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IDENTIFICACIÓN DE GENES DERESPUESTA A SEQUÍA, EN HOJAS Y RAÍCES DE UN GENOTIPO DE *Eucalyptus globulus* TOLERANTE AL ESTRÉS

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DEDICATORIA

Este trabajo se lo dedico, en primer lugar a mi esposa Jessica Arévalo, quien ha sido mi pilar y motor para enfrentar la vida, dándome fuerza y valor para lograr lo que parecía imposible.

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RESUMEN

Eucalyptus globulus es una de las especies forestales más importantes a nivel mundial, debido a su excelente calidad de madera para el uso industrial. Sin embargo, las condiciones ambientales de menor disponibilidad de agua, marcadas por sequías más duraderas y severas, causadas por el calentamiento global, están afectando el rendimiento, el establecimiento y la supervivencia de las plantaciones en todo el mundo. Es urgente desarrollar estrategias para la obtención de nuevas variedades de *Eucaliptus* spp, para asegurar la disponibilidad de recursos forestales en el futuro. Estudiar los mecanismos de respuesta a la sequía a nivel bioquímico, fisiológico y molecular, nos ayudará a comprender cómo las plantas pueden sobrevivir bajo dicho estrés. En el presente trabajo, hemos estudiado los principales cambios a nivel transcripcional, inducidos por el tratamientos de supresión de riego, en un genotipo tolerante de *Eucalyptus globulus*. Nuestro principal objetivo, fue identificar genes candidatos para entender cuáles son los mecanismos de respuesta más importantes en la especie para afrontar al estrés por sequía. Los datos sugieren, que las plantas sometidas al tratamiento, inducen la expresión de factores de transcripción, tales como *MYB*, *NAC* y *WRKY*. Además, otros genes fueron inducidos por las condiciones de estrés, por ejemplo algunos genes involucrados en la eliminación de especies reactivas de Oxígeno ROS (*EuglTRDX*), protección de estructuras celulares (*EuglLEA4-5*, *EuglDHN1*), transporte de lípidos (*EuglLTP2*), regulación osmótica (*EuglVGT1*) entre otros. Los resultados del presente trabajo contribuirán al entendimiento de cuales son las estrategias más importantes en la especie para incrementar sus posibilidades de supervivencia ante estrés por sequía. Por otro lado, también podrán contribuir al desarrollo de estrategias relacionadas al fito-mejoramiento para obtener individuos de mayor tolerancia a la sequía.

ABSTRACT

Eucalyptus globulus is one of the most important forestry specie due to its excellent wood quality for industrial purposes. However, drier environment conditions, marked by longer and harder drought events, caused by global warming are affecting yield, establishment and survival of plantations around the world. Strategies to develop new eucalyptus varieties are urgent to ensure availability of forestry resources in future. Study mechanisms of drought response at biochemical, physiological and molecular level, will help us to understand how plants can to survive under drought stress. We study the main transcripts induced by drought stress in a tolerant *Eucalyptus globulus* genotype in drought stress experiment. Our main objective was to identify candidate genes to get clues about the mechanisms of response to stress in the specie. Data suggest that plants under drought stress induce the expression of many transcription factors such as MYB, NAC and WRKY. Furthemore, other genes were induced by treatment, for example those involved in ROS scavenging (*EuglTRDX*), protection of cellular structures (*EuglLEA4-5, EuglDHN1*), transporting lipids (*EuglLTP2*), Osmotic regulation (*EuglVGT1*) among others. The results present in this work will contribute to development of strategies of plant breeding to obtain varieties with higher drought tolerance.

INTRODUCCION GENERAL

La industria forestal chilena es la tercera actividad económica más importante del país luego de la minería y la pesca. La superficie ocupada por plantaciones forestales a nivel nacional es cercana a los 2.4 millones de hectáreas. Dentro de las especies establecidas *Eucalyptus globulus* corresponde a la segunda especie de mayor importancia económica para el país debido a su calidad de fibra, tiempo de rotación y adaptación a las condiciones edafo-climáticas (INFOR, 2018). Esta especie representa cerca del 37% del área total plantada en Chile. Por otra parte, la industria forestal representa cerca del 2.1% del crecimiento del producto interno, aumentando US $ 6,838 millones de exportaciones durante 2018 (INFOR 2019).

Una de las principales problemáticas, a las que se enfrenta la actividad forestal es la disponibilidad de agua, porque es el factor limitante para el establecimiento de plantaciones, restringiendo el área de distribución, a zonas de mayor pluviosidad, principalmente entre las regiones IV y XI (Infor 2016). Dicha limitación se ve agravada, por la disminución sostenida de la cantidad de agua caída durante los últimos años. Específicamente, Chile se vio afectado por el peor evento de sequía registrado durante la última década (Garreaud et al.,2020). El registro histórico demuestra que existe una disminución, de hasta 300mm por cada 30años, en la zona central del país (Garreaud 2011). El problema adquiere mayor importancia, debido a que se espera, que el cambio global del régimen de precipitaciones, provoque una disminución de hasta un 50 % de las lluvias en chile, hacia fines del presente siglo (Garreaud 2011).

En este contexto, el cambio climático, impactará directamente en la productividad y sanidad de las superficies boscosas, lo cual se observa en muchas facetas, como por ejemplo: la muerte de plántulas, la susceptibilidad a patógenos, el ataque de insectos y la susceptibilidad a incendios forestales (Hogg y Wein 2005; Matusick et al 2016).

Considerando las condiciones actuales de nuestro país, en donde las especies del genero *Eucalyptus* más utilizadas son: *E. globulus* y *E. nitens* , y ambas especies son consideradas susceptibles a eventos de sequía prolongados (White et al 2009, White 1996), se estima

imprescindible estudiar sus mecanismos de respuesta, ante las condiciones de escasez hídrica, con el objetivo de elaborar estrategias de mejoramiento genético, basadas en caracteres relacionados a tolerancia, supervivencia, rendimiento y eficiencia de uso de agua (Hamanishi & Campbell, 2011).

Según el estudio de White (1996), los mecanismos de respuesta en ambas especies son distintos, sin embargo, *E. globulus* posee mayor eficiencia del uso de agua, es decir, mayores tasas de crecimiento en condiciones de estrés hídrico, lo que en términos productivos significa mayores ingresos, por esta razón en zonas de baja pluviometría se prefiere por sobre *E. nitens.* Sin embargo, ambas especies no pueden resistir eventos de sequía prolongados.

En vista de lo anterior, se hace necesario, fortalecer el entendimiento del proceso de adaptación de *Eucalyptus globulus* a condiciones ambientales adversas, porque al conocer los mecanismos de respuesta, se logrará generar estrategias de manejo productivo que permitan obtener productos, de manera sustentable en el futuro.

Actualmente, se realizan grandes esfuerzos para conocer las rutas de señalización, así como los genes que codifican para las proteínas clave en la respuesta al estrés abiótico en especies forestales (Altman y Hasegawa 2012). Por otro lado, el futuro desarrollo de herramientas moleculares de mejoramiento genético y de la ingeniería genética en árboles, permitirán crear variedades más resistentes a la sequía y más eficientes en el uso del agua, lo que se traduce en un incremento de productividad y rendimiento de productos derivados de la madera (Harfouche et al 2014).

A pesar de la importancia económica del género para la economía nacional, existen pocos proyectos de investigación científica para mejorar esta especie, para hacer frente al estrés por sequía.

La tesis doctoral propuesta, se ha desarrollado en el marco del proyecto FONDECYT 1161063, llamado “Wrky TF: are they working for abiotic stress resistance in *Eucalyptus globulus*?” (2016-2019), liderado por la Dra. Sofía Valenzuela, quien participa como profesor guía de la presente investigación.

En dicho proyecto, se ha llevado a cabo un experimento de estrés hídrico, que consistió en someter a un grupo de plántulas de *E. globulus*, correspondientes a un genotipo tolerante a sequía, a un periodo de supresión de riego, para determinar cuáles son los genes más importantes, que participan en el proceso de respuesta a la sequía.

El objetivo de dicho experimento, fue generar bibliotecas para la secuenciación masiva del transcriptoma, de las plantas sometidas al tratamiento, mediante RNA-Seq, de este modo determinar cuáles fueron los principales cambios en la abundancia de transcrito, por efecto del déficit hídrico, en tejido foliar y radicular, respecto a plantas con riego permanente.

En relación a lo anteriormente descrito, es importante mencionar que la técnica de RNA-Seq, se ha empleado para identificar la expresión de genes de respuesta, analizando el transcriptoma de diferentes especies de plantas, en distintos tipos de condiciones ambientales (Vlk y Řepková, 2017). En este sentido, el transcriptoma corresponde al set completo de transcritos en una célula, considerando su cantidad, en un estado de desarrollo o fisiológico específico (Wang et al 2009).

En resumen, las largas hebras de ARN mensajero, expresadas en un momento determinado, son aisladas y convertidas en una biblioteca de ADNcopia, mediate una reacción de retro transcripción y posterior proceso de fragmentación, como resultado se obtienen secuencias de ADN cortas, a las cuales se le añaden pequeños adaptadores (figura 1), que sirven para llevar a cabo la secuenciación masiva en alguna de las plataformas disponibles. Los resultados de la secuenciación, de las pequeñas lecturas, son alineadas contra un genoma o transcriptoma de referencia, para finalmente cuantificar la expresión, según la abundancia de fragmentos obtenidos para cada transcrito (figura 1). Para más detalles acerca de la técnica, revisar el trabajo de Hrdlickova y colaboradores (2017).

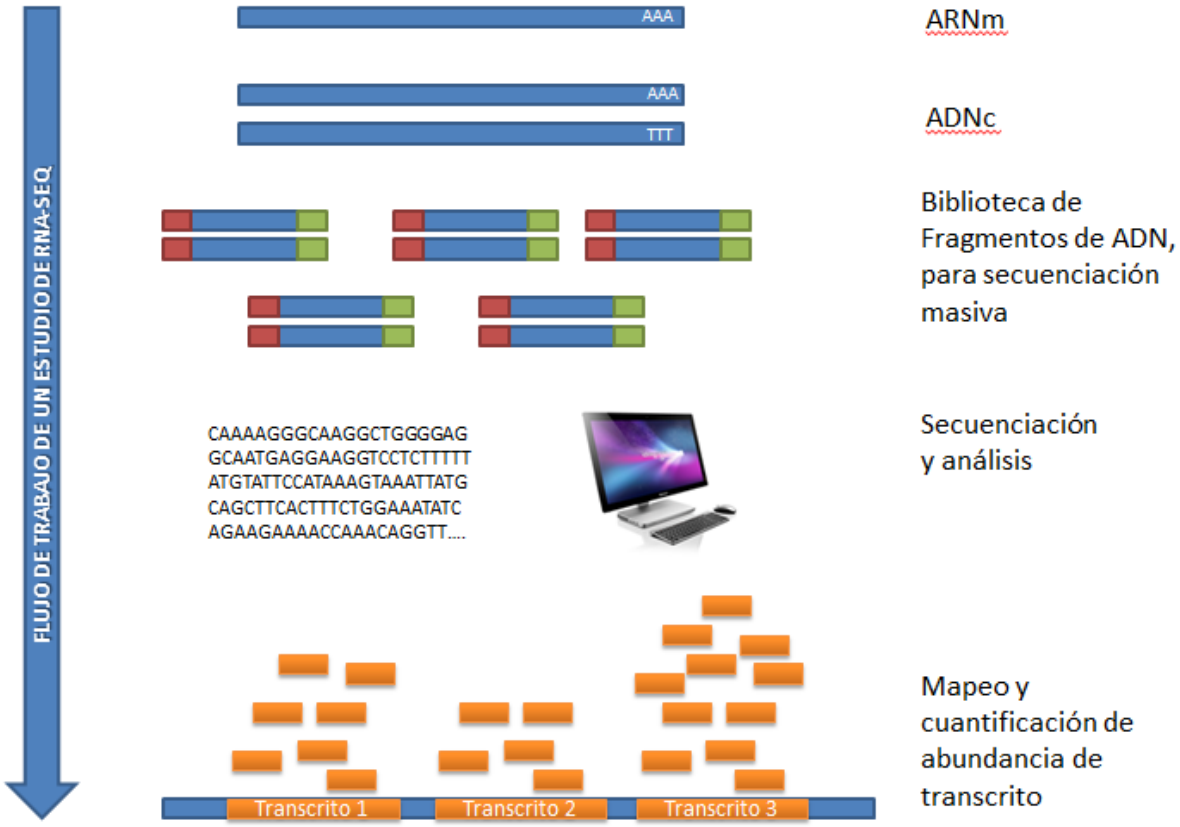


Figura 1 Representación esquemática del flujo de trabajo para el estudio de expresión génica mediante la tecnología de secuenciación masiva por RNA-Seq. A partir de ARN mensajero, se sintetiza ADN copia por una reacción de Retro transcripción. Luego, mediante fragmentación, se obtienen secuencias cortas de ADN, a las cuales se le agregan adaptadores en sus extremos (rectángulos rojos y verdes), que sirven de guía, para iniciar la secuenciación, en una plataforma de secuenciación masiva. Se obtienen millones de secuencias, las cuales se procesan mediante herramientas bioinformáticas, para ensamblar el transcriptoma, en base a una referencia. Luego, se estima la abundancia de cada transcrito, para relacionarlo con la expresión de genes en las condiciones de estudio.

De acuerdo a estudios en otras especies del genero *Eucalyptus,* se ha podido determinar que los principales genes de respuesta a sequía, guardan relación con el proceso de síntesis de lignina y flavonoides, proteínas de shock térmico, aldo/ceto reductasas, proteínas involucradas en la síntesis de etileno, expansinas, factores de transcripción como HB-12, RD26 y ERF110, entre otros (Thumma 2012; Villar et al 2011). En paralelo, también se ha observado una disminución en genes relacionados a fotosíntesis, transporte de agua y azúcar, además de genes involucrados en el metabolismo secundario (Villar et al. 2011).

Por otro lado, mediante un estudio del proteoma de *globulus,* en dos genotipos contratantes, se logró determinar que las principales proteínas expresadas en condiciones de sequía son: Factor de iniciación de la traducción (36), precursor de almidón sintasa, proteína de unión a citokinina semejante a Taumatina, Proteinas de shock térmico (HSP70), Proteína abundantes de embriogénesis tardía (LEA), entre otras (Valdés et al. 2013).

Mientras que a nivel de transcripción, algunos genes de respuesta a sequía en *E. globulus*, han sido inducidos por tratamientos de supresión de riego, entre ellos se encuentran: Deshidrina 3, (Fernández et al., 2012), Malato deshidrogenasa mitocondrial y catalasa peroxisomal (Correia et al., 2018). En contraposición, se ha identificado que los genes relacionados a fotosíntesis, de un genotipo susceptible, en condiciones de sequía, disminuyen significativamente la abundancia de transcrito, por ejemplo Rubisco Activasa, Fosforibulokinasa y Ferredoxina NADPH oxidoreductasa. Mientras que un genotipo tolerante, mantiene constante la expresión de dichos genes en condiciones de tratamiento (Correia et al. 2018).

En base a lo anteriormente descrito se planteó la siguiente Hipótesis.

"Un genotipo tolerante de *Eucalyptus globulus,* induce la expresión de genes de respuesta a sequía, en hojas y raíces, cuando es sometido a un tratamiento de supresión de riego".

Posteriormente se determina el siguiente objetivo general:

"Identificar los principales genes que se inducen en respuesta a un tratamiento de supresión de riego, en un genotipo tolerante de *E. globulus*, en hojas y raíces, mediante la técnica de secuenciación masiva del transcriptoma (RNA-seq)".

Mientras que los objetivos específicos planteados fueron:

“Comparar la expresión de genes de respuesta a sequía en un genotipo tolerante, entre condiciones de estrés por sequía versus control”.

“Identificar diferencias entre la expresión de genes de respuesta a sequía en hojas y raíces de un genotipo tolerante en condiciones experimentales”.

“Validar la expresión de genes de respuesta, mediante experimento mediante qRT-PCR”

“Evaluar la expresión de genes seleccionados en genotipos contrastates de *E. globulus* en experimentos de supresión de riego”.

Experimento en cámaras de crecimiento

Para entender mejor de qué forma se llevaron a cabo los experimentos que forman parte dela presente investigación, en el marco del proyecto de tesis de doctorado, se describe parte del experimento en condiciones de cámara de crecimiento.

En primer lugar, se lleva a cabo un tratamiento de estrés por sequía que consistió en la supresión de riego. Como resultado, el tratamiento de sequía, generó cambios a nivel morfológico y fisiológico en las plántulas del genotipo evaluado. Se reportaron diferencias significativas en la tasa de fotosíntesis, conductancia estomática, transpiración, crecimiento de vástago y raíces (Tabla 1).

Tabla 1 Variables fisiológicas y morfológicas medidas en plántulas de *E. globulus* sometidas a un tratamiento de estrés por sequía en cámaras de crecimiento bajo condiciones controladas. Los asteriscos indican que hubo diferencias significativas entre control y tratamiento, según Test Student (P<0.005).

