three night frost of -2 ºC and one night frost of -6 ºC with a decrease of 2 ºC/h were applied; after the last night frost, samples were collected when the temperature at the chamber reached 8 ºC, and they were frozen in liquid nitrogen and stored at -80 ºC. Root and leaf samples of genotypes T1, T2, T3, S1 and S2 (5 ramets each) were collected at NA, CABF and CAAF treatments for further qRT-PCR assays. At day 52 chamber conditions were changed to long day (14 h) with 12/6ºC day/night temperature, during one week, to determine the survival and leaf damage of each genotype. For the RNA-Seq analysis three ramets (roots and leaves) of the T3 genotype were sampled at each point.

1.3.2 RNA extraction, library construction and sequencing

Total RNA was extracted from 18 samples (roots and leaves tissues from T3 genotype three biological replicates of three treatments: NA, CABF, CAAF were used for RNA-seq analysis,) using the CTAB RNA isolation procedure (Chang et al., 1993). Additionally, RNA samples were passed through an RNeasy MinElute Cleanup (Qiagen, Germany) to remove any residual impurities. Samples were sequenced at ArrayXpress Inc. (http://www.arrayxpress.com). Eighteen cDNA libraries were created from three independent biological samples for each treatment using the NEBNext

Ultra Directional RNA library prep kit for Illumina (New England Biolabs, USA) and indexed with Oligos kit Set 2 (New England Biolabs, USA). The quality and size distribution of the final sequence libraries were assessed using and Agilent 2100 BioAnalyzer and the DNA High Sensitivity Kit Assay. Sequencing was done on the Illumina HiSeq platform generating single end reads and two lanes per pooled library. The raw reads from Illumina were submitted as BioProject (PRJNA486291) to NCBI.

1.3.3 Quality control, the *de novo* assembly and transcriptomes annotation

The raw reads quality was determined with the FastQC version 0.11.3 program (available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Adapters and low quality reads ($Q < 30$) were removed using PRINSEQ version 0.20.4 program (Schmieder et al., 2011). The ambiguous bases *N*, fragments that were less than 50 base pairs (bp) in length, and complex sequences were removed. To obtain the leaf and root transcriptomes, clean reads were assembled into contigs using three short-read assemblers: SOAPdenovo-Trans (Xie et al., 2014), Trinity (Grabherr et al., 2011) and Oases (Schulz et al., 2012). The assemblies were performed varying *k-mer* length and the minimum contig length was set at 300 bp. To generate unigenes and to remove redundant transcripts, the sequences were reassembled using CAP3 (Huang and Madan, 1999) and clustered by the CD-HIT version 4.6.4 tool (Fu et al., 2012). The identity and the word size settings were 0.95 and 10, respectively. Transcriptomes were filtered and contigs with length ≥ 350 bp were selected. The following parameters were considered to analyze the generated assemblies: the average length, contig number and N50. To evaluate the gene coverage of each assembly the transcriptomes were annotated against *E. grandis* genome v2.0 (JGI Phytozome website https://phytozome.jgi.doe.gov/) using BLAST version 2.2.29+ (Altschul et al., 1990) with *p*value $1e^{-10}$ and identity score cut-off ≥ 90 %. The leaf and root transcriptomes were subject to similarity search against public databases: UniProtKB/SwissProt nr protein database and *E. gransdis* RefSeq. To identify putative TFs in the leaf and root transcriptomes all TF protein sequences from the Plant Transcriptional Factor database (PlantTFDB) (Riaño-Pachon et al., 2007) were used. Matches with *E*-value cutoff of $1e^{-10}$ and similarity score of ≥ 45 % and ≥ 90 % were used, for Blastx and Blastn respectively.

1.3.4 Identification of *EglWRKY* gene family members

WRKY gene members in *E. globulus* transcriptomes were identified using two approaches, BLAST and HMM homology search. The amino acid sequences of 79 EgrWRKY (https://phytozome.jgi.doe.gov/) and 67 AtWRKY proteins (PlantTFDB) were used for a Blastx search (parameters: *E*-value cutoff of $1e^{-10}$ and similarity score of ≥ 45 %). The WRKY domain was identified in the putative EglWRKY proteins by HMM search (https://www.ebi.ac.uk/Tools/hmmer/). Additionally, the conserved WRKY domain was predicted using manual inspection in NCBI-CDD web server (https://www.ncbi.nlm.nih.gov/Structure/cdd/). Sequences with an incomplete WRKY domain were removed. Due to the lack of an *E. globulus* genome, the predicted coding sequences (CDS) of the *EglWRKY* genes were compared with the 79 EgrWRKY members using Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/). Proteins with an incomplete CDS were discarded. Finally, the amino acid properties of the predicted EglWRKY proteins were determined using ScanProsite (https://prosite.expasy.org/scanprosite).

1.3.5 Phylogenetic analysis and conserved motif distribution

The EglWRKY proteins and 67 representative domains of AtWRKY were aligned by ClustalX version 2.1 (Larkin et al., 2007) with default parameters (gap opening penalty = 10, gap extension penalty = 0.1). A phylogenetic tree was constructed using Maximum Likelihood method with 1,000 bootstrap replications using RAxML version 8.0.0 program. The phylogenetic analysis was used to classify the WRKY proteins in *E. globulu*s according with the method of Eugelm et al. (2000). The motif location was analyzed by using the Multiple Expectation Maximization for Motif Elicitation (MEME) program version 5.0.2 (http://meme-suite.org/) (Bailey et al., 2009). The parameters employed for motif location were set as follow: minimum motif width six; maximum motif width 50; and maximum number of motifs 10. The conserved Leu zipper motif was identified using 2ZIP program (http://2zip.molgen.mpg.de/), whereas the motifs HARP, LXXLL and LXLXLX were identified manually.

1.3.6 *In silico* differential expression analysis

High quality reads from *E. globulus* were used for *EglWRKY* gene expression analyses. To determine the transcript abundance of each *WRKY*, libraries of three treatments NA, CABF and CAAF were mapped back to the *E. globulus* leaf and root transcriptomes using Bowtie version 2.1.0 (Langmead et al., 2012) software. For the identification of DEG between the treatments, differential expression analysis was performed using EdgeR (Robinson et al., 2010) package implemented in R (Team RC. 2013). Differentially expressed *WRKY* genes were identified based on the negative binomial distribution and pairwise comparison of three treatments. The false discovery rate (FDR) method was used to determine the threshold of the p value using multiple tests (Benjamini et al., 1995). WRKY genes with observed counts ≥ 1 CPM across all samples were selected, and genes with ≥ 2.0-fold change (FC), false discovery rate (FDR) < 0.05, and P-value < 0.05 were characterized as DEGs. A heatmap was generated to show the expression patterns of *EglWRKY* genes in response to cold stress using R program.

1.3.7 Total RNA extraction and qRT-PCR

Total RNA was extracted from root and leaf samples of the five genotypes of *E. globulus* (T1, T2, T3, S1 and S2) plants subjected to CA profile. RNA was extracted using the CTAB method (Chang et al., 1993). One microgram of RNA was used for cDNA synthesis using the High-Capacity cDNA Reverse

Transcription kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. The sequences of the candidate *EglWRKY* and housekeeping genes were obtained from the *E. globulus* transcriptomes and Primer 3 software version 4.0 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) was used for primer design. Primer sequences are shown in Supplementary Table S5.

Quantitative Real-Time (qRT)-PCR was performed using the Detection System (ABI-7300, Applied Biosystems, USA) and EvaGreen Master Mix (Solis Biodyne) according to the manufacturer's instructions. The qRT-PCRs conditions were used as described by Fernández et al. (2015). Each reaction was performed with three biological and three technical replicates. The stability of housekeeping genes was performed using geNorm v3.4 software (Vandesompele et al., 2002). The expression results were analyzed with the $2^{-\Delta\Delta CT}$ method (Livak et al., 2001). Differences between the values were determined by a two-way ANOVA considering treatments and five genotypes. To determine significant differences a Tukey test was applied using the R program. The expression values and patterns, in both RNA-Seq and qRT-PCR analyses were correlated by Spearman correlation coefficient.

1.4 Results

1.4.1 RNA sequencing and *de novo* assembly

Nine libraries from leaf and nine of root were single-end sequenced on the Illumina platform. Around 10 % of raw reads were removed from the libraries due to read length and low quality (Table S1.1, Table S1.2). After adapter and low-quality end trimming a total of 779,563,543 and 711,005,784 high–quality reads were obtained for leaf and root libraries, respectively. To generate the transcriptomes, two different approaches were carried out. First, three initial assemblies were obtained from Oases, SOAPdenovo-Trans and Trinity. To improve the assemblies, all contigs were clustered into transcripts by the assembler CAP3, and to reduce redundancy the preassemblies were clustered using CD-HIT. Each assembly was evaluated using similarity search against the *E. grandis* genome. The highest number of *E. grandis* unigenes was identified for Trinity assemblies (18,076 and 17,914 unigenes for leaf and root transcriptomes, respectively), in contrast to Oases assemblies, which presented the lowest number of unigenes (17,356 – 15,655 unigenes). Based on the assembly statistics (Table S1.3), Trinity was found to be the best among all the assemblers employed for leaf, which generated 149,792 transcripts with > 350 bp length and a mean length of 1,567 bp.

SOAPdenovo-Trans generated the best assembly for root transcriptome, resulting in 102,654 transcripts with a mean length 740 bp. Leaf transcripts ranged between 501-1,000 bp (29%) in length, while root transcripts ranged from 350-500 bp (45 %).

1.4.2 *E. globulus* transcriptomes annotation

The BLAST results showed that 27,404 and 22,517 unigenes from leaf and root transcriptomes were matched in the *E. grandis* RefSeq (Table S1.4). According to prediction of transcription factors in *E. globulus* transcriptomes, a total of 3,624 putative TFs were identified in the leaf transcriptome and classified into 58 TF families. The most represented TF families in leaf were bHLH, MYB related, NAC, ERF and WRKY representing 5% of the total of TFs identified (Fig.1.1a). The root transcriptome presented 2,869 putative TFs, classified into 56 out of the 58 TF families available in PlantTFDB. The largest family was bHLH, followed by NAC, MYB related, MYB, and bZIP, for root the WRKY family represented 4% of the total TFs identified (Fig.1.1b). The major percentage of leaf transcripts matched with TFs from *E. grandis* (16 %), *Malus domestica* (6 %) and *Eucalyptus camaldulensis* (5 %), whereas 53 % (8,091) were aligned to TFs of other plant species. A large proportion of root transcripts presented similarity to TFs from *E. grandis* (10 %), *Malus domestica* (8 %) and *Actinidia chinensis* (6 %).

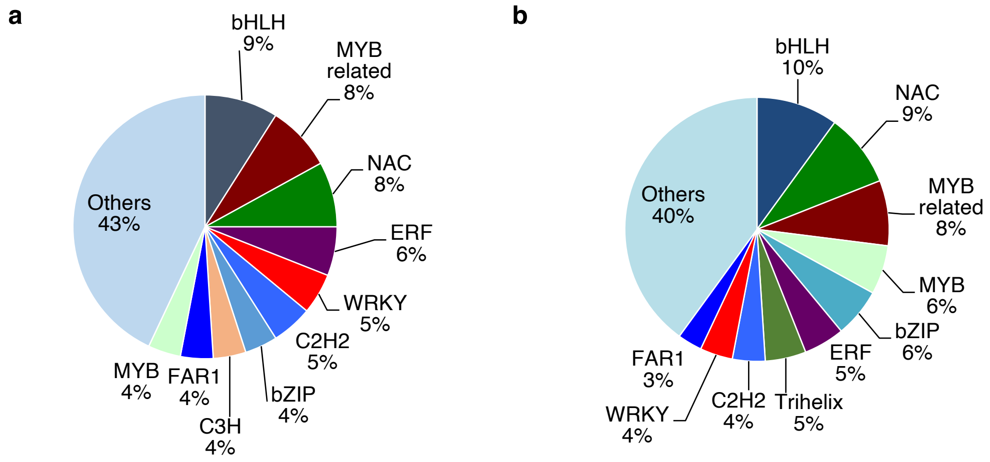


Fig.1.1 Transcription factor families most represented in *E. globulus* transcriptomes; **a** leaf transcriptome, **b** root transcriptome

1.4.3 *EglWRKY* members and structural classification

WRKY genes present in leaf and root transcriptomes of *E. globulus* were identified by BLAST. *Arabidopsis* and *E. grandis* WRKY amino acid sequences were used to identify the EglWRKY proteins. Initially, a total of 75 *EglWRKY* putative genes were identified but a total 51 EglWRKY proteins presented a complete WRKY domain and open reading frame (ORF). The putative *WRKY* genes were termed as *EglWRKY1* to *EglWRKY51*. The CDS length of the 51 *EglWRKY* genes ranged from 339 to 2,256 bp, and the amino acid length ranged from 112 to 751 aa (Table1.1).

Table 1.1 Characteristics of WRKY proteins in *E. globulus*

Gene name	cDNA length	Aa	Mw (KDa)	pI	Group	WRKY domain	Zinc finger motif	AtWRKY homologue
EglWRKY1	1608	535	57.5	6.50	IIb	WRKYGQK	CX$_5$CX$_{22}$HXH	*AtWRKY47*
EglWRKY2	1350	449	48.9	5.80	I	WRKYGQK X2	CX$_4$CX$_{22-23}$HXH	*AtWRKY1*
EglWRKY3	1404	467	51.3	8.80	I	WRKYGQK X2	CX$_4$CX$_{22-23}$HXH	*AtWRKY44*
EglWRKY4	726	241	27.3	8.80	IIc	WRKYGQK	CX$_4$CX$_{23}$HXH	*AtWRKY71*
EglWRKY5	1188	395	42.3	5.61	IIe	WRKYGQK	CX$_5$CX$_{23}$HXH	*AtWRKY27*
EglWRKY6	1038	345	38.6	9.64	IId	WRKYGQK	CX$_5$CX$_{23}$HXH	*AtWRKY21*
EglWRKY7	633	210	23.8	8.94	IIc	WRKYGQK	CX$_4$CX$_{23}$HXH	*AtWRKY75*
EglWRKY8	879	292	32.0	9.73	IId	WRKYGQK	CX$_5$CX$_{23}$HXH	*AtWRKY11*
EglWRKY9	1464	487	52.6	8.75	I	WRKYGQK X2	CX$_4$CX$_{22-23}$HXH	*AtWRKY32*
EglWRKY10	480	159	18.6	5.17	IIc	WRKYGKK	CX$_4$CX$_{23}$HXH	*AtWRKY50*
EglWRKY11	699	232	25.9	9.38	IIa	WRKYGQK	CX$_5$CX$_{23}$HXH	*AtWRKY40*
EglWRKY12	1545	514	53.8	9.08	I	WRKYGQK	CX$_4$CX$_{22-23}$HXH	*AtWRKY32*
EglWRKY13	867	288	32.4	8.52	IIa	WRKYGQK	CX$_5$CX$_{23}$HXH	*AtWRKY60*
EglWRKY14	1020	339	36.4	9.86	IId	WRKYGQK	CX$_5$CX$_{23}$HXH	*AtWRKY11*
EglWRKY15	669	222	24.6	8.21	IIc	WRKYGQK	CX$_4$CX$_{23}$HXH	*AtWRKY12*
EglWRKY16	1227	408	44.6	8.69	IIe	WRKYGQK	CX$_5$CX$_{23}$HXH	*AtWRKY22*
EglWRKY17	372	123	14.4	10.0	IIc	WRKYGQK	CX$_4$CX$_{23}$HXH	*AtWRKY56*
EglWRKY18	1014	337	37.7	5.28	III	WRKYGQK	CX$_7$CX$_{23}$HXC	*AtWRKY41*
EglWRKY19	1083	360	40.3	5.51	III	WRKYGQK	CX$_7$CX$_{23}$HXC	*AtWRKY53*
EglWRKY20	1818	605	64.5	6.58	IIb	WRKYGQK	CX$_5$CX$_{22}$HXH	*AtWRKY6*

EglWRKY21	1305	434	47.1	9.93	IId	WRKYGQK	$CX_5CX_{23}HXH$	*AtWRKY7*
EglWRKY22	1794	597	64.2	6.09	I	WRKYGQK X2	$CX_4CX_{22-23}HXH$	*AtWRKY20*
EglWRKY23	1524	507	54.6	5.85	IIb	WRKYGQK	$CX_5CX_{22}HXH$	*AtWRKY6*
EglWRKY24	2256	751	81.1	5.81	I	WRKYGQK X2	$CX_4CX_{22-23}HXH$	*AtWRKY2*
EglWRKY25	1803	600	65.5	8.61	I	WRKYGQK X2	$CX_4CX_{22-23}HXH$	*AtWRKY20*
EglWRKY26	1020	339	37.6	8.50	IIa	WRKYGQK	$CX_5CX_{23}HXH$	*AtWRKY40*
EglWRKY27	801	266	30.9	9.04	IIa	WRKYGQK	$CX_5CX_{23}HXH$	*AtWRKY40*
EglWRKY28	1326	441	48.3	8.88	IIb	WRKYGQK	$CX_5CX_{22}HXH$	*AtWRKY9*
EglWRKY29	933	310	32.7	6.80	IIc	WRKYGQK	$CX_4CX_{23}HXH$	*AtWRKY57*
EglWRKY30	966	321	36.1	5.27	III	WRKYGQK	$CX_7CX_{23}HXC$	*AtWRKY70*
EglWRKY31	978	325	35.9	6.50	IIc	WRKYGQK	$CX_4CX_{23}HXH$	*AtWRKY23*
EglWRKY32	870	289	32.3	4.68	IId	WRKYGQK	$CX_5CX_{23}HXH$	*AtWRKY35*
EglWRKY33	561	186	21.2	9.00	IIc	WRKYGQK	$CX_4CX_{23}HXH$	*AtWRKY75*
EglWRKY34	786	261	27.6	9.14	IIb	WRKYGQK	$CX_5CX_{22}HXH$	*AtWRKY42*
EglWRKY35	804	267	29.9	8.94	IIc	WRKYGQK	$CX_4CX_{23}HXH$	*AtWRKY13*
EglWRKY36	573	190	21.4	7.76	IIc	WRKYGKK	$CX_4CX_{23}HXH$	*AtWRKY51*
EglWRKY37	576	191	21.8	5.99	IIc	WRKYGKK	$CX_4CX_{23}HXH$	*AtWRKY51*
EglWRKY38	1038	345	37.4	9.61	IId	WRKYGQK	$CX_5CX_{23}HXH$	*AtWRKY15*
EglWRKY39	1230	409	44.6	6.11	IIb	WRKYGQK	$CX_5CX_{22}HXH$	*AtWRKY72*
EglWRKY40	960	319	35.1	5.45	III	WRKYGQK	$CX_7CX_{23}HXC$	*AtWRKY70*
EuglWRKY41	1071	356	38.9	7.02	III	WRKYGQK	$CX_7CX_{23}HXC$	*AtWRKY55*
EuglWRKY42	339	112	12.8	8.56	IIc	WKKYGQK	$CX_4CX_{23}HXH$	*AtWRKY23*
EuglWRKY43	957	318	35.2	8.52	IIc	WRKYGQK	$CX_4CX_{22}HXH$	*AtWRKY33*
EuglWRKY44	948	315	34.9	7.61	IIa	WRKYGQK	$CX_5CX_{23}HXH$	*AtWRKY40*
EuglWRKY45	1113	370	40.5	5.38	III	WRKYGQK	$CX_7CX_{23}HXC$	*AtWRKY41*
EuglWRKY46	1884	627	67.3	6.58	IIb	WRKYGQK	$CX_5CX_{22}HXH$	*AtWRKY6*
EuglWRKY47	828	275	29.6	5.80	IIe	WRKYGQK	$CX_5CX_{23}HXH$	*AtWRKY69*
EuglWRKY48	1008	335	37.4	6.51	IIc	WRKYGQK	$CX_4CX_{23}HXH$	*AtWRKY28*
EuglWRKY49	822	273	30.6	8.24	III	WRKYGQK	$CX_7CX_{23}HXC$	*AtWRKY70*
EuglWRKY50	918	305	33.8	5.20	IIc	WRKYGQK	$CX_4CX_{23}HXH$	*AtWRKY49*
EuglWRKY51	1392	463	50.2	9.37	I	WRKYGQK X2	$CX_4CX_{22-23}HXH$	*AtWRKY44*

According with the number of WRKY domains and the pattern of zinc-finger structure, the EglWRKY proteins were categorized into three major groups: I, II, or III. The detailed alignment of the core WRKY domains showed that the 51 EglWRKY members contained one or two identical WRKYGQK domains followed by a zinc-finger motif (Fig. S1.1). Eight EglWRKY members were assigned to group I; which contained two WRKY domains and the C_2H_2-type zinc-finger motif ($CX_4CX_{22\text{-}23}HXH$). Group II contained the largest number of EglWRKY proteins, which had a single WRKY domain with $CX_{4\text{-}5}CX_{23}HXH$ zinc-finger structure. This group was divided into five subgroups; IIa, IIb, IIc, IId and IIe, with 5, 7, 15, 5 and 4 members, respectively. Seven EglWRKY proteins were classified into group III, which presented a different zinc finger motif structure (CXH) and considered the most evolutionary advanced group and the most adaptable. The EglWRKY protein alignment showed that subgroups IIa, IIb and IId presented a similar structure of WRKY domain, while subgroup IIc and group III showed a major divergence in WRKY domain structure. To determine the phylogenetic relationship between all 51 EglWRKY, a consensus phylogenetic tree was obtained from the alignment of the 197 WRKY protein sequences (51 from *E. globulus*, 67 from *A. thaliana* and 79 from *E. grandis*) (Fig.1.2). EglWRKY, AthWRKY and EgrWRKY members of group I were represented into an independent clade in the phylogenetic tree and showed a close relationship with the majority members of subgroup IIc and subgroup IIa. On the other hand, members of group II showed a relatively close relation between them, while group III diverged from the other groups.

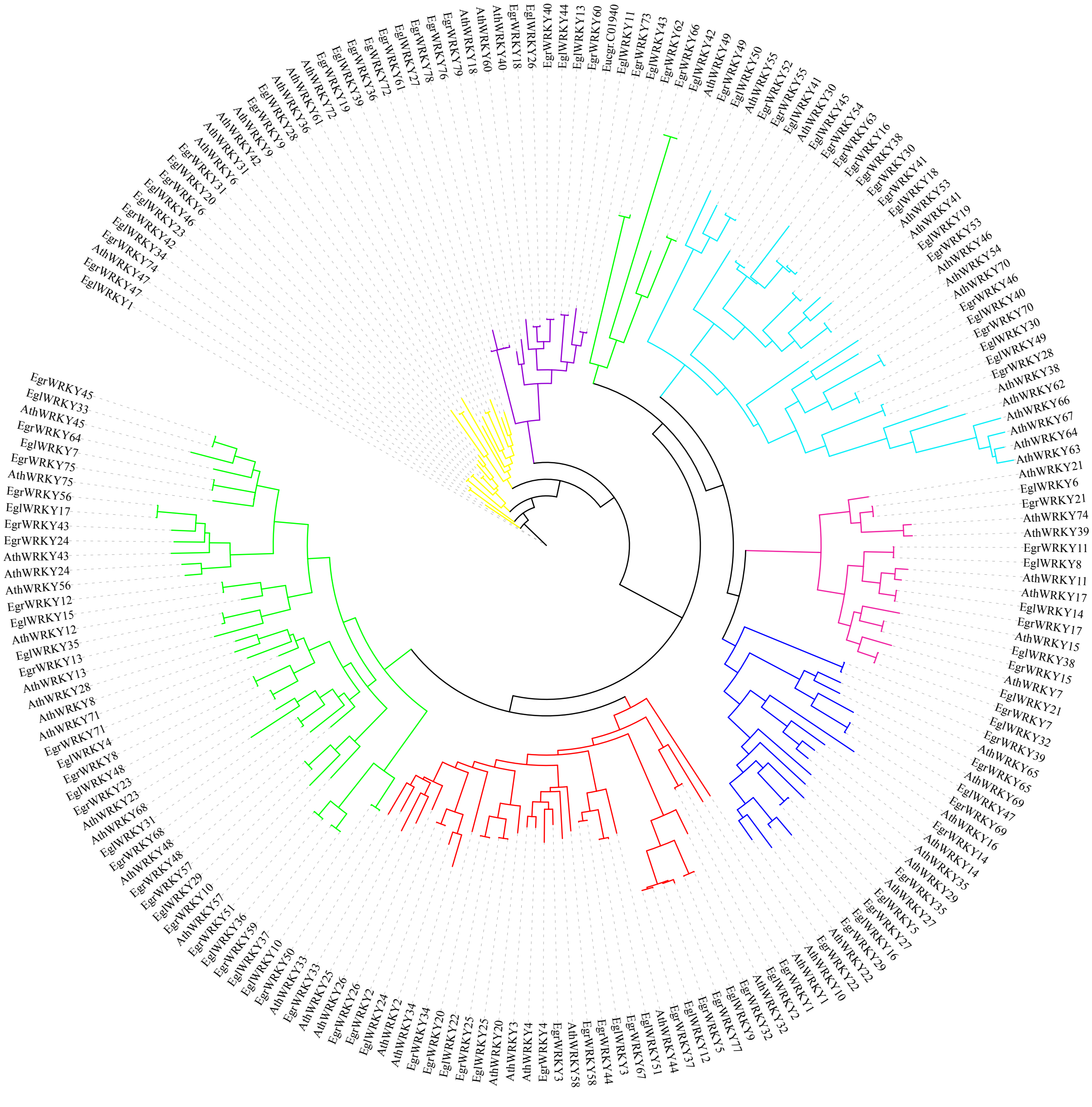


Fig.1. 2 Phylogenetic tree of 51 EglWRKY, 67AthWRKY and 79 EgrWRKY proteins. EglWRKY. Proteins were classified in the groups and subgroups; red (group I), purple

(group IIa), yellow (group IIb), green (group IIc), pink (group IId), blue (group IIe), and white blue (group III).

1.4.4 Conserved motif prediction of *EglWRKY* genes

The conserved shared motifs were determined using the full-length ORFs of EglWRKY proteins by MEME program. Ten motifs were identified in the 51 EglWRKY protein sequences. The pattern of the conserved motifs is listed in Table 1.2 and illustrated in Fig.1.3. Motifs 1, 2 and 3 formed the main structure of the WRKY domain (WRKYGQK); the motifs 1 and 3 were identified in all EglWRKY proteins; while motif 2 was absent in EglWRKY43. Motif 4 was exclusive for group I, whereas motif 8 was only observed in group IIb. Motif 5 was present in members of groups I, IIb, and IIc. The motifs 6, 7 and 10 were identified in groups IIa and IIb. The motif 9 was observed in groups IIe and IId. In addition, other conserved motifs were detected manually in EglWRKY proteins. The Leu zipper motif, was identified in two members of subgroup IIa (EglWRKY27 and EglWRKY44), and one from IIb (EglWRKY1). Four members of EglWRKY subgroup IId (EglWRKY8, EglWRKY14, EglWRKY21 and EglWRKY38) presented the HARF motif. The conserved motif HARF function is unclear (RTGHARFRR[A/G]P), but it has been described in group IId of *Arabidopsis*

(Eulgem et al., 2000; Xie et al., 2005). The co-activator motif LXXLL (L, leucine; X, any amino acid), which has previously been associated with the activation of plant immune responses (Zhang et al., 2014) was found in five proteins distributed in different groups (EglWRKY4, EglWRKY38, EglWRKY16, EglWRKY18, and EglWRKY30); whereas the active repressor motif, LXLXLX, was identified in EglWRKY27, EglWRKY28, EglWRKY39, EglWRKY20, EglWRKY43, EglWRKY8 and EglWRKY16.

