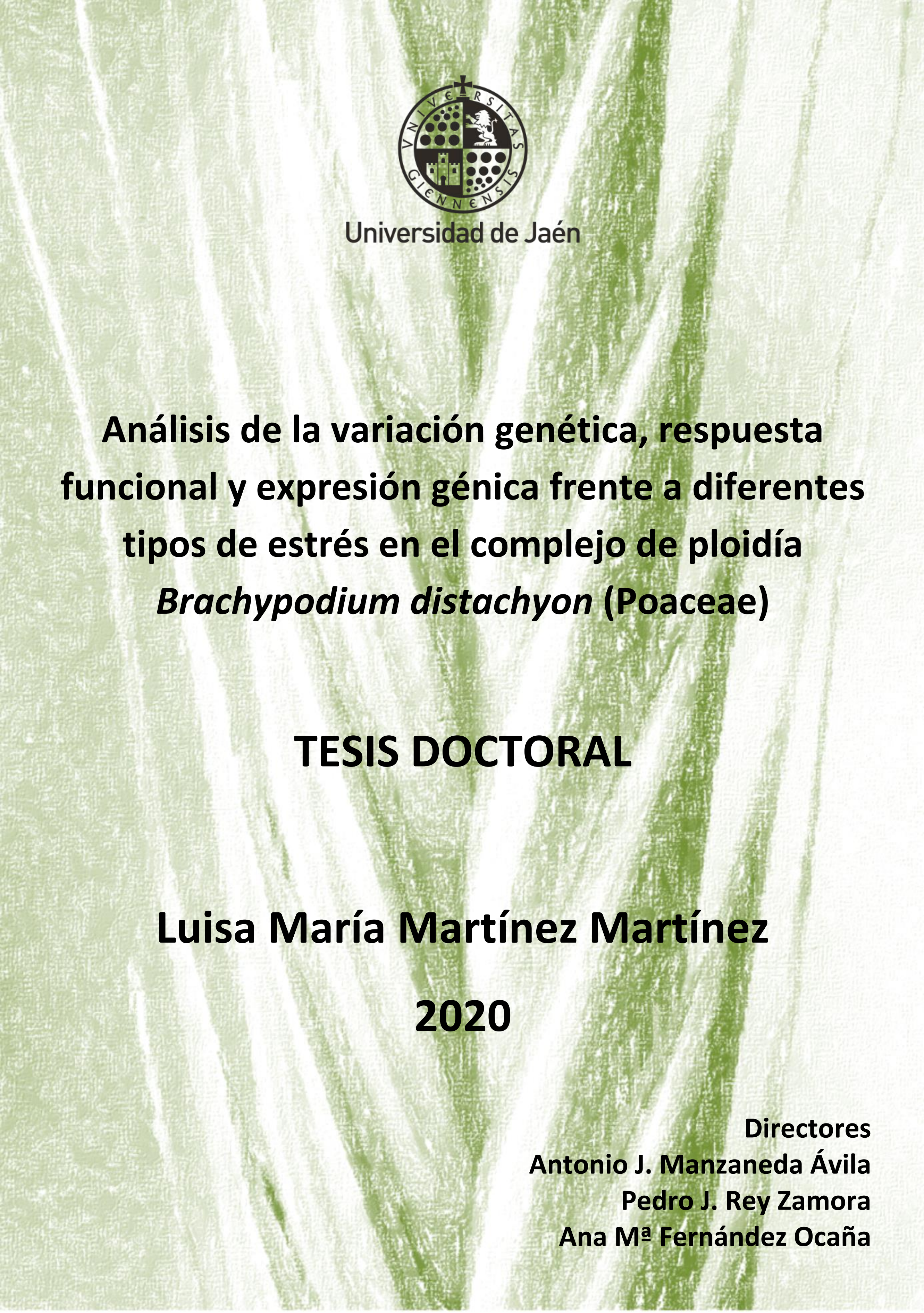

Universidad de Jaén

Análisis de la variación genética, respuesta funcional y expresión génica frente a diferentes tipos de estrés en el complejo de ploidía *Brachypodium distachyon* (Poaceae)

TESIS DOCTORAL

Luisa María Martínez Martínez

2020

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Universidad de Jaén
Escuela de Doctorado

Programa Oficial de Doctorado en Ciencia y Tecnología de la Tierra y del Medio Ambiente

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JAÉN, Febrero 2020

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Departamento de Biología
Animal, Biología Vegetal y
Ecología

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Memoria presentada por la Licenciada en Biología

Dña. Luisa María Martínez Martínez

Para optar al Grado de Doctor por la

Universidad de Jaén

Directores de tesis:

Dr. Antonio José Manzaneda Ávila

Dr. Pedro José Rey Zamora

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El Dr. Antonio José Manzaneda Ávila, Profesor de Ecología; el Dr. Pedro José Rey Zamora, Catedrático de Ecología y la Dra. Ana María Fernández Ocaña, profesora titular del Área de Fisiología Vegetal. Los tres investigadores del grupo de Ecología, Evolución y Conservación de la Vegetación Mediterránea del Departamento de Biología Animal, Biología Vegetal y Ecología de la Universidad de Jaén, hacen constar que el trabajo recogido en la presente memoria de tesis doctoral ha sido desarrollado bajo su dirección y autorizan su presentación y defensa.

Jaén, Febrero 2020

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APOYO A LA INVESTIGACIÓN

Esta tesis doctoral ha sido desarrollada en los laboratorios del grupo de investigación de Ecología, Evolución y Conservación de la Vegetación Mediterránea de la Universidad de Jaén (Departamento de Biología Animal, Biología Vegetal y Ecología) bajo la dirección del Dr. Antonio José Manzaneda Ávila, Dr. Pedro José Rey Zamora y la Dra. Ana María Fernández Ocaña. Parte de los resultados fueron obtenidos en el Departamento de Horticultura de la Universidad de Virginia Tech en Blacksburg (Virginia, Estados Unidos) bajo la supervisión del Dr. Aureliano Bombarely Gomez.

Los trabajos de laboratorio fueron llevados a cabo en el Departamento de Biología Animal, Biología Vegetal y Ecología y en el Centro de Instrumentación Científico-Técnica de los Servicios Centrales de Apoyo a la Investigación de la Universidad de Jaén. Los recursos informáticos empleados fueron los de la Universidad de Virginia Tech.

El desarrollo de esta Tesis se enmarca en los proyectos "Selección natural y significado adaptativo de los genes candidatos asociados a la tolerancia al estrés hídrico en *B. distachyon*" financiado por el Ministerio de Economía y Competitividad (Proyecto No. CGL2012-30838) y "Disentangling the role of plant dehydrin DHN3 on dehydration stress in *Brachypodium distachyon* (Poaceae) an emerging model for temperate grasses" financiado por Grants4TraitsTM Initiative (Bayer AG Crop Science), y fue posible gracias a un contrato pre-doctoral para la formación de doctores asociado al primer proyecto (No. BES-2013-062883). Adicionalmente, se obtuvo una ayuda de movilidad para desarrollar estancias breves de investigación en la Universidad de Virginia Tech (No. EEBB-I-15-09434).

A mi gran familia

“Me enseñaron que el camino del progreso no es ni rápido ni fácil”

“Soy de las que piensan que la ciencia tiene una gran belleza. Un científico en un laboratorio no es solo un técnico: es también un niño colocado ante fenómenos naturales que le impresionan como un cuento de hadas”

- Marie Salomea Curie (1867-1934) -

INDICE

AGRADECIMIENTOS

AGRADECIMIENTOS

Todo llega a su fin, por muy largo que sea el camino. Como todo camino tiene sus obstáculos que hacen que se te pase por la mente querer abandonar pero lo mejor son las cosas que nos hacen olvidar y evitar pensar en lo complicado, así como la ayuda y el apoyo sin el cual nunca podría haber visto el final.

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¡GRACIAS A TODOS!

RESUMEN
SUMMARY

RESUMEN

Los estreses de naturaleza abiótica y biótica afectan a los cultivos agrícolas de todo el mundo siendo los causantes de importantes pérdidas en rendimiento y calidad. Dentro de los estreses abióticos, el estrés hídrico por sequía es uno de los más importantes, considerado además como un determinante principal de la adaptación local de las plantas a las condiciones de aridez actuales o futuras agravadas por el cambio climático. Por otra parte, dentro de los estreses bióticos destaca la herbivoría por insectos causantes de importantes plagas tanto en sistemas naturales como en cultivos, siendo además una fuerza de selección importante de caracteres de las plantas. Por ello, la investigación encaminada a estudiar los mecanismos de control de la tolerancia y la resistencia a estos diferentes tipos de estrés, tiene una gran importancia científica, social y económica.

El complejo *Brachypodium distachyon* ha sido seleccionado como modelo de gramíneas y monocotiledóneas C3 de zonas templadas en la última década. El gran número de recursos genéticos y moleculares que existen, junto a sus características biológicas (simplicidad de los genomas, su amplia distribución nativa circummediterránea y su estrecho parentesco con cereales como el trigo, la cebada o la avena entre otros), lo hacen un modelo ideal para abordar el estudio de numerosas cuestiones de biología evolutiva y genómica funcional de cereales de clima templado y gramíneas que se usan como biocombustibles. La amplia distribución de sus poblaciones naturales en una gran variedad de ambientes, brinda a los investigadores la oportunidad de utilizar su diversidad genética para estudiar los caracteres de adaptación de las plantas a los climas locales y a estreses abióticos. Esto lo convierte en un modelo útil para estudiar la genética y la genómica de la respuesta de tolerancia y resistencia a estrés de cereales y gramíneas bioenergéticas. Además, las tres especies que forman dicho complejo

muestran variación en ploidía: los dos parentales diploides *B. distachyon* (2n=2x=10, x=5) y *B. stacei* (2n=2x=20, x=10), y su derivado alotretraploide *B. hybridum* (2n=4x=30, x=10+5) de naturaleza híbrida. Esto lo convierte en un modelo para investigar la evolución de la (alo)poliploidía debido a los amplios conocimientos de genómica funcional comparativa que se tienen de las tres especies, ampliando así su potencial de aplicación a trigos poliploides.

Esta tesis doctoral aborda el estudio de la respuesta funcional mediada por ploidía e hibridación ancestral (i.e., alopoliploidía), así como la base genética subyacente a la variación natural de la tolerancia al estrés hídrico, entre individuos pertenecientes a diferentes poblaciones naturales de las tres especies del complejo *Brachypodium distachyon*. El material vegetal utilizado para llevar a cabo las investigaciones se ha obtenido de la siembra de semillas procedentes del banco de germoplasma de *Brachypodium* de la Universidad de JAEN, que se encuentra en el Departamento de Biologia Animal, Biología Vegetal y Ecología de la Universidad de Jaén. Dichas semillas fueron recolectadas de 57 poblaciones naturales en la Península Ibérica durante muestreos de campo en 2007 y 2008 por los doctores Antonio J. Manzaneda y Pedro J. Rey, lo que nos ha permitido disponer de un amplio muestreo poblacional dentro del rango de distribución de cada especie.

Para abordar este trabajo, en el **Capítulo 1** se incluye el trabajo que analiza la variación inter-especifica e intra-específica de caracteres funcionales relacionados con la respuesta a la sequía (contenido en agua, área específica de la hoja, daño celular, contenido en prolina y contenido en fitohormonas) en un amplio número de poblaciones naturales de *B. distachyon*, *B. stacei* y *B. hybridum*, hallándose diferenciaciones entre las tres especies (específicamente la respuesta funcional a la sequía de *B. hybridum* y *B. stacei* estuvo correlacionada y diferenciada de la de *B. distachyon*). También se incluye el análisis de la diferenciación de estos caracteres entre diez poblaciones de *B. hybridum* genéticamente diferentes y la relación de esta diferenciación fenotípica y genética con la aridez. Los resultados de este estudio sugieren que las poblaciones de *B. hybridum* se han diferenciado de forma adaptativa a nivel fisiológico y genético, en respuesta a variaciones en aridez durante el último millón de años, tras el proceso de poliploidización.

El trabajo del **Capítulo 2** corresponde al primer análisis transcriptómico comparativo llevado a cabo entre las tres especies del complejo *B. distachyon* en el contexto de respuesta a la sequía y legado parental. Nuestros resultados muestran una mayor respuesta a nivel de expresión génica global en el alopoliploide *B. hybridum* comparado con sus dos ancestros *B. distachyon* y *B. stacei* y una variación significativa de dicha respuesta entre diferentes genotipos de *B. hybridum* en respuesta a la sequía. Destaca la fuerte inducción de proteínas LEA (del inglés Late Embryogenesis Abundant) como las dehidrinas y otras proteínas de choque térmico que protegen a las plantas de la sequía. En cuanto al legado parental en respuesta a la sequía, la mayor parte de pares de genes homoeologos en *B. hybridum* mostró un patrón de expresión estadísticamente similar a *B. distachyon* y un patrón de expresión transgresivo, contrariamente a los resultados encontrados a nivel funcional en el capítulo anterior. También se encontró que la mayoría de pares de homólogos expresados en *B. hybridum* mantuvo la expresión del mismo modo que en los parentales, el 21% de los genes expresados en respuesta a la sequía modificó su expresión con respecto a su expresión en los ancestros.

Para tener una visión más amplia del papel de las dehidrinas, el **Capítulo 3** consistió en la generación de una línea transgénica de *Brachypodium distachyon* que sobreexpresa el gen de la dehidrina3 (BdDHN3), que apareció fuertemente inducido en los análisis transcriptómicos realizados en el capítulo 2 en las tres especies del complejo *Brachypodium*. Se analizó la línea transgénica generada, a nivel transcriptómico y fisiológico, en condiciones de estrés por sequía, hallándose que la sobreexpresión de este gen en dicha línea está correlacionada significativamente con caracteres que potencian la tolerancia a la sequía como el incremento de los contenidos de agua y prolina y un menor daño celular en hoja.

Finalmente, en el trabajo del **Capítulo 4** se realizó el primer análisis de la variación de la respuesta a la herbivoría por insectos en un amplio número de poblaciones naturales diploides y alotetrapolides del complejo *B. distachyon*, además de analizar qué conjunto de caracteres foliares se relacionan con la resistencia y tolerancia al daño por herbívoros. En particular, se analiza a nivel inter-específico e inter-poblacional la variación de caracteres funcionales constitutivos (sílice y SLA) y caracteres modulables (razón C:N y contenido de agua) típicamente relacionados con la resistencia y tolerancia a la herbivoría, hallándose que la herbivoría tiene un impacto significativo sobre la fitness en las especies del complejo *Brachypodium distachyon* y que la condición de poliploidía no mejora la resistencia y tolerancia a la herbívoría en dicho complejo.

El conjunto de estudios de esta tesis y su enfoque ha dado lugar a un incremento en el conocimiento del complejo *Brachypodium distachyon* desde una perspectiva ecológica-funcional. En general, esta tesis realiza una importante contribución al conocimiento de los factores implicados en el origen y control de la variación de la respuesta funcional a la sequía y a la herbivoría en las especies del complejo *B. distachyon*, y al entendimiento de cómo influyen dichos factores en la divergencia de estas especies y en la estructura genética de las poblaciones. Por otra parte, los resultados derivados de esta investigación contribuyen a la identificación de genes clave relacionados con la tolerancia a la sequía en cereales, dotando a este trabajo de un valor añadido importante en la mejora genética de plantas.

SUMMARY

Abiotic and biotic stresses affect crops worldwide causing significant losses in yield and crop quality. Among abiotic stresses, water stress is one of the most important and is also considered as a main determinant of the local adaptation of plants to current and/or future conditions forecasted by climate change. On the other hand, among the biotic stresses, insect herbivory causes important pests in managed and natural vegetation being also an important force for the selection of plant traits. Therefore, the research focused on the study of control mechanisms for tolerance and resistance to these different types of stress has a great scientific, social and economic importance.

The *Brachypodium distachyon* complex has emerged as a grass and monocot C3 model of temperate zones in the last decade. The large number of genetic and molecular resources available, together with its biological characteristics (simplicity of genomes, wide circummediterranean native distribution and close relationship with cereals such as wheat, barley or oats among others), make it an ideal model to approach the study of numerous questions of evolutionary biology and functional genomics of temperate cereals and grasses that are used as biofuels. The distribution of its natural populations in a wide variety of environments, gives researchers the opportunity to use their genetic diversity to study the adaptation features of plants to local climates and abiotic stresses and make it a useful model to study the genetics and genomics of the tolerance and resistance stress responses of cereals and bioenergetic grasses. In addition, the three species that form this complex show a variation in ploidy: the two parental diploids *B. distachyon* ($2n = 2x = 10$, $x = 5$) and *B. stacei* ($2n = 2x = 20$, $x = 10$), and their derived alotretraploid *B. hybridum* ($2n = 4x = 30$, $x = 10 + 5$) with a hybrid nature. This converts the complex in a model to investigate the evolution of (allo) polyploidy

due to the extensive knowledge of comparative functional genomics of the three species, thus expanding its potential for application to polyploid wheats.

This PhD Thesis is focused on the study of the functional response mediated by ploidy and ancestral hybridization (i.e., alopoliploidy), as well as the genetic basis underlying the natural variation of water stress tolerance, among individuals belonging to different natural populations of the three species from the *Brachypodium distachyon* complex. The plant material used in these investigations has been obtained from the *Brachypodium* germplasm bank of the University of JAEN, which is located in the Department of Animal Biology, Plant Biology and Ecology of the University of Jaén. The seeds of this germplasm bank were collected from 57 natural populations in the Iberian Peninsula during field sampling in 2007 and 2008 by two of the supervisors of this work Antonio J. Manzaneda and Pedro J. Rey, which has allowed us to have a large population sampling within the range of distribution of each species.

In this way, **Chapter 1** analyses the inter-specific and intra-specific variation in functional traits related to the drought response (water content, specific leaf area, cell damage, proline and phytohormones contents) in a large number of natural populations of *B. distachyon*, *B. stacei* and *B. hybridum*, finding differences between the three species (specifically the functional response to the drought of *B. hybridum* and *B. stacei* was correlated and differentiated from that of *B. distachyon*). In addition, we included differential analysis of these traits between ten *B. hybridum* populations genetically differentiated of and the relationship of this phenotypic and genetic differentiation with aridity. The results of this study suggest that *B. hybridum* populations have adaptively differentiated at physiological and genetic level in response to variations in aridity during the last million years, after the polyploidization process.

The work of **Chapter 2** corresponds to the first comparative transcriptomic analysis carried out among the three species of the *B. distachyon* complex in the context of drought response and parental legacy. Our results show a higher response in the *B. hybridum* allopolyploid compared to its two ancestors *B. distachyon* and *B. stacei* at overall gene expression level and a significant variation of this response between different *B. hybridum* genotypes in response to drought. They highlight the strong induction of LEA proteins (from Late Embryogenesis Abundant Proteins) such as dehydrins and other heat shock proteins that protect plants from drought. Regarding the parental legacy in response to drought, most pairs of homoeolog genes in *B. hybridum* showed a statistically similar expression pattern to *B. distachyon* and a transgressive expression pattern, contrary to the results found at the functional level in the previous chapter. We also found that the majority of homoeologous pairs expressed in *B. hybridum* maintained the paternal expression, 21% of expressed genes in response to the drought modified their expression with respect to their expression in the ancestors.

To achieve a deeper view of the role of dehydrins, **Chapter 3** consisted in the generation of a transgenic line of *Brachypodium distachyon* that overexpress the dehydrin3 coding gene (BdDHN3), which appeared strongly induced in the transcriptomic analyzes performed in Chapter 2 in the three species of the *Brachypodium* complex. The transgenic line was analyzed, at transcriptomic and physiological level, in drought stress conditions, finding that an overexpression of this gene in this line was significantly correlated with leaf traits that enhance drought tolerance such as an increased in water and proline content and lower cell damage.

Finally, **Chapter 4** shows the first analysis of the variation in the response to herbivory by insects in a high number of diploid and allotetraploid natural populations of *B. distachyon* complex, in addition to analyzing which set of leave traits are related to resistance and tolerance to damage by herbivores. In particular, the variation of constitutive functional traits (silica and SLA) and regulable traits (ratio C: N and water content) typically related to herbivory resistance and tolerance, were analyzed at inter-specific and inter-population level. The results showed that herbivory has a significant impact on fitness in the species of the *Brachypodium distachyon* complex and that the condition of polyploidy does not improve the resistance and tolerance to herbivory in this complex.

In summary, the compilation of studies conduced in this thesis increases the knowledge on the ecological-functional perspective of the *Brachypodium distachyon* complex. In general, our results contribute importantly to the knowledge of the factors involved in the origin and control of variation in the functional response to drought and herbivory in the species of the *B. distachyon* complex and the understanding of how these factors influence on the divergence of these species and the genetic structure of the populations. On the other hand, the results derived from this research contribute to the identification of key genes related to drought tolerance in cereals, giving to this work an important added value in terms of genetic plant improvement.

INTRODUCCIÓN

INTRODUCCIÓN GENERAL

Estreses bióticos y abióticos en plantas

Las plantas en la naturaleza están expuestas a una serie de factores ambientales estresantes que les impiden alcanzar su total potencial genético afectando así a su crecimiento, desarrollo y productividad (Bhatla *et al.*, 2018). Según los factores ambientales que originen el estrés, se puede hablar de estrés abiótico y biótico. El estrés abiótico se puede originar a partir de una alteración de las condiciones climáticas o ambientales físicas (sequía, salinidad, temperatura extrema, irradiación, anaerobiosis y estrés mecánico) o químicas (salinidad, carencia de minerales y contaminantes ambientales) que conducen a desencadenar respuestas de diversa naturaleza en la planta. Por otro lado, el estrés biótico está causado por interacciones directas o indirectas con organismos vivos como pueden ser virus, bacterias, hongos, nematodos, insectos, arácnidos y malas hierbas (Bhatla *et al.*, 2018).

Las plantas responden al estrés con un amplio abanico de cambios a nivel metabólico y a nivel de expresión génica para equilibrar su tasa de crecimiento y desarrollo. Dos importantes formas de respuesta al estrés son la aclimatación y la adaptación fisiológica. La aclimatación fisiológica es un ajuste morfológico y reversible a nivel de individuo que no conlleva a cambios genéticos y que se produce en una escala de tiempo pequeña (días o meses). Esta respuesta consigue recuperar en parte la tasa fisiológica de la planta inicialmente disminuida por el factor estresante. Sin embargo, la adaptación fisiológica es una respuesta de tipo evolutivo a nivel de población, es un ajuste irreversible y conlleva cambios genéticos fijados por la selección natural. Se da a una escala de tiempo muy larga y conlleva que las plantas sobrevivan en condiciones de estrés con tasas fisiológicas similares a las que se podrían encontrar en ausencia del factor estresante (Bhatla *et al.*, 2018).

Las plantas han desarrollado una gran variedad de adaptaciones fisiológicas para ocupar casi todo tipo de hábitats. Sin embargo, una misma planta no presenta el mismo éxito en cualquier hábitat. Son los diferentes tipos de estreses abióticos (e.g., hídrico, frio-congelación, salinidad, etc.) y bióticos (pátogenos, herbivoría, competencia, etc.) los que imponen una presión selectiva a las poblaciones de plantas dando lugar a una adaptación local de las plantas a ese hábitat para poder persistir. Muchos son los estudios que se centran en estudiar tanto los diferentes estreses abióticos y bióticos como los compromisos (*trade-offs*) que ocurren en respuesta a éstos en las plantas (VanWallendael *et al.*, 2019).

Estrés hídrico por sequía

El estrés hídrico es uno de los principales estreses abióticos a los que están expuestas las plantas y ocurre cuando la tasa de transpiración en la superficie de la hoja es mayor que el agua consumida por la raíz debido a que el potencial hídrico en el suelo es menor que el de la raíz (Salehi-lisar *et al.*, 2012). La sequía es la causa más frecuente de dicho estrés y tiene un gran impacto en las plantas a nivel mundial especialmente en hábitats áridos y semiáridos (Madhava Rao *et al.*, 2006). La situación de sequía se ve principalmente afectada por el cambio climático ya que multiplica la frecuencia, la duración y la severidad del estrés hídrico (Mishra & Singh, 2011). Además, existen otras condiciones atmosféricas que incrementan la evaporación del agua del suelo y con ello la sequía, como son las altas temperaturas, la alta intensidad de luz y el viento. También podemos hablar de sequía aunque no haya un déficit hídrico ambiental en casos, por ejemplo, donde hay agua disponible en el suelo pero factores como la salinidad, temperaturas bajas en el suelo o inundaciones alteran la absorción de agua por la raíz dando lugar a un estrés hídrico en las plantas (Salehi-lisar *et al.*, 2012; Arbona *et al.*, 2013).

Mecanismos de respuesta al estrés por sequía en plantas

La respuesta de una misma especie a la sequía puede variar dependiendo del tipo de sequía (moderada o severa, recurrente o puntal), así como del momento y la rapidez con que se establecen dichas respuestas. Por ello, las adaptaciones que presentan las plantas al estrés por sequía son múltiples y pueden ser divididas en tres tipos de estrategias que pueden emplear de forma separada o en combinación: el escape a la desecación, evitación de la deshidratación (resistencia a la sequía) o tolerancia a la deshidratación (Basu *et al.*, 2016) (Figura 1). El *escape* a la desecación involucra tanto caracteres constitutivos (que permiten que las especies sobrevivan en ambientes en los que persiste el estrés) como caracteres inducidos (que se expresan en respuesta a dicho estrés) que permiten que las plantas completen su ciclo vital antes de que comience el periodo de sequía (Franks, 2011; Juenger, 2013). Sin embargo, *la evitación o resistencia* a la sequía implica el mantenimiento del contenido hídrico en la planta aunque se reduzca la disponibilidad de agua en el suelo. Dicho estatus hídrico en condiciones de sequía se mantiene a través del desarrollo de caracteres morfológicos y funcionales constitutivos que minimizan las pérdidas de agua (mecanismos por ahorro de agua) y que maximizan la absorción y/o acumulación de agua en plena sequía siempre que el acceso al agua del suelo y su distribución interna por el xilema no sea limitante (mecanismos por derroche de agua). Entre dichos caracteres se encuentran: sistemas radiculares más profundos y desarrollados, elevada conductancia hidráulica, presencia de cutículas y pelos en las hojas, cierre de estomas rápido y sensible a ligeros descensos del contenido hídrico de los tejidos o al potencial hídrico, valores en general bajos de conductancia estomática, paredes celulares poco elásticas que inducen cambios rápidos de potencial hídrico en respuesta a pequeñas pérdidas de agua, hojas pequeñas, bajas tasas de transpiración, etc. (Fig. 1). Por último, la *tolerancia* a la sequía engloba un rango de adaptaciones y respuestas en las plantas que mitigan el impacto del daño de la desecación y les permite recuperarse tras una deshidratación severa una vez que el agua está de nuevo disponible (Morgan, 1984; Juenger, 2013). Esta última estrategia, implica ajustes osmóticos, acumulación de solutos compatibles, inducción del sistema antioxidante, alteraciones de rutas metabólicas, incremento de las raíces, cierre de estomas, modificaciones en membranas y paredes celulares (Chen *et al.*, 2011).

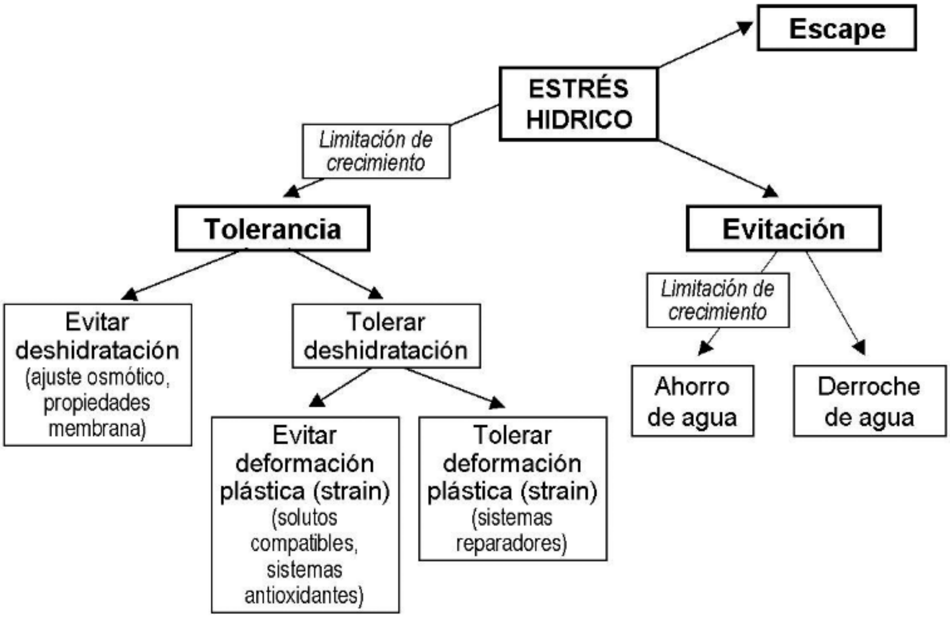

Fig. 1. Las tres posibles estrategias ante el estrés hídrico: escapar, evitarlo o tolerarlo (extraído de Valladares el al., 2004).