Variables fisiológicas

Tratamiento	**Tasa de fotosíntesis ***	**Conductancia estomática ***	**Transpiración ***	**Potencial hídrico de Xilema ***
	µmol $CO_2 m^{-2} s^{-1}$	**mmol $m^{-2} s^{-1}$**	**mmol H_2O $m^{-2} s^{-1}$**	**Ψpd (MPa)**
control	7,59 ± 0,97	0,15 ± 0,03	1,42 ± 0,24	-0,2 ± 0,0
stress	4,21 ± 1,84	0,04 ± 0,02	0,42 ± 0,18	-1,6 ± 0,7

Variables Morfológica

Tratamiento	**Incremento en altura ***	**Diferencia Hojas**	**Verticilos**	**Longitud de Raíces***
	Cm	**N°**	**N°**	**cm**
control	3,4 ± 1,1	5,6 ± 2,1	3,0 ± 0,0	17,8 ± 1,6
stress	-0,4 ± 3,2	2,4 ± 3,0	1,2 ± 1,8	23,2 ± 2,6

De acuerdo con las mediciones fisiológicas realizadas, el genotipo sometido al tratamiento alcanzó el nivel de estrés esperado, reportando una disminución significativa de la tasa de fotosíntesis (P=0,0067). La disminución en la tasa de fotosíntesis observada es similar a las obtenidas en otros estudios, en los cuales se reporta 12 µmolCO_2 /m^2s en condición control versus 8 µmolCO_2 /m^2s bajo Estrés (Gauthier et al 2014), ó 10 µmolCO_2 /m^2s en control versus 5 µmolCO_2 /m^2s en déficit hídrico (Jesús et al 2015).

Respecto a la conductancia estomática, se obtuvo una reducción significativa del 73% (aprox), respecto al control. Por otro lado, la tasa de transpiración se ve reducida en 71% (p<0,0001). Jesús y colaboradores (2015), reportan en *E. globulus*, una reducción de 75% y 50% de la tasa de conductancia y transpiración respectivamente.

En el caso del potencial hídrico de xilema fue significativamente menor en el tratamiento (p=0,026). McKierna y colaboradores (2016) informan de un potencial de xilema cercano a -1.25Mpa para un contenido de humedad del 30%, ellos usaron en su experimento una mezcla de corteza de pino compostada y Arena en proporciones de 8:3, como sustrato, en un vivero, aplicando el tratamiento durante aproximadamente 8 semanas. Otro estudio reporta, valores cercanos a -1.2Mpa para un sustrato compuesto de turba y perlita en proporciones de 3:2, donde aplicó un tratamiento de sequía manteniendo por 2 semanas un porcentaje de humedad de 15%, en cámaras de crecimiento para plantas de 2 meses (Jesus et al 2015).

Además, se observaron cambios morfológicos como la pérdida de turgencia en hojas, disminución del crecimiento, producción de pigmentos, aumento de la densidad de raíces (Figura 2), resultados consistentes con la respuesta de plantas sometidas a condiciones de déficit hídrico.

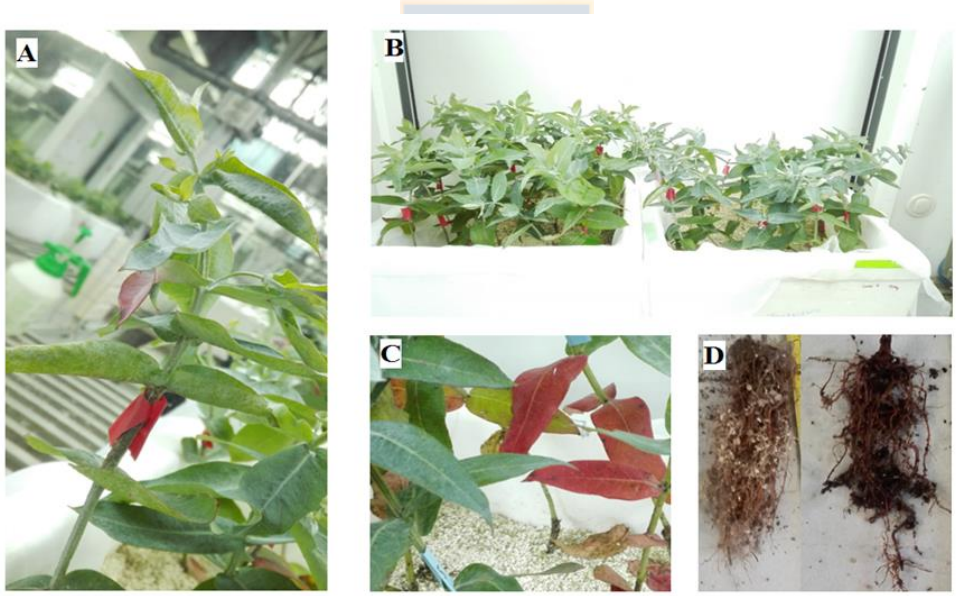


Figura 2. Detalle de los cambios morfológicos observados en las plántulas de *E. globulus* sometidas a tratamiento de estrés por sequía en cámaras de crecimiento. A) detalle de la pérdida de turgencia en hojas. B) Diferencias de crecimiento en plántulas sometidas a tratamiento (derecha) y control (izquierda). C) Cambios en la pigmentación de las hojas (tratamiento). D) Comparación de raíces de plántulas en tratamiento (izquierda) y control (derecha).

En base a los resultados anteriormente descritos se establece que las condiciones de estrés aplicadas, fueron propicias para la evaluación de la respuesta molecular mediante las técnicas de secuenciación masiva.

Se tomaron muestras de hoja y raíz, para la respectiva extracción de ARN y generación de las bibliotecas para secuenciación masiva del transcriptoma a través de la plataforma illumina, mediante la empresa de servicios Arrayexpress (Raleigh, NC, USA; http://www.arrayxpress.com). Para mayores detalles revisar capítulo I.

Experimento en invernadero.

Se llevó a cabo un experimento de invernadero para evaluar el efecto de la sequía sobre diferentes genotipos de *Eucalyptus* spp. Con el objetivo de seleccionar aquellos genotipos con mayores diferencias en cuanto a supervivencia a condiciones de sequía, en esta oportunidad se utilizaron 12 genotipos, 6 correspondientes a *E. globulus* y 6 híbridos *E. nitens x E. globulus*.

A los 2 semanas de iniciado el tratamiento de exclusión de riego, los genotipos *de E. globulus* presentaron perdidas del contenido de agua en el sustrato, entre un 15% y el 60%, respecto a la capacidad de las macetas. En el caso de *E. globulus* es posible diferenciar los genotipos g5 y g6, que mostraron una disminución más rápida que los genotipos g1 y g4, diferencia que es visualizada desde mediados de la segunda semana de experimento (fig.3a). Por otro lado, los genotipos híbridos g7 y g10, fueron los más rápidos en secar el sustrato, mientras que por el contrario, los genotipos g12 y g8 mantuvieron un alto contenido relativo de agua, con pérdidas menores al 40%, respecto de la capacidad de la maceta, en la segunda semana (fig 3b). En el caso de las mediciones a la semana 5, se observa un comportamiento homogéneo entre la mayoría de los genotipos alcanzando valores entre el 70 y 90% de pérdida de agua (fig 3).

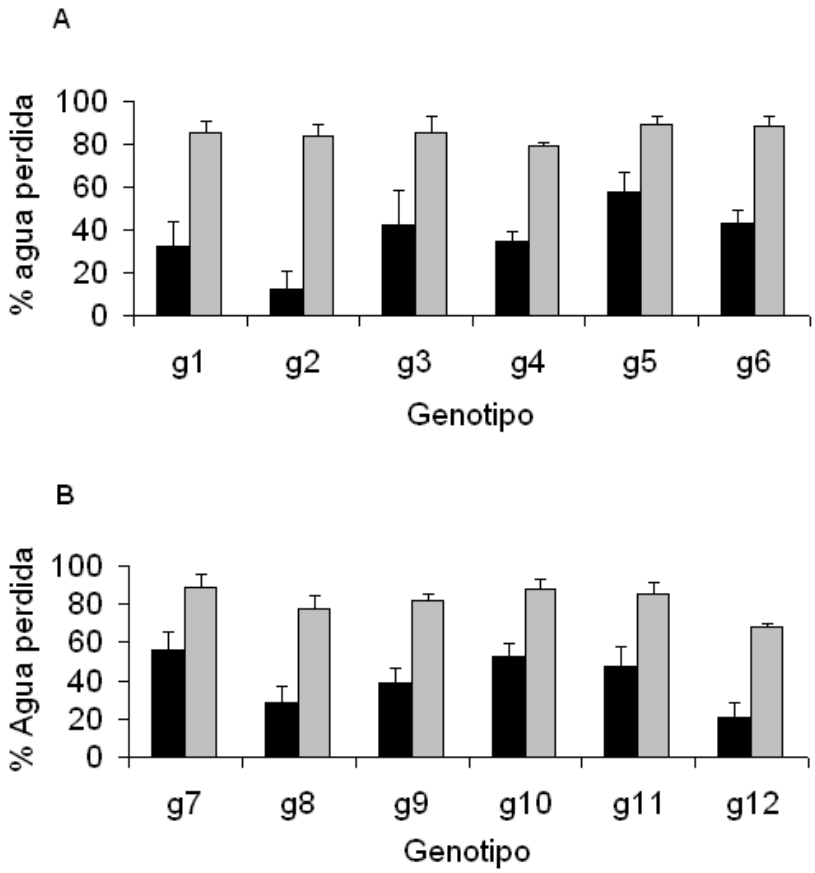
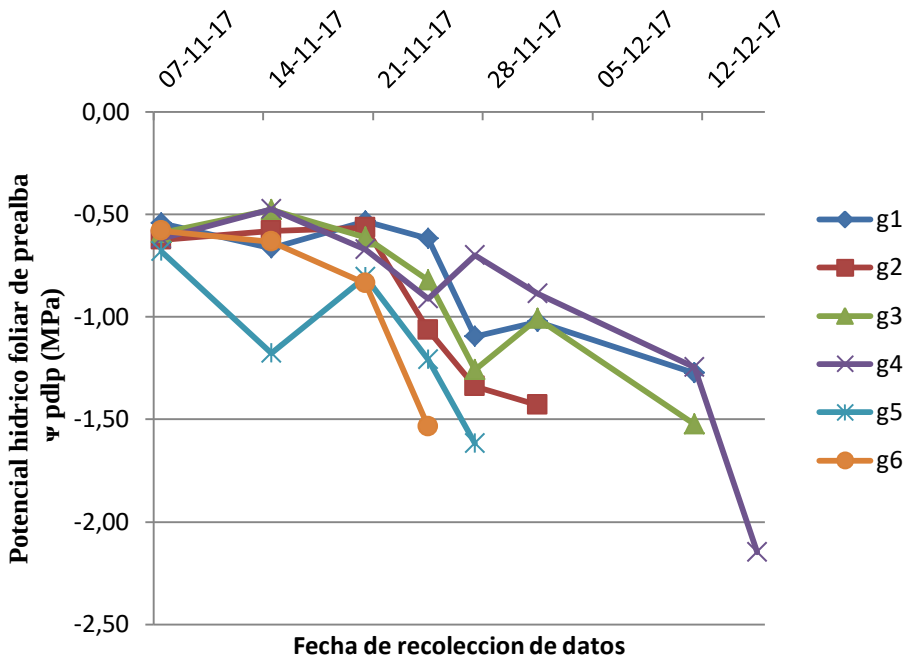


Figura 3 Pérdida del contenido relativo de humedad de sustrato, respecto al inicio del experimento (4 de noviembre) en plantas sometidas al tratamiento de estrés hídrico, medidas con equipo TDR (Time Domain Reflectometry). A) genotipos de Eucalyptus globulus, Barras de color negro corresponde a la semana 2, barras de color gris representa los datos a la semana 5. G1-G6. B) genotipos de Híbridos de E. nitens X E. globulus.

El mismo comportamiento se ve reflejado en el caso de las mediciones de potencial hídrico foliar de prealba, donde se puede observar que los genotipos de *E. globulus* que alcanzan el punto de marchitez permanente de -1,5MPa, antes que el resto coinciden con aquellos que alcanzan menores valores de porcentaje de contenido relativo de agua en el sustrato, es decir, los genotipos g5 y g6 llegan a dicho umbral, prácticamente a las dos semanas de tratamiento, en contraposición a los últimos genotipos, que corresponden al genotipo g1 y g4, que lo alcanzan después de un mes de tratamiento (fig 4 a). Igualmente, los genotipos híbridos, g7 y g10, registran valores de potencial hídrico foliar de -1,5MPa, durante la segunda semana de tratamiento, mientras g8 y g12, después del mes de tratamiento (Fig 4b).

A



B

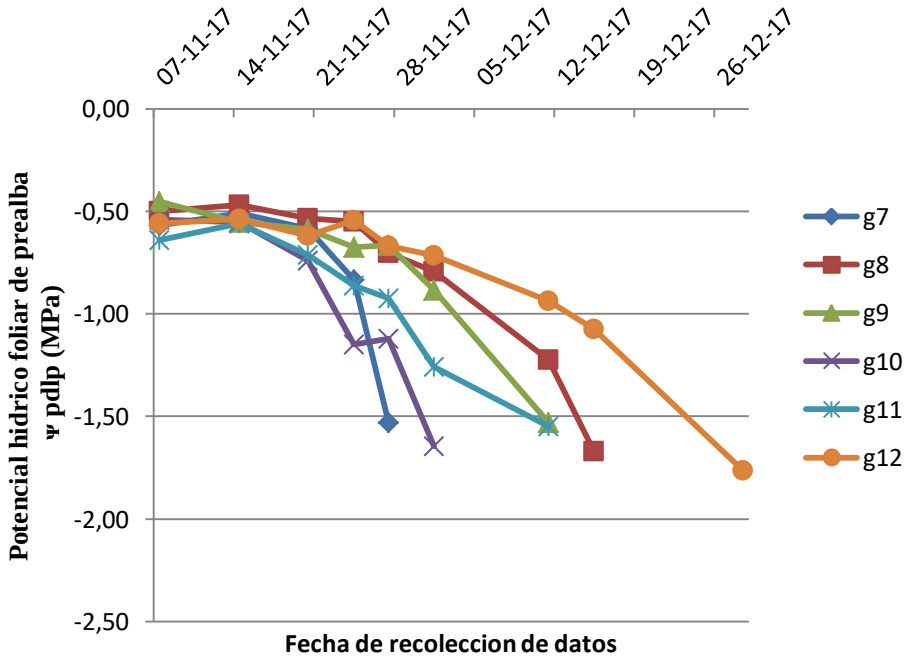


Figura 4 Potencial hídrico foliar de prealba de plantas mantenidas bajo tratamientos de estrés hídrico en condiciones de invernadero. A) Genotipos de *E. globulus.* B) Genotipos Híbridos *E. nitens X E. globulus.*

Al medir la conductancia estomática, de los distintos genotipos evaluados, durante un día, es posible identificar un comportamiento similar, donde se registra los mayores valores, durante las horas de mayor intensidad lumínica y temperatura, mientras que en horario de prealba los valores de conductancia fueron menores (fig 5). Los individuos que mostraron valores más altos, en condición de riego, corresponden a genotipos de *E. globulus* G5, G1, G3 y G6, por otro lado los de menor conductancia fueron los híbridos, G7, G9 y G11. (fig. 6)

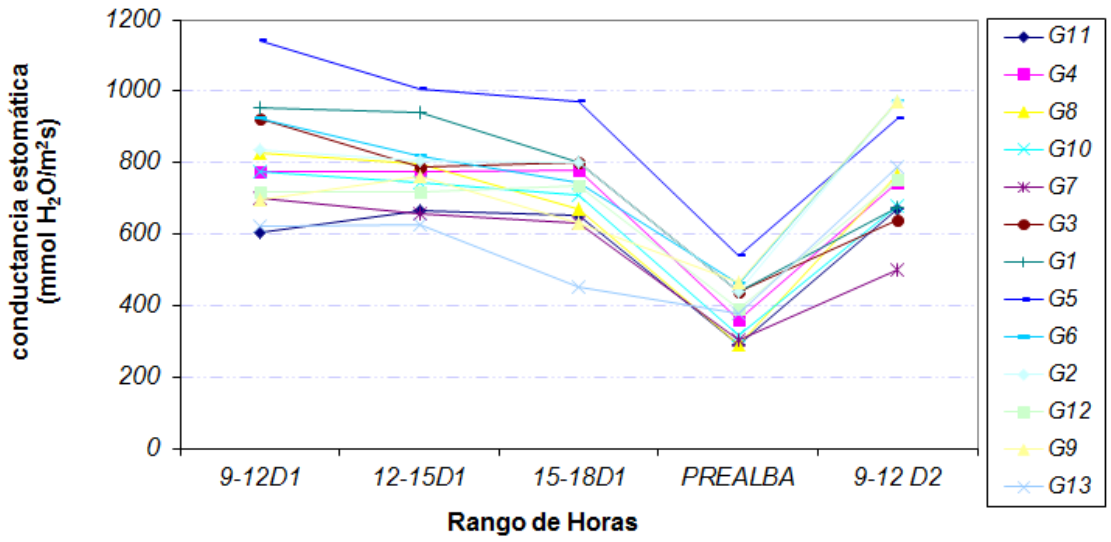


Figura 5 Conductancia estomática de distintos genotipos de *E. globulus* (G1-G6) e Híbridos de *E. globulus X E. nitens* (G7-G12) a lo largo de un día de mediciones. G13 corresponde a un control de E. nitens. En el eje X se encuentra el rango de horas evaluado, acompañado por el día de medición. El rango de prealba, corresponde a mediciones del día 2 desde las 3 AM a las 5 AM.