Table 1.2 Details of conserved motifs from EglWRKY proteins

Motif	Width	Motif sequence
1	21	ILDDGYRWRKYGQKVVKGSPF
2	29	QGCPVKKQVZRSSEDPSILITTYEGEHNH
3	8	PRSYYRCT
4	50	DGYNWRKYGQKQVKGSEYPRSYYKCTHPNCPVKKKVERSPDGHITEIIYK
5	15	IREPRVAFRTRSEVD
6	44	ELGVLKEELDRMSEENKKLKEMLEQVTKNYNALQSHLJELMQKQ
7	28	SLVEQMAASJTSDPNFTAALAAAISSVI
8	25	PLPPAATAMASTTSAAASMLLSGST
9	20	CHCSKKRKHRVKRVVRVPAI
10	15	SASAPFPTITLDLTQ

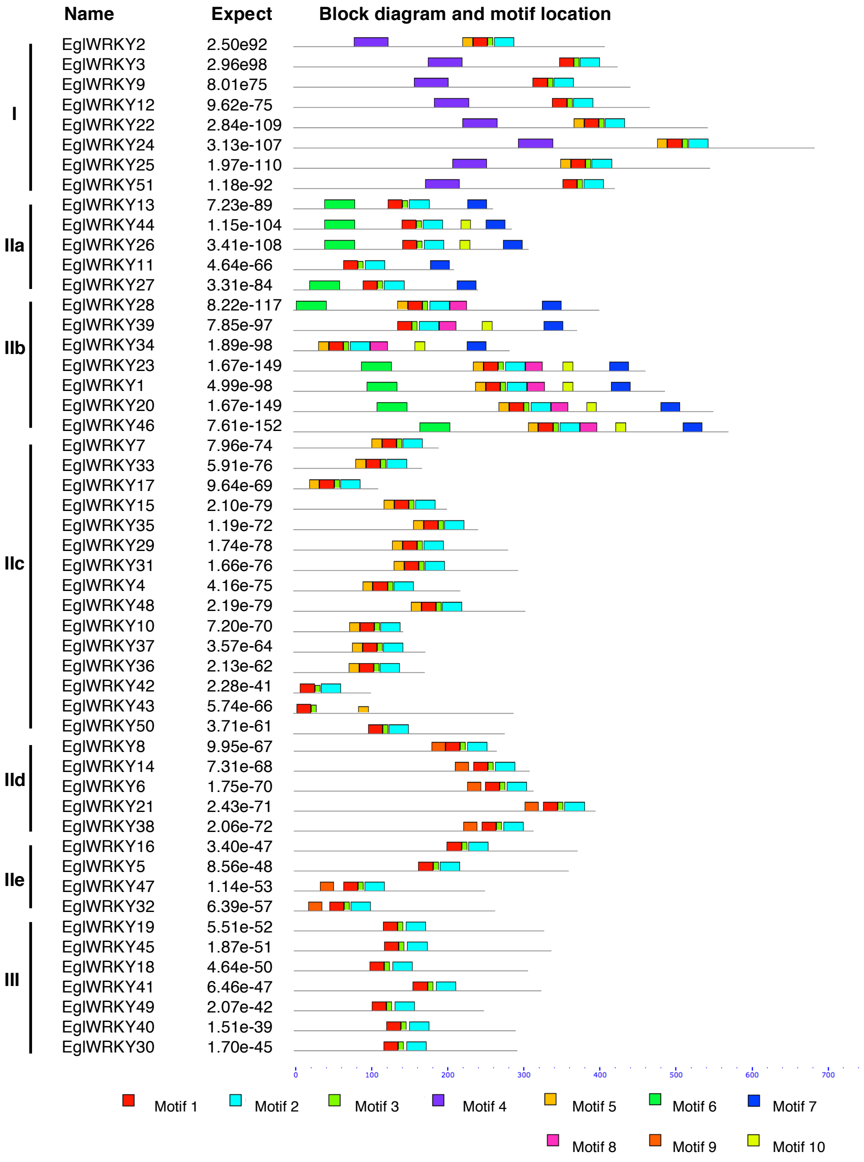


Fig.1. 3 Conserved motifs distribution of 51 EglWRKY amino acid sequences. The motifs were numbered 1-10 and are displayed in different colors.

1.4.5 Identification of cold-response *EglWRKY* genes

To reveal the putative cold-responsive role of WRKY TFs in *E. globulus*, the expression patterns of 51 *EglWRKY* genes under CA profile were determined by RNA-Seq analysis in one cold resistant genotype (T3). The results showed that 42 out of the 51 *EglWRKY* genes were DE under cold stress (Fig.1.4a). Fourteen WRKYs out of 51 were differentially expressed in response to CA in leaf samples ($FC \geq 2.0$, $FDR < 0.05$, and $P < 0.05$) (Fig1.4b). Ten *EglWRKY* genes were up-regulated under CA, where *EglWRKY11* (Group IIa) presented the highest induction detected in all treatments (> 4-fold at CAAF). The expression level of genes *EglWRKY39* (Group IIb), *EglWRKY38* (Group IId), *EglWRKY20* (Group IIb), *EglWRKY16* (Group IIe), *EglWRKY 31* (Group IIc), *EglWRKY26* (Group IIa) and *EglWRKY23* (Group IIb) increased more than 2-fold at the CAAF treatment. *EglWRKY4* (Group IIc) and *EgWRKY18* (Group III) exhibited an early response, their expression levels increased significantly under cold conditions (4 ºC, CABF treatment). The expression level of *EglWRKY13* (Group IIa), *EglWRKY27* (Group IIa), *EglWRKY37* (Group IIc) and *EglWRKY36* (Group IIc) genes decreased significantly in response to CAAF treatment. The expression level of *EglWRKY36* was the strongest inhibited 4-fold at CAAF. None of the studied *EglWRKY* gene was

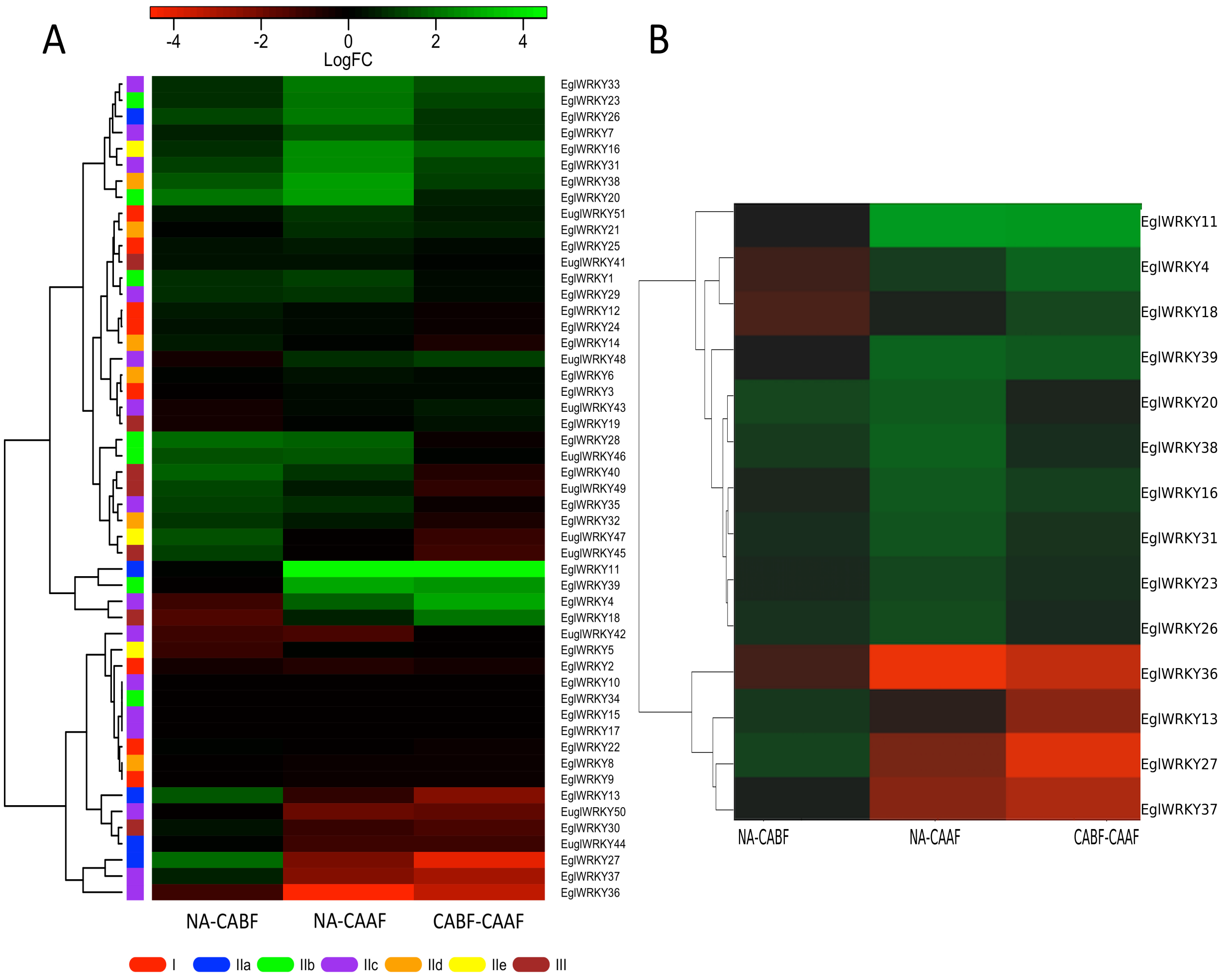
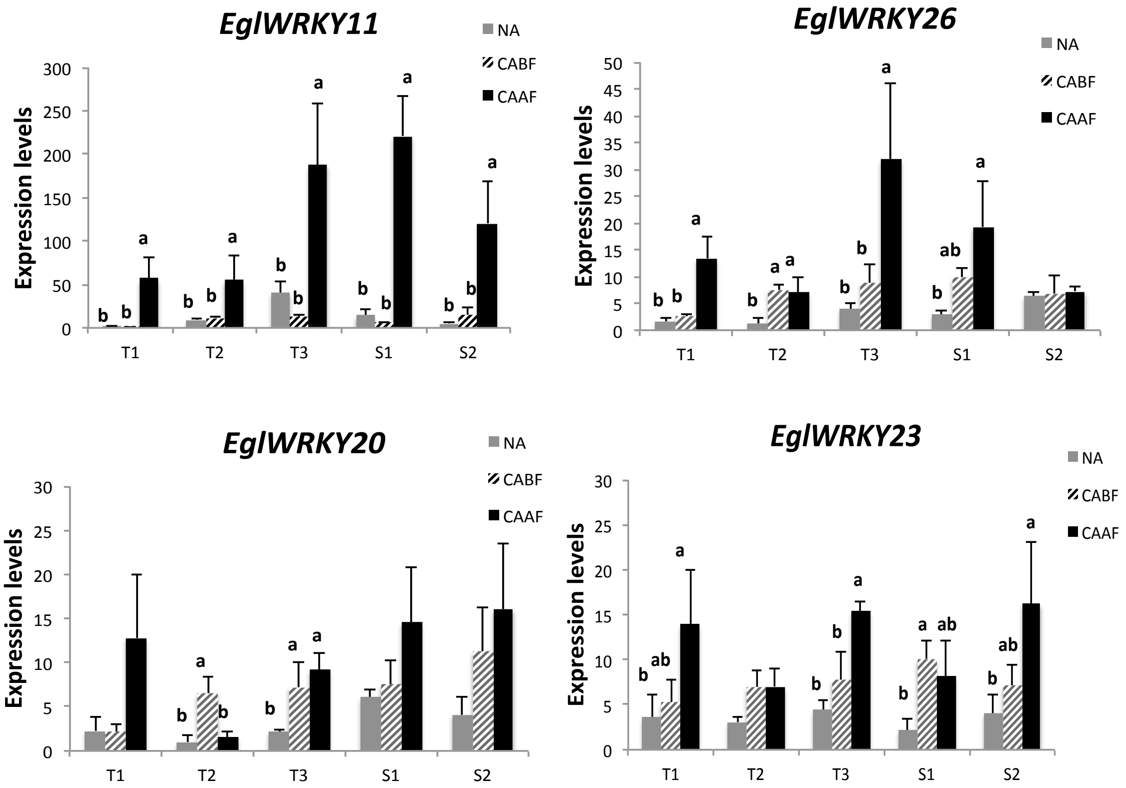


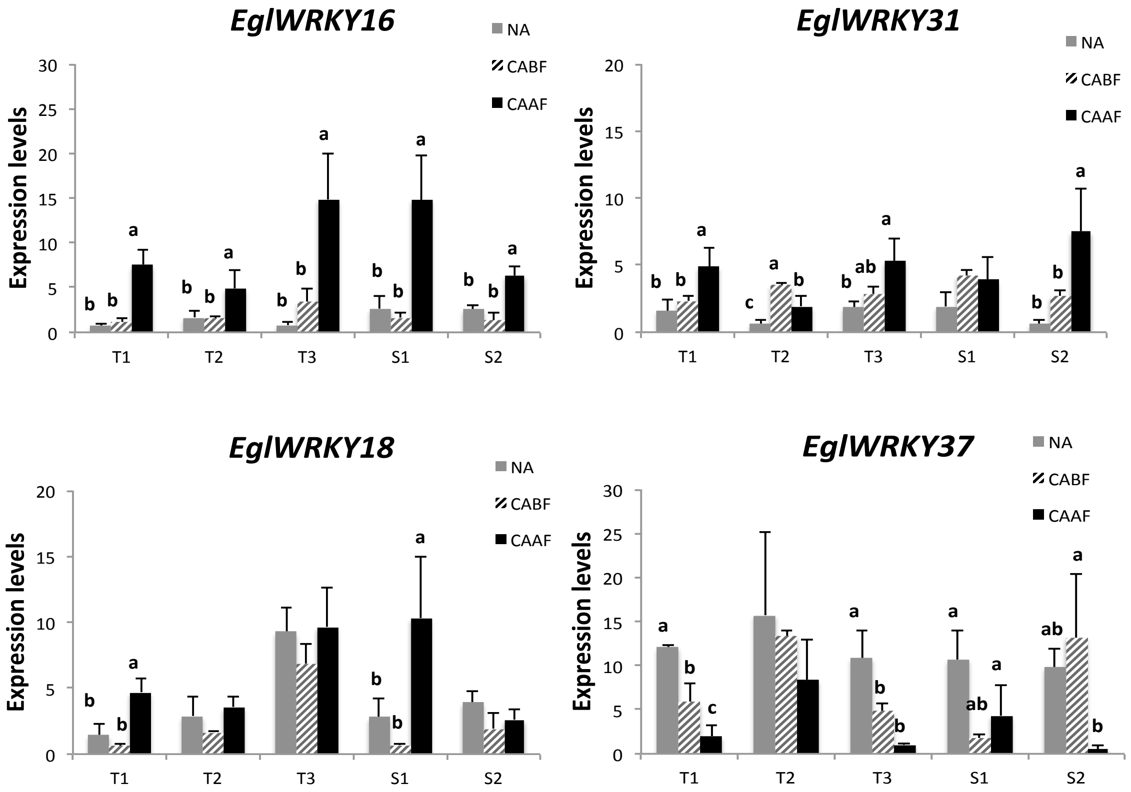
Fig.1. 4 Heatmap of *EglWRKY* genes in leaf under cold acclimation treatment (NA non-acclimated, CABF cold acclimated before frost, CAAF cold acclimated after frost). (a) Expression profiles of 51 *EglWRKY* genes. (b) Expression patterns of the 14 *EglWRKY* selected for qRT-PCR. Green and red boxes indicate high and low expression levels, respectively

1.4.6 Relative expression of cold-responsive *EglWRKY* genes

According to the RNA-Seq results, 14 candidate *EglWRKY* genes were selected to validate their expression patterns in five different genotypes (3 tolerant and 2 susceptible genotypes) by qRT-PCR. The expression levels were determined in leaf and root samples under CA profile (three treatments NA, CABF and CAAF). The results showed that 11 *EglWRKY* genes exhibited significant changes in leaf samples; no significant changes could be observed in root samples. The transcript abundance of *EglWRKY11*, *-26*, -*23* and *-16* genes increased significantly in tolerant and susceptible genotypes under cold stress. Significant difference in the expression level of *EglWRKY39* gene was observed in the tolerant genotypes, while the genes *EglWRKY20*, *-31*, *-18,* and *EglWRKY37* exhibited significant differences in one of the two susceptible genotypes studied. The transcript abundance of *EglWRKY36* was significant different in two tolerant genotypes (T2 and T3), while *EglWRKY38* showed significant changes between treatments of the CA profile. The expression patterns of *EglWRKY11*, *-26*, *-20*, *-23*, *-16*, *-38*, *-31*, *-18*, and *-37* are represented in Fig.1.5 (Supplementary Fig.S1.2 shows the expression patterns of genes *EglWRKY36, -38* and *-39*). The relative expression values ($2^{-\Delta\Delta CT}$) of 11 *EglWRKY* genes were transformed to log-2

FC and compared to RNA-Seq (Supplementary Table S6). Most of 11 *EglWRKY* genes presented a similar expression pattern in both RNA-Seq and qRT-PCR analysis.




EglWRKY16

EglWRKY31

EglWRKY18

EglWRKY37

Fig.1.5. Expression patterns of cold-responsive *EglWRKY* genes in leaves of five genotypes of *E. globulus* under cold acclimation profile (NA, no acclimated; CABF, cold acclimated before frost; CAAF, cold acclimated after frost). The expression levels were determined by qRT-PCR using Ubiquitine and Elongation factor 1-a as reference genes. Letters on top of the bars (mean+SE) indicate statistically significant differences between treatments (a, b and c) evaluated with a Tukey test ($p \leq 0.05$).

1.5 Discussion

1.5.1 *De novo* assembly of *E. globulus* transcriptomes and annotation

Due to the lack of a complete genome, a whole-transcriptome analysis was performed with RNA-seq data from cold acclimated plants of *E. globulus*. In this study, three assemblers were used to construct the leaf and root transcriptomes. The effectiveness of the assembly programs is variable according to the input data set, with no assembler outperforming any other (Visser et al., 2015). Trinity exhibited a high sensitivity in leaf assembly, while SOAPdenovo trans showed uniformity in assembled contigs of root. Oases assembly was discarded due to the high number of large contigs (> 20,000 bp); which could be related to erroneous, miss-assembled and chimeric contigs (Martin and Wang, 2011). The resulting leaf transcriptome contained 149,792 transcripts; while the root assembly had 102,654 transcripts, obtaining more data than our previous study (Fernandez et al., 2015). The *E. globulus* transcriptomes covered a higher proportion of the *E. grandis* genes (> 50 %) (Supplementary, Table S4), indicating that *E. globulus* transcripts were well assembled. When the total transcripts were matched in plantTFDB 58 TF families were identified. Among these, bHLH, MYB-related, NAC, ERF and WRKY TF families were found to be abundant in both transcriptomes, similar result were observed in *V. vinifera* where AP2/ERF, NAC and WRKY were the transcription factor families most commonly observed and differentially regulated under cold stress (Londo et al., 2018).

1.5.2 Phylogenetic analysis of *EglWRKY*s and motif identification

A total of 51 *Egl*WRKY genes were identified in plants of *E. globulus* in response to cold treatment, the amount and group distribution of *EglWRKY* were similar to those reported in other woody plants, such as *E. grandis* (79) (Myburg et al., 2014), *Vitis vinifera* (59) (Wang et al., 2014), *C. sinesis* (50) (Wu et al., 2016) and *P. persica* (61) (Gu et al., 2016).

According to the sequence similarity and distribution of the conserved domains, *EglWRKY* genes were clearly divided among three groups namely I, II and III. Eight *EglWRKY* members were assigned to group I; this group is present in all species, including two non-plant species as *G. lamblia* and *D. discoideum* (Rushton et al., 2010). Members of the WRKY TF group from different plants are variable and not consistent among species (Mohanta et al., 2016). Group II contained the largest number of EglWRKY proteins (36 members), which is comparable with that reported in *E. grandis* (group II contained 50 members) (Fan et al., 2018). This could be explained due to the rapid expansion of this subgroup in dicots that have been associated with segmental and tandem gene duplication in *Arabidopsis* (Cannon et al., 2004; Wu et al., 2005). The group II members, were distributed in five subgroups: IIa, IIb, IIc, IId and IIe in *E. globulus*. The phylogenetic analysis showed that subgroups IIa and IIb are related and have a common ancestor; the same is true for subgroups IId and IIe (De Almeida et al. 2017). Interestingly, subgroup IIc was divided in the phylogenetic tree and related with groups II and I. This was previously shown in species as *Ricinus communis* (Zou et al., 2016), *Oryza nivara* (Xu et al., 2016) and *Theobroma cacao* (De Almeida et al., 2017). Furthermore, previous studies have shown that subgroup IIc had a higher divergence than the other subgroups in the evolution of the WRKY family and is closely related to group III (Wang et al., 2014; Zou et al., 2016; Xu et al., 2016).

The core sequence of WRKY proteins is a conserved WRKYGQK motif in the N-terminus (Rushton et al., 2010; Cheng et al., 2012). However, the WRKYGKK and WKKYGQK motif varieties were also observed (EglWRKY10, -36, -37 and -42, respectively). This phenomenon has been observed in members of subgroup IIc in *Jatropha curcas* L., Arabidopsis, rice and other plant species (Rushton et al., 2010; Cheng et al., 2012; Xiong et al., 2013; Baranwal et al., 2016; Li et al., 2017) suggesting that the WRKY proteins of subgroup IIc may have a variety of biological functions (Song et al., 2016a). Other variants in the WRKY domains have been identified in *Glycine max*, motif such as WRKYGEK, WRKYGKR, WRKYEDK, WKKYGQK, and WHQYGLK (Song et al., 2016b). In gymnosperm plants, such as *Taxus* or *P. abies* only the WRKYGKK variant has been identified indicating that the other variants should be specific in angiosperm plants (Zhang et al., 2018). The variation on the heptad sequence might influence interactions of WRKY genes with some of their downstream target genes (Van Verk et al., 2008; Kumar et al., 2016). In *Nicotiana tabacum*, the WRKYGKK variant in *NtWRKY12* gene binds to a different motif (TTTTCCAC), which is significantly different from the consensus sequence of W-box (Van Verk et al., 2008). Additionally, the predicted proteins EglWRKY1, -27 and -44 contained a Leucine zipper motif, which was shown to be involved in WRKY-WRKY interaction by dimerization and DNA binding in *Arabidopsis* members of subgroups IIa (AtWRKY18, -40 and -60) (Chen et al., 2010) and IIb (AtWRKY6) (Robatzek et al., 2001). Ten different types of domains were found in EglWRKY proteins, acquiring these domains, could explain additional functions, leading to variations in their structural and physiological features (Li et al., 2017).

1.5.3 Expression patterns of *EglWRKY*s under CA

The WRKY family has multiple roles in plant defense; many reports suggested WRKY play a fundamental role in plant response to biotic and abiotic stress especially in angiosperms. This could be essential for the adaptability in flower plants, under different environments (Zhang and Wang 2005). The expression profile generated by qRT-PCR in this work (Fig.1.5) showed different expression patterns for *EglWRKY*, which coincide in most cases, with the expression patterns obtained in the RNA-Seq analysis

Ten of the 11 *EglWRKY* DEG were classified in group (II), several studies have identified that genes of this group play an important role in abiotic stress response in plants (Kiranmai et al., 2016; Zhang et al., 2018; Zhou et al., 2008). Previous studies revealed that WRKY genes present in groups II and III had an important role in abiotic stress conditions (He et al., 2017), in *V. vinifera* for example 15 *VvWRKY*s showed that cold stress-induces their expression patterns and 8 of them belong to group II (Wang et al., 2014).

In this study *EglWRKY11, EglWRKY26*, both members of the WRKY IIa subgroup were up-regulated in the cold tolerant genotypes (T1, T2 and T3) under cold stress (CABF and CAAF treatments). In Arabidopsis *AtWRKY40* putative orthologous of *EglWRKY11* and *EglWRKY26* is up-regulated under drought, salt and cold stresses (Cheng et al., 2010). *EglWRKY11* and *EglWRKY26* showed to be upregulated under cold and freezing conditions, a similar pattern was observed in *V. vinifera* where *VvWRKY28*, also orthologous of *AtWRKY40* in Arabidopsis, presented a strong induction under freezing condition (Londo et al. 2018). These results show a potential role of both genes in response to cold stress and suggest they may have similar functions to the corresponding Arabidopsis orthologous gene.

Among the members of group IIb, *EglWRKY20* and *EglWRKY23* were upregulated under freezing temperatures. *EglWRKY20* despite presenting high levels of expression in the freezing condition in comparison with the control, this gene presented its highest expression level in the sensitive genotypes suggesting a negative role in the regulation of cold stress, similar to what has been described by Li et al. (2017) in Arabidopsis. *AtWRKY6* putative orthologous of *EglWRKY20*, has been involved in cold acclimation under the regulation of BZR1, an other important TF in cold condition, in this case *AtWRKY6* acts as a repressor of BZR1, resulting in a decrease to freezing tolerance in Arabidopsis plants.

EglWRKY31, member of group IIc, was upregulated under freezing temperatures, there is evidence which suggests that WRKY IIc proteins regulate response to abiotic stress (Wu et al., 2017), in Arabidopsis for example, *AtWRKY8* gene (IIc) was highly up-regulated under salt treatment. In transgenic Arabidopsis plants containing two IIc genes of soybean (GmWRKY21/54) confer differential level of tolerance to abiotic stresses (Hu et al., 2013; Zhou et al., 2008). Contrary *EglWRKY36* and *EglWRKY37* genes, also members of group IIc, were the only genes that decreased significantly its response under freezing treatment. These results are similar to those shown in *V. vinefera*, where its putative orthologous *VvWRKY6* had a reduced expression level under cold acclimation treatment (Londo et al., 2018). As described by Gao et al. (2011) *AtWRKY51* putative orthologous of both genes, might serve as positive regulator of SA-mediated signaling, involved in defense response to biotic stress. *EglWRKY16* showed a similar expression pattern as *EglWRKY11,* this gene member of group IIe showed significant differences in freezing condition compared to the control in all the genotypes evaluated, similar to what has been described by Fan et al. (2018)

in *E. grandis,* with its putative orthologous *EgrWRKY39* when submitting *E. grandis* plants to cold stress.

The results obtained in this study show that in general members of the WRKY TFs within the same group or subgroup share a similar gene structure, length and amino acid motif composition, indicating their close evolutionary relationship and a similar function. *EglWRKY* genes showed significant differences in leaf tissue of plants submitted to low temperatures suggesting that they could have a possible role in response to cold stress. Even though only eleven *EglWRKY* genes had significant differences in the qRT-PCR analysis, most genes showed an increase in their expression levels in CABF or CAAF *in silico* analysis. Our result are similar to those described for WRKYs genes in *E. grandis* where most of them were markedly enhanced after cold stress treatment, suggesting an important role of the WRKY family in the Eucalyptus under cold stress.

1.6 Conclusion

The present study used RNA-seq to generate a comprehensive transcriptome resource of leaf and root of *E. globulus* under cold stress, focusing mainly on WRKYs. This data provides the identification, phylogenetic relationship, structure and expression profile of 51 *EglWRKY* genes. Expression patterns derived from the RNA-seq and qRT-PCR analyses revealed that 9 *EglWRKY* are up-regulated during cold acclimation in leaf tissue. These results provide the basis for further studies and analysis of *WRKY* genes to identify their function and molecular mechanisms involves in abiotic stress responses in *E. globulus*.

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1.8 Supplementary material

Table S1.1 *E. globulus* leaf reads statistics

Biblioteca	Number of reads	Filtered reads	Percent
NA_R1_leaf	103,769,722	93,329,886	89.94
NA_R2_leaf	79,428,215	70,467,746	88.72
NA_R3_leaf	77,894,339	70,141,437	90.05
AAH_R1_leaf	59,634,653	52,979,321	88.84
AAH_R2_leaf	132,522,836	119,824,124	90.42
AAH_R3_leaf	127,897,688	114,712,295	89.69
ADH_R1_leaf	59,130,449	53,914,935	91.18
ADH_R2_leaf	70,486,544	63,855,996	90.59
ADH_R3_leaf	68,799,097	62,184,233	90.39

Table S1.2 *E. globulus* root reads statistics

Library	Number of reads	Size	Filtered reads	Percent
NA_R1_root	32,981,816	125	22,172,206	67.23
NA_R2_root	46,457,343	125	32.442,451	69.83
NA_R3_root	40,229,074	125	30,682,517	76.27
CABF_R1_root	53,469,836	125	39,245,508	73.40
CABF_R2_root	49,987,457	125	34,955,949	69.93
CABF_R3_root	10,234,169	125	7,441,826	72.72
CAAF_R1_root	33,998,748	125	24,412,568	71.80
CAAF_R2_root	45,696,827	125	31,522,820	68.98
CAAF_R3_root	62,228,013	125	45,518,625	73.15

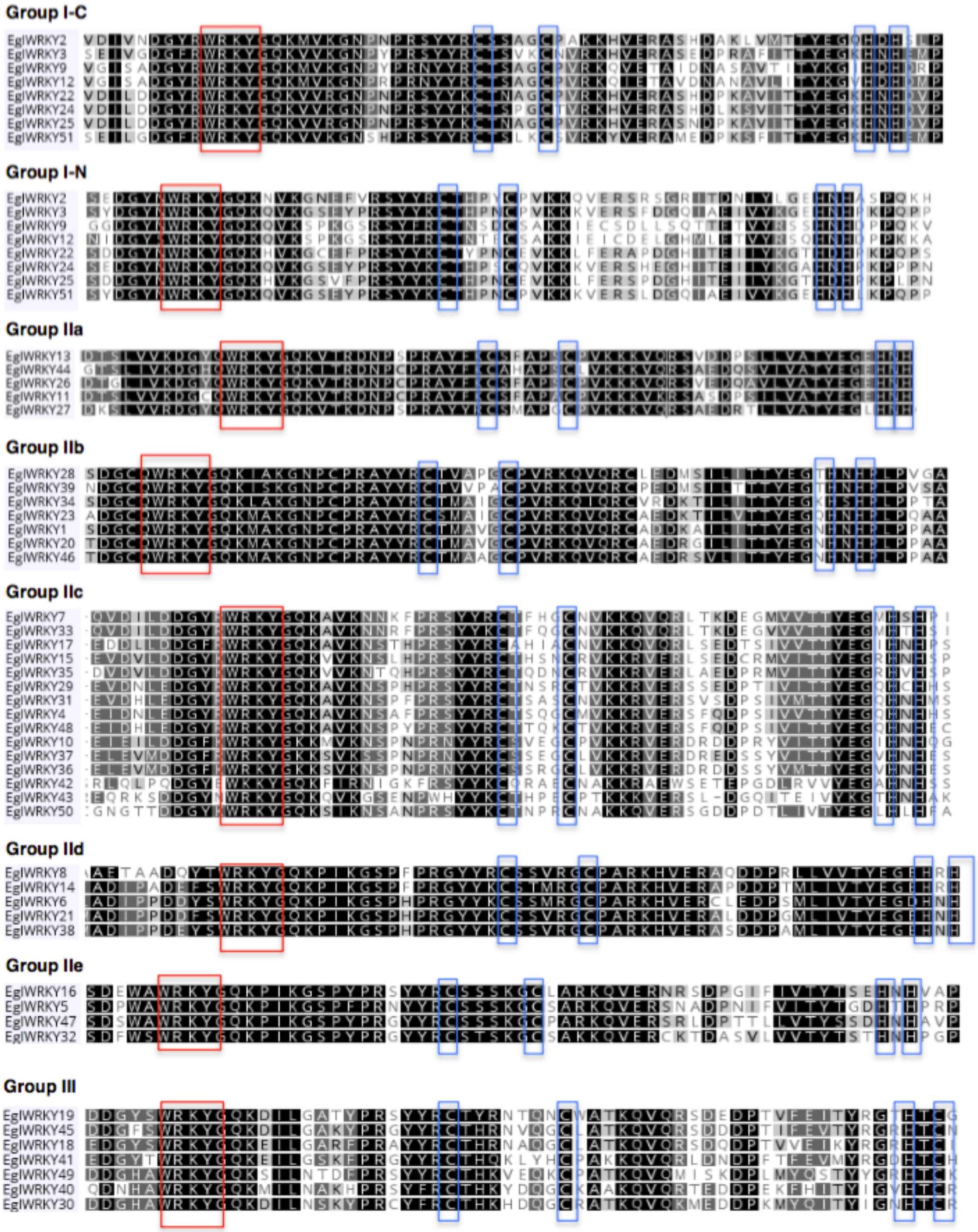

Fig.S1.1 Sequence alignment of the WRKY domains of 51 EglWRKY proteins. The conserved residues were classified into groups I, IIa, IIb, IIc, IId, IIe and III, according to phylogenetic analysis. The names with C or N indicates the C-terminal and N-terminal WRKY domain of group I. The conserved WRKYGQK motif is highlighted in the red rectangular frame, and the zinc-finger motif is highlighted in the blue rectangular frame.