Las tres estrategias de supervivencia en condiciones de escasez de agua implican *trade-offs* clave entre los rasgos complejos que permiten el desarrollo de dichas estrategias, con efectos negativos en la disminución de la tasa de crecimiento y en la productividad en plantas de regiones áridas (Knapp & Smith, 2001; Ciais *et al.*, 2005; VanWallendael *et al.*, 2019). Por ello, el estrés hídrico es considerado como uno de los principales factores ecológicos que actúan como filtro selectivo de especies y genotipos según su tolerancia a este, tanto en poblaciones naturales como en sistemas agrícolas, limitando la distribución y abundancia de las plantas y promoviendo la adaptación local, conduciendo así a la evolución y diversificación (McKay *et al.*, 2003; Knight *et al.*, 2006; Engelbrecht *et al.*, 2007; VanWallendael *et al.*, 2019). Además, afecta negativamente a la reproducción y altera los ritmos de producción de hojas, tallos, flores y frutos. La repetición cada vez más frecuente de los episodios de sequía puede acelerar también los cambios fenológicos a nivel de población (Valladares *et al.*, 2004).

Desde un punto de vista socioeconómico, el estrés por sequía es la causa más importante de disminución del rendimiento de cosechas y su productividad a escala mundial (Bot *et al.*, 2000; Lesk *et al.*, 2016; Matiu *et al.*, 2017). Una de las familias con mayor importancia socioeconómica afectadas por la sequía es la familia de las gramíneas (Poaceae). Globalmente, se han estimado pérdidas en cereales de 1820 millones de toneladas en las pasadas 4 décadas (Lesk *et al.*, 2016). Por lo que la investigación encaminada a obtener un mejor conocimiento de los mecanismos de control de la tolerancia y resistencia a la sequía en las plantas y su significado ecológico y evolutivo tiene una importancia fundamental, no solo desde un punto de vista científico sino también social y económico.

Mecanismos de tolerancia al estrés por sequía

Las plantas tolerantes a la sequía inician diferentes mecanismos morfológicos, fisiológicos y moleculares que atenúan los efectos negativos de la sequía sobre el crecimiento (Hossain *et al.*, 2016).

Entre los mecanismos morfológicos se encuentran los cambios morfológicos por plasticidad fenotípica mediante los cuales las plantas reducen el número y el tamaño de las hojas para reducir el consumo de agua y mejoran el sistema radicular, de forma que las raíces se hacen más profundas y delgadas en respuesta al estrés por sequía para poder absorber agua de las profundidades (Hossain *et al.*, 2016).

Como mecanismo fisiológico clave se encuentra el ajuste osmótico que reduce el potencial osmótico de las células mediante cambios en la elasticidad de la pared o adaptaciones de la presión osmótica para incrementar el potencial hídrico y la turgencia de las células que se ven reducidos en condiciones de deshidratación y así poder llevar a cabo las funciones de expansión y crecimiento (Zare *et al.*, 2011; Bhargava *et al.*, 2013). La acumulación de solutos compatibles (osmolitos) en el citoplasma como la prolina, sacarosa y carbohidratos solubles conlleva la reducción del potencial osmótico atrayendo el agua desde el suelo a las células de la raíz. La prolina es uno de los osmolitos más importantes involucrado en la estabilización de la membrana. Además de osmoprotector, es muy importante en el balance de la energía entre cloroplastos y mitocondrias (Salehi-lisar *et al.*, 2012), también actúa como chaperona preservando la estructura de otras proteínas y la integridad de las membranas (Verbruggen & Hermans, 2008). Cabe destacar también como mecanismos fisiológicos la estabilidad de la membrana, mediante el mantenimiento de la integridad y estabilidad de sus proteínas, y la síntesis y acumulación de fitohormonas reguladoras de los procesos fisiológicos como el ácido abscísico (ABA), auxinas, citoquininas, etileno y giberelinas, que actúan como moléculas señal en condiciones de sequía. El ABA se sintetiza tras las exposición a la sequía y se conoce como hormona del estrés ya que induce el cierre de los estomas (Bernacchia & Furini, 2004; Bray, 2007; Akhtar & Nazir, 2013) y también la expresión de genes involucrados en procesos de adaptación de la sequía (Bernacchia & Furini, 2004; Bhargava *et al.*, 2013; Ding *et al.*, 2013; Nezhadahmadi *et al.*, 2013).

La tolerancia a la sequía es un fenómeno complejo que también comprende mecanismos de adaptación moleculares. Involucra una compleja ruta de transducción de señales (Fig. 2) que comienza activándose mediante la interacción de moléculas señal con receptores que se encuentran en la membrana de las células. De esta forma se generan mensajeros secundarios como las ROS (especies reactivas del oxígeno) entre otros, que causan además daños oxidativos en las células, e inositolfosfatos (IP) que desencadenan una alteración en los niveles de Ca^{2+} intracelular. Esta cascada de señalización finalmente actúa en la regulación de genes diana que se inducen con el estrés. Los productos de estos genes se clasifican en dos grupos: el primero es el grupo de proteínas directamente involucradas en la tolerancia al estrés como proteínas canal, proteínas abundantes en la embriogénesis tardía (LEA), proteínas de protección celular como proteasas,

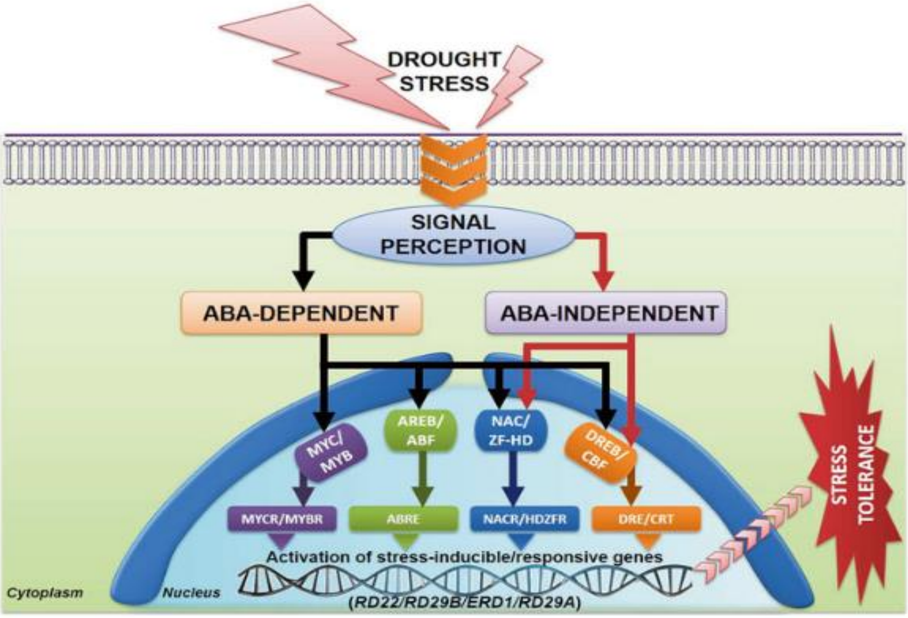

Fig. 2. Representación esquemática de los mecanismos de tolerancia al estrés a la sequía en plantas mediados por varios factores de transcripción dependientes de ABA (AREB/ABC, MYC/MYB y NAC) e independientes de ABA (CBF/DREB) [Extraído de Lata et al. 2015].

enzimas requeridas para la síntesis de componentes osmóticos compatibles (osmoprotectores) y enzimas involucradas en el sistema antioxidante que se encarga de la detoxificación de las ROS (Bray, 2007; Dai, 2011; Arbona *et al.*, 2013). El segundo grupo son la proteínas reguladoras de la señal intracelular como quinasas, fosfatasas, fitohormonas y factores de transcripción (TFs) que regulan a su vez la expresión de otros genes (Lata *et al.*, 2015).

Estrés por herbivoría

La herbivoría es un estrés biótico clave en el ecosistema. Al contrario que los estreses abióticos, los factores estresantes desencadenantes son organismos vivos que evolucionan en respuesta a la adaptación de las plantas (Ehrlich & Raven, 2016; Endara *et al.*, 2017). Los herbívoros se alimentan de diferentes partes de la planta, afectando a la producción primaria, a la translocación de los productos de la fotosíntesis y a la acumulación de productos de defensa (Rehman *et al.*, 2010). La relación planta-herbívoro es una relación antagonista donde el consumidor herbívoro se beneficia de los nutrientes de la planta mientras que ésta muestra una reducción en el fitness debido a la pérdida de tejido fotosintético y reproductivo (Strauss & Zangerl, 2002). Dentro de los herbívoros, los insectos son los más importantes y dependen de las plantas para alimentarse y refugiarse.

Mecanismos de respuesta al estrés por herbivoría en plantas

Tanto plantas como insectos desarrollan estrategias para evitar los sistemas de defensa entre ambos. Por ello, las adaptaciones que presentan las plantas al estrés por herbivoría son múltiples y se clasifican como estrategias de resistencia y de tolerancia (Fig. 3). Ambos tipos de estrategias pueden coexistir en una misma planta o bien aparecer independientemente en algunas especies.

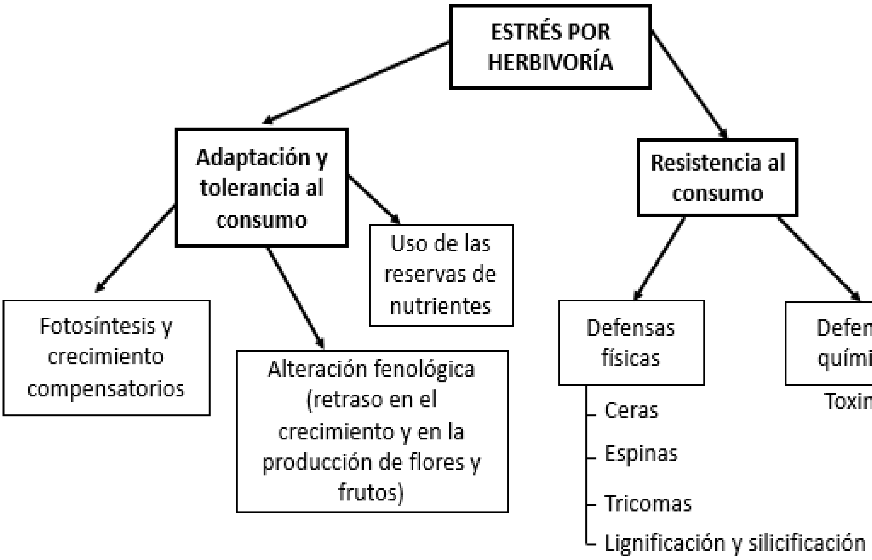

Fig. 3. Estrategias de las plantas frente a los herbívoros (figura construida a partir de información extraída de Mitchell *et al.*, 2016).

Dentro de la estrategia de resistencia a la herbivoría se encuentra el desarrollo de caracteres defensivos que impiden que se produzca el daño o que se produzca en menor cantidad. Por un lado se encuentran las defensas físicas que constituyen la primera barrera frente a los insectos como la presencia de ceras en la cutícula, espinas o tricomas, lignificación en la pared celular o silicificación de los tejidos que confieren dureza a las hojas reduciendo la palatabilidad y la digestibilidad de estas por los insectos. La silicificación es la defensa física más importante en el caso de las gramíneas ya que embastece el pasto y acelera el desgaste dental de los insectos. Por otro lado, se encuentran las defensas químicas, o síntensis *de novo* de metabolitos secundarios, como las toxinas y otros aleloquímicos, que previenen subsecuentes ataques por los insectos. Entre ellos se encuentran los alcaloides, proteínas tóxicas y/o de sabor desagradable, la sílice también se puede considerar una defensa química ya que es no digerible, glucosinolatos, terpenos, etc. (War *et al.*, 2012). Por otro lado la estrategia de tolerancia está basada en la adquisición de caracteres que reducen el efecto negativo del daño y mantienen el fitness tras el daño (Simms & Triplett, 1994; Mitchell *et al.*, 2016).

Al igual que ocurre en el estrés hídrico, se han documentado en varias especies de plantas *trade-offs* entre las estrategias de defensa y la tasa de crecimiento (ver VanWallendael *et al.*, 2019 y referencias). Sin embargo, hay teorías que predicen que las plantas mitigan los *trade-offs* crecimiento-defensa a través de una regulación dinámica entre el medio ambiente y la genética (Hahn & Maron, 2016). Por ello, el estrés por herbivoría también puede ser considerado como un factor ecológico que actúa como filtro selectivo de especies según su tolerancia o resistencia a la herbivoría, tanto en poblaciones naturales como en sistemas agrícolas, limitando la distribución y abundancia de las plantas y promoviendo la adaptación local (Hanley *et al.*, 2007). También hay estudios que revelan que dicha adaptación local se debe a las presiones diferenciales que ejercen los herbívoros a lo largo de un espacio (Stam *et al.*, 2017).

Además la herbivoría también tiene un impacto importante en la productividad vegetal, siendo la responsable de su disminución en un 20% tanto en sistemas naturales como en cultivos (Agrawal, 2011). En cultivos, los daños causados por plagas de artrópodos exceden el 15% anualmente (Mitchell *et al.*, 2016). Por ello, la identificación de caracteres defensivos de las plantas y su inducción es esencial para mejorar la sostenibilidad de la protección de los cultivos (Mitchell *et al.*, 2016).

Mecanismos de tolerancia al estrés por herbivoría

Las plantas responden a la herbivoría a través de mecanismos morfológicos, bioquímicos y moleculares para compensar los efectos del ataque. La expresión de caracteres antes y después del daño puede conferir tolerancia a la herbivoría

(Fornoni, 2011). Estos caracteres están agrupados en aquellos que modifican: (1) los procesos fisiológicos como la actividad fotosintética y el crecimiento, (2) la fenología y (3) el uso de las reservas de nutrientes (Strauss & Agrawal, 1999; Stowe *et al.*, 2000; Tiffin, 2000).

Entre los caracteres que modifican los procesos de fotosíntesis y crecimiento se encuentra la fotosíntesis compensatoria que proporciona tolerancia a la planta tras una defoliación parcial producida por un insecto haciendo que en los tejidos restantes de la planta se incremente la tasa de fotosíntesis (Strauss & Agrawal, 1999; Retuerto *et al.*, 2004). El vigor en las planta también proporciona tolerancia a la herbivoría en algunas especies (Price, 1991) permitiendo la presencia de un alto número de insectos sin afectar a su supervivencia. Asimismo, están los mecanismos de crecimiento compensatorio como la activación del crecimiento brotes latentes para restaurar los tejidos tras el daño o eliminación de flores o meristemos vegetativos (Tiffin, 2000). Estos mecanismos están muy extendidos en gramíneas que contienen meristemos cerca del suelo protegidos por las hojas basales proporcionando una importante estrategia frente a la herbivoría y un éxito evolutivo.

Dentro de los caracteres que alteran la fenología, el retraso en el crecimiento y en la producción de flores y frutos tras el daño por herbívoros podría promover la tolerancia a los herbívoros al posponer el desarrollo de la planta hasta que la amenaza de ataque haya pasado (Tiffin, 2000).

Por último, como caracteres que alteran el uso de reservas de nutrientes están los órganos de almacenaje que son importantes para la recuperación de la planta tras el daño y ofrecen una estrategia efectiva frente a un posible ataque siempre que no afecten a la productividad de la planta (Strauss & Agrawal, 1999).

Poliploidía

La poliploidía en plantas, o duplicación del genoma completo, es un fenómeno que generalmente da lugar a una especiación instantánea incrementando la biodiversidad y proporcionando un nuevo material genético sobre el cual la evolución puede trabajar (Levin, 1983, 2002). Dicha especiación instantánea se produce debido a que las alteraciones del genoma, que tienen lugar en una sola generación durante el proceso de poliploidización, establecen rápidamente unas barreras entre las antiguas especies parentales y las nuevas especies poliploides derivadas de estas que conducen a un aislamiento reproductivo (Ramsey & Schemske, 1998). Los nuevos poliploides contienen copias duplicadas de cada gen (en el caso de los alopoliploides copias presentes en cada una de las dos especies parentales). A través del tiempo, esos genes duplicados en el poliploide pueden mantener la función que tenían en las especies parentales o sufrir una divergencia funcional. Por ejemplo, una de las dos copias puede ser silenciada o pueden diversificar su función y expresión. Una copia se puede perder por deleción o también puede haber una interacción entre los genes duplicados vía recombinación o conversión génica. Esta divergencia funcional de los genes duplicados puede ser una ventaja selectiva a largo plazo.

De acuerdo a su origen, los organismos poliploides pueden clasificarse en dos tipos, autopoliploides y alopoliploides (Fig. 4). Los autopoliploides exhiben una pequeña divergencia citogenética y morfológica de los progenitores diploides y poseen más de dos copias de cada cromosoma (cuatro en el caso de autotetraploides). Por el contrario, los alopoliploides se originan por hibridación y duplicación cromosómica entre dos especies relacionadas, por lo tanto tienen dos genomas parentales divergentes (Stebbins, 1947; Grant, 1975; Lewis, 1980). Un alotetraploide tiene los dos de cada par de cromosomas homólogos derivados de dos especies diferentes: estos se denominan cromosomas homeólogos. Los autotetraploides se forman por duplicación del genoma de un mismo individuo o más probablemente por hibridación entre individuos de la misma especie y duplicación del genoma.

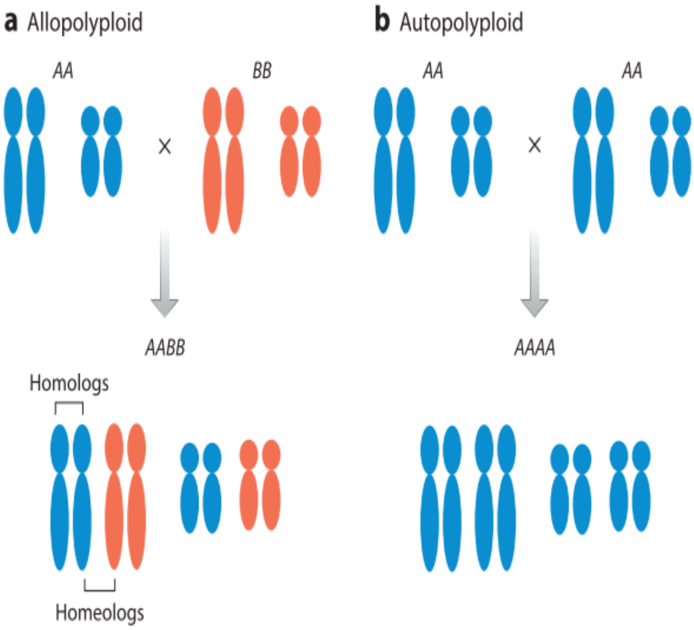

Fig. 4. Dos tipos de poliploides. (a) Los alopoliploides se originan por hibridación entre dos especies parentales diferentes relacionadas y duplicación cromosómica. (b) Los autopoliploides se forman por duplicación cromosómica entre individuos parentales de la misma especie. Por ello, un alotetraploide contiene cromosomas homólogos y homeólogos, donde cada cromosoma del par considerado homeólogo deriva de dos especies parentales diferentes (extraído de Yoo et al., 2014).

Contribución evolutiva de la alopoliploidía

La contribución evolutiva de la alopoliploidía se basa en las ventajas que pueden otorgar dichas alteraciones del genoma a los poliploides con respecto a sus diploides parentales y que pueden llevar a una mejora de los caracteres fisiológicos y con ello incrementar su habilidad de persistir a través del tiempo. Entre las ventajas encontramos que el incremento del número de alelos para un gen determinado en un alopoliploide proporciona una protección frente a alelos recesivos deletéreos y mutaciones recesivas que afectan al *fitness* (Comai, 2005). Otra ventaja sería que la heterosis dada por el genoma híbrido de los alopoliploides les permite conducta transgresiva comparada con las de sus especies parentales (Birchler *et al.*, 2010). La tercera gran ventaja sería que los genes duplicados en el alopoliploide pueden evolucionar y asumir funciones nuevas o ligeramente variadas (neofuncionalización o subfuncionalización) que pueden permitir la expansión del nicho ecológico o incrementar la respuesta funcional de los poliploides a un amplio espectro de condiciones ambientales estresantes, (i.e., funciones que podrían ser evolutivamente adaptativas (Adams & Wendel, 2005; Moore & Purugganan, 2005; Lynch, 2007).

Cambios genómicos y transcriptómicos derivados de la alopoliploidía

Cuando dos genomas parentales están presentes en el núcleo del alopoliploide, el nivel de expresión entre los genes duplicados puede mostrar una desviación de la denominada aditividad parental (considerada como la media aritmética del nivel de expresión de los genes parentales). La aditividad puede considerarse como la conservación de la expresión de los genes parentales o legado parental (Buggs *et al.*, 2014). Sin embargo hay genes que se alejan de este patrón de aditividad y presentan, por el contrario, un patrón de expresión no aditiva que puede derivar de tres posibles escenarios (Fig. 5): (1) que el nivel de expresión total del par de genes homeólogos en el alopoliploide sea similar al nivel de expresión hallado en solo uno de sus parentales (en inglés *expression level dominance,* siglas ELD*)*; (2) que el nivel de expresión total en el alopoliploide sea menor o mayor que el nivel hallado en ambos parentales (*transgressive expression*); y (3) cuando un homeólogo se exprese preferencialmente con respecto al otro homeólogo contribuyendo de forma desigual al nivel de expresión total en el alopoliploide (*homeolog expression bias*) (Grover *et al.*, 2012; Yoo *et al.*, 2014).

La expresión no aditiva es muy importante en términos ecológicos ya que puede incrementar el potencial de plasticidad fenotípica en los alopoliploides recién formados (Doyle *et al.*, 2008; Jackson & Chen, 2010), y esto puede resultar en una mejora de la resistencia al estrés y de la capacidad adaptativa al estrés con respecto a sus especies parentales (Sattler *et al.*, 2016; Shimizu-Inatsugi *et al.*, 2017). Se tiene el conocimiento de que las condiciones de estrés puede alterar la expresión de los genes homeólogos en el alopoliploide (Liu & Adams, 2007; Dong & Adams, 2011; Shimizu-Inatsugi *et al.*, 2017). Sin embargo se sabe muy poco acerca de los efectos que tienen los diferentes estreses ambientales sobre los patrones de expresión no aditiva. Por ejemplo, en un estudio comparativo del transcriptoma del alopoliploide natural *Coffea arabica* con sus especies ancestrales mostró un patrón de ELD que dependió de las condiciones de temperatura de crecimiento (Bardil *et al.*, 2011). En otro estudio comparativo entre el alotetraploide *Arabidopsis kamchatica* y sus especies ancestrales demostró una expresión de los homoeologos bias relacionada con la adaptación a ambientes de metales pesados que fue heredada de las especies ancestrales que eran tolerantes al estrés por metales pesados (Paape *et al.*, 2016).

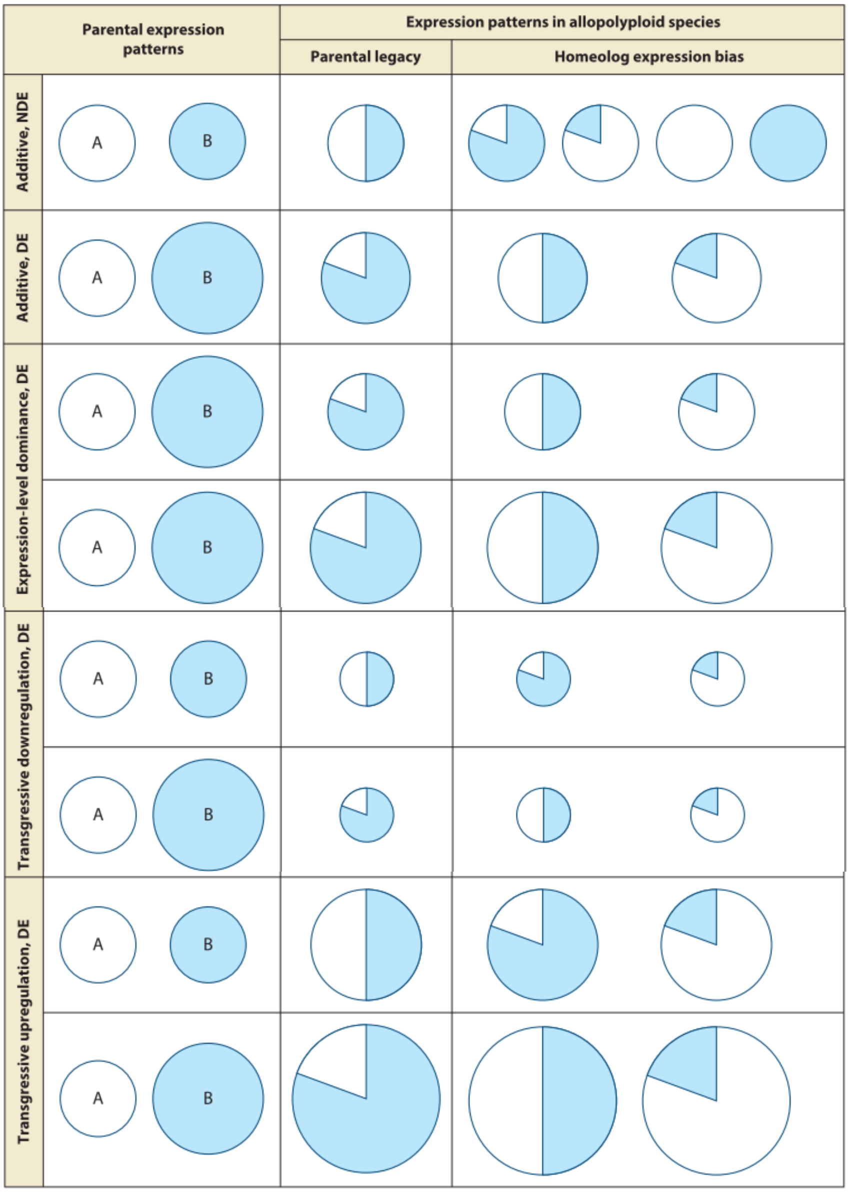

Fig. 5. Patrones de expresión no aditiva en alopoliploides. Considerando el nivel de expression total de los homeólogos, la expresión puede ser aditiva para genes expresados de forma no diferencial (NDE) o diferencial (DE) entre el alopoliploide y sus parentales diploides. La expresión no aditiva puede ser clasificada en expression-level dominance, donde la expresión total de los homeólogos se asemeja a uno de los parentales, y expresión transgresiva donde los genes se regulan disminuyendo o aumentando en relación con los parentales diploides. Para la expresión relativa de los homeólogos, los dos homeólogos en un alopoliploide pueden heredar las diferencias de expresión persistentes en los parentales (legado parental) o un homeólogo puede ser preferencialmente expresado comparando con la expresión del otro homeólogo, esto es conocido como homeolog expression bias. La pérdida de un homeólogo es el caso extremo de homeolog expression bias, mostrado en la primera fila, aunque los mecanismos de expresión reducida son claramente diferentes (extraído de Yoo et al. 2014).

Recientes estudios moleculares proporcionan evidencias de cambios transcripcionales y genómicos en numerosos niveles de regulación que acompañan al proceso de aloploidización, sin embargo en muchos casos no se sabe el efecto que tiene la poliploidía en el *fitness* bajo diferentes condiciones ambientales. Hay pocas evidencias de que los cambios transcripcionales y genómicos observados realmente conduzcan a una mayor adaptación en las poblaciones naturales (ver Madlung, 2013 y referencias). Se necesitan más estudios que integren enfoques moleculares y ecológicos para evaluar el papel de genes específicos en la adaptación en condiciones controladas o en condiciones de campo.