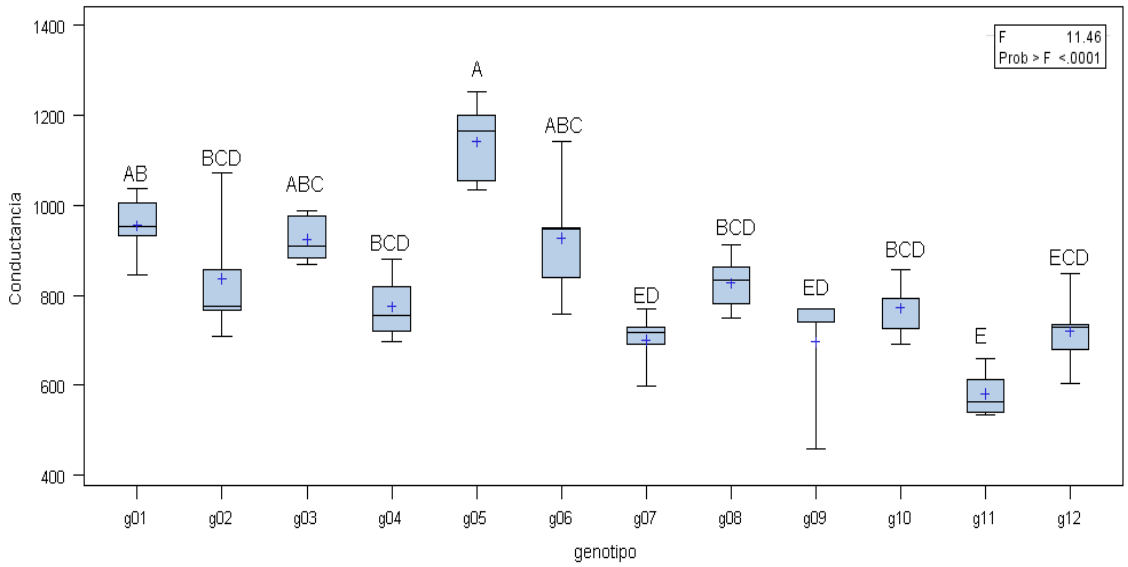


Figura 6. Grafico de cajas, que registra la conductancia estomática (mmol H_2O/m^2s) de distintos genotipos de E. globulus (G1-G6) e híbridos de E. nitens X E. globulus (G7-G12) en condición de riego. La letra sobre la barra de error indica diferencias significativas según test ANOVA y posterior prueba de Tukey, para un valor de alfa = 0.05.

I

En terminos generales la conductancia estomatica se reduce significativamente cuando avanza el nivel de estrés en las plantas, diferenciando claramente las plantas del control respecto a aquellas mantenidas en los tratamientos, sólo en el genotipo g4 no hay una disminución significativa en el tratamiento intermedio (fig 7).

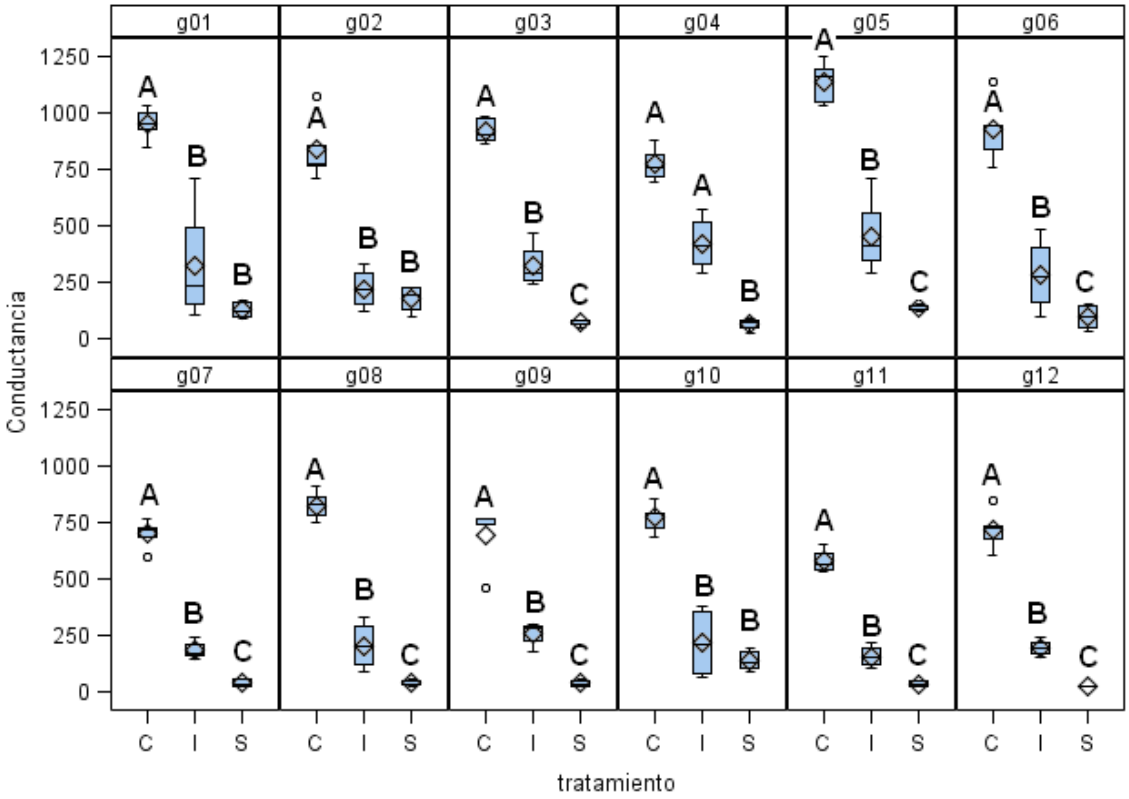


Figura 7. Conductancia estomática expresada como mmol $(H_2O)/m^2s$, en tres puntos de muestreo: Control (C), Estrés Intermedio (I) y estrés Severo (S). Las letras sobre cada caja indican diferencias significativas entre tratamientos según ANOVA y posterior test Tukey ($p<0.05$).

La tasa de fotosíntesis, medida a partir de la eficiencia del foto-sistema II, mediante la relación fv/fm, demuestra la disminución significativa en el genotipo g5 de E. globulus, a 2 semanas del inicio del tratamiento, respecto al control irrigado. Por otro lado los genotipos híbridos g7, g9 y g10 disminuyen su actividad fotosintética de manera similar a g5, en el mismo lapso (fig8).

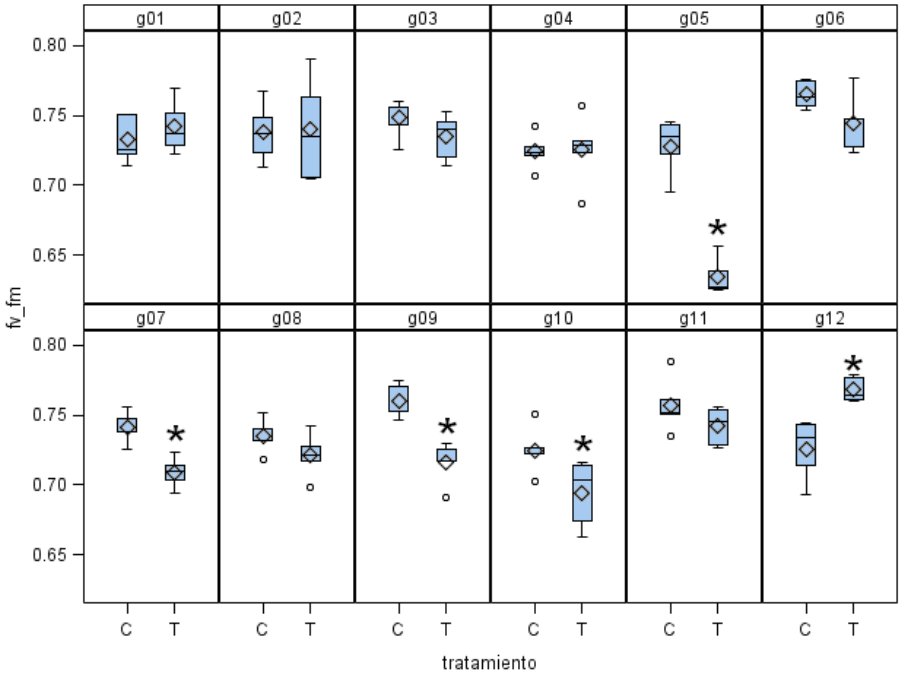


Figura 8 Eficiencia del fotosistema II medida por fluorometría (fv/fm) en plantas sometidas a condición control (C) y tratamiento de estrés hídrico (T) luego de 2 semanas desde el inicio del tratamiento. Los asteriscos indican diferencias significativas respecto al control según Test-Student ($p<0.05$).

La supervivencia de los genotipos, al finalizar 6 semanas de tratamiento, mediante prospección visual, indica que los individuos de *E. globulus*, g3, g5 y g6, poseen una diferenciación significativa entre control y tratamiento, lo que indica que dichos individuos presentaron una menor cantidad de tejido vivo, al finalizar el tratamiento (fig9). En el caso de los híbridos, los genotipos g10 y g11 mostraron menor porcentaje de supervivencia respecto al control (fig9).

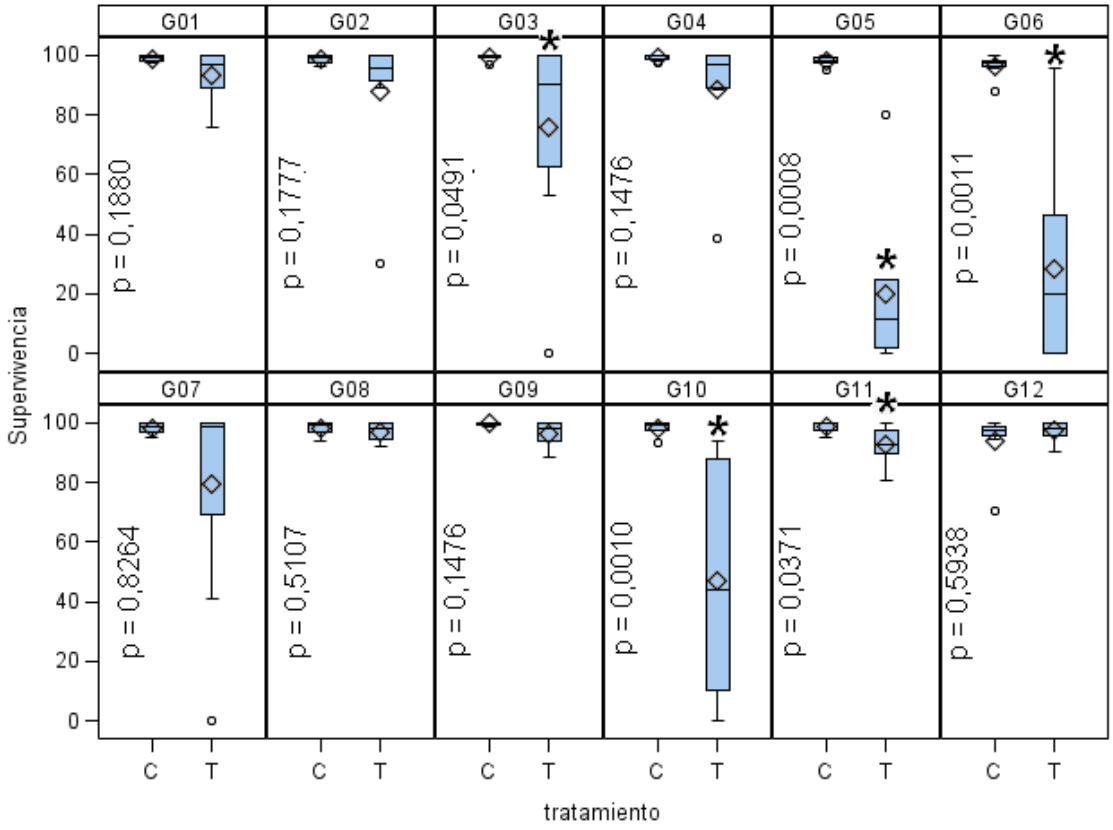


Figura 9. Porcentaje de supervivencia según prospección visual evaluada en 8 plantas de cada condición: Control (C), Tratamiento (T). Los Asteriscos en la figura indican diferencias significativas, respecto a la condición control, según el test no paramétrico de Krukal-Wallis ($p<0.05$). Se indica el valor p, para cada comparación.

Por otro lado, al comparar el crecimiento en altura, entre la condición control y el tratamiento, luego de seis semanas de experimento, se evidencia una reducción significativa, por efecto del estrés hídrico aplicado, en los genotipos de *E. globulus* G1, G2, G3, G5 y G6. Mientras que los híbridos G7 y G11, fueron aquellos que mostraron menor crecimiento, según la prueba T-Student (fig 10).

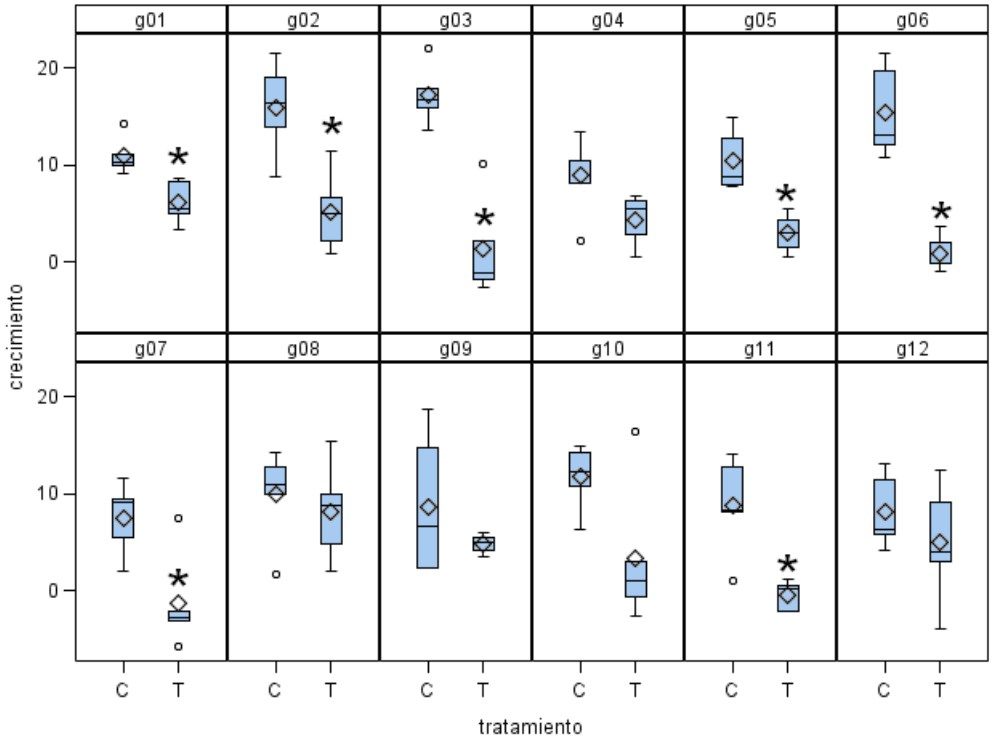


Figura 10. Crecimiento de plantas (centímetros) entre la semana 2 desde el inicio del tratamiento de estrés hídrico y la semana 6. Los asteriscos indican que existen diferencias significativas entre el crecimiento de las plantas del control y el tratamiento, según el análisis de Test-Student ($p<0,05$).

Según el análisis de componentes principales (PCA), se ha determinado que los primeros 3 componentes, explican aproximadamente el 90% de la variación de los datos. En donde el componente 1, posee mayor proporción de la varianza, cercana al 50%, mientras que los componentes 2 y 3 explican el 23 y 17% respectivamente (figura 11).

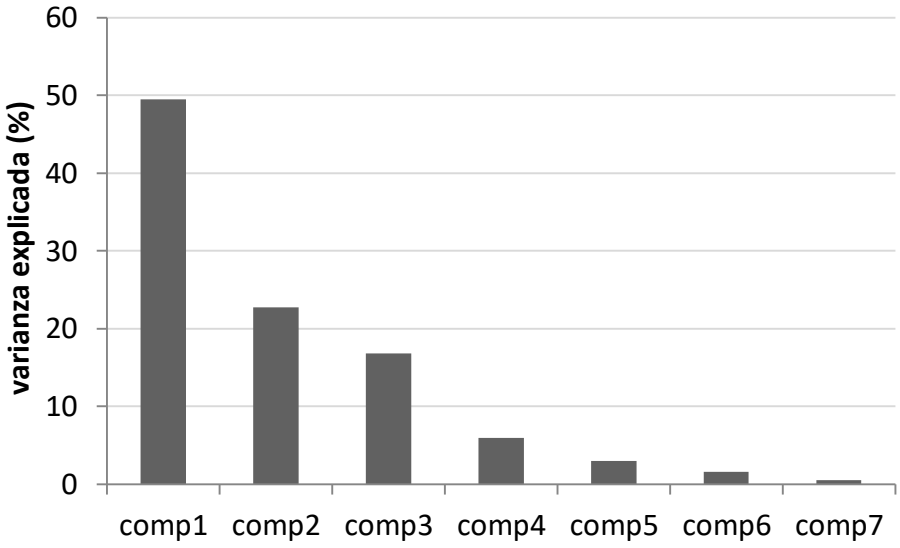


Figura 11 Porcentaje de la varianza explicada por cada uno de los componentes proncipales, resultantes del análisis PCA.