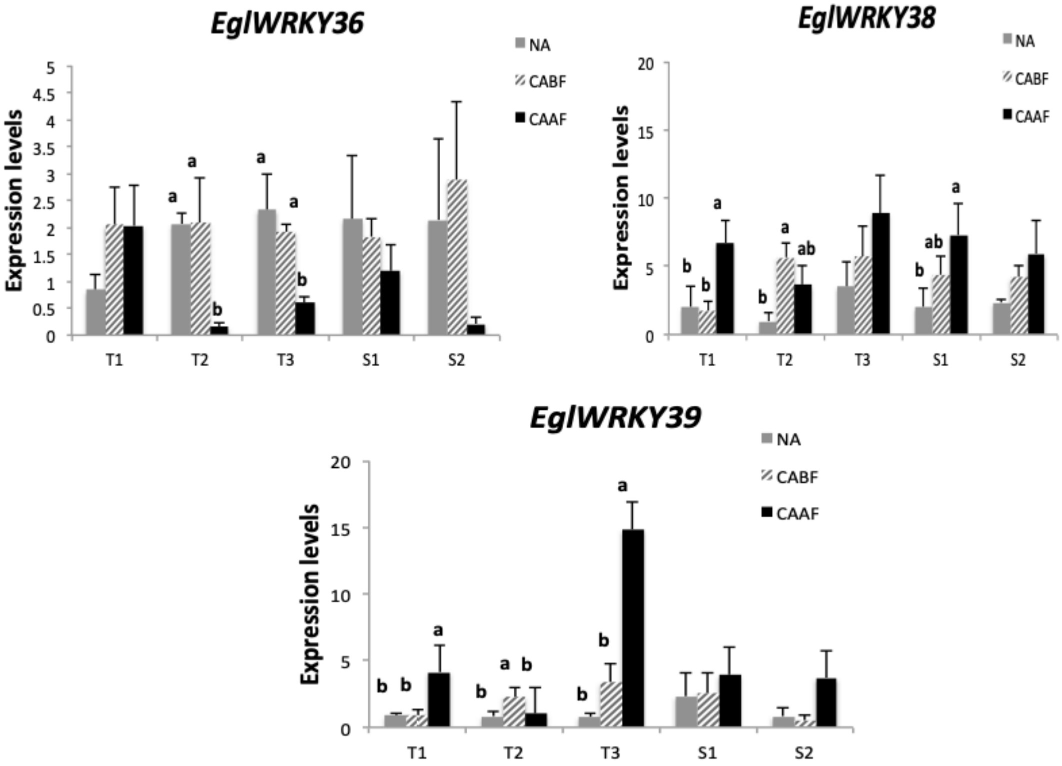

Fig.S1.2. Expression patterns of cold-responsive *EglWRKY36*, *EglWRKY38* and *EglWRKY39* genes in leaves of four genotypes of *E. globulus* under cold acclimation profile (NA, non-acclimated; CABF, cold acclimated before frost; CAAF, cold acclimated after frost). Ubiquitine and Elongation factor 1-a were used as reference genes. Letters on top of the bars (mean+SE) indicate statistically significant differences between treatments (a, b and c) evaluated with a Tukey test ($p \leq 0.05$).

CAPITULO II: Transcriptomic response in foliar and root tissues of a drought-tolerant *Eucalyptus globulus* genotype under drought stress [2]

José Luis Ulloa, Paula Aguayo, Daniel Conejera, Rafael Rubilar, Claudio Balocchi, Sofía Valenzuela

Keymessage: Identification of the potential candidate genes involved in drought tolerance in leaves and roots of *Eucalyptus globulus*.

2.1 Abstract

Eucalyptus globulus is one of the most cultivated forest tree species in the world because of its excellent wood properties; however, climate change is affecting its establishment, survival and productivity due to the presence of new events with abiotic and biotic stresses. Among abiotic stresses, drought is becoming a major issue due to long periods of drought in several countries

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[2] Trabajo publicado 02 diciembre 2021 en revista Trees https://doi.org/10.1007/s00468-021-02241-5

where this species is used in commercial plantations. In this study, RNA-Seq analysis was performed in a tolerant genotype of *E. globulus* to identify the main genes induced by drought stress (DS) in leaves and roots. A total of 686 sequences in leaves and 438 in roots corresponded to differentially expressed genes (DEGs) when comparing DS plants with control plants. Genes involved in protection against reactive oxygen species (ROS), chaperones, transcription factors and secondary metabolism were upregulated by DS in both tissues. Downregulated genes were involved in photosynthesis, generation of precursor metabolites and energy (leaves), and low oxygen and hormone metabolism (roots). A second experiment was carried out to compare some selected candidate genes between genotypes contrasting in drought tolerance. According to qRT-PCR analysis, genes coding for a bifunctional lipid-transporting protein (*EuglLTP2*) and geraniol 8 hydroxylase (*EuglGER*) were induced by drought stress in roots of the tolerant genotype but not in the susceptible genotype. The data generated in this study may form the basis for future research to identify drought tolerance mechanisms in *E. globulus*. They are also useful for use in genetic improvement programs.

Keywords: qRT-PCR, RNA-Seq, Gene Expression, water stress.

2.2 Introduction

Commercial plantations of *Eucalyptus* species cover approximately 20 million hectares in more than 90 countries (Ferreira et al. 2019). Among these species, *Eucalyptus globulus* Labill. is one of the most planted for commercial use due to its rapid growth rate, high yield, and excellent pulp properties and is an important source of biomass for pulp, fiberboard, and fuelwood (Booth 2013; Pita and Pardos 2001).

Drought affects the growth and establishment of commercial *Eucalyptus* plantations (Granda et al. 2014; White et al. 2009). In particular, *E. globulus* is vulnerable to prolonged drought stress since it has a high leaf-to-sapwood area ratio and high leaf conductance during drought (White et al. 1996; White et al. 2009). Reductions in plant biomass and specific leaf area, as well as the allocation of biomass to roots in different *E. globulus* genotypes under drought conditions, have been observed (Coopman et al. 2010; Pita and Pardos 2001).

Correia et al. (2018) reported stomatal regulation in *E. globulus* plants subjected to drought stress by observing a significant decrease in stomatal conductance (gs) in drought-stressed plants compared with the controls. Navarrete et al. (2013) identified a significant reduction in gs in five *E. globulus* genotypes from a total of 6 evaluated genotypes. They reported values ranging from over 200 mmol H2O m-2 s-1 in well-watered plants to less than 100 mmol H2O m-2 s-1 in drought-stressed plants. According to their data, well-watered individuals showed a predawn water potential (Ψpd) higher than -0.5 MPa, while values in stressed plants varied between -2 and -2.7 Consequently, there is a reduction in carbon assimilation and transpiration rate in plants subjected to drought stress due to a reduction in gas exchange because of stomatal closure (Correia et al. 2014b).

Osmotic adjustment is another physiological response to maintain cell hydration and turgor, limiting water efflux from plant tissues (Sanders and Arndt 2012). In drought-stressed *E. globulus* plants, an increase in osmotically active compounds such as proline and sugars of low molecular weight, such as fructose, galactose, and xylose, has been reported (Correia et al. 2016b).

The production of reactive oxygen species (ROS) occurs in plants under drought stress due to an interruption in the electron transport chain in photosynthesis, inducing photorespiration, which turns into H2O2 production (Noctor et al. 2002). In parallel, there is less CO2 fixation, decreasing the regeneration of NADP+ and increasing ROS, which damages membranes and proteins by peroxidation (Hasanuzzaman et al. 2013). Therefore, the antioxidant response is an important mechanism to acquire resistance to drought in all plant tissues. *E. globulus* responds to ROS by increasing the activity of dehydroascorbate reductase (DHAR) associated with a reduction in its cofactor glutathione (GSH) in drought-stressed plants (Correia et al. 2016a).

ROS in plants under drought stress are controlled by specialized proteins such as thioredoxins, antioxidant enzymes or secondary metabolites (for a review, see Laxa et al. 2019). At a molecular level, plants activate a complex network of response signals, changing the transcriptome profiles in different organs or tissues in response to stress (Wang et al. 2003). In fact, certain transcription factors (TFs) are regulated by phytohormones such as ABA, inducing a cascade of stress-responsive genes (Polle et al. 2019). This signaling produces substances to maintain normal function in cells, for example, by producing osmotic active compounds or antioxidant enzymes to scavenge ROS. As specific examples, NAC and MYB TFs have been studied in several plant species due to their role in drought tolerance. Transformation of Arabidopsis thaliana with RD26 from wheat, an NAC transcription factor, improves drought tolerance (Huang et al. 2015). Arabidopsis thaliana plants under osmotic stress induced AtMYB102 (De Vos et al. 2006), whereas Mito et al. (2011) showed improved salt stress tolerance in transgenic *A. thaliana* plants containing a chimeric repressor derived from AtMYB102.

Furthermore, when drought stress begins to cause damage in plant tissues, there are several mechanisms available to protect cell structures and to maintain the structural conformation of proteins. One of these mechanisms implies the protection of client proteins by interaction of hydrophilic residues of late embryogenic abundant proteins (LEA), such as those from group IV acting as chaperons (Cuevas-Velásquez et al. 2017). For example, the overexpression of BnLEA4-1 from Brassica napus enhances tolerance to salt and drought stress in A. thaliana (Dalal et al. 2009). Dehydrins are group II LEA proteins; transgenic rice plants overexpressing OsDhn-Rab16D showed tolerance to drought and osmotic stress when induced by polyethylene glycol (Tiwari et al. 2019).

Villar et al. (2011) studied two hybrids genotypes of Eucalyptus spp., which were contrasting in their growth rate and water use efficiency. On that study, the most productive genotype, shown a downregulation in genes involved in photosystem functioning, water and sugar transport and secondary metabolism, in drought stress treatment. Whereas upregulated genes were involved in primary metabolism and cell organization. In particular, MYB12 and MYB85, genes involved in flavonoid and lignin biosynthesis, were induced by nonirrigated conditions. The genotype with lower productivity induced the expression of genes involved in the abiotic stress response. Genes coding for aldo/keto reductase, proteins of the ethylene-responsive family and heat shock proteins, were highly induced in plants under the nonirrigated treatment.

In drought-contrasting genotypes of *E. calmaldunensis*, the most DEGs corresponded to heat shock proteins, expansins and drought stress-related transcription factors such as HB-12, RD26, and ERF110. The main processes that differ between drought-contrasting genotypes are cell death and apoptosis (Thumma et al. 2012).

In *E. globulus* plants under drought stress, upregulated genes, mainly in leaves, code for proteins such as transcription factors (TFs), molecular chaperones such as LEA, aquaporins, and antioxidant enzymes (Correia et al. 2018; Fernandez et al. 2012; Valdés et al. 2013). Understanding the physiological and molecular responses *of E. globulus* to drought stress can be useful in developing genetic improvement strategies to obtain drought-tolerant genotypes (Berenguer et al. 2018; Correia et al. 2018). The objective of this research was to identify DEGs in the leaves and roots of drought-stressed plants of two *E. globulus* genotypes, contrasting their drought tolerance level.

2.3 Materials and methods

2.3.1 Plant material and drought-stress treatments

Two *Eucalyptus globulus* genotypes, drought tolerant (T) and drought susceptible (S), kindly donated by Bioforest S.A., a Chilean forest research company, were employed in this study. Six-month-old plants with an average height of 30 cm and having at least four to six pairs of leaves were used. Two experiments were established, one in a growth chamber for RNAseq (tolerant (T) genotype, from October to December 2016) and a second experiment in a greenhouse for candidate gene validation by qPCR (tolerant (T) and susceptible (S) genotypes, from October 2017 to January 2018).

For RNAseq, a total of 88 plants (clonal copies) of the T genotype were planted in Styrofoam boxes (volume of 30 L, 22 plants per box, with 2 boxes per treatment) containing 90% composted pine bark and 10% perlite and covered with a 2 cm layer of vermiculite as substrate. Plants were placed in a growth chamber under controlled conditions (16/8 hour photoperiod, 20/12°C day/night) and were well watered for two weeks for acclimation. Afterwards, two treatments were established: a well-watered control (CON) and a drought-stress treatment (DS). The DS treatment consisted of stopping irrigation of plants until predawn leaf water potential (Ψpd) reached levels of -1.5 to -1.8 MPa. When stressed plants reached the expected predawn water potential (after 3 weeks of treatment), roots and leaves of plants from the CON and DS treatments (three biological replicates in each case, it means three entire plants divided in underground and aboveground organs) were collected and stored at -80°C until analysis.

For qRT-PCR analyses, the DS experiment considered the drought-tolerant (T) and drought-susceptible genotypes (S). Plants (88 per genotype) were placed individually in plastic bags filled with a mix of 90% composted pine bark and 10% perlite as substrate and maintained inside a polycarbonate greenhouse for a period of four months (October 2017 to January 2018). Similar to the previous experiment, during the first two weeks, plants were maintained with constant irrigation for acclimation, and after this time, DS was induced by stopping irrigation until Ψpd decreased from -0.8 to -1.2 MPa for an intermediate drought-stress level (IDS), and from -1.5 MPa to -1.8MPa for a severe drought-stress level (SDS). For control plants (CON), constant watering was kept until the end of the experiment. Root and leaf samples of three biological replicates from each genotype at the Ψpd indicated above were collected and stored at -80°C until use.

2.3.2 Measurement of physiological parameters

The physiological parameter used to determine the severity of DS treatments in both experiments was Ψpd, which was measured using a Scholander's Model 1000 chamber (PMS Instrument, Albany, Oregon, USA) as described by Silva et al. (2017). Briefly, a single leaf per plant, from three individuals for each genotype and treatment were cut at their base with a scalpel, and the midrib of leaves was used in place of the petiole, since plants having young leaves lack a petiole.

For the RNA-Seq experiment, stomatal conductance gs (mol H2O m-2s-1), photosynthesis A (µmol CO2 m-2s-1) and transpiration E (mmol H2O m-2s-1) were determined using an infrared gas analyzer (IRGA) model LI-6400XT (Licor®, Bioscience, Lincoln, Nebraska, USA). Three leaves between the second and fourth pair of leaves of 3 plants under the CON or DS treatments were randomly selected. Samples were analyzed using a CO2 concentration of 400 ppm; air flux: 300 µmol s-1; block temperature 21°C and relative humidity of 45–50%.

For genotypes evaluated in the greenhouse experiment used to validate gene expression by qRT-PCR, stomatal conductance (H2O mmol m-2s-1) was measured with a porometer model SC1-leaf Decagon® (Pullman, Washington, USA), and the maximum quantum yield of PSII (Fv/Fm) was measured with a Fluorometer, model OS30p+, Opti-Sciences inc. (Hudson, Nuevo Hampshire, USA). Three biological replicates were employed for each genotype and treatment. Significant differences between treatments were determined by a t-test or ANOVA associated with a Tukey test, according to each case, with a significant alpha equal to 0.05.

The growth of five plants by treatment was measured as the increment in height (cm) from collar to the apex from the first day of water depletion until seventeen days later. The survival rate of plants in both experiments was measured two months after irrigation was stopped. Eight plants per treatment and genotypes were evaluated by visual inspection, determining the percentage of dead versus live tissues (leaves and buds). Significant differences between the control and treatment(s) were determined by a nonparametric Kruskal-Wallis test, with a significant alpha equal to 0.05.

2.3.3 Identification of DEGs by RNA-Seq

For the construction of the 12 RNA-Seq libraries (one genotype*two treatments*two tissues*three biological replicates), samples collected were kept in liquid nitrogen, and plant tissues were sent in polystyrene box covered with dry iced to ArrayXpress (Raleigh, NC, USA; http://www.arrayxpress.com) and sequenced with the Illumina platform in a HiSeq2000 device. The sequencing results were single-end. The raw sequences were selected by a threshold Phred score higher than 30. Sequencing adapters were trimmed to eliminate redundancy at the first 15 bases at the 5' (index), and reads under 30 bp in length were discarded. After filtering size of reads were in average 100 bp. The genome of E. grandis (version 2, downloaded from: https://phytozome.jgi.doe.gov/) was used as a reference for mapping transcript sequences using CLC genomics Workbench software version 10.0.1 (QIAGEN, Aarhus, Denmark). Because reference genome was from a different species, slightly relaxed mapping stringency was setting in order to obtain a greater number of mapped reads. The expression analysis was executed in R studio Software with the Edge–R package to identify DEGs between the CON and DS groups. The output table was filtered to select DEGs according to logarithmic fold change (LFC) values higher than +2 and lower than -2. Data were filtered according to the p-value and false discovery rate (FDR) using a threshold equal to 0.05. A multidimensional scaling analysis to visualize expression differences between samples was performed using Edge-R Package according to the user’s guide.

Venn diagrams were constructed using R software (“Venn Diagram" package). The list of DEGs was extracted from six comparisons, corresponding to root drought stress vs. root control (RDS-RCON); leaf drought stress vs. leaf control (LDS-LCON); leaf drought stress vs. root drought stress (LDS-RDS); root drought stress vs. leaf control (RDS-LCON); leaf drought stress vs. root control (LDS-RCON) and root control vs. leaf control (RCON-LCON). Raw sequences are available in NCBI as Bioproject PRJNA486291 (Table S1).

Clusterprofiler (Yu et al. 2012) was used for the gene ontology analysis (GO), where the overrepresentation analysis of the GO terms in biological processes (BP) was performed. Arabidopsis thaliana was used as background for this analysis. For this, 164 downregulated and 355 upregulated genes in leaves and 196 downregulated and 110 upregulated genes in roots were employed. For visualization, a graph of points was used, which shows the number of genes associated with the GO characteristics through the size of each point. This is associated with the GeneRatio value, which gives the count of genes associated with a GO characteristic compared with the complete list of genes. In addition, p-values are provided that indicate which characteristic is most likely to have biological significance. Therefore, red indicates high enrichment, and blue indicates low enrichment. For all cases, a p-value of 0.01 and a FDR of 0.05 were used. The main pathways and protein domains were identified with the online tool PhytoMine from the database Phytozome V12.1. The list of E. grandis codes for DEGs was uploaded, and the results were used to recognize their main pathways and protein domains.

2.3.4 Validation by qRT-PCR analysis

RNA was isolated as described by Chang et al. (1993), purity was measured using a Nanodrop ND 1000, and its integrity was corroborated by a 1% (w/v) agarose gel and quantified by a Qubit fluorometer (Thermo Fisher Scientific Inc, USA). Enzymatic digestion with the DNAse I kit (Thermo Scientific Inc., USA) was carried out. cDNA was synthesized using the commercial High Capacity kit (Thermo Fisher Scientific Inc., USA) following the manufacturer's instructions. For the real-time PCR Evagreen PCR master mix kit (Solis Biodyne, Tartu, Estonia), a total reaction volume of 20 µL was employed, using cycles of 10 min at 95°C in the denaturing step, 40 cycles consisting of 15 s at 95°C and 1 min at 60°C, and a melting curve step. The housekeeping genes NADP-dependent isocitrate dehydrogenase IDH, translation elongation factor EF-1 alpha/Tu (Cassan-Wang et al. 2012) and eukaryotic initiation factor 4a-1-related EIF4a (Gaete-Loyola et al. 2017) were selected using the G-norm (Vandesompele et al. 2002). Fifteen genes involved in the abiotic stress response were used to compare qRT-PCR values with RNA-Seq data (RPKM). In this case, samples corresponding to leaves from the T genotype in the CON and DS treatments from the experiment established in growth chambers were used (Table S3).

The relative expression of six candidate genes was measured in leaves and roots from the T and S genotypes under drought conditions in the greenhouse experiment, corresponding to bifunctional protease inhibitor/lipid transfer protein (EuglLTP2); late embryogenesis abundant protein 4-5 (EuglLEA4); vacuolar glucose transporter (EuglVGT1); thioredoxin (EuglTRDX); and transcription factors NAC and MYB (EuglNAC66 and EuglMYB58). Additionally, transcript abundance of dehydrin 1 (EuglDHN1) and geraniol 8-hydroxylase (EuglGER) was tested in root samples. The list of primers is available in Table S2. The relative expression values were obtained using the sample maximization method (Hellemans et al. 2007). To compare gene expression between treatment genotypes, ANOVA and Tukey's test were carried out using R studio Software. The Levene's test was applied to verify homoscedasticity in the dataset. The normal distribution of residues was evaluated with the Shapiro-Wilk test. In the case of a nonnormal distribution, log transformation was applied to analyze data, and the Shapiro-Wilk test was applied to ensure normality.

2.4 Results and discussion

2.4.1 Physiological parameters

A drought-tolerant genotype (T) of *E. globulus* was used for RNA-Seq analysis in response to drought stress. Significant differences in the physiological variables measured between plants under the control and stress treatments were observed. The Ψpd in stressed plants was -1.63 ± 0.67 MPa versus the control with -0.24 ± 0.03 MPa. Similar results have been described in other studies on *E. globulus*, with Ψpd values ranging from -1.5 to -2 MPa for DS treatments compared with -0.5 MPa in well-watered plants (Correia et al. 2018; Correia et al. 2014b; Navarrete-Campos et al. 2013). A reduction from 7.59 ± 0.97 µmol CO2m-2s-1 in plants in the CON plants to 4.21 ± 1.84

μmol CO2m-2s-1 in the DS plants was determined in carbon assimilation, similar to the values reported by Correia et al. (2014a) for *E. globulus* genotypes under drought stress with a predawn water potential of ca. -1.5 MPa.

Stomatal conductance decreased from 0.15 ± 0.03 in CON plants to 0.04 ± 0.02 H2O mmol m-2s-1 in DS plants (3.75-fold decrease). These values are lower than those observed in a set of nine genotypes of *E. globulus* under drought stress, showing a 10- to 15-fold decrease in stomatal conductance for plants with Ψpd values near -3 MPa (Granda et al. 2014).

The transpiration rate in CON plants was 1.42 ± 0.24 and decreased to 0.42 ± 0.18 mmol H2O m-2s-1 in DS plants (a 3.4-fold decrease). Navarrete et al. (2013) observed a 10-fold decrease in both transpiration and stomatal conductance when analyzing plants from Eucalyptus clones, where six *E. globulus* genotypes under drought had a Ψpd from -2 to -2.7 MPa.

2.4.2 Transcript abundance of drought response genes by RNA-Seq

Six libraries of leaves and six roots were single-end sequenced on an Illumina platform. After removing low-quality reads and filter analysis, a total of 451,550,233 and 400,503,675 reads were obtained from leaf and root libraries, respectively. After annotation with the reference genome of E. grandis, based on CLC Genomics Workbench software, a total of 30,232 and 31,098 unigenes were identified in leaf and root libraries, respectively. From the comparison between the treatment and control using EdgeR software, a total of 7,048 sequences in leaves and 3,822 sequences in roots were selected by FDR and p-value <0.05 (Supplemental file 1 and file 2). The multidimensional scaling analysis showed that leaf and root samples were separated by the first component, while the second component divided samples under DS and CON treatments (Fig. S1a). A total of 1,124 DEGs between CON and DS were identified, 686 in leaf and 438 in root transcriptomes (Fig. S1 b, c). In the DS treatment, 478 genes were upregulated in leaves and 149 in roots, whereas 208 and 286 genes were downregulated in leaves and roots, respectively. A total of 36 and 17 genes were upregulated and downregulated in both tissues, respectively, while six genes decreased their transcript abundance in roots but were higher in leaves. Upregulated genes in leaves in DS plants were mainly involved in the water deprivation response, secondary metabolic process, response to acid chemicals, regulation of hormone levels, biotic defense, phenylpropanoid and flavonoid biosynthesis, and lipid and carbohydrate transport (Fig. 1a). In this case, genes belonging to the “response to drug” correspond to sequences associated with transcription factors, such as WRKY70, MYBR1, MYB15, heat shock proteins, DREBs, membrane transporters (ABCG40 and ERD6) or phytohormone-related genes (JAR1). Downregulated genes in leaves were associated with processes, such as photosynthesis, generation of precursor metabolites and energy, response to light intensity, and karrikin (Fig.2.1b).

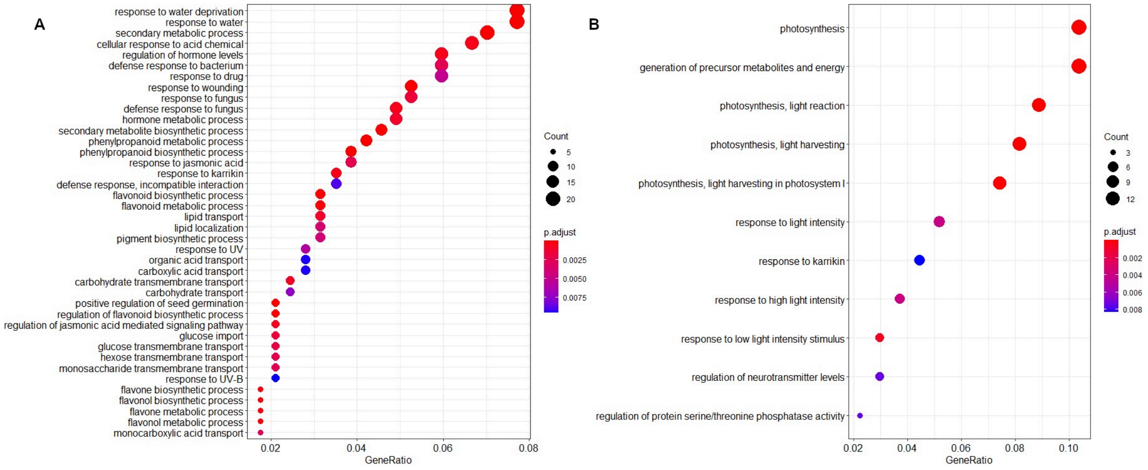

Fig.2.1 A) Gene ontology analysis of the RNA-Seq database, corresponding to upregulated genes from leaves of *Eucalyptus globulus* plants subjected to drought-stress treatment compared with well-watered plants. B) Downregulated genes from leaves of *Eucalyptus globulus* plants subjected to drought-stress treatment compared with well-watered plants

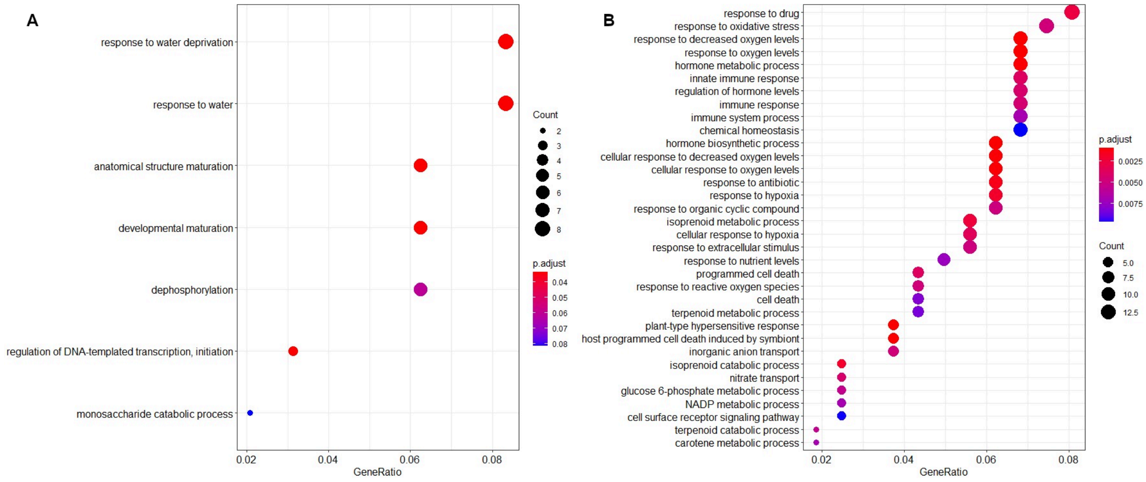

Fig.2.2 A) Gene ontology analysis of the RNA-Seq database, corresponding to upregulated genes from root *Eucalyptus globulus* plants subjected to drought-stress treatment compared with well-watered plants. B) Downregulated genes from roots of *Eucalyptus globulus* plants subjected to drought-stress treatment compared with well-watered plants.