El complejo de especies Brachypodium distachyon

Brachypodium: planta modelo para la investigación en gramíneas

El género *Brachypodium* pertenece a la familia de las gramíneas (Poaceae) donde se incluyen algunas subfamilias que tienen una gran importancia económica ya que en ellas se encuentran cultivos para la alimentación y obtención de combustibles (Opanowicz *et al.*, 2008; Vogel *et al.*, 2010). Entre las subfamilias más ampliamente representadas se encuentran Pooideae (*Triticum aestivum*, *T. turgidum*, trigos; *Hordeum vulgare*, cebada); Panicoideae (*Zea mays*, maíz; *Sorghum bicolor*, sorgo; *Pennisetum glaucum*, *Panicum miliaceum*, *Setaria italica*, mijos) y Ehrhartoideae (*Oryza sativa*, arroz). A día de hoy se encuentran secuenciados los genomas de arroz, maíz y sorgo que se utilizan para la obtención de nuevas variedades con una mejor adaptación a estreses ambientales y una mayor productividad (Kumar *et al.*, 2009; Vogel *et al.*, 2010). Sin embargo, para la mayoría de los genomas de las especies de gramíneas pascícolas templadas, entre las que se incluyen algunas subfamilias de importancia económica como Triticeae, (Poeae-Aveneae), hay una ausencia de recursos de genoma útiles debido a la complejidad de sus genomas (Febrer *et al.*, 2010). La propuesta de la especie

Brachypodium distachyon como modelo funcional de cereales templados y de gramíneas biocombustibles (Vogel *et al.*, 2010; Mur *et al.*, 2011) tuvo una gran aceptación debido a características como ser una planta anual de ciclo de vida corto, ser de fácil propagación y tener un genoma pequeño y compacto (Vogel *et al.*, 2010). Además por ser evolutivamente más próxima a los cereales de clima templado (Triticeae) que *Oryza sativa*, la gramínea modelo subtropical (Catalán *et al.*, 1997; Opanowicz *et al.*, 2008; Mur *et al.*, 2011), facilitaba los estudios genómicos comparativos de *B. distachyon* con trigos, cebadas y otras especies pascícolas y forrajeras templadas (Vogel *et al.*, 2010). Durante más de un siglo *B. distachyon* (Palisot de Beauvois, 1812) fue considerada como la especie representativa del género *Brachypodium* (Schippmann, 1991) y durante más de tres décadas se consideró que había tres citotipos con diferente número de cromosomas 2n = 10, 20 y 30. Recientemente la taxonomía y la separación fenotípica, citogenética y molecular de estos tres citotipos han demostrado que corresponden a tres especies diferentes: dos diploides, *B. distachyon* (2n=2x=10, x=5) y *B. stacei* (2n=2x=20, x=10), y su derivada alotretraploide *B. hybridum* (2n=4x=30, x=10+5) (Catalán *et al.*, 2012)[Fig. 6]. Recientes estudios filogenéticos de las tres especies del complejo *B. distachyon* indican que la especie parental más ancestral, *B. stacei* originada hace 10Ma, y la más recientemente evolucionada, *B. distachyon* originada hace 7Ma, emergieron de dos linajes independientes, confirmando sus contribuciones respectivas como donadores en genomas de la especie alotetraploide *B. hybridum* originada hace 1Ma (Catalán *et al.*, 2012). Análisis de herencia maternal de genes plastídicos demuestran que el alotetraploide *B. hybridum* es el resultado de cruces bidireccionales entre las especies parentales *B. distachyon* y *B. stacei* actuando tanto como progenitores maternos como paternos (López-Alvarez *et al.*, 2012). Estos autores también muestran que la mayoría de las poblaciones circunmediterráneas de *B. hybridum* estudiadas derivan del parental *B. stacei* como progenitor materno, mientras que solo unas pocas poblaciones del mediterráneo occidental derivan del parental *B. distachyon* como progenitor materno.

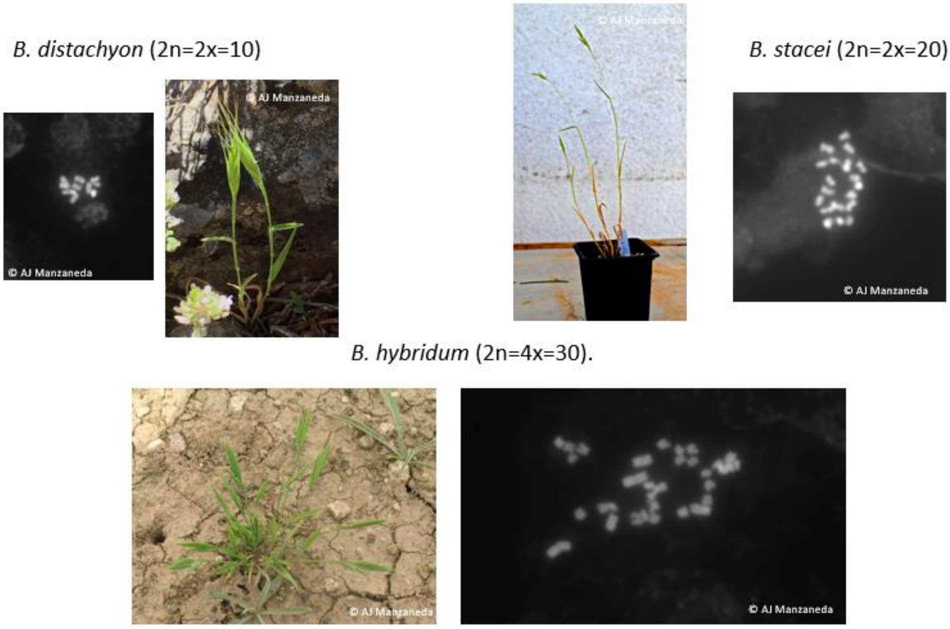

Fig. 6. Complejo de especies *Brachypodium distachyon*. El complejo comprende tres especies diferentes: dos diploides, *B. distachyon* (2n=2x=10, x=5) y *B. stacei* (2n=2x=20, x=10), y su derivada alotetraploide *B. hybridum* (2n=4x=30, x=10+5). [Fotografías de Antonio J. Manzaneda].

Los atributos biológicos y genómicos que hacen que *B. distachyon* sea un modelo de gramíneas óptimo (pequeño tamaño, ciclo de vida corto, se autofecunda, genoma pequeño, poco ADN repetitivo, fácil transformación y filogenéticamente próximo a cereales templados) también son compartidos por *B. stacei* y *B. hybridum* (Catalán *et al.*, 2012, 2016b).

El complejo *B. distachyon* fue propuesto además como un modelo para la (alo)poliploidía y para su potencial aplicación en trigos poliploides debido a los amplios conocimientos de genómica funcional comparativa ya disponibles (Catalán *et al.*, 2014; Gordon *et al.*, 2016).

Distribución y ecología

Se han realizado numerosos estudios ecológicos con las tres especies circunmediterráneas del complejo *B. distachyon*. Los análisis de modelo de nicho ambiental indican que, generalmente, *B. distachyon* crece en zonas de elevada altitud, frías y húmedas; *B. stacei* crece en bajas altitudes, en zonas templadas y secas y *B. hybridum* crece en zonas con características ecológicas intermedias y también a bajas altitudes, zonas templadas y secas, como el parental *B. stacei* (Manzaneda *et al.*, 2012; López-Alvarez *et al.*, 2015). Esto coincide con que la mayoría de las líneas de *B. distachyon* requieren tratamiento de vernalización para florecer, mientras que las líneas de *B. stacei* y *B. hybridum* no (Vogel *et al.*, 2009). Además, *B. stacei* crece en hábitats sombreados, mientras que *B. distachyon* y *B. hybridum* ocurren en hábitats abiertos (López-Alvarez *et al.*, 2015; Catalán *et al.*, 2016a). Los datos de modelado paleoambiental respaldan la idea de que la cuenca mediterránea y las áreas adyacentes sirvieron como refugio a largo plazo para *B. stacei* y *B. distachyon*, y también como posibles zonas híbridas, lo que podría haber favorecido el origen recurrente de *B. hybridum* en el Pleistoceno tardío. Los análisis de similitud de nicho ambiental mostraron evidencias de conservadurismo de nicho para *B. hybridum* y para cada uno de sus parentales en su región autóctona mediterránea; el alotetraploide comparte ocupación de nicho con sus parentales, pero está aislado reproductivamente de ambos. Además, *B. hybridum* tiene el mayor solapamiento con los nichos de los parentales, pero un rango de distribución y una amplitud de nicho similares, lo que puede sugerir que el híbrido no supera a sus parentales en sus rangos nativos posiblemente debido a la competencia de los diploides (López-Alvarez *et al.*, 2015; Catalán *et al.*, 2016b). Sin embargo, estudios de competencia llevados a cabo mediante experimentos de translocación en el sur de España entre poblaciones naturales de *B. hybridum* y *B. distachyon* y bajo condiciones de campo, sugieren un mayor éxito de *B. hybridum* en términos de colonización/invasión en hábitats competitivos áridos, incluyendo las zonas de contacto donde compite con el diploide *B. distachyon* (Rey *et al.*, 2017). Del mismo modo, análisis de campo en el sur del Mediterráneo en Israel han revelado una mayor presencia del alotetraploide *B. hybridum* sobre sus parentales diploides, donde especialmente *B. stacei* es más frecuente (Bareither *et al.*, 2017). Por otra parte, también hay análisis de campo que demostraron que los gradientes de aridez afectan la distribución de *B. distachyon* y *B. hybridum* en la Peninsula Ibérica y que la eficiencia en el uso del agua varía siendo menor en *B. distachyon* y mayor en *B. hybridum* y *B. stacei*, bajo condiciones de crecimiento con restricción de agua (Manzaneda *et al.*, 2012). En condiciones de sequía, los individuos de *B. hybridum* se comportan como escapistas de la sequía, manteniendo una fotosíntesis y una conductancia estomática más altas y mostrando tiempos de floración más tempranos para hacer frente al estrés hídrico que los individuos de *B. distachyon* menos adaptados (Manzaneda *et al.*, 2015).

Todos estos estudios sugieren que el alotetraploide tiene mayor tolerancia ecológica en comparación con los diploides, lo que podría estar asociado con el impulso en la selección diversificada debido al aumento de la expresión genómica y epigenómica, así como a los rápidos cambios en los caracteres fisiológicos y adaptativos, como el fotoperíodo y capacidad adaptativa y de invasión (Bakker *et al.*, 2009; Catalán *et al.*, 2016b).

Recursos genéticos y genómicos

Entre la gran disposición de recursos encontramos el genoma de referencia, completamente secuenciado y anotado, de las dos líneas de *B. distachyon* comúnmente utilizadas (Bd21 y Bd21-3) que están publicados y disponibles en la web de Phytozome (https: // phytozome. Jgi.doe.gov/pz/portal.html), el portal de Genómica Comparativa de Plantas del Departamento de Energía de los EEUU. El ensamblaje de la línea Bd21 se completó en 2010, y las versiones (v) 2.1 y 3.1 están actualmente disponibles públicamente a través de Phytozome. El ensamblaje de la antigua versión v2.1 se refinó en la actualmente completada v3.1. El ensamblaje del genoma la línea Bd21-3 también se ha actualizado. También se encuentra disponible una base de datos de más de 800,000 mutaciones de polimorfismos de un solo nucleótido (SNP) en el ensamblaje recientemente lanzado de Bd21-3. Además de esta línea también se encuentra publicados los genomas de referencia de las otras dos especies del complejo *B. stacei* y *B. hybridum*, que se han completado recientemente y están disponibles en Phytozome. Aunque la secuenciación y la anotación del genoma mitocondrial aún no se han completado, se han secuenciado los genomas plastídicos de *B. distachyon* (54 líneas), *B. stacei* (1 línea) y *B. hybridum* (2 líneas) (Sancho *et al.*, 2018). Juntos, estos genomas del complejo *B. distachyon* son recursos de gran importancia para los estudios de biología evolutiva, poliploidía y especiación de gramíneas.

Otros recursos disponibles que ayudan en el estudio de la función génica y en la identificación de vía metabólicas son los atlas de expresión génica de *B. distachyon* completados recientemente, que mapean la expresión de genes en los principales órganos y en diferentes etapas de desarrollo (Sibout *et al.*, 2017). Se está llevando a cabo otro proyecto de atlas genético, financiado por el Programa de Ciencia Comunitaria (CSP) de JGI, que permitirá la identificación en todo el genoma de respuestas de expresión génica conservadas y únicas que ocurren durante diversas interacciones de *B. distachyon*-microbio, incluidas interacciones patogénicas y beneficiosas. (CSP503406; https://jgi.doe.gov/csp-2018-mandadi-gene-atlases-grass-microbe-communications /). Otras muchas herramientas y recursos genéticos funcionales están disponibles actualmente para la investigación a nivel molecular, citogenético y bioquímico (Scholthof *et al.*, 2018; Tabla 3). Entre ellos se incluyen una colección diversa de líneas de *B. distachyon*, mutantes de T-DNA y EMS, bibliotecas BAC, EST, librerías de levaduras (Y2H) y metodologías para la transformación de *B. distachyon* mediada por *Agrobacterium*.

En cuanto a los métodos de transformación de *B. distachyon* descritos y evaluados se encuentran los mediados por *Agrobacterium tumefaciens* y *Agrobacterium rhizogenes* y las metodologías biolísticas. Estos se basan en la utilización de embriones maduros (Bablak *et al.*, 1995; Vogel, 2016; Ozdemir & Budak, 2017) e inmaduros (Bablak *et al.*, 1995; Draper *et al.*, 2001; Christiansen *et al.*, 2005; Păcurar *et al.*, 2008; Vain *et al.*, 2008; Vogel & Hill, 2008). La eficiencia de transformación (TE), definida como el porcentaje de callos co-cultivados que producen plantas transgénicas con éxito, depende en gran medida de factores como la línea de *B. distachyon* utilizada, etapa embrionaria, morfología del callo, cepa de *Agrobacterium*, así como los promotores y marcadores seleccionables utilizados en el vector binario (Bragg *et al.*, 2015). Aunque se han empleado múltiples cepas de *Agrobacterium* y agentes de selección para la transformación de *Brachypodium*, la cepa de *Agrobacterium* AGL1 y la selección de higromicina son los materiales de elección (Bragg *et al.*, 2015). Los promotores monocotiledóneos de uso común, como el promotor de ubiquitina de maíz (Zea mays), también funcionan bien en *B. distachyon* (ver más detalle en Scholthof *et al.*, 2018 y referencias).

Estrés hídrico en *Brachypodium*

El estudio de los mecanismos de tolerancia al estrés abiótico en plantas es de gran importancia para la agricultura ya que permite el desarrollo de cultivos que puedan resistir el cambio climático y los estreses ambientales. Como ya se ha expuesto *Brachypodium* es un modelo útil para estudiar la genética y la genómica de las respuestas al estrés abiótico que afectan a cereales y gramíneas bioenergéticas. La respuesta al estrés abiótico de *B. distachyon* también se asemeja a la del trigo (*Triticum*), la cebada (*Hordeum*) y el centeno (*Secale*), debido a que tienen una adaptación similar a los climas templados y mecanismos fotosinéticos C3 comunes (Des Marais & Juenger, 2016).

Con el desarrollo de las tecnologías, la detección de caracteres complejos relacionados con la tolerancia al estrés ha avanzado en la última década (Fahlgren *et al.*, 2015). Recientemente se identificaron varias líneas de *B. distachyon* naturales tolerantes a la sequía mediante el cribado de la temperatura de la hoja utilizando técnicas de imagen térmica (Ruíz *et al.*, 2016). Las líneas naturales fueron recolectadas de diferentes regiones geográficas con patrones contrastantes de lluvia y temperatura en la Península Ibérica. Los genotipos adaptados a la sequía tenían temperaturas foliares más altas bajo estrés por sequía, consistente con un mayor cierre estomático y una disminución de la evapotranspiración (Jones, 1999), en comparación con líneas no adaptadas a condiciones áridas. Este estudio también apoya la existencia de mecanismos estomáticos que gobiernan la variación natural a la adaptación a la sequía entre las líneas naturales de *B. distachyon*. La caracterización molecular y genética de estas líneas adaptadas a la sequía y sensibles sin duda ayudará a descifrar los mecanismos subyacentes. La exposición de *B. distachyon* a cuatro grandes estreses abióticos (calor, alta salinidad, sequía y estrés por frío) dio lugar a cambios en el transcriptoma en 22 módulos de genes, 10 de los cuales pertenecen a procesos biológicos definidos (Priest *et al.*, 2014). El análisis de la red de coexpresión génica reveló además que la sequía y la salinidad desencadenan perfiles de coexpresión sinérgicos, mientras que el calor y el frío desencadenan perfiles de coexpresión antagónica. Curiosamente, la sequía y el estrés por salinidad aumentaron la expresión de módulos genéticos con las mismas categorías génicas, mientras que el estrés por frío y calor activaron factores de transcripción y genes supuestos involucrados en el plegamiento y la estabilidad de las proteínas, respectivamente. Estas redes de genes asociadas al estrés se suman a los recursos disponibles de *Brachypodium* para la descripción de los procesos y mecanismos de señalización de estrés abiótico (Priest *et al.*, 2014).

Priest *et al.*, (2014) también determinaron que el estrés por desecación disminuye la expresión de genes en *B. distachyon* (~5000 genes durante el período de recolección de muestras de 24 h), muchos de los cuales están involucrados en la regulación del ciclo celular y la replicación del ADN, lo que sugiere una disminución del crecimiento celular durante el estrés por deshidratación (Priest *et al.*, 2014). Sin embargo, a nivel anatómico, la respuesta al estrés por deshidratación de *B. distachyon* parece contradecir la de *Arabidopsis* y algunos cereales. Mientras que la mayoría de las plantas, incluida *Arabidopsis*, muestran división celular reducida y baja expansión celular bajo estrés por deshidratación, *B. distachyon* no mostró cambios en el número de células en comparación con los controles. El tamaño reducido de la hoja en *B. distachyon* bajo estrés por deshidratación no parece ser el resultado de una expansión celular disminuida (Verelst *et al.*, 2013). Estos resultados apuntan hacia algunos aspectos únicos de *B. distachyon* con respecto a la respuesta a nivel celular y anatómico al estrés por deshidratación.

Un número creciente de estudios ha utilizado *B. distachyon* como sistema modelo para el análisis genético de las respuestas al estrés abiótico. Entre los procesos celulares, la "ubiquitinación" de proteínas es un proceso clave en la adaptación de las plantas al estrés abiótico. Se descubrió que la sobreexpresión del gen de trigo Ta-Ub en tabaco confiere resistencia a la sequía (Guo *et al.*, 2008) y al calor (Tian *et al.*, 2014), lo que demuestra el papel de Ta-UB en la tolerancia al estrés de las plantas en dicotiledóneas. Sin embargo, se sabe poco sobre su papel en las monocotiledóneas. Kang *et al.*, (2016) demostraron recientemente que la sobreexpresión de Ta-Ub2 bajo el control del promotor RD29A inducido por estrés confiere a plantas transgénicas de *B. distachyon* una mayor tolerancia a la sequía, mayor retención de agua y mayor expresión de genes relacionados con la eliminación de especies reactivas de oxígeno. Sin embargo, la expresión constitutiva de Ta-Ub2 impulsada por el promotor CaMV35S tuvo efectos negativos sobre el crecimiento y el desarrollo en *B. distachyon*, lo que sugiere que la expresión génica asociada al estrés debe ser regulada cuidadosamente para evitar consecuencias nocivas para el crecimiento y el rendimiento de las plantas. Sun *et al.*, (2015) mostraron recientemente que el gen WRKY36 en *B. distachyon* regula positivamente la respuesta a la tolerancia a la sequía en transgénicas de *Nicotiana tabacum* (tabaco). La expresión de WRKY36 se induce durante el estrés por sequía en *B. distachyon*, y la sobreexpresión de BdWRKY36 en tabaco transgénico mejora su tolerancia a la sequía, reduciendo de pérdida de electrolitos y la acumulación reactiva de especies de oxígeno. Estos resultados sugieren que las dicotiledóneas y las monocotiledóneas comparten mecanismos conservados de tolerancia al estrés que involucran Ta-Ub2 y WRKY36.

Estrés por patógenos e insectos en *Brachypodium*

Las plagas y enfermedades de las plantas son económicamente devastadoras para los productores, causando pérdidas anuales de rendimiento en los cultivos del 40 al 100%. Gran parte de nuestro conocimiento del sistema de defensa de las plantas se basa en estudios que utilizan la planta modelo dicotiledónea *Arabidopsis* y otras especies como, por ejemplo, *Medicago truncatula*, *Nicotiana atenuata*, *Oryza sativa* (arroz), *Zea mays* (maíz), *Solanum lycopersicum* (tomate) y *Nicotiana benthamiana*. Los estudios de interacción planta-patógeno se han visto obstaculizados por la falta de un sistema modelo de monocotiledóneas manejable. Por supuesto, el arroz y el maíz han sido herramientas invaluables para los biólogos de plantas, pero carecen de varias características clave de los sistemas modelo. Recientemente, como hemos señalado anteriormente, *Brachypodium* ha alcanzado la categoría de especie modelo para la investigación de diversas interacciones planta-patógeno y planta-insecto (Mandadi & Scholthof, 2013).

A diferencia del vasto conocimiento que existe en relación a las interacciones planta-patógeno en *Brachypodium* (Cui *et al.*, 2012; Lee *et al.*, 2012; Mandadi & Scholthof, 2012; Mandadi *et al.*, 2014, 2015; Fitzgerald *et al.*, 2015; Pyle *et al.*, 2017; Irigoyen *et al.*, 2018; Pyle & Scholthof, 2018), que demuestran que *Brachypodium* es un excelente modelo para estudiar la genética y la genómica de patógenos fúngicos biotróficos, hemibiotróficos y necrotróficos de gramíneas (Routledge *et al.*, 2004; Figueroa *et al.*, 2013; Sandoya & Buanafina, 2014; Fitzgerald *et al.*, 2015), y bacterianos (Fitzgerald *et al.*, 2015), sin embargo no se sabe apenas nada de las interacciones insecto-planta en este sistema. Es conocido, no obstante, que *B. distachyon* es una planta huésped para varias especies de insectos, incluidos los áfidos y el noctuido *Spodoptera frugiperdai* (Brem & Leuchtmann, 2001; Azhaguvel *et al.*, 2014; Sandoya & Buanafina, 2014; Tao *et al.*, 2016; Zhou *et al.*, 2016). Los áfidos, además de causar grandes pérdidas de rendimiento debido a que se alimentan del floema, a menudo transmiten virus a las plantas. Por ejemplo, la cepa GAV del virus enano amarillo de la cebada se transmite por *Schizaphis graminum*. Usando *Brachypodium spp*, ahora es posible diseccionar la genética molecular y la bioquímica de las interacciones pulgón-virus y pulgón-planta (Tao *et al.*, 2016). Los resultados de estos estudios deberían ser fácilmente aplicables al desarrollo de medidas de control de plagas basadas en el campo. Sin embargo, el grado de variación natural en la respuesta a la herbivoría por insectos en este sistema es totalmente desconocido, lo que es fundamental para conocer la base genética de la respuesta a la herbivoría.

OBJETIVOS

OBJECTIVES

OBJETIVOS

Objetivos de la tesis doctoral

Objetivo general

Determinar si la variación en la respuesta funcional de resistencia y tolerancia al estrés ambiental, presumible en múltiples poblaciones de las tres especies del complejo de ploidia *Brachypodium distachyon*, tiene una base genética, y si dicha base es cuantificable en términos de variación genética y de expresión génica. En último término, la consecución de dicho objetivo ayudará a entender las causas que subyacen a la variación intra- e interespecífica en la tolerancia al estrés ambiental en cereales de clima templado.

Objetivos específicos:

1. Analizar la variación en la respuesta funcional y la variación genética de la tolerancia al estrés hídrico entre plantas diploides y poliploides del complejo *Brachypodium distachyon* en diferentes niveles de organización (genotipo, población y especie) – *Capítulo 1.*

2. Analizar comparativamente el transcriptoma entre las tres especies del complejo de *Brachypodium distachyon* en respuesta a la sequía, comparando el nivel de expresión de los genes duplicados en el derivado alotetraploide (llamados genes homeólogos) con la expresión de los genes en los ancestros diploides. Ello servirá para determinar el legado de los ancestros diploides en el patrón de expresión del derivado alotetraploide y detectar genes funcionales que subyacen a la respuesta al estrés hídrico en este sistema. Asimismo, analizar la respuesta en expresión génica de genes involucrados en el estrés por sequía en diferentes líneas de *B. hybridum* de diferente origen geográfico y tolerancias al estrés – *Capítulo 2.*

3. Analizar el impacto que el gen codificador de dehidrinas *BdDHN3* produce sobre los caracteres funcionales de tolerancia a la sequía en *Brachypodium distachyon* mediante la transformación y generación de plantas transgénicas que sobreexpresan dicho gen – *Capítulo 3.*

4. Analizar la respuesta funcional y de la variación genética en la tolerancia y resistencia al estrés por herbivoría entre genotipos diploides y poliploides del complejo *Brachypodium distachyon -Capítulo4.*

OBJECTIVES

Objectives of PhD dissertation

General objective

To determine whether the variation in the functional response of resistance and tolerance to environmental stress, presumably in multiple populations of the three species of the *Brachypodium distachyon* ploidy complex, has a genetic basis, and if so, whether is quantifiable in terms of genetic variation and gene expression. Ultimately, the achievement of this objective will help to understand the causes that underlie the intra- and interspecific variation to the tolerance of environmental stress in temperate grasses.

Specific objectives:

1. To analyze the functional response and genetic variation in tolerance to water stress between diploid and polyploid plants in the *Brachypodium distachyon* complex at different levels of organization (genotype, population and species) – *Chapter 1.*

2. To analyze comparatively the transcriptome among the three species of the *Brachypodium distachyon* complex in response to drought, by comparing the level of expression of the duplicated genes in the derived alotetraploid (called homoeolog genes) with the expression of the genes in the diploid ancestors. This will serve to determine the legacy of diploid ancestors in the expression pattern of the derived alotetraploid and detect functional genes that underlie the response to water stress in this system. Likewise, to analyze the response in gene expression of genes involved in drought stress in different lines of *B. hybridum* with different geographical origin and stress tolerances – *Chapter 2.*

3. To analyze the impact of the dehydrine coding gene BdDHN3 produces on functional drought tolerance traits in *Brachypodium distachyon* through transformation and generation of transgenic plants that overexpress this gene – *Chapter 3.*

4. To analyze the functional response and the genetic variation in tolerance and resistance to herbivory stress between diploid and polyploid genotypes of the *Brachypodium distachyon* complex – *Chapter 4.*

METODOLOGÍAS EMPLEADAS

METODOLOGÍAS EMPLEADAS

Las siguientes metodologías, expuestas aquí de forma abreviada, se explican detalladamente en sus capítulos correspondientes.

Material vegetal

Las plantas utilizadas en los diferentes trabajos se obtuvieron a partir de semillas procedentes del banco de germoplasma UJAEN *Brachypodium* que se encuentra en el Departamento de Biología Animal, Biología Vegetal y Ecología de la Universidad de Jaén. Esta colección de semillas fue recolectada de 57 poblaciones naturales en la península ibérica durante muestreos de campo en 2007 y 2008. Además, hemos incluido en todos los trabajos las líneas Bd21 y Bd30-1 de *B. distachyon* como líneas de referencia, las cuales fueron proporcionadas por David Garvin (USDA-ARS, Plant Science Research Unit and Department of Agronomy and Plant Genetics, University of Minnesota, St Paul, MN, USA).

Estudio de la respuesta funcional y variación genética al estrés hídrico

Para el estudio comparativo de la variación de los caracteres fisiológicos relacionados con la tolerancia a la sequía (contenido de agua, área específica de la hoja, daño celular y contenido en prolina) se emplearon un total de 119 líneas procedentes de poblaciones genéticamente diferenciadas y de diferentes regiones climáticas (Fig. 7, y Tabla 1 contenida en el apartado Información suplementaria Introducción y Metodología general): 53 líneas naturales de *B. distachyon* (10 poblaciones), 56 de *B. hybridum* (10 poblaciones) y 10 de *B. stacei* (3 poblaciones). Una vez germinadas las semillas, las plantas fueron sembradas en macetas (cada línea por triplicado), las cuales crecieron en la cámara de cultivo bajo condiciones controladas de luz y fotoperiodo. A las tres semanas de crecimiento las plantas fueron sometidas a estrés hídrico evitando el riego de las mismas durante 21 días y controlando la pérdida de humedad en el suelo. Pasado este tiempo las plantas fueron recolectadas para el estudio de los caracteres fisiológicos. Para el estudio comparativo de la variación en el contenido en fitohormonas (ácido abcísico, ácido jasmónico, ácido indol acético y ácido salicílico) se empleó una selección de 13 líneas procedentes de 12 poblaciones en base a sus diferenciación genética y origen geográfico: 7 líneas (7 poblaciones) de *B. distachyon*, 3 (2 poblaciones) de *B.stacei* y 3 (3 poblaciones) de *B. hybridum*. Consultar el apartado de metodología correspondiente al *Capítulo 1* para más detalle.

Fig. 7. Distribución geográfica de las 23 poblaciones del complejo *Brachypodium distachyon* a través de la Península Ibérica usadas en esta tesis. Con puntos verdes, naranjas y rojos se representan en el mapa las localidades de origen de *B. distachyon*, *B. stacei* and *B. hybridum* respectivamente (Imagen tomada de Google Earth el 14/12/2015).