De acuerdo al PCA, es posible identificar diferencias entre genotipos, principalmente aquellos que mostraron, en promedio, menores niveles de supervivencia, como por ejemplo g5, g6, g10, g3, agrupados a la derecha del gráfico de Scores (figura 12). Los genotipos de mayor grado de supervivencia se agrupan a la izquierda (g12, g1, g8). La diferencia mayor se reporta entre los genotipos g12 y g5, respecto al componente principal 1. Según el gráfico de Loadings, se aprecia que las variables, supervivencia y potencial hídrico foliar de prealba, son importantes en la separación de las muestras. Por otro lado, la variable de eficiencia de fotosistema II (fv/fm), esta inversamente relacionada a ambas mediciones y tiene influencia sobre el agrupamiento de los genotipos (figura 13).

Por otro lado, en el caso del componente 2, la diferencia entre los genotipos, está determinada según la tasa de crecimiento, conductancia, variación del contenido relativo de agua en el sustrato (HUM), y la eficiencia del fotosistema II (figura).

Identificando como más extremos, a los genotipos g2 y g11(figura 1.10).

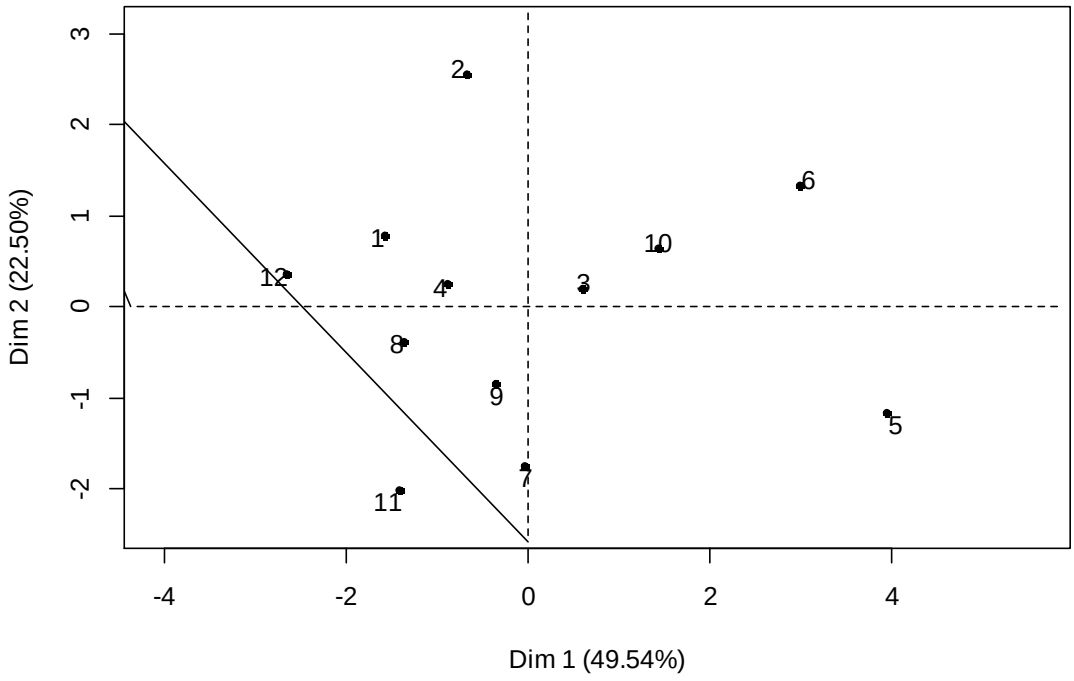


Figura 12 Gráfico de scores resultante del análisis de componentes principales (PCA), utilizado para evaluar de forma exploratoria, el efecto del tratamiento de estrés hídrico, sobre los genotipos de Eucalyptus sp. evaluados. Los rótulos de las muestras corresponden a: E. globulus genotipos g1-g6(identificados en el gráfico con los números 1-6); Híbridos E. nitens X E. globulus g7-g12 (7-12).

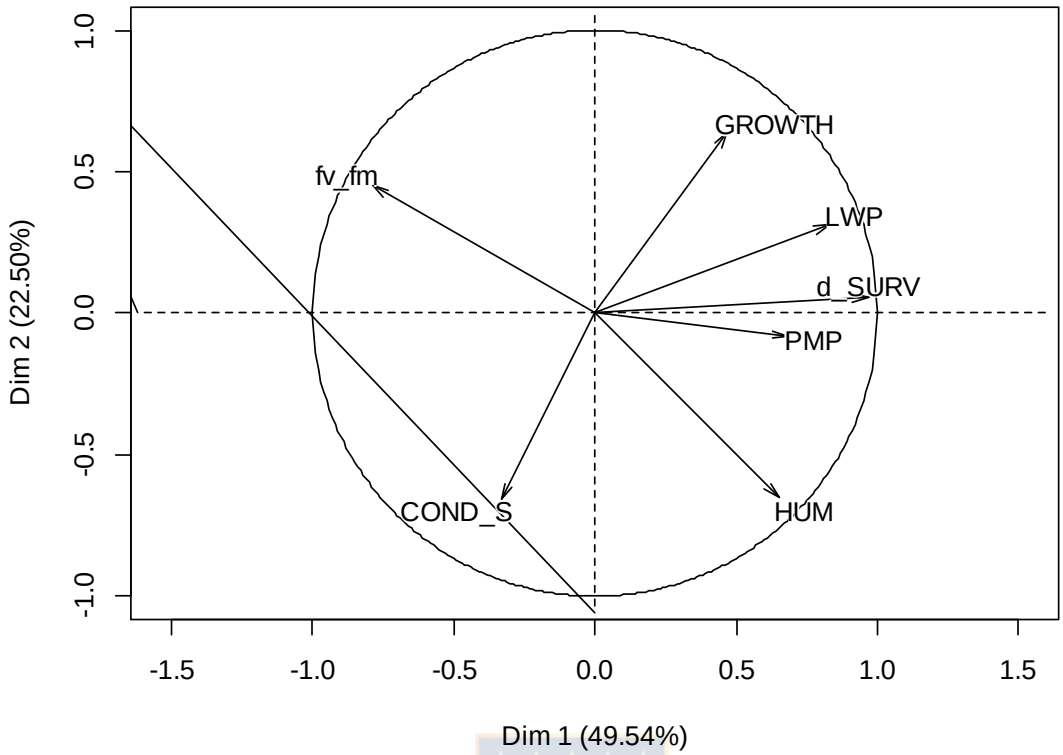


Figura 13. Gráfico de Loadings, correspondiente al análisis de componentes principales (PCA), que relaciona las distintas variables medidas, a los genotipos de Eucalyptus sp., establecidos en el experimento de estrés hídrico. Las variables corresponden a: fv_fm: Relación, entre eficiencia del fotosistema II, de plantas estresadas, respecto a plantas del control, expresada como fluorescencia, mediante la relación fv/fm; GROWTH: Diferencia de crecimiento, entre plantas del control, respecto a plantas del tratamiento a las seis semanas; LWP: potencial hídrico foliar de prealba, a las 2 semanas de iniciado el tratamiento de estrés hídrico; d_SURV: Supervivencia de los genotipos al tratamiento, luego de 2 meses; PMP: punto de marchitez permanente de cada genotipo, de acuerdo a las curvas de presión volumen. HUM: Diferencia del contenido relativo de agua en cada genotipo, entre dos puntos de muestreo; COND_S: Diferencia de conductancia estomática, de los genotipos, entre el tratamiento de -1,5MPa y el control.

Según el análisis desarrollado, es importante identificar, que las principales variables que determinan la tolerancia de los genotipos, basados en la supervivencia, son aquellas que poseen una relación directa con el desempeño fisiológico, a nivel de desarrollo y mantenimiento de las funciones vitales. Por ejemplo se identifica que el potencial hídrico foliar en prealba, medido a las 2 semanas de tratamiento, está correlacionado con el porcentaje de supervivencia del genotipo ($p<0.05$, $R2=0,70$) (figura 13). Mientras que la

relación entre eficiencia del fotosistema II, determinado por la relación de fluorescencia (fv/fm), es inversamente proporcional, a la variación en el contenido relativo de agua en el sustrato, entre los puntos de medición establecidos ($p<0.05$, $R2= 0,55$).

Finalmente, se determinó que fue posible identificar diferencias en cuanto a la supervivencia de los genotipos evaluados en invernadero, determinando la existencia de genotipos contrastantes respecto a la tolerancia a sequía, los cuales son utilizados en los análisis posteriores de expresión génica, para la búsqueda de genes candidatos para supervivencia a condiciones de estrés en *E. globulus*. En la figura 14 se identifica parte del experimento de estrés por sequía al inicio y luego de 2 meses de tratamiento.

Figura 14. Establecimiento de plantas en el invernadero. A) condición control al inicio del experimento, B) plantas del tratamiento de estrés hídrico antes de comenzar la exclusión de riego. C) condición control luego de 2 meses desde iniciado el tratamiento. D) Tratamiento de estrés hídrico luego de 2 meses desde el inicio

CAPITULO I

Transcriptomic response in foliar and root tissues of a drought-tolerant *Eucalyptus globulus* genotype under drought stress

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Key message

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

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 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

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, 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 droughtstressed 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 H2Om−2 s−1 in drought-stressed plants. According to their data, wellwatered 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 H_2O_2 production (Noctor et al. 2002). In parallel, there is less CO_2 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 signalling 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, including Eucalyptus due to their role in drought tolerance (Hussey et al. 2015). 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 the interaction of hydrophilic residues of late embryogenic abundant proteins (*LEA*), such as those from group IV acting as chaperons (Cuevas-Velasquez 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. The most productive genotype, showed a downregulation in genes involved in photosystem functioning, water and sugar transport and secondary metabolism, under 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 (Bezerra-Neto et al. 2019; Correia et al. 2018; Fernandez et al. 2012; Valdes 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.

Materials and methods

Plant material and drought-stress treatments

Two *Eucalyptus globulu*s 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 h 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 droughtstress 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) 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 − 1.8 MPa 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.

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− 2 s− 1), photosynthesis A (μmol CO2 m− 2 s− 1) and transpiration E (mmol H2O m− 2 s− 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− 2 s− 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 the 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.

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 a 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 singleend. 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 by size, reads were in average 100 bp. The genome of *E. grandis* (version 2, downloaded from: https:// phyto zome. jgi. doe. gov/) was used as a reference for mapping transcript sequences using CLC genomics Workbench software version 10.0.1 (QIAGEN, Aarhus, Denmark). Since the reference genome employed was from a different species, slightly relaxed mapping stringency was set to obtain a larger 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 (LDSRCON) 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.

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.

Results and discussion

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. 2014b, 2018; Navarrete-Campos et al. 2013). A reduction from 7.59 ± 0.97 μmol CO2m− 2 s− 1 in plants in the CON plants to 4.21 ± 1.84 μmol CO2m−2 s− 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− 2 s− 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− 2 s− 1 in DS plants (a 3.4-fold decrease). Navarrete et al. (2013) observed a tenfold 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.

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 lowquality 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 7048 sequences in leaves and 3822 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. 1b).

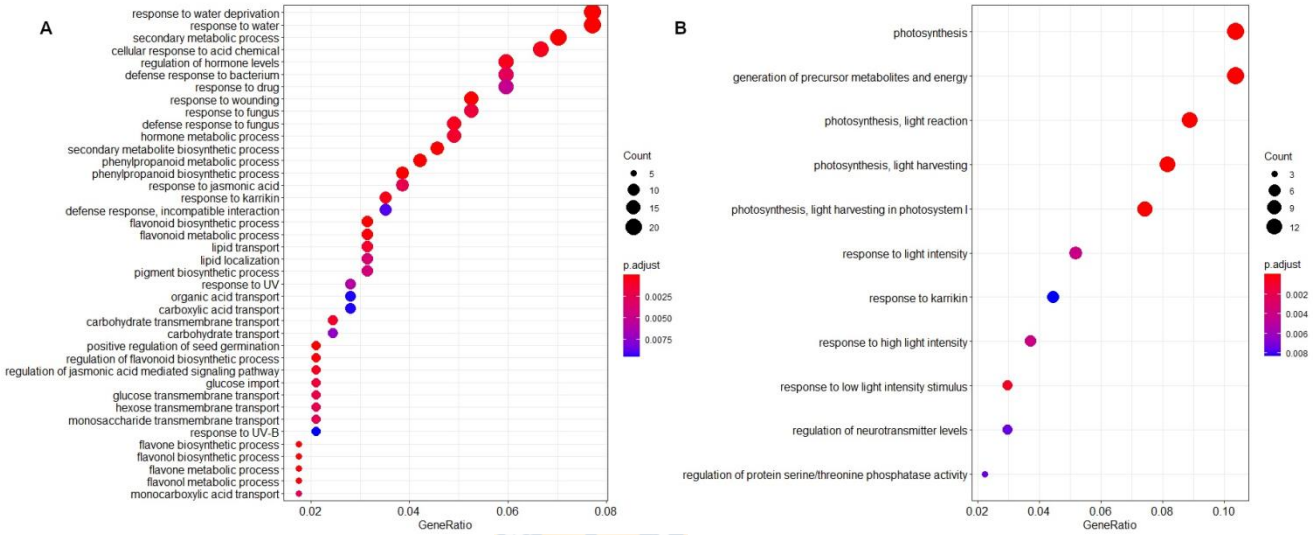

Fig. 1A) Gene ontology analysis of the RNA-Seq database, corresponding to upregulated genes from leaves of *Eucalyptus globulus* plants subjected to drought-stress treatment compared with well-watered plants. B) Downregulated genes from leaves of *Eucalyptus globulus* plants subjected to drought-stress treatment compared with well-watered plants

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. 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*).

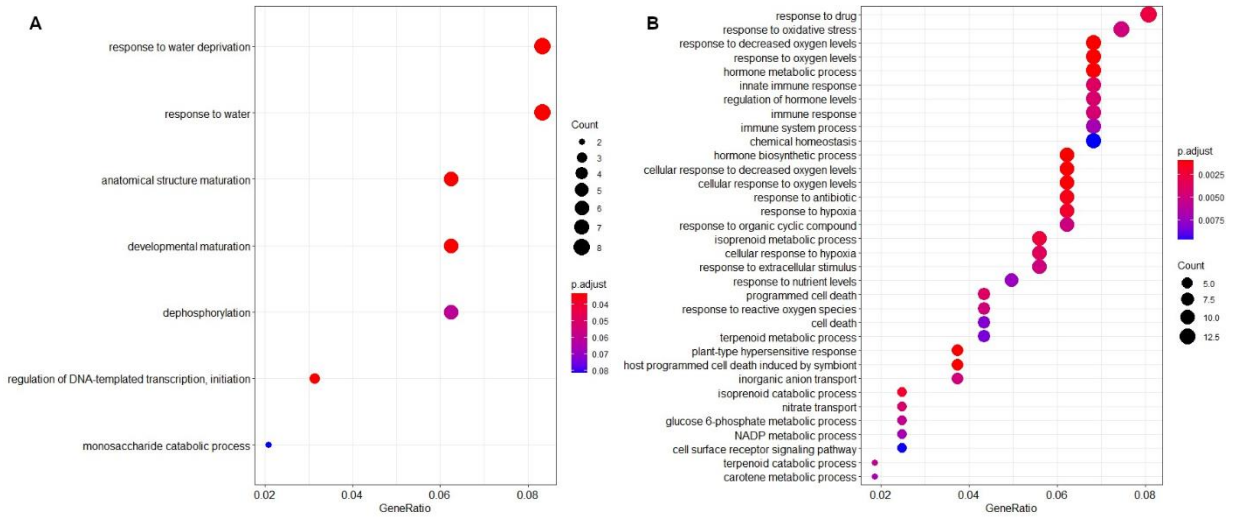

Fig. 2 A) Gene ontology analysis of the RNA-Seq database, corresponding to upregulated genes from root Eucalyptus globulus plants subjected to drought-stress treatment compared with well-watered plants. B) Downregulated genes from roots of Eucalyptus globulus plants subjected to drought-stress treatment compared with well-watered plants.

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* (Kang et al. 2020; 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 dioxygenase *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 and Chen 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). *AtAF*1, 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* (Dhar et al. 2020; 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, 2578 transcript sequences with logFC values higher than 2 were present in all comparisons of tissues (Fig. 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 2610 transcripts with logFC values lower than − 2 that were present in all comparisons (Fig. 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.