The upregulated genes of roots under DS conditions were linked to the response to water deprivation, anatomical structure maturation, dephosphorylation, DNA transcription and monosaccharide catabolic processes (Fig.2.2a). Moreover, downregulated genes in roots were associated with the response to oxidative stress, low oxygen levels, programmed cell death, hormone metabolism and other biological processes (Fig. 2b). The category of genes called “response to drugs” includes sequences related to biotic stress response, such as disease resistance protein

(TIR-NBS-LRR) and pathogenesis-related protein 1 (PR1), phytohormone biosynthesis (ACC oxidase for ethylene), lipid transport protein (LTP), and aquaporin (NIP).

Some of the DEGs with the highest LFC values in leaves and roots corresponded to genes coding for thioredoxins (TRX), dehydrins (DHN) and late embryogenesis-abundant proteins (LEA) (Supplemental file 1 and 2). Since drought produces ROS as a consequence of less carbon assimilation (Pinheiro et al. 2004), higher oxidative environments must be controlled by detoxifying proteins as TRXs (Dos Santos and Rey 2006). Furthermore, dehydration can damage membranes and proteins; therefore, protective chaperones are induced to stabilize the cell components (Bartels and Sunkar 2005). LEA proteins and DHNs have been shown to be expressed in several plant tissues under abiotic stress, conferring tolerance to dehydration in plants (Aguayo et al. 2016; Hong-Bo et al. 2005).

Two genes involved in ABA metabolism, ABA 8 hydroxylase (Eucgr.B02321) and 9-cis epoxycarotenoid dioxigenase NCED9 (Eucgr.F03199) were upregulated in roots of *E. globulus* under DS. The first was similar to CYP707As from *A. thaliana* (69% at the protein level), which codes for an enzyme catalyzing the deactivation of ABA forming a phaseid acid (Yan et al. 2017). NCED9 showed 70% similarity with the corresponding protein from *A. thaliana*, an enzyme that has been shown to participate in ABA biosynthesis in embryo development (Lefebvre et al. 2006). Transgenic *A. thaliana* plants overexpressing NCED from wheat showed enhanced drought tolerance (Tong et al. 2017).

In this study, 51 sequences of the DEGs present in roots and leaves were linked to transcriptional regulation, including transcription factors MYBs and NACs, some with the highest LFC values. For example, in the DS treatment, EuglMYB58 was induced in root tissues, while EuglMYB27 and EuglNAC66 were induced in both tissues (Supplemental files 1 and 2). Members of these TF families have been previously reported to confer drought tolerance in plants (Baldoni et al. 2015; Lu et al. 2018). AtAF1, a member of the NAC family, acted as a positive regulator of drought tolerance mediated by ABA in transgenic *A. thaliana* plants, driving the expression of abiotic stress response genes, such as COR47, ERD10, KIN1, ADH1, RD22 and RD29 (Lu et al. 2007; Wu et al. 2009).

Other genes induced by drought stress in the leaves and roots of *E. globulus* plants corresponded to chalcone synthase CHS (Eucgr.D01632 and Eucgr.D01635) and lipid transfer proteins LTP (Eucgr.G02055 and

Eucgr.L01908). CHS has been associated with flavonoid biosynthesis and salt tolerance stress by ROS amelioration in tobacco (Lijuan et al. 2015). LTPs are involved in the mobilization of fatty acids; however, some genes from this family induce drought tolerance in Arabidopsis, such as LTP3 (Guo et al. 2013).

The decrease in stomatal conductance in DS demonstrates less gas exchange, affecting photosynthesis and reducing the transcript abundance of ribulose bisphosphate carboxylase small chain 1 A (RUBISCO) (Zargar et al. 2017). In *E. globulus* plants under drought, light-harvesting complexes II chlorophyll a/b binding protein 1 LHCB1 (Eucgr.D00322) was downregulated, which is related to carotenoids, protecting photosystem II against photooxidative damage under normal conditions (Dong et al. 2007). The transcript level of glucose 6 phosphate 1 dehydrogenase (Eucgr.K00618) was lower in plants under DS; this enzyme is involved in the production of NADPH and is a key element in plant metabolism as part of the oxidative pentose-phosphate pathway (Lin et al. 2013; Siddappaji et al. 2013).

The transcript abundance of carotenoid cleavage dioxygenase K11159 (Eucgr.C02920) was lower in the roots of *E. globulus* plants under DS. This sequence is an ortholog of CCD1 from A. thaliana and is involved in the degradation of carotenoids (Vogel et al. 2008). This enzyme breaks down double bonds in carotenoid molecules, such as lycopene, δ-carotene, β-carotene, and zeaxanthin, producing volatile compounds from different carotenoids (Vogel et al. 2008).

Comparing leaf and root libraries, 2,578 transcript sequences with logFC values higher than 2 were present in all comparisons of tissues (Fig.2.3a). The most abundant protein domains of these genes corresponded to cytochrome P450, multicopper oxidase, and cupredoxin. The main pathways related to these genes are phenylpropanoid and suberin synthesis. There were 2,610 transcripts with logFC values lower than -2 that were present in all comparisons (Fig.2.3b). The most abundant protein domains related to these sequences were protein kinases, s-locus glycoprotein domains, chlorophyll a/b binding protein domains, among others. The pathways linked to these genes corresponded to phospholipid remodeling, phenylpropanoid methylation and scopolin and esculin biosynthesis.

A total of 15 genes randomly selected from the RNA-Seq data were validated by qPCR in leaves of the T genotype under the control and DS treatments. The results showed similar values in RNA-Seq and qRT-PCR in transcript abundance (Table S3). The linear regression analysis using logFC values showed a high correlation (r-square = 0.82; p-value = 2.4 e-06) between both techniques.

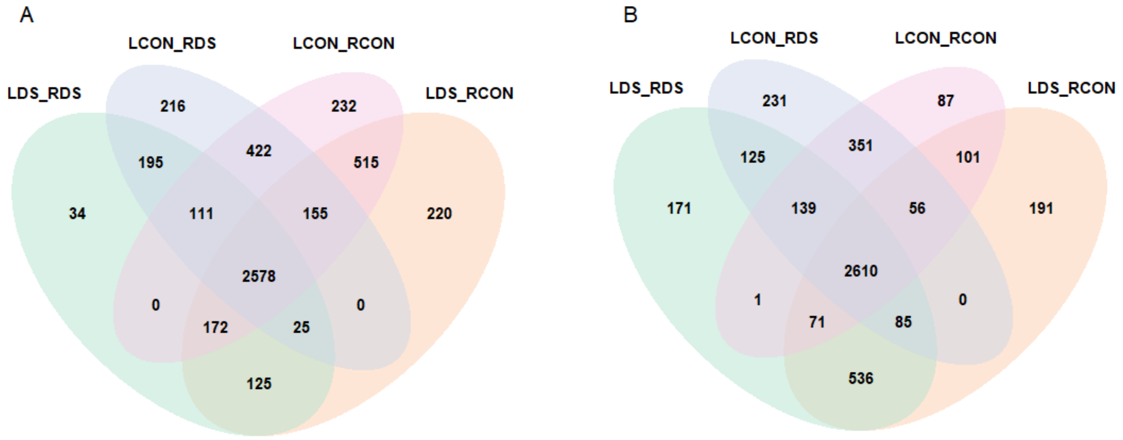

Fig.2.3 Number of DEGs in leaves and roots of *E. globulus* plants under drought stress (DS) and control well- watered conditions (CON) in an RNA-Seq study. The results correspond to comparisons between leaves versus roots. A: Venn diagram with DEGs with LFC values higher than 2. B: Venn diagram showing DEGs with LFC values lower than -2. LDS_RDS: libraries from leaves of plants under the DS regime versus libraries from roots in DS. LCON_RDS: Leaves in the CON versus roots in DS. LCON_RCON: Leaves in the CON versus roots in the CON. LDS_RCON: Leaves in DS versus roots in the CON.

2.4.3 Relative expression of candidate genes in greenhouse experiments

To validate some of the DEGs by qRT-PCR, the drought-tolerant (T) and drought-susceptible genotypes (S) of *E. globulus* were submitted to drought-stress treatment in a greenhouse. Both Ψpd and conductance were significantly reduced at SDS compared with the CON treatment (Table 2.1).

Table 2.1 Physiological measurements in a drought-stress experiment established in a greenhouse using two contrasting *E. globulus* genotypes (tolerant T and susceptible S) for qRT-PCR analysis to evaluate the transcript abundance of selected genes.

Genotype treat	Predawn leaf water potential		Conductance		Maximun quantum yield of PSII		Growth increment		Survival	
	Ψpd (MPa)	sig.	(mmol H_2O $m^{-2}s^{-1}$)	sig.	(fv/fm)	sig.	(cm)	sig.	(%)	sig.
T- CON	-0.54±0.06	a	982±47	a	0.73±0.02		11.0±2.0		76±9	
T- IDS	-0.98±0.30	a	322±271	bc	0.74±0.02		n.a		n.a	
T- SDS	-1.62±0.13	b	126±38	c	n.a		6.2±2.2	*	78±6	
S- CON	-0.68±0.02	a	1,113±81	a	0.73±0.02		10.5±3.2		77±5	
S- IDS	-0.81±0.27	a	456±179	b	0.63±0.01	*	n.a		n.a	
S- SDS	-1.61±0.20	b	135±13	c	n.a		3.0±2.0(*)	*	18±25	*

Letters in the “sig” column correspond to significant differences according to ANOVA and Tukey’s post hoc test ($p<0.05$). Asterisks indicate significant differences according to T-Test (fv/fm and growth) or nonparametric Kruskal-Wallis test (survival) $p<0.05$.

However, for the T genotype, this parameter decreased slower than in the case of the S genotype, reaching a Ψp d of -1.5 MPa almost 2 weeks later (Fig. S2). Similarly, Navarrete et al. (2013) identified differences in Ψpd between Eucalyptus sp. genotypes after two weeks of drought-stress treatment.

Stressed plants significantly reduced stomatal conductance (gs). Both genotypes showed an 8-fold decrease in this variable at SDS compared with the CON treatment, while gs was 3-fold lower than the CON treatment in the T genotype at IDS, but in the case of the S genotype, this value was 2.4-fold lower than the CON treatment (Table 1). Stomatal conductance in SDS was significantly lower than IDS in the S genotype but not in the T genotype. Correia et al. (2014b) reported a decrease in gs in tolerant and susceptible genotypes of *E. globulus* at 25% and 18% of field capacity, respectively, compared with well-watered plants, but no significant differences between treatments were found.

The quantic photochemical efficiency of photosystem II (fv/fm) was significantly reduced (14%) in the S genotype two weeks after beginning the drought-stress treatment with respect to the control (Table 1). However, Correia et al. (2014b) found an increase in this ratio in stressed plants of *E. globulus* versus well-watered plants. Analysis of *E. grandis* showed that drought reduced the ratio of this parameter in drought-stressed plants by 38% compared with control conditions (Tariq et al. 2019).

Both genotypes exhibited a significant reduction in the growth rate at SDS after seventeen days of the beginning of the experiment (Table 2.1), whereas the survival rate after two months was drastically reduced in the S genotype (18%), and it did not affect the T genotype (78%).

2.4.4 Relative expression of candidate genes

EuglLTP2 has 62% identity in translated sequences of amino acids, with bifunctional protease inhibitor/lipid-transfer protein/seed storage 2 from A. thaliana (AT2G37870), which belongs to phylogenetic group V of the nonspecific lipid transfer protein superfamily (Fleury et al. 2019). EuglLTP2 showed a significant increase in transcript abundance in leaves under SDS conditions in the T genotype, but no significant differences were found in the S genotype (Fig.2.4a). The LFC value of the T genotype was almost 2-fold higher than that of the S genotype at SDS (Table S4). In particular, the transcript abundance of this gene was significantly induced in the roots of plants from both genotypes at IDS and SDS; however, in the T genotype, the relative expression was significantly higher than that in the S genotype under the same treatments. Li et al. (2008) reported that this gene is induced by drought in *A. thaliana* and is controlled by NYF5, a TF that enhances tolerance to drought stress. Jülke et al. (2016) identified that this gene is induced by salt stress in roots of *A. thaliana*, and Brinker et al. (2010)

reported an increase in transcript abundance of an ortholog of LTP2 in roots under salt stress in the salt tolerant *Populus euphratica*.

EuglMYB58 is part of subgroup 11 of the MYB R2R3 family in *E. grandis* (Soler et al. 2015). The transcript abundance of EuglMYB58 was significantly induced in leaves at IDS in both genotypes, whereas in roots, this gene was upregulated in SDS in both genotypes (Fig.2.4b). However, according to LFC values, leaf transcript abundance in T was higher than that in S, for example, 1.85 over 1.29 in IDS and 1.36 over -0.04 in SDS (Table S4). EuglMYB58 is orthologous to AtMYB102, a TF that has been reported as a positive regulator of drought tolerance in *A. thaliana* (Denekamp and Smeekens 2003), and it has been shown to be involved in ethylene biosynthesis (Zhu et al. 2018).

EuglLEA4 increased its transcript abundance in the leaves of plants under SDS and IDS in both genotypes. In roots, it was induced only at SDS without significant differences between genotypes (Fig.2.4c). Similarly, LFC values demonstrated that the expression level of this gene was related to the severity of the drought treatment, showing no differences between genotypes (Table S4). In *P. trichocarpa,* the homologous gene ptrLEA85 was upregulated in roots subjected to salt stress, with a 7.25x increase in LFC compared with the control (Cheng et al. 2021). A significant increase in the transcript abundance of ptrLEA85 was reported in leaves and stem, with a peak 12 hours after stress imposition (Cheng et al. 2021). Overexpression of AtLEA4-5 has been demonstrated to improve the recovery of *A. thaliana* transgenic plants subjected to severe drought stress in adult individuals, demonstrating its role in drought tolerance (Olvera-Carrillo et al. 2010). The proteins mentioned above belong to group IV LEA proteins that have high hydrophilicity, small amino acids and scarce hydrophobic residues; they accumulate during water deficit and prevent inactivation and conformational changes of enzymes due to the interaction with the conserved N-terminal region of these proteins (Cuevas-Velazquez et al. 2016, Cuevas-Velazquez et al. 2017).

EuglTRDX showed a significant increase in relative expression in both genotypes in IDS in leaves, showing a significant difference between SDS and the CON in the S genotype (Fig. 2.4d). According to LFC values, the S genotype had a 1.4-fold and 2.6-fold higher value than the T genotype at IDS and SDS, respectively (Table S4). In the case of roots, this gene was upregulated in the both genotypes under treatments. Drought stress induces the formation of ROS in all plant tissues; in this scenario, thioredoxins can ameliorate toxicity by antioxidant activity. EuglTRDX has 42% identity with the NRX1 gene from A. thaliana, which has been shown to be required to provide reductive protection to catalase enzymes under oxidative stress (Kneeshaw et al. 2017).

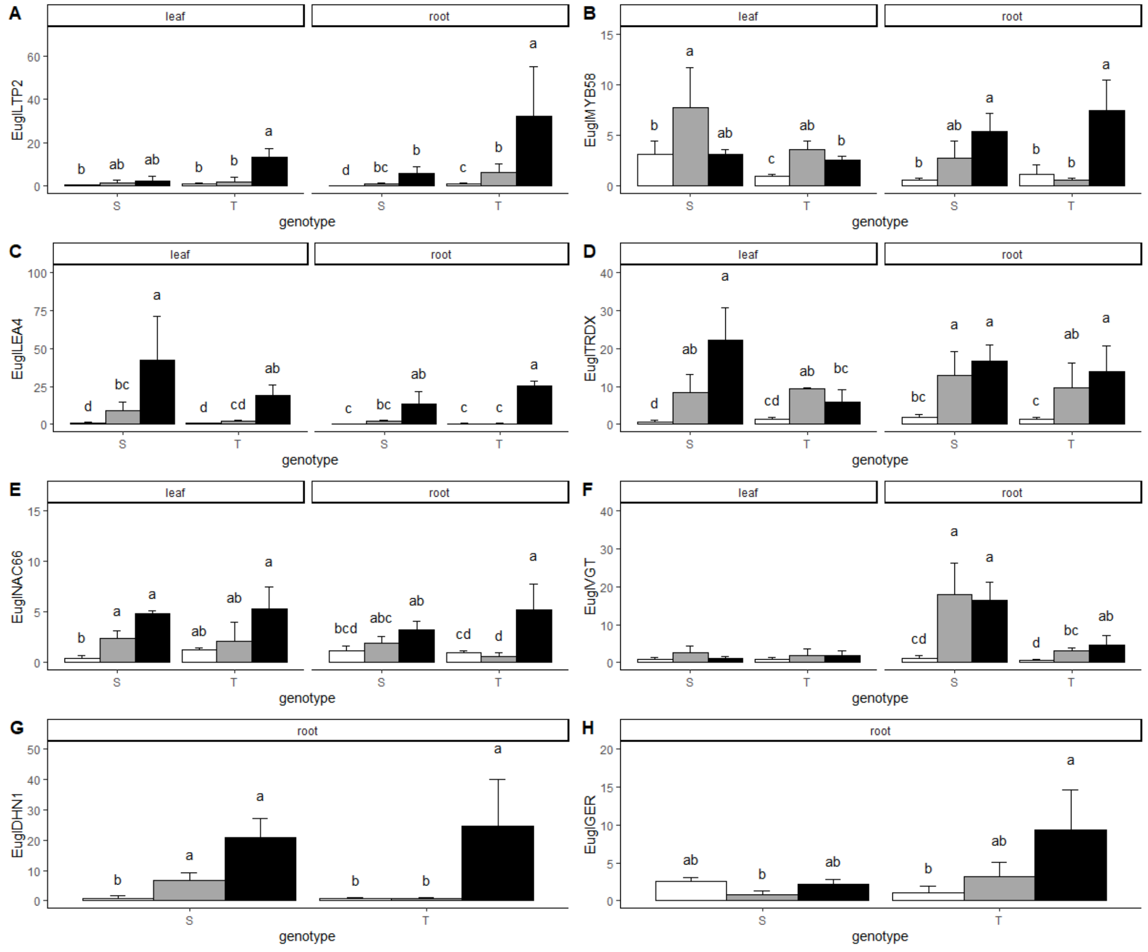

Fig.2.4 Relative expression of genes in leaves and roots of drought tolerance contrasting *E. globulus* genotypes. The experiment consisted of maintaining plants in a well-watered regime (CON; white), intermediate drought stress (IDS; dark gray) and severe drought stress (SDS; black). Letters over bars indicate significant differences according to ANOVA and Tukey’s post hoc test ($p < 0.05$).

The transcription factor EuglNAC66 was significantly induced in leaves of the S genotype at SDS, whereas in the T genotype, a significant increase was observed at SDS in roots (Fig.2.4e). This gene has 62% protein identity with AtRD26 of *A. thaliana.* Overexpression of the orthologous AtRD26 gene from wheat confers drought tolerance to transgenic *A. thaliana* lines and is associated with ABA-dependent signaling in response to abiotic stress (Huang et al. 2015; Puranik et al. 2012).

The relative expression of vacuolar-related gene Vacuolar Glucose Transporter 1 (EuglVGT1) was upregulated in roots of both genotypes. According to LFC values for this gene, the S genotype had 1.8 x and 1.4 x higher relative expression than the T genotype at IDS and SDS, respectively (Table S4). However, no significant differences in transcript abundance of this gene were found in leaf tissues between treatments and the control in either genotype (Fig. 2.4f). This sequence was associated with the Atg162660 gene from A. thaliana, known as AtVGT1, which is expressed in the tonoplast membrane (Aluri and Büttner 2007). It possesses 12 transmembrane domains, and its function is to transport glucose inside the vacuole by a proton antiporter (Patzke et al. 2019). A study of *A. thaliana* revealed that the

Atg162660 locus is associated with some QTLs for invertase activity in soluble extracts and in various organs of seedlings (Sergeeva et al. 2006). Santos et al. (2021) reported that there is an accumulation of vacuolar reducing sugars led by a vacuolar invertase that is highly expressed in a drought-tolerant genotype of Hevea brasiliensis under drought-stress conditions but not in a susceptible genotype, suggesting that osmotic adjustment is important in acquiring tolerance in the species.

EuglDHN1 was induced in roots of the T genotype at SDS; however, in the case of the S genotype, this gene had a significant increase in transcript abundance at IDS and SDS compared with the CON treatment (Fig. 2.4g). The translated peptide sequence has 70% identity with AT2G21490 of *A. thaliana*, identified as dehydrin LEA14 (Hundertmark et al. 2011), which has an adaptive role in seed longevity in the dry state and germination under salt stress (Hundertmark et al. 2011). Aguayo et al. (2016) reported the induction of this gene in a frost-tolerant genotype under cold and frost stress.

An increase in the transcript abundance of the gene coding for geraniol 8 hydroxylase (EuglGER) in roots of the T genotype at SDS was observed, whereas in the S genotype, no significant variation was found between treatments and the CON (Fig. 2.4h). This enzyme belongs to the cytochrome p450 group (Wang et al. 2010) and hydroxylates at position 8 in the geraniol molecule (Sintupachee et al. 2015). Geraniol is a precursor of many compounds belonging to secondary metabolism, particularly the iridoid glycoside (IG) pathway (Ilc et al. 2016). Some IGs have been linked to antioxidant activity and increased concentrations in roots under drought stress (Wang et al. 2010).

2.5 Conclusions

This study reports changes in transcriptomic response in leaves and roots of a drought-tolerant *Eucalyptus globulus* genotype. We identified 1,124 DEGs between the DS and CON treatments in both tissues, which may play an important role in the drought-stress response. The main upregulated genes under drought stress were related to ROS scavenging, protection of molecular structure, transcription factors and osmotic adjustment. However, downregulated genes were involved in photosynthesis, generation of precursor metabolites and energy (leaves), and low oxygen and hormone metabolism (roots). According to qRT-PCR analysis, genes coding for a bifunctional lipid-transporting protein (EuglLPT2) and geraniol 8 hydroxylase (EuglGER) were induced by drought stress in roots of the tolerant genotype but not in the susceptible genotype. The results suggest that these genes could be associated with drought tolerance in *E. globulus* and provide valuable information regarding the transcriptomic response of leaves and roots under drought stress.

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2.7 Supplementary material

Suplemental File 1. Details of DEGs in drought stress treatment compared to control, in leaves of the T genotype.

https://static-content.springer.com/esm/art%3A10.1007%2Fs00468-021-02241- 5/MediaObjects/468_2021_2241_MOESM1_ESM.xls

Suplemental file 2. Details of DEGs in drought stress treatment compared to control, in roots of the T genotype.

https://static-content.springer.com/esm/art%3A10.1007%2Fs00468-021-02241- 5/MediaObjects/468_2021_2241_MOESM2_ESM.xlsx

CAPITULO III: An overview of cold stress responses in Eucalyptus globulus [3]

Paula Aguayo, Marta Fernández, Claudio Balocchi, Sofia Valenzuela

Keymessage: This article summarizes advances in cold tolerance, particularly the signaling pathways of cold genes in *E. globulus*.

3.1 Abstract

Eucalyptus globulus is one of the most important hardwood species used for pulp and paper production worldwide, but its productivity is limited by cold temperatures which affect their optimal development and distribution. Several plants increase their freezing tolerance in response to low temperatures pre-conditioning, this mechanism known as cold acclimation, is a result of a combination of physiological, biochemical and metabolic changes which depend on transcriptome modifications. This review explores the current knowledge about responses of *E. globulus* under cold stress,

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[3] Trabajo publicado 18 octubre 2022 en revista Trees https://doi.org/10.1007/s00468-022-02355-4

focusing in the first section on the physiological damage caused by cold stress in this species, whereas the second part deals with how perception of cold temperature can regulate the induction of cold acclimation, and finally, focusing on the molecular response systems involved in the cold response, in order to synthesize our understanding of the complex response to cold stress in *E. globulus*.

Keywords Abiotic stress, cold signal pathway, low temperature, COR genes

3.2 Introduction

The increasing demand for wood products is the main reason for a significant growth in the hectares of forest plantations in the world, and this trend is predicted to continue to increase as a result of the constant growth of the human population (FAO, 2015; Chang et al. 2019). With a current area of 131 million of hectares of forest plantations and the need to satisfy the wood demand, a variety of tree species have been employed depending on the environmental sites and market demands, which include mainly the genus *Pinus*, *Populus*, *Acacia* and *Eucalyptus*, with the last one the most important hardwood species used for pulp and paper to cover the global demand (FAO 2020; McEwan et al. 2020). *Eucalyptus* has more than 700 species, mostly originating from Australia, which are adapted to a large range of diverse climates and soils, grow fast and have easy to implement breeding programs. Therefore, the genus *Eucalyptus* represents 8% of the forest plantations worldwide and has been distributed in 90 countries with more than 110 species, (Iglesias-Trabado and Wilstermann 2009; Salehi et al. 2019). The current forestry plantations are dominated by nine different species of eucalypts, which cover more than 20 millions of ha. Among these, *E. globulus* is one of the most important species in the forestry industry with extensive plantations in Africa, South America, Asia and southern Europe (Potts et al. 2004; Booth, 2013). *Eucalyptus globulus,* has a natural range around the coasts of Tasmania, southern Victoria and the Bass Strait Islands, with four subspecies, *globulus*, *bicostata*, *maidenii* and *pseudoglobulus* (Dutkowski and Potts 1999; Tibbits et al, 2006). Several advantage have been describe, its high pulp yield, wood density and excellent fiber quality, producing cellulose with higher yields and better strength properties than other species. It has a short rotation time, between 10-12 years, and its ability to adapt to a wide range of sites has made it one of the preferred tree species for the forest industry with approximately 2.5 million ha planted worldwide (Grattapaglia 2004; Carrillo et al. 2018). However, there are extensive areas that exhibit restrictions that limit their optimal development, especially in sites with incidence of low temperatures due to its sensitivity to frost damage (Costa e Silva et al. 2008).

Cold stress is one of the major environmental factors that decrease quality, productivity and restrict growth, of forest plantations. This abiotic stress can be divided in two levels: chilling stress, with temperatures below 15°C and freezing stress, with temperatures below 0°C, in which ice formation may occur within the tissues (Ritonga and Chen 2020).

The degree of damage of cold stress depends on multiple factors such as the tolerance of the species, the chilling rate, exposure time, and the presence of other types of stresses (Nievola et al. 2017). This stress can cause the reduction of photosynthetic levels, decrease the water potential, cell dehydration and low absorption of nutrients, as well as damage in cell membranes, death of tissues, decreased foliar expansion, wilting, chlorosis and even necrosis has been reported in various plant species subjected to cold stress (Arora and Palta 1991; Saxe et al. 2001; Oberschelp et al. 2020). Cold acclimation is a mechanism that allows plants to respond and adapt to cold stress after plants are exposed to a period of time to low, but non-freezing temperatures, this mechanism involves physiological, biochemical, molecular and cellular processes, resulting in an increase of cold tolerance (Thomashow 1999; Miura and Furumoto 2013).

Despite the predicted global warming for the next years, climate models project that cold extreme events will continue to occur and may be more intense than in the past years (Collins et al. 2013), these future events can cause high economic loss for the forestry industry, especially for companies that use frost sensitive species like *E. globulus.* With the need to increase the sites planted with *E. globulus,* new plantations have been established in areas with less optimal environmental conditions for the species, including sites with more frequent frost, where the plantations can suffer severe damage (Costa e Silva et al. 2007). To overcome these limitations through the development of breeding programs, a detailed physiological and molecular information of how this species responds to cold stress is required in order to select the clones for future production (Costa e Silva et al. 2008). A large number of studies have been published in order to understand the signals and responses to cold stress among different *E. globulus* genotypes with different levels of tolerance, in order to develop tools that can be incorporated in the genetic improvement of this hardwood species. In this review, we aim to give a comprehensive overview of the current knowledge about *E. globulus* under cold stress, focusing on the comprehension of the genetic regulation of cold acclimation, discussing molecular mechanisms and how they could contribute to cold stress response in this species.