Análisis comparativo del transcriptoma y del legado parental entre las especies ancestrales diploides B. distachyon y B. stacei y su derivado alotetraploide B. hybridum en respuesta al estrés hídrico

Para este estudio comparativo del transcriptoma entre las tres especies del complejo se utilizaron 3 líneas de tres poblaciones diferentes: 1 línea de *B .distachyon*, 1 línea de *B. stacei* y una línea de *B. hybridum*. Las plantas crecieron en las mismas condiciones y tratamiento de estrés hídrico que en el apartado 4.3.2 pero en este caso se recolectaron las muestras en condiciones de control (capacidad de campo 100%) y en condiciones de sequía severa (capacidad de campo 25%). Las muestras de hoja pertenecientes a las 3 líneas y sus tratamientos fueron procesadas para la extracción y purificación de ARN total con el que se prepararon doce librerías de cDNA que fueron posteriormente secuenciadas usando la tecnología RNAseq de Illumina. Los detalles técnicos de este proceso al igual que los del pre-procesado de los datos derivados de la secuenciación y los análisis posteriores de expresión diferencial se encuentran en la metodología correspondiente al *Capítulo 2*.

Generación de plantas transgénicas que sobreexpresan el gen codificador de dehidrinas BdDHN3 y análisis del impacto de dicho gen en caracteres funcionales de tolerancia a la sequía en B. distachyon.

El gen BdDHN3 que codifica la dehidrina 3 fue el candidato seleccionado para este estudio debido a los altos niveles de expresión significativa hallados en el análisis comparativo de los transcriptomas de las tres especies del complejo en respuesta a la sequía llevado a cabo en el estudio anterior y debido también a la información existente sobre su importante función en mecanismos de estrés hídrico. Dicho gen fue clonado en el vector binario de sobreexpresión pH7WG2D (DNA Cloning Service, Germany) que permitió la posterior transformación en *B.*

distachyon (línea Bd21) mediada por *Agrobacterium tumefaciens* cepa AGL1 siguiendo el protocolo de Alves *et al.*, 2009. Tras la generación y confirmación de las plántas transgénicas, dichas plantas se sometieron a un ensayo de tolerancia al estrés hídrico junto a plantas *wild-type* de la línea Bd21 durante un periodo de 21 días. Tras dicho tratamiento se comparó el nivel de expresión del gen BdDHN3 mediante PCR en tiempo real entre plantas transgénicas y *wild-type* y también se tomaron medidas fisiológicas de tolerancia como el daño celular, el contenido de agua, el área específica de la hoja y la concentración de prolina. Los detalles técnicos de este estudio se encuentran descritos en el apartado de metodología del *Capítulo 3*.

Estudio de la respuesta funcional y variación genética al estrés por herbivoría

Para el estudio comparativo de la variación de los caracteres fisiológicos relacionados con la tolerancia a la herbivoría se emplearon las mismas 119 líneas procedentes de poblaciones de las tres especies del complejo *B. distachyon* utilizadas en el estudio de la respuesta funcional y variación genética al estrés hídrico (Apartado 4.3.2). Las plantas se sometieron al tratamiento de herbivoría utilizando el insecto *Locusta migratoria* (Orthoptera: Acrididae). Cada planta fue aislada de las demás por un cilindro de acetato (6 cm de diámetro y 21 cm de altura) dentro del cual se colocó una langosta previamente pesada por planta durante 24 h. Tras las 24 h de experimentación, se estimó el daño presentado por cada planta (PD) mediante la proporción de hojas dañadas por los insectos y el porcentaje de daño por hoja y la resistencia a la herbivoría (1- PD). Las medidas de caracteres constitutivos que se tomaron en plantas no dañadas fueron: el contenido de agua, el área específica de la hoja, la cantidad de nutrientes en hoja y el contenido en sílice. Consultar el apartado de metodología correspondiente al *Capítulo 4* para más detalle.

Fig. 8. Detalles del método seguido para el tratamiento de las plantas de *Brachypodium* con las langostas. Cada planta con su langosta fue aislada de las demás usando un tubo de acetato (3M®) con la apertura superior cubierta con bolsas de organza (Foto tomada por Luisa María Martínez Martínez).

CAPÍTULOS

CAPÍTULO 1

VARIATION IN FUNCTIONAL RESPONSES TO WATER STRESS AND DIFFERENTIATION BETWEEN NATURAL ALLOPOLYPLOID POPULATIONS IN THE *BRACHYPODIUM DISTACHYON* SPECIES COMPLEX

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ABSTRACT

- **Methods** A comparative functional approach was used to investigate differentiation of drought-tolerance- related traits in the *Brachypodium* species complex, a model system for grass polyploid adaptive speciation and functional genomics that comprises three closely related annual species: the two diploid parents, *B. distachyon* and *B. stacei*, and the allotetraploid derived from them, *B. hybridum*. Differentiation of drought-tolerance-related traits between ten genetically distinct *B. hybridum* populations and its ecological correlates was further analysed.
- **Key Results** The functional drought response is overall well differentiated between *Brachypodium* species. *Brachypodium hybridum* allotetraploids showed a transgressive expression pattern in leaf phytohormone content in response to drought. In contrast, other *B. hybridum* physiological traits correlated to *B. stacei* ones. Particularly, proline and water content were the traits that best discriminated these species from *B. distachyon* under drought.
- **Conclusions** After polyploid formation and/or colonization, *B. hybridum* populations have adaptively diverged physiologically and genetically in response to variations in aridity.

Key words: Adaptation, allopolyploidy, *Brachypodium distachyon*, *Brachypodium stacei*, *Brachypodium hybridum*, drought, genetic differentiation, phytohormones, proline, specific leaf area.

INTRODUCTION

A major proposed advantage of allopolyploidization (hybridization and whole-genome duplication) is that heterosis associated with ancestral hybridization would allow offspring to display transgressive performance compared with its progenitor species, which would enhance allopolyploids' response to environmental shifts and subsequently contribute to successful persistence of allopolyploid populations beyond the progenitors' environmental ranges (Hegarty & Hiscock, 2008; Madlung, 2013; Barker *et al.*, 2016). Thus, numerous studies have investigated the genetic and phenotypic consequences of allopolyploidy (reviewed in Doyle *et al.*, 2008; Chen & Birchler, 2013; Soltis *et al.*, 2016), albeit conclusive evidence for true novel transgressive segregation of ecologically important traits in allopolyploids remains elusive (Hegarty & Hiscock, 2008).

Because allopolyploidization is a novel generator of genetic variation (Doyle *et al.*, 2008; Parisod, 2012), genetic and phenotypic expression analyses are essential for understanding the evolutionary trajectories of ecologically important traits in allopolyploids relative to their progenitors (Schranz & Osborn, 2004; Coate & Doyle, 2013). However, such analyses are rarely conducted in an ecological or physiological context (Madlung, 2013; Soltis *et al.*, 2016). The adoption of an ecophysiological frame in comparative studies of ploidy systems is crucial because the establishment, divergence and evolutionary success of newly formed polyploid lineages may rely on physiological differentiation from diploid progenitors (Hao *et al.*, 2013; Manzaneda *et al.*, 2015; Rey *et al.*, 2017). Additionally, to evaluate the importance of adaptive evolution after polyploidization (e.g. ecological adaptive convergence or divergence of populations) the analysis of polyploid expression patterns should also examine the consistency of genetic and/or phenotypic changes between genetically differentiated natural polyploid populations.

Drought is a widespread abiotic stress and a major determinant of plant adaptation and productivity. Plants respond to water deficit through various physiological, biochemical, anatomical and other phenotypic changes (Hossain *et al.*, 2016). In some ploidy complexes, polyploids have shown enhanced physio- logical tolerance of drought compared with their progenitors by optimizing gas exchange physiology and/or plant hydraulic function under water limitation (e.g. Li *et al.*, 1996; Maherali *et al.*, 2009; Del Pozo & Ramirez-Parra, 2014; Manzaneda *et al.*, 2015). Such physiological differentiation of polyploids with respect to water stress is thought to have a pivotal role underlying the establishment of polyploids in drier hábitats (Ramsey, 2011; Hao *et al.*, 2013; Manzaneda *et al.*, 2015; but see Buggs & Pannell, 2007). At the molecular level, some stud- ies have revealed that abscisic acid (ABA) and reactive oxygen species (ROS) are augmented in polyploids compared with diploids (Allario *et al.*, 2013; Del Pozo & Ramirez-Parra, 2014). However, unlike autopolyploids, allopolyploids (in which two distinct genomes are present) show transgressive expression, and the physiological responses of an allopolyploid could be correlated to that of one of its parents if expression-level dominance occurs (Yoo *et al.*, 2014).

Within species, environmental aridity (an ecological proxy of soil water availability) is a driver of adaptive population divergence in many plant species (e.g. Lee & Mitchell-Olds, 2011; Brouillette *et al.*, 2014). However, how aridity influences drought-response trait differentiation and genetic divergence across populations within the same polyploid species is not well known (but see Manzaneda *et al.*, 2015), although it has been shown that the enhanced ability of polyploids to cope with drought stress may have evolved after polyploidization (Maherali *et al.*, 2009).

Here, we use a comparative functional approach to investigate differentiation of drought-tolerance-related traits in the *Brachypodium* species complex. This species complex is a model system for grass polyploid speciation and for comparative functional genomics of monocots (Catalán *et al.*, 2016). It comprises three closely related annual species; the two diploid parents, *B. distachyon* and *B. stacei*, and their derived allotetra- ploid, *B. hybridum* (2n = 2x = 10, 2n = 2x = 20 and 2n = 4x = 30, respectively; Catalán *et al.*, 2012, 2016). Phylogenetic analyses indicate that *B. hybridum* has a polyphyletic origin, which arose recurrently across the Mediterranean basin during the last ~1 million years from bidirectional and reciprocal crosses, although *B. hybridum* lineages derived from an ancient cross between maternal *B. stacei* and paternal *B. distachyon* parents seem prevalent (López-Alvarez *et al.*, 2012; Catalán *et al.*, 2016). These species are ecologically differentiated; *B. distachyon* is found in higher, cooler and wetter places than *B. stacei*, which grows at low altitude in warmer and drier environments, whereas *B. hybridum* grows in zones with intermediate values, yet also appears frequently in low altitude, warmer and drier places, like its *B. stacei* progenitor (Manzaneda *et al.*, 2012; López-Alvarez *et al.*, 2015). Drought response has been investigated mainly in the diploid *B. distachyon*, given its emerging role as a plant model for temperate grasses (e.g. (Luo *et al.*, 2011; Verelst *et al.*, 2013; Des Marais & Juenger, 2016; Fisher *et al.*, 2016). However, the adaptive significance of natural variation observed in the drought physiology of this species complex is not well understood yet (but see Manzaneda *et al.*, 2015; Des Marais *et al.*, 2017).

We have recently demonstrated differentiation in gas exchange physiology between two species of the complex, *B. distachyon* and *B. hybridum*, and also that trait divergence among *B. hybridum* populations is associated with inter-population variations in soil moisture deficit and precipitation (Manzaneda *et al.*, 2015). While that previous study was limited to only two species of the complex, here we firstly deepen the study of the significance of trait divergence among the three species by analysing specific differentiation in leaf traits typically related to drought response (e.g. Fisher *et al.*, 2016; Des Marais *et al.*, 2017), such as water content, specific leaf area, electrolyte leakage, proline content and phytohormone content. Secondly, we analyse the differentiation of drought-tolerance-related traits between *B. hybridum* Iberian populations that are genetically distinct, and whether such phenotypic differentiation between *B. hybridum* populations is related to aridity. We finally investigate whether existing *B. hybridum* genetic divergence at the population level is explained by geography or environmental factors (climate) and/or is associated with differentiation in drought-tolerance traits, which would indicate ecological adaptive divergence of *B. hybridum* populations.

MATERIALS AND METHODS

Plant material, growth conditions and experimental procedure

All the plant material (seeds) used in this study came from the UJAEN *Brachypodium* germ plasm collection (Departamento de Biología Animal, Biología Vegetal y Ecología, University of Jaén, Spain). The collection is derived from a field collection conducted in 2008 across 57 natural populations in the Iberian Peninsula (Manzaneda *et al.*, 2012). In addition, we included as reference lines the *B. distachyon* diploid lines Bd21 and Bd30- 1, which were provided by Dr David Garvin (USDA-ARS, Plant Science Research Unit and Department of Agronomy and Plant Genetics, University of Minnesota, St Paul, MN, USA). For the physiological differentiation experiments we selected 53 *B. distachyon* natural accessions (inbred lines, ‘genotypes’ hereafter), 56 *B. hybridum* genotypes and 10 *B. stacei* genotypes from ten, ten and three different Iberian populations, respectively (Supplementary Data Table S1). We chose two to six genotypes from each population. Genotypes come from populations genetically differentiated and with contrasting climate conditions (Supplementary Data Fig. S1; Manzaneda *et al.*, 2012).

Seeds were put on moist filter paper in sealed Petri dishes, and then stratified at 4 ºC for 1 week to facilitate uniform seed germination. Once germinated, seeds were individually planted in pots (7 × 7 × 8 cm) and grown on sterile soil containing perlite, sand and organic substrate (0.5:0.5:1, v/v). Plants were grown in a growth chamber at 21 ºC, 65 % relative humidity, under long-day conditions (16 h light/8 h dark; 120–200 $\mu Em^{-2}\ s^{-1}$), and were regularly watered (by bottom-watering until reaching field saturation) for 3 weeks. Then, nine individual plants of each genotype were grown in separate flats (i.e. blocks) and after 3 weeks of growing in the same conditions three replicates of each genotype were allocated to one of the three following dry-down experimental treatments following a randomized block design: 100 % (control; plants grown without any watering restriction for 21 d), 50 % (moderate drought conditions, reached 13 d after watering ceased) and 25 % (severe drought conditions, reached 21 d after watering ceased) of field capacity. Drought treatments simulate the natural onset of a progressive seasonal drought. Soil field capacity was monitored daily during the whole experimental period using a volumetric soil moisture device (TDR100 Time-Domain Reflectometer, Campbell® Scientific). Plant physiological responses (see below) were measured in the three replicates per genotype and treatment.

Similar reductions in soil water content have been shown previously to impact significantly on soil water potentials and physiology of Brachypodium species under similar growing experimental conditions (Manzaneda *et al.*, 2015; Des Marais *et al.*, 2017).

Physiological measurements

From each plant and irrigation treatment, we made the following measurements. (1) above-ground plant water content (WC) was calculated as 100 × [(FW − DW)/FW], where FW is fresh weight and DW is dry weight. The FW was determined by weighing the leaves on a precision scale immediately just after plant harvest. To estimate DW, leaves were dried for 48 h at 70 ºC and weighed again. (2) The specific leaf area (SLA) of the largest leaf was leaf area/DW (m^2 kg^{-1}). Leaf area was assessed using a leaf area meter (LI-3000C Portable Area Meter from LI-COR® Biosciences). (3) Electrolyte leakage (EL) was determined to evaluate cell membrane integrity by measuring electrical conductivity (Bouchabka *et al.*, 2008). For this purpose, 0.1 g of plant material (leaves) was incubated in 20 mL of deionized water and then shaken at room temperature for 5 h. The initial conductivity (Ci) was measured using a conductimeter (HI9033 Multi-Range Conductivity Meter, Hanna® Instruments). The solution was then boiled for 20 min, cooled at room temperature and the conductivity of the tissues (Cmax) was determined again. We calculated EL (%) as (Ci/Cmax) × 100. (4) Free proline content was measured using a colorimetric assay (Bates *et al.*, 1973; Colton-Gagnon *et al.*, 2014; for details see appendix in Supplementary Data). Proline content of leaves is expressed as mg g^{-1} dry leaf weight.

Finally, during the whole study period all the plants were scored daily for flowering. A flowering time for each plant was then obtained as the number of days from germination until the emergence of the first spikelet.

Phytohormone content

Leaf concentrations of abscisic acid (ABA), jasmonic acid (JA), indole-3-acetic acid (IAA) and salicylic acid (SA) were determined for plant extracts (aerial parts) by liquid chromatography–tandem mass spectrometry (LC–MS/MS) following the procedures described in (Durgbanshi *et al.*, 2005). Phytohormone analyses were conducted at the Ecophysiology and Biotechnology Laboratory of the Universitat Jaume I (http://www.ecofisiologia.uji.es). In this case, from each irrigation treatment we harvested and froze in liquid nitrogen two samples (~500 mg per sample of fresh plant material) from the following genotypes: *B. distachyon*, Villa13, CER5, RON-11, CANU14, Jhin13, Bd21 and Bd30; *B. stacei*, Alsur1, Alsur4 and Altab5; *B. hybridum*, RUI11, LARVA20 and Faro3. Genotype selection was made on the basis of their genetic dif- ferentiation (Supplementary Data Fig. S1) and geographical origin (Supplementary Data Table S1). Two technical replicates per sample were conducted.

Data analysis

To analyse differentiation of physiological traits and phytohormone content between the *Brachypodium* species, we conducted both multivariate analyses and general linear mixed models (GLMMs) with maximum likelihood estimates. First, we performed a multivariate analysis of variance (MANOVA) to test the effects of species, soil moisture treatment and their interaction on overall drought-tolerance-related traits and phytohormone content. Second, we fitted separate mixed models for each physiological trait and phytohormone to test the effect of species, soil moisture and their interaction on each variable. In these models, we included genotype nested within species and block as random factors. The block effect was negligible and consistently non-significant and was thus removed from the models.

Next, we used canonical discriminant analysis (CDA) to determine which traits best discriminated between *Brachypodium* species and between *B. hybridum* populations. Trait differentiation between *B. hybridum* populations was also analysed by performing a MANOVA and separate GLMMs for each trait. In this case, factors included in the model were population, soil moisture, their interaction, and genotype nested within population. In addition, we examined the rela- tive contributions of genotype and population to trait variation by conducting a variance decomposition following a hierarchical design and using restricted maximum likelihood estimates. Phenotypic traits and expression data were log-transformed to improve normality and homoscedasticity. Analyses were conducted using JMP 9.0.1 and SAS 9.3 (SAS Institute, Cary, NC, USA). Finally, to investigate between-species differentiation in flowering time we fitted a proportional hazards Cox regression model (Proc PHREG, SAS).

To analyse the ecological correlates of inter-population physiological variation in *B. hybridum*, we first conducted partial correlation analyses between the physiological trait canonical variates (CVs) best discriminating among *B. hybridum* populations and variations in aridity of each population (Supplementary Data Table S1), controlling by geographical origin (latitude and longitude). We used the ppcor package in R (Kim, 2015). Second, we conducted distance-based redundancy analyses (dbRDA; Legendre & Fortin, 2010) using the capscale function in the Vegan package in R (Oksanen *et al.*, 2012) to examine whether variation in drought tolerance related traits, climate and/or geography explain the neutral genetic variation observed between *B. hybridum* populations. Thus, the genetic distance matrix (pairwise FST; Supplementary Data Table S2) was tested against latitude, longitude, aridity and the population's CV scores (from moderate and severe drought conditions) for the first two axes from CDA analyses previously conducted (see above). The significance of the predictors was assessed using multivariate F statistics with 9999 permutations using the anova.cca function in R. We firstly analysed the relationship between the genetic distance matrices and each set of variables separately (marginal test), and then we performed a partial dbRDA (conditional test) for each set of variables while controlling for the influences of latitude and longitude (included as covariates).

RESULTS

Differentiation in drought-tolerance-related traits

Multivariate (Supplementary Data Table S3) and univariate (Table 1) analyses showed interdependent effects of species and soil water content on drought-tolerance-related leaf traits (i.e. significant interaction effects of both factors). In particular, univariate tests showed no significant differences between species in any trait when species were well watered (i.e. 100 % of soil water content; Fig. 1A–D). However, reductions in soil water content decreased average SLA and WC in all species; the magnitude of such decrease depended upon species (Fig. 1A, B). At moderate drought levels, SLA and WC were on average significantly lower for *B. distachyon* than for *B. stacei* and *B. hybridum* (Fig. 1A, B). However, under severe drought conditions, *B. distachyon* had higher SLA and WC than *B. stacei* and *B. hybridum* (Fig. 1A, B). Average EL increased linearly according to diminutions in soil water content for *B. distachyon* (Fig. 1C). Average EL of *B. stacei* and *B. hybridum* did not vary significantly between 100 % and 50 % of soil field capacity; however, under severe drought conditions EL increased significantly in both species (Fig. 1C). Thus, under severe drought, *B. stacei* showed the highest and *B. distachyon* the lowest EL, whereas *B. hybridum* EL was intermediate (Fig. 1C). Free pro- line content did not respond to soil water content variation in *B. distachyon* (Fig. 1D). Contrarily, proline increased significantly for *B. stacei* and *B. hybridum* in a similar manner under moderate and severe drought conditions (Fig. 1D). Genotypic variation within each species was also significant for all traits examined (Table 1; Fig. 2). Water content was the physiological trait with the highest weight for the canonical variate CV1 both in well-watered and severe drought conditions (Supplementary Data Table S4), accounting for 96.3 and 86.5 % of between-species variation, respectively. In moderate drought conditions proline content was the trait contributing most to CV1 (Supplementary Data Table S4), accounting for 93.9 % of between-species variation. The CDAs showed that *B. hybridum* was not differentiated physiologically from *B. stacei* under any soil water condition (Fig. 1), whereas *B. distachyon* always appeared well-differentiated physiologically from *B. hybridum* and *B. stacei* (Wilks' λ = 0.52, P < 0.0001; Wilks' λ = 0.59, P < 0.0001; Wilks' λ = 0.71, P < 0.0001 for well-watered, moderate and severe drought conditions, respectively; Fig. 1).

Table 1. Results of general linear mixed models testing the effects of species, soil moisture content and their interaction on variation of four physiological traits related to water-stress response in the *Brachypodium* species complex. The effect of genotype nested within species was incorporated as a random factor in the models. Significant values ($P < 0.05$) are in bold.

	SLA (m^2 kg^{-1})			EL (%)			WC (%)			Proline (µmol g^{-1})		
Source of variation	df	F	P	df	F	P	df	F	P	df	F	P
Species (S)	2,125	1.40	0.251	2,146	0.09	0.91	2,137	6.22	**0.0026**	2,130	30.58	<**0.0001**
Soil Moisture (T)	2,653	90.43	<**0.0001**	2,643	246.3	<**0.0001**	2,655	337.7	<**0.0001**	2,241	45.05	<**0.0001**
S x T	4,654	9.04	<**0.0001**	4,644	15.58	<**0.0001**	4,657	33.75	<**0.0001**	4,600	17.51	<**0.0001**
Random effects		Z	P		Z	P		Z	P		Z	P
Genotype (species)		4.95	<**0.0001**		4.16	<**0.0001**		2.96	**0.003**		4.48	<**0.0001**

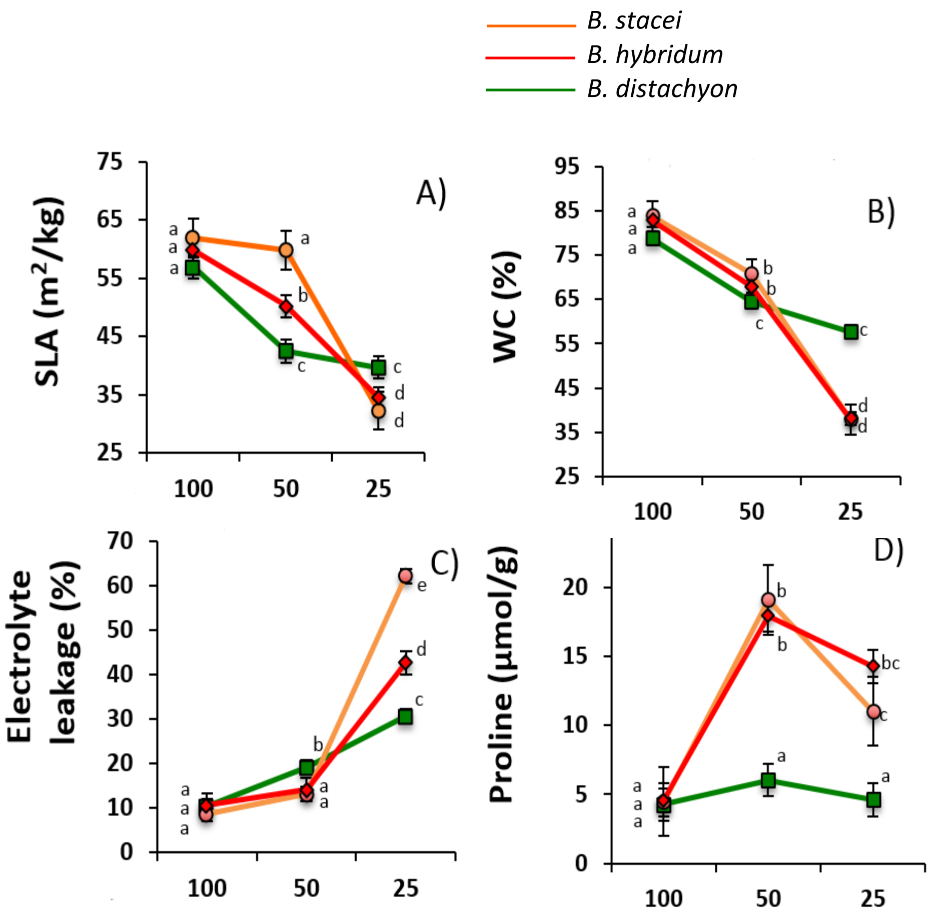

Fig. 1. (A, B, C, D panels) Variation (average values: model-adjusted least squares means ± 1 s.e.) in four drought-tolerance-related physiological traits between the three species of the *Brachypodium distachyon* ploidy complex under three different soil moisture conditions. In each figure, different letters mean significant differences ($P < 0.05$) in post hoc comparisons. (Panels on the next page) Canonical physiological trait variation. Position of the three species of the *Brachypodium distachyon* species complex over the plane defined by the first two canonical variates, CV1 and CV2, obtained from multivariate canonical discriminant analyses conducted on four physiological traits related to water-stress response. Shaded ellipses are the 95 % confidence intervals around the centroid (mean) for each species. Green, *Brachypodium distachyon* (Bd); red, *Brachypodium hybridum* (Bh); orange, *Brachypodium stacei* (Bs). Data points are omitted for clarity.

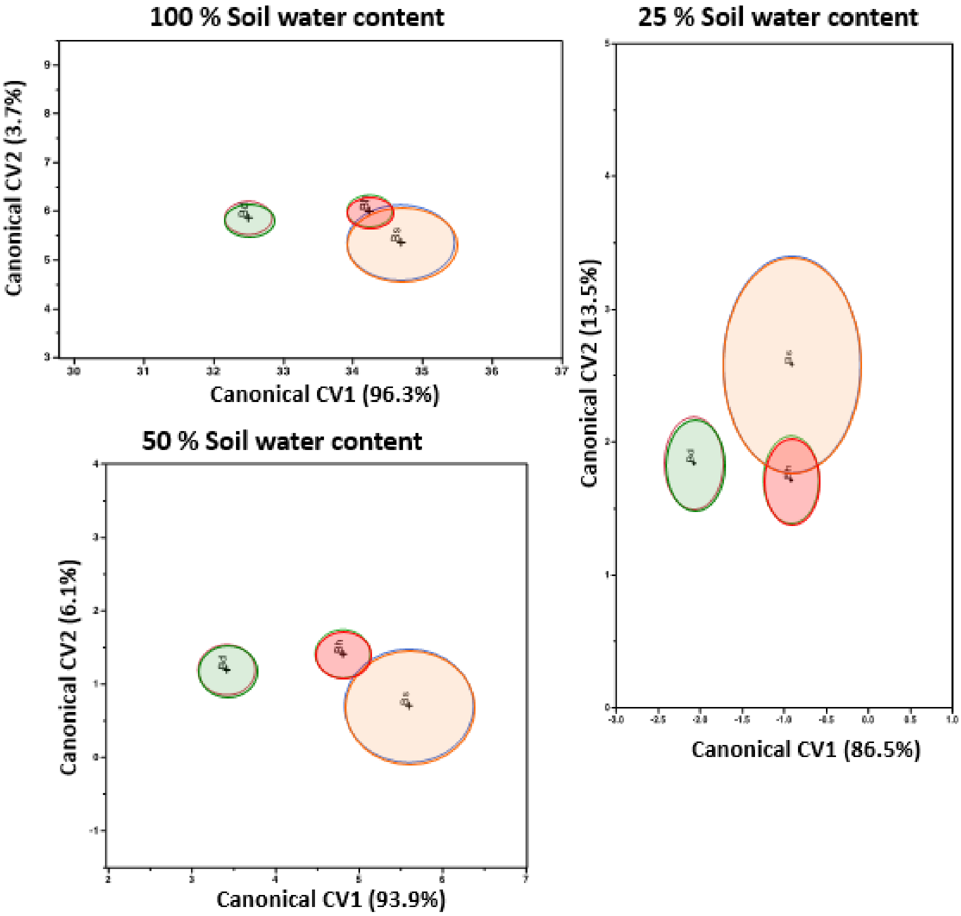

Fig. 1 (continued).