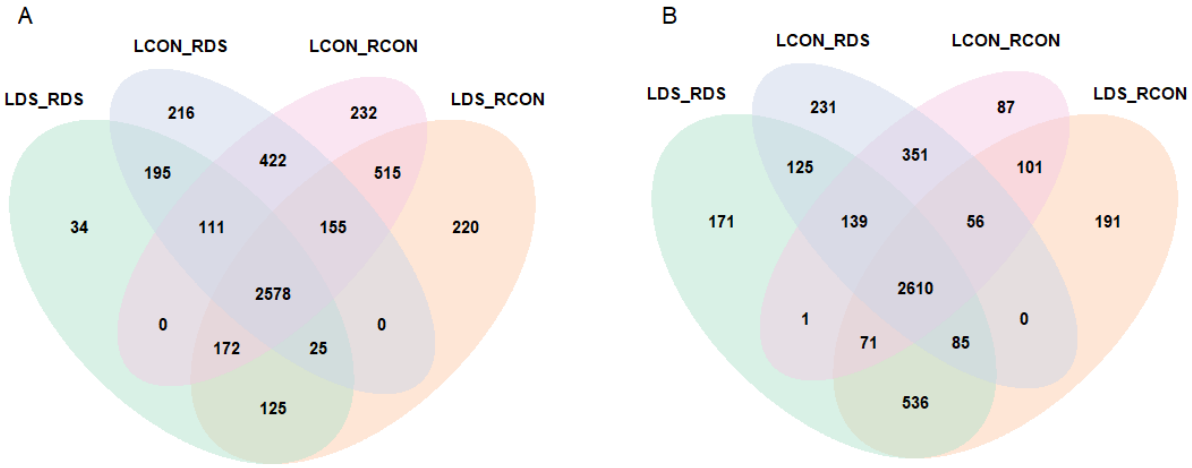

Fig. 3 Number of DEGs in leaves and roots of *E. globulus* plants under drought stress (DS) and control well-watered conditions (CON) in an RNA-Seq study. The results correspond to comparisons between leaves versus roots. **A:** Venn diagram with DEGs with LFC values higher than 2. **B:** Venn diagram 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.

Relative expression of candidate genes in greenhouse experiments

To validate some of the DEGs by qRT-PCR, the droughttolerant (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 1). However, for the T genotype, this parameter decreased slower than in the case of the S genotype, reaching a Ψpd 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 eightfold decrease in this variable at SDS compared with the CON treatment, while gs was threefold 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).

Table 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		Maximum 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) $p < 0.05$

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 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%).

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. 4a). The LFC value of the T genotype was almost twofold 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. Julke et al. (2016) identified that this gene is induced by salt stress in the 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. 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. 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 7.25 . 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 h 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. 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 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).

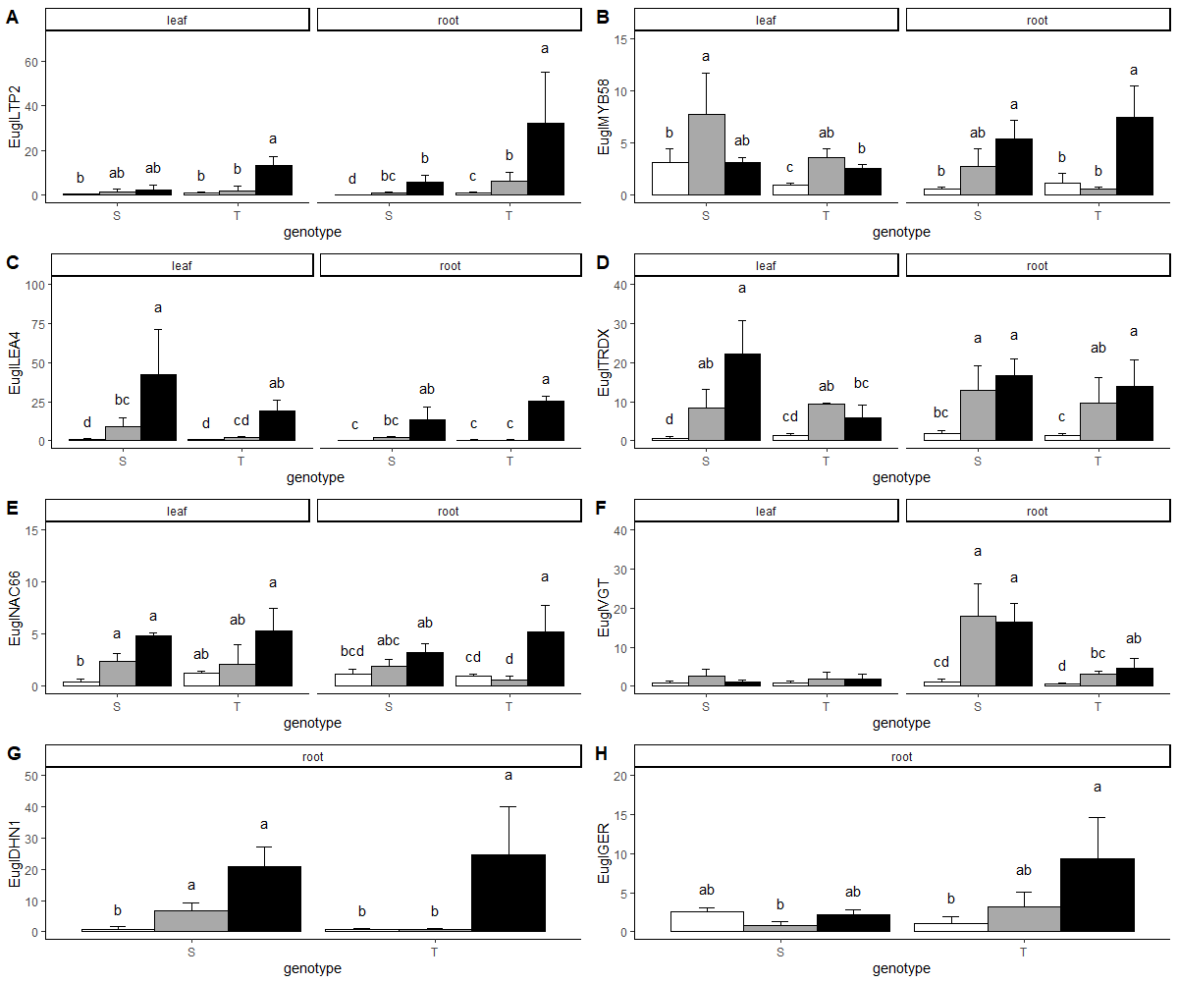

Fig. 4 Relative expression of genes in leaves and roots of drought tolerance contrasting E. globulus genotypes. The experiment consisted of maintaining plants in a well-watered regime (CON; white), intermediate drought stress (IDS; dark gray) and severe drought stress (SDS; black)). Letters over bars 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. 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 signalling 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 the roots of both genotypes. According to LFC values for

this gene, the S genotype had 1.8 . and 1.4 . 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. 4f). This sequence was associated with the *Atg162660* gene from *A. thaliana*, known as *AtVGT1*, which is expressed in the tonoplast membrane (Aluri and Buttner 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. 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. 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).

Conclusions

This study reports changes in transcriptomic response in leaves and roots of a drought-tolerant *Eucalyptus globulus* genotype. We identified 1124 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.

Author contribution statement JLU designed the experiments for drought stress; JLU, PA contributed in the manuscript draft, data analysis and interpretation, performed the molecular experiments, collected the samples, performed RNA extractions and qRT-PCR analysis. DC performed part of the bioinformatic analysis. RR and CB helped with the physiological and genetic data analysis. SV is the PI conceived and supervised the research.

Supplementary Information The online version contains supplementary material available at https:// doi. org/ 10. 1007/ s00468- 021- 02241-5.

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Availability of data and material The data have been submitted to the NCBI. The Bioproject ID related to this paper is PRJNA48629. Code availability Not applicable.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval Not applicable.

Consent to participate All the authors contributed in the manuscript elaboration.

Consent to publication All authors have read and approved the manuscript for publication.

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Supplemental 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

Table. S 1 Accession numbers of SRA files used for RNA-Seq study on drought-stressed samples of six-month-old seedlings from the tolerant genotype T of *E. globulus*.

Biosample	Description	SRA Accession	Replicate
SAMN12401664	Plant root sample DS - from *Eucalyptus globulus*	SRR10768429	r1
		SRR10803491	r2
		SRR10958733	r3
SAMN12401737	Plant leaves sample DS - from *Eucalyptus globulus*	SRR9887586	r1
		SRR9888129	r2
		SRR9888508	r3
SAMN09843459	Plant sample NA - Leaf from *Eucalyptus globulus*	SRR7726470	r1
		SRR7726471	r2
		SRR7726474	r3
SAMN09843446	Plant sample NA - Root from *Eucalyptus globulus*	SRR7726477	r1
		SRR7726466	r2
		SRR7726467	r3

Table. S 2 Sequence of primers used in qRT-PCR for gene expression analysis

Accesion	Forward	Reverse	Code
Eucgr.F02901	GGTTTGGATCGCTTGGCTTG	TGATGGACCCGATAATGGCG	*EuglIDH*
Eucgr.C03418	TCTCTGCCACAATGCCACCTG	TCATCACGCTTCACAAGAATCCTAAC	*EuglEIF4A1*
Eucgr.F01693	TGGTGCGTCCCCTTTCGTC	GCTGGTGGTCTCGTCGTCTC	*EuglTRDX*
Eucgr.D02029	GGCGGAACCCTACCCAAGC	CACGACCAGCAACACGGAAAG	*EuglVGT1*
Eucgr.J01345	GGTAGCCACTGACACCAAAGC	ATTGTTTCTTACCCTGCCAGTATCG	*EuglNIP*
Eucgr.I01201	CTGCTTGCGGTTCTCATCTTCC	AAGTGCTTGCTTCCTGTGTTCG	*EuglHNL*
Eucgr.D02045	CCACCATCAAAGCCGCACAAG	CGCACACGCCCAGTTTAAGG	*EuglDRN1*
Eucgr.B03987	TCGAGCCCAATCTCTTGACT	ATAGCATCGCCAAAACAAGG	*EuglGOLS*
Eucgr.A02070	CTTCCATGAGGTTGGACGAT	AACGTGATTGTTGGGGATGT	*EuglNAC19*
Eucgr.K01836	GCCCAGGAGAAGATGGAGA	CTTGTTGAGCTCGGCCTGAT	*EuglLEA4*
Eucgr.F01091	GTGGGTCCTGTGCCGAATCTAC	CCCGTGGCTCTGTTCCTTGC	*EuglNAC65*
Eucgr.F03054	CCATAGTGCTTGCTTGCTCCTC	GGCGTAGACACAGTAGACCAATGC	*EuglTIP*
Eucgr.I02395	GGCGGTGGCGGAATGCTC	TGCTGCTGCTGCTCATCGTC	*EuglDHN1*
Eucgr.F01093	GTGGGTCCTGTGCCGAATCTAC	CCCGTGGCTCTGTTCCTTACTC	*EuglNAC66*
Eucgr.D00751	ATGGGAAGGACGCCGTGTTG	CGTAGCCGTGCCTCTGGATG	*EuglMYB58*
Eucgr.I00058	GAGAGAGAAGCAAGTGTTGAGGTG	ACAAGAGGTGACATGATTCCAAGC	*EuglNAC126*
Eucgr.G02486	GCTGGATGATTGGGTGCTGTG	CGCCTGGACATTATGGTTGGATTC	*EuglNAC115*
Eucgr.D01633	GGGCAATCACAACCATAGTCATCTC	AGCCACACAACGGACTCAGC	*EuglGER*
Eucgr.L01908	ACACAAGAATGACTCACAACTCTCG	AAGATGGACCAGGGCACAACC	*EuglLTP2*

Table. S 3 Comparison of gene expression obtained by RNA-Seq and qRT-PCR using 15 putative genes that respond to drought stress in leaves of a tolerant *Eucalyptus globulus* genotype (T) subjected to drought stress treatment (DS) compared to control well-watered(CON). The data used for statistical analysis were reads per kilobase per million mapped reads (RPKM) values in case of RNA-seq and relative units of transcript abundance in case of qRT-PCR.

		RNA-Seq							qRT-PCR						
Accession	**Gene**	**CON**		**DS**					**CON**		**DS**				
Phytozome	**name**	**mean**	**st. Dev.**	**mean**	**st. Dev**	**logFC**	**p**		**mean**	**st. Dev.**	**mean**	**st. Dev**	**logFC**	**p**	
Eucgr.F01693	*EuglTRX*[3]	78	40	11777	4682	7.24	0.0495	*	1.11	0.61	48.79	9.54	5.46	0.013	*
Eucgr.D02029	*EuglVGT1*[4]	803	157	22486	10089	4.81	0.0204	*	1.017	0.22	8.48	2.97	3.06	0.048	*
Eucgr.J01345	*EuglNIP*[5]	288	202	545	227	0.92	0.22		1.013	0.21	1.76	0.23	0.81	0.014	*
Eucgr.I01201	*EuglHNL*[9]	6871	1378	332	85	-4.37	0.014	*	1.21	0.84	0.19	0.03	-2.68	0.169	
Eucgr.D02045	*EuglDRN1*[10]	513	317	9	8	-5.89	0.11		1.03	0.3	0.01	0	-6.73	0.027	*
Eucgr.F01093	*EuglNac66*[1]	229	82	1717	116	2.91	<0.0001	*	1.04	0.32	20.38	15.36	4.29	0.065	
Eucgr.I00058	*EuglNac126*[1]	321	97	1872	391	2.54	0.0026	*	1.01	0.18	5.08	1.91	2.33	0.065	
Eucgr.G02486	*EuglNac115*[1]	359	102	1950	860	2.44	0.03	*	1.08	0.55	5.81	4.33	2.43	0.19	
Eucgr.E01031	*EuglMyb68*[2]	61	38	324	262	2.42	0.16		1.19	0.68	4.37	0.37	1.88	0.002	*
Eucgr.K01836	*EuglLEA4*[7]	54	17	998	599	4.21	0.11		1.17	0.75	3.89	2.63	1.74	0.16	
Eucgr.F01091	*EuglNAC65*[1]	411	85	748	219	0.87	0.016	*	1.71	1.9	8.39	8.17	2.29	0.002	*
Eucgr.A02070	*EuglNAC*[1]	2654	300	6034	1435	1.19	0.016	*	1.16	0.68	3.97	1.25	1.78	0.002	*
Eucgr.B03987	*EuglGOLS*[6]	10532	3742	29753	2752	1.5	0.002	*	1.27	0.92	3.2	3.16	1.33	0.37	

Eucgr.I02395	*EuglDHN1*[8]	36455	9928	67136	55759	0.88	0.4	1.19	0.8	8.86	9.44	2.89	0.29	
Eucgr.F03054	*EuglTIP*[5]	2108	318	1628	1166	-0.37	0.52	1.01	0.17	0.36	0.04	-1.47	0.003	*

(*) significant differences according to T-Test Analysis, p-value (<0.05).

Nomenclature according to Hussey et al 2015 [1], Soler et al 2015 [2], Kang et al 2020[3], Aluri and Büttner 2006[4], Bezerra-Neto et al 2019[5], Sengupta et al. 2012 [6], Cuevas-Velasquez, et al. 2017 [7], Fernández et al 2012 [8],Rao et al 2021[9], Dhar et al 2020[10]

Table. S 4 Log fold change (LFC) values of the relative expression of drought-stress response genes from E. globulus plants subjected to a drought stress experiment in a greenhouse. The measures were performed using the qRT-PCR analysis. Two sources of plant material were evaluated: leaf and root. Two genotypes of E. globulus were used, a tolerant (T) and a susceptible (S) genotype, regarding their drought survival measures. Two treatments were established: intermediate drought stress (IDS) and severe drought stress (SDS). Each treatment was compared with a wellwatered control (CON). Eight DEGs were selected according to RNA-Seq study.

