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# Caracterización de genes *CBF* (c-repeat binding factors) de *Eucalyptus globulus* y su validación como genes candidatos que regulan la resistencia al frío mediante transformación genética en *Arabidopsis thaliana*

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**CARACTERIZACIÓN DE GENES CBF (C-REPEAT BINDING FACTORS) DE *Eucalyptus globulus* Y SU VALIDACIÓN COMO GENES CANDIDATOS QUE REGULAN LA RESISTENCIA AL FRÍO MEDIANTE TRANSFORMACIÓN GENÉTICA EN *Arabidopsis thaliana***

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**DEDICATORIA**

*A mis padres, Eliana y Pedro*

*A mis hermanos, Vanessa y Pedro Arturo*

*A mi madrina, Gloria*

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# RESUMEN

*Eucalyptus globulus* es la principal especie latifoliada de maderas duras y de rápido crecimiento utilizada en la industria forestal chilena para la producción de pulpa de celulosa, por su alto rendimiento y excelente calidad de fibra. Sin embargo, esta especie presenta una alta sensibilidad a temperaturas de congelamiento, que afectan a su establecimiento, crecimiento y productividad. Hace tres décadas, varios investigadores han focalizado sus estudios en explicar los mecanismos que regulan la repuesta al frío y congelamiento en plantas, utilizando para ello el análisis de expresión génica y transformación genética.

En este estudio se identificó nuevos genes que codifican a factores de transcripción C-repeat binding factor (CBF), como principales elementos de señalización en respuesta al estrés por frío en plantas. Se determinó su expresión génica enfocándose sobre el proceso de aclimatación al frío en tres diferentes genotipos de *E. globulus*, contrastantes en su supervivencia y daño frente a exposición a heladas. También, se validó la función putativa los genes mediante transformación genética en *Arabidopsis thaliana*. Adicionalmente, se evaluó la regulación de regiones promotoras de dos genes deshidirna 2 (*DHN2*) de dos especies, con diferencias en su resistencia al frío, *E. globulus* (*Eugl*) y *E. nitens* (*Eni*), generando como hipótesis que el promotor putativo Eni*DHN2* presenta una mayor inducción del gen bajo estrés por frío, como un rol importante en conferir resistencia al frío para la especie.

El aislamiento de secuencias homologas a genes *CBF* permitió identificar y caracterizar tres nuevos genes en *E. globulus*. Estos fueron denominados Egl*CBF1a*, Egl*CBF1c* y Egl*CBF1d*, los cuales codifican para tres diferentes proteínas de 220, 229 y 196 residuos de aminoácidos, respectivamente. Estos presentan un dominio AP2/ERF de unión a DNA, como también dos motivos con residuos de aminoácidos PKKP/RAGRxKFxETRHP y DSAWR que flaquean el dominio, siendo característicos de estos factores de transcripción.

Los análisis de expresión de genes *CBF* determinan que el gen Egl*CBF1c* se expresa constitutivamente en plantas no aclimatadas y en plantas aclimatadas expuestas a heladas nocturnas de –2 ºC, en respuesta al congelamiento. Por otra parte, los genes Egl*CBF1a* y

Egl*CBF1d* se inducen en respuesta al estrés por frío y congelamiento. La mayor acumulación de transcritos ocurre en plantas aclimatadas expuestas a heladas nocturnas, disminuyendo notablemente en el tratamiento de desaclimatación. Además, uno de los genotipos categorizados como resistentes al estrés por frío, presentó mayores niveles de transcritos para los tres genes evaluados en plantas aclimatadas, en comparación con el genotipo sensible al congelamiento. Estos resultados respaldan que los factores de transcripción CBF tendrían un importante rol en la resistencia al congelamiento en *E. globulus*, lo cual ha sido reportado en varias especies de plantas, tanto herbáceas como leñosas.

La transformación genética en *Arabidopsis* de los tres diferentes genes Egl*CBF1*, induce la acumulación de transcritos de genes *COR* (*COR6*.6, *COR15a* y *ERD10*) de respuesta a frío, en plantas no aclimatadas. Además, induce la expresión de un gen de señalización por vía ABA-dependiente (*RAB18*). La expresión relativa del gen *COR15a* al disminuir las temperaturas de 23 ºC a 4 y –6 ºC, fueron mayormente inducidos en plantas transformadas que en plantas no transformadas. La sobreexpresión de los tres genes Egl*CBF1* en *Arabidopsis* no sólo mejoró la tolerancia al congelamiento en plantas transformadas, sino que también inhibe el crecimiento y retrasa la floración. Estos resultados evidencian la función putativa de los genes Egl*CBF1* como factores de transcripción, mejorando la resistencia al frío y congelamiento en plantas.

Finalmente, los resultados de expresión del gen marcador (*GUS*) asociado a los promotores de los genes deshidrina 2 de *E. globulus* y *E. nitens*, determinaron que el promotor del gen Eni*DHN2*, presento mayor acumulación de transcritos de *GUS* que el promotor del genes Eugl*DHN2*, cuando se expone a temperaturas frías, sugiriendo que estas características diferenciales podrían otorgar mayor resistencia al frío por parte de la especie *E. nitens*.

## ABSTRACT

*Eucalyptus globulus* is the main hardwood, fast growing specie used in the Chilean forestry industry for pulp production, due to its high yields and fiber quality. However, this species presents a high sensitivity to freezing temperatures, which affect its establishment, growth and productivity. Three decades ago, several researchers have focused their studies on explaining the mechanisms that regulate the cold response and freezing tolerance in plants, using gene expression analysis and genetic transformation.

In this study, new genes coding for C-repeat binding transcription factors (CBF) were identified, as the main elements in response to cold stress in plants. Their gene expression was determined by focusing on the process of cold acclimation in three different *E. globulus* genotypes, contrasting in their survival and damage level against frost exposure. Additionally, the putative functions of the genes were validated by genetic transformation in *Arabidopsis thaliana*. Also, the regulation of the promoter regions of two dehydrin 2 (*DHN2*) genes of two related species with differences in their resistance to cold, *E. globulus* and *E. nitens*, were evaluated, raising the hypothesis that the putative Eni*DHN2* promoter drives a higher gene induction under cold stress, a key role in conferring to cold resistance for this specie.

The isolation of *CBF* homologous sequences allowed the identification and characterization of three new genes in *E. globulus*. These were named Egl*CBF1a*, Egl*CBF1c* and Egl*CBF1d*, which encodes for three different proteins of 220, 229 and 196 amino acid residues, respectively. All sequences present an AP2/ERF DNA binding domain and the motifs PKKP/RAGRxKFxETRHP and DSAWR franking this domain, which are characteristic for this transcription factors.

*CBF* gene expression analyzes identified that the Egl*CBF1c* gene is constitutively expressed in non-acclimated and acclimated plants exposed to night frosts of –2 °C as freezing response. On the other hand, the Egl*CBF1a* and Egl*CBF1d* genes are induced by chilling and freezing temperature stresses. The highest accumulation of transcripts occurs in acclimatized plants exposed to night frosts, steeply decreasing in the treatment of de-acclimation. In addition, one of the genotypes previously categorized as cold resistant,

showed higher levels of transcripts for the three genes evaluated in cold acclimated plants, compared to the freeze-sensitive genotype. These results suggest that these CBF transcription factors could play an important role in the freezing resistance *E. globulus* specie, in agreement with reports for several herbaceous and woody plants.

*Arabidopsis* plants transformed with the three different Egl*CBF1* genes induces the accumulation *COR* gene transcripts (*COR6.*6, *COR15a* and *ERD10*) of cold response in non-acclimated plants and additionally induces the expression of the ABA-dependent gene *RAB18*. The relative expression of the *COR15a* gene when lowering temperatures from 23 °C to 4 °C and – 6 °C, were more strongly induced in transformed than in non-transformed plants. The overexpression of the three Egl*CBF1* genes in *Arabidopsis* not only improved their freezing tolerance, but also inhibited growth and delayed flowering. These results evidence the putative function of Egl*CBF1* genes as transcription factors that improves cold and freezing resistance in plants.

Finally, the results associated to marker gene expression (*GUS*) for the promoters of the dehydrin 2 genes of *E. globulus* and *E. nitens*, determined that the promoter of the Eni*DHN2* gene had greater *GUS* transcript accumulation than the putative promoter of the Eugl*DHN2* genes, when exposed to chilling and freezing temperatures, suggesting that these different characteristics could give greater cold resistance to *E. nitens* species.

## ABREVIACIONES

| | |
|---|---|
| **ABA** | Ácido abscísico |
| **ABRE** | (*ABA binding responsive element*) |
| | Elemento regulador de respuesta a ABA |
| **AP2/ERF** | (*APETALA 2/ Ethylene response factor*) |
| **At*CBF1/DREB1B*** | Gen *CBF1* identificado en *A. thaliana* |
| **At*CBF2/DREB1C*** | Gen *CBF2* identificado en *A. thaliana* |
| **At*CBF3/DREB1A*** | Gen *CBF3* identificado en *A. thaliana* |
| **At*CBF4/DREB1D*** | Gen *CBF4* identificado en *A. thaliana* |
| **AtDDF1** | Gen *Dwarf and delayed flowering 1* identificado en *A. thaliana* |
| **AtDDF2** | Gen *Dwarf and delayed flowering 2* identificado en *A. thaliana* |
| **CBF/DREB1** | (*C-repeat binding factor / Dehydration response element binding 1*) |
| | Factor de transcripción en respuesta a frío y deshidratación |
| ***CBF*-target** | Genes regulados vía de señalización factores de transcripción CBF |
| ***CBF* regulon** | Genes regulados vía de señalización factores de transcripción CBF |
| **COR** | (*Cold-response gene*) Genes de respuesta a frío |
| **CRT/DRE** | (*C-repeat / Dehydration responsive element*) |
| | Elemento regulador de respuesta a frío y deshidratación |
| **Egl*CBF1a*** | Gen *CBF1a* identificado en *E. globulus* |
| **Egl*CBF1c*** | Gen *CBF1c* identificado en *E. globulus* |
| **Egl*CBF1d*** | Gen *CBF1d* identificado en *E. globulus* |
| **ERD** | (*Early dehydration-inducible*) |
| | Gen inducible por deshidratación temprana |
| **ERF** | (*Ethylene responsive element*) |
| | Elemento regulador de respuesta a etileno |
| **FT** | Factor de transcripción |
| **kDa** | Kilo Dalton |
| **KIN** | (*Cold-inducible*) Gen inducible por frío |
| **LEA** | (*Late embryogenesis abundant proteins*) |
| | Proteínas abundantes de embriogénesis tardía |

| | |
|---|---|
| **LTI** | (*Low temperature-induced*) Gen inducido por bajas temperaturas |
| **Mha** | Miles de hectáreas |
| **qPCR** | PCR cuantitativa o *real time PCR* |
| **RD** | (*Responsive to desiccation*) Gen de respuesta a la desecación |

# 1. CAPÍTULO I: INTRODUCCIÓN GENERAL

## 1.1. Importancia del genero *Eucalyptus*

### 1.1.1. *Eucalyptus* en el mundo

*Eucalyptus* L' Hérit (Myrtaceae) es un género de plantas leñosas de maderas duras extensamente plantado en el mundo. Sus plantaciones superan las 20 millones de hectáreas a nivel mundial, siendo Brasil el país que lidera con 21%, le siguen India con 19%, China con 13%, Australia con 5% y Chile con 3% (Iglesias-Trabado y col. 2009). Originario de Australia, Tasmania e islas al norte (Nueva Caledonia, Nueva Guinea y archipiélago indonesio), cuenta con más de 700 especies, dividiéndose en 13 subgéneros, incluyendo *Corymbia* y *Angophora* (Brooker 2000; Ladiges y col. 2003 Poke y col. 2005). Del total de plantaciones en el mundo, el 80% se encuentra representado por las especies *E. grandis*, *E. globulus* y *E. camaldulensis* (Potts 2004). A éstas les siguen *E. nitens*, *E. saligna*, *E. deglupta*, *E. urophylla*, *E. pilularis, Corymhia citriodora* y *E. tereticornis* (Potts 2004). La principal característica de importancia en estas especies es su rápida tasa de crecimiento (Turnbull 1999), además de características morfo-anatómicas de sus maderas con fibra corta, estructuras de fustes rectos y cilíndricos, escasa presencia de nudos, lo que las hace ideales para algunos procesos industriales. Algunos de los usos de estas especies en la industria forestal son la producción de maderas aserradas, pasta de celulosa, papelera (papeles y cartones), materiales de construcción (postes y paneles), calefacción (leña, carbón vegetal), y aceites esenciales (Jacobs 1981). En el caso de pasta de celulosa, las especies preferidas son *E. grandis*, *E. urophylla* y sus híbridos para regiones tropicales y subtropicales, y *E. globulus* para regiones templadas (Potts 2004).

### 1.1.2. Plantaciones de *Eucalyptus* en Chile

Chile se ubica en el quinto lugar de plantaciones de *Eucalyptus* en el mundo con 841 miles de ha (Mha) (Iglesias-Trabado y col. 2009; INFOR 2016). Las principales especies del genero *Eucalyptus* cultivadas en el país son *E. globulus* y *E. nitens*, con 574 Mha y 255 Mha, respectivamente (INFOR 2016). *E. globulus* es la segunda especie forestal más plantada después de *Pinus radiata*, siendo utilizada principalmente para la producción de

pulpa de celulosa, debido a su rápido crecimiento, alto rendimiento de celulosa y excelente calidad de fibra (Kibblewhite y col. 2000; Ramírez y col. 2009; Costa e Silva y col. 2009). Durante el año 2015, el incremento de 3,8% en la producción de pulpa blanqueada de eucalipto, vino a atenuar la pérdida en la producción de pulpa de celulosa de –1,8%, producto de la caída de 7,7% en la pulpa blanqueada de *Pinus radiata*, llegando a 5,12 millones de toneladas (INFOR 2016). En ese mismo año las exportaciones forestales cayeron un 10,8%, llegando a los US$ 5.439,1 millones, aun cuando productos como la pulpa blanqueada de eucalipto registraron un moderado incremento (INFOR 2016).

### 1.1.3. *Eucalyptus globulus*

*E. globulus* Labill es la principal especie plantada en climas templados libres de heladas severas, debido a su excepcional calidad de madera, combinada con su rápido crecimiento (Teulières y Marque 2007). Sus plantaciones en Chile se concentran en la zona centro-sur del país, entre las regiones del Biobío y Araucanía, aunque existen plantaciones desde Copiapó, zona norte en la región de Atacama, hasta la isla grande de Chiloé, zona sur en la región de los Lagos (INFOR 2011). En la zona centro-sur, el clima predominante es templado cálido con una estación seca de 4 a 5 meses y templado lluvioso con influencia mediterránea, climas idóneos para las plantaciones de *E. globulus*. En la última década se ha registrado un incremento en las plantaciones de *E. globulus* siguiendo un aumento anualmente por la alta demanda de papel en el mundo (Costa e Silva y col. 2009; INFOR 2016). Dicha tendencia resulta en la necesidad de optar por sitios con condiciones climáticas no óptimas para la especie, sitios donde episodios de heladas (temperatura < 0 ºC) se presentan con cierta frecuencia y limitan la expansión de estas plantaciones. Tales condiciones afectan principalmente al establecimiento de plantas juveniles, ya que son menos resistentes a condiciones ambientales extremas que plantas adultas (Close y col. 2004), también reducen la fotosíntesis, y por ende la productividad de plantas adultas (Davidson y col. 2004). Las temperaturas mínimas toleradas por *E. globulus* han sido registradas en variados estudios, Almeida y col. (1994) informaron que plantas de siete meses de edad no endurecidas al frío, presentaban temperaturas letales al 50% de daño en sus tejido ($TL_{50}$) entre – 5,3 a – 5,6 ºC. Por otra parte, Volker y col. (1994) determinaron que plantas de cuatro meses de edad fueron severamente afectadas a – 7 ºC, infiriendo que

bajo el criterio de $TL_{50}$ estaría alrededor de los – 6 ºC. En este ámbito, Kellison (1999) clasifica varias especies de *Eucalyptus* según su resistencia al frío, indicando que *E. globulus* soporta heladas de poca intensidad. Sin embargo, existen reportes que indican que mediante métodos de endurecimiento en vivero pueden inducir una resistencia al congelamiento en plantas, registrando índices de $TL_{50}$ de – 8,5, – 8 y – 9 ºC para tres subespecies de *E. globulus*, subespecie *globulus*, *bicostata* y *maidenii*, respectivamente (Moraga y col. 2006). En ensayos de laboratorio, con plantas de 6 meses de edad de dos genotipos operacionales crecidos en cámaras de cultivo en condiciones normales, se ha determinado índices de $TL_{50}$ entre – 4,2 y – 4,9 ºC (datos no publicados). Tibbits y col. (2006) estudiaron la variación genética en la resistencia al congelamiento durante invierno de *E. globulus*, determinando que los patrones de resistencia al congelamiento de diferentes procedencias, no fueron claros y presentaron poca consistencia, llegando a obtener índices de $TL_{50}$ de hasta – 8,8 ºC en algunas procedencias. Todo esto sugiere que la resistencia al congelamiento en *E. globulus* es una característica variable dentro de la especie, y entre familias, indicándose también que está bajo un fuerte control genético aditivo (Volker y col. 1994; Tibbits y col. 2006).

## 1.2. Estrés y resistencia al frío en plantas

Las plantas en su área de distribución natural necesitan de un gradiente en temperaturas óptimas para un adecuado crecimiento y desarrollo. El frío (o bien las bajas temperaturas) es clasificado en temperaturas de enfriamiento, inferiores a los 15 ºC, y en temperaturas de congelamiento, inferiores a los 0 ºC (Chinnusamy y col. 2007; 2010), condiciones que limitan la distribución geográfica, crecimiento y desarrollo de las plantas, afectando directamente la calidad y rendimiento de los cultivos. Las temperaturas de enfriamiento, generan daño en plantas, exhibiendo síntomas tales como la disminución de la expansión foliar, marchitamiento, clorosis e inclusive necrosis en sus tejidos (Wang 1990). Por otra parte, las temperaturas de congelamiento provocan la muerte de los tejidos, dañando las membranas celulares, lo que causa la pérdida de electrolitos en el apoplasto y el desbalance de las propiedades de transporte de las membranas (Arora y Palta 1991; Saxe y col. 2001).

### 1.2.1. Efecto de las temperaturas congelantes

En la mayoría de las plantas, al disminuir las temperaturas bajo los 0 ºC, presentan los primeros indicios de daños por congelación, sobre el sistema de membranas lipídicas a nivel celular (Steponkus 1984). Las membranas lipídicas son estructuras fluidas que mantienen la organización y función celular, el daño sobre éstas provoca la salida de electrolitos y la pérdida en la capacidad de transporte de la célula (Saxe y col. 2001). Estas se ven conformadas principalmente por dos clases de ácidos grasos, saturados e insaturados. Diferencias estructurales entre estos, como la presencia de enlaces dobles entre átomos de carbono para los insaturados, permiten un mayor grado de fluidez de las membranas a bajas temperaturas (Steponkus y col. 1993). Existe una transición entre ácidos grasos saturados e insaturados, esta función es realizada por las enzimas denominadas ácido graso desaturasas. Estas enzimas remueven dos átomos de hidrogeno desde la cadena de carbonos en el ácido graso, generando enlaces dobles carbono/carbono. Si la célula no tiene la capacidad de realizar la transición de ácidos grasos en sus membranas, estas se verán afectadas por una pérdida en su fluidez al descender las temperaturas.

Por otro parte, el daño por congelación induce una deshidratación celular con la formación de cristales de hielo, generalmente iniciada en el apoplasto, debido a que el fluido extracelular tiene un mayor punto de congelación por presentar menor concentración de solutos que el fluido intracelular. Esto provoca la caída del potencial químico fuera de la célula, generando un movimiento de agua no congelada por gradiente, desde el interior de la célula al espacio extracelular o apoplasto (Thomashow 1999; Kaminska-Rozek y Pukacki 2005), lo cual contribuye al crecimiento de cristales de hielo existentes en el apoplasto. La formación y crecimiento de estos cristales de hielo genera una tensión mecánica entre la pared celular y la membrana citoplasmática, generando finalmente la ruptura celular (Olien y Smith 1977; Mahajan y Tuteja 2005). Se han reportado proteínas anticongelantes (AFPs *antifreeze proteins*) que se asocian a la superficie del cristal de hielo, inhibiendo su crecimiento y re-cristalización (Griffith y Yaish 2004; Deswal y Sharma 2014), y de esta manera previniendo su expansión y la ruptura de estructuras celulares. Normalmente, un cristal de hielo crece como un disco redondo, sin embargo, en presencia de AFPs no crece homogéneamente, presentando una forma hexagonal. Está inhibición del

crecimiento de cristales de hielo ha sido observado en extractos apoplásticos de *Deschampsia antarctica* aclimatadas y no aclimatadas al frío (Bravo y Griffth 2005). También la deshidratación celular inducida por congelación da lugar a múltiples formas de daño a la membrana celular, incluyendo lisis celular inducida por expansión y transición de fase lamelar a hexagonal-II (Sakai y Larcher 1987; Uemura y Steponkus 1997). Otros elementos que pueden contribuir al daño inducido por heladas son las especies reactivas de oxigeno (ROS), con la consecuente pérdida en la actividad metabólica, compartimentalización celular, reducción de la fotosíntesis y desnaturalización de proteínas (Kaminska-Rozek y Pukacki 2005).

### 1.2.2. Aclimatación al frío y mecanismos de resistencia a estrés

Las plantas de clima templado que experimentan frío prolongado durante el invierno, se han adaptado a temperaturas frías y de congelamiento, alternando entre procesos de activo crecimiento y dormacia vegetativa, de acuerdo a los cambios climáticos estacionales a los cuales se ven expuestas. La mayoría de estas plantas, son capaces de aumentar su resistencia al frío, mediante el proceso de aclimatación al frío. Esto ocurre cuando las plantas se exponen a temperaturas frías no congelantes (Levitt 1980; Thomashow 1999), lo que induce diversos cambios fisiológicos y/o bioquímicos en respuesta a bajas temperaturas, adquiriendo una mayor resistencia a este tipo de estrés (Beck y col. 2007). En este proceso de aclimatación pueden estar relacionados otros factores ambientales, tales como la longitud del día y/o la disponibilidad hídrica que afecta a las plantas, para generar una mayor tasa de endurecimiento a heladas (Beck y col. 2004; 2007). Aunque se ha sugerido que la condición de días cortos y bajas temperaturas puede promover la aclimatación al frío a través de vías independientes (Welling y col. 2002), solo la estimulación combinada de días cortos y bajas temperaturas, proporciona un alto grado de aclimatación al frío, tanto en plantas herbáceas como en leñosas (Christersson 1978; Li y col. 2003; Puhakainen y col. 2004).

Diversos estudios han demostrado la capacidad de desarrollar mecanismos de respuesta a cambios ambientales. En álamo y abedul, la disminución en la longitud del día al final de la temporada de crecimiento sirve como primera señal ambiental del inicio de la dormancia vegetativa y del desarrollo de aclimatación al frío (Welling y col. 2002; Li y col. 2003). Por

otra parte, en un genotipo de *E. globulus* (CN5) crecido a temperaturas de 10/5 ºC día/noche, se ha observado un aumento de azúcares solubles y lípidos totales en hojas, como efecto del proceso de aclimatación a bajas temperaturas, pudiendo esto conferir a este genotipo mejor rendimiento bajo temperaturas de enfriamiento (Shvaleva y col. 2008). En tabaco, las bajas temperaturas (15 ºC) activan la biosíntesis de ácido graso trienoico en hojas, en comparación con plantas crecidas a 25 ºC, sugiriendo que la producción de ácido trienoico durante la aclimatación por enfriamiento es uno de los requisitos previos para el desarrollo de la resistencia al frío en esta especie (Kodama y col. 1995). Estudios a nivel celular en *Arabidopsis* aclimatadas a bajas temperaturas (5°C), reportan una disminución en el contenido de agua celular, seguido de incremento de proteínas en el volumen y densidad citoplasmática, además de una disminución en la contribución de la vacuola al volumen total de la célula (Strand y col. 1999). De esta manera, la capacidad de desarrollar estos mecanismos de respuesta y adaptación a cambios ambientales, dependerá de la habilidad de las plantas en aclimatarse a frío (Xin y Browse 2000).

Por lo tanto, una importante función en el proceso de aclimatación al frío en plantas es la estabilización de membranas y componentes celulares. Optimizar la transición de ácidos grasos saturados a insaturados en la membrana lipídica, mejoraría la fluidez de esta. A su vez la acumulación de moléculas crio-protectoras tales como azúcares solubles (sacarosa, rafinosa, trehalosa), componentes nitrogenados de bajo peso molecular (prolina, glicina betaina) y polipéptidos de la familia de proteínas LEA (*late embryogenesis abundant*), influirían en mantener una mayor estabilización de proteínas y fosfolípidos de membranas, proteínas citoplasmáticas, como también mantener interacciones hidrofílicas y la homeostasis celular (Janská y col. 2010). Adicionalmente, la acumulación de antioxidantes enzimáticos y no enzimáticos protegen a la célula del daño oxidativo (Shao y col. 2008). Todas estas respuestas fisiológicas y bioquímicas en las plantas son mecanismos altamente regulados por la expresión génica. Por lo tanto, la habilidad de las plantas de aclimatarse al frío es un rasgo cuantitativo que involucra la acción de muchos genes con pequeños efectos aditivos (Thomashow 1990).

Las respuestas de aclimatación a bajas temperaturas gatillan diversos procesos que otorgaran resistencia al frío (Beck y col. 2007). Dichos procesos dependerán del

mecanismo por el cual las plantas generan tal resistencia, que está dada por mecanismos de evasión y tolerancia. Los mecanismos evasivos tienen por función evitar el frío o congelamiento en los tejidos, como por ejemplo: dormancia de brotes y semillas, sensibilidad al fotoperíodo, vernalización y sobre-enfriamiento (Janská y col. 2010). Particularmente, la respuesta de sobre-enfriamiento evitan la formación de cristales de hielo en los tejidos, disminuyendo la temperatura de nucleación del hielo (Wisniewski y Fuller 1999), lo que constituye una respuesta evasiva al congelamiento. Esto ha sido reportado en plantas de *Colobanthus quitensis* aclimatadas al frío, con índices de $TL_{50}$ superiores (–5,8 ºC) a las del punto de nucleación de hielo (–9,4 ºC), determinando que la especie no tolera la congelación, y que su principal mecanismo para sobrevivir a bajas temperaturas es sobre-enfriando (Bravo y col. 2001). Por otra parte, los mecanismos de tolerancia tienen relación con soportar temperaturas congelantes y los efectos que estas condiciones generan, como la formación de hielo y la consecuente deshidratación celular, sin sufrir daños irreversibles en sus tejidos (Larcher 2003). Esto ha sido evidenciado en plantas de *D. antarctica* aclimatadas al frío, al registrar temperatura de nucleación de hielo de –10,4 ºC, con índices de $TL_{50}$ de hasta –26,6 °C. Esto determina que *D. antarctica* es capaz de tolerar la congelación más allá de los índices de $TL_{50}$ registrados por Bravo y col. (2001). Se considera que el dilucidar qué elementos se activan en el proceso de aclimatación al frío, proporcionara estrategias potenciales para mejorar la resistencia al congelamiento en plantas.