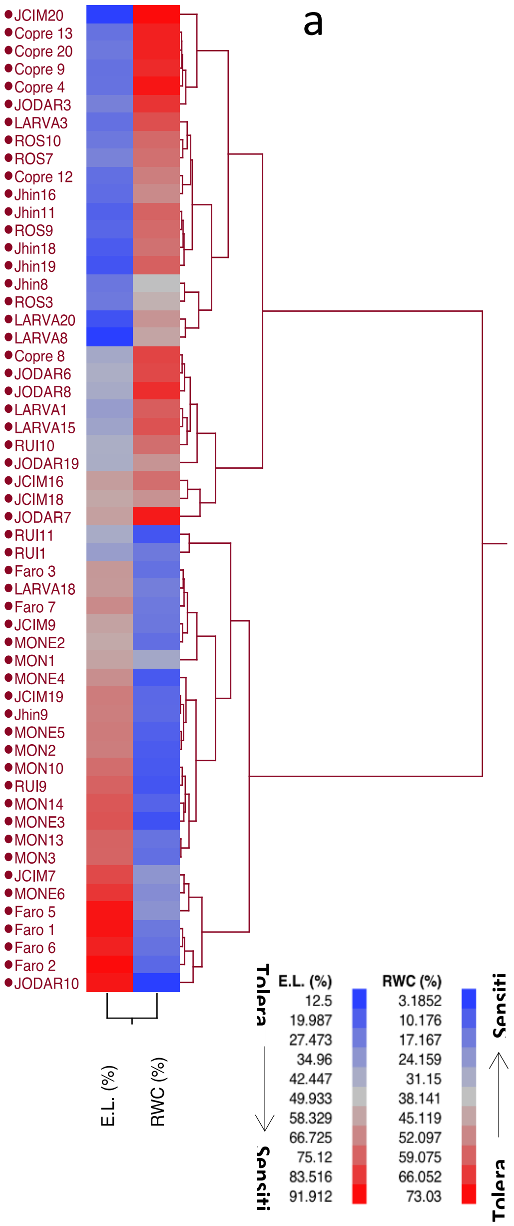

Fig. 2. Hierarchical clustering by Euclidean distance of Electrolyte leakage (E.L.) and plant water content (RWC) (the two traits that best discriminate among genotypes) in the three species of the *Brachypodium distachyon* ploidy complex under severe conditions of water stress (25 % of soil moisture). Inbred lines are sorted from the most drought tolerant to the most drought sensitive from up to bottom. (A) (red), *B. hybridum* allotetraploid lines; (B) (green), *B. distachyon* lines; (C) (orange), *B. stacei* lines.

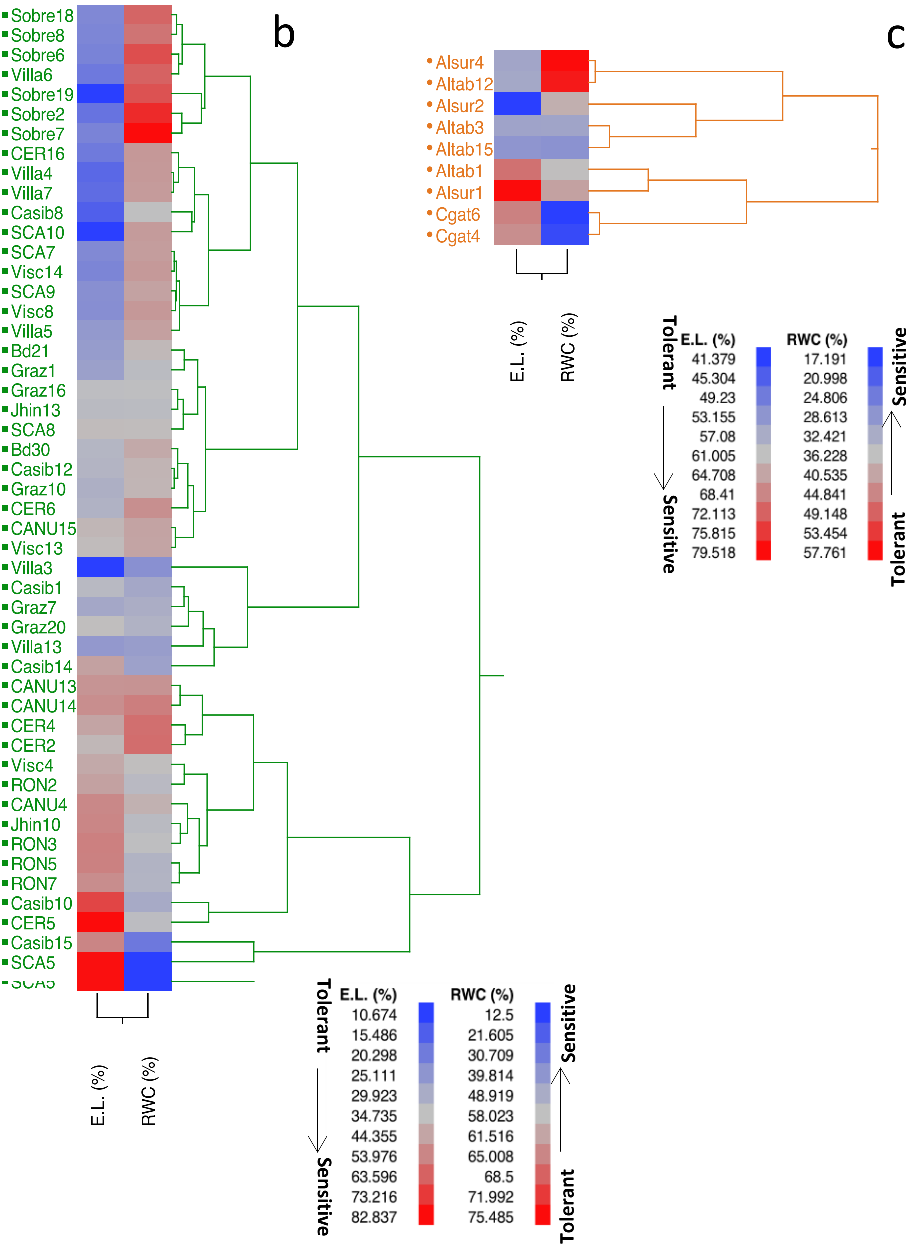

Fig. 2 (continued)

Flowering time

During the trials, 100 % (N = 10) of *B. stacei* lines and 90 % (N = 56) of *B. hybridum* flowered, whereas only 10 % (N = 53) of *B. distachyon* did (Supplementary Data Fig. S2). In fact, the Cox regression model showed a strong, significant effect of species on the probability of flowering along the study period ($\chi2 = 198.67$, d.f. = 2, $P < 0.0001$; Supplementary Data Fig. S2). Observed average flowering times were 39.4 ± 0.32, 43.5 ± 0.51 and 62.7 ± 0.47 d for *B. stacei*, *B. hybridum* and *B. distachyon*, respectively.

Differentiation in phytohormone content

Multivariate (Supplementary Data Table S5) and univariate (Table 2) analyses again indicated interdependent effects of species and soil moisture in phytohormone content. In particular, in all cases the phytohormone content of *B. hybridum* was significantly higher than that of *B. distachyon* and *B. stacei* in moderate and/or severe drought conditions (Fig. 3A–D), suggesting a transgressive pattern in the phytohormone production of *B. hybridum* plants under water stress. Constitutive ABA content of *B. distachyon* was significantly higher than that of *B. hybridum* and *B. stacei* (Fig. 3A). Yet, for *B. distachyon* the ABA concentration was unresponsive to soil water moisture variation whereas in *B. hybridum* and *B. stacei* it increased significantly in moderate and severe drought conditions (Fig. 3A). For the rest of the phytohormones, we detected low or no variation in the phytohormone content in response to soil water content for *B. distachyon* and *B. stacei*. For *B. hybridum* the phytohormone response to soil water content variation was the strongest and significantly higher than for other species (Fig. 3B–D). Multivariate CDAs showed that *B. hybridum* plants were significantly differentiated from *B. distachyon* and *B. stacei* in terms of phytohormone content in all the experimental watering conditions

(Wilks’ λ = 0.045, P = 0.0013; Wilks’ λ = 0.075, P = 0.016; and Wilks’ λ = 0.038, P = 0.0027 for well-watered, moderate and severe drought conditions, respectively; Fig. 3). The SA concentration contributed largely to CV1 in well- watered conditions, and ABA was the phytohormone whose concentration contributed to the largest extent to CV1 under water restriction (Supplementary Data Table S6). This CV1 accounted for 97.26, 88.7 and 96.3 % of between-species variation, respectively (Fig. 3).

Trait differentiation between B. hybridum populations

Multivariate (Supplementary Data Table S7) and univariate (Table 3) analyses showed interdependent effects of population and soil moisture on trait differentiation between *B. hybridum* populations. In general, trait variation was minimal under well- watered conditions, notable in severe drought conditions and intermediate in moderate water limitation (Fig. 4). For example, COPRE genotypes under water stress showed relatively high values of WC and SLA and low EL, which is concordant with a drought-tolerant response (Fig. 4A). In contrast, FARO, MONE and MON genotypes in severe drought conditions showed relatively low WC and SLA values and high EL, concordant with a drought-sensitive response (Fig. 4A). Similarly, free proline content in COPRE and JODAR genotypes was unresponsive to soil water restriction, whereas in MONE, MON, FARO and JCIM genotypes proline increased significantly under water stress (Fig. 4A). For all traits, there was a significant effect of genotype within population (Table 3), although its relative importance was dependent on the drought level. Overall, under well-watered and moderate drought conditions there was higher variance among genotypes within populations than among populations (Fig. 4B). On the contrary, in severe drought condi- tions variance among populations was higher (Fig. 4B). Multivariate CDAs indicated that *B. hybridum* populations were significantly differentiated when soil water was limited, but not under well-watered conditions (Wilks' $\lambda = 0.08$, $P < 0.0001$; Wilks' $\lambda = 0.061$, $P < 0.0001$; and Wilks' $\lambda = 0.376$, $P = 0.1095$ for moderate, severe drought and well-watered conditions, respectively; Supplementary Data Fig. S3). Canonical variates CV1 and CV2 in moderate and severe drought accounted for 89.51 and 93.93 %, respectively, of between-population variation (Supplementary Data Fig. S3). Proline content had the largest weight for the canonical variate CV1, while SLA had the largest weight for CV2 in moderate drought conditions (Supplementary Data Table S8). In severe drought, SLA and EL had the highest weight for CV1 and CV2, respectively (Supplementary Data Table S8).

Table 2. Results of general linear mixed models testing the effects of species, soil moisture content and their interaction on variation in content of four phytohormones in the *Brachypodium distachyon* species complex. The effect of genotype nested within species was incorporated as a random factor in the models. Significant values (P < 0.05) are in bold.

	ABA (ng g^{-1})			JA (ng g^{-1})			IAA (ng g^{-1})			SA (ng g^{-1})		
Source of variation	df	F	P	df	F	P	df	F	P	df	F	P
Species (S)	2,8	3.67	0.074	2,11	0.53	0.601	2,13	6.94	**0.009**	2,10	4.8	**0.032**
Soil Moisture (T)	2,52	154.7	**<0.0001**	2,53	4.2	**0.02**	2,55	3.16	0.052	2,53	3.97	**0.024**
S x T	4,51	59.4	**<0.0001**	4,53	11.7	**<0.0001**	4,54	3.58	**0.011**	4,53	2.43	0.058
Random effects		Z	P		Z	P		Z	P		Z	P
Genotype (species)		0.54	0.586		1.83	0.067		1.23	0.219		1.78	0.075

Table 3. Results of general linear mixed models testing the effects of population, soil moisture content and their interaction on variation of four physiological traits related to water-stress response in *B. hybridum*. The effect of genotype nested within population was incorporated as a random factor in the models. Significant values (P < 0.05) are in bold.

	Specific Leaf Area (m^2 kg^{-1})			Electrolyte Leakage (%)			Plant wáter content (%)			Proline (µmol g^{-1})		
Source of variation	df	F	P	df	F	P	df	F	P	df	F	P
Population (P)	9,54	4.01	**0.0006**	9,57	2.19	**0.0354**	9,59	9.30	**<0.0001**	9,62	8.1	**<0.0001**
Soil Moisture (T)	2,302	99.53	**<0.0001**	2,295	220.3	**<0.0001**	2,299	464.3	**<0.0001**	2,270	70.96	**<0.0001**
P x T	18,301	4.78	**<0.0001**	18,295	5.22	**<0.0001**	18,298	11.32	**<0.0001**	18,267	10.84	**<0.0001**
Random effects		Z	P		Z	P		Z	P		Z	P
Genotype (Population)		2.81	**0.0025**		2.96	**0.0015**		2.01	**0.0223**		2.64	**0.0041**

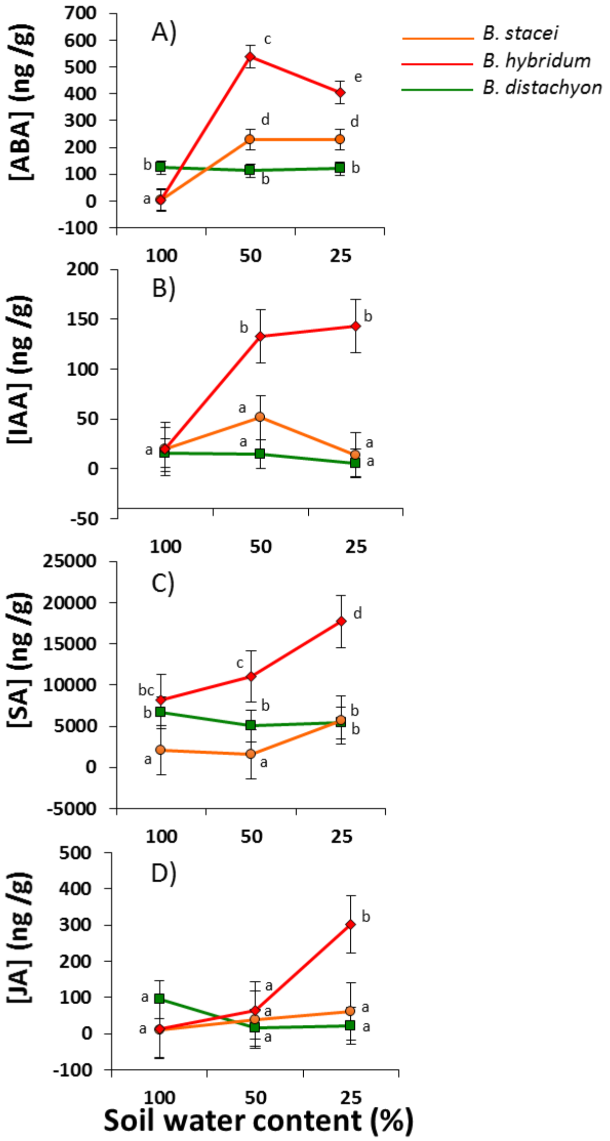

Fig. 3. Variation (average values: model-adjusted least squares means ± 1 s.e.) in the concentration of four phytohormones (expressed as nanograms per gram of fresh tissue) between the three species of the *Brachypodium distachyon* ploidy complex under three different soil moisture conditions. In each figure, different letters mean significant differences (P < 0.05) in post hoc comparisons. (Panels on next page) Position of the three species of the *Brachypodium distachyon* species complex over the plane defined by the first two canonical variates obtained from multivariate canonical discriminant analyses conducted on four phytohormones. Shaded ellipses are the 95 % confidence intervals around the centroid (mean) for each species. Green, *Brachypodium distachyon* (BD); red, *Brachypodium hybridum* (BH); orange, *Brachypodium stacei* (BS).

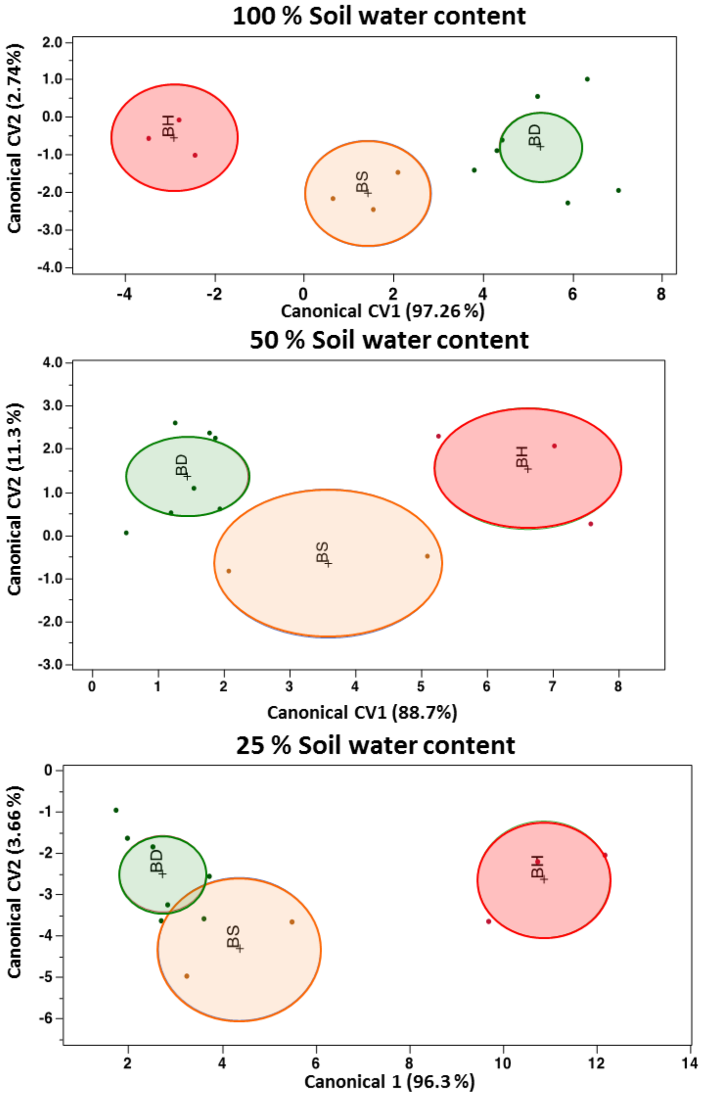

Fig. 3 (continued)

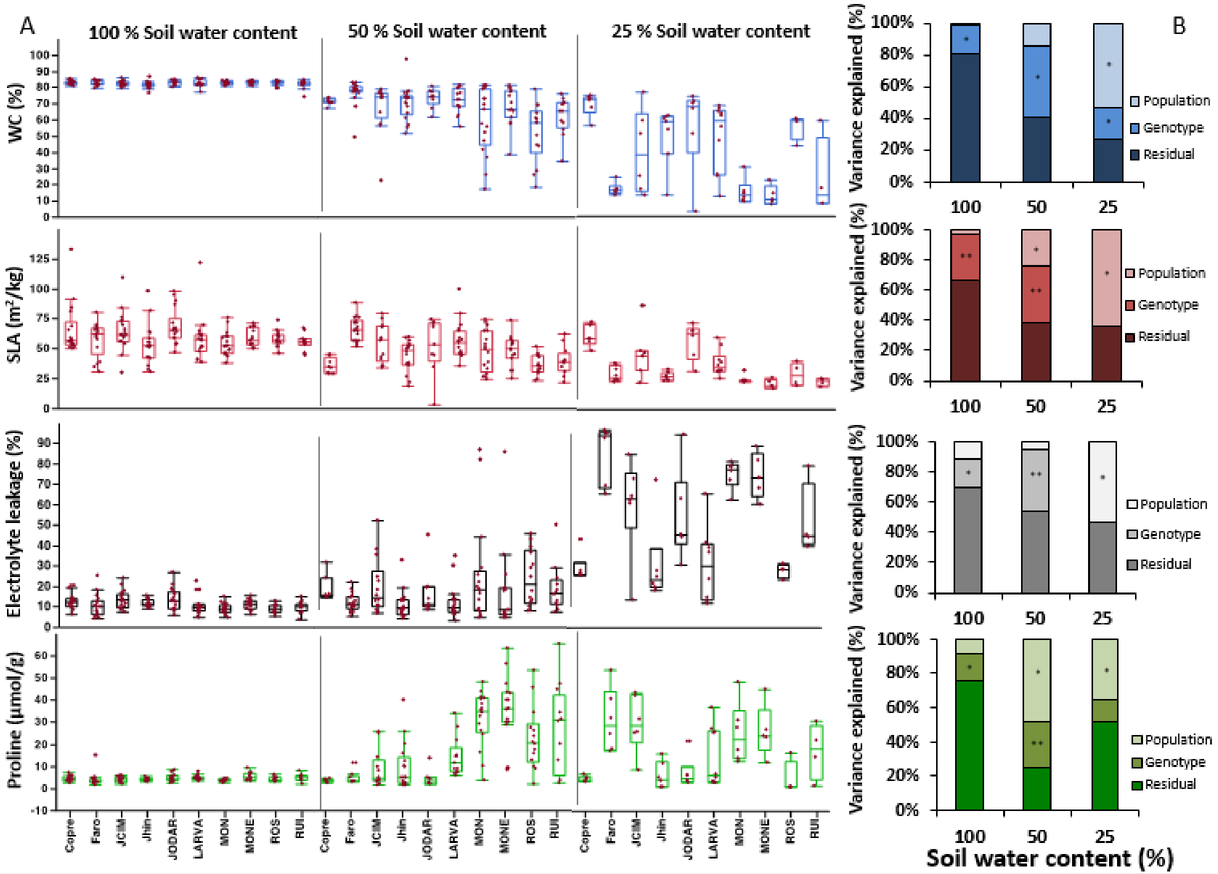

Fig. 4. (A) Box-plot diagrams depicting variation of four drought-tolerance-related physiological traits (plant water content, specific leaf area, electrolyte leakage and free proline content) between ten *B. hybridum* populations under three experimental watering conditions. (B) Variance partitioning of drought-tolerance related physiological traits between *B. hybridum* populations and genotypes within populations. *P < 0.1; **P < 0.001. Residual variance was high and significant in all cases.

Ecological correlates of physiological variation and influence of *B. hybridum* genetic differentiation

Partial correlation analyses indicated that only CV1 was significantly correlated to variation in aridity under moderate water stress (r = 0.74, P = 0.036, N = 10; P > 0.1 for the rest of the cases). Finally, either aridity or trait differentiation significantly explained genetic differentiation between *B. hybridum* populations (Table 4, Fig. 5). Importantly, after controlling by latitude and longitude, trait variation was significantly associated with genetic structure, and this was especially true for trait differentiation in severe drought conditions (Table 4).

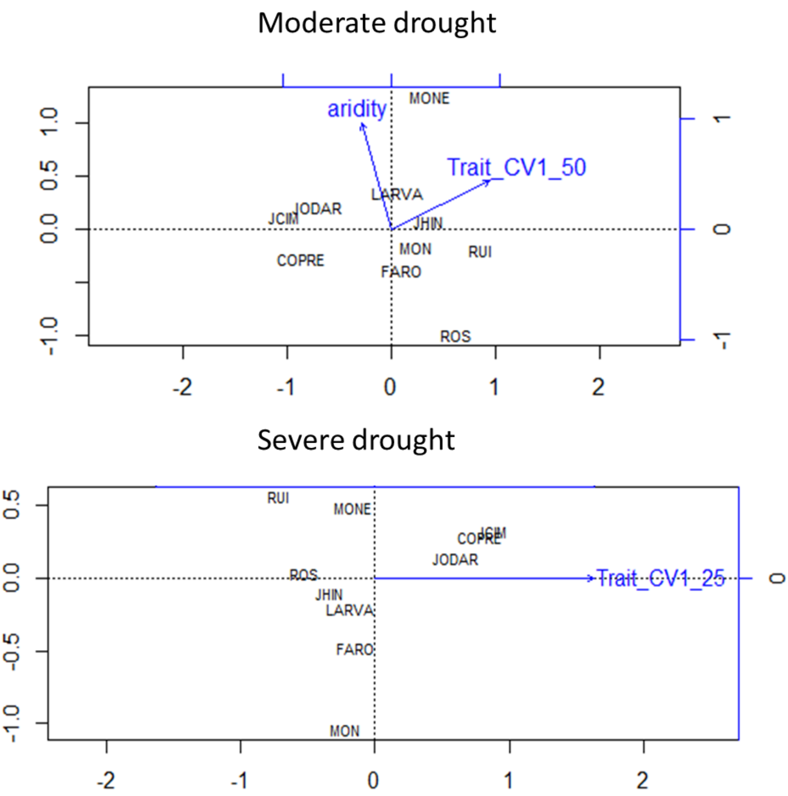

Fig. 5. Distance-based RDA plots (by multidimensional scaling) showing the relative position to the two main RDA axis of 10 *B. hybriudm* populations according to their genetic differentitation. In graphs, the closer the populations are the lower genetic dissimilarity is. Significant explanatory variables (P<0.1) are depicted by blue vectors.

Table 4. Results of distance-based redundancy analyses (dbRDA) testing the effects of geography (latitude and longitude), aridity and trait differentiation (main canonical variates from moderate and severe drought experimental treatments, CV1-50, CV2-50, CV1-25, CV2-25 respectively) on genetic differentiation between ten *B. hybridum* populations across the Iberian Peninsula. The proportion of multivariate genetic variation explained (%var) by a given predictor is indicated. Predictors with P < 0.1 are highlighted in bold.

Marginal tests								Conditional tests							
Variable	*F*	*P*	*%var*	Variable	*F*	*P*	*%var*	Variable	*F*	*P*	*%var*		*F*	*P*	*%var*
Moderate drought				*Severe drought*				*Moderate drought*				*Severe drought*			
Latitude	0.86	0.519	22.2	Latitude	0.73	0.661	4.6								
Longitude	1.15	0.341	7.8	Longitude	1.29	0.258	4.8								
Trait CV1-50	1.98	**0.074**	43.2	Trait CV1-25	2.94	**0.016**	27.6	Trait CV1-50	1.98	**0.086**	43.1	Trait CV1-25	3.09	**0.027**	27.6
Trait CV2-50	1.05	0.408	0.7	Trait CV2-25	1.001	0.444	1.1	Trait CV2-50	1.05	0.400	0.7	Trait CV2-25	0.93	0.507	1.1
Aridity	3.02	**0.012**	30.1	Aridity	0.89	0.554	1.1	Aridity	3.017	**0.023**	30.1	Aridity	1.03	0.402	1.1

DISCUSSION

Our results indicate that the functional response to water stress is overall well differentiated between *Brachypodium* species, although the extent of such differentiation depends on the water stress level and the specific trait considered. We detected a transgressive expression pattern in phytohormone content for *B. hybridum* allotetraploids in response to drought, whereas the leaf-level physiological response to water stress of *B. hybridum* was essentially correlated to the *B. stacei one*. Under drought conditions, Iberian *B. hybridum* populations appear physiologically differentiated. Environmental aridity was associated with *B. hybridum* trait differentiation between populations at moderate levels of water stress. Notably, we detected a link between aridity, physiological differentiation and neutral genetic differentiation at the population scale, which suggests an adaptive origin of genetic divergence between *B. hybridum* populations across the Iberian Peninsula (see also Manzaneda *et al.*, 2015).

Functional differentiation in drought response between Brachypodium species

Different studies have recently demonstrated extensive natural variation in drought-response traits across the native ranges of *B. distachyon* (e.g. Luo *et al.*, 2011; Fisher *et al.*, 2016). However, much less is known about the adaptive significance of this variation in this species (but see Des Marais *et al.*, 2017) and its close relatives, particularly in the context of allopolyploidization and post-polyploidization evolution (but see Manzaneda *et al.*, 2015). Here, we document significant between-species variation in leaf physiological traits in response to water limitation, indicating species-level trait differentiation. Trait expression depended largely upon water treatment, suggesting also trait plasticity, which is an expected response of ecophysiological traits when exposed to environments with contrasting water availability (Nicotra & Davidson, 2010).