3.3 Effect of cold stress in *Eucalyptus globulus*

Different definitions of abiotic stress have been made over the years, for example Lichtenthaler, (1998) defined stress as “any substance or condition which stops a plant’s metabolism, growth or development”; Ben-Ari and Lavo (2012) defined abiotic stress as “the negative impact of non-living factors on living organisms in a specific environment”; Kaur et al. (2020), defined it as the factor that negatively influences plant survival and plant productivity. Cold stress, defined as chilling with temperatures between 0°C and 15°C and freezing with temperatures below 0 °C, is one of the main abiotic stress factors limiting the geographical distribution of plants and decreasing the yield of several species (Kazemi-Shahandashti and Maali-Amiri 2018; Ritonga and Chen 2020). Since cold stress is one of the important environmental factors that affects the optimal development of plants and 64% of the surface of the earth has temperatures below 0°C, studies about freezing damage mechanisms are very important for producing cold-tolerant varieties (Rihan et al. 2017).

Depending on the frost tolerance of different plant species, the damage caused by cold stress may occur in a few hours or days. In the case of *E. globulus,* several studies have reported the minimum temperature of cold damage and have additionally determined that young plants are less tolerant to cold stress conditions than adult ones (Costa e Silva et al. 2008). Skolmen and Leding (1990) reported that seedling exposed to temperatures below -5°C can suffer severe damage that lead to their deaths, setting -5°C as lethal temperature for the species. Almeida et al. (1994) showed a LT_{50}, (which corresponds to the minimum temperature at which 50% of the seedlings are damaged), between -5.3 to -5.6 °C in 7 month old seedlings. Volker et al. (1994) determined that the temperature of severe damage on seedlings of 7 month of age occurred at -7°C, with the 50% damage criteria probably met at about -6°C. Wisniewski et al. (2014) described *E. globulus* as highly sensitive to freezing temperatures in the range of -6 to -8 °C. Some studies have reported that natural genetic diversity affects cold tolerance. A CSIRO provenance trial of the four subspecies of *E. globulus* planted in Canberra in 1994 (Floyd et al. 2002) had high survival rate despite experiencing several frosts event each year and a minimal temperature annual of -6°C. Moraga et al. (2006), evaluated the resistance to freezing in *E. globulus* subspecies, previously hardened, from difference provenance of Australia. Results showed that the most resistant provenances were Bolaro Mountain of *E. maidenii ssp* and Moogora of *E. globulus ssp.* with a LT_{50} of -9°C and -8.5°C, respectively.

These studies suggest that minimum temperature of cold damage is a variable characteristic within the species and depends on factors such as the tolerance of the sub-species or genotypes, the chilling rate, age and exposure time.

In several species, it has been reported that cold stress disrupts photosynthetic components including electronic transport in the thylakoids, transpiration rate, stomatal conductance and carbon fixation, resulting in a retardation in photosynthetic efficiency or rate, in less extreme cases, or damage to foliar area and plant death in severe condition (Davidson et al. 2004; Sharma et al. 2020). In *E. globulus* photoinhibition induced by cold has been studied. In 2000 Close et al., showed that 23 week-old seedlings transplanted in sites considered marginal for the species, due to the constant presence of low temperatures, exhibited a severe photoinhibition. Also, in the same study comparative photoinhibition damage between *E. globulus* and *E. nitens,* a resistant cold species, was analyzed. Result showed an increased photoinhibition in *E. globulus* seedlings result in a 20% mortality and a less affect in *E. nitens.* Similar results were reported by Davidson et al. (2004). In order to determine the damage in photosynthetic rates, subjected the two different species of *Eucalyptus*, *E. nitens* and *E. globulus* to temperatures between -1°C and -2°C. Results showed a reduction of 42% in the photosynthetic rate in *E. globulus* exposed to -1,5°C compared to the control conditions, and 7% more than *E. nitens* plants exposed to the same temperatures.

Costa e Silva et al. (2008) obtained similar results, where plants of *E. globulus* decreased around 79% in photosynthesis rate when they were subjected to freezing temperatures (-2 °C) compared with the control plants. Chang et al. (2021), have reported that a decrease in the level of photosynthetic pigments, due to cold contributes with the reduction in photosynthetic rate. For example, Close et al. (2000) showed that seedlings of *E. globulus* subjected to cold conditions had significantly low levels of chlorophyll and carotenoids when compared with seedlings of resistant cold species (*E. nitens*). These results were confirmed by Shvaleva et al. (2008) where clones of *E. globulus* with different levels of tolerance to cold stress showed a reduction of total chlorophyll content, between 22% and 48%, compared to control plants. Therefore, the authors agree that the reduction in the content of the photosynthetic pigments, especially the chlorophyll, results in the decrease in the level of photosynthetic rates. Davidson et al. (2004) indicated that *E. globulus* has a reduced capacity for photosynthetic recovery after a cold stress event, which can contribute to limiting its geographic distribution.

One of the most important damages related to cold stress and that has been widely studied is the damage to the plasma membrane composition and function. In non-cold tolerant species, the membrane is composed of high percentage of saturated fatty acids chains and tends to solidify at temperature between 10°C to 0°C affecting the permeability, reducing the movement of membrane lipids resulting in a less fluid membrane (Takahashi et al. 2013; Kazemi-Shahandashti and Maali-Amiri 2018). The damage by cold stress on the cell membrane can cause a series of functional decline; water and intracellular solute loss and inactivation of transport channels have been reported in various species (Nievola et al. 2017). Loss of fluidity and changes in the permeability produce a loss in the selectivity of the membrane causing an uncontrolled exchange of ions and metabolites followed by a loss of the cellular content towards the outside of the cell and over-accumulation of reactive oxygen species (ROS) inside the cell, which can lead to the collapse of cellular functions and cause its death (Kamiñska-Rozek and Pukacki 2005; Kazemi-Shahandashti and Maali-Amiri 2018). Costa e Silva et al. (2008) measured the membrane injury in leaf discs of *E. globulus* subjected to artificial freezing showing that 50% of the relative damage was reached at -3.8 ± 0.1°C, similar results were reported by Almeida et al. (1994), Tibbits et al. (2006) and Fernández et al. (2012a) for the same species.

Low temperatures can reduce water absorption through decreasing the water potential, which can lead to dehydration and a reduction in plant growth (Nievola et al. 2017). In *E. globulus,* Prado et al. (1994) evaluated the foliar damage for plants subjected to artificial freezing, concluding that there are damages that compromise around 30% of the foliage of the plants at temperatures between -1°C and -4 °C. This foliar damage decreases their growth but does not threaten their survival. Almeida et al. (1994) showed a decrease in the water potential in seedlings of *E. globulus* after 16 nights of cold treatment compared with the controls, also, the production of new leaves and height growth were less in plants subjected to cold. Costa e Silva et al. (2007) studied the effect of cold stress (10/5°C, day/night) in the growth of ramets of two *E. globulus* clones, previously characterized has resistant and susceptible to other abiotic stress. A reduction of ~34% in mean total biomass compared to controls (24/16°C, day/night) was reported, as well a 61% decrease in total leaf area and a 39% decrease in the ratio of leaf area to total biomass. Also, significant difference was observed between the two clones, where the chilling treatment affected less the growth of clones previously characterized has resistant. Similar results were obtained by Shvaleva et al. (2008), where seedlings submitted to the same temperatures (10/5°C, day/night) for forty-two days showed a negative effect on growth with reduction of total biomass, leaf area ratio and total root length in relation to control values.

It is important to point out that the degree of damage of low temperatures depends on multiple factors, being the chilling rate and exposure time the most relevant (Nievola et al. 2017). If the temperature keeps dropping and the exposure time continues, in the extracellular spaces of plant cells ice is formed, reducing the water potential of the apoplastic solution, leading to water flowing from the cells; and if freezing continues, osmotic forces can produce cellular dehydration, generating intracellular ice formation which can produce a mechanical tension between the cell wall and the cytoplasmic membrane that finally produces cell rupture and ultimately causes the plants to die (Pearce 2001; Mahajan and Tuteja 2005).

3.4 Cold acclimation in *Eucalyptus globulus*

In plants, resistance mechanisms to abiotic stress are divided into two adaptive strategies; 1-avoidance, which consists in minimizing the presence of stress, where the plant can have morphological adaptations to evade adverse conditions, and in this way preventing that an external stress factor triggers responses that modify plant functioning, 2- tolerance, which is the ability to resist the alterations caused by stress and involves changes of physiological mechanisms, gene expression and changes involved in developmental activities with the purpose to tolerate the effect of stresses (Rihan et al. 2017; Nievola et al. 2017). In the absence of any strategy to avoid cold stress, the survival of some species depend on their ability to cold acclimate, a tolerance strategy in response to environmental changes, such as short-days and low temperatures (Levitt 1980). This takes place on the time scale of days or weeks when plants are exposed to chilling temperatures, as a result of a combination of physiological, biochemical and metabolic changes depending on transcriptome modifications (El Kayal et al. 2006; Thomashow 1999). In general, plant response to this abiotic stress can be divided in three phases, i) cold acclimation known as pre-hardening, occuring at low, but above zero temperatures; ii) hardening, where the full degree of tolerance is acquired, and needs exposure to sub-zero temperatures and iii) plant recovery occurring at the end of winter (Li et al. 2008).

Studies have shown that activation of resistance mechanisms in cold acclimated species compared with the non acclimated, includes changes i) structural levels, such the existence of preferential sites for ice formation in woody plants, ii) cellular level, such as the stabilization of membranes through the activation of mechanisms of protection of the fluidity for ensuring their optimal activity in acclimated species (Wisniewski et al. 2014; Wisniewski et al. 2018), iii) in the concentration of various metabolites, including carbohydrates, protective proteins as well as modifications in the cell membrane, iv) in phytohormone levels, accumulation of cytosolic Ca2+, activation of ROS detoxification systems, accumulation of proline and expression of genes associated with cold response and transcription factors (TFs) (Hughes and Dunn 1996; Thomashow 1999) allowing plants to tolerate the associated cell desiccation with the formation of extracellular ice and freezing temperatures have been some of the differences reported in the acclimated species.

In woody plants during cold acclimation several resistance mechanisms are activated in order to be resistant to a hard winter with temperatures below the freezing point. The process of acclimation in woody plants takes place when plants are exposed to chilling temperatures and occurs gradually with the progressive drop in temperatures until the trees become tolerant to freezing temperatures (Nievola et al. 2017). Over the years several studies have reported the differences between the acclimated versus non acclimated plants in various species. Compared to deciduous woody plants, *Eucalyptus* lacks endodormancy or leaf senescence mechanisms occurring in the same environmental conditions, therefore it is considered an excellent model for studying cold acclimation (Keller et al. 2009). The process of acclimation varies among species and environmental conditions, being the damage higher or lower depending on its ability to acclimate (Oberschelp et al. 2020). In *E. globulus* considerable efforts have been made to understand the principal mechanism involved in cold acclimation response including the biochemical and molecular basis and the changes that take place throughout this process (Travert et al. 1997; Moraga et al. 2006; Fernández et al. 2006; Rasmussen-Poblete et al. 2008). Costa e Silva et al. (2008), evaluated the effect of cold stress in acclimated and unacclimated clones of *E. globulus*, where acclimated plants had a lower damage at membrane level, higher water potential and photosynthetic rates than unacclimated clones. Similar results were obtained by Shvaleva et al. (2008), where clones of *E. globulus* subjected to chilling temperatures after 10 days of acclimation showed changes in different metabolic response as in growth, osmotic potential, leaf pigments, soluble proteins and lipid composition of membranes and mainly in the content of soluble sugar. The levels of glucose, sucrose and fructose increased in leaves and roots of the acclimated clones compared with the non-acclimated. Castillo et al. (2010) performed the classification of the cold tolerance degree in leaves of different genotypes of *E. globulus*, using carbohydrates content and Near-infrared spectroscopy (NIR spectra), showing that NIR spectral data correctly assigned the samples in the modeling sets, according to their response to cold acclimation treatments. Previous works in several plant species have shown a high accumulation of soluble sugars during exposure to low temperatures, and it has been reported that carbohydrate metabolism plays a key role in cold acclimation of plants functioning as osmoprotectants and cellular membrane protector (Yuanyuan et al. 2008; Nievola et al. 2017). Under cold stress, the interaction of sugars with the phospholipids of cell membranes can reduce the membrane permeability, increased by cold stress, suggesting the increase in sugar levels as a mechanism for maintaining integrity and stability under freezing conditions (Fürtauer et al. 2019). Also, several carbohydrates in synergism with other components have been shown to be important components of the cellular ROS scavenging system (Tarkowski and Van den Ende 2015).

A fundamental function described in the cold acclimation process in plants is the stabilization of cell membranes and components, therefore the lipid membrane could improve its fluidity by optimizing the transition from saturated to unsaturated fatty acids. The accumulation of cryoprotective molecules such as soluble sugars, low molecular weight nitrogenous components (proline, glycine betaine) and LEA protein family (late abundant embryogenesis), help to stabilize membrane components, as well as, maintain hydrophilic interactions and cell homeostasis (Janská et al. 2010). In 1990, Thomashow defined the ability of cold acclimation as a quantitative trait, meaning that the action of several genes with small additive effects triggers cold response in plants; in the past years most of these mechanisms have been associated with reprogramming gene expression in different species.

3.5 Molecular response to cold stress in *Eucalyptus globulus*

The acclimation process involves the reprogramming of gene expression with the purpose to adjust the metabolism of the plants to face cold stress (Oberschelp et al. 2020). More than a thousand cold induced genes have been reported in different species, among them, about 170 genes have been described as TFs (Nievola et al. 2017). For *E. globulus* a timeline with the cold induced genes and its publish date is shown in Table 3.1.

Table 3.1 Timeline summary of experimental cold stress studies in *E. globulus* published to date

Family or gene	Low temperatures	Tissue	Analysis	Reference
CBFs	4°C	Leaves	RT-PCR	Gamboa et al. (2007)
UBC, a-TUB, EF1a and ELIP	4°C, -2°C and -6°C	Leaves	qRT-PCR	Fernández et al. (2010)
IDH, SAND, ACT, A-Tub, and UBQ	6.1°C and -9.1°C	Leaves	RT-PCR	Moura et al. (2012)
DHNs	4°C, -2°C and -6°C	Leaves	qRT-PCR	Fernández et al. (2012a)
DHNs	4°C, and -2°C	Leaves and stems	qRT-PCR	Fernández et al. (2012b)

NACs	7°C	Leaves, primary stems, secondary stems and roots	RNA-seq and qRT-PCR	Hussey et al. (2015)
DHN1, OEP, LTP, VDAC1, TIL, PCP, EIF-5A, LEA, ARP and CAX3	4°C, -2°C and -6°C	Leaves and apex	RNA-seq and qRT-PCR	Fernández et al. (2015)
DHNs	4°C, -2°C and -6°C	Leaves	qRT-PCR	Aguayo et al. (2016)
CBF1	4°C, -2°C and -6°C	Leaves	qRT-PCR	Navarrete-Campos et al. (2017)
NAC, MYB, CBF and KIN	4°C	Stems	RNA-seq	Araújo et al. (2018)
WRKYs	4°C, -2°C and -6°C	Leaves	RNA-seq and qRT-PCR	Aguayo et al. (2019)
MYB64 and MYB68	4°C, -2°C and -6°C	Leaves	qRT-PCR	Solis et al. (2019)
KIN10	10°C	Leaves and stems	qRT-PCR	Domingues-Junior et al. (2019)

As described in the previous sections cell membranes are the first site to be damaged by cold stress, studies in different species postulate that the perception of this type of stress occurs first at the plasma membrane level which triggers expression of Cold Regulated Genes (*COR)* (Knight and Knight 2012). Changes in cell membranes act as a signal to activate pre-existing TF, which can induce the expression of C-repeat binding factor (CBF), which are the primary regulators of the transcriptional cold response network in higher plants, these activate the expression of genes that have C-repeat (CRT)/dehydration responsive element (DRE) in their promoter, as is the case of many *COR* genes (Wisniewski et al. 2014; Chang et al. 2021). The CBF TF family, represents a highly conserved pathway of ABA-independent TF and most studied in relation to the induction of gene expression in response to cold stress with a prominent role in stress tolerance by freezing (Navarro et al. 2009; Thomashow 2010). Described originally in *Arabidopsis*, several CBF orthologs have been discovered in woody plants, including *Eucalyptus.* For example, El Kayal et al. (2006) identified two *CBF* genes (*CBF1a* and *CBF1b)* induced by low temperatures in *E. gunni*, a cold tolerant species. They also showed the specific response of regulation for both genes, where *CBF1a* had a more transient response to severe cold conditions while *CBF1b* showed a longer response to progressive cold conditions.

In the same species, Navarro et al. (2009) isolated two additional *CBF* genes (*CBF1c* and *CBF1d*) and reported their responses to different abiotic stress, results showed *CBF1c* is a constitutive and non-specific gene, while *CBF1d* was durably induced during chilling exposure. Navarro et al. (2011), overexpressed two *CBF1* in a cold-sensitive *Eucalyptus* hybrid (*E. urophylla* x *E. grandis*), the transgenic lines exhibited improvement on cold tolerance and other changes such as, reduced growth, decrease in stomatal density and better water retention capacity. In the reference genome of *E. grandis* Cao et al. (2015), identified a total of 17 CBF members participating mainly in cold response. Particularly for *E. globulus,* Gamboa et al. (2007) isolated one full-length CBF cDNA, observing the expression of *EgCBF1* in seedlings under different abiotic stress condition, showing a response only to low temperatures after plants were exposed at 4°C. Similar results were obtained by Navarrete-Campos et al. (2017), three *CBF* genes (*CBF1a*, *c* and *d*) were identified, isolated and characterized from *E. globulus*, relative expression analysis in different genotypes of *E. globulus* subject to cold acclimation process showed that these genes were highly related to acclimation especially at temperatures below 0°C. Navarrete-Campos et al. (2017) observed that the transgenic *Arabidopsis* plants overexpressing each CBF gene had high transcript levels of endogenous *COR* genes, enhancing its tolerance to freezing.

Cold acclimation has been associated with induction of *COR* genes mediated by the cold-and dehydration-responsive DNA cis-elements (Jaglo et al. 2001). Among these *COR* genes, dehydrins (DHNs), a sub-group of LEA (late embryogenesis abundant) protein family, are induced by diverse abiotic stresses that involve cold stress and cellular dehydration (Kosová et al. 2007; Layton et al. 2010). In trees, DHNs play an important role in cold acclimation, performing diverse functions associated with osmoprotection and cryoprotection, which has generated great interest in the study of these proteins. Keller et al. (2009; 2013), generated a cDNA library from clones of *E. gunnii* subject to cold acclimation process, among the most abundant ESTs sequences dehydrins were identified and they proposed these genes as good candidates for improving frost tolerance. In *E. globulus*, Fernández et al. (2012a, b) isolated and compared the seasonal regulation of four dehydrin genes, finding that three of them (*DHN1, 2* and *10*) increased their relative expression levels during cold acclimation in leaf and stem tissue and decreased when cold treatment stopped, having a higher transcript abundance in a freezing resistant genotype of *E. globulus* compared to a freezing sensitive one. The same study determined the cis-element in each *DHN* gene, identifing CRT regulatory elements, which are a vital part of ABA-independent and dependent gene expression in abiotic stress response (Shinozaki et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2005). *DHN2* was used to obtain transgenic *Arabidopsis thaliana* lines (Aguayo et al. 2016), results showed that the overexpression of this gene increased the survival rate to cold treatment of the transgenic lines compared to the non-transform plants, showing the significant role in cold acclimation and frost tolerance that this protein has in *E. globulus*.

One of the mechanisms to overcome cold stress is the accumulation of soluble sugars. The important role played by the carbohydrate metabolism in cold stress in plants of *E. globulus* has been reported recently by Domingues-Junior et al. (2019). Plants subjected to 10°C showed a differential expression level of kinase 10 (*KIN10*), gene part of the sucrose-nonfermentation1-related protein kinase1 (SnRK1) genes system, associated with the activation of metabolic processes. The induction of high *KIN10* transcript levels under low temperatures, resulted in a stimulation of the photosynthesis and promoted the carbon substrate production, deriving in a better growth rate according to the authors.

Among the different next generation sequencing (NGS) techniques, RNA-seq has become a key tool in transcriptome studies, being often used for analyzing differential gene expression between experimental groups. RNA-seq helps to characterize the transcriptional structure of genes, determine and compare the expression levels of transcripts in biological samples at a specific tissue and a certain moment with high efficiency, speed and low cost (Wang et al. 2009). The first RNA-seq study described in *Eucalyptus* that investigated response of gene expression under abiotic stress was reported by Villar et al. (2011), using two *Eucalyptus* genotypes subject under water deficiency. The transcript data showed that a higher number of genes were expressed in the genotype with better growth rates. Particularly for *E. globulus,* Fernández et al. (2015), used RNA-seq to study genes being up- and down-regulated between two genotypes of *E. globulus*, contrasting on their frost tolerance/sensitivity. Around 14,200 non-redundant transcripts, out of which 163 were up-regulated and 537 down-regulated genes were found. Among the up-regulated genes various DHNs proteins were identified, confirming the role described previously. As well several transcriptional factors were identified, finding that 12% of upregulated genes corresponded to TF in the tolerant genotype. Recently Araújo et al. (2018), used RNA-seq identifying 3,300 and 1,370 differentially expressed genes in stems of *E. urograndis,* a hybrid of *E. urophylla* x E. grandis, and *E. globulus* under low temperature stress conditions, family members of TFs like NAC and MYB presented the highest expression levels detected.

Transcription factors play an important role regulating the expression of target genes, interacting with specific elements of the promoter sequences and acting as an on or off switch of gene expression in the stress response in plants (Kiranmai et al. 2016; Wang et al. 2016). In *A. thaliana,* it has been reported that between 6 to 10% of the genes that make up the genome of the species code for TFs (Lindemose et al. 2013; Wisniewski et al. 2018). Over the years, CBF TFs have been one of the most studied in the cold acclimation process, but recently other TFs have gained importance. One of them are NAC proteins which are one of the most numerous TF families specific to plants and are present in a large variety of species (Olsen et al. 2005). In most cases it has been seen that the response of these TFs is strong and rapidly induced by abiotic stress, such as drought, salinity or cold, which indicates that they have a regulatory function, allowing the plants to tolerate stress. The exact role of each of the members of this superfamily related to cold stress is not clear yet and few studies have been addressed in tree species. In the case of *Eucalyptus*, Hussey et al. (2015) characterized 189 NAC in *E. grandis* arranged into 22 subfamilies associated with stress response including cold stress. Also, with the purpose to better understand NAC gene functions, the authors analyzed the expression level of NAC orthologs in *E. globulus* in response to chilling temperatures of 7°C, finding ten NAC genes with response to cold treatment.

Using RNA-seq Aguayo et al. (2019), identified 3,624 and 2,869 TFs in *E. globulus* leaf and root transcriptomes respectively. The most represented families were, NAC, bHLH, bZIP, MYB, ERF and WRKY, these results coincide with those described by Liu et al. (2014) which used the same technique to investigate the response of plants of *E. dunni* under cold acclimation treatment.

One of the TF families reported with abundant transcription levels in *Eucalyptus* species under cold stress are MYB proteins (Liu et al. 2014; Leonardi et al. 2015; Gaete-Loyola et al. 2017). Like the other TFs mentioned in this review, MYB proteins have been reported to have an adaptive response to cold temperature (Agarwal et al. 2006; Su et al. 2014). Solís et al. (2019), reported twenty R2R3-MYB in the transcriptome of *E. nitens* in response to cold acclimation and two of them showed higher transcript accumulation according to *in silico* analysis (*MYB64* and *MYB68*). To validate these results, authors analyzed the relative expression of these *MYB* genes in leaf tissue of *E. nitens, E. globulus* and hybrid subjected to cold treatment. The higher expression levels were obtained in *E. nitens,* which is a cold tolerant species (able to tolerate temperatures of −15°C), but significant levels were observed also in *E. globulus* when temperatures reached −2°C.

Another important TFs family are WRKY which have been involved in different biological processes and in biotic and abiotic stress response, such as regulation of different physiological programs, as plant acclimation process, hormone signaling or secondary metabolism (Rushton et al. 2010). In the genome of *E. grandis* 79 *WRKY* genes have been identified by Fan et al. (2018), a cold stress treatment was performed where plants of *E. grandis* were subject to 4°C during different period of times (0, 1, 6, 24, and 48h). Results showed five WRKY cold-respond groups (cold 1, 2, 3, 4 and 5) which were significantly upregulated at different times. In the same study of Aguayo et al. (2019), a total of 51 *WRKY* genes were found in *E. globulus* plants and eleven of them showed significant differences in the relative expression levels measured in leaf tissues during cold acclimation treatments, suggesting that these TFs have a significant role regulating gene expression in response to cold temperatures.

Cold response is now known to be a mechanism that integrates a complex network of signaling pathways; a number of genes involved in these mechanisms have been described for different species but only a few have been reported for *E. globulus*. Based on the information available for *E. globulus* and *A. thaliana* a schematic representation of signaling pathways that respond during cold stresses is shown in Fig.3.1. This hypothetical network explains i) the possible interaction between the different TFs, for example, NAC genes could interact upstream with CBF genes and regulate the ICE-CBF-COR pathway (Diao et al. 2020) ii) the different pathways that these genes can take to respond to cold stress, such as, *AtMYB12* the putative orthologous of *EglMYB64* in *A. thaliana*, which has been reported in the regulation of genes involved in flavonoid biosynthesis and ABA biosynthesis under abiotic stress (Wang et al. 2016) iii) and the response of different genes to several mechanisms in which they have been involved, for example *KIN10* has been described as playing a key role in the increase of carbon assimilation, whereas other genes such as *DHNs* are involved in stabilizing cell membranes and preventing cell dehydration.

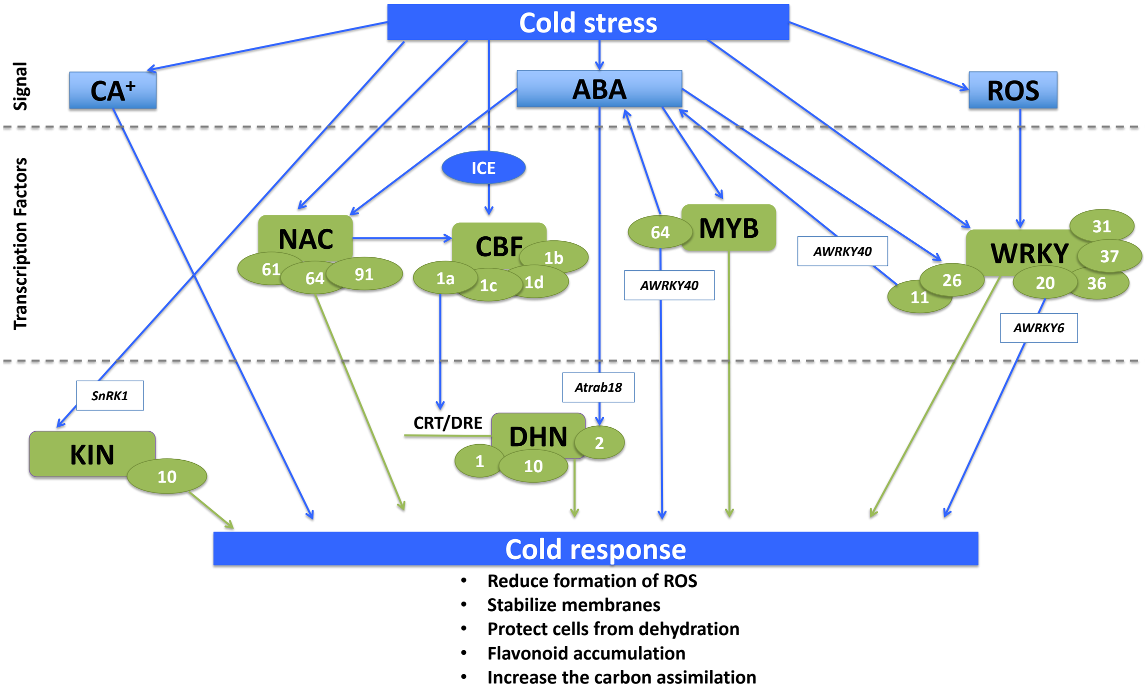

Fig.3.1 Schematic representation of signaling pathway under cold stress in *E. globulus*. Cold stress (CS) can cause the increase of abscisic acid (ABA), cytosolic Ca^{2+} ion and ROS accumulation, which can act as the first signal for the activation of genes related to CS. In *E. globulus*, CS induces the expression of some TFs, such as, NAC, MYB and WRKY, also, the ICE-CBF-COR pathway has been reported, where DHNs are the main COR genes described for this species. Green boxes indicate the genes reported in E. globulus; green circles indicates genes with significant differences in their expression levels under cold treatments in *E. globulus*; blue circles, indicate genes described in *A. thaliana* related to cold responses pathway in E. globulus; blue arrows, indicate the direction in the response according to the described in *A. thaliana*; white boxes, indicate the orthologous genes of *E. globulus* in *A. thaliana*; green arrows indicate the genes with validated response to CS in *E. globulus*.