### 1.2.3. Estrés por frío y vías de señalización cruzada con otros estrés

Una característica notable en las plantas es su grado de adaptabilidad a condiciones ambientales adversas, con múltiples respuestas involucrando complejas redes interconectadas. Estas redes son activadas por diversos estímulos, ya sea fotoperiodo, frío, salinidad, sequía, etc., y donde las plantas pueden aumentar su grado de resistencia al estrés ambiental mediante adaptaciones físicas, interacciones moleculares y cambios celulares que comienzan depués de la aparición del estrés (Knight y Knight 2001). Sin embargo, para simplificar la respuesta de las plantas a este estrés ambiental, es que típicamente se han estudiado en condiciones controladas de crecimiento. En el ambiente, la exposición simultánea a distintos tipos de estrés y posiblemente una combinación de estos, implicaría

una respuesta integral de las plantas a tales condiciones, aunque su interpretación muchas veces pudiese ser compleja. De hecho, varias vías de señalización poseen elementos en común que actúan como nodos de regulación cruzada a varios tipos de estrés (Chinnusamy y col. 2004). Consecuentemente a lo anterior, recientemente se ha reportado que la respuesta de las plantas a combinaciones de dos o más condiciones de estrés es única, y no puede extrapolarse directamente desde la respuesta de las plantas a cada uno de los diferentes estrés aplicados por separado (Suzuki y col. 2014).

Diversas condiciones abióticas producen efectos tanto generales como específicos sobre el crecimiento y desarrollo de las plantas. Por ejemplo, la sequía limita el crecimiento de las plantas debido al declive fotosintético, las restricciones osmóticas impuestas por el estrés y la interferencia con la disponibilidad de nutrientes a medida que el suelo se seca (Chaves y col. 2003). La salinidad interfiere con el crecimiento de las plantas, ya que conduce a la sequía fisiológica y toxicidad iónica (Zhu 2002). El frío también puede causar estrés osmótico, además de su efecto directo sobre el metabolismo (Thomashow 1999). De esta manera, el estrés por frío, estrés hídrico y estrés salino, pueden causar el mismo efecto sobre los tejidos, y en particular sobre las células, como es la disminución del potencial osmótico y la activación de la respuesta al estrés, operando mediante dos vías de señalización, ácido abscísico (ABA) – dependiente y ABA – independiente (Fig. 1.1) (Huang y col. 2012).

La biosíntesis de ABA se produce bajo condiciones de déficit hídrico en plantas, incrementando significativamente sus niveles endógenos en condiciones de sequía y alta salinidad, revelando un rol importante en la respuesta de las plantas a estos tipos de estrés (Shinozaki y Yamaguchi-Shinozaki 1997), pero no en respuesta al estrés por frío (Shinozaki y col. 2003). La expresión génica regulada por ABA se relaciona a factores de transcripción tipo AREB, MYB y MYC (Shinozaki y Yamaguchi-Shinozaki 2000). Los factores de transcripción AREB (*ABA responsive element binding*) de la familia de factores bZIP (*basic leucine zipper*), presentan unión a elementos en *cis* ABRE (*ABA responsive elements*). Diversos estudios han demostrado que factores de transcripción AREB requieren de una señal mediada por ABA, reportando una reducción en la actividad de proteínas AREB en mutantes *aba2* (deficientes a ABA) y en mutantes *abi1* (insensibles a ABA),

como también una mayor actividad de estas proteínas en mutantes *era1* (hipersensibles a ABA) (Choi y col. 2000; Uno y col. 2000). Otros reguladores transcripcionales importantes son las proteínas MYB y MYC, siendo activadores en la vía regulada por ABA, que son inducibles también por sequía y alta salinidad, y su sobreexpresión no sólo da lugar a un fenotipo hipersensible a ABA, sino también mejora la tolerancia al estrés osmótico en plantas de *Arabidopsis* (Abe y col. 2003).

Por otra parte, los estudios sobre la regulación del estrés por frío en *Arabidopsis* han dado como resultado el descubrimiento de una familia de factores de transcripción conocidos como CBF (*C-repeat binding factor*) o DREB (*dehydration reponsive element binding*) que controlan la expresión de genes en respuesta al estrés por frío mediante la vía ABA–independiente (Thomashow 2001; Huang y col. 2012). Existen genes *CBF*/*DREB1A* y *DREB2A* que se unen específicamente a elementos en *cis* CRT/DRE (*C-repeat/ dehydration reponsive element*), desempeñando la función de activadores transcripcionales en plantas (Tomashow 2001). Se ha determinado que la expresión del gen *CBF3*/*DREB1A* y sus dos homólogos (*CBF1*/*DREB1B* y *CBF2*/*DREB1C*), es inducida por estrés a baja temperatura, mientras que la expresión de los dos genes *DREB2* (*DREB2A* y *DREB2B*) se induce por deshidratación (Gilmour y col. 1998; Liu y col. 1998). Estos resultados sugieren que las proteínas CBF/DREB1 están involucradas en la expresión de genes específicos en respuesta a frío, mientras que las proteínas DREB2 se expresan en respuesta específica a deshidratación. Sin embargo, otros genes que son inducibles por sequía y frío, también son inducidos mediante tratamiento de ABA exógeno, por ejemplo: los genes *RD29A* y *RD29B*, que en sus regiones promotoras contienen elementos *cis* CRT/DRE y ABRE, lo que da lugar a la regulación por ambas vías de señalización ABA–dependiente y ABA–independiente (Yamaguchi-Shinozaki y Shinozaki 1993; 1994; Msanne y col. 2011; Jia y col. 2012). También los genes *COR* (*cold-regulated*): *COR6*.6, *COR15a* y *COR47* son inducidos por frío, deshidratación y ABA (Thomashow 1999; Mahajan y Tuteja 2005). Por otra parte, se ha identificado sitios de reconocimiento a factores MYB y MYC en la región promotora del gen *RD22*, que funcionan como elementos en *cis* en la expresión génica inducida por sequía y ABA (Abe y col. 1997). Todo esto indica la complejidad de la respuesta molecular a diversos estreses ambientales, pudiendo estar mediada tanto por

complejos sistemas reguladores de expresión génica, como por diferentes sistemas de transducción de señales, y/o por regulación cruzada entre estos sistemas.

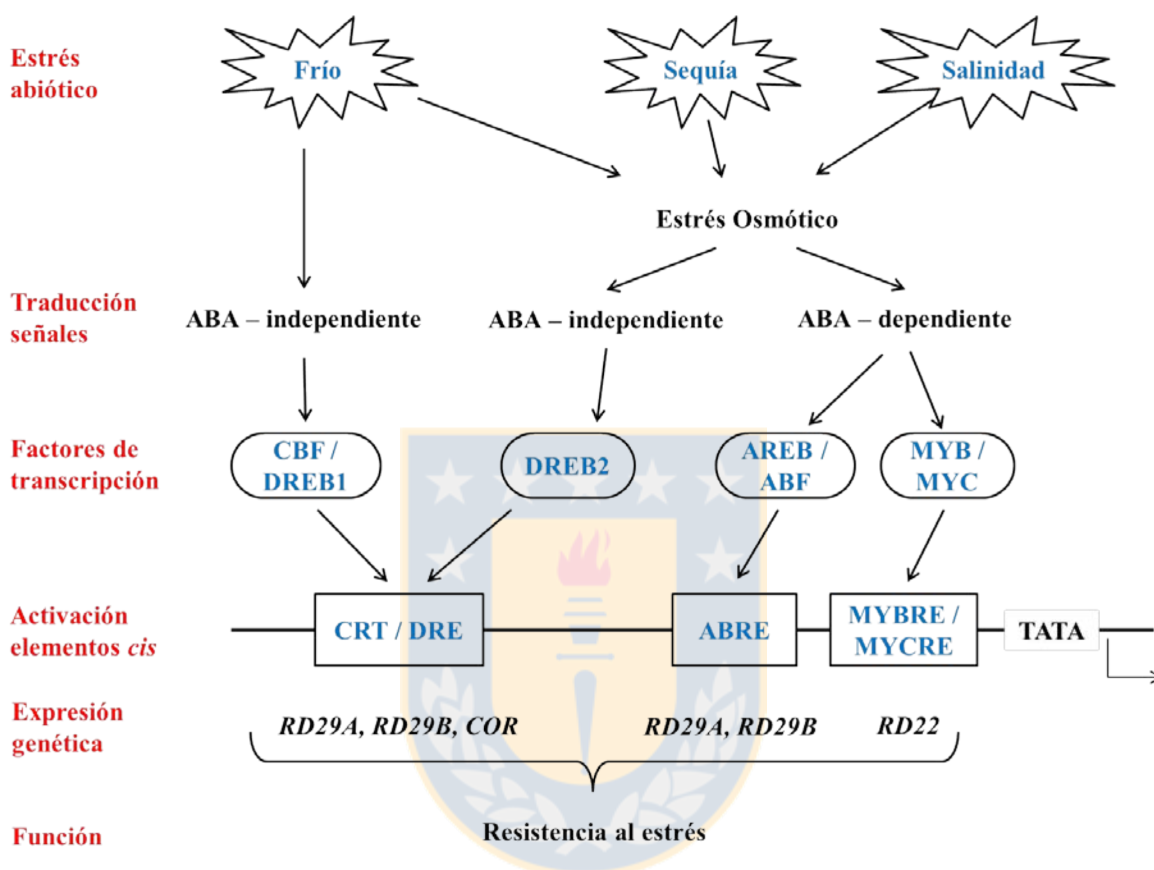



**Fig. 1.1** Regulación cruzada de la señalización al estrés por frío, sequía y salinidad. Activación de vías de regulación ABA–dependiente y ABA–independiente, en respuesta al estrés osmótico. Factores de transcripción CBF/DREB1 y DREB2, componentes claves en la regulación al estrés por frío y deshidratación por vía ABA–independiente (modificado de Huang y col. 2012).

### 1.2.4. Genes en respuesta al estrés por frío

En general, los genes de respuesta al estrés pueden clasificarse principalmente en dos grupos, genes reguladores y genes funcionales. Los genes reguladores corresponden a

factores de transcripción (FT), también denominados elementos en *trans*, que desempeñan un rol esencial en la respuesta al estrés abiótico, regulando la abundancia de transcritos (ARNm) de un amplio espectro de genes diana. Estas proteínas interactúan con los elementos en *cis* presentes en las regiones promotoras de los genes que regulan, activando o reprimiendo su transcripción. Hasta el momento, varias familias de FT y sus elementos interactivos de acción en *cis*, denominados colectivamente como "regulones", han sido bien caracterizados como actores claves implicados en las respuestas al estrés abiótico. Dentro de los genes funcionales, hoy en día varios son los genes reportados que pueden contribuir con la resistencia al frío en plantas. Algunos ejemplos se mencionan a continuación: *FAD8* (*fatty acid desaturase 8*) de *A. thaliana* codifica un ácido graso desaturasa que facilita una mejor transición de ácidos grasos saturados a insaturados en los lípidos de membranas, manteniendo el grado de fluidez de estas a medida que descienden las temperaturas (Gibson y col. 1994). Por otro parte, la inducción de algunos genes *HSP* (*heat shock proteins*) durante la aclimatación al frío, en *Spinacia oleracea HSP70* y en *Brassica napus HSP90,* codifican moléculas chaperonas que pueden estabilizar proteínas contra la desnaturalización provocada por el congelamiento (Anderson y col. 1994; Krishna y col. 1995). Estos y otros genes regulados en respuesta al frío son denominados genes *COR* (*cold-regulated*). Si bien los genes *COR,* algunos denominados como *LTI* (*low temperature-induced*), *KIN* (*cold-inducible*), *RD* (*responsive to desiccation*) y *ERD* (*early dehydration-inducible*), se inducen principalmente en respuesta a frío y deshidratación, también pueden ser inducidos por alta salinidad e incluso por acción de fitohormonas como ABA. En *Arabidopsis* se ha reportado que los genes *COR6.6*, *COR15a*, *COR47* y *COR78/RD29A*, codifican para polipéptidos hidrofílicos (Thomashow 1999). El gen *COR6*.6, codifica un polipéptido de 6,6 kDa rico en alanina (Gilmour y col. 1992; Kurkela y Borg-Franck 1992), el cual también se denomina *KIN2*, por presentar alta similitud en su secuencia a la proteína *KIN1*, esta última similar a proteínas anticongelantes de peces (Kurkela y Franck 1990). El gen *COR15a*, codifica un polipéptido de 15 kDa con región N-terminal similar a proteínas LEA. Se ha reportado que la proteína COR15A presenta una señal de dirección al compartimento del estroma en los cloroplastos, donde es procesada a una proteína de 9,4 kDA (Lin y Thomashow 1992; Zhou y col. 2009). Al ser modificada, esta proteína COR15AM participa en la estabilización de membranas lipídicas como

mecanismo de tolerancia a la deshidratación provocado por el congelamiento (Steponkus y col. 1998). Estudios de transformación genética en *A. thaliana* con la expresión constitutiva de la proteína COR15a, indican un incremento de la tolerancia al congelamiento de cloroplastos y protoplastos congelados en 1 a 2 ºC sobre el rango de temperaturas –5 a –8 ºC (Artus y col. 1996; Thomashow 1999). El gen *COR47* codifica un polipéptido de 30 kDa, identificado como una proteína LEA del grupo II (Gilmour y col. 1992; Welin y col. 1995), pertenece a la familia de proteínas deshidrinas. Como función se ha determinado su capacidad crioprotectora *in vitro*, sobre membranas de tilacoides aisladas de espinacas durante ciclos de congelación-descongelación (Bozovic y col. 2013). Por último, el gen *COR78*, también denominado *RD29A*, codifica un polipéptido de 78 kDa, siendo inducido en respuesta al estrés por frío y desecación. Los primeros estudios describen esta proteína con estructura semejante a proteínas LEA, suponiendo una función de protección celular frente a la desecación (Yamaguchi-Shinozaki y Shinozaki 1993). Sin embargo, aún no es clara su función fisiológica durante el estrés abiótico, pues actualmente algunos autores cuestionan que esta proteína sirva directamente como molécula protectora (Msanne y col. 2011).

Sobre la cascada de señales de los genes *COR*, existen genes reguladores que codifican a factores de transcripción CBF, también inducidos por frío u otras condiciones asociadas al déficit hídrico. Esta ruta de señalización mediada por factores CBF, es la vía de regulación al frío más importante y altamente estudiada, que imparte resistencia al congelamiento en las plantas (Gilmour y col. 1998; Thomashow 2001; Maibam y col. 2013).

### 1.2.5. Factores de transcripción CBF y la resistencia al frío en plantas

Los genes *CBF* (Stockinger y col. 1997) o también denominados *DREB* (Liu y col. 1998), codifican factores de transcripción (FT) de la familia AP2/ERF (*APETALA 2/Ethylene Response Factor*), clasificados dentro del grupo A1 de la subfamilia DREB, único en plantas (Sakuma et al., 2002). Esta familia de FT CBF (grupo DREB-A1) se caracteriza por presentar un dominio AP2 de unión a ADN, altamente conservado de 60 aminoácidos, junto a dos motivos localizados inmediatamente río arriba y ría abajo del dominio AP2, con residuos de aminoácidos de PKKP/RAGRxKFxETRHP (abreviado PKKPAGR) y DSAWR, respectivamente (Jaglo y col. 2001). Canella y col. (2010) han reportado que

estos motivos característicos de proteínas CBF son necesarios para la correcta función y actividad de los estos FT. El dominio AP2 tiene como función unirse al ADN en sitios con elementos activadores en *cis* dentro de las regiones promotoras de genes funcionales, activando su transcripción río abajo. Este dominio reconoce sitios en el ADN con repeticiones de nucleótidos C como elementos en *cis* CCGAC (Baker y col. 1994). Estos elementos en *cis* han sido identificados como elementos en respuesta a deshidratación, llamados CRT/DRE (Stockinger y col. 1997). Específicamente los elementos CRT/DRE son secuencias ricas en repeticiones de citosina de 6 pb (A/GCCGAC), que contienen la secuencia core de 5 pb CCGAC, y se encuentran en regiones promotoras de los genes *COR* (Baker y col. 1994; Sakuma y col. 2002; Yamaguchi-Shinozaki y Shinozaki 2005), los cuales como se indicó anteriormente, son genes que se inducen como respuesta al estrés por frío y deshidratación (Stockinger y col. 1997). La relación de estos FT CBF/DREB1 en respuesta a bajas temperaturas se ha demostrado en varias especies. En *Arabidopsis*, se han caracterizado cuatro FT CBF/DREB1: AtCBF1/DREB1B, AtCBF2/DREB1C, AtCBF3/DREB1A y AtCBF4/DREB1D (Stockinger y col. 1997; Liu y col. 1998; Haake y col. 2002), los cuales en su secuencia nucleotídica no poseen intrones (Medina y col. 1999; Haake y col. 2002). A nivel de proteínas, las secuencias CBF1-2-3 presentan una alta similitud entre sus secuencias, mayor a un 85%, y con un peso molecular cercano a 24 kDa (Medina y col. 2011). Se ha reportado que la acumulación de transcritos de estos genes At*CBF1-2-3* ocurre entre los primeros 15 a 30 minutos de haber expuesto las plantas a 2,5 ºC, con la subsecuente inducción de genes *COR* (*COR15a* y *COR78*), ocurriendo después de 2 a 4 h (Gilmour y col. 1998).

En la actualidad se han descrito FT CBF en plantas leñosas, como por ejemplo: *Populus* spp. (Benedict y col. 2006), *Prunus avium* (Kitashiba y col. 2002), *Malus x domestica* (Wisniewski y col. 2011), *Citrus* spp. (Champ y col. 2007), *E. grandis* (Cao y col. 2015), *E. globulus* (Gamboa y col. 2007), *E. gunnii* (El Kayal y col. 2006; Navarro y col. 2009), entre otras especies. Recientemente el genoma de *E. grandis* ha sido secuenciado (Myburg y col. 2014), con lo cual se han podido determinar un mayor número de genes con homología a FT tipos CBF, encontrando 17 genes tipo CBF en *E. grandis* (Cao y col. 2015). En el caso particular de *E. globulus* se ha descrito un solo FT CBF (Egl*CBF1*), en el cual se induce su expresión solo en respuesta a bajas temperaturas, no siendo inducido ni por sequía ni por

una alta salinidad (Gamboa y col. 2007). Esta expresión se induce tempranamente al exponer plantas juveniles de *E. globulus* a temperaturas de 4 ºC. Por otra parte, en *E. gunnii* se han descrito cuatro FT CBF: EguCBF1A, EguCBF1B, EguCBF1C y EguCBF1D (El Kayal y col. 2006; Navarro y col. 2009), reportándose que la proteína EglCBF1 descrita para *E. globulus* presenta mayor similitud con la proteína EguCBF1B descrita para *E. gunnii* (Navarro y col. 2009). Adicionalmente estos investigadores proponen una función putativa para cada gen descrito, en relación al estrés por frío y congelamiento. Así, el gen Egu*CBF1A* de inducción rápida y de corta duración, es relacionado en respuesta a un golpe de frío extremo. En cambio, el gen Egu*CBF1B* con una inducción más duradera, se relaciona con una respuesta de aclimatación al frío, pudiendo participar en la protección basal durante los meses fríos y en respuesta a heladas progresivas. Por otra parte, el gen Egu*CBF1C* presenta una inducción más bien constitutiva y poco específica, vinculándolo en la protección permanente a la célula y no en una respuesta al estrés ambiental. Finalmente, el gen Egu*CBF1D* presenta una mayor inducción en respuesta a heladas, pudiendo ayudar a la célula a tolerar episodios de heladas, con o sin aclimatación previa al frío y asociado a cambios diurnos de temperatura. Con el conocimiento proporcionado, sería de gran utilidad poder identificar genes homólogos en *E. globulus*, ya que es una especie filogenéticamente cercana a *E. gunnii* (Steane y col. 2011), y por ello se esperaría encontrar genes altamente conservados con funciones similares para otorgar resistencia a este tipo de estrés.

### 1.2.6. Deshidrinas, proteínas de respuesta al frío en plantas

Como ya se ha informado, diferentes tipos de estrés abiótico, como estrés por frío, hídrico y salino, convergen en un estrés osmótico sobre los tejidos de las plantas, produciendo deshidratación celular. Se ha observado que durante el proceso de aclimatación al frío se inducen una serie de elementos protectores, dentro de los cuales las proteínas deshidrinas (DHN) presentan un papel fundamental en respuesta a bajas temperaturas (Close 1996; Strimbeck y col. 2015). Las DHN son proteínas hidrofílicas pertenecientes al grupo II de proteínas abundantes de embriogénesis tardía (LEA), las cuales se acumulan en respuesta a deshidratación celular, impuesta por condiciones de estrés abiótico y donde varios autores les otorgan un rol importante en la protección celular contra el estrés (Kosová y col. 2007;

Hanin y col. 2011; Graether y Boddington 2014). Dentro de sus funciones, se propone que actúan como emulsionantes o chaperonas, protegiendo estructuras celulares, ya sea proteínas o membranas plasmáticas, frente a los cambios estructurales desfavorables causados por la deshidratación (Kosová y col. 2007). Estas proteínas fueron identificadas en 1990s, siendo clasificadas según sus características estructurales, con la presencia de secuencias altamente conservadas denominadas segmentos K, Y y S (Close 1996). Por definición, una proteína deshidrina debe contener al menos un segmento K, pudiendo presentar de 1 – 11 copias cerca del extremo C-terminal (Close 1996; Battaglia y col. 2008). El segmento–K, es una secuencia de 15 aminoácidos ricos en lisinas [EKKGIME/DKIKEKLPG] que puede formar una α-hélice anfipática responsable de la interacción con componentes lipídicos de membranas e interacciones hidrofóbicas de proteínas chaperonas con proteínas parcialmente desnaturalizadas (Close 1996). El segmento–S, es una secuencia conservada que se encuentra en el centro de la proteína, formado por un tramo de 4 – 10 residuos de serina contiguos [$LHRSGS_{4-10}(E/D)_3$], el cual puede ser fosforilado, y esta fosforilación estaría implicada en la unión de un péptido señal de localización nuclear (Campbell y Close 1997; Hanin y col. 2011). El segmento–Y, rico en residuos de tirosina presenta una región conservada [T/VDE/QYGNP] cerca del extremo N-terminal. También, existen otras regiones menos conservadas denominadas segmentos Φ, las cuales son altamente variables entre diferentes DHNs y habitualmente ricos en aminoácidos polares (Rorat 2006). De acuerdo con la presencia y número de combinaciones de los diferentes segmentos K, Y y S, las DHNs se pueden dividir en cinco subgrupos estructurales: $K_n$, $SK_n$, $K_nS$, $Y_nK_n$ y $Y_nSK_n$ (Close 1996; Battaglia y col. 2008).

Con la finalidad de identificar la función de estas proteínas, varios estudios *in vitro* han demostrado que las deshidrinas de diferentes especies (COR85 de espinaca, DHN1 de maíz, WSC120 de trigo, PCA60 de durazno y CuCOR19 de *Citrus unshiu*) pueden proteger a enzimas lábiles al frío como lactato deshidrogenasa (LDH, EC 1.1.1.27), de la desactivación por ciclos de congelación-descongelación (Kazuoka y Oeda 1994; Houde y col. 1995; Close 1996; Wisniewski y col. 1999; Hara y col. 2001). Por otra parte, Bozovic y col. (2013) han demostrado *in vitro* la capacidad crioprotectora de algunas proteínas deshidrinas de *Arabidopsis* (COR47, ERD14 y LT29), sobre membranas de tilacoides aislados de espinacas durante ciclos de congelación-descongelación. Además, Rinne y col.

(1999) han indicado que una fracción purificada de deshidrinas de abedul fue capaz de mejorar la actividad de α-amilasa (EC 3.2.1.1) respecto a la fracción donde las deshidrinas fueron eliminadas por inmunoprecipitación. Si bien, el rol *in vivo* de las proteínas DHNs se desconoce, esta evidencia experimental *in vitro*, permite inferir una función de protección en respuesta al estrés por deshidratación impuesto por la sequía, congelamiento y alta salinidad, y donde los mecanismos de acción estarían relacionados con la protección de membrana, crioprotección de enzimas y protección contra estrés oxidativo (Graether y Boddington 2014). Se conoce que, durante el proceso de aclimatación a bajas temperaturas, se induce la expresión y acumulación de deshidrinas en muchas especies de plantas herbáceas, como trigo y cebada (Guo y col. 1992; Fowler y col. 2001; Kosová y col. 2007), como también en especies leñosas, *Betula pubescens* (Rinne y col. 1999), *Cornus sericea* (Sarnighausen y col. 2002), *Pinus sylvestris* (Kontunen-Soppela y Laine 2001), *Picea glauca* (Liu y col. 2004) y *Picea obovata* (Kjellsen y col. 2013). Respecto a la especie *E. globulus*, Fernández y col. (2012a; 2012b) han informado la presencia de cuatro genes deshidrinas, denominados Eugl*DHN1*, Eugl*DHN2*, Eugl*DHN3* y Eugl*DHN10*. La expresión relativa de estos, evidenció que los genes Eugl*DHN1*, Eugl*DHN2* y Eugl*DHN10* se inducen y acumulan en condiciones de bajas temperaturas, con mayor abundancia de transcritos en un genotipo resistente que en uno susceptible a heladas, mientras que Eugl*DHN3* se expresó principalmente en condiciones de deshidratación. Últimamente se ha reportado que los genes Eugl*DHN2* y Eugl*DHN10* están principalmente involucrados en el proceso de aclimatación al frío (Fernández y col. 2015). Por otra parte, se ha reportado en numerosos estudios que la expresión transgénica de deshidrinas que mejoran la resistencia al frío y sequía en plantas (Hara y col. 2003; Puhakainen y col. 2004; Yang y col. 2014).

La regulación específica de los genes *DHNs* es compleja, se ha reportado en *Arabidopsis* que la expresión de estos genes estaría regulada por deshidratación, aunque también se inducirían por frío, sequía, salinidad y tratamientos con ABA exógeno (Thomashow 1999). Sin embargo, estudios han reportado la expresión del gen Bpu*DHN1* (*Betula pubescens*), en respuesta a un efecto combinado de fotoperiodo y bajas temperaturas (Welling y col. 2004), sugiriendo que su expresión es potenciada por una exposición previa de las plantas a cambios en la longitud del día. No obstante, esto no ha sido corroborado en otras especies de leñosas como *Prunus persica* y *E. globulus*, donde el fotoperiodo no afecta la expresión

de genes *DHNs*, siendo estos inducidos principalmente por baja temperatura (Wisniewski y col. 2006; Fernández y col. 2012b). Un promotor regulado por el estrés abiótico puede ser un importante interruptor molecular implicado en la regulación transcripcional de una red dinámica de genes asociados al proceso de aclimatación y resistencia al estrés. Sin embargo, muchas veces la estructura y las funciones de un promotor son ambiguas, pudiendo ser regulado por varios estímulos. Estudios de regiones promotoras de los genes *DHNs* han identificados elementos activadores en *cis* regulados por ABA (ABRE) y factores de transcripción CBF (CRT/DRE). En particular para genes *DHNs* de *E. globulus*, se han reportado estos elementos *cis* a cada uno de los cuatro genes descritos (Tabla 1.1).