Transgressive performance of allotetraploids compared with their diploid progenitor species is a predicted yet elusive outcome of allopolyploidization (Hegarty & Hiscock, 2008; but see Coate & Doyle, 2013). Our results show that the response to water stress of *B. hybridum* allotetraploids was clearly transgressive only for phytohormone content (Fig. 3), while expression of other leaf physiological traits corresponded to *B. stacei* (Fig. 1). Thus, under water restriction *B. hybridum* plants showed a higher concentration of phytohormones in leaves than *B. distachyon* and *B. stacei* plants, which overall showed no or low variation in response to water stress. The CDAs indicated that ABA was the phytohormone that best discriminated *B. hybridum* from its diploid ancestors under water limitation. Elevated endogenous ABA concentration is a well-known primary response of plants to drought stress and is directly implicated in stomatal closure and ROS production, and is also associated with leaf senescence and carbon remobilization under water limitation (Burgess & Huang, 2016). While we are not aware of any previous study comparing phytohormone variation between allopolyploids and their lower-ploidy relatives in the context of drought, augmented ABA concentration, enhanced stomatal closure and reduced transpiration rates in response to water stress have been recently reported in *Arabidopsis* autotetraploid plants compared with diploids (Del Pozo & Ramirez-Parra, 2014). Because we have already shown that under drought stress *B. distachyon* plants reduce stomatal conductance to a larger extent than *B. hybridum* plants (Manzaneda *et al.*, 2015), the higher ABA concentration exhibited by *B. hybridum* allotetraploids in drought conditions might be not related directly to leaf gas exchange in these species. Interestingly, drought-induced increase in endogenous ABA concentration has also been linked to leaf senescence and carbon allocation from stems and leaves, accelerating grain filling in the hexaploid wheat under water stress (Yang *et al.*, 2003), which is concordant with the drought-escape strategy (i.e. rapid development, flowering and seed set) exhibited by *B. hybridum* allotetraploids in drought conditions (Manzaneda *et al.*, 2015). Univariate analyses also show higher IAA, SA and JA concentrations in *B. hybridum* compared with its diploid relatives in water stress. While the effect of drought-induced IAA on plant physiological response is variable depending on species and/ or tissue (Burgess & Huang, 2016), there is growing evidence supporting important roles of SA and JA in regulating plant responses to drought stress (Burgess & Huang, 2016). Thus, SA accumulation has been related recently to drought tolerance enhancement by reducing stomatal conductance and by improving antioxidant capacity and water content in tolerant ecotypes of *Avena sativa* (Sánchez-martín *et al.*, 2015). Endogenous SA and JA levels have also appeared to be elevated in *B. distachyon* in extreme water stress (Fisher *et al.*, 2016). Such phytohormone accumulation, which does not seem directly related to stomatal physiology in *Brachypodium*, could be involved in alternative drought-induced regulatory mechanisms related to the maintenance of primary metabolism (Fisher *et al.*, 2016). Unlike the aforementioned study, we did not find here any drought induction of SA and JA in our *B. distachyon* lines, although our water stress treatments did not reach values as extreme as those reached in their experiments (<15 and 0 % of soil water content; Fisher *et al.*, 2016). In any case, given the importance of phytohormones in regulating drought stress, the higher levels of phytohormones in *B. hybridum* allotetraploids when subjected to drought might have a protective function in enhancing drought response and subsequently contributing to successful persistence of *B. hybridum* populations in drier environments (Manzaneda *et al.*, 2012).

In contrast to phytohormone content, the *B. hybridum* physiological response (including flowering response) to drought was overall correlated to the *B. stacei* one. The CDAs showed that proline in moderate water stress and WC in severe drought were the traits that best discriminated these species from *B. distachyon*. Both traits are indeed negatively correlated across genotypes within each species under water restriction, indicating that the lower the WC the higher the proline content (Fig. 6A). Proline accretion is considered a protective adaptive cellular mechanism against environmental stresses, including drought (Verbruggen & Hermans, 2008). In particular, proline may act primarily as an osmoprotectant, and also may function as a molecular chaperone and as part of the stress signal influencing adaptive responses (Verbruggen & Hermans, 2008). Proline may also play a role in flowering and development, both as a metabolite providing the cell with enough energy to sustain rapid growth and as a signal molecule (Mattioli *et al.*, 2009). However, proline accumulation also depends on the metabolic context (i.e. the relative flux of proline synthesis and catabolism; Bhaskara *et al.*, 2015), drought susceptibility (Rampino *et al.*, 2006; Fisher *et al.*, 2016) and/or complex interactions between genotype and others stressors (e.g. temperature; Des Marais *et al.*, 2017). However, it is still unclear how proline metabolism fits into different drought-tolerance strategies (Bhaskara *et al.*, 2015). Thus, our results show that drought-induced proline accumulation was strong in *B. hybridum* and *B. stacei* plants, which are drought-escapers (both show accelerated flowering at the onset of severe drought; Supplementary Data Fig. S2) and may also be considered as a drought-susceptible species, since for both species the average WC in severe water stress was <55 % (Fig. 1B; Fisher *et al.*, 2016). In contrast, in *B. distachyon*, which is a dehydration avoider (Manzaneda *et al.*, 2015) and may be classified as a drought-intermediate or drought-tolerant species (~ 64 % of the genotypes show WC values >55 %; Fig. 2) (Fisher *et al.*, 2016), proline content did not vary in response to soil drying (Fig. 1D). Our results coincide with observations on other Pooideae, such as *Aegilops*, *Triticum* and *Avena*, in which the proline levels of resistant genotypes were overall lower than those of sensitive ones under drought stress(Rampino *et al.*, 2006; Sánchez-martín *et al.*, 2015). Similarly, Fisher *et al.*, (2016) showed recently that drought-induced proline was significantly elevated in drought-tolerant or intermediate *B. distachyon* ecotypes but not in susceptible ones, which are particularly underrepresented in our samples (Fig. 2). In fact, the three most drought-sensitive *B. distachyon* genotypes in our samples, SCA5, Casib15 and Villa3, exhibited proline levels similar (>20 µmol g^{-1}; Fig. 6A) to those accumulated on average by *B. hybridum* and *B. stacei* (Fig. 1D). How proline accumulation is specifically involved in drought tolerance in sensitive *Brachypodium* lines is beyond the scope of this paper; however, we found a significant negative relationship between proline accumulation and SLA (i.e. light-capturing surface area per unit of dry biomass) across *B. hybridum* and *B. stacei* genotypes under severe drought (Fig. 6B). Reduced SLA is presumed to be a way to improve water-use efficiency in drought stress because photosynthetically active tissue that is more tightly packed (which proline could help preserve by maintaining its osmotic potential) allows more efficient use of a relatively smaller surface (Wellstein *et al.*, 2017, and references therein). Although this link between proline accumulation, SLA reduction and water-use efficiency enhancement still would require experimental evidence, this hypothesis is consistent with the higher water-use efficiency, through the maintenance of photosynthesis, exhibited by *B. hybridum* genotypes under drought stress (Manzaneda *et al.*, 2015).

Thus, our results agree with the findings of a recent metabolomic study conducted on this species complex that reported a closer constitutive metabolomic affinity of *B. hybridum* to its *B. stacei* parent than to its *B. distachyon* parent (López-álvarez *et al.*, 2017). Because the first two species share common xeric ecological niches whereas *B. distachyon* grows in mesic ones (López-Alvarez *et al.*, 2015), such differentiation possibly has an adaptive basis, which is concordant with our previous observations on this system (Manzaneda *et al.*, 2015).

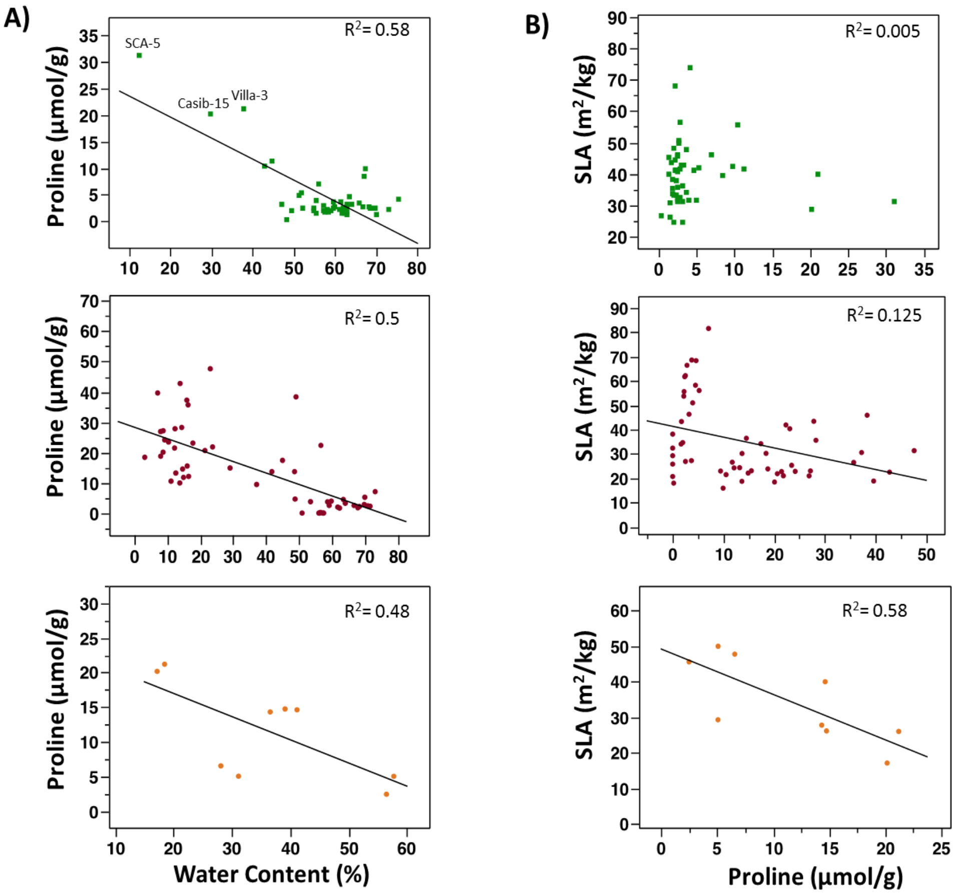

Fig. 6. (A) Relationship between plant water content (genotypic mean) and proline accumulation in severe drought (25 % of soil water content) across genotypes in the three species of the *Brachypodium distachyon* complex. (Top) *B. distachyon* diploids. (Middle) *B. hybridum* allotetraploids. (Bottom) *B. stacei* diploids. Lines depict a significant simple regression fit between variables (summary of fit: *B. distachyon*, $y = 27.5 - 0.39x$, $F_{1,48} = 65.19$, $P < 0.0001$; *B. hybridum*, $y = 28.35 - 0.38x$; $F_{1,54} = 53.02$, $P < 0.0001$; *B. stacei*, $y = 23.7 - 0.33x$; F1,8 = 6.58, P = 0.037). Labels in top left graph correspond to the three *B. distachyon* genotypes most susceptible to drought stress. (B) Relationship between proline accumulation (genotypic mean) and SLA in severe drought (25 % of soil water content) across genotypes in the three species of the Brachypodium distachyon complex. (Top) *B. distachyon* diploids (grey square dots) (Middle) *B. hybridum* allotetraploids (black dots). (Bottom) *B. stacei* diploids (grey dots). Lines depict a significant simple regression fit between variables (summary of fit: *B. hybridum*, $y = 41.35 - 0.44x$, $F_{1,54} = 7.61$, $P = 0.008$; *B. stacei*, $y = 49.21 - 1.28x$; $F_{1,8} = 9.71$, $P = 0.017$).

Differentiation among *B. hybridum* populations

Although rarely addressed, the analysis of the consistency in trait expression among genetically differentiated allopolyploid populations is crucial for the evaluation of the evolutionary trajectories of allopolyploid natural populations after polyploid formation (Soltis *et al.*, 2016). This is especially relevant in allopolyploids with a polytopic origin, like *B. hybridum* (López-Alvarez *et al.*, 2012). Iberian *B. hybridum* populations are genetically differentiated (Hammami *et al.*, 2014) (Supplementary Data Fig. S1C) and our results reveal that the expression of all physiological traits analysed varied significantly among *B. hybridum* populations, yet the magnitude of this effect was dependent on water availability. In relative terms, at constitutive level, inter-population variations did not account for trait expression variation, whereas in severe drought conditions the influence of between-population variation on trait expression was maximal (Fig. 4B), indicating substantial population differentiation in drought-response physiology among *B. hybridum* populations (Manzaneda *et al.*, 2015). The CDAs showed that proline and SLA in moderate drought and SLA and EL in severe drought were the traits that discriminated among Iberian populations (Supplementary Data Fig. S3). Interestingly, in moderate drought inter-population variations in aridity were positively associated with variations in CV1, which was mainly correlated to proline (Supplementary Data Table S8). This indicates that *B. hybridum* genotypes from arid localities express higher free proline when subjected to moderate drought than those from less arid localities, suggesting that such trait differentiation may originate adaptively in response to mild water stress. Although inter-population variation in CV1was not directly associated with aridity under severe water stress, aridity and trait differentiation (either CV1 or CV2) did explain a significant fraction of multivariate neutral genetic variation among *B. hybridum* populations (ranging from 27.6 to 43.2 %; Table 4) in both levels of water stress. Again, this result coincides with previous observations on this species that proved the existence of a clinal differentiation among *B. hybridum* populations in gas-exchange traits across an aridity gradient (Manzaneda *et al.*, 2015). Together, our findings suggest that after polyploid formation and/or colonization Iberian *B. hybridum* populations have adaptively diverged physiologically and genetically in the last ~1 million years in response to local variations in aridity.

CONCLUSIONS

Our previous research on this species complex suggested adaptive differentiation of the allotetraploid *B. hybridum* in dry environments compared with one of the diploid species, *B. distachyon* (Manzaneda *et al.*, 2012, 2015; Rey *et al.*, 2017). However, because the functional response was analysed only in two species of the complex, we could not evaluate in full the expression pattern of drought-functional response of *B. hybridum* compared with both its ancestors. Here we have shown that part of the drought response of allopolyploids is transgressive, which is concordant with the expected benefits derived from ancient heterosis for allopolyploids. On the other hand, we have also demonstrated that, at species level, *B. hybridum* leaf-level functional responses to water stress are essentially correlated to one of its ancestors, *B. stacei*, which concurs with post-polyploidization adaptive evolution. How these two contrasting findings can be reconciled with each other and with the nucleolar dominance described for *B. hybridum*, in which only rRNA loci belonging to the *B. distachyon*-like genome seems to be transcriptionally active (Idziak & Hasterok, 2008; Borowska-zuchowska *et al.*, 2016), will require further attention. Thus, the existence of post-transcription regulation (miR- NAs, cis and trans regulation, etc.) and/or epigenetic factors may also influence drought expression in *Brachypodium* (Des Marais & Juenger, 2016, and references therein), which could alter in turn the expression of gene transcription in *B. hybridum*, yielding functional/phenotypic responses convergent with *B. stacei*. Future studies examining the parental legacy of the *B. hybridum* drought–transcriptome and the phenotype–genotype pattern will be necessary to elucidate this question.

Our understanding of expression in allopolyploids is still limited due to a lack of a precise knowledge of parental identity (i.e. haplotypes) of allopolyploid genotypes from different populations and the specific date of the polyploidy events for many ploidy complexes (Soltis *et al.*, 2016). Here we have shown that Iberian *B. hybridum* populations have adaptively diverged physiologically and genetically in response to local variations in aridity. Futures studies in *B. hybridum* should still relate variation in polyploid gene and/or phenotypic expression to specific polyploidization events, which is a major but elusive question in the evolution of polyploidy (Soltis *et al.*, 2016). In addition, a comparison between synthetic and natural multiple polyploid origins is now possible in *B. hybridum* since stable synthetic allotetraploids have recently been developed (Dinh Thi *et al.*, 2016), which will make it possible to analyse variation in the repeatability of gene and phenotypic expression patterns across natural polyploids and these synthetic lines.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup. com/aob and consist of the following. Table S1: *Brachypodium* ID accessions and geographical origin of the plants included in the study. Table S2: genetic differentiation between ten Iberian *B. hybridum* populations. Table S3: MANOVA to test the effects of species (S), soil moisture content (T) and their interaction on four physiological traits related to water-stress response in the *Brachypodium* species complex. Table S4: weights of the two first canonical variates (CVs) for the four physiological traits included in the multivariate canonical discriminant analysis (standardized scoring coefficients) in each experimental treatment. Table S5: MANOVA to test the effects of species (S), soil moisture content (T) and their interaction on variation of four phytohormones in the *B. distachyon* species complex. Table S6: weights of the two firsts canonical variates (CVs) for the four phytohormones included in the multivariate canonical discriminant analysis (standardized scoring coefficients) in each experimental treatment. Table S7: MANOVA to test the effects of population (P), soil moisture content (T) and their interaction on four physiological traits related to water-stress response in the allotetraploid *B. hybridum*. Table S8: weights of the first two canonical variates (CVs) for the four physiological traits included in the multivariate canonical discriminant analysis (standardized scor- ing coefficients) in the experimental treatments. Figure S1: unrooted neighbour-joining trees of *B. distachyon*, *B. stacei* and *B. hybridum* populations based on Nei's genetic distance. Figure S2: probability of flowering for the three *Brachypodium* species during the study period. Figure S3: canonical physiological trait variation.

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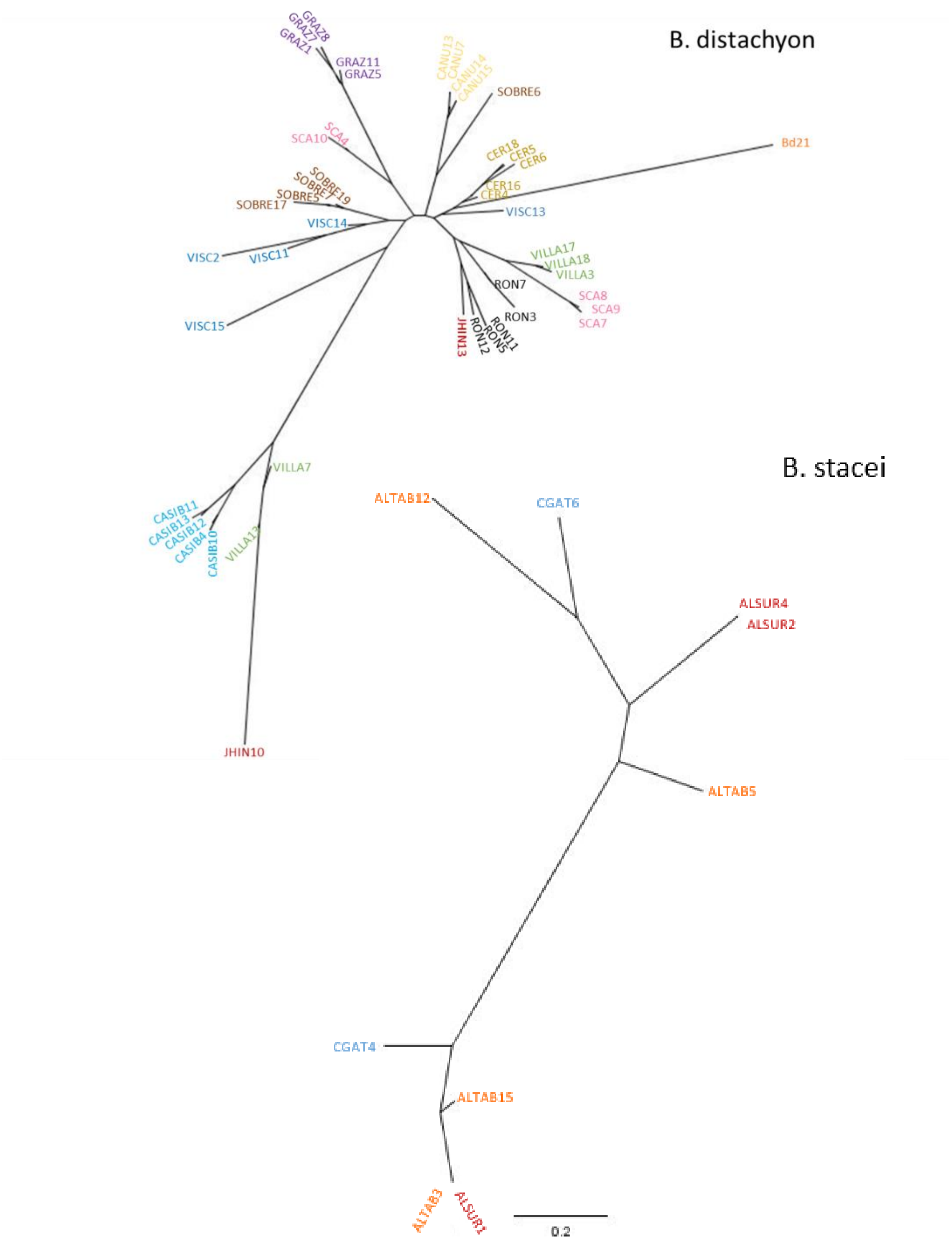
SUPPLEMENTARY MATERIAL

FIGURES



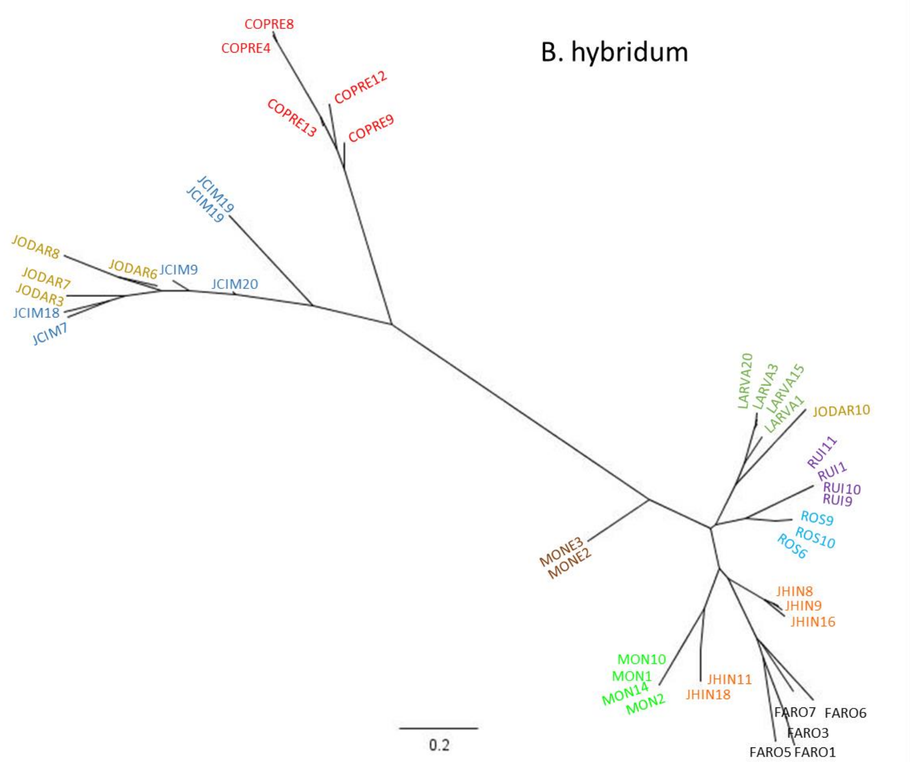


Fig. S1. Unrooted neighbor-joining trees of *Brachypodium distachyon*, *Brachypodium stacei* and *Brachypodium hybridum* populations based on Nei's genetic distance. Unrooted neighbor-joining tree of each specie were constructed using 15 SSR markers showing genetic relationships among individuals from different populations: (A) individuals from 11 *B. distachyon* populations, (B) individuals from 3 *B. stacei* populations and (C) individuals from 10 *B. hybridum* populations. PolySat, ADEGENET and Poppr R packages were used to calculate Nei's genetic distance. Genotypes names and locations names are indicated in Table S1.

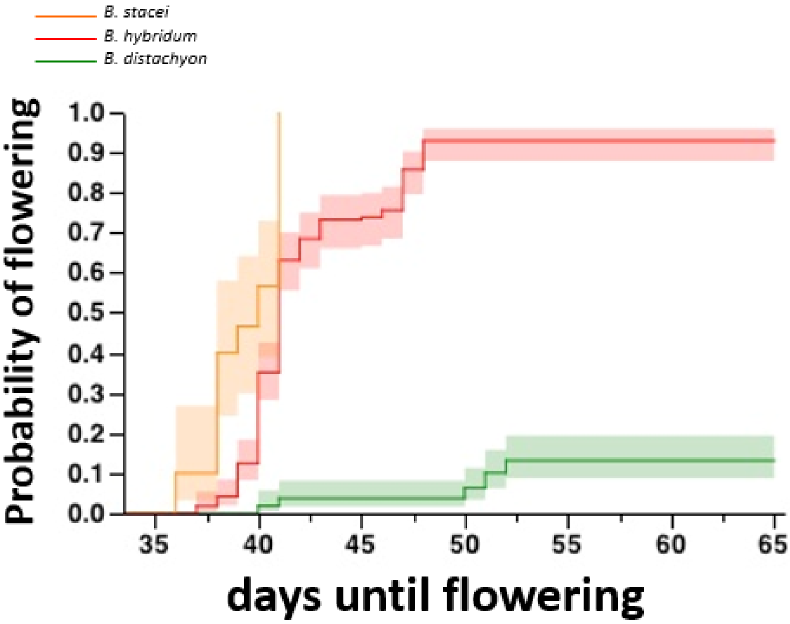


Fig. S2. The probability of flowering for the three *Brachypodium* species along the study period. Shading indicates 95% confidence intervals. No vernalization treatment was applied in the experiment.

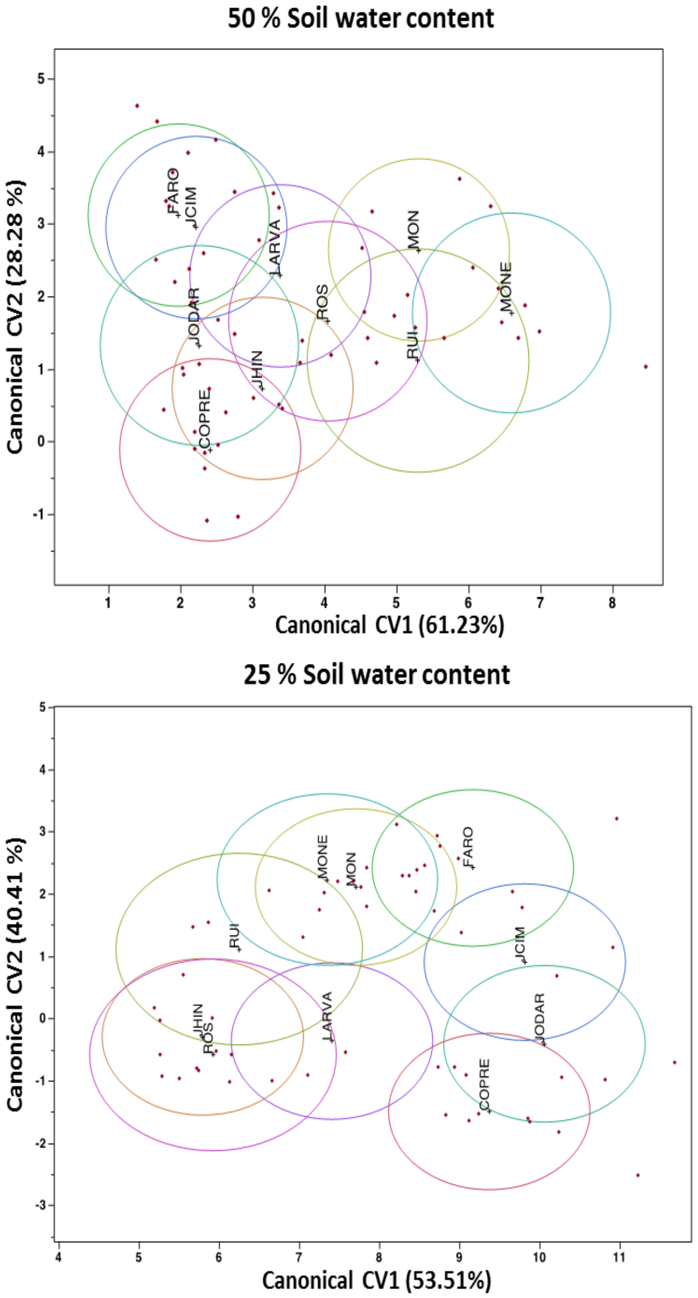


Fig. S3. Canonical physiological trait variation. Position of *Brachypodium hybridum* populations over the plane defined by the first two canonical variates, CV1 and CV2, obtained from Multivariate Canonical Discriminat Analyses conducted on four physiological traits related to water-stress response. Shaded ellipses are the 95% confidence intervals around the centroid (mean) for each population.

TABLES

Table S1. *Brachypodium* ID accessions and geographical origin of the plants included in the study. For *B. hybridum* populations, aridity data from www.cgiar-csi.org are provided in brackets.