3.6 Conclusions

Climate change predicts an increase in extreme environmental conditions and consequently, the need to identify species and genotypes that are better adapted to these extreme conditions. For species such as *E. globulus*, an important species for industrial applications, this is an important aspect to consider, since its productivity and distribution is limited by low temperatures.

In early studies, the description of physiological damages of plants of *E. globulus* under cold stress was the main objective. Decrease in photosynthesis, reduced water absorption, membrane injury and reduction in growth, were some of the reported effects. Although the physiological consequences of exposure to low temperature are well characterized, the main molecular actors responsible for sensing low temperature and triggering cold response in *E. globulus* are still unclear and only a few studies of the molecular response have been published. Our present knowledge, based principally on herbaceous species, indicate that responses to cold stress cover a wide range of molecular pathways that can trigger processes such as hormone responses, activation of TF and COR genes and the reprogramming of a large proportion of the transcriptome (Estravis-Barcala et al. 2020). Several articles studying *E. globulus* under cold stress were summarized in this review and a putative signaling pathway was proposed (Fig.3.1), including the ICE-CBF-COR pathway which is known to play a significant role in cold acclimation in various species (Chinnusamy et al. 2010). This information is important to understand and build gene networks that can allow us to predict their contribution in cold tolerant phenotypes, as well as to select candidate genes to be employed in the development of cold-tolerant genetic engineered trees; increasing the capacity to design several strategies to predict and potentially obtain trees with better response to extreme temperatures under changing climate conditions.

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CAPITULO IV: EglNAC34: a transcription factor involved in cold and drought stress in Eucalyptus globulus[4]

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4.1 Abstract

Transcription factors are associated with various types of abiotic stresses in different plant species. The NAC protein family constitutes one of the most numerous and specific families of transcription factors found in a wide variety of species. This family has been implicated in the regulation of diverse processes, including responses to biotic and abiotic stress, mostly in agricultural crops, with few reports on woody species of forest interest. *Eucalyptus globulus*, one of the most important species in the forest industry, has been affected by climate change, particularly due to more frequent drought periods and extreme cold events. In this study, 101 *NAC* transcription factor genes were identified in the transcriptome of a genotype of *E. globulus* characterized as tolerant to drought and cold stress. Among these genes, 13

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[4] Trabajo enviado 17 de junio 2024 en revista Trees TSAF-D-24-00201

EglNAC genes exhibited significant differential expression under both treatments. In particular, the expression level of *EglNAC34* was significantly greater than that of all the other EglNAC strains analyzed. To validate these results, this gene was selected for comparative quantitative real-time polymerase chain reaction analysis using *E. globulus* genotypes previously characterized as tolerant or susceptible to drought and cold stress, where similar results were observed. To further understand the role of EglNAC34, a network interaction analysis of the response to drought and cold stress was conducted.

Keywords: NAC family, abiotic stress, transcriptome, water stress

4.2 Introduction

Due to current climate change, the occurrence of extreme environmental events that affect the adaptation and growth of forest trees is increasing. Higher intensity and frequency of different types of abiotic stress result in decreased productivity, increased tree mortality and, ultimately, economic losses for the forest industry (Xu and Fu 2022; Li et al. 2023). Most climate

models predict an increase in the frequency of drought periods and extreme cold events in subsequent years (Collins et al. 2013; Li et al. 2023).
Drought stress is the main abiotic stress that disturbs the normal balance of vital processes in plants, such as an imbalance between water uptake from soil and water loss through transpiration and perturbations in the carbon balance, which are mainly associated with a decrease in carbon assimilation and hydraulic failure resulting from cavitation (Puglielli et al. 2023; Li et al. 2023; Pavanetto et al. 2024). All these interconnected processes result in a reduction in the growth and survival of plants exposed to drought stress.

Under cold stress, the main damage in plants is caused by damage to the cellular plasma membrane, loss of the permeability and fluidity of the membrane occur at temperatures below 10°C, and alterations in its function cause a decrease in a series of processes that can lead to the collapse of cellular functions, including cell death (Thomashow 1999; Kazemi-Shahandashti and Maali-Amiri 2018). Additionally, plants subjected to cold stress show a reduction in the ability to absorb water since low temperatures in the soil reduce the amount of available water and inhibit the activity of proteins related to water absorption through roots. Subzero temperatures lead to ice formation in plant tissues, reducing the water potential and leading to dehydration and a reduction in plant growth (Ritonga and Chen 2020; Li et al. 2023).

One of the pathways to protect plants from abiotic stress and to re-establish the normal balance of vital processes is reprogramming genetic expression (Kazemi-Shahandashti and Maali-Amiri, 2018). In recent decades, extensive research has been conducted to identify and characterize genes associated with the response to abiotic stress in different plant species, and in recent years, transcription factors (TFs) have been the focus of this research (Sharma et al. 2020; Baoxiang et al. 2023). Transcription factors are regulatory members that are responsible for transferring external stress signals to the nucleus and regulating the stress response at the transcriptional level, interacting with the cis elements present in the promoter regions of target genes and acting as activators or repressors in the stress response in plants (Hoang et al. 2017). In plant genomes, approximately 7% of the coding sequences are TFs (Wisniewski et al. 2018). The most relevant TF families described in plants include AP2/EREBP, MYB, WRKY, bZIP and NAC (Wang et al. 2016; Trono and Pecchioni 2022; Zhang et al. 2023). These TFs are associated with various types of abiotic stresses in different plant species (Hoang et al. 2017; Yao et al. 2021; Shinozaki and Yamaguchi-Shinozaki 2022). The NAC protein family constitutes one of the most numerous and specific families of TFs in plants and is found in a wide variety of species (Olsen et al. 2005). These proteins have a highly conserved NAC domain, originally characterized from consensus sequences from petunia (**N**AM) and Arabidopsis (**A**TAF1/2 and **C**UC2), located in the N-terminal region that comprises approximately 150 amino acids that are divided into five subdomains (A to E), and their functions have been associated with nuclear localization, DNA binding and the formation of functional homodimers or heterodimers (Singh et al. 2021; Han et al. 2023). In contrast, the C-terminal region functions as a transcription regulatory region, acting as a transcriptional activator or repressor, and has been described as a highly divergent region (Shao et al. 2015; Nagahage et al. 2018). Over the past few years, several research groups have focused on understanding the role of NAC TFs under abiotic stress. The main results revealed a strong and rapid response induced by different types of stresses, such as salinity, heat, drought or cold. These have been studied mainly in agricultural crops, and only a few have been reported in woody species of forest interest. Hu et al. (2019)

showed that *BpNAC012*, an NAC gene characterized in *Betula platyphylla*, is upregulated in response to osmotic stress. In Populus spp., Yao et al. (2020) evaluated the expression patterns of the *PtNAC* family present in *Populus simonii*×*Populus nigra* under salt stress, and 20 NAC TFs were found to be involved in this response. In *E. grandis*, Hussey et al. (2015) characterized 189 EgrNACs, some of which were involved in the regulation of opposing and tension wood development and in response to low temperatures.

Eucalyptus sp. is one of the most planted types of forest species in the world and is distributed in more than 90 countries due to its short rotation, excellent wood quality and high adaptative capacity (Booth 2013). Within these more than 700 species of eucalyptus, *E. globulus* is one of the most important for the forest industry, with a rotation time between 10 and 12 years, a high pulp yield, high wood density and excellent fiber quality (Carrillo et al. 2018; Picoli et al. 2021). Despite its high adaptive capacity, it has been affected by climate change, especially due to the occurrence of more frequent drought periods and extreme cold events. These conditions limit the adaptation of *E. globulus,* restricting its distribution in plantation areas that allow the optimal development of the species. The appropriate establishment of *E. globulus* plantations in areas under abiotic stress depends on the combination of several silvicultural systems and the selection of appropriate genotypes (Rubilar et al. 2020).

Reprogramming of gene expression under abiotic stress, such as drought and cold, allows the selection of susceptible and tolerant plants considering phenotypic traits of interest. Using the available RNA-seq data from leaf tissues of *E. globulus* by Aguayo et al. (2019), this work identified the NAC gene family in *E. globulus* (*EglNAC*) under drought and cold stress. In this study, 101 NAC TF genes were identified, and phylogenetic relationships, conserved motifs, and *in silico* characterization of their expression patterns were analyzed under stress conditions. The results showed that the *EglNAC34* gene was significantly induced under both conditions. To validate these results, this gene was selected for comparative quantitative real-time polymerase chain reaction (qRT–PCR) analysis using *E. globulus* genotypes previously characterized as tolerant or susceptible to drought and cold stress.

4.3 Materials and methods

4.3.1 Plant Materials and Datasets

The *E. globulus* datasets used in this study were published by Aguayo et al. (2019), and one genotype characterized as tolerant to drought and cold stress was used for RNA-seq analysis. Libraries were constructed from mRNA isolated from foliar material from six-month-old plants of *E. globulus*. The accession code available in NCBI is PRJNA486291. To validate the qRT–PCR results obtained from the analysis of differentially expressed genes (DEGs) of the *NAC* genes identified, eight *E. globulus* genotypes provided by Bioforest S.A. were used. These genotypes exhibited contrasting responses to drought or cold under field conditions. A total of 44 plants (ramets) per genotype were evaluated in each assay.

4.3.2 Drought assays

The same drought-stress assays described by Ulloa et al. (2022) were used in this research. In a greenhouse, 44 plants per genotype were transplanted to individual plastic bags with a mixture of substrate containing 90% pine bark and 10% perlite. Two treatments were established: a control (CON), in which plants were constantly watered until the end of the assay, and a drought stress treatment (DS). For plants subjected to DS treatment, after two weeks of constant irrigation, DS was induced by stopping irrigation until the predawn leaf water potential (Ψpd) reached − 1.5 MPa for an intermediate drought stress level (IDS) and −1.8 MPa for a severe drought stress level (SDS). Leaf samples of three biological replicates from each genotype were collected at the CON, IDS and SDS points. All samples were stored immediately in liquid nitrogen and kept at –80°C until processing. The survival rate (percentage of live plants) and damage (percent of necrotic leaves and buds) of the plants were measured two months after irrigation stopped, and ten plants per genotype and treatment were evaluated.

4.3.3 Cold assays

Cold assays were carried out as described by Aguayo et al. (2019). All plants (44 per genotype) were transferred to polystyrene boxes and covered with vermiculite to maintain substrate humidity. The cold experiment was conducted in a completely randomized design in a cold chamber. Three treatments were established; for the control condition, nonacclimated (NA) plants were maintained for 10 days under 14 h day at temperatures of 20/12°C (day/night). In treatment two, which involved cold acclimation before frost (CABF), plants of each genotype were maintained on a 14 h day at a temperature of 8/4°C (day/night), and leaf samples were collected after 7 days. Finally, for cold acclimation after frost (CAAF), a three-night frost of -2°C and a single-night frost of -6°C, with a decrease rate of 2°C per hour, samples were collected when the temperature in the chamber reached 8°C. Leaves of three biological replicates for each genotype at the end of each treatment were collected, frozen in liquid nitrogen and stored at –80°C until use. To measure the survival and damage rate, after the treatments, the chamber conditions were changed to 14 h day at 12/6°C (day/night) for one week, and ten plants per genotype were used to determine the percentage of dead vs live tissues.

4.3.4 Identification of the *EglNAC* gene and conserved motif

To identify NAC genes in the transcriptome of *E. globulus* in response to cold and drought stress conditions, the genome of *E. grandis* v2.0 available in the Phytozome database (https://phytozome.jgi.doe.gov/) was used as a reference. In addition, the annotations of NAC TFs in *E. grandis* described by Hussey et al. (2015) and the amino acid sequences of 138 AtNAC from *A. thaliana* (PlantTFDB v5.0) were used for a BLASTX search (parameters: *E* value cutoff of 1e−10 and similarity score of ≥ 45%). Open reading frame prediction for NAC genes from the coding sequences of *E. globulus*, which shares high identity with *E. grandis*, the closest species to the available genome, was performed. The NAC domain was predicted by an HMM search (https://www.ebi.ac.uk/Tools/ HMMER/) and in Prosite (https://prosite.expasy.org). Sequences lacking an initiation or termination codon and sequences with an incomplete NAC domain were removed. Motif searching was performed using the Multiple Expectation Maximization for Motif Elicitation (MEME) tool version 5.5.5 (http://meme- suite.org/), and the parameters used for motif location were a minimum motif width of 6, a maximum motif width of 50 and a maximum number of motifs of 10.

4.3.5 Phylogenetic analysis

Phylogenetic analysis was carried out in MEGA7, and protein sequences of EglNAC identified in the transcriptome of *E. globulus* under both stress treatments were aligned using MUSCLE with default parameters (gap opening penalty = 10, gap extension penalty = 0.1). An unrooted phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstraps. The MEGA7 output tree was edited using the Interactive Tree of Life online tool (iTOL; https://itol.embl.de/).

In silico differential expression analysis and functional association

The assessment of *EglNAC* gene expression utilized high-quality reads obtained from *E. globulus* samples. The four libraries sequenced after treatment with NA, CABF, CAAF and DS and aligned using Bowtie2 (Langmead and Salzberg 2012) were used for the analysis. For the identification of DEGs between treatments, the EdgeR package was used (Robinson et al. 2010) within the R statistical environment tools (Team RC, 2013). Differentially expressed *NAC* genes were identified based on the negative binomial distribution and pairwise comparison treatments. Genes with a count ≥ 1 CPM, a fold change (FC) ≥ 2.0, a false discovery rate (FDR) <0.05, and a *p* value <0.05 across all samples were considered as DEGs. Furthermore, to visually represent the expression patterns of *EglNAC* genes in response to both abiotic stresses, a heatmap was generated using the R program.

The NAC gene with the highest expression under both stress conditions was selected for network interaction analysis. The biological database STRING (https://string-db.org/), a web resource of known and anticipated protein–protein interactions in the species *A. thaliana*, was used to identify NAC proteins and their target genes with putative roles in response to abiotic stress.

4.3.6 Expression validation by qRT-PCR

Total RNA from leaf samples of six genotypes of *E. globulus* previously characterized as having contrasting responses to drought or cold under field conditions was extracted using the cetyltrimethylammonium bromide (CTAB)-based method (Chang et al. 1993) and quantified by UV spectrophotometry (NanoDrop ND-1000, Thermo Scientific, USA). RNA integrity was visualized on a 1% agarose gel.

For cDNA synthesis, one microgram of RNA was treated with DNAseI to eliminate genomic DNA contamination, followed by cDNA synthesis using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA) according to the manufacturer's protocol. Primers for the selected genes (*EglNAC,* target and housekeeping genes) were designed using Primer3 software version 4.1.0 (http://bioinfo.ut.ee/primer3). Quantitative real-time PCR was performed using a detection system (ABI-7300, Applied Biosystems, USA) and Evagreen Master qPCR Mix Plus (ROX) under the following conditions: initial denaturation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, in 96-well optical reaction plates. Relative expression levels were determined using the 2−ΔΔCT method (Livak and Schmittgen 2001), with *EF1α* and *UBC* as endogenous reference genes for cold treatment samples (Aguayo et al. 2019) and *EF1α* and *EIF4* genes for drought treatment samples (Ulloa et al. 2022). Statistical analysis was performed using two-way ANOVA considering treatments and six genotypes, followed by Tukey's test to identify significant differences. R software was utilized for both analyses. The correlation between expression values obtained from RNA-Seq and qRT–PCR analyses was assessed using the Spearman correlation coefficient calculated with R tools (Team RC, 2013).

4.4 Results

4.4.1 Identification of putative *NAC* genes and conserved motifs in *Eucalyptus globulus*

A total of 101 *NAC* genes were identified in the leaf transcriptomes of *E. globulus* via BLAST. The annotation was made using the information available in the Phytozome database for *E. grandis, Arabidopsis*, and the NAC genes of *E. grandis* reported by Hussey et al. (2015). A total of 113 *NAC* putative genes were identified, but only 101 proteins presented a complete NAC domain. The putative *NAC* genes were named *EglNAC1* to *EglNAC101*. The identified NAC domains were found within a position range of 4 to 200 amino acids, consistent with the findings of Ooka et al. (2003), who reported the presence of NAC domains in the N-terminal region. The characteristics of the NAC sequence greatly varied in *E. globulus*. The CDS lengths of the 101 *EglNAC* genes ranged from 471 to 3973 bp; the smallest protein was EglNAC82, with 141 amino acids, and the largest was EglNAC57, with 660 amino acids. The MWs of the proteins ranged from 16.68 to 74.52 kDa. and the pIs ranged from 4.66 to 9.98 (Table S4.1).

To determine the conserved shared motifs among the 101 EglNAC proteins, the MEME program was used. Ten motifs were identified and classified as general motifs that presented a consensus sequence described for subdomains A to E (the NAC domain) and as specific motifs outside of this region. Using the motif sequences described by Ooka et al. (2003) and Hussey et al. (2015), we assigned the amino acid sequence of motif 1 to subdomain A, motif 6 to subdomain B, motif 3 and motif 5 to subdomain C, motif 2 and motif 4 to subdomain D, and motif 7 to subdomain E (Table 4.1). Ninety-six EgloNAC proteins had motif 1, 99 EgloNAC proteins had motif 2, 87 EgloNAC proteins had motif 3, 96 EgloNAC proteins had motif 4, 70 EgloNAC proteins contained motif 5, 87 EgloNAC proteins included motif 6, and 95 EgloNAC proteins had motif 7. The most conserved motifs with ~96% identity were 1, 2 and 4, which represented subdomains A and D, respectively (Fig.S4.1).

Table 4.1 Details of conserved motifs from EglNAC proteins

Motif name	Sequence	Length	Subdomain
Motif 1	GFRFHPTDEELVNHY	15	A
Motif 2	PKGEKTBWVMHEYRL	15	D
Motif 3	RPNRATGSGYWKATGKDKPIY	21	C
Motif 4	VIGMKKTLVFYKGRA	15	D
Motif 5	AKSGEKEWYFFSPRDRKYPNG	21	C
Motif 6	VDJYKFEPWDL	11	B
Motif 7	RLDEWVLCRIF	11	E
Motif 8	NKKGTIEKHFPSTHNKTAEFPEMETKPQIVMPAALNPYTTP	41	-
Motif 9	LRRKVAGKPISVDII	15	-
Motif 10	PMTTDHMYMDSSESVPRLHTDSSCSEHAV	29	-

4.4.2 Phylogenetic analysis of EglNACs

The phylogenetic classification of NAC members identified in the transcriptomes of *E. globulus* was performed based on the classification described by Hussey et al. (2015) for *E. grandis* (Table S4.2). The unrooted phylogenetic tree of EglNAC proteins identified and separated the NAC protein family into 2 groups divided into 17 subfamilies (Ia, Ib, Ic, II, IIIa/b, IIIc, IVa, IVb, IVc, IVd, Va, Vb, VIa, VIb, VIc, VII and XI). Among these groups, group I was divided into three subfamilies, Ia, Ib and Ic, with 8, 2 and 6 members, respectively. Seven EglNAC proteins were classified into group/subfamily II; group III was divided into two subfamilies, IIIa/b, which included 8 members, and IIIc, which included 6 members. Group IV was divided into four subfamilies, IVa, IVb, IVc and IVd, with 25, 2, 6 and 3 members, respectively, representing the group with the most members identified. Group V was divided into two subfamilies, Va (1 and 2), with 1 and 5 members, respectively, and Vb with 12 members. Group VI was divided into three subfamilies, VIa, VIb and VIc, with 3, 1 and 2 members, respectively. Two EglNAC proteins were classified into group/subfamily VII. Group XI contained three members (Fig.4.1).

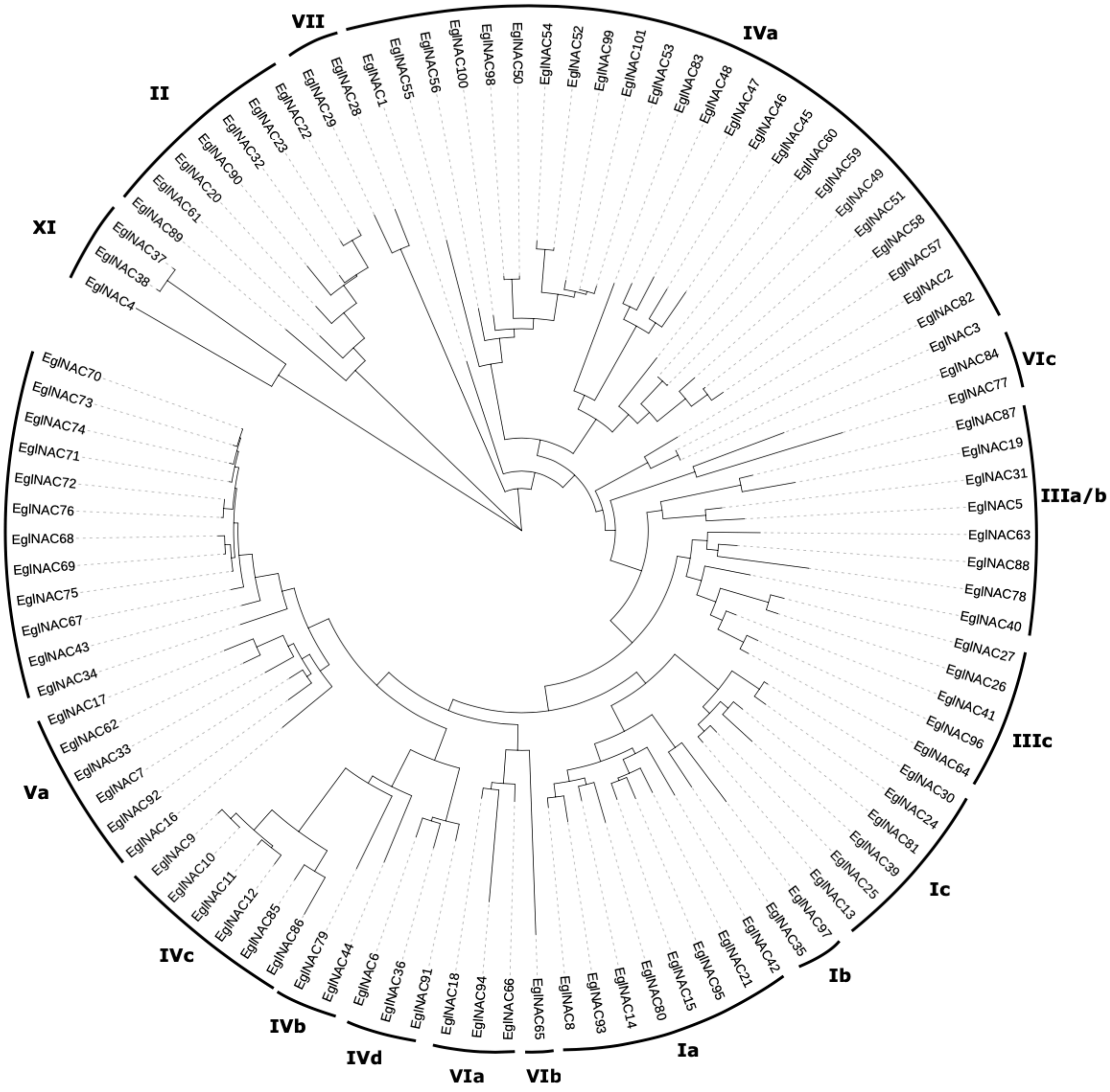

Fig.4.1 Phylogenetic tree of NAC proteins in *E. globulus*. The phylogenetic tree was constructed by MEGA 7 software using the maximum likelihood method with 1000 bootstraps. The numbers and letters indicate the 17 subfamilies represented in the transcriptome of *E. globulus.*

4.4.3 Identification of the response of *EglNAC* genes to drought and cold stress

To understand the putative role of the NAC genes identified in *E. globulus* under abiotic stress, the expression patterns of 101 *EglNAC* genes under drought and cold stress treatment were identified by RNA-Seq analysis in a genotype characterized as tolerant to drought and cold stress. The genotype utilized for library construction was labeled GDC. Fig.4.2 shows the heatmap of the 101 *EglNAC* genes. The results showed that under drought stress treatment (DS), six genes were upregulated, and the expression of *EglNAC18* increased more than fivefold, followed by that of EglNAC34 (>fourfold at DS). The expression levels of the genes *EglNAC43*, *EglNAC62*, *EglNAC69* and *EglNAC72* increased more than twofold under DS conditions.

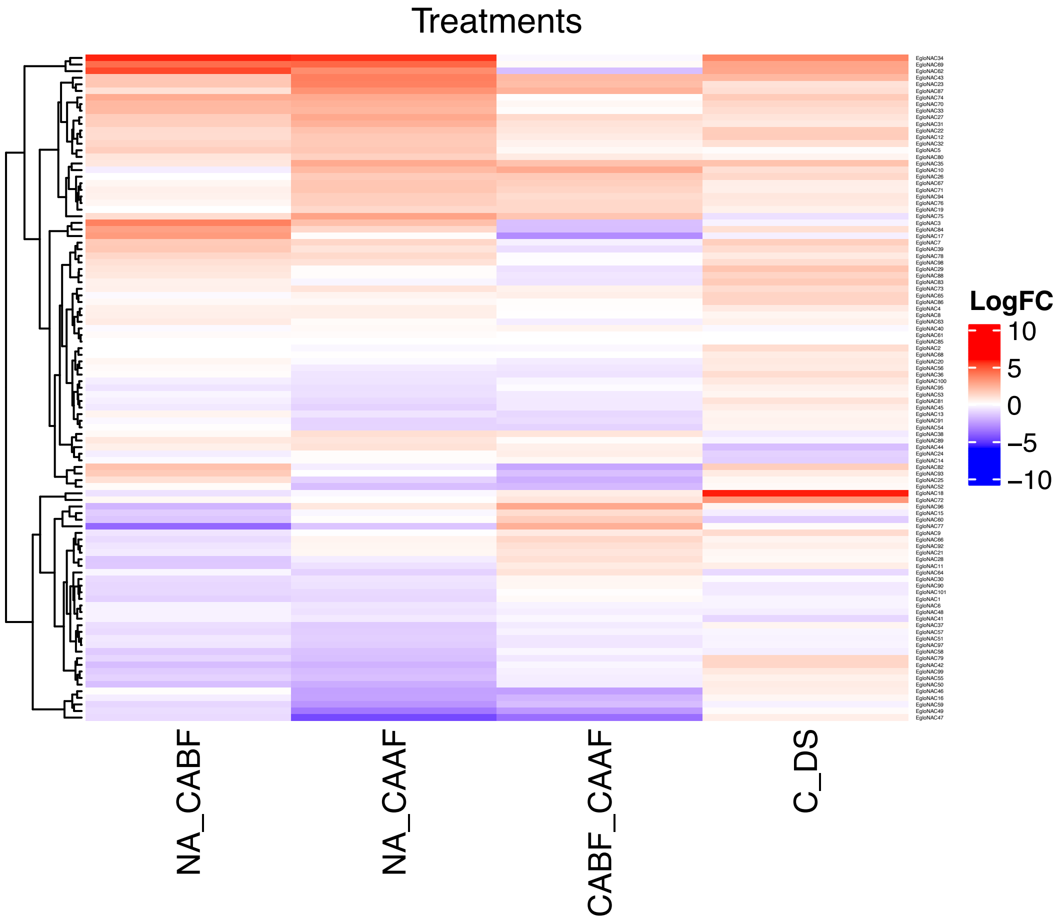

Fig.4.2 Heatmap of the expression profiles of 101 EglNAC genes identified in leaves under drought and cold stress treatment (CON control, DS drought stress, NA nonacclimated, CABF cold-acclimated before frost, CAAF cold-acclimated after frost). The red and blue boxes indicate high and low expression levels, respectively.

Of the total *EglNAC genes* evaluated for cold acclimation, five genes were upregulated under CABF conditions, and the expression level of EglNAC34 increased more than fivefold compared to that under the control condition (NA-CABF), followed by that of *EglNAC62* (> fivefold at CABF). Compared with those under the control conditions, the *EglNAC18* expression levels under the CABF treatment increased more than threefold, and the expression of the genes *EglNAC70* and *EglNAC74* increased more than twofold. A comparison between the control and cold acclimation after frost (NA-CAAF) groups revealed 13 genes whose expression significantly differed; twelve *EglNAC* genes were upregulated, and the expression level of EglNAC34 increased more than fivefold compared with that in the control group, followed by that of *EglNAC62* (> fourfold at CAAF). The expression levels of the genes *EglNAC23, 43*, and 69 were threefold greater than those under the control condition, while the expression levels of the genes *EglNAC31, 33, 35, 70, 73, 74*, and *75* increased more than twofold under the CAAF condition. *EglNAC47* was the only gene with DE detected with negative expression in the CAAF group compared with the control group. To validate the *in silico* expression results, three genes (*EglNAC18, EglNAC34, and EglNAC62*) with a DE response under both stress conditions and *EglNAC47,* the only gene with a downregulated response, were selected for further analysis by qRT–PCR.