**Tabla 1.1** Detalles de elementos de acción *cis* involucrados en la expresión de respuesta al estrés de cuatro Eugl*DHNs* en *E. globulus* (modificado Fernández y col. 2012b)

| **Elementos *cis*** | **Secuencia** | **Frecuencia elementos *cis* *E. globulus dehydrins*** | | | | **Función** | **Reference** |
|---|---|---|---|---|---|---|---|
| | | *DHN1* | *DHN2* | *DHN3* | *DHN10* | | |
| **ABRE** | TACGTG | 3[a] | 1 | 3[a] | 0 | Respuesta ABA | Baker y col. (1994) |
| **CRT** | GCCGAC | 4[a] | 2[a] | 0 | 0 | Respuesta a baja temperatura | Stockinger y col. (1997) |
| **LTR** | CCGAAA | 0 | 0 | 0 | 1 | Respuesta a baja temperatura | White y col. (1994) |

[a] La frecuencia observada es más alta de lo esperado para el elemento en acción en *cis* en la región promotora

Se observa que los niveles de transcritos reportados de los genes Eugl*DHNs* en condiciones de baja temperatura, no están relacionados directamente con el número de elementos CRT encontrado en las regiones promotoras putativas (Fernández y col. 2012a; 2012b), dado que el gen Eugl*DHN1*, con 4 elementos CTR, tiene una menor acumulación de transcrito a bajas temperaturas que el gen Eugl*DHN2*, el cual tiene 2 elementos CRT. Por otra parte, el gen Eugl*DHN10* es inducido por bajas temperaturas tanto en hojas como en tallo, no presenta elementos CRT, pero si elementos LTR (*low-temperature responsiveness*), que han sido reportados como similares a CRT por contener la secuencia similar a CCGA (Sakuma y col. 2002).

Otra de las especies de eucalipto plantada en Chile es *E. nitens*. También es una especie de rápido crecimiento, que puede crecer en áreas de gran altitud, presentando mayor resistencia a las heladas que *E. globulus* (Tibbits y col. 1997). En Chile, ha sido introducida

con éxito para ser plantada en áreas donde las heladas son frecuentes, pero ha demostrado tener menor rendimiento pulpable que *E. globulus*, además de presentar otros inconvenientes como la baja capacidad de enraizar, lo que impide el uso esquejes como principal método de reproducción vegetativa.

Existen pocos estudios de la identificación de genes en respuesta al estrés por frío en *E. nitens*. Recientemente, se ha reportado un perfil transcriptómico sobre el mecanismo molecular del proceso de aclimatación y desaclimatación en frío (Gaete-Loyola y col. 2017). Si bien esta especie es más resistente al frío que *E. globulus*, surgen algunas interrogantes: 1) ¿La especie *E. nitens* presentará genes deshidrinas, que tengan relación con el proceso de aclimatación al frío o inducción por bajas temperaturas? además, 2) ¿qué diferencias podrían identificarse en los elementos activadores en *cis* en las regiones promotoras de algunos de estos genes deshidrinas?, y 3) ¿estas diferencias proporcionaran algún cambio en la expresión de los genes que controlan, los cual pudiesen atribuirle una mayor resistencia al frío por parte de la especie?. Las respuestas a estas interrogantes permitirían describir posibles aplicaciones, que comparen y exploren la regulación al frío de regiones promotoras de un mismo gen, en especies de eucaliptos diferentes, pero relacionadas filogenéticamente.

### 1.3. Métodos de cuantificación y validación génica

Como se ha mencionado, diversos son los genes propuestos que pueden intervenir en la resistencia al estrés por frío y al congelamiento en plantas. Dichos genes se proponen como genes candidatos en especies donde aún no se han descrito y validado su función. La validación de genes hace referencia a describir, probar y reconocer su función putativa, a partir de especies modelos, extrapolando dicha función a especies de interés. Hoy en día, existen métodos para relacionar y validar la función de un gen, algunos de estos recurren a la cuantificación relativa de transcritos de un determinado gen o a la transformación genética con el gen de interés. La cuantificación relativa de transcritos o PCR cuantitativa (qPCR), da luces, en términos relativos, del nivel de transcripción de un gen, relacionando la importancia en la expresión de transcritos a la proteína que codifica y el posible cambio en la característica fenotípica generada. Por otro lado, la transformación genética corresponde a la inserción o modificación de secuencias genómicas funcionales, tanto

secuencias codificantes como de regiones promotoras, con la obtención de un organismo genéticamente modificado, el cual se define como un organismo que su material genético ha sido manipulado con el fin de otorgarle nuevas características genéticas específicas, únicas y diferentes al organismo original, no transformado.

### 1.3.1. Análisis de expresión génica y método de cuantificación de transcritos.

El método qPCR es una técnica sensible y precisa, que permite la cuantificación de transcritos presentes en una muestra. La técnica consiste en la medición de producto acumulado durante la fase exponencial en la reacción de PCR luego de un número de ciclos umbral ($C_T$, cycle threshold), mediante la detección por fluorescencia (Gibson y col. 1996). Los dos principales sistemas de detección por fluorescencia corresponden, a un agente de unión al ADN y sondas de hibridación especificas marcadas con fluoróforos. El agente de unión al ADN, es un fluoróforo el que aumenta notablemente la emisión de fluorescencia cuando se unen a la molécula de ADN de doble hebra. El principal inconveniente de esta metodología es su baja especificidad, debido a que se unen indiscriminadamente a cualquier producto de doble hebra presente en la reacción. Esto puede sobreestimar la cuantificación de ADN amplificado al detectar productos inespecíficos, como dímeros de partidor, que frecuentemente son generados en la reacción PCR. En cambio, las sondas de hibridación específicas, denominadas Taqman®, corresponden una secuencia específica de ADN de hebra simple, la cual hibrida con el ADN templado permitiendo la cuantificación del producto amplificado. La principal ventaja de las sondas Taqman® es que incrementan significativamente la especificidad de la detección en el análisis.

La técnica de qPCR se ha convertido en una de las preferidas para la cuantificación de pequeñas cantidades de ácidos nucleicos, por ser una técnica rápida, especifica y con un alto grado de sensibilidad (Valasek y Repa 2005). Esta permite cuantificar diferencias en los niveles de expresión de un gen específico entre diferentes muestras y tratamientos. El método más utilizado es el método comparativo de los valores $C_T$ o bien denominado método $\Delta\Delta C_T$. Este método utiliza cálculos aritméticos para obtener el resultado de la cuantificación relativa, considerando la expresión del gen en estudio, normalizado contra uno o varios genes de referencia endógena y comparado con una muestra calibradora (muestra control) (Livak y Schmittgen 2001). Un gen de referencia endógeno, es un gen

que participa en funciones básicas de mantenimiento celular y es utilizado como control interno, para representar una expresión estable, en el tiempo y bajo diferentes condiciones de tratamientos del organismo en estudio. Los datos generados por el análisis de qPCR son expresados como la diferencia en número de veces (fold-change) de los niveles de expresión, en comparación con la muestra calibradora.

Considerado todas estas limitantes, es posible contar con un análisis robusto y reproducible para la cuantificación de la expresión génica, que permita comparar la expresión de genes de interés, en distintas condiciones experimentales y en particular para este estudio de la aclimatación al frío y estrés por congelamiento en *E. globulus*.

### 1.3.2. Transformación genética en plantas.

En la aplicación de esta metodología, un ensayo ampliamente utilizado es realizar la sobreexpresión de un gen de interés obtenido de una determinada especie, sobre una especie modelo como: *Arabidopsis*, *Nicotiana tabacum*, *Oryza sativa*, *Populus* spp., entre otras o bien utilizar la misma especie de estudio, este fue el caso de los genes *CBF* que en un principio se describieron en *Arabidopsis*. La expresión constitutiva de FT At*CBF1-2-3* en plantas transformadas, induce la expresión de genes *COR* en plantas no aclimatadas, confiriendo resistencia a congelación, sequía y alta salinidad (Gilmour y col. 1998; Jaglo-Ottosen y col. 1998; Haake y col. 2002). De igual manera, para mejorar la resistencia al frío en plantas de uso agrícola, se ha introducido el gen *CBF* de *Arabidopsis* en diversas especies de interés agronómico, informado que una sobreexpresión de los genes At*CBF1-3* mejora la tolerancia al estrés por frío, congelamiento, sequía y salinidad en plantas transgénicas de canola (Jaglo y col. 2001), tomate (Hsieh y col. 2002), tabaco (Kasuga y col. 2004), trigo (Pellegrineschi y col. 2004) y arroz (Oh y col. 2005). Por lo tanto, dicha metodología nos permitiría corroborar la función putativa de los genes *CBF* de *E. globulus*.

## 1.4. Propuesta de investigación

Como principal problemática, la alta demanda en plantaciones de eucaliptos en el mundo, está llevando a especies como *E. globulus* a ser cultivada en sitios no óptimos para su crecimiento. Sitios con frecuencia de heladas en primavera, están siendo ocupados para el

cultivo de esta especie. Tales condiciones afectan al establecimiento, crecimiento y producción de las plantaciones, por efectos del frío. Esto ha llevado a la necesidad de reconocer y estudiar que mecanismos están asociados en otorgar una mayor resistencia al estrés por frío, para así identificar y seleccionar el material vegetal con estas características, o bien realizar modificaciones genéticas, que aumenten dicha resistencia. En este trabajo, se plantea identificar, caracterizar y validar la función de genes *CBF* de *E. globulus*, como uno de los principales factores de transcripción que regulan la señalización y respuesta al frío, otorgando mayor resistencia al estrés. Para determinar esto, se propone identificar y aislar secuencias de genes *CBF* en *E. globulus*, homólogos a los *CBF* actualmente descritos para especies de *Eucalyptus* filogenéticamente relacionadas. La correcta identificación y aislación de estas secuencias genéticas implicó estrategias de secuenciación, con el uso de partidores secuencia-específica, y el desarrollo de análisis bioinformáticos para caracterizar las secuencias obtenidas. Además, relacionar la expresión de estos factores de transcripción con el proceso de aclimatación al frío en plantas juveniles de *E. globulus*, identificando las condiciones que inducen estos genes. Para ello, se desarrolló metodologías adecuadas en la cuantificación relativa de transcritos, mediante la detección por sondas de hibridación tipo Taqman®. Una vez determinada la relación en el proceso de aclimatación al frío en plantas de *E. globulus,* se validó la función putativa de los genes *CBF* en plantas de *Arabidopsis thaliana*, desarrollando vectores de clonación y expresión, para aislar y transferir las secuencias obtenidas. Adicionalmente, se analizó las regiones promotoras putativas de los genes Eugl*DHN2* y Eni*DHN2*, mediante análisis bioinformáticos y por validación en plantas *Arabidopsis* transformadas, observando la expresión relativa del gen marcador *GUS* en diferentes líneas transgénicas.

## 1.5. HIPOTESIS

**i.** *Eucalyptus globulus* posee más de un gen que codifica factores de transcripción CBF y su expresión se encuentra relacionada con el proceso de aclimatación al frío.

**ii.** La expresión heteróloga de factores de transcripción CBF de *E. globulus* otorgan un aumento de la resistencia al frío en *Arabidopsis thaliana*.

**iii.** Región promotora putativa del gen *DHN2* de *E. nitens* opera como un promotor fuerte inducido por frío.

## 1.6. OBJETIVOS

### 1.6.1. Objetivo general

Identificar y caracterizar genes *CBF* en *Eucalyptus globulus*, estudiar su expresión relativa en relación con el proceso de aclimatación al frío en plántulas de *E. globulus*, y validar su función mediante la expresión heteróloga en *Arabidopsis thaliana*. Además, analizar la expresión del gen marcador (*GUS*) inducido por regiones promotoras putativas de los genes *DHN2* de dos especies de *Eucalyptus* que difieren en su resistencia al frío, *E. globulus* y *E. nitens.*

### 1.6.2. Objetivos específicos

**i.** Identificar y caracterizar secuencias génicas que codifiquen factores de transcripción CBF en *E. globulus.*

- ✓ Buscar y examinar genes *CBF* disponibles en bases de datos genéticas y/o genómicas de especies emparentadas a *E. globulus*.

- ✓ Obtener, secuenciar y reportar la estructura de las secuencias codificantes y no codificantes de los genes *CBF*, mediante la utilización de herramientas bioinformáticas.

**ii.** Relacionar los niveles de expresión de los diferentes factores CBF con el aumento de la resistencia al frío en plantas juveniles de *E. globulus*.

- ✓ Estandarizar una metodología adecuada para la cuantificación relativa de genes *CBF*, mediante PCR en tiempo real.

- ✓ Someter plantas juveniles de *E. globulus* a experimentos de aclimatación al frío, en cámaras de crecimiento con control de temperatura y fotoperiodo.

- ✓ Examinar los niveles de expresión de los genes *CBF* identificados con el estado de aclimatación al frío de plantas juveniles de *E. globulus*.

**iii.** Validar la función de aquellos genes *CBF* de *E. globulus* (Egl*CBF*) que presenten una marcada relación con el estrés por frío, mediante su expresión heteróloga en *A. thaliana*.

- ✓ Diseñar plásmidos para la expresión constitutiva de genes Egl*CBF* en plantas de *A. thaliana*.

- ✓ Obtener plantas genéticamente transformadas de *A. thaliana* con plásmidos de expresión constitutiva, mediante la transfección del T-DNA en un proceso denominado inmersión floral.

- ✓ Someter plantas transformadas de *A. thaliana* a experimentos de temperaturas congelantes, con el uso de cámara de cultivo con control de temperatura y fotoperiodo.

- ✓ Examinar los niveles de expresión de los genes Egl*CBF* con la resistencia al congelamiento de plantas de *A. thaliana* transformadas.

- ✓ Estudiar la función de genes Egl*CBF*, mediante la cuantificación relativa de genes efectores (*CBF target*) de respuesta al estrés por congelamiento, inducida por la sobreexpresión de genes Egl*CBF*.

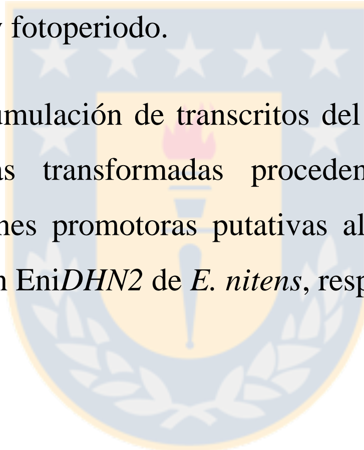
**iv.** Analizar la expresión relativa del gen marcador *GUS* en plantas de *Arabidopsis* transformadas, inducido por regiones promotoras putativas de los genes *DHN2* de dos especies de *Eucalyptus* que difieren en su resistencia al frío, *E. globulus* y *E. nitens.*

- ✓ Obtener las secuencias promotoras del gen *DHN2* de las especies *E. globulus* (Eugl*DHN2*) y *E. nitens* (Eni*DHN2*).

- ✓ Diseñar plásmidos para la expresión inducida por frío del gen marcador *GUS* en plantas de *A. thaliana*, utilizando regiones promotoras putativas del gen *DHN2* de dos especies de *Eucalyptus*.

- ✓ Obtener plantas genéticamente transformadas de *A. thaliana* con plásmidos de expresión inducida por frío, mediante la transfección del T-DNA en un proceso denominado inmersión floral.

- ✓ Someter plantas transformadas de *A. thaliana*, a experimentos de temperaturas frías y congelantes, con el uso de cámara de cultivo con control de temperatura y fotoperiodo.



- ✓ Examinar la acumulación de transcritos del gen *GUS* inducido por frío, en diferentes líneas transformadas procedentes de dos constructos con diferentes regiones promotoras putativas al gen *DHN2*, Eugl*DHN2* de *E. globulus* y al gen Eni*DHN2* de *E. nitens*, respectivamente.

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## CAPÍTULO II: OVEREXPRESSION OF THREE NOVEL CBF TRANSCRIPTION FACTORS FROM *Eucalyptus globulus* IMPROVES COLD TOLERANCE ON TRANSGENIC *Arabidopsis thaliana*

Darío Navarrete-Campos, Regis Le Feuvre, Claudio Balocchi, Sofía Valenzuela

### ABSTRACT

Three new *CBF* genes were isolated from *E. globulus* denominated Egl*CBF1a*, *c* and *d*, coding for proteins of 220, 229 and 196 amino acids, respectively. The sequence analysis showed that the three predicted proteins contain an AP2 DNA-binding domain and two CBF signature sequences. Phylogenetic analysis demonstrated that these proteins were highly similar to those described in *E. grandis* and *E. gunnii*. Transcript abundance analysis in three different *E. globulus* genotypes exposed to a cold acclimation treatment, showed that these *CBF* genes were highly related to the acclimation process and presented the highest relative expression at freezing temperatures. Egl*CBF1a* showed the highest expression level (1,311-fold change) in the cold tolerant genotype (R1). Egl*CBF1a* and *d* genes were induced by chilling and freezing temperatures, while Egl*CBF1c* was constitutively expressed, increasing its transcript level when plants were exposed to freezing temperatures. The constitutive overexpression of each *E. globulus CBF* gene in *Arabidopsis* plants induces the endogenous *CBF* regulon gene expression of *Arabidopsis* and enhanced its tolerance to freezing, with additional phenotypic effects including growth inhibition and delayed flowering. These results indicate that the three Egl*CBF* genes analyzed play important roles under cold acclimation processes in *E. globulus* and are involved in the signaling pathway of cold stress and the freezing tolerance phenotype observed on specific genotypes of this species and in transgenic *Arabidopsis* lines.

Keywords: *Freezing tolerance • qPCR Taqman® probe • C-repeat binding factor*

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## INTRODUCTION

Eucalypts are among the fastest growing tree species in the world, representing about 8% of the forest plantations, with more than 20 million hectares of plantations distributed in 90 countries (FAO 2007; Iglesias-Trabado et al. 2009). In Chile, *E. globulus* is the main hardwood cultivated species used for pulp production, with 541,859 hectares (INFOR 2014), and is characterized by its fast growth, straightness, high wood density and good fiber quality (Pita and Pardo 2001; Grattapaglia 2004). This species grows well on temperate regions, with temperatures between 10 and 15 ºC, but it is sensitive to low temperatures, although it has been reported that it can tolerate frosts of – 4.5 to – 6 ºC during short periods of time (Almeida et al. 1994; Tibbits et al. 2006). The most damaging effects of freezing on eucalypts take place in the establishment phase of the young trees during the late winter–early spring, especially on regions with frosts of –7 to –9 ºC (Volker et al. 1994); the damage is characterized by injuries on stems, leaves and apical buds. The final consequence of this stress is a decrease on yield or even the lost of plantations (Geldres and Schlatter 2004).

Cold is an adverse abiotic factor, with severe negative impacts on plant productivity (Ruelland et al. 2009). In temperate regions it has been observed that the exposure of plants to low, non-freezing temperatures, can increase their freezing tolerance, by triggering a genetic response that allows them to tolerate cold or freezing temperatures (Thomashow 1999), in a process known as cold acclimation (Levitt 1980; Thomashow 1990), but this ability is mainly associated to some species (Rodziewicz et al. 2014).

Several physiological and molecular changes take place during the acclimation process and are associated to changes on gene expression (Thomashow 1999; Chaves et al. 2003). There are some genes that have been reported as responsive to cold, known as *COR* genes (*cold-regulated*), some of them are the LEA (*late embryogenesis abundant*) proteins, LTI (*low temperature induced*) proteins, and dehydrins (DHNs), associated to a cold signal pathway dependent of the CBF transcription factors. This pathway is considered as a *CBF* regulon (Thomashow 1999; Thomashow et al. 2001), and includes essential regulatory elements in response to cold (Vinocur and Altman 2005; Chinnusamy et al. 2010).

CBF/DREB1 (*C-repeat Binding Factor/Dehydration Responsive Element Binding*) proteins are members of the AP2/ERF (APETALA2/Ethylene-Response Factor) protein family of transcription factors (Riechmann & Meyerowitz 1998), defined by containing a conserved 60-aminoacid region, the AP2/ERF DNA-binding domain (Jofuku et al. 1994; Ohme-Takagi and Shinshi 1995). This domain binds to the specific *cis*-element sequences CRT/DRE (*C-Repeat/Dehydration Responsive Element*) present in the promoter regions of *COR* genes (Yamaguchi-Shinozaki and Shinozaki 1994; Thomashow 2010). The primary feature that distinguishes the CBF proteins from others of the AP2/ERF family members corresponds to two signature sequences flanking the AP2/ERF domain (Jaglo et al. 2001). These sequences, PKKP/RAGRxKFxETRHP (abbreviated PKKPAGR) and DSAWR, are motifs located immediately up and downstream from the AP2/ERF domain, respectively, and are required for the correct activity of the protein (Canella et al. 2010). These motif sequences and some differences in amino acid residues of the AP2/ERF domain have been essential in the classification within the A1 group of DREB subfamily of AP2/ERF family transcription factors (Sakuma et al. 2002). The first *CBF/DREB1* genes described were studied in *Arabidopsis thaliana*, with four sequences reported known as *CBF1* to *4* (Stockinger et al. 1997; Liu et al. 1998; Gilmour et al. 1998; Haake et al. 2002), which are expressed under cold temperatures and water deficit conditions (Gilmour et al. 1998; Haake et al. 2002). However, in *Arabidopsis* the A1 group has six members, with other two atypical *CBF* homolog genes, named *DDF1* and *2* being induced by high salinity stress (Magome et al. 2004; 2008). Regarding the overexpression of *CBF1* to *4* genes in *Arabidopsis*, an increase in the freezing tolerance of non-acclimated plants, accompanied by an increased tolerance to drought and high salinity conditions has been reported (Jaglo-Ottosen et al. 1998; Haake et al. 2002; Gilmour et al. 2004). To date, there are many studies that have identified *CBF* genes in numerous herbaceous and woody plants (Zhang at al. 2004; Xiao et al. 2006; Benedict et al. 2006; Champ et al. 2007; Welling and Palva 2008), as well as its role in cold tolerance (Medina et al. 2011; Zhou et al. 2011). For eucalypts, four *CBF* sequences in *E. gunnii* have been reported (Egu*CBF1a-b-c-d*), which are differentially induced by cold and freezing temperatures (El Kayal et al. 2006; Navarro et al. 2009). An *in silico* screening on the reference genome of *E. grandis* identified a total of 17 *CBF* homologous sequences (Azar et al. 2011), that were later annotated by Cao et al.

(2015). In the case of *E. globulus*, Eg*CBF1* is the only *CBF* gene reported to date (Gamboa et al. 2007).

Recently, several studies have found that the overexpression of *CBF* genes improved cold and freezing tolerance in plants, including model species such as *Arabidopsis* and *Nicotiana tabacum* (Siddiqua and Nassuth 2011; Li et al. 2013; Zhou et al. 2014; Wang et al. 2014; Xue et al. 2014; Fang et al. 2015; Wang et al. 2015), monocotyledonous species such as rice (Xu et al. 2011; Byun et al. 2015), and dicotyledonous woody species such as eucalypts (Navarro et al. 2011), apple (Wisniewski et al. 2011, 2015) and grape (Tillett et al. 2012). Additionally, it has been reported in a transcriptome of a frost tolerant *E. globulus* genotype, that 12% of the differentially expressed genes correspond to transcription factors, but none of these have been identified as a *CBF* gene (Fernández et al. 2015). In this work, three new Egl*CBF* genes are identified in *E. globulus* and their differential transcript abundances for three genotypes under cold acclimation treatments are reported. Also, the overexpression of Egl*CBF* gene in *Arabidopsis* is evaluated, showing a remarkable increase in cold tolerance correlated with the expression of the Egl*CBF* genes.

## MATERIAL AND METHODS

### *Plants materials and cold acclimation treatment in Eucalyptus*

Three different *E. globulus* genotypes were used, previously characterized as cold resistant (R1 and R2) and cold sensitive (S1). The level of resistance/susceptibility was assessed under field conditions and collecting historical data provided by the forest company. For each genotype, thirty biological replicates (ramets) of 6-month-old plants were used, planted in Styrofoam boxes with (1:1) vermiculite and perlite substrate. Thirty ramets were distributed in three growth chambers (ten ramets of each genotype in each chamber), under a completely randomized design with controlled temperature and photoperiod. The clonal identity of each ramet was verified using microsatellite markers (data not shown).

The plants were exposed to four different treatments varying in temperature and photoperiod: non-acclimated (NA), cold acclimated before night frosts of -2 ºC (CABF), cold acclimated after night frosts of -2 ºC (CAAF), and de-acclimated (DA) as described by

Fernández et al. (2010). For each treatment, three ramets per genotype were sampled, collecting its stem and leaves between 08:00 - 09:00 a.m. on days 7, 14, 21 and 28, respectively. All samples were immediately frozen in liquid nitrogen and kept at -80 ºC until used. Finally, a last treatment was incorporated consisting in a night frost of −6 °C (NF) on day 29, which was applied to the 18 remaining ramets per genotype, to verify the freezing tolerance of the different genotypes assayed. The ramets were kept for a recovery during 10 days under long-day photoperiod (14 h light) and 12/6 ºC day/night temperature, with periodic irrigation. For each ramet, the survival and leaf damage caused by freezing temperatures was measured, and the information obtained from live and dead organs, including leaves, buds and apical buds, were used to calculate the survival and damage, according to Fernández et al. (2012).

***CBF genes sequencing and data analysis***

To sequence the *CBF* genes of *E. globulus*, specific primers for the four *CBF* gene sequences reported in *E. gunnii* (El Kayal et al. 2006; Navarro et al. 2009) were designed. The genes were amplified by PCR using cDNA templates from leaves of *E. globulus* plants subjected at 8/4 ºC day/night temperature and 10 h photoperiod for one week, with DNA polymerase *PfuUltra II Fusion HS* (Agilent Technologies) and the primers described on Supplemental data Table S2.1.

Sanger DNA sequencing was carried out at Macrogen (Korea). The corresponding amino acid sequences for each gene were analyzed by Geneious 6.1 software and the putative DNA binding domains were identified by PROSITE (http://prosite.expasy.org/). The molecular weight (MW) and theoretical isoelectric point (pI) of the deduced proteins were analyzed by using the ProtParam tool (http://web.expasy.org/protparam/). The analysis of protein sequence homology was performed by multiple alignment using ClustalW with default parameters and assembled by Geneious 6.1 software. For the phylogenetic tree construction, the protein multiple alignment was performed by MEGA 6.0 software using ClustalW, and the tree was constructed by using the neighbor-joining method with a bootstrap test calculated on 1,000 replicates. The full-length *CBF* nucleotide sequences for *E. globulus* were deposited in Genbank.

***EglCBF sequence cloning and vector construction***

Each Egl*CBF* gene was isolated and amplified from cDNA samples of *E. globulus* plants subjected at 8/4 ºC day/night temperature, using high fidelity *Pfu*Ultra II Fusion HS DNA Polymerase (Agilent Technologies) and specific primers for each gene (Table S2.1), the products were cloned into pGEM-T Easy vector (Promega). Based on this material, the open reading frame (ORF) sequence of three Egl*CBF* genes were amplified using specific primers adding *attB* recombination sites for Gateway® cloning (Invitrogen) (Table S2.1). Each amplified product was recombined by BP reaction with the pCC1155 vector, corresponding to the pDONR221 vector with an ampicillin resistance gene, modified by Bonawitz et al. (2012). The resulting vectors were recombined for the LR reaction separately with the pMDC32 destination vector (Curtis and Grossniklaus 2003), obtaining three different expression vectors, driven by the constitutive CaMV35S promoter and identified as 35S::Egl*CBF1a*, 35S::Egl*CBF1c* and 35S::Egl*CBF1d*, respectively. Each construct was verified by sequencing, and introduced on *Agrobacterium tumefaciens* strain GV3101 by electroporation (Weigel and Glazebrook 2002), and used to transform *Arabidopsis* plants by the floral dip method (Clough and Bent 1998).