Species	Accession	Population	Geographical coordinates	Origin
B. distachyon	Bd21	Mosul	N/A	Iraq (USDA)
	Bd30-1	Sierra Nevada	36º 59' 43" N 3º 34' 18 W	Spain (USDA)
	SCA-5, SCA-7, SCA-8, SCA-9, SCA-10	Sierra Nevada	36º 59' 43" N 3º 34' 18 W	Spain (UJAEN)
	CANU-4, CANU-13 CANU-14, CANU-15	Algeciras	36º 08' 37" N 5º 30' 43" W	Spain (UJAEN)
	CER-2,CER-4.CER-5 CER-6,CER-16,CER-18	Cervera	42º 49' 32" N 4º 29' 27" W	Spain (UJAEN)
	JHIN-10, JHIN-13	Hinojares	37º 43' 41" N 2º 58' 52" W	Spain (UJAEN)
	VILLA-3, VILLA-4, VILLA-5, VILLA-6, VILLA-7, VILLA-13	Villatoya	39º 20' 32" N 1º 20' 47" W	Spain (UJAEN)
	RON-2, RON-3, RON-5 RON-7, RON-11 RON-13	Roncal	42º 46' 50" N 0º 57' 48" W	Spain (UJAEN)
	GRAZ-1, GRAZ-5, GRAZ-7, GRAZ-10, GRAZ-16, GRAZ-20	Grazalema	36º 45' 21" N 5º 26' 30" W	Spain (UJAEN)
	CASIB-1,CASIB-8, CASIB-10, CASIB-12, CASIB-14, CASIB-15	Casas Ibañez	39º 16' 09" N 1º 29' 22" W	Spain (UJAEN)
	SOBRE-2, SOBRE-6, SOBRE-7, SOBRE-8, SOBRE-18, SOBRE-19	Sobrado	42º 31' 54" N 6º 51' 7" W	Spain (UJAEN)
	VISC-4, VISC-8, VISC-13, VISC-14	Íscar	41º 22' 12" N 4º 32' 18" W	Spain (UJAEN)
B. stacei	ALTAB-1, ALTAB-3, ALTAB-5, ALTAB-12, ALTAB-15	Tabernas	37º 02' 28" N 2º 24' 22" W	Spain (UJAEN)
	CGAT-4, CGAT-6	Cabo de Gata	36º 43' 51" N 2º 12' 4" W	Spain (UJAEN)
	ALSUR-1, ALSUR-2, ALSUR-4	Sorbas	37º 05' 79" N 2º 06' 35" W	Spain (UJAEN)
B. hybridum	JHIN-8, JHIN-9, JHIN-11, JHIN-16, JHIN-18, JHIN-19	Hinojares (**0.616**)	37º 43' 41" N 2º 58' 52" W	Spain (UJAEN)
	COPRE-4, COPRE-8, COPRE-9, COPRE-12, COPRE-13, COPRE-20	Los Pedroches (**0.567**)	38º 36' 38" N 5º 9' 3" W	Spain (UJAEN)
	FARO-1, FARO-2, FARO-3, FARO-4, FARO-5, FARO-6	Faro (**0.547**)	37º 0' 52" N 7º 58' 35" W	Portugal (UJAEN)
	MON-1, MON-3, MON-10, MON-13, MON-14	Monfrague (**0.631**)	39º 49' 20" N 6º 02' 52" W	Spain (UJAEN)
	RUI-1, RUI-9, RUI-10, RUI-11	Ruidera (**0.629**)	38º 57' 42 N 2º 52' 17" W	Spain (UJAEN)

	JODAR-3, JODAR-6, JODAR-7, JODAR-8, JODAR-10, JODAR-19	Jodar (**0.65**)	37º 48' 53" N 3º 19' 09" W	Spain (UJAEN)
	LARVA-1, LARVA-3, LARVA-8, LARVA-15, LARVA-18, LARVA-20	Larva (**0.66**)	37º 48' 38" N 3º 12' 53" W	Spain (UJAEN)
	MONE-2,MONE-3,MONE-4 MONE-5,MONE-6	Monegros (**0.678**)	41º 30' 50" N 0º 30' 30" W	Spain (UJAEN)
	ROS-3,ROS-6,ROS-7,ROS-9 ROS-10	Rosas de Mar (**0.347**)	42º 14' 45" N 3º 11' 1" E	Spain (UJAEN)
	JCIM-7, JCIM-9, JCIM-16 JCIM-18, JCIM-19, JCIM-20	Cimbarra (**0.62**)	38º 23' 28" N 3º 22' 13" W	Spain (UJAEN)

Table S2. Genetic differentiation between ten Iberian *B. hybridum* populations. Pairwise F_{st} values are shown below the diagonal. PolySat, ADEGENET and Poppr R packages were used to calculate genetic distance values using 15 SSRs markers. Locations names are indicated in Table S1.

	LARVA	JCIM	COPRE	MON	JODAR	FARO	JHIN	RUI	MONE	ROS
LARVA	-									
JCIM	0.390	-								
COPRE	0.413	0.264	-							
MON	0.344	0.444	0.440	-						
JODAR	0.319	0.138	0.253	0.378	-					
FARO	0.280	0.368	0.353	0.270	0.310	-				
JHIN	0.283	0.358	0.358	0.230	0.298	0.162	-			
RUI	0.353	0.443	0.447	0.443	0.378	0.324	0.245	-		
MONE	0.333	0.372	0.377	0.418	0.315	0.303	0.258	0.294	-	
ROS	0.291	0.400	0.405	0.321	0.334	0.243	0.216	0.208	0.338	-

Table S3. MANOVA to test the effects of species (S), soil moisture content (T), and their interaction on four physiological traits related to water-stress response in the *Brachypodium* species complex.

Source	Wilks' λ	df	F	P
Species (S)	0.876	8, 1130	9.63	**<0.0001**
Soil Moisture (T)	0.412	8, 1130	78.71	**<0.0001**
S x T	0.732	16, 1726	12.1	**<0.0001**
Genotype (species)	0.275	464, 2262	1.85	**<0.0001**

Table S4. Weights on the two firsts canonical variates (CV) of the four physiological traits included in the multivariate canonical discriminant analysis (standardized scoring coefficients) in each experimental treatment (i.e., 100%, 50% and 25% of soil moisture content). The most important trait value in each canonical variate is in bold.

	Soil moisture content (%)					
	100		50		25	
Trait	CV1	CV2	CV1	CV2	CV1	CV2
SLA	-0.183	-0.233	0.361	**-1.174**	0.302	0.011
EL	-0.077	**1.01**	-0.313	-0.064	0.004	**1.195**
WC	**1.06**	0.121	0.362	0.796	**-0.988**	0.069
Proline	-0.097	-0.195	**1.025**	0.559	0.189	-0.943

Table S5. MANOVA to test the effects of species (S), soil moisture content (T), and their interaction on variation of four phytohormones in the *Brachypodium distachyon* species complex.

Source	Wilks' λ	df	F	P
Species (S)	0.296	8, 96	10.02	**<0.0001**
Soil Moisture (T)	0.084	8, 96	29.32	**<0.0001**
S x T	0.096	16, 147	10.60	**<0.0001**
Genotype (species)	0.082	40, 184	4.29	**<0.0001**

Table S6. Weights on the two firsts canonical variates (CV) of the four phytohormones included in the multivariate canonical discriminant analysis (standardized scoring coefficients) in each experimental treatment (i.e., 100%, 50% and 25% of soil moisture content). The most important trait value in each canonical variate is in bold.

	Soil moisture content (%)					
	100		50		25	
Trait	CV1	CV2	CV1	CV2	CV1	CV2
ABA	1.263	1.66	**0.879**	-0.472	**1.038**	-0.721
JA	**2.635**	-1.318	-0.538	-0.774	-0.071	-1.385
IAA	1.415	**-1.818**	0.362	0.796	0.886	**2.48**
SA	**-3.846**	1.242	0.156	**1.118**	0.134	-1.586

Table S7. MANOVA to test the effects of population (P), soil moisture content (T), and their interaction on four physiological traits related to water-stress response in the allotetraploid *Brachypodium hybridum*.

Source	Wilks' λ	df	F	P
Population (P)	0.363	36, 950	8.18	**<0.0001**
Soil Moisture (T)	0.184	8, 506	83.97	**<0.0001**
P x T	0.274	72, 997	5.40	**<0.0001**
Genotype (Population)	0.263	184, 1012	2.18	**<0.0001**

Table S8. Weights on the two firsts canonical variates (CV) of the four physiological traits included in the multivariate canonical discriminant analysis (standardized scoring coefficients) in the experimental treatments of 50% and 25% of soil moisture content. The nost important trait value in each canonical variate is in bold.

	Soil moisture content (%)			
	50		25	
Trait	CV1	CV2	CV1	CV2
SLA	-0.443	**1.498**	**0.968**	-0.243
EL	-0.358	0.405	0.899	**0.483**
WC	0.633	-0.891	0.478	-0.252
Proline	**1.261**	-0.004	0.402	0.312

NOTES 1

Proline extraction and quantification.

Proline was extracted by grinding 100 mg of dried plant material with 600 ml of 3% (w/v) sulfosalicylic acid. The extract was centrifuged at 13 000 rpm for 15 min at room temperature. A 0.3 ml aliquot of the extract was mixed with 1.5 ml of a solution of acidic ninhydrin [40% acidic ninhydrin (8.8 mM nihydrin, 10.5 M glacial acetic acid, 2.4 M orthophosphoric acid), 40% glacial acetic acid and 20% of 3% sulfosalicylic acid]. The samples were then incubated for 60 min at 96 ºC and the reaction was terminated by incubating the samples on ice for 5 min. Samples were then extracted by adding 3 ml of toluene and vortexing for 20 s. Absorbance at 520 nm was measured using toluene as a reference. A standard curve was made using L-proline in a range of 0 – 57.5 mg ml^{-1}. Free proline content was calculated according to Bates et al., (1973): Free proline = (mg proline ml^{-1} × ml toluene) /115 x 5 mg $mmol^{-1}$/ (g sample/5).

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CAPÍTULO 2

EXPRESSION LEVEL DOMINANCE IN RESPONSE TO WATER STRESS AND HOMOEOLOGOUS EXPRESSION BIAS IN THE ALLOTETRAPLOID *BRACHYPODIUM HYBRIDUM* (POACEAE).

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Manuscrito inédito

Abstract

Allopolyploidy involves massive genetic changes in the newly formed allopolyploids, including nonadditive expression of homoeologous genes that may increase phenotypic plasticity and adaptation to environments. However, our knowledge about the effects of abiotic stresses on nonadditive expression patterns is scarce. Here, we conduct the first comparative drought transcriptome analysis in the *Brachypodium distachyon* species complex, a tractable polyploid system to investigate sub-genome dominance and origin of adaptive responses of allopolyploids.

By performing transcriptome analyses and RT-qPCR, we analyze whole-genome differential expression between *B. hybridum* allopolyploids and its diploid closest ancestors *B. distachyon* and *B. stacei* in response to drought, variation in gene expression among *B. hybridum* genotypes and parental legacy in the allopolyploid.

B. hybridum exhibited a stronger transcriptional response to drought compared to its diploid ancestors. Drought-gene expression among *B. hybridum* genotypes varied, although, a strong drought-induction was recorded for late embryogenesis abundant proteins. *B. distachyon* expression-level dominance and transgressive expression were the most common expression patterns of the homoeologs genes in *B. hybridum* under water stress.

The ability of *B. hybridum* to tolerate drought did not result from differential use of *B. stacei*-inherited homeologs, but differential gene expression is probably dependent on a small number of regulators, which are themselves probably dependent on aridity.

Keywords: *Brachypodium*, expression bias, gene expression, late embryogenesis abundant proteins, parental legacy, polyploidy, transcriptome, water stress.

INTRODUCTION

Allopolyploidization (hybridization and whole genome duplication) has long been recognized as a prominent mode of plant speciation and adaptation across a wide range of environments (Adams & Wendel, 2005; Madlung, 2013). Allopolyploid plants involve the fusion of distinct parental genomes which can lead to numerous genetic and epigenetic modifications and often the formation of new phenotypes (Doyle *et al.*, 2008; Parisod, 2012). Duplicate gene copies in the new allopolyploid (termed homoeologs) may alter their expression patterns compared to their ancestors showing expressions deviated from parental additivity (i.e., deviated from the arithmetic average of the parental expression level; Buggs *et al.*, 2014; Yoo *et al.*, 2014). Several studies have shown that nonadditive expression is widely observed in different allopolypoid species (Wang *et al.*, 2006; Stupar *et al.*, 2007; Pumphrey *et al.*, 2009; Chagué *et al.*, 2010; Kim & Chen, 2011; Takahagi *et al.*, 2018; Wu *et al.*, 2018), which may involve at least three possible scenarios: (1) the total expression level of a homologous gene pair in an allopolyploid is statistically similar to only one of the two diploid parental (expression level dominance [namely ELD]); (2) the total gene expression is lower or higher compared to both parents (transgressive expression); and (3) preferential expression of one homoeolog relative to the other in the allopolyploid contributing unequally to the total gene expression (homoeolog expression bias) (Grover *et al.*, 2012a; Yoo *et al.*, 2014).

Nonadditive expression is biologically significant because it may increase the phenotypic plasticity potential in the newly formed allopolyploid (Doyle *et al.*, 2008; Jackson & Chen, 2010), which could result in enhanced abiotic stress resistance and its adaptive response to stress compared to their parental species (Sattler *et al.*, 2016; Shimizu-Inatsugi et al., 2017). Thus, it is known that abiotic stress conditions (e.g., drought, temperature, metal toxicity, etc.) may alter the homoeologous gene expression in allopolyploids (Liu & Adams, 2007; Dong & Adams, 2011; Shimizu-Inatsugi et al., 2017), however, we still know very little about the effects of environmental stresses on nonadditive expression patterns (but see Bardil *et al.*, 2011; Paape *et al.*, 2016).

In the *Brachypodium distachyon* (Poaceae) species complex, the natural allotetraploid *Brachypodium hybridum* (2n = 4x = 30) is derived from bidirectional crosses of its parents *Brachypodium distachyon* (2n=2x=10) and *Brachypodium stacei* (2n=2x =20) that occurred repeatedly approximately 1 million years ago (Catalán *et al.*, 2016). In this complex, *B. stacei* is the oldest linage that arose ~10 million years ago, while *B. distachyon* emerged ~ 7 million years ago (Scholthof *et al.*, 2018). These three annual species have been recently proposed as a tractable grass polyploid system to investigate the effects of subgenome dominance and the origin of phenotypic, physiological, and adaptive responses of allotetraploid hybrids (Catalán *et al.*, 2016). This is due to the notorious subgenome integrity of the allopolyploid and the knowledge accumulated on this species complex, at the genetic, cytogenetic and phylogenetic level. These species grow across the Mediterranean region are ecologically and genetically differentiated (Vogel *et al.*, 2009; Manzaneda *et al.*, 2015). *B. distachyon* grows in higher, cooler and wetter areas than *B. stacei*, which grows at low altitude in warmer and drier environments, whereas *B. hybridum* occurs in zones with intermediate conditions and occupies the largest growth area compared to its ancestors (Manzaneda *et al.*, 2012; López-Alvarez *et al.*, 2015). This distribution pattern suggests that species diversification may be associated to particular adaptations to climate (López-Alvarez *et al.*, 2015). In particular, previous research suggests that Iberian *B. hybridum* populations may have adaptively diverged physiologically and genetically in response to variations in aridity (Manzaneda *et al.*, 2015; Martínez *et al.*, 2018). On the other hand, (Idziak &

Hasterok, 2008), and more recently (Borowska-zuchowska *et al.*, 2016) have described nuclear dominance in which only *B. distachyon*-like 35S rDNA loci were transcriptionally active in *B. hybridium* (Borowska-zuchowska *et al.*, 2016). This expression pattern contrasts with the drought-functional response of *B. hybridum* which is correlated to *B. stacei* response, or shows a transgressive pattern for some other traits (Martínez *et al.*, 2018).

Previous studies have analyzed the drought transcriptome of *B. distachyon* by exploring the variation in the whole-genome expression level among different *B. distachyon* genotypes, which has allowed to detect some candidate genes involved in the drought response of temperate cereals (Gordon *et al.*, 2014; Priest *et al.*, 2014; Shaar-Moshe *et al.*, 2015; Chen *et al.*, 2016). However, to our knowledge, only one comparative transcriptome analysis between the three species of the *Brachypodium* complex exits related to heat acclimation (Takahagi *et al.*, 2018), and no study on water stress exists, which is essential for understanding the genetic basis of ecological differentiation in this species complex (Manzaneda *et al.*, 2012; López-Alvarez *et al.*, 2015). While the first type of aforementioned studies are key to reveal gene function, multispecific global comparative transcriptome analyses are necessary to understand the origin of genetic and physiological drought response in the allotetraploid *B. hybridium*, and to evaluate whether homeolog gene expression changes in the *B. hybridum* drought-transcriptome may have contributed to phenotypic variation and differentiation of this species in drier environments (Manzaneda *et al.*, 2015).

Here, we have conducted the first comparative drought whole transcriptome analysis of allotetraploid *B. hybridum* and its ancestors. In particular, we investigate: (1) whole-genome differential expression pattern of *B. hybridum* and its diploid ancestors in response to drought; (2) variation in gene expression, by RT-qPCR, among *B. hybridum* genotypes with different tolerances to water stress and from different geographical origin; (3) global patterns of differential expression in the allotetraploid *B. hybridum* by comparing the total expression of the homoeologs in *B. hybridum* relative to the expression levels found in the ancestor species; and (4) the magnitude and directionality of homoeolog expression bias (defined as relative contribution of homoeologs to the transcriptome; Yoo *et al.*, 2013). Because the drought-functional response of *B. hybridum* is correlated to the one of *B. stacei*, we hypothesize that homoeologs inherited from *B. stacei* may have contributed more to the transcriptional drought-response in *B. hybridum*.

MATERIAL AND METHODS

Plant material

To analyze transcriptome variation and parental legacy in allopolyploid gene expression, we used 3 different inbred lines from the *B. distachyon* species complex: the allotetraploid *B. hybridum* line LARVA-20, the *B. stacei* diploid line ALSUR-1, and *B. distachyon* diploid line Bd30-1. All these lines derived from natural accessions collected from different Iberian Peninsula populations in 2007-2008 (UJAEN *Brachypodium* germplasm collection located in Departament de Biología Animal, Biología Vegetal y Ecología, University of Jaén, Spain). These lines come from nearby populations located at the southeast of Iberian Peninsula (within a radius of 120 km; Table S1, Fig. S1) growing under Mediterranean climate. Bd30-1 occurs naturally in a pure *B. distachyon* population at Sierra Nevada (Table S1), while LARVA-20 and ALSUR-1 grow in *B. hybridum* / *B.distachyon* and *B.hybridum* / *B.stacei* mixed populations respectively, and where *B. hybridum* is the most abundant species (Manzaneda *et al.*, 2012; López-Alvarez *et al.*, 2015). Bd30-1, belongs genetically to the group of *B. distachyon* western lines (*S+;* sensu (Gordon *et al.*, 2017) that does not show a delayed-flowering phenotype. ALSUR1 is genetically well-differentiated from other *B. stacei* lines from southern Spain and shows an early flowering phenotype that does not require vernalization (Martínez *et al.*, 2018). Finally, LARVA-20 clusters along with other southeastern Iberian genotypes and similarly does not require vernalization to flower (Martínez *et al.*, 2018). Genetic studies based on barcoding nuclear loci have shown that *B. distachyon* and *B. stacei* subgenomes of *B. hybridum* are overall intact compared to the studied genomes of current ancestor diploid species (López-Alvarez *et al.*, 2012).

Growth conditions and treatments

Seeds were incubated on wet filter paper in sealed Petri dishes, and then stratified at 4 ºC for 1 week to facilitate uniform seed germination. Once germinated, four seedlings per line were individually planted in pots (7x7x8 cm) in a randomized complete block design and grown on sterile soil containing perlite, sand, and organic substrate (0.5:0.5:1, v/v). Seedlings were grown in a growth chamber at 21ºC with a photoperiod of 16/8 h (day/night), humidity at 65% and luminosity 120-200 µmol photons m^{-2} s^{-1}. After three weeks of growing in well-watered conditions, plants selected for the drought treatment were subjected to water stress conditions by ceasing irrigation completely during 21 days, and reaching 25 % of field capacity. Plants in control treatment were watered weekly during this period. After 21 days of treatment, 0.1 g of fresh plant leaves from both treatments were collected and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA isolation, sequencing and gene expression analysis

Total RNA was isolated from the aerial parts (100 mg of homogenized tissue) using TRIzol® Reagent (Gibco-BRL, Life Technologies, Paisley, UK). RNA samples were treated with DNase RNase-Free (Invitrogen^{TM}) and purified using RNeasy Plant Mini Kit (Qiagen). We extracted RNA samples from two independent biological replicates per line and treatment. The RNA quality was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) using a RNA 6000 Pico assay kit (Agilent Technologies). Sequencing was performed at the Centro de Investigación Biomédica de la Rioja (https://www.cibir.es/es/). Twelve sequencing libraries were prepared using the TruSeq mRNA sample preparation kit (Illumina) following the manufacturer's protocol. The resulting libraries were loaded onto an Illumina Genome Analyzer IIX instrument and subjected to 150 cycles of single-end sequencing.

Raw Illumina RNA-seq reads were first preprocessed using Fastqmcf (Aronesty, 2011) by discarding primers and reads with adaptors, unknown nucleotides, and poor quality or short-length reads, increasing the Q score above 30 for all the libraries and lengths greater than 50 bp (Q30L50). A thorough quality control of sequencing was then performed twice using FastQC software (version 0.10.1, http://www.bioinformatics.bbsrc. ac.uk/projects/fastqc; (Schmieder & Edwards, 2011) to provide a summary and compare statistics files before and after preprocessing. Firstly, all high-quality reads from each sample that passed the quality threshold were aligned to the annotated *Brachypodium distachyon* reference genome *Brachypodium distachyon* v3.1 (Phytozome) using Bowtie2 software (Langmead & Salzberg, 2013). These mapped reads were statistically evaluated for differential gene expression analyses between treatments (control and drought stress) within each species. In addition, secondly, differences in gene expression across species were statistically evaluated comparing the allopolyploid *B. hybridum* to their diploid progenitors. In this case, processed reads from *B.distachyon*, *B.stacei* and *B.hybridum* were mapped to the *Brachypodium distachyon* v3.1, *Bracypodium stacei* v1.1 and *Brachypodium hybridum* v1.1 reference genomes respectively (Phytozome) using Bowtie2.

After alignments, transcript abundance of each gene was estimated with the RSEM software (Li & Dewey, 2011) generating RPKM data (*Reads Per Kilobase of exon model per Million mapped reads).* For differential gene expression analyses, the read count files of each species (including biological replicates per treatments) were combined and normalized to a gene expression matrix. Thus, differentially expressed genes (DEGs) and its statistical significance, between treatments (drought vs. well-watered), and between species, was calculated by analyzing mapped sequence counts using the DESeq2 package (Love *et al.*, 2014) in R software (R Core Development Team, 2015). For each gene, a fold-change value of 2 between experimental conditions (i.e., treatments and species) was used to identify DEGs. Statistical significance was estimated using an adjusted p-value ≤ 0.05 for multiple testing with a Benjamini-Hochberg correction.

Hierarchical clustering of expression profiles was performed for DEG shared genes between the three species. Log2-fold change values from the well-watered to the water stress conditions were used based on the mean results of biological replicates. Genes with standard deviations ≥1.5 over three species were selected to analyze interspecific gene expression differences. In total, 710 genes were used for the clustering analysis, using Ward's hierarchical clustering method with Euclidean distance.

RT-qPCR analyses of gene expression

Total RNA was isolated from 8 *B. hybridum* genotypes, FARO-3, FARO-5, JCIM-20, JHIN-8, LARVA-20, MONE-3, ROS-9 y RUI-11. *B. hybridum* genotype selection was made according to their genetic differentiation (Martínez *et al.*, 2018), geographical origin and differential tolerance to water stress (drought-sensitive: FARO-5 and MONE-3; drought-tolerant: JCIM-20, JHIN-8, ROS-9 and LARVA-20; intermediate: FARO-3 and RUI11; Martínez *et al.*, 2018). First-strand cDNA was synthesized from 1µg using the First-Strand cDNA Synthesis Kit for RT-PCR (AMV, Roche). cDNAs were then diluted at 1:5. Gene-specific primers of 26 drought-response candidate genes (Table S2) were designed using Oligo Primer Analysis software v. 6.65. Selected genes are involved in six key biological processes: osmoregulation, molecular chaperones, photosynthesis, ROS scavenging, regulatory functions and flowering time (Table S2). Influence of geography and genetic differentiation on drought-gene expression was analyzed by using distance-based redundancy analyses (dbRDA; Legendre & Fortin, 2010). Real-time quantitative PCR amplification was performed following procedure detailed in Supplementary Material Appendix 1. For each gene, calculation of normalized relative quantities (i.e., relative mRNA expression data) from Cq values were computed following the procedures described in Hellemans et al., (2007). Three technical measurements were conducted.

Analyses of ELD and homoeolog expression bias

To study the changes in ELD and homoeolog expression bias in the allopolyploid *B. hybridum*, we monitored the expression levels of the 12952 *B. distachyon*-*B. stacei*-*B. hybridum* orthologous genes. To identify homologous genes between the three species, cluster analysis of the transcripts with a sequence similarity greater than 85% were performed. Of the 12952 homoeolog pairs, 10468 pairs were expressed

(FPKM ≥1) in the three species. We then assigned the homoeologs in *B.hybridum* LARVA20 to their appropriate subgenome by comparing the individual homoeolog sequences from the polyploid to their orthologous counterparts in the diploid ancestors (two orthologs from the progenitors and one from each of the homoeologs). For ELD analysis, considers the total expression level of a homologous gene pair in the allopolyploid compared with the relative expression levels in its two parents. Thus, twelve possible outcomes of differential expression, including ELD, additivity and transgressive response, were classified according to Yoo *et al.*, 2014. We defined genes showing conserved inheritance if the fold change was < 1.5 (Zhang *et al.*, 2018). Accordingly, genes were classified as the follows: (1) Additivity: the total expression level in hybrid was an intermediate (i.e., average) between parents ; (2) Bd-ELD: the total expression level in hybrid was similar (fold change < 1.5) to the *B. distachyon* subgenome parent, but different from the *B. stacei* subgenome parent (fold change > 1.5); (3) Bs-ELD: the total expression level in hybrid was similar to the *B. stacei* subgenome parent (fold change < 1.5) but either higher or lower *B. distachyon* subgenome parent; (4) Transgressive regulation: the total expression level in hybrid was 1.5-fold higher or lower than both parents; (5) Conserved: the total expression level in hybrid was similar to both parents. In addition, to evaluate how expression of individual homoeologs relates to joint homoeolog expression and to explain the phenomenon of ELD, we investigated the individual homoeolog expression levels relative to those of the diploid parents. To validate the ELD-transcriptome results we performed RT-qPCR on the same samples used for the RNA-seq experiments. Gene-specific primers of a subsample of 8 candidate genes (Bradi2g23507, Bradi1g52740, Bradi3g31410, Bradi1g60050, Bradi1g19940, Bradi1g52230, Bradi1g08340 and Bradi1g48830 in Table S2) were used. Real-time quantitative PCR amplification was performed following the procedure described previously.

For homoeolog expression bias analysis, we compared the expression level of each homoeolog pair in the diploid parents and the allopolyploid. In both cases we conducted Student's t-tests ($P \leq 0.05$) to infer statistical significance.

Gene ontology enrichment analysis

An enrichment analysis of gene ontology (GO) terms was conducted to study the biological significance of DEGs using the agriGO toolkit (Du *et al.*, 2010) by comparing the number of GO terms in the test sample to the number of GO terms within the background *Brachypodium distachyon* reference. Significance was evaluated using a Fisher statistical test with an FDR corrected p-value < 0.05 and the minimum number of mapping entries with a particular GO term set to 5.

RESULTS

Transcriptome sequencing and mapping efficiency

A total of 118.6 million reads were obtained after excluding reads with low mapping quality scores with an average of 9.9 million reads (1.3 Gb) in each sample (Table S3). First, the reads from all samples were mapped to the *B. distachyon* reference genome (Bdystachyon_307_v3.0 Phytozome) with an average of 72.6%. These mapped reads were evaluated for differences in expression between treatments (control and drought stress) in each species separately. From a total of 34310 genes described in the B.distachyon genome reference, 10404, 9894 and 19471 genes were expressed in *B. distachyon*, *B. stacei* and *B. hybridum*, respectively. Differences in gene expression across species were also assessed by comparing the alloplyploid *B. hybridum* to their diploid progenitors. The reads from *B. distachyon*, *B. stacei* and *B.*

hybridum were also mapped to the *B. distachyon* genome with an average of 72.6% (see Table S3). 39769 genes were surveyed for their expression.

Secondly, for the homoeolog expression analysis (ELD and expression bias), differences in gene expression across species were also assessed by comparing the alloplyploid *B. hybridum* to their diploid progenitors. The reads from *B. distachyon*, *B. stacei* and *B. hybridum* were mapped in this case to the *B. distachyon*, *B. stacei* and *B. hybridum* reference genomes with an average of 87.38%, 86.42% and 83.76 % respectively (see Table S4). Of the 12952 homologous genes identified in the reference genomes of the three species, we found a total of 10468 genes in stress samples and 10619 genes in constitutive samples that were surveyed for analyzing the expression of the homoeologs.

Differential gene expression between treatments

B. hybridum exhibited overall stronger gene expression in response to drought compared to their diploid ancestors (Fig. 1). In particular, *B. hybridum* showed 6825 significantly DEGs, while *B. distachyon* and *B. stacei* presented 4929 and 1386 significantly DEGs, respectively (Fig. 1). This pattern was constant either for upregulated and downregulated expression (Fig. 1). Noticeably, 51.2 % of overall DEGs *in B.hybridum* were unique (Fig. 1). For *B. hybridum*, GO analysis of top ten significantly unique enriched biological process terms revealed that gene groups related to molecule transport, response to heat and catabolic processes were up-regulated, whereas unique down-regulated genes in this species belonged mainly to chloroplast organization (Fig. 2). For *B. distachyon*, intracellular and nuclear protein transport, and RNA methylation genes were significantly up-regulated under water stress, whereas genes related to chromosomal structural changes and DNA conformation were significantly down-regulated (Fig. 2). For *B. stacei*, lipidic metabolic processes were uniquely up-regulated, whereas molecular phosphorylation genes were significantly down-regulated (Fig. 2).