4.4.4 Validation of *EglNAC* gene expression in *E. globulus* by qRT–PCR

Expression levels were determined in leaf samples of genotypes of *E. globulus* under drought and/or cold stress. Four *NAC* genes, *EglNAC18, EglNAC34, EglNAC62* and *EglNAC47,* were evaluated by qRT–PCR.

Four genotypes of *E. globulus* with contrasting responses to drought stress were used: two drought-tolerant genotypes, designated GDT1 and GDT2, and two drought-susceptible genotypes, designated GDS1 and GDS2. Similarly, an equivalent number of genotypes were subjected to cold stress: two cold-tolerant genotypes, designated GCT1 and GCT2, and two cold-susceptible genotypes, designated GCS1 and GCS2. Additionally, the genotype utilized for library construction was labeled GDC and was included in both expression analysis assays.

Under drought treatments, the transcript abundance of EglNAC18 increased under IDS and SDS conditions in three of the five genotypes evaluated (GDT1, GDT2 and GDS2). A significant difference in the expression level of this gene was detected under SDS conditions for the GDT1 and GDS2 genotypes compared to the control condition; for genotype GDS1, an increase in transcript abundance was observed only in the IDS compared with the CON condition, but no significant difference was observed (Fig.4.3). For genotype GDC, a decrease in the transcription accumulation levels with no significant difference was shown, presenting a similar expression pattern as that observed in the RNA-Seq analysis.

The transcript accumulation of the *EglNAC34* gene increased in all genotypes when comparing the treatment conditions (IDS and SDS) to that of the CON group, and a significant difference in the expression level was observed for the GDT2 and GDS2 genotypes (Fig.4.3). For genotype GDC, an increase in the transcript accumulation levels with a significant difference was observed, and a similar expression pattern was obtained via RNA-Seq analysis when comparing the CON to the DS conditions. For *EglNAC62*, no significant difference was detected among the genotypes evaluated (Fig.4.3).

The transcript accumulation of the *EglNAC47* gene increased under IDS condition in genotypes GDT1 and GDS2, showing significant differences between both genotypes. Compared with that in the CON group, the transcript abundance in the GDT decreased significantly. For the GDC genotype, an increase in the transcription accumulation levels with significant differences was detected under the SDS condition (Fig.4.3), and a similar expression pattern was detected via RNA-Seq analysis.

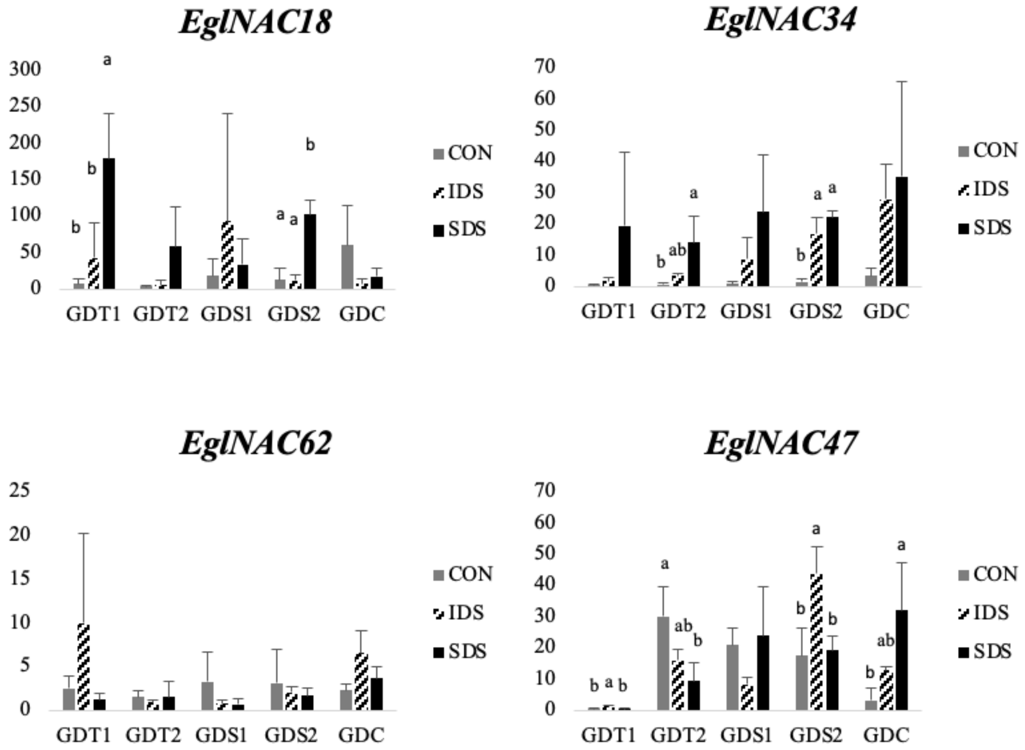

Fig.4.3 Expression patterns of *EglNAC* genes in the leaves of five genotypes of *E. globulus* under drought stress treatment (CON control, IDS intermediate drought stress and SDS severe drought stress). The expression levels were determined by qRT–PCR using the *EF1α* and *EIF4* genes as reference genes. Letters on top of the bars (mean + SE) indicate statistically significant differences between treatments (a and b) according to Tukey's test ($p \leq 0.05$).

Under cold stress treatment, the transcript accumulation of the *EglNAC18* gene decreased in all genotypes when comparing the NA condition with the CABF and CAAF conditions. Significant differences in the expression level of this gene were detected under CAAF conditions for the susceptible genotypes (GCS1 and GDS2) compared to the control condition (Fig.4.4).

For the GDC genotype, a decrease in transcription accumulation with no significant difference was detected, and RNA-Seq analysis revealed a similar expression pattern between the NA and CAAF conditions. However, when comparing NA to CABF conditions, an increase in transcription accumulation was observed via RNA-Seq analysis but not via qRT–PCR analysis.

For *EglNAC34*, transcript abundance increased in all the genotypes when comparing the CABF and CAAF treatment conditions to the NA treatment, and a significant difference in the expression level was observed for the tolerant and susceptible genotypes compared to the NA treatment condition (Fig4.4). For the GDC genotype, a significant increase in transcription accumulation at these conditions was detected, and a similar expression pattern was detected via RNA-Seq analysis.

The transcript accumulation of the *EglNAC62* gene increased under CABF and CAAF conditions only in genotypes GCT1 and GCS2 compared with the NA condition. For GCT2, a decrease in the expression level was observed, while for GCS1, a significant increase was observed between the NA condition and CAAF (Fig.4.4). For the GDC genotype, a significant decrease in transcription accumulation was detected under the treatment conditions compared with the control condition. These results do not correlate with what is obtained by RNA-seq analysis.

For *EglNAC47*, transcript abundance decreased in all genotypes, with significant differences in genotypes GCT1, GCT2 and GDS1 under CABF and CAAF conditions compared to those under NA condition (Fig.4.4). Similar results were observed for the GCS1 genotype; the transcript abundance decreased under treatment conditions compared with that in the control, but no significant difference was observed, and a comparable expression pattern was also obtained via the *in silico* approach.

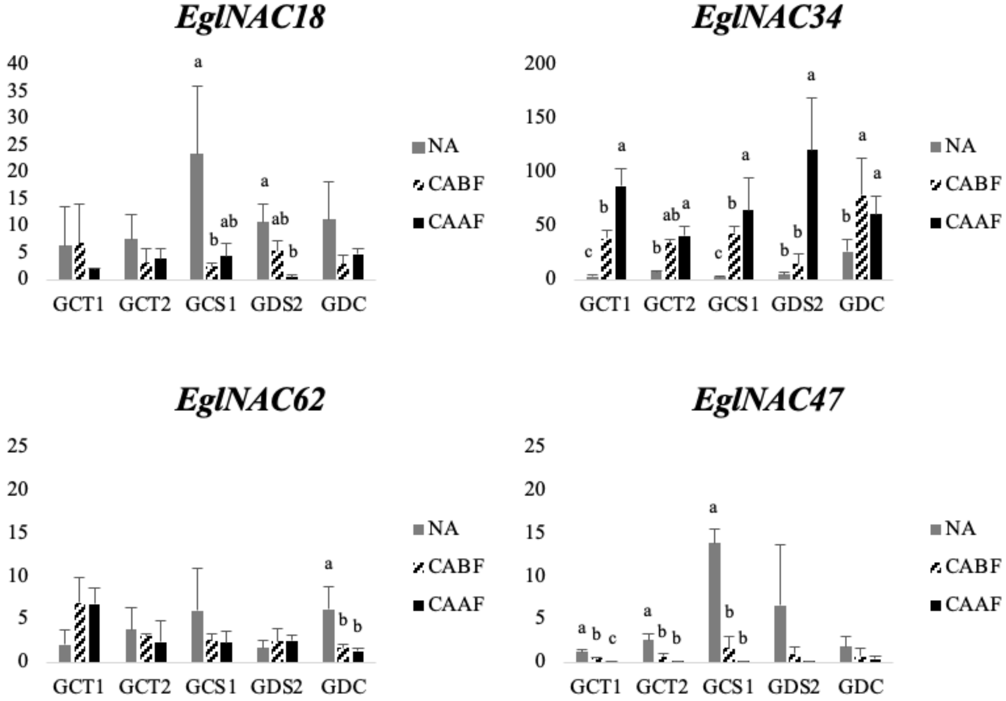

Fig.4.4 Expression patterns of *EglNAC* genes in the leaves of five genotypes of *E. globulus* under drought stress treatment (NA, nonacclimated; CABF, cold-acclimated before frost; CAAF, cold-acclimated after frost). The expression levels were determined by qRT–PCR using the *EF1α* and *EIF4* genes as reference genes. Letters on top of the bars (mean + SE) indicate statistically significant differences between treatments (a, b and c) according to Tukey's test (p ≤ 0.05).

4.4.5 Characterization and network interaction analysis of EglNAC34

To understand the potential role of NAC proteins in the response to abiotic stress in *E. globulus*, the gene with the highest expression response under both stress treatments was selected (EglNAC34). Multiple alignments were generated using Geneious Prime® 2024.0.2 software. Four sequences of NAC proteins present in other species were used. EgloNAC34 showed 97.2% identity with *E. grandis* (Eucgr. F01093), 66.7% sequence identity with *Vitis vinefera* (RVX15765), 61.8% sequence identity with *Populus thricocarpa* (NC_037295), and 62.3% sequence identity with *A. thaliana* (AT4G274410). The comparison of the full length of the EgloNAC34 protein and the domain sequences of NAC proteins present in other species indicated a high degree of similarity in the conserved NAC domain located in the N-terminal region (Fig.4.5).

```
     EglNAC34   MGVPGTDPLAQLSLPPGFRFYPTDEELLVQYLCRKVAGYEFSLEIIGEIDLYKFDPWVLP
Eucgr.F01093   MGVPGTDPLAQLSLPPGFRFYPTDEELLVQYLCRKVAGYEFSLEIIGEIDLYKFDPWVLP
    RVX15765   -------MTAELQLPPGFRFHPTDEELVMHYLCRKCASQSIAVPIIAEIDLYKFDPWQLP
   NC_037295   MGLQETDPLAQLSLPPGFRFHPTDEELLVQYLCKKVAGHHFSLQIIGEIDLYKFDPWFLP
  AT4G274410   MGVREKDPLAQLSLPPGFRFYPTDEELLVQYLCRKVAGYHFSLQVIGDIDLYKFDPWDLP

    EglNAC34   S-----------------KAMFGEKEWYFFSPRDRKYPNGSRPNRVAGTGYWKATGTDKV
Eucgr.F01093   S-----------------KAMFGEKEWYFFSPRDRKYPNGSRPNRVAGTGYWKATGTDKV
    RVX15765   E-----------------MALYGEKEWYFFSPRDRKYPNGSRPNRAAGTGYWKATGADKP
   NC_037295   G-----------------KAIFGEKEWYFFSPRDRKYPNGSRPNRVAGSGYWKATGTDKV
  AT4G274410   SKQTCFTFVGEYNCNYLGKALFGEKEWYFFSPRDRKYPNGSRPNRVAGSGYWKATGTDKI

    EglNAC34   ISTEGRQVGIKKALVFYIGKAPKGNKTNWIMHEYRLLA----STRKSGSTKLDEWVLCRI
Eucgr.F01093   ISTEGRQVGIKKALVFYIGKAPKGNKTNWIMHEYRLLA----STCKSGSTKLDEWVLCRI
    RVX15765   IG-HPKPVGIKKALVFYAGKAPRGEKTNWIMHEYRLADVDRSARRKNNSLRLDDWVLCRI
   NC_037295   ITTEGRKVGIKKALVFYVGKAPKGTKTNWIMHEYRLLE----SSRKSGSTKLDEWVLCRI
  AT4G274410   ITADGRRVGIKKALVFYAGKAPKGTKTNWIMHEYRLIE----HSRSHGSSKLDDWVLCRI

    EglNAC34   YKKSSGGQKPSTSTSSS--KEQ-----SHG--------------------SHS-PSSSLD
Eucgr.F01093   YKKSSGGQKPSTSTSSS--KEQ-----SHG--------------------SHS-PSSSLD
    RVX15765   YNKKGIVEKQHTAARKS--DCSDVEDQKPGPLALSRKVGAMPPPPPPSSSTAPTATAALD
   NC_037295   YKKNSSAAQKSMSSVSS--KEY----STNG--------------------SCSSSSSHLE
  AT4G274410   YKKTSGSQRQAVTPVQACREEH----STNG--------------------SSSSSSSQLD

    EglNAC34   NML-----ETLPEINDQRFALPHANSLRIAQHQQQQQQQQQQQRQKPSL-QALMGSGSFD
Eucgr.F01093   NML-----DTLPEINDRRFALPHANSLRIAQQQQQQQQQRQRHEQKPSL-QALMGSGSFD
    RVX15765   DLVYFDSSDSVPRLHTDSSCSEHVVSPEFTCEREVQSEPKWKEWENPMDFSYNYMDATVD
   NC_037295   DVL-----DSLTEIDDRLFALPRTNSLKQMQ-----------HEEKINL--ANLGSGSFD
  AT4G274410   DVL-----DSFPEIKDQSFNLPRMNSLRT------------------------ILNGNFD

    EglNAC34   WATLASLNLEPQQRLVPVSQPQPQGLVNY---GGNDVFVPQMNRLDGLQ--RQQLGGSAN
Eucgr.F01093   WATLASLNLEPQQQLVPVSQPQPQGLVNY---GGNDVFVPQMNRLDGLQ--RQQLGGSAN
    RVX15765   NAFLSQF---PNNQMSPLQ-----------------------------------------
   NC_037295   WATLAGLNSLPE--LLQ---T--QPGANYSNTNVNGVHVPSMPPLCHADSSTGRMGTSVE
  AT4G274410   WASLAGLNPIPE--LAPT-----NGLPSY---GGYDAFR------------------AAE

    EglNAC34   EEVQSGIRTQPP-APAVGLYQPSSTGFAQNLANS-LDPYGFRYSAH-NVGLGFRQ
Eucgr.F01093   EEVQSGIRTQPPPAAAAGLYQPSSTGFAQNLANS-LDPYGFRYSAH-NVGLGFRQ
    RVX15765   ----------------------------DMFMYL-----------------QKPF
   NC_037295   EEVQSGVRTQLR-DGNSGAFQQNSGVMTPNFSSTLLDPYELRYSTQPGSGYGFRQ
  AT4G274410   GEAESGHVN----------RQQNSSGLTQSFGYS-SSG--FGVS---GQTFEFRQ
```

Fig.4.5 Conservation of the NAC domain and protein sequence alignment of the EglNAC domain from homologous sequences between *Eucalyptus globulus* (EglNAC34), *Eucalyptus grandis* (Eucgr. F01093), *Vitis vinefera* (RVX15765), *Populus thricocarpa* (NC_037295) and *Arabidopsis thaliana* (AT4G274410). The five subdomains of the NAC domain are denoted by lines designated with the letters A–E, and the red box indicates the nuclear positioning signal (NLS).

Network interaction analysis of the EgloNAC34 amino acid sequence was conducted via the *A. thaliana* database in STRING. This protein mainly has several functional roles in various biological processes related to abiotic stress in plants, including interactions with zinc-finger homeodomain protein (ZHD11), low-temperature-induced 65 kDa protein (LTI65), low-temperature-induced 78 kDa protein (RD29A), dehydration-responsive element-binding protein 2A (DREB2A)*,* 9-cis-epoxycarotenoid dioxygenase, chloroplastic (NCED3), ABSCISIC ACID-INSENSITIVE 5-like protein 7 (ABF4), transcription factor MYC2, transcriptional activator (MYC2) and chaperone protein ClpD (CLPD) (Fig.4.6). To validate the role of the target genes in response to abiotic stress, four genes, *ZHD11, LTI65, NCED3*, and *CLPD,* were selected. The expression levels under both drought and cold treatments were assessed using qRT–PCR.

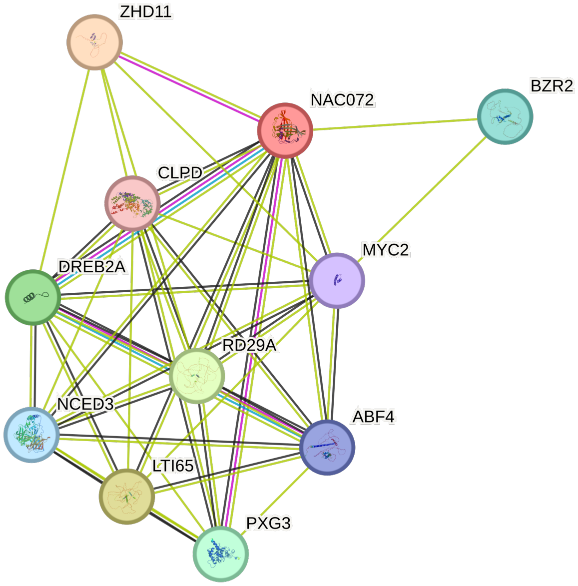

Fig.4.6 Protein–protein interaction networks for the AT4G274410 (AtNAC072) transcription factor of Arabidopsis thaliana homologs to EglNAC34 from *Eucalyptus globulus*.

4.4.6 Expression analysis of target genes under drought and cold stress

The same genotype used in the EglNAC analysis was employed for the target gene assays of EgloNAC34. In the drought treatment group, the transcript accumulation of the *ZHD11* gene increased under SD conditions in the GDT1, GDT2, and GDS2 genotypes, with significant differences observed between GDT1 and GDS2. In GDS1, an increase in transcript abundance was observed compared to the IDS condition under the CON condition. The transcript accumulation of the *LTI65* gene increased in all genotypes when comparing SDS to CON conditions, with significant differences in expression levels observed only in the tolerant genotypes GDT1 and GDT2. For *NCED3*, transcript abundance increased in all evaluated genotypes, with significant differences observed in genotypes GDT1, GDT2, and GDS1 under SDS conditions compared to CON. The *CLPD* gene exhibited increased transcript abundance in genotypes GDT1, GDT2, and GDS2 compared to CON under IDS and SDS conditions, but no significant differences were observed. For GDS1, a susceptible genotype, transcript abundance decreased under treatment conditions compared to the control, with significant differences observed between CON and ISD conditions (Fig.4.7).

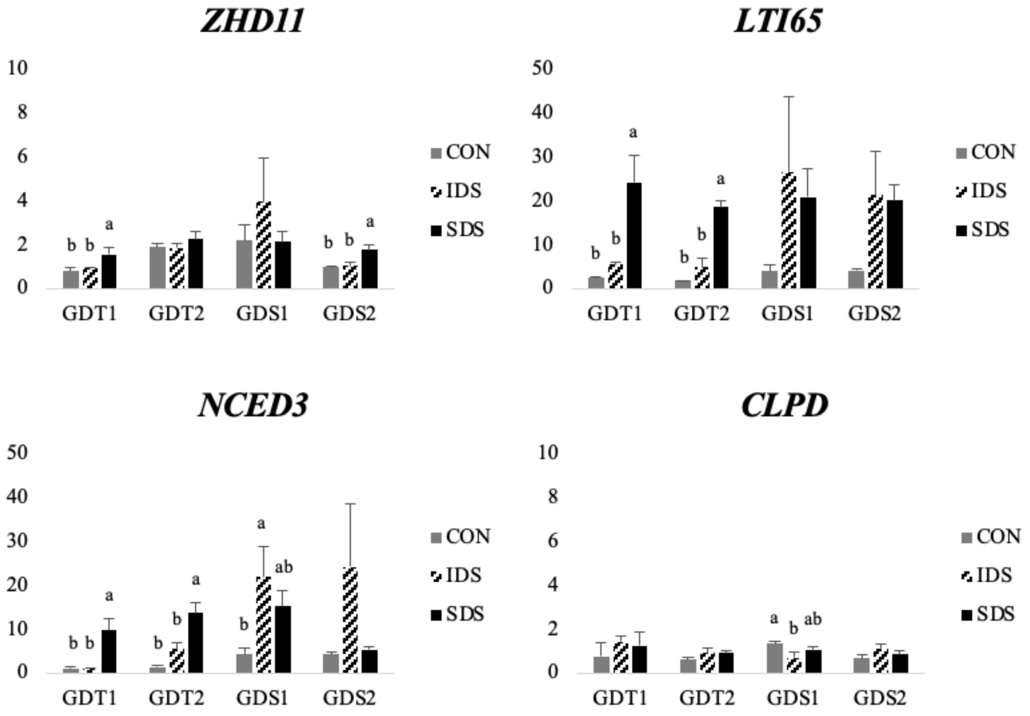

Fig.4. 7 Expression patterns of target genes in the leaves of four genotypes of *E. globulus* under drought stress treatment (CON control, IDS intermediate drought stress and SDS severe drought stress). The expression levels were determined by qRT–PCR using the *EF1α* and *EIF4* genes as reference genes. Letters on top of the bars (mean + SE) indicate statistically significant differences between treatments (a and b) according to Tukey's test ($p \leq 0.05$).

Under cold treatment, the transcript accumulation of *ZHD11* decreased in all the genotypes compared to that under NA condition with CABF or CAAF. Significant differences in the expression level of this gene were detected among the GCT1, GCS1, and GCS2 genotypes. For *LTI65*, an increase in transcript accumulation was observed in genotypes GCT1, GCT2, and GDS1, with significant differences shown for genotype GCT2 at CAAF compared to the NA. Decreases in transcript levels were detected for the GCS2 genotype, but they were not significant. For *NCED3*, transcript abundance decreased in both tolerant genotypes (GCT1 and GCT2) but did not significantly differ, while for the susceptible genotypes (GCS1 and GSC2), an increase in transcript levels was observed, with only GSC2 showing significant differences compared to CABF to CAAF. Finally, for *CLPD,* an increase in transcript accumulation was observed in the tolerant genotypes GCT1 and GCT2, with significant differences observed between GCT1 and NA-CABF. The susceptible genotypes showed a decrease in transcript accumulation, but no significant differences were detected (Fig.4.8).

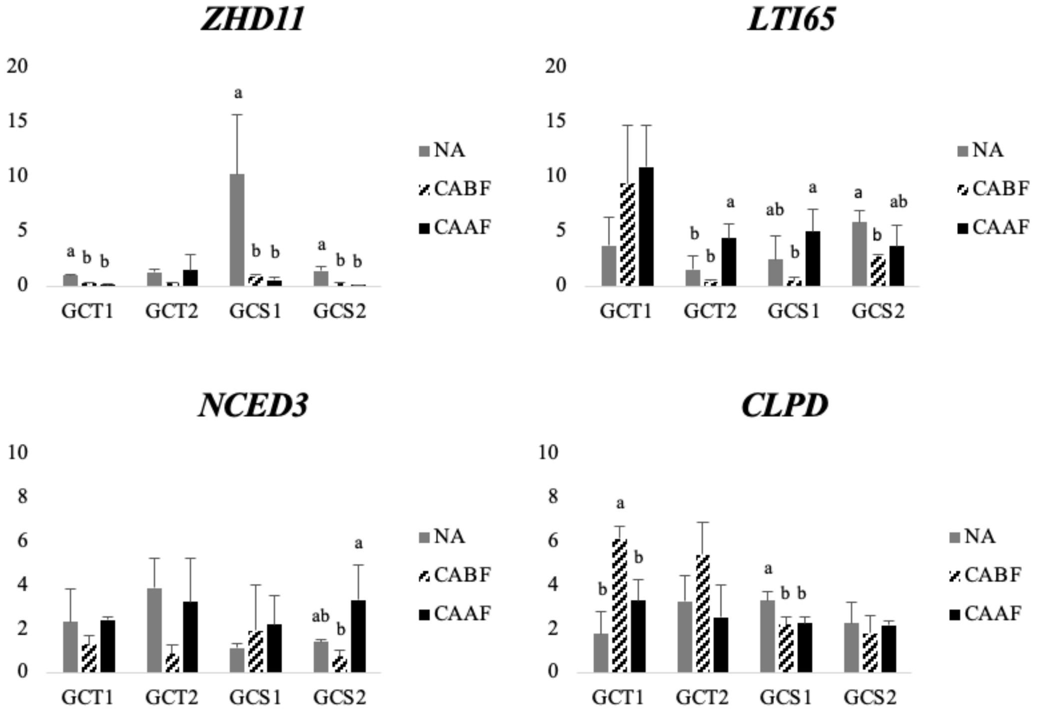

Fig.4.8 Expression patterns of target genes in the leaves of four genotypes of **E. globulus** under drought stress treatment (NA, nonacclimated; CABF, cold-acclimated before frost; CAAF, cold-acclimated after frost). The expression levels were determined by qRT–PCR using the *EF1α* and *EIF4* genes as reference genes. Letters on top of the bars (mean + SE) indicate statistically significant differences between treatments (a and b) according to Tukey's test ($p \leq 0.05$).

4.5 Discussion

NAC proteins represent one of the largest and most plant-specific families of transcription factors (Olsen et al. 2005) and are present across a diverse range of species. This TF family has been implicated in the regulation of a wide variety of processes, such as seed and embryo development, shoot apical meristem formation, fiber development, leaf senescence, cell division, and response to biotic and abiotic stress (Nakashima et al. 2012; Singh et al. 2017). In this study, a total of 101 *EglNAC* genes were identified in the leaf transcriptome of *E. globulus* under drought and cold stress. Based on the phylogenetic analysis of the NAC, the domains were classified into two large groups (I and II) subdivided into 17 subfamilies. Group I is divided into 14 subfamilies, and group II is divided into 3 subfamilies. According to the results of *E. grandis* Hussey et al. (2015), all subfamilies except (Nakashima et al. 2012; Singh et al. 2017), subfamily VIII, were represented by at least one member. The biological functions of members within the subfamilies were examined based on the available literature. Members within the same subfamily exhibited comparable biological processes (Munir et al. 2020); for example, most of the members of subfamily IV, containing the largest number of members identified, presented homology with the NAC transcription factor-like 9, NTL9 (AT4G35580) described for *A. thaliana,* which has recently been described as being involved in the regulation of cambial activity (Sugimoto et al. 2022), suggesting a potential role in the development of secondary growth for members of this subfamily. In relation to the response to abiotic stress, members of subfamilies IVd and Vb showed homology with ANAC042 (AT2G43000) and ANAC002 (AT1G01720), respectively, which have been previously associated with responses to heat, high salinity and drought stress in *A. thaliana* (Wu et al. 2009; Shahnejat-Bushehri et al. 2012; Ebrahimian-Motlagh et al. 2017; Alshareef et al. 2022). To perform a structural analysis of the EglNAC members, we analyzed the conserved motifs in relation to phylogenetic relationships, and the results showed that NAC proteins sharing similar structural motifs were clustered within the same subfamily, suggesting evolutionary conservation (Fig. S4.1). Despite this structural similarity among proteins within subfamilies, molecular characterization revealed diversity in the length, molecular weight (MW), and isoelectric point (pI) of the NAC genes (Table S4.1).