***Arabidopsis transformation, growth conditions and freezing treatment***

*Arabidopsis thaliana* ecotype Col-0 plants were used for transformation of three *CBF* genes from *E. globulus*. Seeds collected from *A. thaliana* were germinated in Petri dishes containing half strength MS medium and 2.43 g/L Phytagel (Sigma), selecting the transformed plants with hygromycin B at 15 µg/ml, according to the method described by Harrison et al. (2006). At seven days, the selected plants were transplanted into pots and maintained in a growth chamber at 23 ºC and 16/8 h day/night photoperiod. Growth and phenotypic development was measured in *Arabidopsis* transformed lines and untransformed wild type (WT) plants, collecting information of rosette diameter and plant height of 35, 40 and 60 days-old plants.

All T0 and T1 transformed plants were selected by hygromycin B resistance and verified by PCR amplification of the gene of interest (data not shown). Transformed lines were designated as lines A, C and D, with correlative numbering for each construct, namely

35S::Egl*CBF1a*, 35S::Egl*CBF1c* and Egl*CBF1d*. Ten T2 transformed lines and WT plants of 5 weeks age were exposed to the freezing treatment. Thirty plants per line were subjected to a temperature decrease in a Percival® LT-36VL phytotron, starting at 23 ºC, with a 2 ºC decrease per h until reaching -6 ºC, and kept at this temperature for 3 h. Three plants per each line were sampled at 23, 4 and -6 ºC and immediately stored in liquid nitrogen and then transferred to -80 ºC freezer until further analysis. After this assay, twenty-one plants were kept at 23 ºC for seven days in order to visually estimate the survival rate by assessing the plant recovery.

***RNA extraction and gene expression analysis***

The total RNA was extracted from the collected plant material using the CTAB method described by Chang et al. (1993), in the case of *Eucalyptus*, and the protocol described by Weigel and Glazebrook (2002) for *Arabidopsis* samples. The RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific). RNA purity of the samples was determined by its $A_{260/280}$ ratio of between 1.9 and 2.1, and an $A_{260/230}$ ratio higher than 2.0. RNA integrity was checked by electrophoresis in 2% agarose gels. For each sample, 1 µg of RNA was treated with DNaseI (Fermentas) to remove genomic DNA contamination. First strand copy DNA (cDNA) was synthesized by reverse transcription using the High-Capacity cDNA Reverse Transcription kit (Life technologies) according to the manufacturer's instructions.

Gene expression analysis in *Eucalyptus* was measured in samples under four acclimated treatments previously described and was determined by quantitative real-time PCR (qPCR) using *Taqman*® probes for detection with a StepOne Plus system (Applied Biosystems). Total reaction volume was 20 µl with 10 ng cDNA template, 10 µl *TaqMan Gene Expression Master Mix* (Applied Biosystems) and a concentration of primers and probe of 200 nM and 250 nM, respectively. Two endogenous (housekeeping) genes were used (*UBC* and *a-TUB*), previously reported by Fernández et al. (2010). The probes and primers used were designed for each gene by Primer Express 2.0 software (Table S2.1). All qPCR reactions were carried out under the following conditions: 2 min at 50 ºC, 10 min at 95 ºC, 40 cycles of 15 s at 95 ºC, and 1 min at 60 ºC in 96-well optical reaction plates (Applied Biosystems). The calibrator sample corresponded to one ramet of the S1 genotype under

NA treatment. For constitutive relative expression of *CBF* genes in *E. globulus* each expression levels were normalized with respect to the *UBC* gene, which has a single copy in the *E. grandis* genome.

In transformed and WT *Arabidopsis* plants, the gene expression of Egl*CBF* and endogenous *Arabidopsis* genes (At*CBF2,* At*CBF3* and four *COR* genes) was determined by qPCR analysis using the specific primers listed on Supplemental data Table S2.2 and the Evagreen® fluorophore (Solis BioDyne). The detection was performed on a StepOne Plus system with a qPCR reaction mixture of 1X Evagreen®, 200 nM primers and 10 ng cDNA templates, under the following conditions: 10 min at 95 ºC, 40 cycles of 15 s at 95 ºC and 1 min at 60 ºC in 96-well optical reaction plates. Two endogenous genes were used as controls, the elongation factor 1-alpha (*Ef1-α*, AT5G60390) and the protein phosphatase 2A subunit A3 (*PP2AA3*, AT1G13320). To normalize the relative expression of the transgenes samples, the transformed line with the lower expression was used. However, to normalize the relative expression of endogenous genes in *Arabidopsis,* a WT plant sample was used as a reference.

For all genes, the PCR efficiency was determined by measuring the $C_T$ to a specific threshold for a serial dilution of cDNA samples. The specificity of the amplified products was determined by the dissociation curve, with 118 cycles increasing +0.3 ºC per cycle from 60 to 95 ºC. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), including three technical replicates for each of the three biological replicates.

***Statistical analysis***

Before data analysis, the assumptions of normality and homogeneity of variance for each variable were verified. In *Eucalyptus*, survival and leaf damage data were subjected to one-way analysis of variance (ANOVA) to test the effect of genotypes. For relative gene expression, data was subject to two-way ANOVA to test the effect of cold acclimation treatments and genotype. In *Arabidopsis*, the relative gene expression of Egl*CBF* transgenes and endogenous genes data were subjected to one-way ANOVA to test the

effect of different T2 transformed lines and WT plants. A Tukey test was applied to determine significant differences between samples.

## RESULTS

### ***Sequencing and characterization of the three CBF genes from E. globulus***

Three *CBF* homologous sequences were identified in *E. globulus*, corresponding to the paralog genes of *E. gunnii*. These sequences were named Egl*CBF1a*, Egl*CBF1c* and Egl*CBF1d*, and deposited on GenBank with the accession numbers KX669025, KX669026 and KX669027, respectively. In the case of Egl*CBF1b*, there were some problems in the Taqman probe for its relative expression detection, for this reason this gene was discarded for further analysis. The full-length sequence obtained for Egl*CBF1a* was 736 bp; including a CDS of 660 bases corresponding to 220 amino acid residues, with a predicted MW of 24.24 kDa and an isoelectric point (pI) of 5.73. The full length obtained for Egl*CBF1c* gene was 996 bp; including a CDS of 687 bases coding for a 229 amino acid protein, with a predicted MW of 24.98 kDa and a pI of 5.10. Finally, the full length obtained for Egl*CBF1d* was 1,285 bp; including a CDS of 588 bases corresponding to 196 amino acid residues, with a predicted MW of 21.59 kDa and a pI of 5.87.

Multiple alignment over the amino acid sequences determined the identity of the sequences as CBF transcriptional factors, with the presence of the AP2/ERF domain and two characteristic motifs of CBF proteins (Fig. 2.1). These motifs and domains have highly conserved amino acid residues, the comparison of the full length of the three EglCBF proteins and the domain sequences of other *Eucalyptus* species indicate a high degree of similarity. At the amino acid residue level, the complete sequence for EglCBF1a protein shows 77.7% similarity to EglCBF1c and 76.6% to EglCBF1d, and the EglCBF1c protein was 76.0% similar to EglCBF1d. At the domain residue level, EglCBF1a and c were 100% identical to EgrCBF6 and 1 of *E. grandis* sequences, respectively, and EglCBF1d was 100% identical to EguCBF1d of *E. gunnii*. The full-length predicted proteins have more than 94% similarity with their paralogs of *E. grandis* and *E. gunnii*.

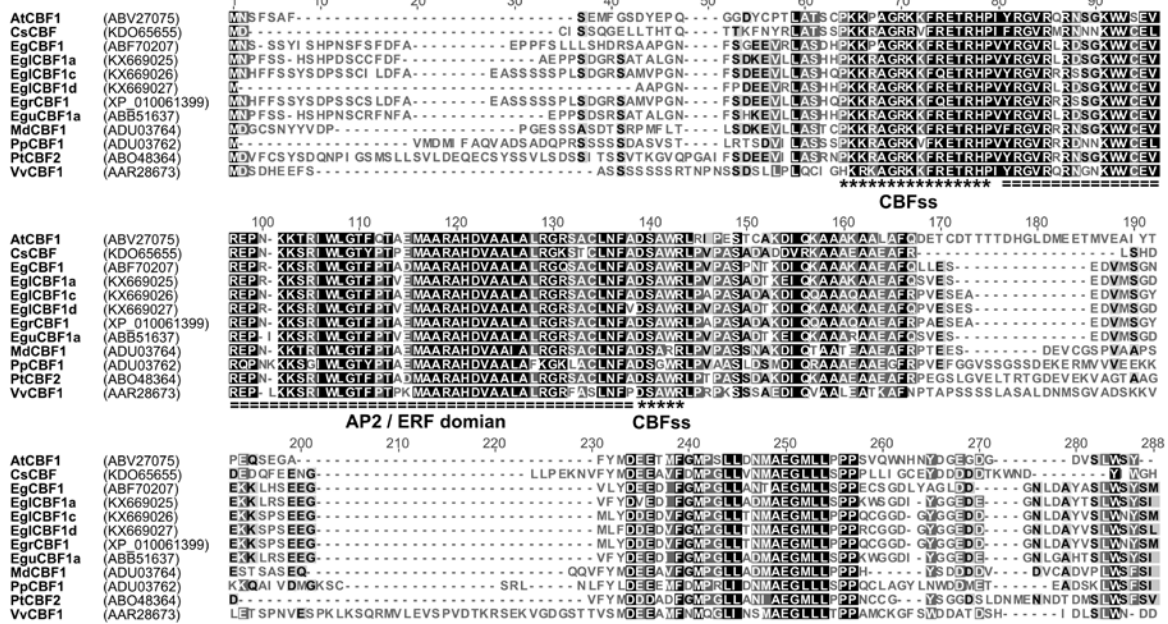


**Fig. 2.1** Multiple sequence alignment of CBF proteins in *Arabidopsis* (AtCBF1), *Citrus sinensis* (CsCBF), *E. globulus* (EgCBF1), *E. globulus* (EglCBF1a-c-d), *E. grandis* (EgrCBF1), *E. gunnii* (EguCBF1a), *Malus domestica* (MdCBF1), *Prunus persica* (PpCBF1), *Populus trichocarpa* (PtCBF2) and *Vitis vinifera* (VvCBF1); the GenBank accession number is shown in parentheses; ***black shading*** indicate identical amino acid residues; ***asterisk*** on the alignment indicate the CBF signature sequences (CBFss); ***double underline*** indicate AP2/ERF domain

Multiple alignment of CBF protein sequences from *Arabidopsis* and *Eucalyptus* species (including *E. globulus*, *E. grandis* and *E. gunnii*) were used to generate the phylogenetic tree. The CBF paralogs were distributed in seven clades, five for *Eucalyptus* CBFs proteins, one for *Arabidopsis* CBFs proteins and one for the *Arabidopsis - Eucalyptus CBF* (Fig. 2.2). EglCBF1a grouped to CBF proteins reported in *E. gunnii* (EguCBF1a) and in *E. grandis* (EgrCBF6, 8, 10 and 12). In another clade, the *CBF* sequences of *E. grandis* (EgrCBF7, 9, 11, 13 and 14) were grouped with one sequence of *E. gunnii* (EguCBF1b) and to the only CBF currently reported for *E. globulus* (EgCBF1). The EglCBF1c identified has a high similarity to EgrCBF1 and EguCBF1c, but a distant clustering to the EglCBF1d

protein; which groups with the EgrCBF2 and EguCBF1d paralogs. For *Arabidopsis*, one clade of CBFs protein was obtained (AtCBF1, 2, 3, and 4), and additionally a small clade of an atypical CBF protein (AtDDF1 and 2) was grouped with some CBF proteins of *E. grandis* (EgrCBF15-16), demonstrating an apparent phylogenetic relationship between these distant species.

***Freezing tolerance in different cold acclimated E. globulus genotype***

The application of the night frost treatment of -6 °C, allowed the assessment of freezing tolerance of the three genotypes of *E. globulus* studied and the determination of the survival rate and leaf damage. For survival rate, the values were 14, 35 and 10% for the R1, R2 and S1 genotypes, respectively, but considering the leaf damage, the two resistant genotypes (R1 and R2) showed less than 50% damage, while the sensitive genotype (S1) had a leaf damage of 63.5%, this difference being statistically significant (Supplementary table S2.3).

***Expression analysis of EglCBF genes in response to cold acclimation treatments***

The data obtained by gene expression analysis showed that Egl*CBF1a, c* and *d* genes showed an increased transcript accumulation in cold acclimated plants, when compared to non-acclimated plants (Fig. 2.3). For the three genes analyzed, the highest transcript accumulation was observed in the CAAF treatment.

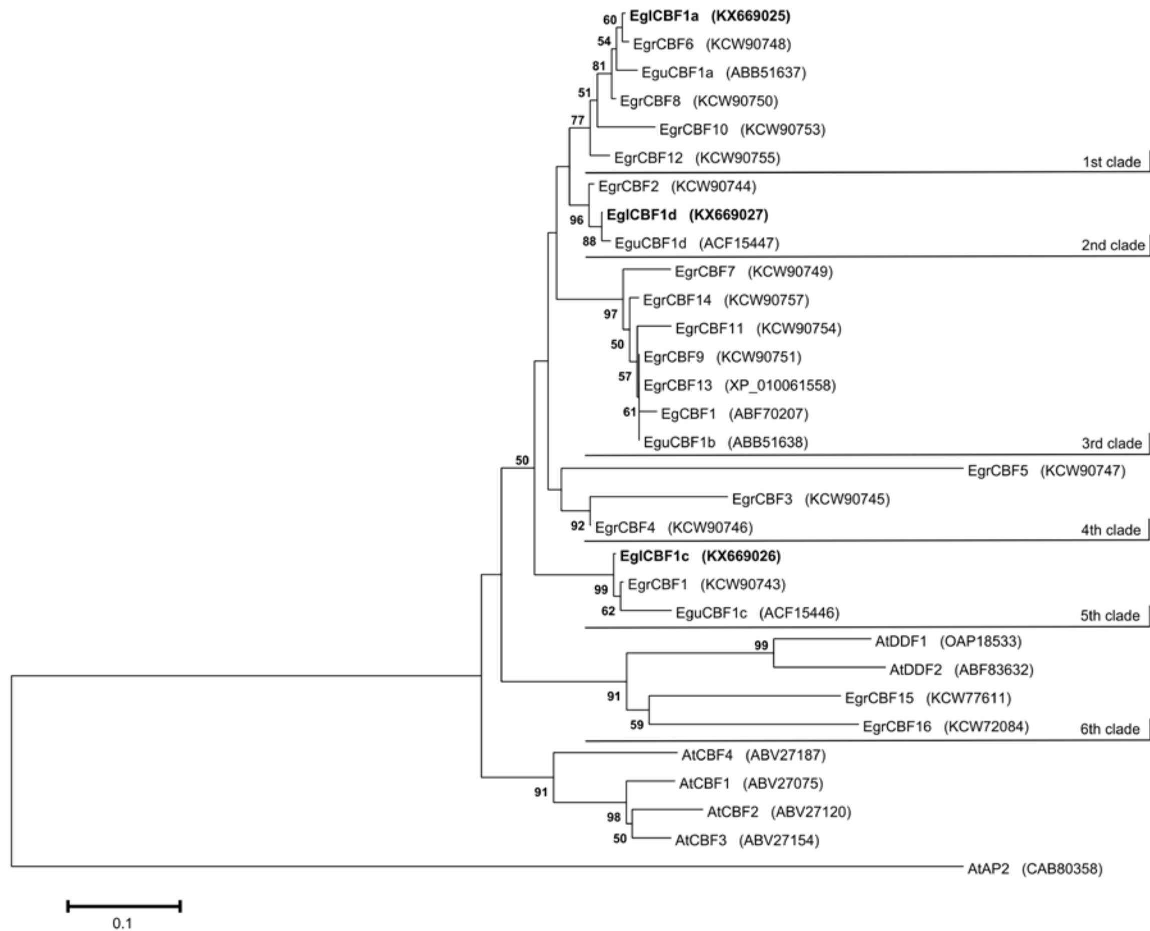


**Fig. 2.2** Phylogenetic tree of CBF proteins generated by the Neighbor-Joining method using MEGA 6.0, multiple alignment full-length amino acid sequences of EglCBF1a-c-d and CBFs from: *Arabidopsis thaliana* (AtCBF1-4 and AtDDF1-2), *E. globulus* (EgCBF1), *E. grandis* (EgrCBF1-16) and *E. gunnii* (EguCBF1a-b-c-d) were used. AthAP2 is a member of the AP2 family used for rooting the phylogenetic tree. Each protein has the GenBank accession number in parenthesis. A line separated each *Eucalyptus* clade. ***Bootstrap values*** are indicated for each branch and low values (<50) were removed from the tree

The transcript abundance of Egl*CBF1a* gene increased during the CABF treatment for the genotypes S1 and R2 (Fig. 2.3a). The highest transcript abundance was obtained in the CAAF treatment, reaching a fold change of 1,311 for genotype R1, and 340 and 445 for R2

and S1, respectively. In plants exposed during one week to the DA treatment, the expression of this gene falls significantly with the increase of temperature (12/6 ºC), reaching fold changes of 3.8, 4.4 and 3.5 in genotypes R1, R2 and S1, respectively. The transcript abundance of Egl*CBF1c* during the CAAF treatment increased to values of 28, 18 and 5-fold change in R1, R2 and S1 genotypes, respectively (Fig. 2.3b). For the DA treatment, Egl*CBF1c* gene expression decreases below the levels observed for the control treatment (NA). The transcript abundance of Egl*CBF1d* gene showed an increase in the CABF treatment in the sensitive genotype (3.4-fold) compared to the resistant genotypes (Fig. 2.3c). This gene presents the highest relative expression observed in the CAAF treatment, with values of 823, 590 and 180-fold for R1, R2 and S1 genotypes, respectively. These transcript accumulation levels were significantly higher in the resistant genotypes (R1-R2) when compared to the sensitive genotype (S1). As it has been observed in the previous gene assessed, Egl*CBF1d* gene expression in DA treatment falls significantly with values of 2.6, 3.3 and 2.5-fold in R1, R2 and S1 genotypes, respectively. Additionally, the constitutive relative expression of the three *EglCBF* genes were calculated by normalizing each expression level, with respect to the UBC gene present into a single copy in the genome of *E. grandis*.

***Growth and phenotypic development in transformed Arabidopsis lines***

The rosette diameter and plant height, on 35, 40 and 60 days-old plants were measured in the WT and ten transformed lines of *A. thaliana*. Between 35 and 40 days, both transformed and WT plants showed rosette diameters between 4.6 (±0.2) and 7.0 (±0.7) cm, without any evident development of the inflorescence (Fig. 2.4a). At 60 days, the plants showed rosette sizes between 7.5 (±0.1) and 12.2 (±0.4) cm length, with evident induction of flowering in eight of the ten lines tested (Fig. 2.4a). The transformed lines with the smaller rosette diameter were A17 and C09, presenting significant differences when compared to the WT (Fig. 2.4b). At the same time, inflorescence development was observed in eight lines, with the exception of A17 and D32. The line A17 showed an abnormal development, with absence of the inflorescence (Fig. 2.4a, 2.4c, 2.4d). In the case of C09 and D32 lines, a slow floral development was observed, with well-developed stems and siliques at 80 days, while the WT have the same development at 60 days (Fig. 2.4d).

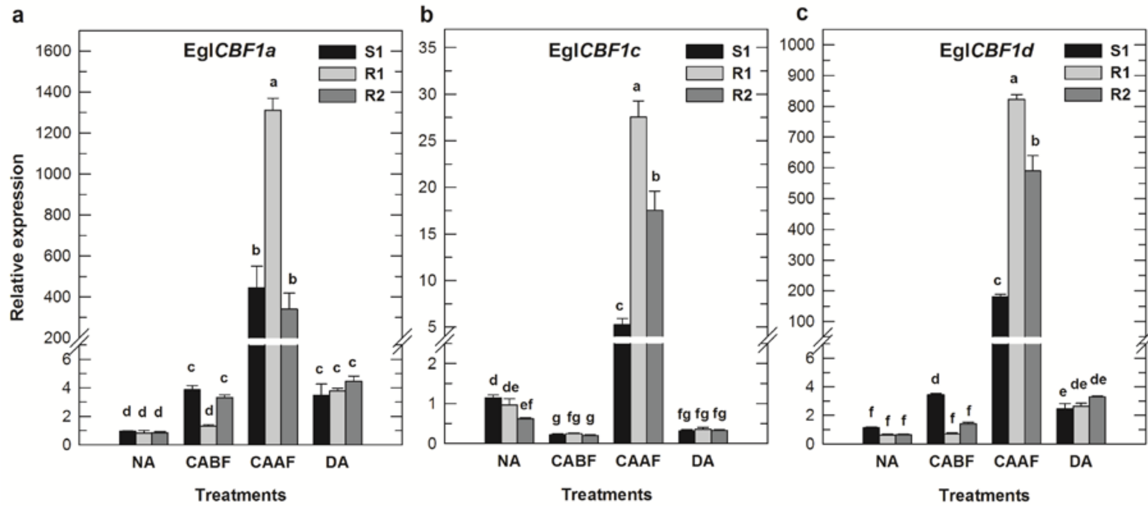


**Fig. 2.3** Relative expression analysis of three *CBF* genes in *E. globulus* plants. **a** Egl*CBF1a*. **b** Egl*CBF1c*. **c** Egl*CBF1d*, for four treatments of cold acclimation assay **NA** (non-acclimated), **CABF** (cold acclimated before night frosts of −2°C), **CAAF** (cold acclimated after night frosts of −2°C) and **DA** (de-acclimated) using Taqman® probes with internal controls *UBC* and *α-TUB* genes. Calibrator sample corresponds to one ramet of S1 genotype at NA treatment; ***bars*** indicate fold change mean *n=3*; ***error bars*** represent SE; ***lowercase letters*** on top of the bars indicate statistically significant differences between treatments and genotype evaluated with *Tukey* test ($p<0.05$)

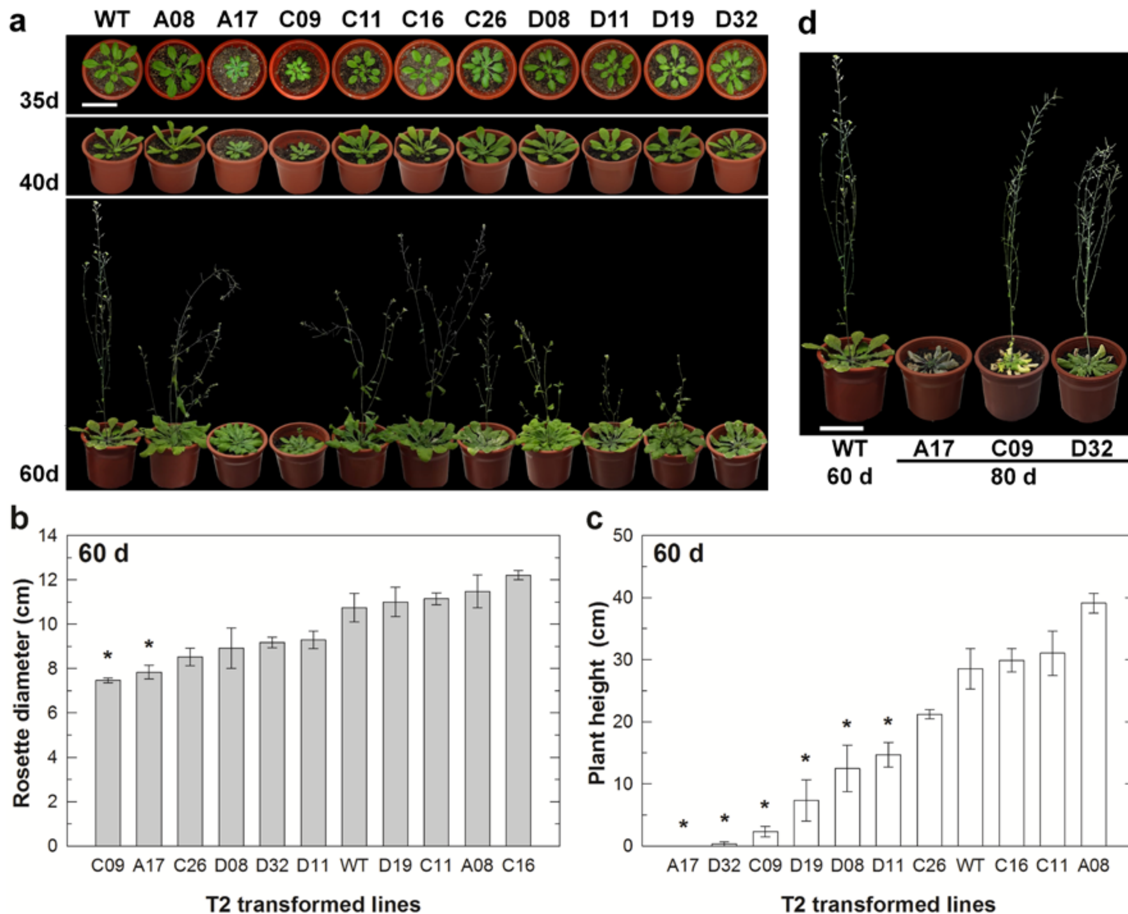


**Fig. 2.4** Growth and phenotypic development of transformed and WT plants. **a** plant growth at 35, 40 and 60 days after transplant to pots. **b** rosette diameter of different lines at 60 days. **c** plant height of different lines at 60 days. **d** lines with delayed development growth at 80 days compared to WT plant 60 days old; ***bars*** indicate fold change mean, *n=10*; ***error bars*** represent SE; ***asterisk*** on top of the bars indicate significant differences between each transformed line compared to WT by *Tukey* test ($p<0.05$)

***Survival rate to freezing stress in Arabidopsis plants***

WT plants of *A. thaliana* showed 0% survival rate when exposed to -6ºC freezing temperatures (Fig. 2.5). The transformed lines that showed the highest survival rates were A17 and C09, reaching 90.5%. Additionally, the transformed lines C26 and D32 showed a survival rate above 50%.