Tissue	genotype	treatment	*EuglTRDX*	*EuglVGT1*	*EuglLTP2*	*EugMYB58*	*EuglNAC66*	*EuglLEA4*	*EuglDHN1*	*EuglGER*
Leaf	T	IDS	2.72	0.92	1.20	1.85	0.74	1.63	NA	NA
Leaf	T	SDS	2.05	0.87	3.88	1.36	2.08	4.67	NA	NA
Leaf	S	IDS	3.87	1.41	1.58	1.29	2.56	2.96	NA	NA
Leaf	S	SDS	5.28	0.26	2.05	-0.04	3.56	5.18	NA	NA
Root	T	IDS	2.84	2.31	2.73	-0.99	-0.78	0.27	-0.30	1.55
Root	T	SDS	3.38	2.90	5.06	2.73	2.34	5.79	4.80	3.09
Root	S	IDS	2.71	4.09	3.87	2.26	0.63	2.96	2.83	-1.74
Root	S	SDS	3.07	3.95	6.05	3.23	1.41	5.80	4.48	-0.26

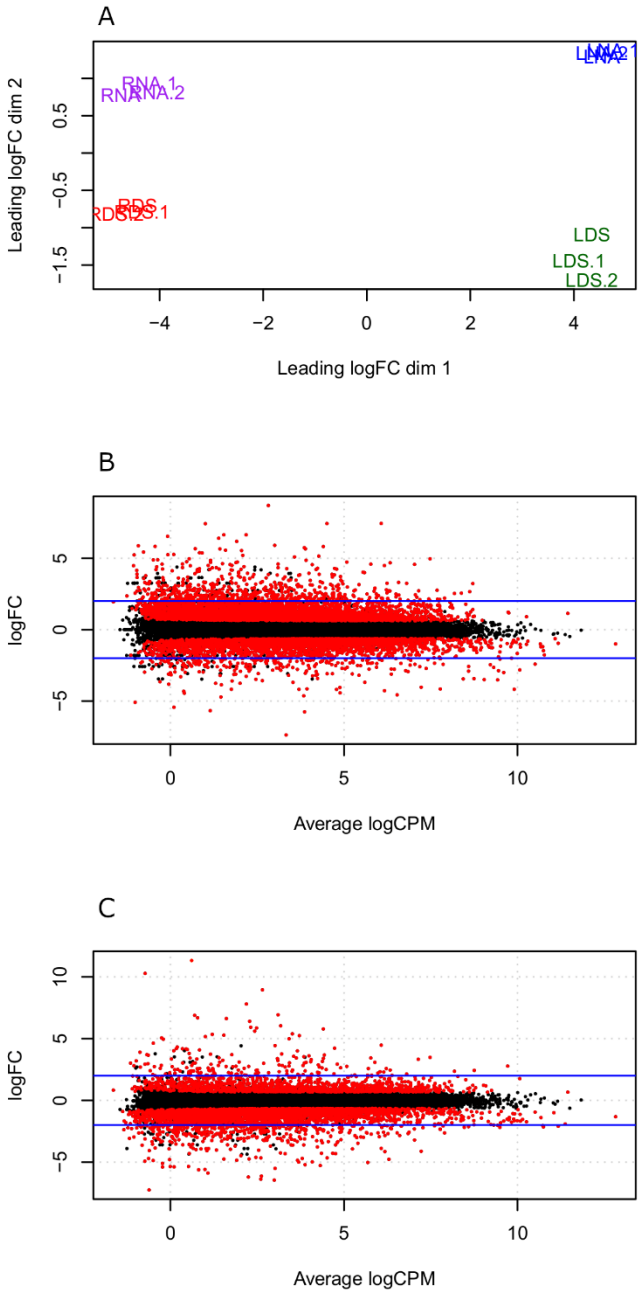

Fig.S 1 A) Multidimensional scaling analysis of the drought-stress experiment using RNA-Seq technology. Samples correspond to E. globulus seedlings. From 4 libraries, leaf control (LNA, blue), leaf drought-stress treatment (LDS, green), root control (RNA, purple) and root drought-stress treatment (RDS, red) are plotted. B) and C): Smear plots that relate log fold change values (logFC) against log counts per million (logCPM), representing differentially expressed genes (DEGs) for leaves and roots, respectively. Blue lines show the threshold for logFC values <-2 or > 2. Red dots are genes with FDR and p-values higher than 0.05.

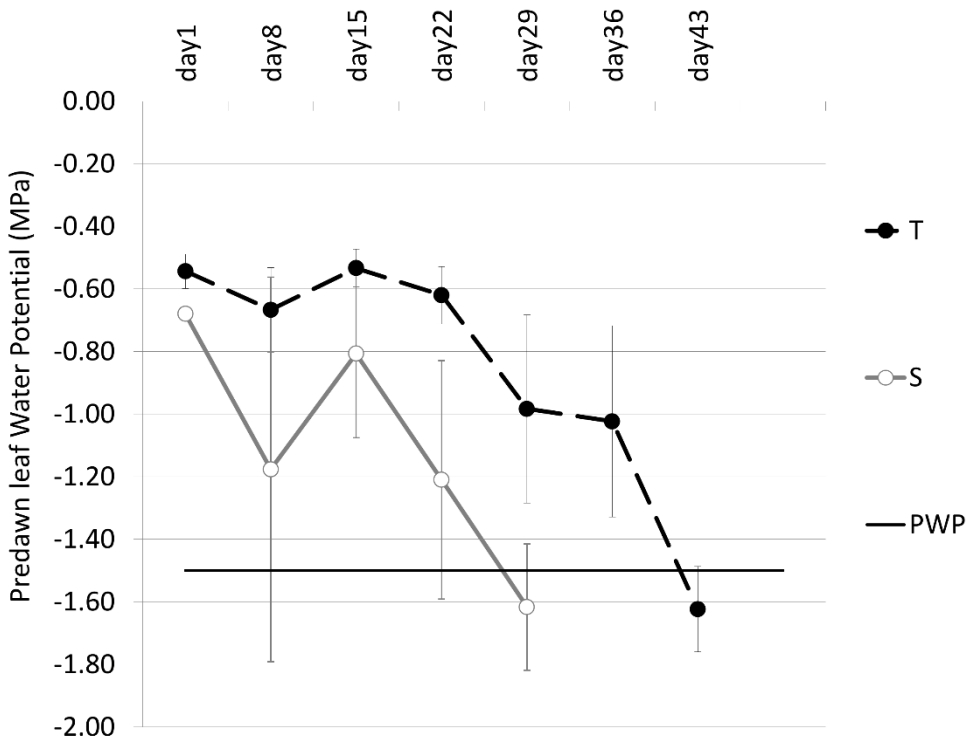

Fig.S 2. Predawn water potential (Ψpd) for each E. globulus genotype subjected to drought-stress treatment. Since the beginning of the measurements with a Scholander pressure chamber, the water potential is presented in Mega Pascals, and the date of sampling is displayed on the X axis. Gray lines indicate the susceptible genotype (S), and dashed black lines correspond to the tolerant genotype (T). The continuous horizontal black line is the theoretical threshold for the permanent wilting point (-1.5 MPa).

CAPITULO II

Transcription factors related to drought stress response in *Eucalyptus*

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Declarations

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Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' contributions JLU wrote the first draft of the manuscript, and all authors contributed to all versions of the publication. All authors have read and approved the manuscript for publication. SV is the PI and supervised the written review.

Ethics approval: Not applicable

Consent to participate: All the authors contributed to the manuscript elaboration.

Consent to publication: All authors have read and approved the manuscript for publication.

Key message: Summarize information regarding to drought tolerance mechanisms associated to transcription factors families in forestry hardwood species, especially *Eucalyptus*

Abstract

Eucalyptus is one of the most important genera of hardwood forestry species, mainly due to its excellent wood properties for pulp and other industrial purposes. However, climate change is affecting the establishment, yield, health and survival of plantations, due to the incidence of longer and more severe droughts caused by global warming. For this reason, to ensure forestry resources for the future, genetic improvement is key to develop productive genotypes that can tolerate abiotic and biotic stress. Therefore, it is necessary to understand the mechanisms involved in drought response in trees to develop strategies to obtain more drought tolerant varieties of this species. One of the mechanisms associated with drought tolerance is related to signaling and "switching on" the genetic response in different tissues by transcriptional factors (TFs), proteins that control expression of target genes, which can be involved in drought stress tolerance acquisition. Some of the most important TFs in abiotic stress response are NAC, MYB, WRKY, bZIP, bHLH, DREBs, among others. The interaction between different TFs is a complex network that induces morphological or biochemical changes to improve plant survival against drought stress. The main objective of this review is to give an overview of the main mechanisms and role of TFs in response to drought stress in *Eucalyptus* spp. and other hardwood species.

Introduction.

Eucalyptus sp. is one of the most planted forest species in the world due to its excellent wood quality for the pulp industry, short rotation period and adaptability to a wide edaphoclimatic range. There are over 700 eucalyptus species known to date, but only nine

have been used as commercial plantations (de Toledo et al., 2021). Among them, *E. globulus*, *E. grandis*, *E. urophylla*, *E. nitens* and their hybrids are the most important (Booth, 2013; Saadaoui et al., 2017), although some of them are susceptible to extreme climate conditions such as drought or salt stress (Mitchell et al., 2013; Rubilar et al., 2018). In this context, rising temperatures caused by an increase in CO_2 due to anthropogenic activity, produce changes in the pattern of rain globally, causing that some areas will have more and longer periods of droughts (Ault, 2020; Zhang et al., 2016). Consequently, decrease in precipitation and rise in evaporative demands have an impact on the establishment and survival of plant species (Vicente-Serrano et al., 2020). The main impacts of drought on forest plantations are loss of productivity due to the reduction in growth and tree survival, higher susceptibility to insect attack, diseases, fires and tree mortality (Choat et al., 2012; Hamanishi and Campbell, 2011).

The study of drought stress mechanisms involves molecular responses, being the genomic information crucial to comprehend the pathways being part of the plant response to stress. In the past decades sequencing and next-generation sequencing have been powerful tools to sequence genomes, *Populus trichocarpa* was the first tree species sequenced in 2006, since then more than 50 species have been sequenced, including *E. grandis* in 2014 (Myburg et al., 2014; Tuskan et al., 2006; Wegrzyn et al., 2019). This same technology has been employed to identify gene expression by using RNAseq in different plant species under a specific environmental or growth condition (Vlk and Řepková, 2017). In the case of trees under drought conditions, Villar et al. (2011) studied the response of gene expression in two eucalypts genotypes subject under water deficiency, where the transcript data showed that a larger number of genes were highly expressed in the most productive genotype,

which also has a better water use efficiency. Although there are a large number of publications regarding drought and molecular response in plants, in trees, studies are limited, being the main aim of this review is give an overview of the main mechanisms of response to drought regarding TFs in Eucalyptus spp. and other hardwood species.

How do woody plants respond to drought?

According to Ault (2020), drought is a period of time in which the supply of moisture is insufficient to satisfy its demand. There are various definitions of drought, depending on the objective of study. In particular, agricultural droughts imply economic losses and can last for months, whereas, hydrological droughts are characterized by depletion of stream flows or reservoir levels and occur on a scale of seasons or years (Ault et al., 2020). Droughts induce water limitation in plants, for this reason they must carefully manage this resource to survive (Kapoor et al., 2020). Water is essential to maintain almost all biochemical reactions for life, it is the solvent and transport media for nutrients, keeping plant morphology and architecture, among other functions (Baker et al., 1992).

Plants respond to drought by avoidance or tolerance strategies (Polle et al., 2019). Avoidance consists in keeping the normal physiological activity, to tolerate a moderate stress, adjusting plant structures at morphological level or setting growth rate to maintain higher water potential inside the tissues, reducing transpiration or keeping intake of water (Khan et al., 2016). Some examples of avoidance are stomata closing, accumulation of cuticle waxes, and changes in proportion of aerial–root tissues, secondary growth and wood development (Van Oosten et al., 2016). On the other hand, tolerance is the process in which plants keep their growth and productivity under low water potential in plant tissues. This

involves expression on thousands of transcripts, and activation of gene networks to reduce or repair damage caused by water limitation (Khan et al., 2016). Some examples are osmotic adjustment, scavenging of reactive oxygen species (ROS) by production of antioxidant enzymes and compounds, synthesis of protective protein such as chaperons like LEA or dehydrins, changes in membrane composition (Bartels and Sunkar, 2005).

Finally, another strategy adopted by some species is known as escape, which consists in the adjustment of maturity rate, accelerated phenological cycle and mobilization of assimilated carbohydrates and nutrients, to grow and reproduce before the severe drought occurs (Choat et al., 2005). Examples of this strategy are drought-deciduous species, which survive in arid regions however they posses morphological structures of a drought susceptible plant, since they change their growth period only if there is water availability stopping the metabolism in drier seasons by losing leaves (Choat et al., 2005).

Tissue specific response to drought

Foliar tissues of plants under drought stress suffer many changes that modify their metabolism, for example photosynthesis rate is affected by low water availability, the stomatal closure reduces transpiration rate with less net carbon assimilation as consequence (Kapoor et al., 2020). Leaves start to synthesize osmoprotectant compounds such as sugars with low molecular weight, proline and other compatible solutes (Correia et al., 2014). ROS produced by drought induce up-regulation of enzymes as Superoxide dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX) or other protectants such as glutathione (Figure 1)(Correia et al., 2018; Shvaleva et al., 2006; Silva et al., 2004).

Conductive tissues on woody plants are determinant to acquire resistance to drought stress, since decreasing water potential in soil can induce extreme forces inside the water columns in the xylem vessels producing its disruption, phenomena known as cavitation (Figure 1), that is one of the main causes of hydraulic failure and death in adult trees (Choat et al., 2012). Therefore, some species increase thickening of vessels, decreasing the lumen for water flow, which reduces the risk of this problem (Hacke et al., 2001; Pineda-García et al., 2016) In young individuals, green stem cortex helps seedlings to keep physiological activities to produce photosynthates, given that stomatal closing inhibits photosynthesis in leaves. The products assimilated by this green phelloderm are translocated to different organs (Cernusak and Cheesman, 2015).

In the case of roots, taproots develop the growth of fine roots to increase higher water uptake from deeper soil (Brunner et al., 2015), whereas the increments in osmoprotectant compounds decrease water potential in root tissue to keep water absorption (Figure 1). When drought is severe, plants produce antioxidant compounds and changes in gene expression occur, which augment the production of protecting proteins (Van Oosten et al., 2016).

In sum, the information available for woody species regarding morphological changes during drought stress response helps us to understand the specific mechanisms at tissue levels that determine the most important strategies to overcome stress. At early stages of drought, plants limit efflux of water by avoidance strategy for example by stomata closure, synthesis of ABA, cuticles (Figure 1). But, when drought stress becomes severe, tolerance response is the strategy to induce expression of genes coding for proteins to reduce cell damage and allow them to survive with lower water potential inside tissues, those

mechanisms are ROS scavenging, protectant chaperons, osmotically active compounds, among others (Figure 1). Some of these morphological and biochemical changes have been previously reported in eucalypts plants under drought stress (Costa e Silva et al., 2004; Valdés et al., 2014; Granda et al., 2014).

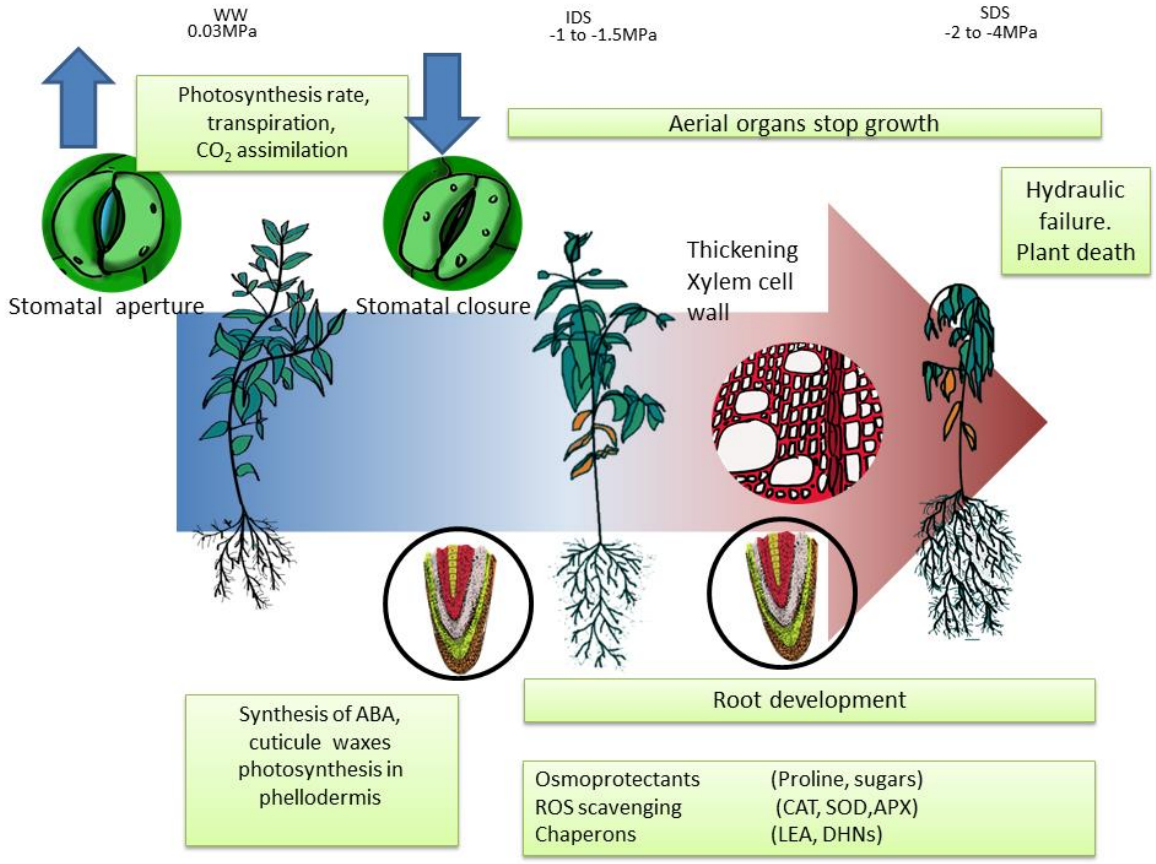

Figure 1 Schematic representation of successive events that occur in drought stress imposition in *Eucalyptus* seedlings to illustrate morphological and biochemical changes, according to water potential inside the plant tissues. Abbreviations: CAT, Catalase, SOD: Superoxide dismutase, APX: Ascorbate peroxidase, LEA: Late embryogenesis abundant proteins; DHN: Dehydrins; WW: Well watered; IDS: Intermediate Drought Stress; SDS: Severe Drought Stress. MPa: Water potential in Mega Pascals.