Members of the NAC family of transcription factors have been widely studied in response to diverse stress conditions, such as heat, flooding, salinity, cold, and drought (Han et al. 2023). The precise role of each member in response to environmental signals is still under investigation, with only a limited number of studies conducted in woody species. In this study, RNA-seq analysis of E. globulus genotypes under either drought or cold stress conditions revealed that a total of six and thirteen NAC genes, respectively, were upregulated (>twofold change). Seven of these genes are members of subfamily Vb (*EglNAC34, 43, 69, 70, 73, 74,* and *75)*, and previous studies revealed that NAC proteins in this subfamily play an important role in the response to abiotic stress. For example, members of the same subfamily Vb in *A. thaliana* (called the ATAF group) have been mostly reported to respond to abiotic stress compared to other NAC subfamilies (Ooka 2003; Christianson et al. 2010; Nuruzzaman et al. 2013). Among the members of subfamily Vb, *EglNAC34* demonstrated significantly higher expression levels under both evaluated abiotic stresses. Under drought stress, the *EglNAC34* gene was significantly upregulated in two of the five genotypes evaluated under SDS conditions. Similar results have been reported in *E. camaldulensis*, where plants were subjected to water stress by maintaining soil moisture at 30% of field capacity for two months. Differential gene expression analysis revealed that Eucgr. F01093, which is homologous to *EglNAC34*, was among the genes with the most significant differential expression compared with the control (Thumma et al. 2012). In Arabidopsis, the gene *AtNAC072 (*AT4G274410), a putative ortholog of *EglNAC34,* has been shown to be upregulated in response to salt and drought treatments (Tran et al. 2004). Huang et al. (2015) demonstrated that overexpressing the

TaNAC29 gene, an ortholog of *AtNAC072* from wheat, in transgenic *A. thaliana* lines resulted in enhanced drought tolerance. The transcript accumulation of *EglNAC34* reached its highest level under cold stress treatment, was upregulated in all the genotypes evaluated and significantly different between the NA treatment and the CABF or CAAF treatments, suggesting that EglNAC34 plays an important role in the response to cold stress. These results are in accordance with the study described by Miao et al. (2021), who reported significant increases in the expression levels of thirteen NAC genes from *Capsicum annuum L*. after exposure to 4°C for 72 hours. In their study, *NAC72*, a putative ortholog of *EglNAC34* (with 54% id), exhibited particularly high transcript levels under cold stress conditions. Recently, Huang et al. (2024) investigated the response of *PwNAC31,* a homolog of *AtNAC072* in Arabidopsis, to drought stress. *Picea wilsonii seedlings* were subjected to drought conditions by being placed on absorbent paper without water. This study revealed a significant upregulation of *PwNAC31* expression in response to drought stress.

Sequence analysis of the predicted EglNAC34 protein revealed a conserved N-terminal region consisting of five subdomains (A–E) and a highly variable C-terminal region. Additionally, a nuclear localization signal (NLS) was identified within the C subdomain (Fig.4.5). This NLS plays a crucial role in facilitating the translocation of the protein from the cytoplasmic matrix into the nucleus, which represents its active state for DNA binding (Bian et al. 2020). The functional *relationship of EglNAC34 with its orthologs in A. thaliana* according to the STRING database revealed its role in the response to abiotic stress. Four target genes were selected for gene expression analyses via qRT–PCR. Under drought stress, the transcription level of the gene ZHD11 increased under SD condition in three of the four genotypes evaluated (GDT1, GDT2, and GDS2). These results are similar to those described in barley, where the overexpression of the *HvZFHD1* gene in *A. thaliana,* which acts as a transcriptional activator of *ERD1,* has been reported in response to drought and salinity stress (Abu-Romman and Al-Hadid 2017). The transcript accumulation of the target gene *LTI65* increased in all genotypes evaluated under both conditions, and these results are similar to those described by several authors who reported that this gene is involved in the response to low temperatures, drought treatment and the exogenous addition of abscisic acid (ABA) in several plant species (Narusaka et al. 2003; Msanne et al. 2011; Jia et al. 2012). The transcript abundance of *ZHD11* increased in all evaluated genotypes only in plants of *E. globulus* subjected to drought stress. These results are consistent with those described by Tong et al. (2017), who reported that the overexpression of *TaNCED* from wheat increased drought tolerance in Arabidopsis transgenic lines. This increase was attributed to elevated levels of endogenous ABA and free proline, resulting in reduced water loss and stomatal conductance. Finally, for the target gene *CLPD,* a significant decrease in transcript abundance under both stress conditions was observed in one susceptible genotype. These results contrast with those obtained by Huang et al. (2024), who reported that the heterologous expression of *PwNAC31* in Arabidopsis mutant lines restored the hypersensitive phenotype of *AtNAC072* under drought stress through activation of the *CLPD* (called *ERD1*) gene.

NAC proteins constitute a prominent family of transcription factors implicated in diverse abiotic stress responses across plant species, including *E. globulus.* A recent study indicated that EglNAC TFs in the same subfamilies generally share structural and functional characteristics. EglNAC34 plays an important role in the response to drought and cold stresses in *E. globulus*. Furthermore, analyzing the interaction network of *EglNAC34* enhances our understanding of how this gene could respond to abiotic stress.

4.6 Conclusion

In this study, 101 NAC TF genes related to the response to drought and cold stress were identified in the transcriptome of *E. globulus*. *In silico* analysis revealed that 13 genes were upregulated under abiotic stress conditions. The relative expression of *EglNAC34* increased in response to both stresses, suggesting that this gene plays a positive role in abiotic stress responses. The identification of the structure, functional characterization and interaction networks of EglNAC34 provides a basis for understanding the molecular mechanisms underlying the adaptation of *E. globulus* to abiotic stress conditions, and we propose that EglNAC34 is a candidate gene for future molecular breeding studies in *E. globulus*.

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4.8 Supplementary material

Table S4.1 Characteristics of 101 NAC proteins identified in *E. globulus*

Gene name *E. globulus*	**CDS length (pb)**	**Aa**	**Mw (kDa)**	**pI**	**Group**	**AtNAC homolog**
EglNAC1	927	309	34.976	6,16	IVa	AT4G35580
EglNAC2	927	307	34.867	5,16	IVa	AT4G35580
EglNAC3	1157	316	35.910	5,15	IVa	AT4G35580
EglNAC4	1123	373	41.608	4,92	XI	AT1G65910
EglNAC5	1794	597	66.711	4,9	IIIa/b	AT4G35580
EglNAC6	939	312	35.563	6,84	IVd	AT2G43000
EglNAC7	1158	385	42.941	8,24	Va	AT3G04070
EglNAC8	1233	410	45.597	5,36	Ia	AT5G18270
EglNAC9	801	266	30.797	6,73	IVc	AT5G22380
EglNAC10	801	177	20.669	9,51	IVc	AT5G22380
EglNAC11	795	264	30.199	5,66	IVc	AT5G22380
EglNAC12	1144	268	30.031	6,01	IVc	AT5G22380
EglNAC13	1047	348	40.537	6,02	Ic	AT1G12260
EglNAC14	1086	361	40.637	7,1	Ia	AT5G61430
EglNAC15	1074	357	39.975	6,67	Ia	AT3G18400
EglNAC16	839	279	31.695	9,07	Va	AT1G61110
EglNAC17	768	255	29.437	9,38	Va	AT1G69490
EglNAC18	1386	251	28.554	9,37	VIa	AT5G13180
EglNAC19	1116	372	41.397	7,16	IIIa/b	AT3G44290
EglNAC20	1452	483	53.606	6,38	II	AT4G29230
EglNAC21	981	326	36.265	8,92	Ia	AT2G24430
EglNAC22	888	296	33.841	8,71	II	AT1G28470
EglNAC23	903	300	33.948	9,04	II	AT1G28470
EglNAC24	1152	383	42.867	7,24	Ic	AT2G46770
EglNAC25	1011	336	39.116	6,67	Ic	AT1G12260
EglNAC26	1074	357	39.995	4,8	IIIc	AT4G17980
EglNAC27	1041	346	39.525	5,58	IIIc	AT4G17980
EglNAC28	957	318	35.935	5,17	VII	AT5G46590
EglNAC29	748	249	28.303	8,56	VII	AT3G10480
EglNAC30	1200	399	44.968	6,73	Ic	AT2G46770
EglNAC31	1953	650	71.909	4,97	IIIa/b	AT1G33060
EglNAC32	942	313	34.921	8,14	II	AT4G28500
EglNAC33	1101	366	40.409	8,8	Va	AT3G15510

EglNAC34	1059	352	39.243	9,39	Vb	AT4G27410
EglNAC35	1005	334	37.304	7,56	Ib	AT1G56010
EglNAC36	951	316	35.980	7,21	IVd	AT3G12910
EglNAC37	1206	401	44.366	5,37	XI	AT5G07680
EglNAC38	1206	401	44.367	5,37	XI	AT5G61430
EglNAC39	963	320	36.856	6,01	Ic	AT1G71930
EglNAC40	1701	566	63.345	4,78	IIIa/b	AT1G34190
EglNAC41	1855	618	70.766	5,22	IIIc	AT1G65910
EglNAC42	1152	383	43.622	6,11	Ia	AT1G76420
EglNAC43	903	300	33.589	6,46	Vb	AT1G01720
EglNAC44	1299	432	48.530	6,77	IVb	AT2G02450
EglNAC45	1704	567	63.881	5,01	IVa	AT4G17980
EglNAC46	1334	444	50.445	7	IVa	AT4G35580
EglNAC47	1587	528	60.498	5,99	IVa	AT5G22290
EglNAC48	1617	538	60.962	6,12	IVa	AT4G35580
EglNAC49	1776	591	67.136	5,04	IVa	AT4G35580
EglNAC50	876	291	32.649	5,25	IVa	AT4G35580
EglNAC51	645	214	24.793	7,16	IVa	AT1G33060
EglNAC52	831	276	31.472	5,52	IVa	AT4G35580
EglNAC53	762	253	28.753	5,66	IVa	AT4G35580
EglNAC54	666	221	24.832	5,48	IVa	AT4G35580
EglNAC55	998	249	27.962	7,66	IVa	AT4G35580
EglNAC56	1255	151	17.563	5,96	IVa	AT1G33060
EglNAC57	2396	660	74.518	5,8	IVa	AT1G33060
EglNAC58	1578	525	59.373	6,19	IVa	AT1G33060
EglNAC59	426	142	16.677	8,67	IVa	AT4G35580
EglNAC60	1473	487	55.510	5,82	IVa	AT4G35580
EglNAC61	1290	429	48.205	5,17	II	AT1G25580
EglNAC62	870	289	33.233	8,98	Va	AT1G69490
EglNAC63	1239	412	46.507	6,62	IIIa/b	AT3G10490
EglNAC64	741	246	28.239	4,76	IIIc	AT3G17730
EglNAC65	729	242	26.628	9,98	VIb	AT5G13180
EglNAC66	780	259	29,346	9,49	VIa	AT5G13180
EglNAC67	867	288	33.251	7,65	Vb	AT1G01720
EglNAC68	882	293	33.620	6,51	Vb	AT1G01720
EglNAC69	885	294	33.361	6,26	Vb	AT1G01720
EglNAC70	882	288	33.273	5,74	Vb	AT1G01720
EglNAC71	885	294	33.759	6,46	Vb	AT1G01720
EglNAC72	861	286	32.708	6,25	Vb	AT1G01720

EglNAC73	882	293	34.014	6,16	Vb	AT1G01720
EglNAC74	882	293	33.415	6,15	Vb	AT1G01720
EglNAC75	1543	359	40.831	6,23	Vb	AT1G01720
EglNAC76	696	231	26.436	7,63	Vb	AT1G01720
EglNAC77	732	204	23.332	4,92	VIc	AT5G64530
EglNAC78	1890	630	69.475	4,66	IIIa/b	AT5G64060
EglNAC79	906	301	34.800	7,08	IVb	AT2G17040
EglNAC80	918	305	34.956	6,62	Ia	AT5G61430
EglNAC81	1059	352	40.492	5,23	Ic	AT2G18060
EglNAC82	958	141	16.770	9,23	IVa	AT5G13180
EglNAC83	553	184	22.053	8,26	IVa	AT4G35580
EglNAC84	579	192	21.859	4,92	VIc	AT5G64530
EglNAC85	660	219	25.334	9,05	IVc	AT5G22380
EglNAC86	720	239	27.020	6,35	IVc	AT5G22380
EglNAC87	1260	419	46.952	5,47	IIIa/b	AT2G27300
EglNAC88	1617	538	60.436	4,66	IIIa/b	AT5G04410
EglNAC89	1292	423	48.236	5,61	II	AT3G01600
EglNAC90	891	296	33.600	9,29	II	AT4G28500
EglNAC91	894	297	34.183	7,57	IVd	AT2G43000
EglNAC92	990	329	37.530	8,26	Va	AT3G04070
EglNAC93	1098	356	40.839	5,79	Ia	AT5G18270
EglNAC94	736	217	23.940	7,69	VIa	AT2G33480
EglNAC95	927	309	34.603	7,65	Ia	AT3G18400
EglNAC96	708	235	27.333	5,08	IIIc	AT3G17730
EglNAC97	3973	283	31.868	6,05	Ib	AT4G28530
EglNAC98	882	293	33.111	5,04	IVa	AT4G35580
EglNAC99	810	271	30.773	5,11	IVa	AT4G35580
EglNAC100	813	271	31.115	4,98	IVa	AT4G35580
EglNAC101	471	157	18.100	5,97	IVa	AT4G35580

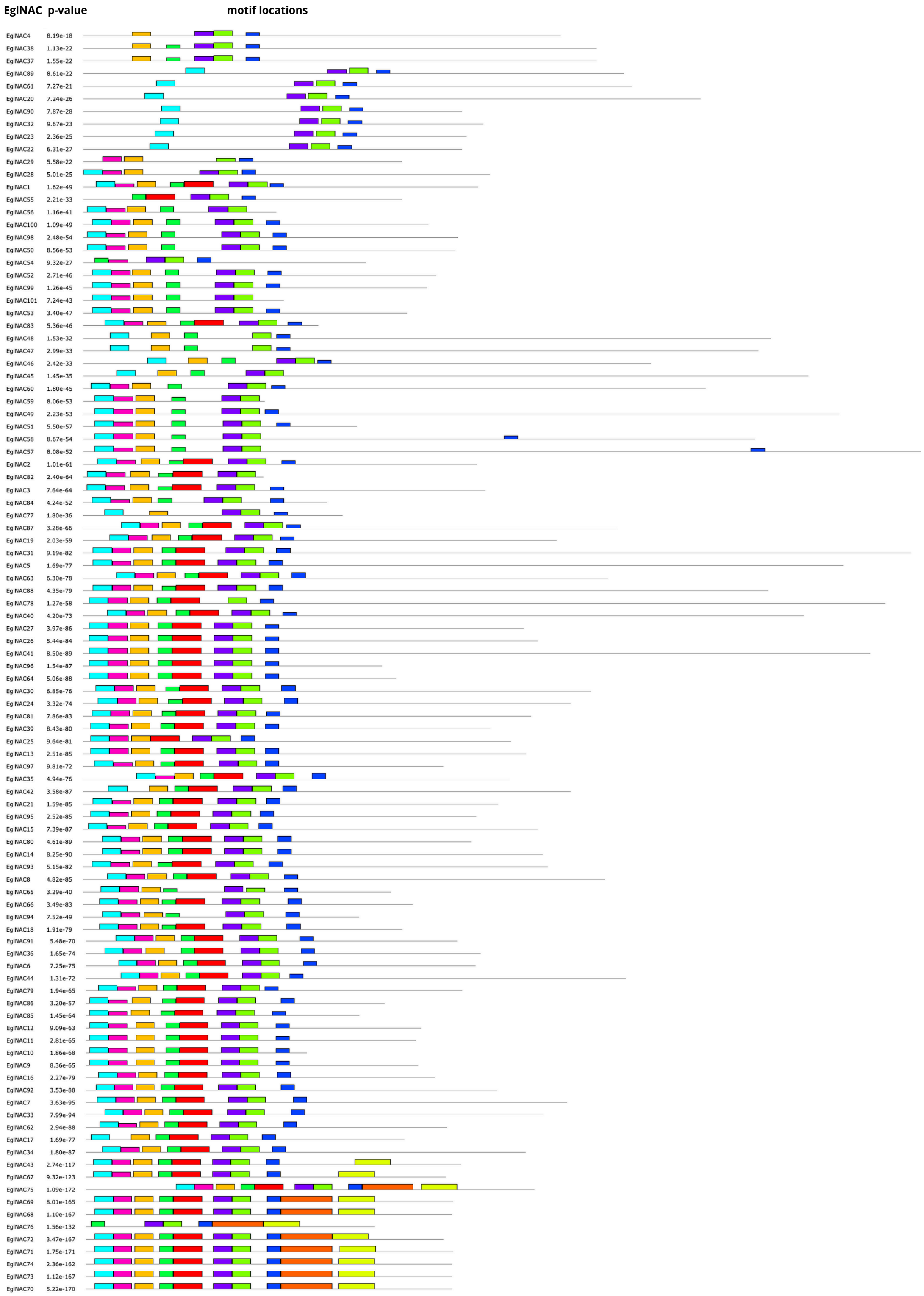

Fig. S4.1 Conserved motifs distribution of 101 putative NAC protein identified in *E.*

globulus using MEME 5.5.5. The motifs were numbered 1–10 and are displayed in different color.

Table S4.2 Phylogenetic classification of NAC members.

NAC in *Eucalytus grandis* by Hussey et al. (2015)			***Eucalyptus globulus***
Gene name	gene simbol	subfamily	Gene name
Eucgr.A00357	EgrNAC1	IVa	EglNAC1
Eucgr.A00368	EgrNAC9	IVa	EglNAC2
Eucgr.A00435	EgrNAC13	IVa	EglNAC3
Eucgr.A00494	EgrNAC15	XI	EglNAC4
Eucgr.A00969	EgrNAC16	IIIa/b	EglNAC5
Eucgr.A02028	EgrNAC18	IVd	EglNAC6
Eucgr.A02070	EgrNAC19	Va	EglNAC7
Eucgr.A02074	EgrNAC20	Ia	EglNAC8
Eucgr.A02635	EgrNAC21	IVc	EglNAC9
Eucgr.A02637	EgrNAC23	IVc	EglNAC10
Eucgr.A02638	EgrNAC24	IVc	EglNAC11
Eucgr.A02639	EgrNAC25	IVc	EglNAC12
Eucgr.A02887	EgrNAC26	Ic	EglNAC13
Eucgr.B00529	EgrNAC27	Ia	EglNAC14
Eucgr.B00724	EgrNAC28	Ia	EglNAC15
Eucgr.B02485	EgrNAC32	Va	EglNAC16
Eucgr.B03208	EgrNAC33	Va	EglNAC17
Eucgr.B03537	EgrNAC35	VIa	EglNAC18
Eucgr.B03823	EgrNAC39	IIIa/b	EglNAC19
Eucgr.C00958	EgrNAC40	II	EglNAC20
Eucgr.C02105	EgrNAC42	Ia	EglNAC21
Eucgr.D00594	EgrNAC46	II	EglNAC22
Eucgr.D00595	EgrNAC47	II	EglNAC23
Eucgr.D01671	EgrNAC49	Ic	EglNAC24
Eucgr.D02027	EgrNAC50	Ic	EglNAC25
Eucgr.D02182	EgrNAC51	IIIc	EglNAC26
Eucgr.E00298	EgrNAC52	IIIc	EglNAC27
Eucgr.E00542	EgrNAC54	VII	EglNAC28
Eucgr.E00551	EgrNAC57	VII	EglNAC29

Eucgr.E01053	EgrNAC61	Ic	EglNAC30
Eucgr.E01095	EgrNAC62	IIIa/b	EglNAC31
Eucgr.E03226	EgrNAC64	II	EglNAC32
Eucgr.F01091	EgrNAC65	Va	EglNAC33
Eucgr.F01093	EgrNAC66	Vb	EglNAC34
Eucgr.F01449	EgrNAC68	Ib	EglNAC35
Eucgr.F01463	EgrNAC69	IVd	EglNAC36
Eucgr.F01538	EgrNAC73	XI	EglNAC37
Eucgr.F01539	EgrNAC74	XI	EglNAC38
Eucgr.F02615	EgrNAC75	Ic	EglNAC39
Eucgr.F02771	EgrNAC76	IIIa/b	EglNAC40
Eucgr.F02910	EgrNAC77	IIIc	EglNAC41
Eucgr.F03588	EgrNAC78	Ia	EglNAC42
Eucgr.F04341	EgrNAC82	Vb	EglNAC43
Eucgr.G00054	EgrNAC83	IVb	EglNAC44
Eucgr.G01047	EgrNAC84	IVa	EglNAC45
Eucgr.G01049	EgrNAC85	IVa	EglNAC46
Eucgr.G01052	EgrNAC86	IVa	EglNAC47
Eucgr.G01053	EgrNAC87	IVa	EglNAC48
Eucgr.G01060	EgrNAC89	IVa	EglNAC49
Eucgr.G01061	EgrNAC90	IVa	EglNAC50
Eucgr.G01066	EgrNAC93	IVa	EglNAC51
Eucgr.G01074	EgrNAC98	IVa	EglNAC52
Eucgr.G01075	EgrNAC99	IVa	EglNAC53
Eucgr.G01077	EgrNAC100	IVa	EglNAC54
Eucgr.G01078	EgrNAC101	IVa	EglNAC55
Eucgr.G01507	EgrNAC105	IVa	EglNAC56
Eucgr.G01548	EgrNAC106	IVa	EglNAC57
Eucgr.G01550	EgrNAC107	IVa	EglNAC58
Eucgr.G01551	EgrNAC108	IVa	EglNAC59
Eucgr.G01758	EgrNAC112	IVa	EglNAC60
Eucgr.G02349	EgrNAC114	II	EglNAC61
Eucgr.G02486	EgrNAC115	Va	EglNAC62
Eucgr.G02740	EgrNAC117	IIIa/b	EglNAC63
Eucgr.H00614	EgrNAC119	IIIc	EglNAC64
Eucgr.H03362	EgrNAC121	VIb	EglNAC65

Eucgr.H03387	EgrNAC122	VIa	EglNAC66
Eucgr.H05089	EgrNAC123	Vb	EglNAC67
Eucgr.I00057	EgrNAC125	Vb	EglNAC68
Eucgr.I00058	EgrNAC126	Vb	EglNAC69
Eucgr.I00059	EgrNAC127	Vb	EglNAC70
Eucgr.I00060	EgrNAC129	Vb	EglNAC71
Eucgr.I00060	EgrNAC130	Vb	EglNAC72
Eucgr.I00099	EgrNAC133	Vb	EglNAC73
Eucgr.I00100	EgrNAC134	Vb	EglNAC74
Eucgr.I00101	EgrNAC135	Vb	EglNAC75
Eucgr.I00102	EgrNAC136	Vb	EglNAC76
Eucgr.I00191	EgrNAC137	VIc	EglNAC77
Eucgr.I00213	EgrNAC140	IIIa/b	EglNAC78
Eucgr.I01494	EgrNAC143	IVb	EglNAC79
Eucgr.I01958	EgrNAC145	Ia	EglNAC80
Eucgr.I02366	EgrNAC146	Ic	EglNAC81
Eucgr.I02571	EgrNAC147	IVa	EglNAC82
Eucgr.I02578	EgrNAC151	IVa	EglNAC83
Eucgr.I02695	EgrNAC152	VIc	EglNAC84
Eucgr.J00509	EgrNAC155	IVc	EglNAC85
Eucgr.J00520	EgrNAC164	IVc	EglNAC86
Eucgr.J00531	EgrNAC166	IIIa/b	EglNAC87
Eucgr.J01038	EgrNAC168	IIIa/b	EglNAC88
Eucgr.J02254	EgrNAC169	II	EglNAC89
Eucgr.K01061	EgrNAC170	II	EglNAC90
Eucgr.K01228	EgrNAC171	IVd	EglNAC91
Eucgr.K01471	EgrNAC172	Va	EglNAC92
Eucgr.K01472	EgrNAC173	Ia	EglNAC93
Eucgr.K02205	EgrNAC175	VIa	EglNAC94
Eucgr.K02225	EgrNAC176	Ia	EglNAC95
Eucgr.K02303	EgrNAC177	IIIc	EglNAC96
Eucgr.K03256	EgrNAC178	Ib	EglNAC97
Eucgr.L01925	EgrNAC191	IVa	EglNAC98
Eucgr.L02201	EgrNAC193	IVa	EglNAC99
Eucgr.L02202	EgrNAC194	IVa	EglNAC100
Eucgr.G01073	EgrNAC213	IVa	EglNAC101

DISCUSION GENERAL

El sector forestal es actualmente un pilar fundamental en el desarrollo económico del país, de ser una actividad secundaria en décadas previas 1970 hoy ha logrado posicionarse, no solo como uno de los sectores más importante en la economía del país, aportando con cerca de 300.000 empleos y representando el 2% del PIB nacional, sino que se ha consolidado como uno de los mayores sectores exportadores, con más de 370 productos que llegan a 126 países (INFOR, 2023).

La creciente demanda de productos madereros en los últimos años, causada por el aumento continuo de la población humana, ha generado un aumento sostenido a nivel global, en la superficie de terreno destinada a plantaciones forestales (FAO, 2015; Chang et al., 2019). En América del Sur, Chile está dentro de los países con mayor superficie de plantaciones, especialmente con *Pinus radiata, Eucalyptus nitens* y *Eucalyptus globulus* (FAO, 2006; INFOR, 2020). Estas especies han sido ampliamente utilizadas a nivel comercial al menos en el último medio siglo, siendo parte de diversos programas de mejoramiento genético conducidos por las principales empresas forestales del país.

Eucalyptus globulus se introdujo al país a finales del siglo XIX y cobró real importancia en la década de los 80, donde pasó a ser un actor primario para las plantas de celulosa debido a su rápido crecimiento, adaptabilidad y propiedades de la madera muy apetecidas por la industria de celulosa (Prado, 2015; Carrillo et al., 2018). Originario del sur de Australia, actualmente cubren una superficie cercana a los 483.000 ha, con una distribución entre las regiones de Valparaíso y Los Lagos (Banco Mundial, 2020).

Las plantaciones forestales de Chile han tenido un crecimiento aproximado de 100.000 hectáreas por año hasta el 2013, donde las plantaciones de *P. radiata* y *Eucalyptus spp.* sustentan el 95% de la economía forestal, llegando a cubrir el 99% de los requerimientos de madera industrial del país. Sin embargo, en los últimos años, la expansión de las plantaciones ha disminuido a debido a la limitación en la disponibilidad de terrenos aptos para plantar, entre estos la fuerte competencia para otros usos como la urbanización y agricultura, además de enfrentar restricciones producto del cambio climático que limita la expansión de las plantaciones forestales a nuevas áreas.

A pesar de su capacidad de adaptación a una amplia gama de sitios cómo se mencionó anteriormente, *E. globulus* es vulnerable a condiciones de estrés hídrico y sensible a las bajas temperaturas lo que se ve reflejado en una disminución en las tasas de crecimiento y además de un aumento en la mortalidad (Davidson et al. 2004; Battaglia y Bruce 2017).

Lo anterior se ha traducido en nuevos desafíos para el sector forestal para mantener la productividad de la actual superficie o bien en el desarrollo de nuevos genotipos que se adapten mejor a las condiciones de estrés biótico y abiótico (Payn et al., 2015; Chang et al., 2019; Banco Mundial, 2020).

Las especies vegetales han desarrollado varios mecanismos de defensa en múltiples niveles para responder a condiciones desfavorables, tales como estrés abiótico. Comprender estos mecanismos son una herramienta fundamental en el desarrollo de nuevas técnicas de mejoramiento genético. La identificación y caracterización de genes involucrados en la respuesta al estrés abiótico es un requisito fundamental para la generación de nuevas técnicas de mejoramiento, tales como modificación genética y edición génica (Anwar and Kim 2020; Yadav et al. 2023). Mas allá de la selección de un solo gen la compresión de ciertos grupos de genes reguladores se ha convertido en el foco de atención actual, como una posible estrategia eficaz para controlar la expresión de muchos genes que respondan a determinado estrés (Wang et al. 2016). Es así como los factores de transcripción (FT), nacen como buenos candidatos para futuras estrategias de mejoramiento, debido a su papel como reguladores de varios genes con respuesta a estrés abiótico (Nievola et al. 2017; Fuertes-Aguilar y Matilla 2024)

Los FT NACs representan una de las familias de factores de transcripción más grandes y específicas de plantas presentes (Wei et al. 2024). Presente en una gran gama de especies esta familia de FT participa en la regulación de una amplia variedad de procesos, como el desarrollo de semillas y embriones, la formación de meristemas apicales de brotes, el desarrollo de fibras, la senescencia de las hojas, la división celular y la respuesta al estrés biótico y abiótico (Nakashima et al. 2012; Singh et al. 2017). En este estudio, 101 FT NAC fueron identificados en el transcriptoma de un genotipo de *E. globulus* previamente caracterizado como tolerante a estrés hídrico y frio. Entre estos, 13 genes *EglNACs* exhibieron una expresión diferencial significativa en ambos tratamientos, destacando *EglNAC34* que presentó niveles de expresión significativamente mayor en ambos tratamientos. Estos resultados proveen de información importante en la compresión de las redes de genes involucradas en la respuesta al estrés abiótico que pueden ayudarnos a generar nuevas estrategias de mejoramiento genético para potencialmente obtener árboles con mejor respuesta a condiciones climáticas cambiantes futuras.

CONCLUSION GENERAL

Se identificaron 101 factores de transcripción NACs con dominio completo en el transcriptoma de *E. globulus* sometido a tratamiento de estrés hídrico y frio. En base a los análisis filogenéticos, dos grandes grupos (I y II) subdivididos en 17 subfamilias fueron encontrados. El análisis *in silico* mostró que 13 factores de transcripción *NAC* tuvieron una mayor abundancia de transcritos en respuesta a ambos tratamientos de estrés abiótico. La expresión relativa por qRT-PCR evidenció que el gen *EglNAC34* posee un importante rol en la respuesta a los tratamientos de estrés analizados, mostrando la mayor expresión relativa en todos los genotipos evaluados.

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