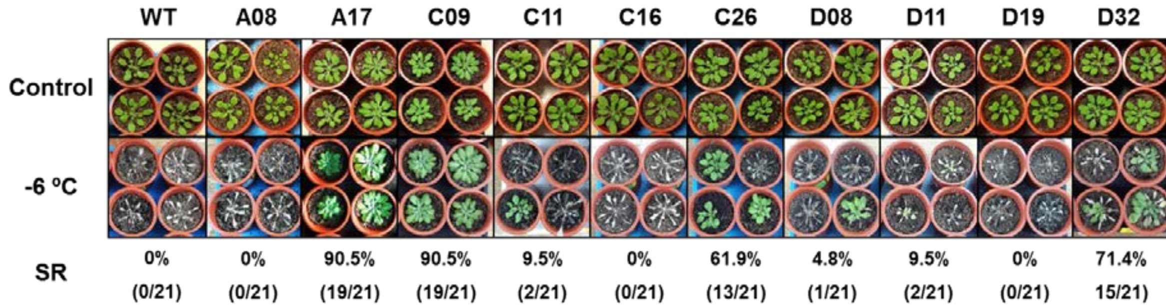


**Fig. 2.5** Freezing tolerance of WT and ten transformed lines that overexpress independently Egl*CBF1a*, Egl*CBF1c* and Egl*CBF1d* genes, respectively. **Control**, 5-week-old plants growing under normal conditions at 23ºC; **-6 ºC** 5-week-old plants under freezing treatment and then returned to normal condition for 7 days; **SR** survival rate calculated as recovered plants over total plants treated

***Transcript abundance of EglCBF transgenes and endogenous genes in transgenic Arabidopsis lines***

Several *Arabidopsis thaliana* transgenic lines containing the coding region for each of the three CBF transcription factors from *E. globulus* were generated. The T0 transformed lines for each construct were selected, with 20 lines for Egl*CBF1a*, 30 lines for Egl*CBF1c* and 40 lines for Egl*CBF1d*. All T0 lines were verified for the inserted gene integrity by PCR analysis (data not shown). On the T1 generation, four lines with low, medium and high expression levels were selected for further analysis. In the case of Egl*CBF1a* construct, two lines were discarded due to abnormal phenotypes at T1, which lacked flowers and seeds. The higher transcript abundance of the corresponding constructs 35S::Egl*CBF* in T2 *Arabidopsis* transformed lines were found in A17, C09 and D32 lines, respectively (Fig. 2.6a, 2.6b and 2.6c).

Two endogenous *CBF* genes (At*CBF2,* Ath*CBF3*) and four *COR* genes (*COR15a, COR6.6, ERD10* and *RAB18*) of *Arabidopsis* were evaluated at 23 ºC in the transgenic and WT plants. In the case of At*CBF2*, the highest transcript levels were found in A17 and D08 lines, showing significant differences compared to the WT (Fig. 2.6d). For At*CBF3*, the

highest relative expression lines were A17, C09, C11, C26, D08 and D32, also showing significant differences when compared to WT. Of the four *COR* genes evaluated, the lines A17, C09 and D32 showed the highest increase on transcript accumulation compared to WT (Fig. 2.6e).

The effect of the temperature on the transcript abundance of *COR15a*, the most induced gene from all *COR* genes tested, was measured on three transformed *Arabidopsis* lines showing high frost tolerance (A17, C09 and D32), at three different levels representing control (23 ºC), cold (4 ºC) and frost (-6 ºC) temperatures, respectively (Fig. 2.7). In lines A17 and C09, the transcript accumulation increased with a decrease in the temperature from 23 °C to -6 ºC, with significant differences compared to the WT, while in lines D32 the transcript levels were similar at all tested temperatures but significantly different when compared to the WT on their respective treatment.

**DISCUSSION**

Three *CBF* homologous sequences were identified in *E. globulus*, containing the main signatures that characterize CBF transcriptional factors, including an AP2/ERF domain and two flanking motifs. Previous studies reported that the AP2/ERF domain is needed for the DNA binding specificity (Sakuma et al. 2002) and that the PKKPAGR motif is a nuclear localization signal (Stockinger et al. 1997). Canella et al. (2010) have demonstrated that the AP2/ERF domain is needed for nuclear CBF protein localization while the PKKPAGR motif is essential for the CBF-specific protein binding to CRT/DRE elements.

The CBF proteins of *E. globulus* showed high similarity to previously characterized proteins in *E. gunnii* (El Kayal et al. 2006; Navarro et al. 2009), and to proteins recently annotated on the *E. grandis* genome (Wisniewski et al. 2014; Cao et al. 2015), that are grouped on the same clade based on a phylogenetic analysis. The high similarity and conservation of sequences suggests that EglCBF1a-c-d proteins could have an important role on the transcriptional regulation in a similar manner as it has been proposed for other plants (Chinnusamy et al. 2010; Thomashow 2010).

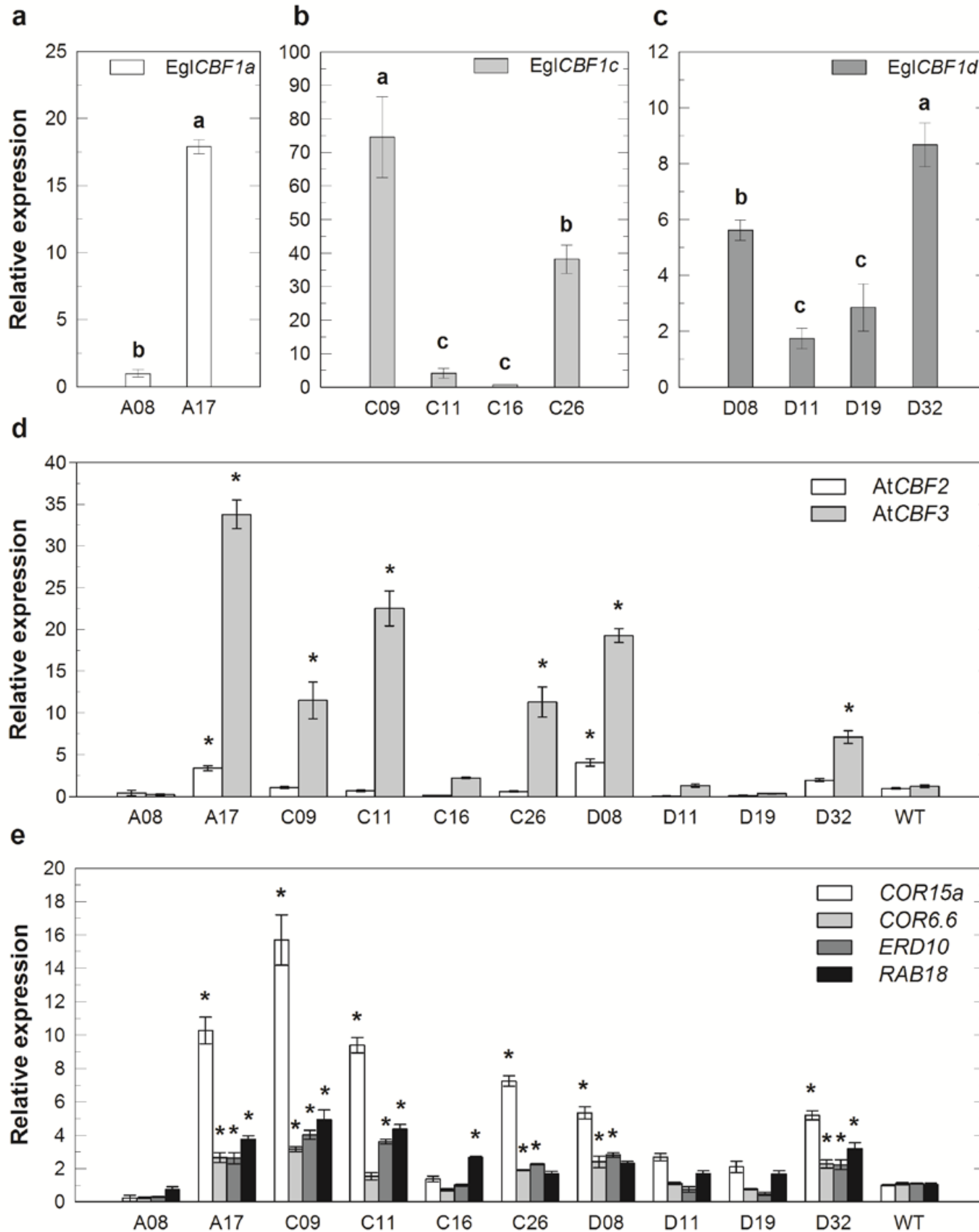


**Fig. 2.6** Relative expression levels of three Egl*CBF1* transgenes and six endogenous genes, in ten transformed lines and WT plants. **a** expression of Egl*CBF1a* in two independent overexpressing Egl*CBF1a* lines. **b** expression of Egl*CBF1c* in four independent overexpressing Egl*CBF1c* lines. **c** expression of Egl*CBF1d* in four independent overexpressing Egl*CBF1d* lines; ***bars*** indicate fold change mean, *n=3*; ***error bars*** represent

SE; ***lowercase letters*** on top of the bars indicate significant differences between the respective transformed lines determined by *Tukey* test ($p<0.05$). **d** expression levels of two *CBF* endogenous genes. **e** expression levels of four *COR* endogenous genes. The data was normalized data with the two internal control genes *EF1-α* and *PP2AA3*; ***asterisk*** on top of the bars indicate significant differences between each transformed line compared to WT determined by *Tukey* test ($p<0.05$)

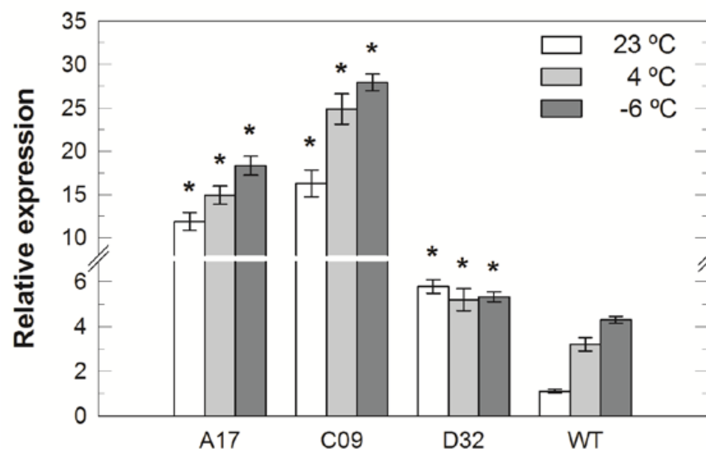


**Fig. 2.7** Relative expression levels of *COR15a* in three transformed lines and WT plants under three different temperatures: control 23 ºC, cold 4 ºC and freezing -6 ºC. The data was normalized with the two internal controls genes *EF1-α* and *PP2AA3*; ***bars*** indicate fold change mean, $n=3$; ***error bars*** represent SE; ***asterisks*** on top of the bars indicate significant differences between each transformed line compared to WT in the respective temperature treatment, determined by *Tukey* test ($p<0.05$)

Although several sequences to *CBF* homologs have been recently described in *E. grandis* (Cao et al., 2015). In this study, we have focused on the analysis of three Egl*CBF* sequences, similar to the *CBF*s described by El Kayal et al. (2006) and Navarro et al. (2009). Nevertheless, when we screened cold expression libraries of *E. globulus*, we were able to find a total of 15 *CBF* homologous sequences to *E. grandis* (data not shown), but the effect of the other *CBF* homologs present in this species remains to be determined.

The results of transcript abundance for Egl*CBF1a-c-d* genes (1,311, 28 and 823-fold change) in the R1 genotype during the CAAF treatment compared to the non-acclimated genotype, are similar to those obtained in *E. gundal*, which is more cold tolerant than *E. globulus*, where the paralogs of Egu*CBF1a-c-d* showed values of 1,690, 91 and 436-fold change respectively, after a 5 h exposure at 4 ºC (Navarro et al. 2009). To date, the only other *CBF* gene reported for *E. globulus* is Eg*CBF1* (Gamboa et al. 2007). This gene is similar to Egu*CBF1b*, considered as a gene that participates on the cold acclimation process, with a prolonged expression over time at cold temperatures when tested on acclimation experiments (El Kayal et al. 2006; Navarro et al. 2009).

In the case of Egl*CBF1a*, there was a high transcript abundance at -2 ºC (CAAF), this information is on agreement with the results for *E. gunnii* and its paralog, Egu*CBF1a*, showing an early induction between 2 to 5 h after exposure of plants to 4 ºC, and this expression is intensified if the experiment is conducted at short photoperiods of 8 h light/day (El Kayal et al. 2006). Navarro et al. (2009) replicated this phenomenon, observing high expression levels being reached at 2 h (1,760-fold change) and 4 h (1,690-fold change) after exposing the plants to a gradual change in temperature from 22 to 4 ºC. The authors propose that this gene is involved in an early response to sharp changes in cold temperature, in accordance with its early expression pattern.

The expression levels of Egl*CBF1c* gene in the three genotypes of *E. globulus* studied were lower than the other two *CBF* genes analyzed in CABF and DA treatments. In this case, the expression level falls below 1-fold, suggesting that the sample used to normalize the relative quantification levels (NA condition, the calibrator sample), showed a higher basal expression compared to the Egl*CBF1a-d* genes in the NA treatment. This was observed in quantifying constitutive transcripts levels in samples under NA treatment, where Egl*CBF1c* gene was constitutively expressed unlike the other two genes, Egl*CBF1a* and Egl*CBF1d*. Although several studies report that *CBF* genes are induced by stress conditions in plants, in addition to low-temperature, drought and high-salinity genes (Gilmour et al, 1998; Ryu et al, 2014; Fang et al, 2015), other reports indicate that there are some CBF transcription factors that are constitutively expressed in several plants species (Tang et al, 2005; Xiao et al. 2008; Peng et al. 2013). In *E. gunnii*, one *CBF* gene, Egu*CBF1c*, has a basal expression

of 2.8 copy number $ng^{-1}$ cDNA, when compared with other weakly expressed genes of the same family, Egu*CBF1a-d*, under non-stress conditions (Navarro et al. 2009). These researchers reported a putative role of Egu*CBF1c* gene with a constitutive expression, suggesting that it may be involved in a permanent cell stress protection in response to various stimuli.

Egl*CBF1d* presented high expression levels during the CAAF treatment, showing significant differences on resistant genotypes, this is in accordance with previous observations for the *E. gunnii* paralog Egu*CBF1d,* where the expression levels increased with colder freezing temperatures of -4, -6 and -8 ºC, reaching a 1,367-fold change at the lowest temperature tested (Navarro et al. 2009). The proposed role for this gene is the response to frost, providing tolerance to cells with or without previous acclimation. Both eucalypt resistant genotypes assayed presented differential relative expression levels for the three *CBF* genes studied (in the case of R1), or for two *CBF* genes (in the case of R2 genotype) on the more severe cold treatment with freezing temperatures (CAAF). The phenotypic data supports this observation, since young plants of the resistant genotypes presented significantly less leaf damage when compared to the susceptible genotype, when confronted to a -6 ºC frost treatment.

In order to validate the proposed function of these genes, in this work the overexpression of three *E. globulus CBF* genes was performed separately in *A. thaliana*. These Egl*CBF1a-c-d* genes were annotated recently in the *E. grandis* genome and correspond to *CBF*-like *6 – 1 – 2*, respectively (Cao et al. 2015). Ten transformed lines were selected for three constructs, two lines for EglCBF1a and four lines for each Egl*CBF1c-d* gene. Of these ten lines, only five showed a large transcript accumulation, with four of them having high survival rates to freezing treatments, suggesting that a high transcript accumulation of the Egl*CBF* gene is correlated with an increased survival to freezing stress. The same correlation has been reported in several studies, where high survival rates were observed in transformed lines with high amounts of transcript accumulation of *CBF* (Siddiqua and Nassuth 2011, Wisniewski et al. 2011; Tillett et al. 2012; Li et al. 2013). Additionally, the overexpression of *CBF* genes from different plant species, has led to increased survival rates to cold and freezing stress in *Arabidopsis* (Tong et al. 2009; Xue et al. 2014; Fang et al. 2015) and in

other herbaceous or woody species (Navarro et al. 2011; Xu et al. 2011; Zhou et al. 2014; Byun et al. 2015). The role of these transcription factors is to regulate gene expression in response to environmental stresses, by binding to the *cis*-elements CRT / DRE (Stockinger et al. 1998), present in the promoter regions of cold response genes (*COR*). It has also been reported that a large accumulation of *CBF* genes induces the expression of *COR* genes (Jaglo-Ottosen et al. 1998; Gilmour et al. 2004; Zhou et al. 2014; Xue et al. 2014). Some broadly reported *COR* genes responding to cold and freezing stresses are *COR6.6*, *COR15a*, *COR47*, *COR78* and *ERD10* (Kasuga et al. 1999; Thomashow et al. 2001; Seki et al. 2001). In this work, the induction of endogenous genes in *Arabidopsis* was evaluated on two *CBF* genes (At*CBF2 – 3*), three *CBF*-target genes (*COR6.6* - *COR15a* - *ERD10*) and one non-*CBF* target gene (*RAB18*), an ABA-dependent pathway gene (Mäntylä et al. 1995). In the case of the endogenous *CBF* genes At*CBF2* and At*CBF3*, two and six lines with high transcript accumulation were observed, respectively, with At*CBF3* showing the highest expression values. Interestingly this work reports that the constitutive expression of Egl*CBF* transgenes induces increased expression levels of the At*CBF* endogenous genes in *Arabidopsis*, but these transcription factors do not have the regulatory sites CRT/DRE on their promoter regions, required for activation with the CBF proteins (Gilmour et al. 1998). This also has been reported by Zhou et al (2014), where the overexpression of Cb*CBF* from *Capsella bursa-pastoris* in tobacco, not only increased the transcript levels of Nt*ERD10a – b* genes for cold response, but also participated in the up-regulation of the *CBF* genes Nt*DREB1 – 3*, providing a likely mechanism for the enhanced cold acclimation due to Cb*CBF*. On the other hand, the phenomenon of regulation between CBF transcription factors has been reported in *Arabidopsis*, where At*CBF1* and At*CBF3* gene expression are negatively regulated by At*CBF2* (Novillo et al. 2004; 2007), and the effect has been recently validated by Kim et al. (2015) who reported that the relative expression of At*CBF1* and At*CBF3* genes in an *Arabidopsis cbf2* mutant, defective in the At*CBF2* gene, presented higher At*CBF3* relative expression levels, while At*CBF1* expression was not affected. Additionally, they verified that At*CBF2* indirectly regulates At*CBF3* expression but does not bind to their promoter region. Moreover, other authors reported that plants of the *Arabidopsis* mutant *erd10* exposed to cold for 24 h showed a significant loss of cold tolerance, related to the absence of induction on the CBF transcription factors, proposing

that this cold signaling pathway could present a more complex level of regulation (Kim et al. 2010).

Regarding the induction of *CBF*-target genes, part of the so-called *CBF* regulon (Thomashow 1999), five *Arabidopsis* transformed lines showed increased expression levels of the three *CBF*-target genes in non-acclimated plants, among them, four presented an overexpression of the Egl*CBF* transgene and high survival rates, suggesting that the overexpression of Egl*CBF* activates the transcript accumulation of *CBF* regulon genes, improving the constitutive tolerance to freezing stress. There are additional reports confirming that the overexpression of *CBF* genes increases the accumulation in transcripts of *CBF*-target genes, correlated to an enhanced freezing stress tolerance (Tong et al. 2009; Siddiqua and Nassuth 2011; Li et al. 2013; Fang et al. 2015). Additionally, we found that on five lines the overexpression of the transgene increased the transcript accumulation of *RAB18* gene, a dehydrin that is not regulated by CRT/DRE *cis*-elements (Wang et al. 2008). This could indicate that the constitutive expression of the Egl*CBF* gene activates other metabolic pathways different to the *CBF* regulon, in this case an ABA-dependent pathway, an effect previously reported by other authors, with an overexpression of *CBF* in *Arabidopsis* resulting in the transcription of ABA-dependent pathway genes (Fang et al. 2015; Wang et al. 2008).

Additionally, we evaluated the relative gene expression of *COR15a* at different temperatures: control, cold and freezing. The results showed that the relative transcript levels increased with decreasing temperatures, in two of three lines evaluated and in the WT plants. This effect is well correlated with the cold acclimation phenomenon, where decreasing temperatures induce an increase on transcript abundance of genes required for the cold signaling pathway, and even more if these genes are expressed in control temperature (Zhuang et al. 2015). Accordingly, three Egl*CBF1* constitutively activated the expression of *COR15a* in transgenic plants and up-regulated their expression under cold stress. It is notable that the fold change of *COR15a* expression in transgenic plants was greater than other *COR* genes under normal and freezing stress conditions, suggesting that *COR15a* could play a prominent role in freezing tolerance of transgenic plants overexpressing Egl*CBF1s*.

The growth and phenotype development of transformed plants in some lines had a slower growth rates than the WT, leading to a delay of flowering, and even in one line (A17) abolishing completely its presence. This phenomenon was correlated with high levels of transgene expression and a high survival to freezing treatment in these lines, suggesting that growth inhibition was an additional effect due to the high transcript abundance of Egl*CBF* transgene, which has been observed by other authors, who point out that the overexpression of *CBF* genes in *Arabidopsis* causes dwarfism and delayed flowering (Liu et al. 1998; Gilmour et al. 2004), the same effect was found on some woody species (Navarro et al. 2011; Tillett et al. 2012). The effects of the overexpression of *CBF* genes was studied in more depth by Achard et al. (2008), who found that the constitutive expression of At*CBF1* in *Arabidopsis* induces the accumulation of DELLA proteins, which restricts growth by interfering with the gibberellin (GA) signaling pathway. Under normal growth conditions, plants produce bioactive GA, which in turn degrades DELLA proteins by the ubiquitin-proteasome pathway (McGinnis et al. 2003), but when the levels of bioactive GA decreases, DELLA proteins accumulate and inhibit growth, causing dwarfism and delayed flowering (Thomashow 2010). Achard et al. (2008) found that the overexpression of At*CBF1* induces the expression of two genes that encode for GA 2-oxidases, enzymes that catalyze the inactivation of bioactive GA. The same effect was reported by other authors, where the constitutive expression of *CBF* genes induces the accumulation of genes encoding enzyme GA oxidases, reducing the amount of bioactive GA, thus accumulating the DELLA proteins and producing growth inhibition in transformed plants (Tong et al. 2009; Siddiqua and Nassuth 2011; Zhou et al. 2014). Recently, Zhou et al. (2017) working in *Arabidopsis* suggested that CBF3 promotes the accumulation of DELLA proteins by repression of gibberellin biosynthesis and also DELLA contribute to cold induction of At*CBF* genes through interaction with jasmonate signaling. One possibility for the positive regulation of the At*CBF*2 – 3, that lacks a CBF target *cis* element on their promoters, by the overexpression of Egl*CBF*s observed in this work, is that positive regulation between CBF and DELLAs occurs not only at low temperatures, but also there are alternative pathways of regulation for warm temperatures and cold induction. This possibility, or the interaction with other factors that does not depends on low temperatures, requires further research.

In conclusion, the three genes that codes for CBF transcription factors described here for *E. globulus* are believed to participate actively on the cold acclimation process, and showed a strong relationship with freezing tolerance for this species. Accordingly, the coldest tolerant *E. globulus*, used in this study, had an increased relative expression of these genes when compared with the most sensitive one; this knowledge would be of great value for guiding future breeding programs for cold tolerance in *E. globulus*. Furthermore, the overexpression of the different Egl*CBF* provide freezing tolerance in four *Arabidopsis* transgenic lines, by increasing the gene expression levels of cold response genes (*CBF* regulon), and could be useful for future genetic modification strategies in plants, but a further characterization of the effects on growth inhibition and flowering delay on transformed plants is required.

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## SUPPLEMENTARY DATA

**Table S2.1** List of primers used to amplify and clone different *E. globulus* sequences

| **Analysis** | **Gene** | **Primers / probe name** | **Sequence (5' – 3')** | **Annealing temperature (ºC)** | **Amplicon length (bp)** |
|---|---|---|---|---|---|
| *CBF* gene sequence | Egl*CBF1a* | CBF1a_fwd | AACACTAGCCCCACCACATTCA | 61 | 793 |
| | | CBF1a_rev | CGTCAAAGTCGTCCCTAACAGCA | | |
| | Egl*CBF1c* | CBF1c_fwd | CCAGAACTCAAGCGCAGA | 54 | 1023 |
| | | CBF1c_rev | GCCTTATACATTATACATG | | |
| | Egl*CBF1d* | CBF1d_fwd | GTAAACACTCATCACTGCTC | 57 | 1351 |
| | | CBF1d_rev | ACGACCTTTCCTGATAGA | | |
| qPCR analysis *Taqman* primers and probe | Egl*CBF1a* | qCBF1a-fwd | TCGGAAGAGGGAGTGTTCTACG | 60 | 140 |
| | | qCBF1a-rev | TCCAAATTCTCCTCGTCCTCTCC | | |
| | | CBF1a-p | ATCTCCGCCCCATTTT | | |
| | Egl*CBF1c* | qCBF1c-fwd | AGACGTACTCCAATGAACCACTTC | 60 | 147 |
| | | qCBF1c-rev | CACCTCCTCATCAGAAAAGTTCCC | | |
| | | CBF1c-p | ACCATAGCACTCCT | | |
| | Egl*CBF1d* | qCBF1d-fwd | GATGGAGTGCGGATGAAAGGC | 60 | 128 |
| | | qCBF1d-rev | CATCAGAGGCCCAACATCAGAAG | | |
| | | CBF1d-p | CGTGCAGTCTCTATT | | |
| | *UBC* | qUBC-fwd | GACGGACAGGAACAAGTATGAGAC | 60 | 93 |
| | | qUBC-rev | CCCTCCACGGAATAATGATCGC | | |
| | | UBC-p | CTGAACCCAGAAGTATG | | |
| | *α-TUB* | qα-TUB-fwd | GGAAGATGCTGCCAACAACTTTGC | 60 | 152 |
| | | qα-TUB-rev | AACCAGTGCCTCCACCAACAG | | |
| | | α-TUB-p | TTGGGAAGGAAATTGT | | |
| Amplify complete coding region | ORF-Egl*CBF1a* | CBF1a-*attB1* | *attB1* - ATGAACCCTTTCTCTTCTCATTCCCAT | | 724 |
| | | CBF1a-*attB2* | *attB2* - TCAGATCGAATAGCTCCATAATGACGT | | |
| | ORF-Egl*CBF1c* | CBF1c-*attB1* | *attB1* - ATGAACCACTTCTTCTCTTCTTACTCAGA | | 751 |
| | | CBF1c-*attB2* | *attB2* - CTACATGGAATAGTTCCATAACGACACG | | |
| | ORF-Egl*CBF1d* | CBF1d-*attB1* | *attB1* - ATGGCAGCCCCCGGGAAC | | 652 |
| | | CBF1d-*attB2* | *attB2* - CTACAGGGAATAGCTCCATAACGACA | | |

* *attB1 – attB2* correspond to specific sequences used as recombination sites in Gateway cloning.