Gene network and signaling under drought

Drought stress response in plants depends on hundreds of genes with a small contribution to the global reconfiguration of the plant transcriptome to tolerate water loss (Lindemose et al., 2013). The molecular response to drought in plants consists of a complex network of genes that starts with the perception of the stress in plant tissues. There are two main pathways in signaling to drought stress, the first is led by abscisic acid (ABA) and the second is an ABA independent route. In the first place, ABA interacts with a protein complex formed by Pyrabactin resistance 1 (*pyr1*)/pyr1-like (*pyl*)/regulatory *PYR/PYL* protein, and C-2 domain ABA receptor *rCAR*, inducing a phosphorylation reaction to stimulate SNF1 related kinase s2 (*snRKLs)*, activating bZIP transcription factors (Figure 2), which control gene expression of target genes that are involved in processes such as root development or in the increment of cells and chloroplast present in leaves, among others (Diaz et al., 2016; Yoshida et al., 2014). On the other hand, ABA-independent route involves the expression of a TF known as DREB2 (Yoshida et al., 2014) which controls drought stress response genes (Figure 2). For example, in *P. euphratica* (an arid adapted specie) the gene *PeDREB2* is expressed by abiotic stress but not by ABA (Dong et al., 2014). Transgenic plants of *Nicotiana tabaccum* were more tolerant to drought, and were associated with the increment in transcript abundance of *ERD10c* and *ERD10d* genes (Figure 2), which code for LEA proteins (Chen et al., 2009).

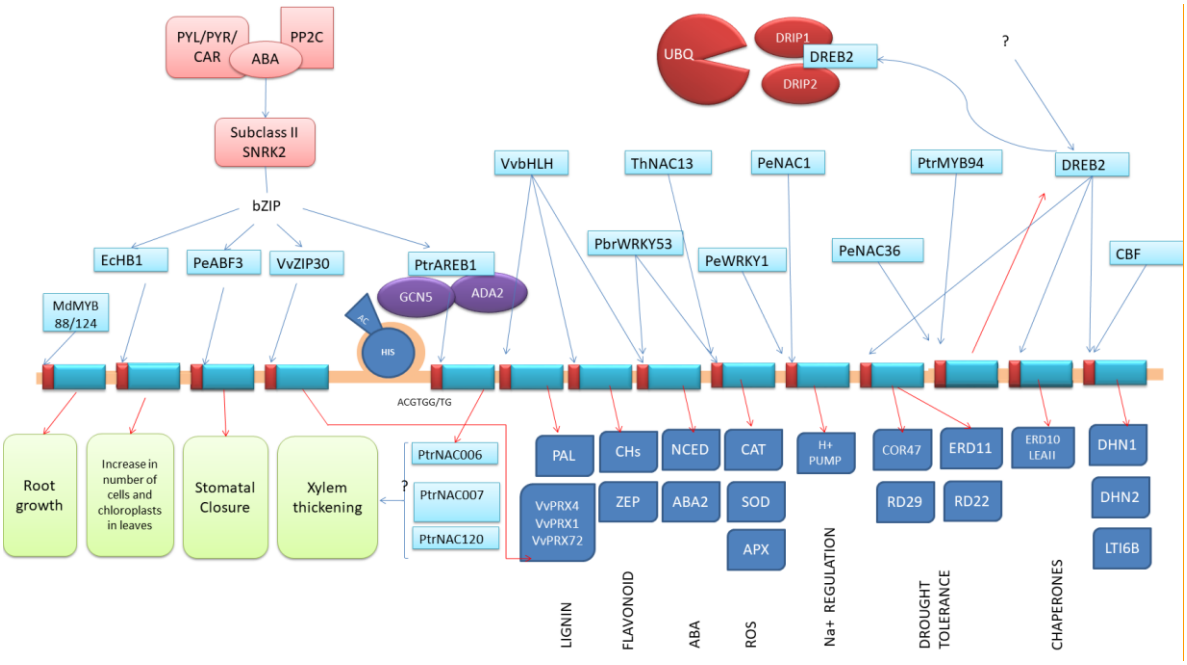

Figure 2 Gene network of drought stress response, describing the main molecular routes involving transcription factors (TFs) in woody species. Boxes represent genes formed by a coding region (sky blue) and promoters (red rectangles). TFs are represented by light blue squares, and specific binding sites are connected by a blue arrow. Signaling proteins are illustrated by red rectangles (ABA dependent signaling pathway). Green squares show morphological changes induced by TFs. Blue squares at bottom represent the products of target genes that are expressed by the control of TFs. Red arrows connect coding sequences with products of translation of mRNA. Elliptical elements represent proteins that interact with TFs.

TFs are proteins that control the expression of genes, regulating many processes in woody plants, from seed development, morphology, hormonal signaling, biotic and abiotic stress, among others (Pascual et al., 2018). They can recognize specific nucleotide sequences (cis elements) in DNA, binding to the promoter region leading the induction or repression of transcription, determining when, how much, and where, genes must be expressed (Pascual et al., 2018). Thousands of genes coding for TFs have been described in woody species, according to the plant transcription factor database (Jin et al., 2016). In *Populus* there are

ca. 2,900 TF genes, which represents 6% of the total genes identified in this specie (Hu et al., 2010); in *E. grandis* there are 2,163 genes coding for TF located in 1,731 loci and in *E. camaldulensis* 1,937 (1730 loci) have been described (Jin et al., 2016). Many TFs families, such as *MYBs, NAC, WRKY, bHLH, bZIP, DREB, CBF*, among others have been associated with drought stress response in several plant species (Supplementary Table 1).

TFs in Woody Species.

In general TFs present in plants are represented by several genes, which share common protein domains, which give them their characteristics. The NAC family (**N**on **A**pical Meristem, *ATAF 1*, **C**up shaped), which were the first genes identified of this kind, is one of the largest TF families in plants (Riechmann and Ratcliffe, 2000). They have a NAC domain consisting of ca. 160 amino acid (aa) residues in the N-terminal, and they are classified in five sub families in *A. thaliana* (Christianson et al., 2010). Studies in *P. trichocarpa* identified 163 full-length *NAC* domain proteins, grouped in 18 subfamilies according to phylogeny (Hu et al., 2010). These genes have been associated with control of morphological characteristics, abiotic stress response, senescence and wood formation, among others (Pascual et al., 2018). In *E. grandis* a *EuglNAC31* and *EuglNAC141* belonging to subfamily IVc, which are not present in herbaceous genomes such as *A. thaliana*, have been associated with secondary growth (Hussey et al., 2015). In *E. globulus* NACs are induced in leaves and roots under drought stress, such as *EuglNAC66* which is closely related with gene RD26 that is a well known drought induced gene of *A. thaliana* (Ulloa et al., 2021). Likewise, gene *EuglNAC126* was induced by drought stress in roots and leaves (unpublished data). This gene is related to *AtAF1* and is involved in drought tolerance induced by ABA (Lu et al., 2007).

Other over-represented TFs in plants under drought stress conditions correspond to the basic Leucine zipper (bZIP) family. These TFs are present in plants, animals and yeast, they are characterized by the presence of a conserved bzip domain of 40 to 80 aa and a highly conserved basic region composed by 16 aa with an invariant N-x7-R/K motif that binds to DNA and contributes to a leucine zipper dimerization (Zhao et al., 2016). In *A.*

thaliana there are 13 phylogenetic groups designated from A to M, where *ABF,* a bZIP TF belonging to subgroup A has been shown to be induced by ABA, drought and salinity (Yang et al., 2020). By direct binding to ABRE (ACGTGG/TC) cis-elements, *ABFs* regulates the expression of stress responsive target genes (Nakashima and Yamaguchi-Shinozaki, 2013; Uno et al., 2000). In *E. grandis,* 40 bZIP genes were identified, grouped in four families regarding a phylogenetic comparison with rice and *A. thaliana*. *EgHD-Zip27* and *EgHD-Zip37* were induced in eucalypt plants under salt stress (NaCl 100 mmol/L), suggesting an important role in saline stress response (Zhang et al., 2020)

WRKYs, a family of TFs characterized as having drought response elements, contain one or two highly conserved domains composed by 60 aa, having an hepta-peptide WRKYGQK followed by a zinc finger motif at the N terminus (Eulgem et al., 2000; Rushton and Somssich, 1998). WRKYs are classified in three groups and further subgroups according to the number of domains and the Zn-finger motif type (Eulgem et al., 2000). In *E. globulus* 51 complete sequences of WRKY transcription factors were identified (Aguayo et al., 2019), of which *EuglWRKY26 and EuglWRKY18* were highly induced by drought stress in leaves of *E. globulus* plants under drought stress in greenhouse experiments (unpublished data). *AtWRKY40,* orthologous gene of *EuglWRKY26* in *A. thaliana,* acts as a prerequisite for ABA signaling regulating the expression of other transcripts related to drought response (Geilen and Böhmer, 2015; Lindemose et al., 2013). *N. benthamiana* plants over-expressing *GhWRKY41* (ortholog to *EuglWRKY18*) increased drought and salt stress tolerance, when the transgenic plants were exposed to osmotic stress, acting as a positive regulator by enhancing stomatal closure and regulating ROS (Chu et al., 2015).

The MYB superfamily is the largest TF family in plants (Wu et al., 2019). They are characterized by having one to three helix-turn-helix motif in their structure, composed by 32 aa approximately, which has three tryptophan residues with certain distance that have regulatory function (Soares-Cavalcanti et al., 2008). MYB proteins can be classified into four subfamilies R1/2, R2R3, R1R2R3, and 4R (Wu et al. 2019). R2R3 corresponds to the most diverse subgroup and they are involved in several processes such as morphology, development and stress response (Dubos et al., 2010). The sub-family R2R3, is composed of 190 genes predicted in *P. trichocarpa*, (Wilkins et al., 2009). In *E. grandis* there are 141 MYB transcription factors (Soler et al., 2015). *In E. globulus* plants under an intermediate drought stress treatment, *EuglMYB58* showed higher transcript abundance in a tolerant than in a susceptible genotype (Ulloa et al., 2021). *EuglMYB58* is the ortholog gene of AtMYB102, which has been associated with osmotic stress response (Denekamp and Smeekens, 2003).

bHLH is the second largest class of TFs, present in yeast, animals and plants (Pires and Dolan, 2010)). In *A. thaliana* there are 162 genes coding *bHLH* TFs (Bailey et al 2003). The structure of this TFs is defined by a *bHLH* domain, that is highly conserved and comprises 60 aa with 2 functional basic regions at the N-terminus, which contains 13-17 basic aa and binds to the DNA motif CANNTG. In *E. grandis* 153 *bHLH* sequences, clustered in 16 groups according to evolutionary relationship have been identified. Wang et al. (2016), identified a TF which contained a *bHLH-MYC* domain in *Vitis vinifera*, called *VvbHLH*. *A. thaliana* plants over-expressing *VvbHLH1* were associated with higher activity of ROS scavenging enzymes, flavonoid and ABA biosynthesis resulting in higher drought tolerance than the WT plants. On the other hand, overexpression of *PebHLH35* from *P.*

euphratica, in *A. thaliana* plants showed an increase in the chlorophyll content and photosynthetic rate, while, under drought stress a stronger induction of genes *FAMA* and *PLC1* on OE plants were identified (Dong et al., 2014).

A schematic representation of the interaction of TFs involved in drought stress is presented in figure 2. This map was built with information available of TFs in trees (supplemental table 1). Briefly, TFs are activated and interact with cis elements present in the promoter of target genes, controlling the expression of genes coding for proteins such as those involved in osmoregulation, chaperons, ROS, Na+ homeostasis, among others (figure 2). Furthermore TFs are regulated by proteins that activate its function, for example PtrAREB1 needs the interaction with the proteins known as alteration/deficiency inactivation 2 (ADA2) and general control non depressible 5 (GCN5) to form a complex that increases acetylation in histones located close to the promoter region of its target genes (figure 2) (Li et al 2019). On the other hand, DREB2 interacts with proteins DRIP1 and DRIP2, facilitating their degradation by the Ubiquitin proteasome (figure 2) (Qin et al., 2008). Some of these TFs have been used in transgenic plants, mainly in *A. thaliana* or *P. trichocarpa* conferring drought tolerance (Supplementary table 1), being a promising strategy to obtain genetic engineered trees with higher drought tolerance in the future.

Conclusion

Studies presented here suggest that TFs are main regulators of molecular response to drought in trees, controlling the expression of target genes involved in mechanisms to avoid or tolerate drought stress such as morphological or biochemical changes in woody species, mainly in *Eucalyptus spp.* and *Poplar spp*. However, further studies are needed to better

understand drought stress response to obtain varieties that will ensure forestry production, using in a more efficient way the scarce water resources available under the current scenario of climate change.

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Supplementary Table 1

Supplementary Table 1**.** TFs involved in drought stress response in woody species. Abbreviations **DS**: Drought stress; **OE**: Over Expression; **RNAi**: Supression of transcription via RNAi; **WT:** Wild type.

Gene Name	Family	Specie	Target genes	Regulation	Observation	Reference
PeNAC036	NAC	*P. Euphratica*	*COR47, RD29B, ERD11, RD22, DREB2A*	Induced by DS	OE in *A. thaliana* confers Drought tolerance.	Lu et al. (2017)
PeNAC1	NAC	*P. Euphratica*		Induced by abiotic stress. There are MYB, MYC,W boxes, GA-responsive elements, NAC in its promoter.	Higher drought tolerance in OE in *A. thaliana* plants. OE in *A. thaliana* showed down-regulation of *AtHKT1*	Wang and Wang (2013), Wang et al. (2016b)
PeNAC070	NAC	*P. Euphratica*		*peu-miR164* (cleavage)	DS t and salt stress susceptibility	Lu et al., (2017)
PtrNAC007 *PtrNAC007* *PtrNAC120*	NAC	*P. trhichocarpa*		Abiotic stress, *PtrAREB1/GCN5/ADA2* (tertiary complex), Epigenetic control	OE results in smaller lumen area in xylem, higher tolerance to DS.	Li et al. (2019)
ThNAC13	NAC	*Tamarix*		Salt stress	Confers tolerance to Salt stress in OE *A.*	Wang et al. (2017)

		hispida			*thaliana*	
PtrAREB1-2	bZIP	*P. trichocarpa*		Induced by DS	Induce Expression of NAC genes	Li et al. (2019)
VlbZIP30	bZIP	*hybrid (Vitis labrusca × V. vinifera)*	*VvNAC17*	Induced by DS. *VlbZIP30.* Binding site ACGYGKC	OE plants higher drought tolerance and induce Lignin pathway genes: *VvPRX1,VvPRX4, VvPRX72* and *VvCCoAOMT*	Tu et al. (2020)
PeABF3	bZIP	*P. tormentosa*	*PeADF5*	Induced by DS	Actin depolymerization cytoskeleton oclusive cells,OE showed lower stomatal aperture	Yang et al. (2020)
EcHB1	bZIP	*E. camaldulensis*		Induced by DS	OE in Hybrid *E. grandis x E- urophylla*, modify mesophyll anatomy, more cells and chloroplasts	Sasaki et al. (2019)
PeWRKY1	WRKY	*P. euphratica*	*PeHA1*	Binding sites TTGAC and GTCAA	Induce Proton Pump, involved in balance of Na+	Yao et al. (2020)
PbrWRKY53	WRKY	*Pyrus betulaefolia*	*PbrNCED1*	Induced by DS and ABA. Repressed by cold. Binding site TTGATC	Involved in ABA biosynthesis, OE result in more chlorophyll, ABA, Ascorbic Acid and ROS scavenging enzymes (SOD, POD and CAT)	Liu et al. (2019)
PtrMYB94	MYB r2r3	*P. trichocarpa*	*ABA1,DREB2B, P5CS2, SRK2C, ADH1, KRP2*	Induced by DS	transcriptional activator, OE lines mor tolerant to DS	Fang et al. (2020)

MdMYB88 and MdMYB124	MYB r2r3	*Malus domestica*	*MdVN6 MdMYB46*	Expressed in roots. Binding site AACCG.	Related to DS tolerance by modeling root architecture, OE plants showed higher hydraulic conductivity. RNAi plants decrease expression of genes involved in secondary cell wall synthesis	Geng et al. (2020)
VvHLH	bHLH	*Vitis Vinifera*		Induced by Salt and Osmotic Stress	OE in *A. thaliana* plants induce: *AtPAL, AtCHS,AtCHI,AtZEP,AtNCED, AtABA2*	Wang et al. (2016a)
PebHLH	bHLH	*P. euphratica*		Induced by DS and ABA	OE in plants lower transpiration, slower *fv/fm* decrement in DS treatment compared to WT.	Dong et al. (2014)
DREB2	DREB2	*P. euphratica*	*ERD10c* and *ERD10d*	*DRIP1* and *DRIP2* induce degradation by ubiquitin proteasome.	OE in plants induce genes *FAMA* and *PLC1* *N. tabaccum* overexpression get more tolerant plants	Chen et al. (2009)
PagWOX11 /12a	WOX	*Populus alba* × *Populus glandulosa*		It is controlled by *PagERF35* TF (Induced by Drought)	OE plants more and longer roots, higher tolerance than WT	Wang et al. (2020)
PeNF-YB7	NF	*P. Euphratica*	*CBF4, COR15B, LEA76, BAM5,* and *GST*	induced by drought in leaves	OE in *A. thaliana* showed higher water use efficiency , lower transpiration, higher photosynthetic rate and longer roots than WT	(Han et al., 2013; Li et al., 2008)

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DISCUSIÓN GENERAL

En relación al potencial hídrico foliar de prealba, es posible evidenciar que los genotipos de mayor susceptibilidad a la sequía, disminuye más rápido respecto a genotipos tolerantes, esto se ve reflejado también en los trabajos de Silva, y colaboradores (2004). Donde se observa, que desde la tercera semana de tratamiento de estrés por sequía, el genotipo de menor tolerancia posee menor Ψpd. Por otro lado, también se reportan diferencias de comportamiento, en cuanto a la adaptación a sequía, que varía entre genotipos dentro de la especie *E. globulus* (coopman et al 2008, Pita et al 2001).

En cuanto a la conductancia estomática, es posible identificar en otras especies de *Eucalyptus* sp. un comportamiento similar al observado en *E. globulus*, donde la noche se evidencia el cierre estomático, y durante el día hay mayores niveles de conductancia, especialmente durante la mañana (Lewis et al 2011). Igualmente, en otras especies, se reportan variaciones diurnas de la conductancia estomática, evidenciando una mayor actividad durante las horas de la mañana y disminuyendo hacia el atardecer, como un mecanismo adaptativo en la especie *Aristolochia macrophylla*, para prevenir el embolismo de los vasos xilemáticos en condiciones de estrés (Miranda et al, 2013). Es común encontrar, que durante el ciclo circadiano, las plantas experimenten cambios en cuanto a su balance hídrico, que varía de favorable durante las horas nocturnas a desfavorables durante el día, lo cual rige un patrón de crecimiento diário (Caldeira et al 2014). Por otra parte, es evidente en algunas especies leñosas, que la conductancia estomática se reduce en relación al potencial hídrico en la planta, con el fin de reducir el riesgo de embolismo o cavitación en condiciones de estrés (Vilagrosa et al 2003).

En algunas especies, como híbridos de *Populus deltoidesX Populus nigra*, existe una amplia variabilidad genotípica relacionada a la tolerancia a estrés hídico y rendimiento, generalmente aquellos genotipos de mayor productividad poseen menos tolerancia a sequía y viceversa(Monclus et al 2006), además existe una relación directa entre discriminación isotópica de carbono 13 (una variable muy usada en fisiología vegetal, dado a su relación con la eficiencia del uso de agua, respecto a la asimilación de carbono), con la conductancia estomática, revelando que las diferencias entre genotipos, podrían ser usadas para predecir el comportamiento hídrico, en base a estas dos variables (Monclus et al 2006).

En el caso de *E. globulus*, podemos identificar que el genotipo de mayor tasa de conducción estomática (g5), posee menor supervivencia, sin embargo, g4, uno de los genotipos tolerantes, posee una tasa de conductancia estomática inferior en condición control. Silva et al (2017), demuestra que existe una relación negativa entre variables de intercambio gaseoso con la supervivencia de distintos genoptipos de *Eucalyptus*, dado a la capacidad de disminuir la pérdida de agua, lo que determian su éxito ante el estrés hídrico.

Cuando se relaciona la eficiencia del fotosistema II, como un equivalente a la tasa de fotosíntesis, en conjunto a la conductancia estomática, es posible identificar que existe una relación que determina la supervivencia de los genotipos. En el caso de la fotosíntesis, se identifica al genotipo 5 como aquel que posee una disminución significativa de la tasa de fotosíntesis a las 2 se manas de iniciado el experimento, que se relaciona a su bajo nivel de supervivencia. Algo similar ocurre con el genotipo 10 de los híbridos. En el caso de *Eucalyptus* es posible identificar que la disminución de la tasa de fotosíntesis es un indicador del daño provocado por el estrés, y se identifican cambios significativos después de los cambios evidenciados a nivel de conductancia estomática. (Silva et al 2017).

Estudio de transcriptoma de *E. globulus* en condiciones de sequía

Se han reportado resultados de RNAseq comparables, en experimentos de estrés por sequía en otras especies, por ejemplo, en *Citrus sinensis*, se identificaron 41.827 transcritos únicos, de los cuales 1.764 se expresaron diferencialmente entre sequía y control (Gonçalves et al. 2019). En *Populus trichocarpa* se expresaron 33.044 genes como lecturas de RNA-Seq, de esas secuencias 935 se expresaron específicamente en condiciones de limitación de agua (Tang et al. 2015). En *E. globulus* se identificaron 27,404 y 22,517 unigenes en hojas y raíces en bibliotecas de estrés por frío (Aguayo et al.2019), mientras que cuando se utilizó el mismo genotipo de *E. globulus* bajo estrés por sequía se identificaron un total de 30,232 y 31,098 unigenes en hojas y raíces, donde se identificaron un total de 686 y 430 DEG en hojas y raíces, respectivamente. La mayoría de los genes regulados positivamente informados en este estudio, se han identificado como parte de la respuesta al estrés abiótico, en otras especies leñosas, por ejemplo, TRX en *V. vinifera* (Haider et al. 2017); genes implicados en la señalización y biosíntesis de ABA en la raíz de variedades de *C. sinensis* resistentes a la sequía (Gonçalves et al. 2019); Proteínas LEA y

DHN en *Populus alba x Populus glandulosa* (Yoon et al. 2014) y en *Eucalyptus sp*. (Valdés et al. 2013). Los genes regulados negativamente son una parte importante del mecanismo de defensa contra el estrés por sequía, ya que implica una reordenación drástica de la asignación de recursos en plantas estresadas (Estravis-Barcala et al. 2019). Algunos genes regulados a la baja descritos en este estudio están involucrados en la fotosíntesis, resultados que se han descrito para otras especies leñosas como *V. vinifera* y *Populus deltoides* en condiciones de estrés por sequía (Haider et al.2017; García et al.2018). Quince Genes diferencialmente expresados, obtenidos a partir de datos de RNAseq se correlacionaron con el análisis qRT-PCR (r^2 = 0,82), similar a los resultados obtenidos por Ksouri et al. (2015) para *Prunus persica* en condición de sequía (r^2 = 0,89).

Según PlantTFDB4.0, se identificaron un total de 1.731 y 1.730 loci que codifican TF en los genomas de *E. grandis* y *E. camaldulensis*, respectivamente (Jin et al. 2016). En el caso de *E. globulus* se identificaron un total de 3.624 y 2.869 TF putativos utilizando datos del transcriptoma de hojas y raíces respectivamente. En la planta modelo *A. thaliana* se han identificado 1.600 secuencias como TFs, estando entre las más representadas MYB (9%), NAC (7%) y WRKY (4%) (Li et al.2015; Shao et al.2015; Wang et al.2011). Estas familias son las más abundantes en plantas y se han relacionado con múltiples funciones. De los genes diferencialmente expresados los FTs MYB, NAC y WRKY fueron los más representados, de manera similar a los resultados descritos por Yıldırım y Kaya (2017) en *P. nigra* bajo estrés por sequía y Aguayo et al. (2019) en *E. globulus* bajo estudios de aclimatación al frío.

El gen *EuglMYB58* posee identidad mayor al 90% con el gen *EugrMYB58* de E grandis, que es parte del subgrupo 11, identificado por Soler et al. (2015) y son ortólogos de *AtMYB102*, un FT que ha sido reportado como un elemento de respuesta a condiciones de deshidratación en *A. thaliana* (Denekamp y Smeekens, 2003). Los datos de qRT-PCR mostraron un incremento significativo de la abundancia de transcripciones de *EuglMYB58* en hojas de dos genotipos tolerantes a la sequía en la condición de IDS, mientras que *EuglMYB27* incrementó su nivel de expresión en plantas de *E. globulus* estresadas por sequía (datos no mostrados). *EuglMYB27* tiene un 52% de identidad con *AtMYB62* de *A. thaliana*, que es inducida por la falta de fosfato (Devaiah et al. 2009). Los estudios en

Carthamus tinctorius indican que *CtMYB62* podría ser uno de los principales genes tolerantes a la sequía, teniendo un papel clave en los genotipos tolerantes (Wei et al. 2020).

EuglNAC66 y *-126* fueron regulados positivamente en hojas de plantas de *E. globulus* estresadas por sequía. En *E. grandis*, estos TF están estrechamente relacionados filogenéticamente, agrupados en grupos Vb, respondiendo al estrés abiótico (Hussey et al. 2015). *EuglNAC66* mostró un 62% de identidad con *RD26* de *A. thaliana*, que está involucrada en la senescencia foliar (Li et al. 2016). La abundancia de transcripción de *EuglNAC66* aumentó en todos los genotipos en este estudio en SDS en hojas y raíces (Figura 6c). La sobreexpresión de *TaNac29* en *A. thaliana*, un gen ortólogo de *RD26* del trigo, confiere tolerancia a la sequía a las diferentes líneas transgénicas y se asoció con la señalización dependiente de ABA que responde al estrés abiótico (Huang et al. 2015). *EuglNAC126* tiene un 61% de identidad con *AtAF1* de *A. thaliana* que se ha informado como un regulador positivo de la tolerancia a la sequía mediada por ABA (Lu et al. 2007).

Por otro lado, el gen *EuglWRKY18* perteneciente al grupo III, mostró cambios significativos en respuesta al estrés por sequía. Se ha demostrado que el grupo III de WRKY tiene un papel en el estrés biótico, como se informó anteriormente en *A. thaliana* (Kalde et al. 2003). Sin embargo, las plantas transgénicas de *N. benthamiana* que sobreexpresan *GhWRKY41* (homólogo *EuglWRKY18*) aumentaron la tolerancia al estrés por sequía y sal, cuando las plantas estuvieron expuestas al estrés osmótico, este gen actuó como un regulador positivo mejorando el cierre de los estomas y regulando las especies reactivas de oxígeno (Chu et al. 2015).). *EuglWRKY26* fue inducido en estrés por sequía severo (Datos no mostrados) y su ortólogo de *A. thaliana AtWRKY40* actúa como un prerrequisito para la señalización funcional de ABA al facilitar la expresión de la señalización de ABA (Geilen y Böhmer, 2015). Cuando se induce ABA, se expresa *AtWRKY40* y podría actuar como un efector transcripcional temprano para regular la expresión de otros genes relacionados con la respuesta a la sequía (Lindemose, 2013). Además, y al igual que en nuestro trabajo, un estudio reciente mostró que este gen es inducido por la sequía, alta salinidad y altas temperaturas y la sobreexpresión de *ZmWRKY40* del maíz mejora la tolerancia a la sequía en *A. thaliana* regulando genes relacionados con el estrés y ROS (Wang et al. al.2018).

En cuanto a los genes de respuesta como *EuglLEA*4-5 en los genotipos evaluados en este trabajo, se puede sugerir que este gen corresponde a proteínas protectoras, las que son importantes para la tolerancia al estrés por sequía, ya que ayudan a mantener la función de las células cuando el agua escasea (Olvera-Carrillo, et al. 2011). Se ha demostrado que los genes que codifican las proteínas *LEA* incrementan su expresión en condiciones de sequía en muchas especies leñosas como: *P. sylvestris* (Joosen et al. 2006), *Populus alba x Populus glandulosa* (Yoon 2014), *Eucalyptus sp*. (Valdés et al. 2013, Villar et al. 2011, Thumma 2012). *EuglLEA*4-5 se induce por estrés por sequía severo, por ello se puede relacionar a la protección de estructuras celulares del daño.

En el caso de *EuglDHN1* hubo un incremento en plantas bajo estrés. Los genes deshidrina han sido reportados previamente como elementos protectores en condiciones de estrés abiótico como estrés por frío (Fernández et al. 2012b; Fernández et al. 2015). Específicamente, esos genes pueden proteger las estructuras celulares que actúan como acompañantes, dando un correcto plegamiento de proteínas cuando el agua comienza a faltar (Hanin, et al. 2011).

Por otro lado el gen *EuglVGT1* está relacionada con el gen *Atg162660* de *A. thaliana*, conocido como *AtVGT1*, que se expresa en la membrana del tonoplasto (Aluri y Buttner 2007). Posee 12 dominios transmembrana y su función es transportar glucosa dentro de la vacuola mediante un antiportador de protones (Patzke et al. 2019). Un estudio de *A. thaliana* reveló que el locus *Atg162660* está asociado con algunos QTL para la actividad invertasa en extractos solubles y en varios órganos de plántulas (Sergeeva et al. 2006).

Santos y col. (2021) informaron que hay una acumulación de azúcares reductores vacuolares liderada por una invertasa vacuolar que se expresa altamente en un genotipo tolerante a la sequía de *Hevea brasiliensis* en condiciones de estrés por sequía, pero no en un genotipo susceptible, lo que sugiere que el ajuste osmótico es importante para la tolerancia en la especie.

En particular, la abundancia de transcripciones del gen *EuglLTP2* se indujo significativamente en las raíces de plantas de ambos genotipos en IDS y SDS; sin embargo, en el genotipo T, la expresión relativa fue significativamente mayor que en el genotipo S

bajo los mismos tratamientos. Li y colaboradores (2008) informaron que este gen es inducido por la sequía en *A. thaliana* y está controlado por NYF5, un FT que mejora la tolerancia al estrés por sequía. Este gen es inducido por estrés salino en las raíces de A. thaliana (Julke et al, 2016). Por otro lado, se ha reportado un aumento en la abundancia de transcrito de un ortólogo de *LTP2* en raíces bajo estrés salino en el *Populus euphratica* tolerante a la sal.(Brinker et al., 2010).

Finalmente. El gen *EuglGER* codifica para una enzima que pertenece al grupo del citocromo p450 (Wang et al. 2010) e hidroxila en la posición 8 la molécula de geraniol (Sintupachee et al. 2015). El geraniol es un precursor de muchos compuestos que pertenecen al metabolismo secundario, en particular la vía del glucósido iridoide (IG) (Ilc et al. 2016). Algunos IG se han relacionado con la actividad antioxidante y el aumento de las concentraciones en las raíces bajo estrés por sequía (Wang et al. 2010).

Respecto a proyecciones futuras basadas en la información de la presente investigación, se plantea que la identificación de genes de respuesta a sequía es el primer paso para determinar los mecanismos específicos en los que dichos genes tienen participación para incrementar la posibilidad de sobrevivir ante periodos de escasez hídrica. En este sentido la generación de árboles modificados genéticamente podría ser una alternativa ante el acelerado cambio en el patrón de precipitaciones causado por el cambio climático, que el futuro cercano limitará el recurso hídrico y por ende la capacidad productiva de las generaciones futuras respecto a productos y servicios forestales.

CONCLUSIONES GENERALES

En primer lugar, a partir de la presente investigación fue posible determinar que existe variabilidad genética en la especie *E. globulus*, respecto a la tolerancia a sequía, dado a las diferencias en cuanto a supervivencia determinadas en condiciones de invernadero, que permitieron seleccionar genotipos contrastantes para la evaluación de genes de respuesta a sequía en la especie. Además, las variables fisiológicas, tales como potencial hídrico de prealba, eficiencia de fotosistema II mediante la relación *Fv/fm o* conductancia estomática, son parámetros útiles para identificar genotipos de mayor tolerancia a estrés por sequía en condiciones de invernadero.

Fue posible determinar que, la especie *E. globulus* responde a la supresión de riego, mediante la inducción de la expresión de genes de respuesta a sequía, dentro de los cuales se destacan factores de transcripción tales como *EucglMYB58*, *EuglMYB27*, *EuglNAC66*, *EuglNAC126*, *EuglWRKY18* y *EuglWRKY26*, entre otros. Mientras que también hubo un incremento en la abundancia de transcrito en otros genes como por ejemplo proteínas LEA *EuglLEA4-5*, deshidrinas *EuglDHN1*, Geraniol 8 hidroxilasa *EuglGER*, proteínas de transporte de lípidos *EuglLTP2* y proteínas de regulación osmótica como transportador de glucosa vacuolar *EuglVGT1*.

Dentro de los genes anteriormente descritos se destacan los gene *EuglLTP2* y *EuglGER* debido a que son inducidos principalmente en el genotipo tolerante, por sobre el susceptible.

Respecto a la compleja red de interacción de genes, es posible identificar mecanismos de señalización bioquímica y molecular que activan finalmente a factores transcripcionales, los cuales a su vez son los responsables de inducir la expresión de genes target, mediante la unión a un sitio específico en el promotor de la secuencia de ADN correspondiente a dichos genes. De ese modo se induce la expresión de un mecanismo de defensa que finalmente determinará si el individuo sobrevive a la sequía o no. Dentro de los mecanismos identificados en especies leñosas, se puede mencionar, la producción de pared secundaria en tejido xilemático, para evitar embolismo o cavitación, aumento de sustancias osmóticamente activas, eliminación de especies reactivas de oxígeno, cambios

morfológicos como aumento de crecimiento de raíces, cambio en la densidad de células en hojas, entre otras estrategias que en conjunto contribuyen a determinar la tolerancia al estrés por sequía..

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