**Table S2.2** Primers designed for qPCR analysis of transformed lines and WT *Arabidopsis* plants

| Gene | Primers name | Sequence (5' – 3') | Amplicon length (bp) |
|---|---|---|---|
| Egl*CBF1a* | cbf1a-ins_fwd | TTTGCCGAGCCGCCATCTG | 173 |
| | cbf1a-ins_rev | ACCCATTTGCCCGAGTCACG | |
| Egl*CBF1c* | cbf1c-ins_fwd | AGCGTCGTCCTCCTCGTC | 76 |
| | cbf1c-ins_rev | CACCTCCTCATCAGAAAAGTTCCC | |
| Egl*CBF1d* | cbf1d-ins_fwd | GAGTCCGAGTCCGAGGAC | 152 |
| | cbf1d-ins_rev | CCGCCGCATCGTGGTGGAG | |
| At*CBF2* | q-cbf2_fwd | CTGCCCCAAGAAACCAGCGGGAAG | 128 |
| | q-cbf2_rev | TCCTCGTTTTCTTGTTTGGCTCTCTCAAC | |
| At*CBF3* | q-cbf3_fwd | CGATGAGGCGATGTTTGAGATGCCG | 90 |
| | q-cbf3_rev | TTATGATTCCACTGTACGGACGGAAG | |
| *COR6.6* | cor6.6_fwd | GCCAAGGATGCTGCTGCTG | 136 |
| | cor6.6_rev | CCAAAGTTGACTCGGATCGCTAC | |
| *COR15a* | cor15a_fwd | CAGCGGAGCCAAGCAGAGC | 119 |
| | cor15a_rev | GGATGTTGCCGTCACCTTTAGC | |
| *ERD10* | erd10_fwd | CGAGTGATGAAGAAGGTGAAGACG | 152 |
| | erd10_rev | CCCTGGTTTCTCTCCGAGTGG | |
| *RAB18* | rab18_fwd | CCGATGGGAGGAGGAGGATACG | 163 |
| | rab18_rev | CGCCAGTTCCAAAGCCTTCAG | |
| *EF1-α* | ef1-α_fwd | TGAGCACGCTCTTCTTGCTTTCA | 76 |
| | ef1-α_rev | GGTGGTGGCATCCATCTTGTTACA | |
| *PP2AA3* | pp2aa3_fwd | AGGAAACTTGCGTGAGGGAGAAAG | 144 |
| | pp2aa3_red | ATGCTGATACTCTGGCTGTGAACC | |

**Table S2.3** Survival percentage, leaf damage percentage and ratio between survival and leaf damage of three genotypes of *E. globulus* treated with a -6ºC night frost (F-6) after of cold acclimation profile treatments

| Genotype | Survival (%) | Leaf damage (%) |
|---|---|---|
| R1 | 14.0 (± 2.6) *ns* | 35.8 (± 2.5) *ns* |
| R2 | 34.8 (± 6.1) ** | 40.3 (± 4.9) *ns* |
| S1 | 10.1 (± 2.6) *ns* | 63.5 (± 2.7) ** |

Each value represents the means ± standard error (SE), n=18 ramets per genotype, ** significant differences using *Tukey* test (*P<0.05*) and *ns* no significant differences

# CAPÍTULO III: OVEREXPRESSION OF AN SKn-DEHYDRIN GENE FROM EUCALYPTUS GLOBULUS AND EUCALYPTUS NITENS ENHANCES TOLERANCE TO FREEZING STRESS IN ARABIDOPSIS

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## ABSTRACT

Dehydrins play a fundamental role in plant response and adaptation to abiotic stresses, having an important role in seed desiccation, response to abscisic acid, low temperatures, drought and salinity conditions. *Eucalyptus nitens* has a greater tolerance to cold than *Eucalyptus globulus*, which in part can be due to the role of dehydrins present in this species. This work reports the identification of four *DHN* genes in *E. nitens* and examines their response under low temperature, comparing them to those previously described in *E. globulus*. Transcript abundance of dehydrins increased when plants were cold acclimated, being higher in a freezing-resistant family of *E. nitens* than in a freezing-sensitive family. The relative levels of *DHNs* in *E. nitens* were higher than the corresponding of *E. globulus* under the same conditions. The analysis of the promoter region for the four Eni*DHNs* showed that they contained several cold-or dehydration inducible *cis* elements, such as ABRE, MYC and CRT. The analyzes in the genomic sequence of *DHN* from *E. globulus* and *E. nitens*, together with the results of transcript abundance under cold acclimation, can be in part explained by differences found at the *cis* elements in the several *DHN* promoters studied. The most responsive gene to cold tolerance in both species was *DHN2*, which was used to obtain transgenic *Arabidopsis thaliana* containing either the coding region or the putative promoter. The transgenic lines with coding region, Eni*DHN2* and Eugl*DHN2,* had a higher survival rate to cold than the untransformed *A. thaliana*. In addition, the Eni*DHN2* putative promoter drive induced higher expression levels of the gene (*GUS*) marker than the one of *E. globulus*, when exposed to cold temperatures. Therefore, we hypothesize that the putative promoter of Eni*DHN2* and its coding region has a key role in conferring cold tolerance to this species.

Keywords: *Cold acclimation • Freezing tolerance • cis-regulatory element • Transgenic plants • Dehydrin 2*

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## INTRODUCTION

Chile is one of the main country growing eucalypts, which is widely used in forest plantations, covering a total surface of 700,000 ha, corresponding to *Eucalyptus globulus* (75 %) and to a less extent *Eucalyptus nitens* (25 %). *E. globulus* is the main species grown in temperate climates free of severe frosts due to its exceptional wood quality, combined with fast growth (Teulières and Marque 2007). Of the fast-growing species *E. nitens* is considered suitable for planting on high-altitude sites where severe frosts and snow occur (Turnbull and Eldridge 1984). During the past years considerable effort has been directed towards understanding how *E. globulus* responds and adapts to low temperature due to its low freezing resistance, by different approaches, going from cold acclimation to transcriptomic analysis (Moraga et al. 2006; Costa e Silva et al. 2008; Fernández et al. 2006, 2015; Rasmussen-Poblete et al. 2008). Recently, Fernández et al. (2015) reported the identification of the main mechanisms likely to participate at cold acclimation in *E. globulus*, proposing that some of the differentially expressed genes responding to cold acclimation are a good source to discovering candidate genes for improving frost tolerance and potentially drought tolerance in this species such as LEA-like proteins including dehydrins. In a similar approach, Keller et al. (2013) identified more than 2500 unigenes involved in cold acclimation of *E. gunnii*, being some transcription factors as C-repeating factors (CBFs).

Plants are subjected to environmental variations that determine and limit their geographic distribution. Low temperature is an environmental factor limiting plant growth, affecting their development and reducing their productivity (Guzmán 2009; Amolkumar and Arun 2008). Cold resistance is one of the main mechanisms studied in plants, in some species, the phenomenon known as cold acclimation increase their cold tolerance as a response to environmental stimuli such as low non-freezing temperatures (Thomashow 1999; Fowler et al. 2007). Cold acclimation (CA) triggers a cascade of signals with physiological and biochemical changes in response to stress, providing the ability to acclimate to cold temperatures (Thomashow 1999). Gene induction in response to cold (*COR*) leads to the synthesis of proteins. An important group of these elements are LEA (Late Embryogenesis Abundant) proteins (Wise 2003), with a subset of proteins called dehydrins (DHNs) has

been described in angiosperms (Close 1996, 1997). These proteins are involved in seed desiccation in response to low temperatures, drought, salinity and response to abscisic acid (Muñoz-Mayor et al. 2012; Layton et al. 2010; Kosová et al. 2007), and have been attributed important roles during cell dehydration, and stabilization of cell membrane contributing to stress tolerance (Close 1997). DHNs are characterized by possessing conserved domains known as K, Y and S segments (Close 1996, 1997), as well as less conserved regions rich in polar amino acids, called ϕ segments (Rorat 2006). According to the presence and number of Y-, S- and K-segments, DHNs can be classified into five different subclasses: $Y_nSK_n$, $K_n$, $K_nS$, $SK_n$, and $Y_2K_n$ (Close 1996). Several works suggest that the K-segment is involved in establishing hydrophobic interactions with other proteins during dehydration stress, mainly stabilizing cell membranes (Campbell and Close 1997; Danyluk et al. 1998; Koag et al. 2003). However, Perdiguero et al. (2014) recently identified dehydrin proteins with truncated K-segments and a dehydrin protein completely lacking K-segments in *Pinus pinaster*, being the role of these unusual dehydrins remains unrevealed.

Several studies have shown that dehydrins can protect cells against dehydration, Navarro et al. (2011) reported that the overexpression of *DHN2* in transgenic lines of *E. gunnii* (EguCBF1a-OE) responded in a similar way than non-transformed lines previously acclimated to cold, increasing their frost tolerance. Fernández et al. (2012a, b) reported the presence of four dehydrin genes in E. globulus, namely *DHN1*, *DHN2*, *DHN3* and *DHN10*. Relative expression analyses of these four genes in two contrasting genotypes of *E. globulus* (susceptible- and frost-tolerant) performed under low temperature, dehydration and at different photoperiods, showed that *DHN1*, *DHN2* and *DHN10* are mainly induced by low temperature, with higher transcript abundance in the frost-tolerant genotype than in the susceptible one, while *DHN3* was mainly expressed under dehydration conditions. Further studies have demonstrated that Eugl*DHN2* and Eugl*DHN10* are mainly involved in the cold acclimation process of *E. globulus* (Fernández et al. 2015).

In the present study, we identify genes belonging to group II LEA/dehydrin family present in *E. nitens*, which has been described as a more cold tolerant species. Young plants from two half-sib families of *E. nitens* and cuttings from one genotype of *E. globulus* were

subjected to a cold treatment to determine the dehydrins that play a major role in CA. The dehydrin having a major role, measured as transcript abundance, were cloned and used to make transgenic *Arabidopsis thaliana* lines to assess cold tolerance. In parallel the putative promoter of these gene were used with a marker gene (GUS) to observe if it is cold induced and to provide evidence if the expression of genes regulated by these promoters under cold stress is tissue specific.

## MATERIAL AND METHODS

### *Plant materials and cold treatments*

Two half-sibs families, represented by 6-month-old rooted seedlings of *E. nitens* and one genotype of *E. globulus* (16 plants) previously characterized as frost resistant were used. All plants were transferred to polystyrene boxes covered with vermiculite to maintain the substrate humidity, placed in a cold chamber and subjected to four treatments as described by Fernández et al. (2012a). Briefly, non-acclimated (NA, control condition) plants were maintained under 14 h day length at 20/12 ºC day/ night temperature for 10 days; on day 10, leaves were collected and the condition of the growth chamber was changed as follows: treatment I, cold acclimated before night frosts of -2 ºC (CABF), short days (10 h day) at 8/4 ºC for 7 days; treatment II cold acclimated after night frosts of -2 ºC (CAAF): plants were maintained at 8/4 ºC where a night frost of -2 ºC during four nights was applied (using a temperature decrease rate of 2 ºC/h), and leaves were collected after the last frost (when the chamber reached 8 ºC). For treatment III, deacclimated (DA), seedlings were exposed to long day length (14 h day length) and 12/6 ºC day/night temperature for one week. A night frost of -6 ºC during DA treatment was applied to determine the survival and percent of leaf damage for each genotype/family to assess their tolerance to a simulated late spring frost. Ten plants were exposed to the night frost and survival (percentage of live ramets), and leaf damage (percent of necrotic area per plant or ramet) was visually evaluated after 10 days. The growth chamber experiment was arranged as a completely randomized design. Three replicates (seedlings or ramets) were collected and stored at -80 ºC until further use for qRT-PCR analysis.

Wild type and transgenic *A. thaliana* ecotype Columbia were germinated on 1X Murashige and Skoog media (MS) agar plates supplemented with 2 % sucrose, pH 5.8. After 4 days of incubation at 4 ºC in the dark, the seeds were transferred to the growth room at 23 ºC under short-day photoperiods (12/12 day/night) and cultivated in soil for 3 weeks. To assess frost tolerance, 19 plants per line were subjected to a temperature decrease, in a phytotron (Percival LT-36VL), starting at 23 ºC and reaching -6 ºC, with a 2 ºC decrease per hour and kept at this temperature for 3 h. Three plants per each line were collected at 23, 4 and -6 ºC. After this assay, plants were kept at 23 ºC for 7 days and the survival rate was estimated by visually assessing the plant recovery. When each of the tested temperatures was reached, leaves from three plants per line were collected for qRT-PCR analysis, stored at -80 ºC until further use and leaves were stained for *GUS* activity at the end of each treatment.

***EniDHNs gene identification***

DNA was extracted from *E. nitens* leaf tissue according to the protocol described by Doyle and Doyle (1987). For the identification of *DHNs* genes in *E. nitens* four pairs of primers were designed using a reference the dehydrin sequences previously described for *E. globulus* (Fernández et al. 2012a). The details of the primers employed for each sequence are: Enidhn1_F 5'-TCGTCTCATCATTTAGTGCATCGG-3', Enidhn1_R 5'-GCAGCTTATCCATGATCTTGTCCA-3' (expected size 1325 bp); Enidhn2_F 5'-CAGCACGCCTAACTTGAATATA-3', Enidhn2_R 5'-GGCGGCGGAGGAGAATAGAC-3' (1671 bp); Enidhn3_F 5'-CTAGTGAGGTGGGTGAGGATAGAG-3', Enidhn3_R 5'-AGTCTCGGTATCTCTACTGTGTCG-3' (1473 bp) and for Enidhn10_F 5'-TAATCAACCGTGCTACGTTTGTCC-3', Enidhn10_R 5'-AATATAACCTGACTGCGAAACGGG-3' (552 bp). The PCR conditions were 0.9 µM primers, 1 U Taq platinum DNA polymerase (Invitrogen, USA), 200 mM dNTP mix (Invitrogen, USA), 2.5 mM $MgCl_2$ and 2 ng of DNA in a total volume of 25 µl. The amplification process was performed following a cycle at 95 ºC for 5 min, 40 cycles of 15 s at 95 ºC, 30 s at 60 ºC and 30 s at 72 ºC, and a final cycle of 7 min at 72 ºC. PCR products for each primer pair were analyzed on a 2 % agarose gel electrophoresis and purified using

the QIAquick PCR Purification Kit and under BigDye terminator cycling condition (Macrogen, Korea).

***Multiple sequence alignment, cis-elements identification and phylogenic tree construction***

The full-length DHNs nucleotide sequences were translated using ExPASy translate tool (http://web.expasy.org/translate/). The S-, K- and Y segments of DHNs proteins were identified using the ExPASy prosite server (http://prosite.expasy.org/). The molecular weight and isoelectric point of the four dehydrins were predicted using the ExPASy-Compute pI/MW tool (http://web.expasy.org/compute_pi/) based on their amino acid composition. The comparison of 9 or 10 DHN proteins sequences from different species per DHNs type were aligned using CLUSTAL-W version 2.1 and a phylogenetic tree was generated using PAUP version 4.0, with Neighbor-Joining method on 1000 bootstrap iterations. The putative promoter regions were analyzed for plant regulatory *cis* elements using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html)

***RNA extraction, cDNA synthesis and real time qRT-PCR***

Total RNA was extracted from leaves either of *E. globulus* or *A. thaliana* using the CTAB method according to Chang et al. (1993) and quantified by UV spectrophotometry (NanoDrop ND-1000, Thermo scientific, USA). RNA integrity was visualized on a 1 % agarose gel. For cDNA synthesis, samples containing 1 μg of total RNA were pretreated with DNAse I to remove DNA contamination, and then the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) was used according to the manufacturer's instructions.

Real-time qRT-PCR was carried out using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, USA), with the following conditions: initial denaturation 10 min at 95 ºC, followed by 40 cycles of 15 s at 95 ºC and 1 min at 60 ºC, in 96-well optical reaction plates. Two housekeeping genes *Ubiquitin C* and *α-Tubulin* were used as described by Fernández et al. (2010). The DHNs primers employed for the relative quantification in *E. nitens* were: EniDHN1-F/R: 5'-AGACCAGCAGCAGCAGATGAG-3' and 5'-

CTTCTTCCTCCTCCCGCCTTG-3'; EniDHN2-F/R: 5'-CCCTGTGGAGAAGTGCGACGAG-3' and 5'-CGGGGGCGGAGGAGAATAGAC-3'; EniDHN3-F/R: 5'-CGCATTTCCAGAACCACTACGC-3' and 5'-ATGCTCCGCCCTGACACC-3', and EniDHN10-F/R: 5'-CGAGAACAAGGGAGGCTTCACC-3' and 5'-CTGTCGCTGCTGCTGCTGTC-3'. For *E. globulus*, primers used were those described by Fernández et al. (2012a, b). For *A. thaliana*, two housekeeping genes *EF1α* and *PP2A3* were used: EF1a-F/R: 5'-TGAGCACGCTCTTCTTGCTTTCA-3' and 5'-GGTGGTGGCATCCATCTTGTTACA-3'; PP2A3-F/R: 5'-AGGAAACTTGCGTGAGGGAGAAAG-3' and 5'-ATGCTGATACTCTGGCTGTGAACC-3'. The At*ERD10*, At*RAB18* and *GUS* primers employed for the relative quantification in *A. thaliana* were: AtERD10-F/R: 5'-CGAGTGATGAAGAAGGTGAAGACG-3' and 5'-CCCTGGTTTCTCTCCGAGTGG-3'; AtRAB18-F/R 5'-CCGATGGGAGGAGGAGGATACG-3' and 5'-CGCCAGTTCCAAAGCCTTCAG-3' and for GUS: 5'-TGGCCTGGCAGGAGAAACT-3' and 5'-CGTATCCACGCCGTATTCG-3'. The fragment lengths varied between 79 and 173 bp and their specificity was verified by checking the melting curve (60–95 ºC) after 40 cycles. Three technical replicates were analyzed for each sample. For each gene, PCR efficiency was determined by measuring the $C_T$ to a specific threshold for a serial dilution of cDNA.

***EuglDHN2 gene isolation and plant expression vectors construction***

With the purpose of studying the response of Eugl*DHN2* and Eni*DHN2* genes to low temperature exposure, the coding and promoter regions were isolated using specific primers previously detailed. Gateway® Clonase Technology was used to facilitate the cloning of PCR-amplified fragments into adequate transformation vectors. Forward and reverse primers containing *attB* sites were designed with the Geneious Pro 6.0 software (Biomatters LTD). The PCR amplification was performed following a 95 ºC cycle for 5 min, 40 cycles of 15 s at 95 ºC, 30 s at 60 ºC and 30 s at 72 ºC, and a final cycle of 7 min at 72 ºC. Amplified fragments were then electrophoresed on 0.8 % agarose gels and then purified using a PCR purification kit (Qiagen Gel Extraction Kit).

Amplified fragments were cloned into pDONR221 vector by BP reaction and then into destination vectors by LR reaction following standard protocols. The destination vector pMDC32, for constitutive expression under the CaMV35S promoter, were used to drive the coding region of Eugl*DHN2*, while the promoter region was cloned on the vector pMDC163 containing the β-glucuronidase gene (*GUS*) as reporter gene. Both cloning vectors were developed by Curtis and Grossniklaus (2003) and obtained by the Addgene service (www.adgene.org). The resulting plasmids were named *35S-EuglDHN2*, *35S-EniDHN2*, *5'EuglDHN2-GUS* and *5'EniDHN2-GUS*.

***Arabidopsis thaliana transformation***

The transformation of *A. thaliana* was carried out according to Clough and Bent (1998). Purified plasmids were electroporated to *Agrobacterium tumefaciens* strain GV3101 using a Biorad MicroPulser (Biorad, USA) apparatus set at 2.2 kV, recovered for 1 h at 28 ºC in LB medium, and then the cells were plated and grown overnight at 28 ºC in a LB medium supplemented with gentamycin, rifampicin and kanamycin at 100 mg/l each. Confirmed *Agrobacterium* transformants were grown on LB media supplemented with antibiotics and used for *Arabidopsis* transformation using the floral dip technique (Clough and Bent 1998). The seeds were collected from individual plants and plated on MS medium containing hygromycin at 15 µg/ml. Transformed seedlings were selected according to the method described by Harrison et al. 2006. Transformed seedlings were transplanted into pots containing a mixture of sterile peat moss and vermiculite in the ratio 1:1 and maintained on growth chamber at 23 ºC and 16/8 photoperiod.

***Molecular analysis of transgenic plants***

DNA was extracted from the leaves of putative transgenic plants. PCR was carried out using sets of primers specific to each interest region. The primer pair HYGRF/R 5'-CTACACAGCCATCGGTCCAG-3' and 5'-AAAAAGCCTGAACTCACCGC-3' were used to amplify a 961 bp region of the hygromycin phosphotransferase (*HPH*) gene while for GUS the primers used to amplify the full-length gene corresponded to GUS-F/R: 5'-CAACGTCTGCTATCAGCGCGAAGT-3' and 5'-TATCCGGTTCGTTGGCAATACTCC-3'.

### *GUS staining*

Histochemical GUS staining was performed according to the method described by Clough and Bent (1998). Leaves and flowers were incubated with GUS staining solution containing 0.5 M sodium phosphate buffer pH 7, 10 % Triton X-100, 100 mM potassium ferrocyanide, 100 mM potassium ferricyanide and 1 mM 5-bromo-4-chlore-3-in-dolyl-β-D-glucuronic acid (X-gluc). The material was incubated in the dark at 37 ºC overnight. Finally, the staining solution was removed with ethanol and the tissues were cleared and fixed with 70 % ethanol for further analysis.

### *Statistical analysis*

Software SAS version 9.2 (SAS Institute Inc., USA) was used for statistical analysis. *T* test for comparison of sample *E. nitens* resistant and susceptible families, and *E. globulus* resistant genotype, on each treatment and comparing each treatment with their respective control. For *A. thaliana,* the effects of the treatments were analyzed with ANOVA and a Turkey's test for means comparison. $p$ value $\leq 0.05$ was considered significant.

## RESULTS

### *Isolation and characterization of EniDHNs*

A total of four dehydrins were identified in *E. nitens*. The isolated fragment for Eni*DHN1* (KU67482) corresponded to a 1325 bp fragment, containing an intron of 237 bp and a coding sequence of 539 bp corresponding to 179 amino acids with a calculated isoelectric point of 5.3 and molecular mass of 17.23 kDa which differed in 30 amino acids with the previously described EuglDHN1. The sequenced DNA segment of Eni*DHN2* (KU674822) is 1671 bp long with an intron of 228 bp and a coding sequence of 663 bp coding for 221 amino acids with a deduced molecular mass of 25 kDa and an isoelectric point of 5.23, having a difference of 46 amino acids with the EuglDHN2. In the case of Eni*DHN3* (KU674823) it was possible to isolate a single fragment of 1473 bp containing one intron of 73 bp and a coding sequence of 441 bp corresponding to 147 amino acids with a calculated isoelectric point of 8.76 and molecular mass of 16.07 kDa, differing in six amino acids. For Eni*DHN10* (KU674824) the sequenced DNA segment was 552 pb long with a coding

sequence of 297 bp corresponding to 99 amino acids showing no difference with the EuglDHN10. The calculated isoelectric point for EniDHN10 was 6.59 with a deduced molecular mass of 10.93 kDa.

The dehydrin protein sequences identified in *E. nitens* were compared with existing sequences in other plant species by Clustal W. Eight dehydrin sequences showed high percentages of amino acid identity (up to 70 %) when searching in BLASTP for each EniDHNs (Supplemental data, Fig. S3.1 and Table S3.1). Although an 88 % identity of EniDHN10 with a sequence of *E. grandis* (XP_010036245) was retrieved from NCBI, this does not correspond to a dehydrin as described in the latest version of the *E. grandis* genome (Phytozome).

In addition, a phylogenetic tree including all EniDHNs proteins isolated and dehydrins described for other species of *Eucalyptus* was created (Fig. 3.1). Based on the phylogenetic results, the EniDHNs proteins were clustered in different groups, the EniDHN1 clustered with the *Eucalyptus* $SK_n$-type, the amino acid sequence showed one S and two K-segments, for EniDHN2 the results showed a cluster with the *Eucalyptus* $SK_n$-type, the amino acid sequence showed one S and two K-segments. EniDHN3 clustered with the *Eucalyptus* $Y_nSK_n$-type protein containing three Y, one S and two K-segments, while for EniDHN10 clustered in the KS-type, the amino acid sequence showed one K and one S segment.

The analyzes of the putative promoter regions for the four Eni*DHNs* studied, showed that the *cis*-elements can be classified mainly into hormone-responsive and stress-responsive elements are shown in Table 3.1 and Fig. 3.2. Among the main elements found are: for Eni*DHN1* three ABRE (ABA responsive elements) elements and one G-box were identified, in the case of Eni*DHN2* four ABRE elements and seven G-boxed were found, while in Eni*DHN3* three ABRE elements and five G-box elements were identified, and for Eni*DHN10* only one ABRE element was found. Other *cis*-elements responding to the ABA-independent pathway were found, in Eni*DHN1* and Eni*DHN2* four and two low temperature responsiveness element (CRT) were identified, respectively, in the same position described by Fernández et al. (2012b) for *E. globulus*. In Eni*DHN3* four MYC elements were found, these elements are related with dehydration-responsive gene promoter elements.

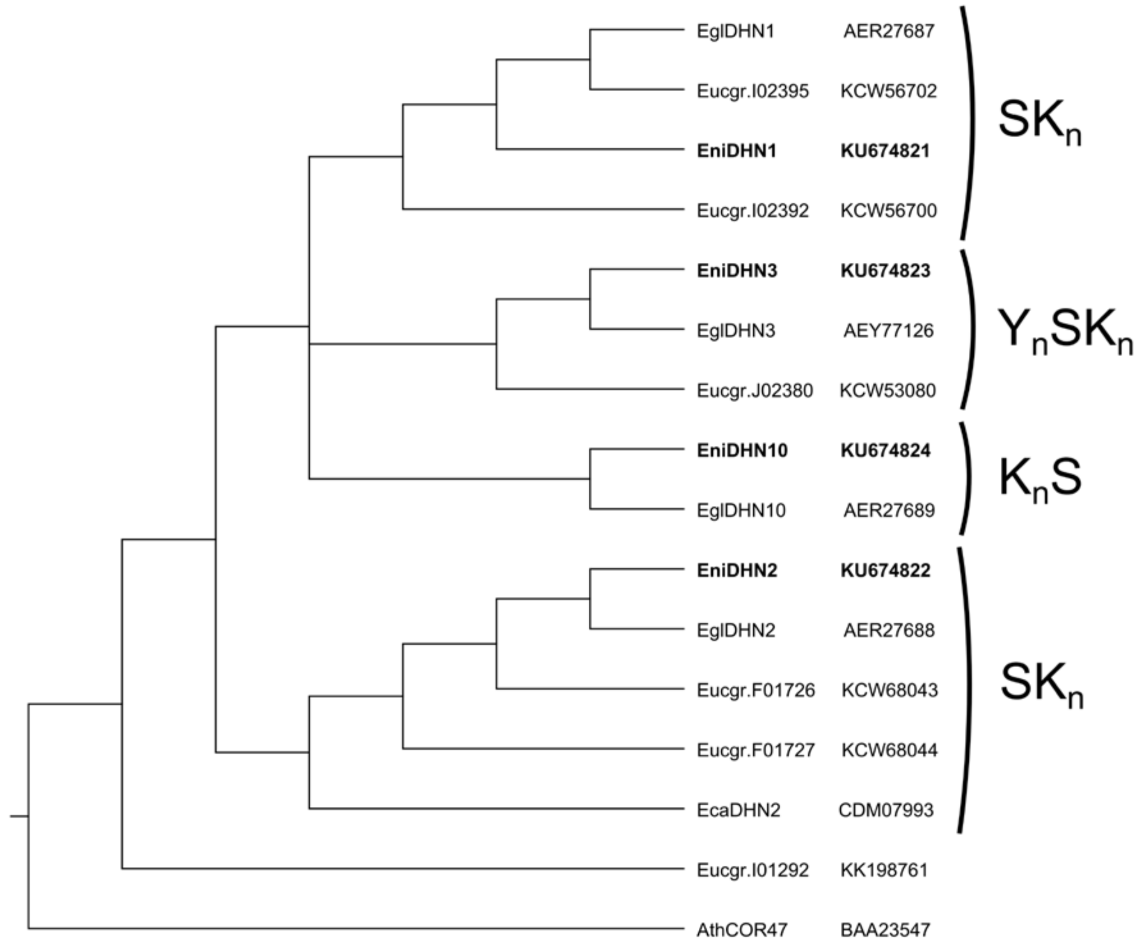


**Fig. 3.1** Phylogenetic analysis of dehydrin proteins from *Eucalyptus* species. Phylogenetic tree was generated using PAUP program. Proteins were arranged according to their sequence similarities

**Table 3.1** Putative ***cis*****-**acting regulatory elements involved in stress-responsive expression in the promoter regions of four *EniDHNs* in *E. nitens*

| ***cis*** **elements** | **Sequence** | **Frequency of the *cis*-element present in the *DHNs* of *E. nitens*** | | | | **Function** | **Reference** |
|---|---|---|---|---|---|---|---|
| | | *DHN1* | *DHN2* | *DHN3* | *DHN10* | | |
| **ABRE** | TACGTG | 4[a] | 4[a] | 4[a] | 1 | ABA responsiveness | Allagulova et al. (2003) |
| **AuxRR-core** | GGTCCAT | 0 | 0 | 0 | 0 | Auxin responsiveness due to stress | Sakai et al. (1996) |
| **Box-4** | ATTAAT | 1 | 0 | 1 | 0 | Light responsiveness | Lois et al. (1989) |
| **CAAT-Box** | CAAT | 6 | 3 | 19[a] | 3[a] | Common in promoter and enhancer regions | Straub et al. (1994) |
| **CGTCA-motif** | CGTCA | 1 | 0 | 1 | 0 | MeJA responsiveness | Rouster et al. (1997) |
| **Circadian** | CAANNNNATC | 2[a] | 1[a] | 0 | 0 | Involved in the circadian mRNA accumulation | Pichersky et al. (1985) |
| **CRT** | GCCGAC | 4[a] | 2 | 0 | 0 | Low-temperature responsiveness | Qin et al. (2004) |
| **G-Box** | CACGTG | 4 | 7[a] | 0 | 0 | Promoter regulatory element involved in dehydration responsiveness | Maruyama et al. (2012) |
| **GARE-motif** | AAACAGA | 1 | 0 | 0 | 0 | GA-responsive element | Pastuglia et al. (1997) |
| **GC-motif** | CCCCCG | 0 | 2 | 0 | 0 | Enhacer-like anoxic specific inducibility | Viret et al. (1994) |
| **HSE** | AAAAAATTTC | 0 | 1[a] | 1 | 0 | Heat stress responsiveness | Pastuglia et al. (1997) |
| **MBS** | CGGTCA | 1 | 0 | 1 | 0 | Drought responsiveness | Nash et al. (1990) |
| **MYC** | CACGTC | 2[a] | 0 | 4[a] | 0 | Dehydration responsiveness | Abe et al. (1997) |
| **TCA-element** | CCATCTTTTT | 0 | 0 | 1 | 3[a] | SA responsiveness | Pastuglia et al. (1997) |
| **TGA-motif** | TGACG | 1 | 1 | 0 | 1 | Auxin responsiveness | Pastuglia et al. (1997) |

[a] The frequency observed is higher than expected for *cis*-acting element in the promoter region

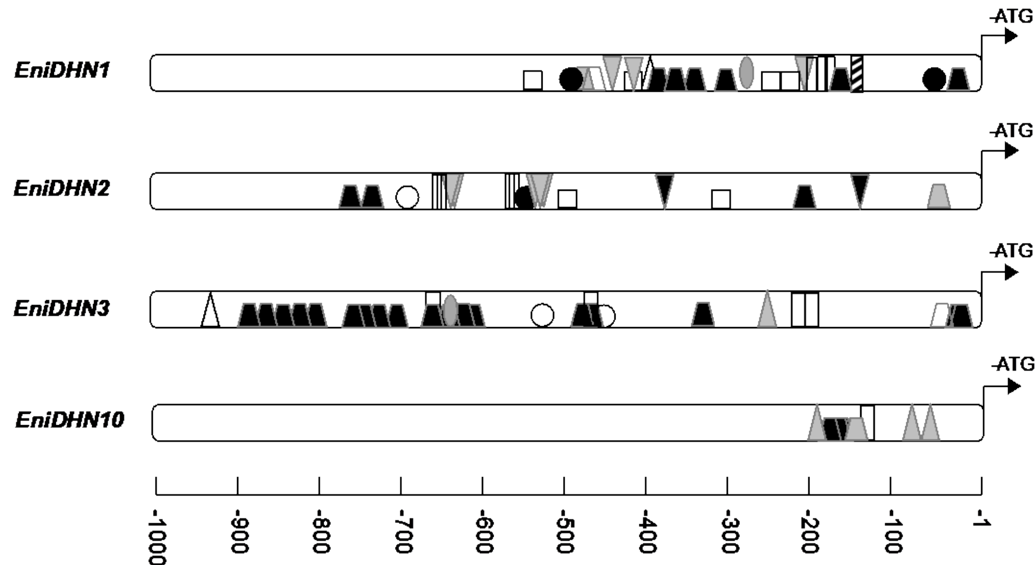


**Fig. 3.2** Location of *cis*-elements associated with the four dehydrins promoter region. ***Negative numbers*** indicate the position of nucleotides relative to the translation start site. ***Gray triangle*** = TCA element, ***white triangle*** = Box 4, ***inverted gray triangle*** = G-Box light responsive element, ***inverted black triangle*** = GC motif, ***white circle*** = heat stress-responsive element, ***black circle*** = regulatory element involved in circadian control, ***white square*** = CRT low temperature response motif, ***white trapezoid*** = CGTCA-motif, ***gray trapezoid*** = TGA motif, ***black trapezoid*** = CAAT-BOX enhancer region, ***white rectangle*** = ABRE motif involved in abscisic acid responsiveness, ***hatched rectangles*** = GARE-motif gibberellin-responsive element

***Gene expression of EuglDHNs and EniDHNs in response to cold acclimation***

Ten days after the night frost of -6 ºC, the survival rate resulted in 56 % and leaf damage was 61 % for the resistant to frost (RF) *E. nitens* and 33 and 81 % of survival and leaf damage, respectively, for the frost susceptible (FS) *E. nitens* family, while for *E. globulus* it was 15 and 33 % of survival and leaf damage, respectively. The results obtained for *E. globulus* showed that all Eugl*DHNs* increased the transcript accumulation when comparing the CAAF to the NA condition: Eugl*DHN1* presented a ~10-fold increase, while Eugl*DHN2* showed the highest transcript accumulation increasing ~62-fold, with Eugl*DHN10* transcripts level increased only

~8-fold (Fig. 3.3a). Transcript abundance of the four dehydrins described in *E. nitens* was assessed in both families. The results of cold treatments showed that in the case of Eni*DHN1*, the transcript level increased ~27-fold (RF) and ~42-fold (SF) at the CAAF condition compared to the NA. For Eni*DHN2* both families increased their gene expression at CAAF compared to NA, with the RF showing the highest transcript accumulation of ~180-fold, while for the SF, only a ~39-fold was observed. The transcript abundance for Eni*DHN10* showed a ~32-fold for the RF when comparing the CAAF with the NA treatment, while for the SF a ~17-fold increase was observed (Fig. 3.3b, 3.3c). Since in all cases the highest transcript accumulation was detected at the CAAF condition, the transcript level for all dehydrins was compared between both *E. nitens* families (Fig. 3.4a), finding only a significant difference for Eni*DHN2*, which had a ~141-fold change when comparing the RF to the SF. Similar to the results for *E. nitens*, only Eugl*DHN2* showed a significant difference in the transcript accumulation from NA to CAAF treatments, however, the transcript abundance for Eni*DHN2* was highest (Fig. 3.4b). Eni*DHN3* and Eugl*DHN3* were not detectable in any of the conditions tested.

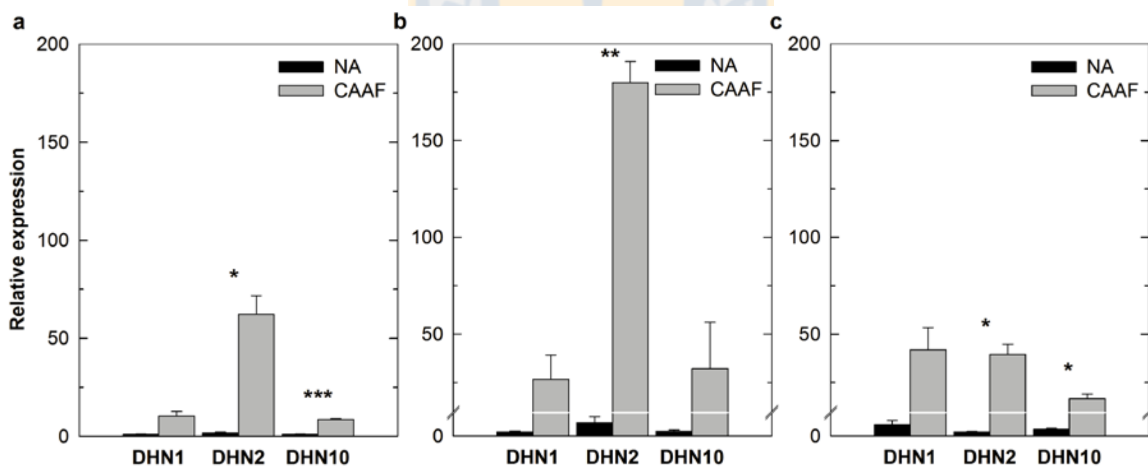


**Fig. 3.3** Gene expression of three *DHN* genes in leaves of **a** *Eucalyptus globulus* **b** frost resistant *Eucalyptus nitens* and **c** frost susceptible *Eucalyptus nitens* under low temperature using two internal controls *UBC* and *α-TUB*. The ***asterisks*** on top of the bars (mean + SE) indicate statistically significant differences among NA and CAAF treatment (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$)

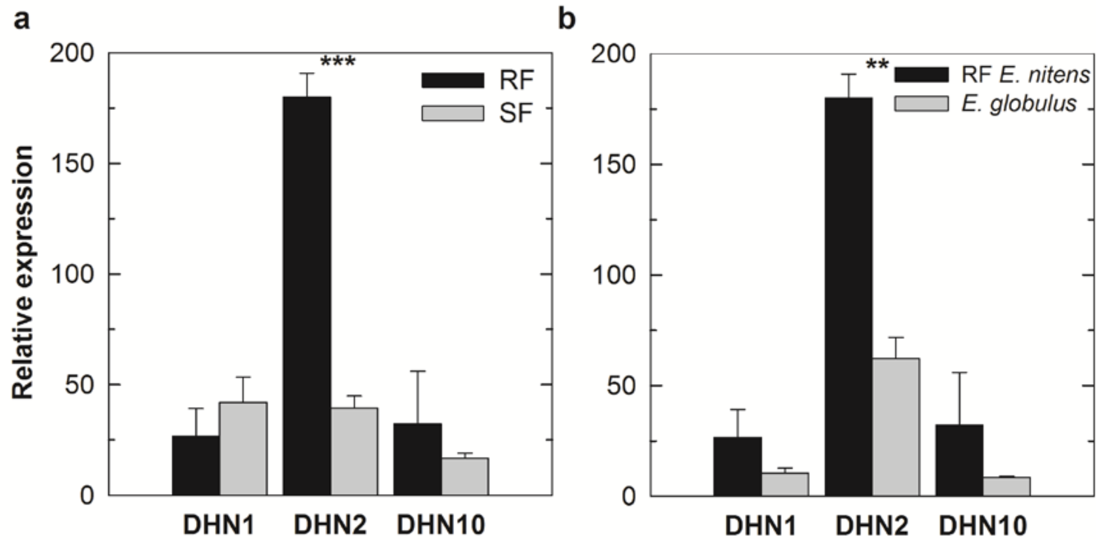


**Fig. 3.4** Transcript abundance of three DHN genes measured in leaves of **a** two *Eucalyptus nitens* families, resistant (RF) and susceptible (SF) and **b** comparison of the transcript abundance between an *Eucalyptus globulus* frost resistant genotype and the frost resistance family (RF) of *Eucalyptus nitens* under low temperature using two internal controls *UBC* and *α-TUB*. The ***asterisks*** on top of the bars (mean + SE) indicate statistically significant differences in the CAAF treatment (**$p \leq 0.01$; ***$p \leq 0.001$)

The results showed that Eugl*DHN2* and Eni*DHN2* were the only genes that had a significant difference in transcript accumulation at CAAF compared to the control conditions. In the case of Eni*DHN2* the transcript abundance (as fold change) was three times higher than the one observed for the same dehydrin in *E. globulus* (Fig. 3.4b). Based on these results the corresponding coding sequence for *DHN2* was cloned from *E. globulus* and *E. nitens* under the constitutive promoter *35SCaMV* and the putative promoters of these genes were cloned and used to determine *GUS* as a marker gene, to determine if it is induced by cold and if it is tissue specific.

***Effect of EuglDHN2 and EniDHN2 in response to cold acclimation in Arabidopsis thaliana***

Several transgenic lines containing either the coding region for Eugl*DHN2* or Eni*DHN2* were generated. The expression of the gene under the constitutive promoter *35SCaMV* for all T0 lines was measured using qRT-PCR (data not shown). For the case of Eugl*DHN2* three lines each representing low, medium and high expression levels were selected for further analysis, however, two lines were discarded since they had an abnormal phenotype at T1 (dwarf or slow growth rate). For the Eni*DHN2* lines three low and three high expressors were selected. For further analysis Eni*DHN2* were selected on hygromycin B and it was observed that all lines assayed were abnormal, showing a dwarf phenotype or a very slow growth rate, despite this, they were still considered and included in the cold tolerance assay. The T1 lines were subjected to a cold treatment as previously described and the survival rate for each line was determined. In most cases a higher survival rate was observed when compared to non-transformed *A. thaliana* (50 %), with the lines transformed with Eugl*DHN2* reaching 50–100 % survival, and the lines transformed with Eni*DHN2* reached 10–90 %. Due to the small phenotype and the lower survival rate observed in the Eni*DHN2* lines, no qRT-PCR analysis was carried out, however, data was collected for the Eugl*DHN2* transgenic lines and sampling was carried out at 23 ºC (control), 4 and -6 ºC and the transcript abundance was measured in each case (Fig. 3.5a). No significant differences at the transcript abundance of the gene were observed when plants were submitted at different temperature, although there were significant differences between the seven Eugl*DHN2* T1 lines assayed. There was no correlation between the relative transcript level of the transgene and the observed survival rate. The transcript levels for the orthologous genes At*ERD10* (Fig. 3.5b) and At*RAB18*, were also measured observing that in the case of At*ERD10* the transcript level increased significantly with low temperatures in all lines tested, including the wt *A. thaliana*, while for At*RAB18* no significant differences in the transcript abundance at the three temperatures analyzed could be observed for any of the lines tested (transgenic and wt). The data of these endogenous genes did not correlate with the survival rate observed. For example, L5 had a high expression level of both genes and a survival rate of 80 % at -6 ºC, while L2 and L27 had a 100 % of survival rate and the expression levels of these genes were lower than the ones observed for L5 and the wt.

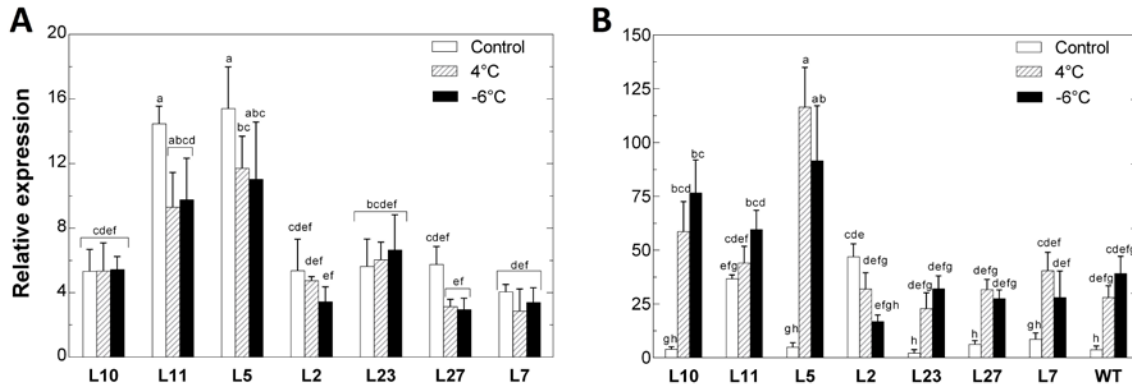


**Fig. 3.5** Transcript abundance of the Eugl*DHN2* (**a**) and At*ERD10* (**b**) in seven transgenic lines of *Arabidopsis thaliana*, subjected to three different temperatures (23, 4 and -6 ºC). L represent the transgenic lines and WT is the untransformed *Arabidopsis thaliana*

***Role of the putative promoters of EuglDHN2 and EniDHN2 in response to cold acclimation in Arabidopsis thaliana***

The putative promoter of Eugl*DHN2* and Eni*DHN2* was used with the *GUS* gene to determine if it was cold induced, by transgenic lines of *A. thaliana*. In the case of the putative promoter of Eugl*DHN2* five T0 lines were assayed, which had a low germination rate when tested with hygromycin B (10–37 %), while for the T0 lines containing the putative promoter of Eni*DHN2* the germination rate was high (70–80 %). The *A. thaliana* T1 lines were tested under the same cold profile used previously and sampled at 23 ºC (control), 4 and -6 ºC. For the T1 lines of Eugl*DHN2* putative promoter three of the four lines tested, the expression of the transcript abundance of *GUS* was significantly increased with temperature compared to the control (two-fold change), while in the fourth line there was no significant differences. For the Eni*DHN2* putative promoter, in the five T1 lines tested, the transcript abundance of *GUS* increased significantly with lower temperatures in all cases, at much higher levels (7–40 fold change) than those observed for the Eugl*DHN2* putative promoter (Fig. 3.6a, 3.6b). The histochemical staining of the samples, in both cases, showed that the expression was not tissue specific (data not shown).

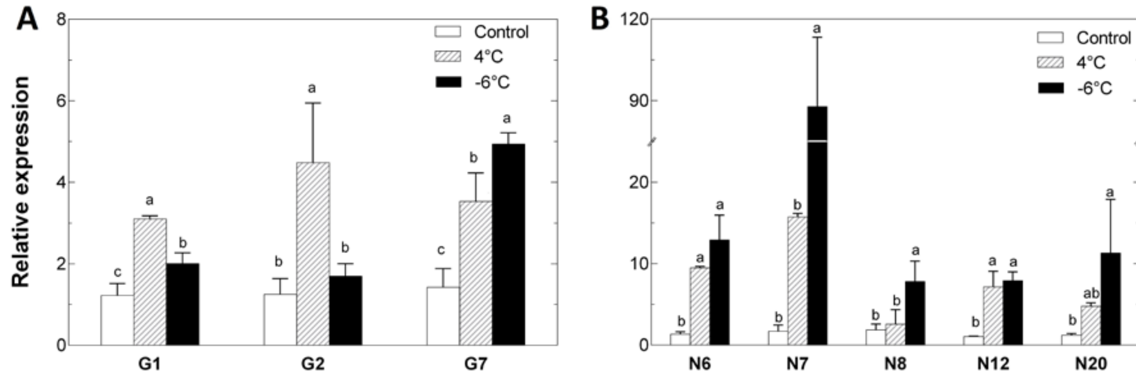


**Fig. 3.6** Transcript abundance of *GUS* under the putative promoter of *5'EuglDHN2* (**a**) and *5'EniDHN2*, measured at 23, 4 and -6 ºC

## DISCUSSION

It has been suggested that different types of DHNs proteins are involved in responses to various growth conditions (Xu et al. 2014). Improvement of plant response to abiotic stress would enhance plant adaptation mainly because of the function of DHNs, which can protect cells from dehydration, stabilize the cell membrane, eliminate free radicals by binding metal ions or act as molecular chaperones (Close 1996). In this study four dehydrins were identified in *E. nitens*, corresponding to Eni*DHN1*, Eni*DHN2*, Eni*DHN3* and Eni*DHN10*, with a potential function in the response to cold acclimation. The Eni*DHN1* and Eni*DHN2* showed the most interesting results, since they had a high transcript level at the acclimated state compared to the control (non-acclimated), being similar to the results reported by Fernández et al. (2012b) in *E. globulus*. Interestingly the transcript level was much higher in *E. nitens* than in *E. globulus* for both genes, taking into account that the later is more cold tolerant than *E. globulus*.

Based on the phylogenetic tree constructed by EniDHNs and other available sequences, these dehydrins belong to the $SK_n$-type, previous studies suggest that the synthesis of these kind of dehydrins are preferentially accumulated in plants in response to low temperatures (Allagulova et al. 2003). Fernández et al. (2012a) reported that the hydrophilic nature of $SK_n$-type polypeptides is well suited to replace water and stabilize membranes through polar

interactions during dehydration. Rorat (2006) showed that $SK_n$-type DHNs in *Solanum* species accumulate in response to low temperature and are correlated with the capacity of the plants to cold acclimate and develop frost tolerance. In *Eriobotrya japonica* a freezing treatment resulted in up-regulation of the expression levels of two dehydrins of the $SK_n$-type, these levels were more pronounced in the freezing-tolerant cultivar than in the sensitive cultivar (Xu et al. 2014). Additionally, Welling et al. (2004) reported that the transcript levels for $SK_n$-type DHNs from *Betula pendula* decreased during autumn and started to increase in winter. Eni*DHN10* showed a variation in transcript levels from a non-acclimated to acclimated state being higher in the acclimated state, this dehydrin belongs to the $K_nS$ type (Rorat 2006). Fernández et al. (2012a) suggested that EuglDHN10 protein ($K_nS$ types in *E. globulus*) might be involved in the iron transport in phloem mediated long- distance transport. It has been shown that the lipid peroxidation as a result from reactive oxygen species (ROS) is generated in stressed plants. Xu et al. (2014) suggest that decreases in membrane fluidity, as a result of peroxidation, may be one cause of cold stress, while Thompson et al. (1987) found lipid peroxidation in plant membranes to be caused primarily by free-radical attacks. It has been frequently shown that DHNs can alleviate oxidative damage stressed plants by scavenging hydroxyl and peroxyl radicals or binding metals (Hara et al. 2004; Sun and Lin 2010). Also it was reported that the presence of KS-type dehydrins can reduce the formation of reactive oxygen species from Cu and inhibit the generation of hydrogen peroxide and hydroxyl radical Cu-ascorbate system (Hara et al. 2013). Eni*DHN3* was not detectable in any of the assays; previous studies suggest that the synthesis of $Y_nSK_n$ dehydrins is induced by drought but not by low temperatures (Allagulova et al. 2003; Fernández et al. 2012b). In wheat $YSK_n$ DHNs are mostly induced in dehydrated seedling leaves, which suggest their roles in wheat dehydration tolerance (Wang et al. 2014), in barley seedlings $YSK_n$ dehydrins are accumulated in response to ABA treatment and dehydration, but their content remains unchanged during low temperature (Choi et al. 1999).

As a first attempt to isolate the promoter region putatively responsible for the cold expression of dehydrins, a computational search over the whole sequence was performed to look for previously described conserved DNA elements (Komarnytsky and Borisjuk 2003). The analysis of the 5' upstream region of each of the dehydrins identified in *E. nitens*, contained most of the putative promoter, showing that the four Eni*DHNs* had several cold or dehydration

inducible *cis*-elements (Table 3.1). These elements are involved in both the ABA-dependent and ABA-independent pathways of gene regulation of dehydrins (Shinozaki and Yamaguchi-Shinozaki 2000). Within the putative promoter region of Eni*DHN1* it was possible to identify four CRT (C-repeat) regulatory elements while in Eni*DHN2* two CRT regulatory elements were found, these elements are part of an ABA-independent pathway. The high transcript levels of accumulation observed for Eni*DHN1* and Eni*DHN2* could be related to the presence of the CRT regulatory element, since this sequence is present in the promoters of many cold-induced structural genes, with several studies pointing to CBF (C-repeat Binding Factor) transcription factors that interact with DRE/CRT-elements and regulate the transcription activity of dehydrin genes (Thomashow 1999; Allagulova et al. 2003; Kosová et al. 2007; Shinozaki et al. 2003; Stockinger et al. 1997). For Eni*DHN10* no elements involved in the ABA-independent pathways were identified, indicating that Eni*DHN10* is more responsive to stress by dehydration than to cold, however, in Eugl*DHN10* Fernández et al. (2012b) identified one LTR element (responsiveness to low temperature) in the putative promoter region. Since Eni*DHN10* showed a variation at the transcript level from a non-acclimated to acclimated state, being higher in acclimated state, it could be possible that this type of motif is present in the putative promoter region but was not identified within the region sequenced (224 bp). For Eni*DHN3* four MYC and three ABRE elements, which are mainly involved in dehydration and ABA-responsive gene expression were identified (Vornam et al. 2011). All putative promoter regions of Eni*DHNs* studied here contained ABRE elements, concluding that the response of dehydrins to cold can be mediated by several ABA-dependent and ABA-independent mechanisms. The ABRE elements contain two sequences known as G-box and GC-motif and the presence of a single copy in the promoter region is not enough for ABA-dependent gene expression (Choi et al. 1999; Kosová et al. 2007). In barley ABA-inducible *DHN1* contains a G-box and GC-motif; its removal was accompanied by the loss of promoter sensitivity to ABA (Robertson et al. 1995). When comparing the number of *cis*-elements present in Eni*DHNs* and in Eugl*DHNs*, significant differences were found between ABRE elements especially in the case of *DHN2* where the number of ABRE and G-box elements in *E. nitens* were higher than those present in *E. globulus*. This is consistent with the high expression levels for this dehydrin, which is the only gene studied that showed a significant transcript accumulation in both species, at CAAF compared with the NA, and being higher the

transcript abundance in *E. nitens* than in *E. globulus*. The presence of this kind of elements suggests that Eni*DHN3* may be more related to dehydration caused by water deficit than by cold stress, this is in agreement with the reports of Eugl*DHN3*, an $Y_nSK_2$- type DHN from *E. globulus*, presenting high levels of transcript in plants subject to water deficit treatment while plants subject to low temperature had a low transcript level compared to the control (Fernández et al. 2012b), and also with Shen et al. (2004), that reported the transcripts of Bc*Dh2* (an $Y_nSK_2$- type DHN from *Boea crassifolia*) highly accumulated when the plants were treated with drought, salinity, exogenous ABA and moderated heat shock, but showed only a slight accumulation in response to low temperature stress.

Based on these results, and since the main dehydrin responding to cold acclimation corresponded to *DHN2*, transgenic lines of *Arabidopsis thaliana* transformed with the genes Eni*DHN2* and Eugl*DHN2* under the constitutive promoter *35SCaMV* that were generated. The low germination rate observed for the Eugl*DHN2* lines (less than 30 %) has also been observed in plants over-expressing dehydrins as in the case of *A. thaliana* with At*ERD*10 and Cu*COR*19 in *Nicotiana tabacum* (Kim and Nam 2010; Hara et al. 2003). The aberrant phenotype observed in all the Eni*DHN2* lines assayed has not been observed in other *DHN* over-expressing transgenic lines (Puhakainen et al. 2004; Houde et al. 2004; Brini et al. 2011), however, this aberrant phenotype has been observed in when other genes involved in cold stress tolerance, such as *CBF* are overexpressed in *A. thaliana* (Gilmour et al. 2004; Sharabi-Schwager et al. 2010).

The transgenic *A. thaliana* T1 lines obtained containing either the Eugl*DHN2* showed that they had a higher cold tolerance, at least in a 40 %, than the untransformed *A. thaliana*, when subjected to a cold tolerance assay. Similar results have been observed in *N. tabacum* over-expressing Zm*DHN2b* (Xing et al. 2011) and in *Fragaria* × *ananassa* with the WCOR410 (Houde et al. 2004), therefore, suggesting that this gene can play a key role in the cold acclimation of eucalypts. Nevertheless, this is not true for the Eni*DHN2* lines, in which case only the high expressing lines had a higher survival rate than the wt *A. thaliana*, but they showed an aberrant phenotype. It is worth noting that this heterologous system, using *Arabidopsis*, does not necessarily represent the role that these genes will have in eucalypts. The endogenous gene At*ERD10*, was induced by cold temperatures in all transgenic lines

tested to similar levels as the ones observed in the non-transformed plants. This gene is also a $SK_n$ having a 36 % nucleotide identity with Eni*DHN2* or Eugl*DHN2* and they respond to cold, high salinity and water stress (Nylander et al. 2001). Overexpression of At*ERD10* increases cold tolerance in *A. thaliana* as reported by Kim and Nam 2010. In L5 the expression of this gene was significantly higher than for the rest tested, however, it did not have a role in conferring a higher cold tolerance to this line. For At*RAB18*, no significant difference was observed in transcript abundance due to cold in any of the lines tested (transgenic or wild type). This gene has been shown to increase under abiotic conditions such as cold, water stress or by ABA (Lång and Palva 1992).

The putative promoter of Eni*DHN2* was strongly induced by low temperatures (4 and -6 ºC) in all lines of the transgenic *A. thaliana* tested, while the one from Eugl*DHN2* was induced to a lower extent. This is an interesting result, since the putative promoter of Eni*DHN2* can have a key role in a faster and stronger response to cold stress, the characteristic that makes this species to be more frost-tolerant. The differences observed at the *cis*-elements of each promoter can as well explain the difference in the fold changes observed at the same temperature of the corresponding transgenic lines. Zhu et al. (2014) observed that the putative promoter of a $SK_n$ dehydrin in wheat, (wzyl-2) was induced by several factors involved in abiotic stress such as cold (4 ºC), phytohormones (ABA, SA, MeJA) as well as PEG6000. In the case of cold temperature, the promoter was induced at early times 12 h, having a peak at 24 h. These results are similar to ones obtained in this study, where the promoter was induced at 4 ºC and increased when temperatures reached -6 ºC. Compared with other studies (Rodríguez et al. 2005; Nylander et al. 2001) we did not observe any transcript accumulation in a specific tissue or organ of the *GUS* gene under the control of the putative promoter of *DHN2*, rather a constitutive expression that was induced by temperature was observed. However, Nylander et al. (2001) reported a tissue specific accumulation for five different dehydrins in *A. thaliana*, the most similar dehydrin tested, compared to the *DHN2* of eucalypts, was *ERD14* ($SK_n$-type dehydrin), in which case they found the protein in all tissues tested. Zhu et al. (2014) did not observe any staining in calli treated by cold, but they assume that this can be due to the duration of the experiment. However, gus staining is observed, at different levels, when the calli are treated with different phytohormones.

As a conclusion, the analysis in the genomic sequence of *DHN* genes from *E. globulus*, a frost sensitive tree, and *E. nitens*, a frost-tolerant tree, in combination with gene expression under cold acclimation, can explain the differences in this trait. The presence of different *cis*-acting elements identified in the putative *DHN* promoter regions and the results of the relative expression data demonstrate the existence of an association between the role of *DHNs* of both species and the process of cold acclimation, allowing to infer that they have a key role in tolerance to low temperatures in *Eucalyptus*. The evidence showed that Eni*DHN2* putative promoter acted as a strong promoter induced by cold temperatures, therefore, this could have a key role in conferring frost tolerance to this species.

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# SUPPLEMENTARY DATA

**a**

| Species | Accession |
|---|---|
| *E. nitens* DHN1 | EniDHN1 |
| *E. globulus* DHN1 | AER27687 |
| *E. grandis* | KCW56702 |
| *E. grandis* | KCW56700 |
| *Coffea canephora* | ABC55670 |
| *Coffea canephora* | ABC55671 |
| *Camelia sinesis* | ACJ65691 |
| *Vitis riparia* | AAW58105 |
| *Vitis vinifera* | ABN79618 |
| *Cucumis melo* | XP_008452109 |

**b**

| Species | Accession |
|---|---|
| *E. nitens* DHN2 | EniDHN2 |
| *E. globulus* DHN2 | AER27688 |
| *E. grandis* | KCW68043 |
| *E. camaldulensis* DHN2 | CDM07993 |
| *Citrus sinensis* | AAP56259 |
| *Citrus x paradisi* | AAN78125 |
| *Populus maximowiczii* | ABS12346 |
| *Populus x canadensis* | ABS12348 |
| *P. alba x P. glandulosa* | ABH11546 |
| *Eriobotrya japonica* | ACL01289 |

**Fig. S3.1** Multiple sequence alignment of EniDHNs with DHNs homologous proteins. Identical residues are highlighted in ***black***, residues with conservative substitutions are ***shaded in gray*** and ***dashes*** indicate gaps in a sequence. The ***broken lines*** indicate the S segments, ***solid lines*** indicate the K segments and ***dotted lines*** indicate the Y segments. **a** EniDHN1, **b** EniDHN2, **c** EniDHN3 and **d** EniDHN10. The alignment was generated using the Clustal W program

**Table S3.1** Identity percentage of four dehydrins of *E. nitens* with homologous dehydrins proteins of other species.

| *Eucalyptus nitens* dehydrins | Species | Accession | Identity % |
|---|---|---|---|
| Eni*DHN1* | *Eucalyptus globulus* | AER27687 | 98 |
| | *Eucalyptus grandis* | KCW56702 | 93 |
| | | KCW56700 | 60 |
| | *Camellia sinensis* | ACJ65691 | 74 |
| | *Coffea canephora* | ABC55670 | 35 |
| | | ABC55671 | 37 |
| | *Vitis vinifera* | ABN79618 | 56 |
| | *Vitis riparia* | AAW58105 | 53 |
| | *Cucumis melo* | XP_008452109 | 42 |
| Eni*DHN2* | *E. globulus* | AER27688 | 84 |
| | *E. grandis* | F01726 | 67 |
| | *Eucalyptus camaldulensis* | EcC013097.40 | 44 |
| | *Populus alba x glandulosa* | ABH11546 | 44 |
| | *Citrus sinensis* | AAP56259 | 31 |
| | *Citrus paradise* | AAN78125 | 36 |
| | *Populus maximowiczii* | ABS12346 | 43 |
| | *Eriobotya japonica* | ACL01289 | 37 |
| Eni*DHN3* | *E. globulus* | AEY77126 | 96 |
| | *E. grandis* | KCW53080 | 90 |
| | *Ricinus communis* | XP_002521516 | 79 |
| | *Corylus manshurica* | AER13140 | 51 |
| | *Quercus robur* | AAU06816 | 37 |
| Eni*DHN10* | *E. globulus* | AEY77126 | 98 |
| | *Solanum commersonii* | ACJ26759 | 65 |
| | *Glicine max* | ABQ81887 | 52 |
| | *Citrus trifoliate* | AAA99963 | 54 |

## DISCUSIÓN GENERAL

*Eucalyptus* es uno de los géneros de plantas leñosas más cultivadas en el mundo, sus especies poseen valiosas características con un alto interés comercial y donde la investigación tanto del sector productivo como académico, ha proporcionado el conocimiento necesario para mejorar rendimientos y productividad de estas.

En Chile, particularmente la especie *E. globulus* ha sido seleccionada y cultiva desde hace 30 años, principalmente para la producción de pasta de celulosa, alcanzando un consumo total anual de 56%, con 2.323,4 miles de toneladas durante el año 2015 (INFOR 2016). Sin embargo, esta especie es altamente sensible a bajas temperaturas, siendo en un impedimento para la expansión de sus plantaciones (Turnbull y Eldridge, 1983). Actualmente en zonas con alta incidencia de heladas, la especie ha sido reemplazada por otra especie de *Eucalyptus* resistente a heladas, como es *E. nitens*. Estas plantaciones poseen un mayor riesgo en reducir las ganancias industriales, debido a propiedades pulpables inferiores que presenta esta última especie, tales como, menor densidad básica, fibras más cortas, mayor contenido de lignina, entre otros factores que influyen en el proceso de obtención de pasta de celulosa, con una reducción de hasta un 8,3% en su rendimiento (Kibblewhite y col. 2000; Escalona y col. 2000).

Dentro de los primeros estudios generados en especies de *Eucalyptus,* se destacan aspectos fisiológicos relacionados con la aclimatación al frío y tolerancia a heladas (Almeida y col. 1994; Shvaleva y col. 2008), y donde los autores sugieren que la protección al frío pudiese estar determinada por la síntesis de azúcares, entre estos: sacarosa, glucosa y fructosa. Hacía un enfoque más genético, se destacan estudios en la construcción de mapas genéticos (Grattapaglia y Sederoff 1994; Freeman y col. 2006; Hudson et al 2012), expresión de secuencias EST (Rasmussen-Poblete y col. 2008; Keller at al. 2009), como también la reciente publicación del genoma de *E. grandis* (Myburg y col. 2014) y su asociación a mapas de ligamiento (Gion y col. 2014). Por otra parte, estudios sobre el transcriptoma en respuesta a la aclimatación al frío y tolerancia a heladas han sido generado en especies como *E. gunnii* (Keller y col. 2013), *E. globulus* (Fernández y col. 2015) y *E. nitens* (Gaete-Loyola y col. 2017). La recopilación de información tanto del genoma como el transcriptoma en especies del

género *Eucalyptus*, ha facilitado el estudio de genes en respuesta al estrés ambiental, permitiendo el uso de herramientas bioinformáticas para el diseño de partidores que accedan al aislamiento de las secuencias y su caracterización.

El estudio genómico involucra la caracterización tanto estructural como funcional de un gen, identificando regiones altamente conservadas, junto a la determinación de su patrón de expresión, incluyendo la manipulación genética a través del uso de plantas transgénicas o mutantes, para demostrar su función putativa (Kemmer y col. 2008). Actualmente, el análisis de expresión de un gen se realiza mediante la metodología de PCR en tiempo real o PCR cuantitativo (qPCR), donde los ácidos nucleicos de doble hebra son detectados en cada ciclo de amplificación mediante la unión de una molécula fluorescente, proporcionando una técnica altamente sensible y rápida, con capacidad de cuantificar pequeñas cantidades de ADN en el transcurso de horas. La combinación de esta técnica con la utilización de sondas de hibridación Taqman®, permite una técnica altamente específica que no detecta productos inespecíficos (Holland y col. 1991), característica que fue necesaria para el estudio de expresión relativa de los genes Egl*CBF* de *E. globulus*, que al poseer secuencias altamente homólogas, imposibilitaron el de partidores específicos, siendo imprescindible el diseño de sondas de hibridación. Como genes de control endógeno se emplearon alpha-Tubulina (*α-TUB*) y Ubiquitina C (*UBC*), descritos con anterioridad por Fernández y col. (2010), por presentar una alta estabilidad en el estudio de expresión génica de *E.* globulus en condiciones de aclimatación al frío, estos también se detectaron mediante sondas Taqman®.

De acuerdo a los resultados encontrados por otros grupos de investigación en expresión génica y estrés por baja temperatura en plantas, se han identificado genes que inducen su expresión durante el proceso de aclimatación, los cuales han demostrado tener un rol importante en la adaptación a baja temperatura y la resistencia al congelamiento (Kreps y col. 2002; Dhanaraj y col. 2004; Joosen y col. 2006). Dentro de estos, los genes *CBF* (o *DREB*) se han descrito como una ruta de señalización ABA–independiente de respuesta al estrés por frío y deshidratación (Stockinger y col. 1997; Thomashow 1999).

***Los factores de transcripción CBF***

En los noventa se comienzan a dilucidar los mecanismos que regulan la activación de genes inducidos por bajas temperaturas y déficit hídrico en plantas, reportando secuencias activadoras en *cis* tipo CRT/DRE dentro de regiones promotoras de algunos genes (Yamaguchi-Shinozaki y Shinozaki 1994; Baker y col. 1994; Jiang y col. 1996). Stockinger y col. (1997) describen por primera vez un gen *CBF* en *Arabidopsis*, demostrando que la expresión *in vitro* e *in vivo* de la proteína CBF1 permite la unión a elementos *cis* descrita previamente, que se ubican en el promotor del gen *COR15a*, que corresponde a un gen efector de la respuesta a frío, funcionando la proteína CBF1 como un activador transcripcional. Seguidamente, Jaglo-Otttosen y col. (1998) demostraron que la sobreexpresión del gen *CBF1* induce la expresión de genes regulados por frío (*COR – cold regulated*) e incrementa la tolerancia al congelamiento. Actualmente, los genes *CBF* han sido clasificados dentro del grupo A1 de la subfamilia DREB de factores de transcripción AP2/ERF (Sakuma y col. 2002), siendo ampliamente estudiados en diferentes especies de importancia comercial, como arroz (Nakano y col. 2006), maíz (Zhou y col. 2012), uva (Licausi y col. 2010), manzano (Girardi y col. 2013), álamo (Zhuang y col. 2008), eucalipto (El Kayal y col. 2006; Navarro y col. 2009; Cao y col. 2015), entre otras especies. En el presente trabajo se identificaron, aislaron y caracterizaron tres secuencias de genes *CBF* en *E. globulus*, de acuerdo al dominio y motivos conservados que los identifican. Se demostró que estos genes son expresados durante el proceso de aclimatación al frío, siendo fuertemente inducidos por temperaturas congelantes. Además, se observó que uno de los genotipos resistentes, R1, acumula mayores niveles de transcritos a temperaturas congelantes, comparado con el genotipo susceptible S1 a heladas, lo cual sugiriere que estos factores transcripcionales desempeñan un importante rol en el proceso de resistencia al congelamiento en *E. globulus*. Estos resultados de expresión son similares a los observados en *E. gunnii,* donde se han reportado cuatro genes tipo *CBF*, relacionados con el proceso de aclimatación y de respuesta al estrés por frío y congelamiento (El Kayal y col. 2006; Navarro y col. 2009). Estos resultados también fueron semejantes a lo encontrado previamente para el único gen *CBF* descrito para *E. globulus* a la fecha, Eg*CBF1*, en el cual su expresión es inducida por bajas temperaturas (4 ºC) (Gamboa y col. 2007). Análisis preliminares de secuencias realizados en librerías de expresión de *E. globulus* han identificado 15 secuencias homologas a genes tipo *CBF* (comunicación poster IUFRO Tree Biotechnology

2015 Conference, Navarrete-Campos y col. 2015), todas estas con un alto grado de similitud entre sus secuencias. Recientemente para *E. grandis* se han anotado 17 secuencias homólogas a genes *CBF* (Cao y col. 2015), de las cuales el grupo de genes Egr*CBF2 – 14* ha sido involucrado en respuesta al frío (4 ºC), y los genes Egr*CBF1*, Egr*CBF15 – 17*, sin especialización, podrían ser responsables de la respuesta al calor y sequía (Nguyen y col. 2017). De igual forma, nueve genes *CBF* (*CBF2*, *CBF4*, *CBF6*, *CBF8 – 12* y *CBF14* de acuerdo anotación en *E. grandis*) han sido identificados mediante análisis de expresión diferencial en el transcriptoma de *E. nitens* aclimatado al frío, informando que todos, con excepción del gen *CBF14*, fueron altamente regulados por frío (Gaete-Loyola y col. 2017). En el presente trabajo se encontró que los genes Egl*CBF1a* (*CBF6*), Egl*CBF1c* (*CBF1*) y Egl*CBF1d* (*CBF2*) fueron altamente expresados en condiciones de temperaturas congelantes (CAAF, – 2 ºC), mientras que los genes Egl*CBF1a* y *d* fueron inducidos por bajas temperaturas (CABF, 8/4 ºC día/noche), siendo reprimida la expresión del gen Egl*CBF1c* en tal condición. Adicionalmente este último gen en condición no aclimatado (NA), presento el doble de acumulación de transcritos que los otros dos genes evaluados (datos no publicados), sugiriendo que su expresión es más bien constitutiva a diferencia de los genes Egl*CBF1a* y *d*, que son inducidos por frío (4 ºC) y congelamiento (– 2 ºC). Estos resultados fueron similares a los presentados con anterioridad para los genes *CBF* de *E. gunnii*, donde los autores proponen que el gen tipo *CBF1a* presenta una inducción rápida y de corta duración, en respuesta a un golpe de frío extremo. El gen tipo *CBF1c* tiene una expresión constitutiva pudiendo estar o no involucrado en la protección permanente a la célula, y no en una respuesta al estrés. Y finalmente el tipo *CBF1d* tiene una expresión relacionada con la respuesta a heladas intensas, con o sin aclimatación previa (Navarro y col. 2009). Además, la proteína putativa del gen Egl*CBF1c* es similar a la proteína EgrCBF1 descrita para *E. grandis*, y como se ha mencionado, el gen Egl*CBF1c* no se induce por temperaturas frías en *E. globulus*, este pudiese estar asociado a una respuesta al estrés por sequía, como se ha descrito para el gen Egr*CBF1* (Nguyen y col. 2017).

### *Transformación de genes CBF y resistencia al frío*

Una excelente metodología para validar la función de estos genes es su expresión heteróloga, mediante transformación genética de *A. thaliana* con los tres genes *CBF* descritos para *E.*

*globulus*. Como resultado, se obtuvieron 10 líneas transformadas (generación T2), de las cuales cinco presentaron una alta expresión de los Egl*CBF*, junto a una mayor acumulación de transcritos de los genes *COR*. Cuatro de estas cinco líneas lograron un alto porcentaje supervivencia, superior al 60% a temperaturas congelantes (– 6 ºC), en comparación a plantas control no transformadas, que presentaron un 0% de supervivencia, demostrando que los genes *CBF* de *E. globulus* incrementaron la resistencia al congelamiento en *Arabidopsis* mediante la inducción de genes *COR*. Similares resultados han sido descritos por numerosas investigaciones, donde la transformación genética tanto de *Arabidopsis*, como de *N. tabacum*, *Populus* spp. y otras especies, con genes *CBF* aislados tanto de especies herbáceas como leñosas, otorgan resistencia al frío y congelamiento (Benedict y col. 2006; Tong y col. 2009; Siddiqua and Nassuth 2011; Zhou y col. 2014; Fang y col. 2015). También existen estudios que han reportado la transformación genética en especies de *Eucalyptus* con genes *CBF* (o *DREB*). Al respecto, Navarro y col. (2011) realizaron la transformación genética con dos factores de transcripción CBF, Egu*CBF1a* y *b*, aislados de *E. gunnii* y expresados constitutivamente en un genotipo híbrido *E. urophylla* × *E. grandis* altamente sensible a heladas. Además del incremento en la resistencia al congelamiento en las líneas transformadas, estas presentaron diversos cambios anatómicos y fisiológicos, incluyendo una disminución en la densidad estomática, el incremento en la acumulación de antocianinas, deposición de cera en cutícula, reducción del área foliar, reducción del tamaño celular, disminución del crecimiento y mayor capacidad de retención de agua durante el estrés por frío en comparación con plantas no transformadas. Estos investigadores no sólo han generado líneas transgénicas de eucalipto tolerantes al frío, si no también han informado el rol de factores de transcripción CBF, como mecanismo adaptativo en la regulación de genes *CBF*-target, proponiendo nuevas alternativas en la mejora genética de eucaliptos con tolerancia al frío. Por otra parte, empresas dedicadas a la producción de plantas como ArborGen®, han desarrollado *Eucalyptus* transgénicos con el objetivo de obtener nuevas variedades resistentes al frío. Estos han informado de la transformación de un genotipo hibrido *E. grandis* × *E. urophylla* altamente productivo, en el cual se ha insertando la secuencia del gen *CBF2* de *A. thaliana*, bajo el promotor del gen At*RD29a*, como elemento inductor al estrés por frío por que posee cuatro elementos *cis* CRT (Hinchee y col. 2009; 2011). Esta nueva variedad transgénica, ha logrado resistir temperaturas en campo de hasta –7 a –9 ºC, durante la

temporada de invierno 2009/2010 en los estados de Alabama y Carolina del Sur, al sureste de los EE.UU., manteniendo su productividad (Hinchee y col. 2011) y corroborando la importancia de los genes aquí aislados para la resistencia a temperaturas congelantes.

***Efectos secundarios en la transformación con genes CBF***

Se observó que dos de las líneas transformadas de *Arabidopsis* con máximos niveles de expresión de Egl*CBF*, incrementaron su resistencia congelamiento, pero además presentaron un fenotipo con una tasa de crecimiento disminuida y un retardo de la floración. Esta alteración del desarrollo ha sido previamente reportada como una severa inhibición de crecimiento, que a menudo es referida como enanismo después de la sobreexpresión de genes *CBF* en diversas especies vegetales como *Arabidopsis* (Liu y col. 1998; Gilmour y col. 2000), tabaco (Kasuga y col. 2004) y tomate (Hsieh y col. 2002), entre otras especies. Respecto a plantas leñosas, la expresión heteróloga de genes *CBF* en eucalipto, manzano y uva, también ha resultado en la obtención de líneas transgénicas con disminución del crecimiento (Navarro y col. 2011; Wisniewski y col. 2011; Tillett y col. 2012). Algunos de estos autores indican que a mayor nivel de transcritos del transgén *CBF*, mayor es el efecto de enanismo en las plantas transformadas (Liu y col. 1998; Wisniewski y col. 2011). Estos efectos de la sobreexpresión de genes *CBF* fueron estudiados en profundidad por Achard y col. (2008), quienes reportan que la expresión constitutiva del gen At*CBF1* en *Arabidopsis* induce la expresión de genes que codifican para GA 2-oxidasas, enzimas que catalizan la inactivación de ácido giberélico (GA) bioactivo, principal fitoregulador de acción hormonal del crecimiento y desarrollo en plantas. La presencia de GA bioactivo en condiciones normales de crecimiento, promueve la degradación de proteínas DELLA por la vía ubiquitina – proteasoma (McGinnis y col. 2003). Por lo tanto, la disminución de GA bioactivo, genera la acumulación de proteínas DELLA y consecuentemente, la inhibición del crecimiento, causando enanismos y retraso de la floración (Tong y col. 2009; Thomashow 2010; Siddiqua y Nassuth 2011; Zhou y col. 2014). Adicionalmente, y dada la naturaleza aleatoria de la transformación mediada por *Agrobacterium*, un efecto posicional de los transgenes insertados en alguna zona donde pueda interferir con la regulación endógena del crecimiento en las plantas transformadas de *Arabidopsis* no puede ser descartado, aun cuando a la posibilidad que esto ocurra en dos de

133 plantas transformadas en total es remoto, por lo cual la explicación mas simple sería un efecto directo de la sobreexpresión de los *CBF* sobre el desarrollo de dichas plantas.

***Promotor de gen DHN2 en Eucalyptus***

En la mayoría de los casos, la inducción de genes deshidrinas ocurre frecuentemente en respuesta al estrés por deshidratación, muchas veces estimulada por efectos de congelación y sequía. Sin embargo, se ha observado que las regiones promotoras exhiben elementos activadores en *cis* MYC, ABRE y CRT, siendo frecuentemente asociados a las respuestas por deshidratación, biosíntesis de ABA y factores de transcripción CBF, respectivamente.

En esta investigación, la expresión del gen *GUS* inducida por los promotores putativos de los genes *DHN2* tanto de *E. nitens* (Eni*DHN2*) como de *E. globulus* (Eugl*DHN2*) expone diferencias en la expresión relativa del gen reportero, al ser inducido por frío y congelamiento. Los resultados indican que el promotor del gen Eni*DHN2* fue altamente activado por bajas temperaturas (4 y –6 °C) en todas las líneas de la *Arabidopsis* transformadas, mientras que el promotor del gen Eugl*DHN2*, presentó una menor respuesta. Esto sugiere que el promotor putativo del gen Eni*DHN2* puede tener un rol importante en mejorar la respuesta a frío y congelamiento en *E. nitens*, promoviendo una rápida y fuerte expresión del gen *DHN2*, como gen efector de la respuesta a frío, lo cual puede estar relacionado a las características que hacen que la especie sea más tolerante a heladas. Estos resultados son similares a estudios que reportan la activación de regiones promotoras de dos genes deshidrinas por bajas temperaturas (4 ºC), donde los promotores de los genes *WZY1-2* y *WZY2* inducen la expresión del gen *GUS*, obteniendo un máximo en su expresión a las 24 y 12 h, respectivamente (Zhu y col. 2014a; 2014b). Las diferencias observadas en los elementos activadores *cis* de cada promotor pueden explicar también la diferencia en los cambios de expresión observados en el gen reportero. Estudios de transformación con genes *CBF* en *Eucalyptus*, han demostrado que plantas transformadas inducen la expresión de genes regulados por elementos *cis* CRT en sus regiones promotoras, entre estos los genes *DHN*, que también pueden ser activados vía estrés hídrico (DREB2) y vía estrés por frío (DREB1/CBF) (Navarro y col. 2011).

Este trabajo confirma la importancia de la investigación sobre procesos moleculares de la resistencia al frío y congelamiento en especies de interés comercial, como es *E. globulus*. Los

resultados aquí obtenidos corroboran la importancia de factores de transcripción CBF, como también de genes efectores o elementos reguladores que faciliten comprender mejor la regulación de las vías de señalización y respuesta al frío en plantas. Debido a la gran importancia de la especie *E. globulus* en la industria forestal de nuestro país, es que se ha podido ampliar su conocimiento con el fin de mejorar aspectos en su crecimiento, rendimiento y productividad, que aporten al desarrollo de recursos genéticos y biotecnológicos, en la investigación del sector forestal, tanto académica como del sector productivo.

## CONCLUSIONES GENERALES

Las tres secuencias identificadas, aisladas y caracterizadas de *E. globulus*, corresponden a genes tipo *CBF*, siendo descritos como: Egl*CBF1a*, Egl*CBF1c* y Egl*CBF1d*. Estos codifican factores de transcripción, mostrando en sus secuencias residuos de aminoácidos altamente conservados a un dominio y dos motivos que los caracterizan. Además, análisis de secuencias mediante bibliotecas de expresión permitió identificar 15 secuencias homologas a factores de transcripción tipo CBF, de las cuales tres son descritas en esta investigación.

El estudio de expresión génica demostró que los tres genes *CBF* son inducidos en el proceso de aclimatación al frío en plantas juveniles de *E. globulus*. Diferentes genotipos de *E. globulus* con respuesta contrastante al congelamiento, presentan diferentes patrones de acumulación de transcritos, cuando son expuestos a temperaturas frías (4 ºC) y a heladas nocturnas (–2 ºC), siendo mayor su expresión luego de exponerse a temperaturas congelantes.

De acuerdo a la transformación heteróloga de los tres genes *CBF* en *Arabidopsis*, se logra aumentar la resistencia al frío en cuatro líneas transformadas, resistiendo temperaturas congelantes de hasta –6 ºC. Estas líneas transformadas corresponden a las mismas líneas que presentan mayor acumulación de transcritos a cada uno de los transgenes insertados, sugiriendo que esta mayor acumulación permite una mayor resistencia al estrés por temperaturas congelantes.

En los análisis de expresión del gen marcador *GUS* conducidas por los promotores de los genes *DHN2* de las especies *E. globulus* y *E. nitens*, se determina que el promotor de *E. nitens* presento una expresión más intensa del gen marcador, sugiriendo que es un promotor fuerte en respuesta al estrés por congelamiento.

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