B. hybridum expression shared a higher number of genes with *B. distachyon* compared to *B. stacei*, whereas the common number of DEGs between *B. distachyon* and *B. stacei* was relatively low (Fig. 1). Overall, the three species shared only 5.4 % (N= 13140) of the total of genes expressed (Fig.1). Among the top ten significantly enriched biological process terms for shared up-regulated genes we identified genes that are involved in the proline biosynthesis and response to water stress (Fig. 3). By contrast, among the top ten significantly enriched biological process terms for shared down-regulated genes we found that genes involved in photosynthesis and amino acid metabolic processes (Fig. 3).

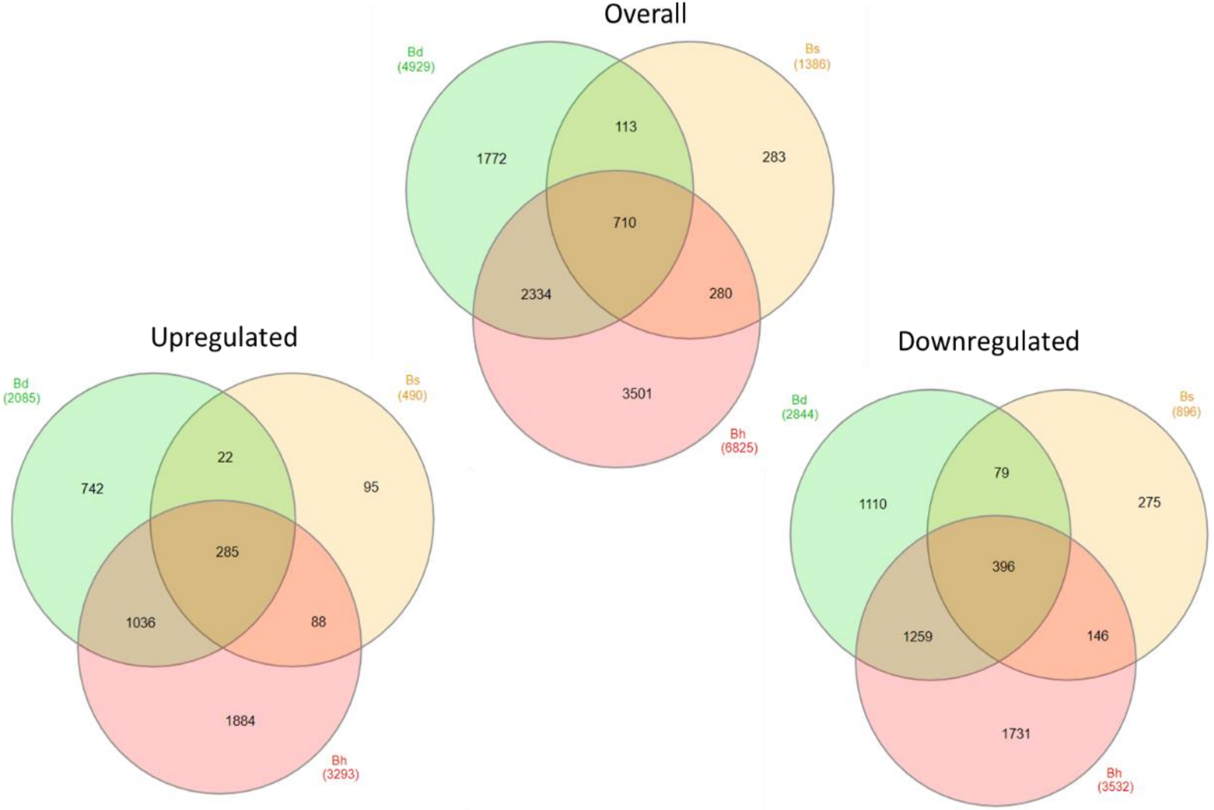


Fig. 1. Venn diagram showing the extent of the overlap between *B. distachyon* (Bd), *B. stacei* (Bs) and *B. hybridum* (Bh) for all genes differentially expressed in response to drought in each species. Sets of upregulated and downregulated genes are considered separately.

Differential gene expression between species

The percentage of genes with differential expression between *B. hybridum* and their two parents was asymmetric (false discovery rate [FDR] ≤ 0.05; Benjamini–Hochberg adjustment) under both water conditions. Thus, constitutively, global gene expression in *B. hybridum* was more similar between *B. hybridum* and its species ancestors than between ancestors (Fig. 4). In total, 3607 (2187+1420) DEGs were identified (18.5% of expressed genes) under control conditions in *B. hybridum*. Among these DEGs, 2187 were significantly expressed in *B. hybridum* compared to *B. distachyon,* and 1420 compared to the *B. stacei* genome (Fig. 4), suggesting that constitutive expression pattern in *B. hybridum* was closer to *B. stacei* than to *B.distachyon* . Significantly enriched biological process terms of genes showing significantly constitutive higher expression in *B. hybridum* than in *B. distachyon* belong to saccharide biosynthesis, oxidation-reduction process, anion transport, response to wounding and amino acid biosynthesis and metabolic process (Fig. 4). Conversely, enriched GO analyses of genes showing constitutive higher expression in *B. distachyon* than in B. *hybridum* underlying DNA conformation, chromatin and nucleosome reorganization. Enriched GO analyses of genes showing higher expression in *B. hybridum* compared to *B. stacei* were mainly related to photosynthesis, whereas enriched GO analyses of genes showing higher expression in *B. stacei* than *B. hybridum* were involved in molecular phosphorylation (Fig. 4).

Under water stress conditions, the global gene expression was more similar between *B. distachyon* and *B. stacei* than global gene expression between *B. hybridum* and its ancestor species (Fig. 5). A total of 10428 (4761+5667) DEGs were identified (53.5% of expressed genes) in *B. hybridum*. Among these DEGs, 4761 were significantly expressed in *B. hybridum* compared to *B.distachyon,* and 5667 compared to the *B. stacei* genome (Fig. 5). Expression pattern in *B. hybridum* was more similar to *B. distachyon* progenitor than to *B. stacei* one. Thus, *B. hybridum* showed higher expression than *B. distachyon* in genes related to phosphorylation, transport and response to water (Fig. 5) while genes showing higher expression in *B. distachyon* than in *B. hybridum* underlie nitrogen metabolic process (Fig. 5). On the other hand, genes showing higher expression in *B. hybridum* compared to *B. stacei* were mainly related to heat and water stress, whereas genes showing higher expression in *B. stacei* than *B. hybridum* were involved in plastid organization, and photosynthesis among other processes (Fig. 5).

The hierarchical cluster analysis of the shared genes with significantly expression changes produced by water stress revealed that the gene expression pattern of *B. hybridum* clustered with the ancestor *B. distachyon* (Figure S2).

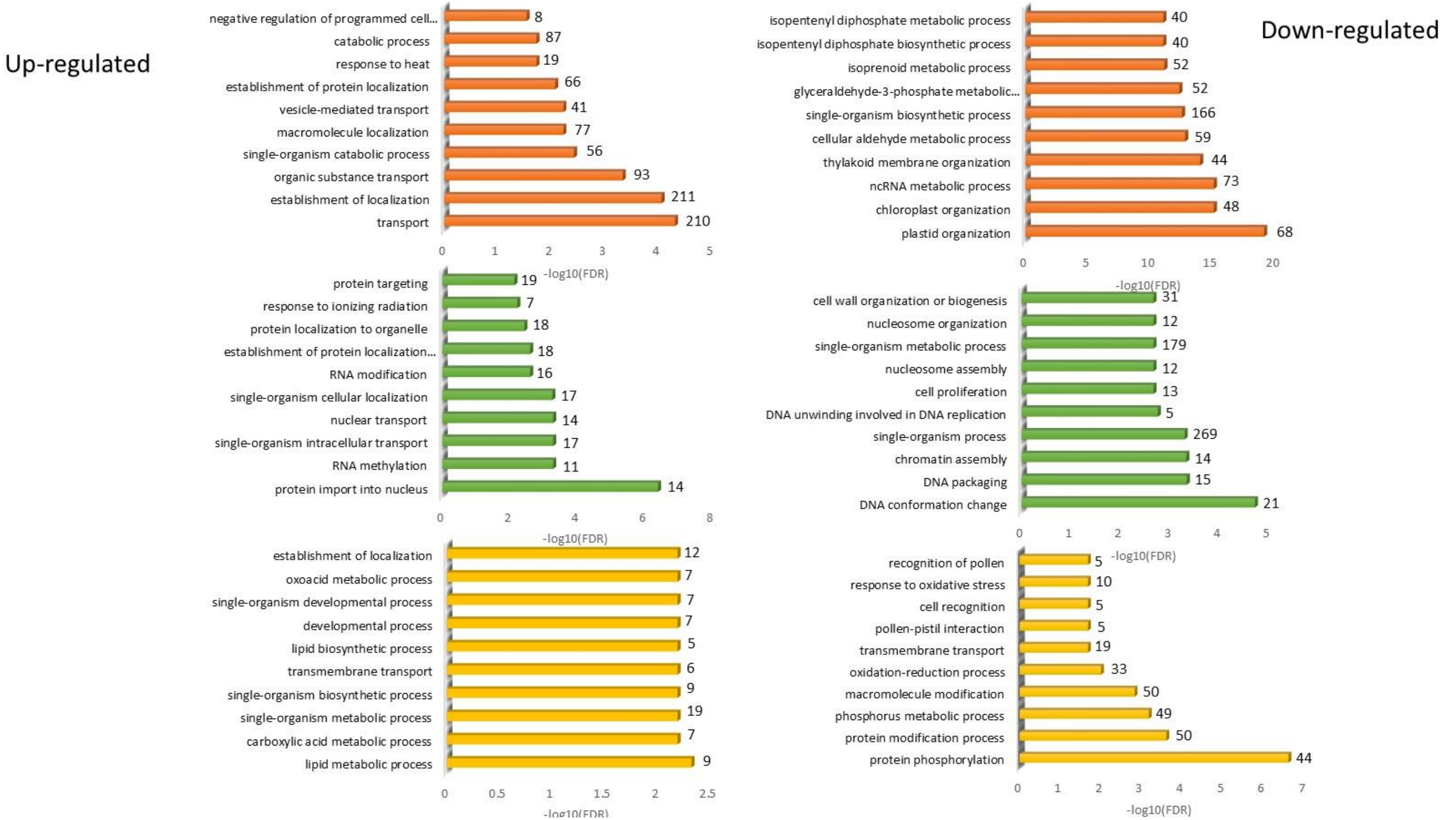


Fig. 2. Top ten unique up-regulated (left) and down-regulated (right) significantly enriched GO terms that are expressed after 21 days of water stress in the three species of the *Brachypodium distachyon* species complex. The number of genes that are identified in each biological process. Above (orange bars) data for *B. hybridum*, middle (green bars) data for *B. distachyon* and bottom (yellow bars) data for *B. stacei.*

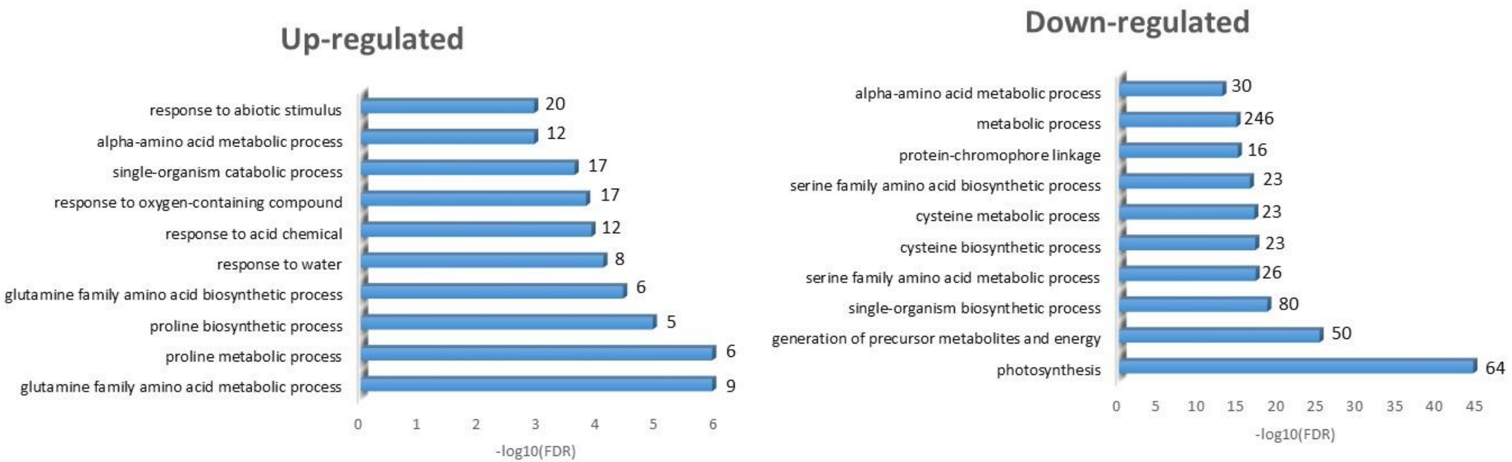
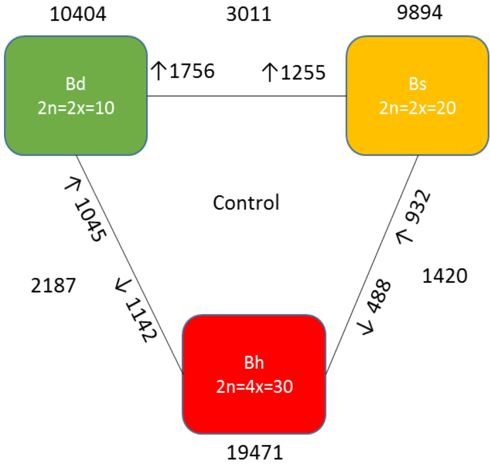


Fig. 3. Top ten significantly shared enriched GO terms in the biological process ontology of 285 up-regulated and 396 down-regulated that are expressed after 21 days of water stress in the three species of the *Brachypodium distachyon* species complex. Number on bars are the number of genes that are identified in each biological process.



Genes showing higher expression in Bh than in Bd (1045)

GO-ID	Term	FDR
GO:0009312	oligosaccharide biosynthetic process	3.20E-02
GO:0046351	disaccharide biosynthetic process	3.40E-02
GO:0055114	oxidation-reduction process	5.60E-02
GO:0005992	trehalose biosynthetic process	5.60E-02
GO:0005991	trehalose metabolic process	5.60E-02
GO:0006820	anion transport	1.90E-01
GO:0009611	response to wounding	1.90E-01
GO:0009073	aromatic amino acid family biosynthetic process	3.70E-01
GO:0009072	aromatic amino acid family metabolic process	3.80E-01
GO:0006576	cellular biogenic amine metabolic process	3.80E-01

Genes showing higher expression in Bd than in Bh (1142)

GO-ID	Term	FDR
GO:0071103	DNA conformation change	6.90E-08
GO:0006323	DNA packaging	1.70E-06
GO:0031497	chromatin assembly	8.40E-06
GO:0034728	nucleosome organization	1.80E-05
GO:0065004	protein-DNA complex assembly	2.80E-05
GO:0051276	chromosome organization	0.00025
GO:0006325	chromatin organization	0.00045
GO:0071840	cellular component organization or biogenesis	0.00049
GO:0007017	microtubule-based process	0.00049
GO:0007018	microtubule-based movement	0.00049

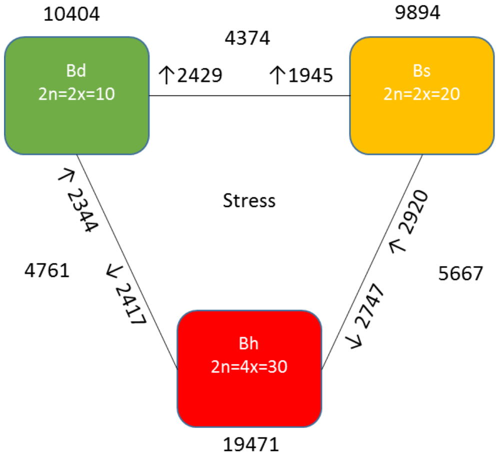
Genes showing higher expression in Bh than in Bs (932)

GO-ID	Term	FDR
GO:0015979	photosynthesis	7.50E-06
GO:0048513	animal organ development	3.10E-05
GO:0019725	cellular homeostasis	1.40E-04
GO:0045454	cell redox homeostasis	1.40E-04
GO:0006739	NADP metabolic process	4.10E-04
GO:0009311	oligosaccharide metabolic process	4.10E-04
GO:0006098	pentose-phosphate shunt	1.40E-03
GO:0019684	photosynthesis, light reaction	2.00E-03
GO:0051156	glucose 6-phosphate metabolic process	3.50E-03
GO:0042592	homeostatic process	3.50E-03

Genes showing higher expression in Bs than in Bh (488)

GO-ID	Term	FDR
GO:0006468	protein phosphorylation	7.50E-06
GO:0016310	phosphorylation	3.10E-05
GO:0006796	phosphate-containing compound metabolic process	1.40E-04
GO:0006793	phosphorus metabolic process	1.40E-04
GO:0036211	protein modification process	4.10E-04
GO:0008152	metabolic process	4.10E-04
GO:0043412	macromolecule modification	1.40E-03
GO:0019538	protein metabolic process	2.00E-03
GO:0009698	phenylpropanoid metabolic process	3.50E-03
GO:0046274	lignin catabolic process	3.50E-03

Fig. 4. Transcriptome sequencing of derived allotetraploid *B. hybridum* and its diploid parents. DEGs between *B. hybridum* (Bh) and its diploid progenitors (Bd and Bs) in well wáter conditions; the number of upregulated genes is adjacent to Bd or Bs, and the number of downregulated genes is near Bh. Numbers close to the species represent the total number of expressed genes. Top 10 enriched GO terms in the biological process ontology of genes showing significantly higher expression in one specie than other.



Genes showing higher expression in Bh than in Bd (2344)

GO-ID	Term	FDR
GO:0006468	protein phosphorylation	4.30E-06
GO:0044712	single-organism catabolic process	8.90E-04
GO:0006793	phosphorus metabolic process	7.50E-03
GO:0001101	response to acid chemical	7.50E-03
GO:0006796	phosphate-containing compound metabolic process	8.10E-03
GO:0006810	transport	1.20E-02
GO:0002679	respiratory burst involved in defense response	1.50E-02
GO:0009415	response to water	1.60E-02
GO:0006464	cellular protein modification process	1.90E-02
GO:0016042	lipid catabolic process	1.90E-02

Genes showing higher expression in Bd than in Bh (2417)

GO-ID	Term	FDR
GO:1901566	organonitrogen compound biosynthetic process	1.40E-42
GO:0006807	nitrogen compound metabolic process	8.20E-41
GO:0022613	ribonucleoprotein complex biogenesis	6.60E-28
GO:0034660	ncRNA metabolic process	1.20E-27
GO:1901576	organic substance biosynthetic process	1.50E-26
GO:0043604	amide biosynthetic process	5.30E-26
GO:0009058	biosynthetic process	5.40E-26
GO:0006412	translation	6.10E-26
GO:0015979	photosynthesis	1.00E-25
GO:0043043	peptide biosynthetic process	1.60E-25

Genes showing higher expression in Bh than in Bs (2920)

GO-ID	Term	FDR
GO:0009408	response to heat	3.20E-05
GO:0010035	response to inorganic substance	1.90E-02
GO:0009266	response to temperature stimulus	7.40E-02
GO:0009415	response to water	7.40E-02
GO:0052542	defense response by callose deposition	7.40E-02
GO:0033037	polysaccharide localization	7.40E-02
GO:0052545	callose localization	7.40E-02
GO:0006914	autophagy	7.40E-02
GO:1901700	response to oxygen-containing compound	8.20E-02
GO:0009620	response to fungus	8.20E-02

Genes showing higher expression in Bs than in Bh (2747)

GO-ID	Term	FDR
GO:0009657	plastid organization	1.60E-50
GO:0044711	single-organism biosynthetic process	3.10E-49
GO:0015979	photosynthesis	3.50E-48
GO:0019682	glyceraldehyde-3-phosphate metabolic process	7.40E-46
GO:0006081	cellular aldehyde metabolic process	4.60E-44
GO:0044281	small molecule metabolic process	9.10E-44
GO:1901566	organonitrogen compound biosynthetic process	1.80E-42
GO:0034660	ncRNA metabolic process	3.80E-40
GO:0019684	photosynthesis, light reaction	1.00E-39
GO:0006720	isoprenoid metabolic process	1.40E-39

Fig. 5. Transcriptome sequencing of derived allotetraploid *B. hybridum* and its diploid parents. DEGs between *B. hybridum* (Bh) and its diploid progenitors (Bd and Bs) in control (A) and stress (B); the number of upregulated genes is close to Bd or Bs, and the number of downregulated genes is near Bh. Numbers close to the species represent the total number of expressed genes.

Variation in gene expression between *B. hybridum* genotypes

The 26 selected genes were significantly regulated in response to the drought treatment in any of the eight genotypes analyzed (Table 1). Bradi1g66470, Bradi1g44230 and Bradi3g10010 were responsive to water limitation only in a genotype (Table 1). Transcript regulation to drought was highest in LARVA-20, FARO-5 and FARO-3 in which 18 out 26, 15 out 26 and 14 out of 26 genes regulated respectively showed a significant differential expression under water stress (Table 1; Fig. S3-S4). By contrast, few genes were regulated in ROS-9, JHIN-8 and MONE-3 with 7, 11 and 11out of 26 genes respectively (Table 1; Fig. S3-S4).

Strong drought induction was recorded for late embryogenesis abundant proteins DHN3 and LEA3, and some of the heat shock proteins genes (e.g., Bradi2g23250, Bradi1g68440; Table 1), although the magnitude of such induction was also dependent on genotype (e.g., induction was especially strong for LARVA-20, FARO-3 and JCIM-20*;* see Table 1). Osmoprotectans as proline biosynthesis related-genes were moderately up-regulated in FARO-3, FARO-5, LARVA-20 and RUI-11, whereas Bradi2g07420 only was induced significantly in FARO-3 and LARVA-20 (Table 1). Strong drought down-regulation was detected in some of the photosynthetic and plastidic genes (e.g., Bradi3g26391, Bradi1g26880), for the metabolic gen Bradi1g60050 and for the flowering promoter Bradi1g48830 in FARO-3 (Table 1). For the rest of the genes, expression was more variable depending upon gene group and genotype (Table 1).

We detected a significant or marginally significant ($P<0.1$) association between genetic differentiation (see genetic population analysis in Martínez *et al*., 2018) and gene expression in 7 out of 26 genes under well-watered conditions (9 out 26 genes after controlling by latitude and longitude; Table S6). This was especially evident for photosynthetic and plastidic genes (Table S6). Under water stress, db-RDA analyses indicated a significant association between gene expression dissimilarities among populations and genetic differentiation in 9 out of 26 genes (Table S6), while after controlling by geography significance of such relationship was somewhat reduced (7 out of 26 genes, Table S5,). Influence of geographical variables on genetic expression was overall non-significant (Table S5).

Table 1. Transcript level variation determined by qRT-PCR analysis of 26 drought-response candidate genes in *B. hybridum* genotypes after 21 days of experimental water restriction. Values above 1 denote gene up-regulation, values below zero denote gene down-regulation *P<0.05; ** P<0.001; ***P<0.0001; n.s. = non-significant at P<0.05 (statistical significance come from tests of the main effects of univariate General Lineal Mixed Models performed on each gene). Folds come from back-log transformed normalized transcription data.

		Average fold change values (treatment/control)							
Gene ID	GO annotation	*FARO-3*	*FARO-5*	*JCIM-20*	*JHIN-8*	*LARVA-20*	*MONE-3*	*ROS-9*	*RUI-11*
Cell- stress osmoprotection genes									
Bradi2g23507	Proline synthesis (P5CS1)	29.58^{***}	10.70^{***}	0.20^{*}	$1.56^{n.s.}$	58.73^{***}	3.71^{*}	$2.62^{n.s.}$	8.52^{*}
Bradi2g54920	Proline synthesis (P5CS2)	32.40^{***}	11.87^{*}	$1.36^{n.s.}$	0.23^{*}	220.08^{***}	$0.53^{n.s.}$	$0.46^{n.s.}$	52.71^{***}
Bradi2g07420	Proline synthesis (BAC2)	874.53^{***}	$4.18^{n.s.}$	$2.51^{n.s.}$	$2.09^{n.s.}$	40.19^{*}	$0.55^{n.s.}$	$4.63^{n.s.}$	$4.47^{n.s.}$
Bradi3g31410	Trehalase-1	24.43^{***}	3.83^{*}	$1.47^{n.s.}$	0.19^{*}	13.21^{*}	$0.47^{n.s.}$	$0.31^{n.s.}$	$1.03^{n.s.}$
Chaperones genes									
Bradi2g18090	LEA3	446.07^{***}	54.82^{*}	$4.03^{n.s.}$	$1.85^{n.s.}$	19135.58^{***}	9.68^{*}	$3.48^{n.s.}$	50.23^{*}
Bradi1g37410	Dehydrin DHN3	1136.61^{***}	49.82^{*}	$0.73^{n.s.}$	10.41^{*}	14141.51^{***}	$0.62^{n.s.}$	$3.13^{n.s.}$	462.72^{**}
Bradi1g66470	Heat shock protein	$7.91^{n.s.}$	$14.19^{n.s.}$	$23.19^{n.s.}$	8.22e-4*	$113.63^{n.s.}$	$8.96^{n.s.}$	$14.55^{n.s.}$	$0.56^{n.s.}$
Bradi2g23250	Heat shock protein	31153.17^{***}	$3.70^{n.s.}$	423.27^{**}	76.61^{*}	0.03^{*}	0.03^{*}	-	-
Bradi1g44230	Heat shock protein	$1.44^{n.s.}$	$2.56^{n.s.}$	$0.37^{n.s.}$	$2.29^{n.s.}$	19.07^{*}	$3.81^{n.s.}$	$2.19^{n.s.}$	$0.99^{n.s.}$

Bradi1g68440	Heat shock protein	4.33^{*}	$0.95^{n.s.}$	3244.28^{***}	$1.92^{n.s.}$	4.12^{*}	$2.42^{n.s.}$	$1.98^{n.s.}$	$0.85^{n.s.}$
Photosynthetic and plastidic genes									
Bradi3g26391	Photosynthesis (Rubisco metabolism)	0.006^{***}	0.004^{***}	0.20^{*}	$0.64^{n.s.}$	$1.98e-4^{***}$	$1.78^{n.s.}$	$1.20^{n.s.}$	0.002^{***}
Bradi4g08500	Photosynthesis (Rubisco metabolism)	$1.11^{n.s.}$	0.11^{**}	$1.10^{n.s.}$	$0.54^{n.s.}$	0.09^{**}	$1.34^{n.s.}$	$0.83^{n.s.}$	0.07^{***}
Bradi4g08800	Photosynthesis (Rubisco metabolism)	$3.09^{n.s.}$	13.22^{*}	38.37^{**}	9.62^{*}	$2.28^{n.s.}$	5.27^{*}	10.62^{*}	0.07^{*}
Bradi1g12130	Photosynthesis	0.13^{*}	0.20^{*}	107.13^{***}	0.16^{*}	0.07^{*}	$3.81^{n.s.}$	$2.01^{n.s.}$	0.003^{***}
Bradi1g26880	Stomal protein	0.16^{**}	0.09^{***}	$0.59^{n.s.}$	0.18^{**}	0.003^{***}	0.38^{*}	0.08^{***}	0.05^{***}
ROS scavenging and metabolic genes									
Bradi1g26470	Ascorbate peroxidase	$0.84^{n.s.}$	0.12^{*}	$2.22^{n.s.}$	$0.85^{n.s.}$	$0.64^{n.s.}$	$0.77^{n.s.}$	$1.33^{n.s.}$	0.24^{*}
Bradi1g60050	G6PDH2	0.03^{***}	0.13^{**}	$0.96^{n.s.}$	$1.55^{n.s.}$	0.008^{***}	$1.88^{n.s.}$	$0.85^{n.s.}$	0.07^{***}
Bradi3g42780	Acyl-CoA Reductase	$2.15^{n.s.}$	$0.45^{n.s.}$	7.01^{*}	9.87^{*}	$2.17^{n.s.}$	5.49^{*}	31.79^{***}	0.06^{**}
Regulatory control and hormone metabolism genes									
Bradi1g66360	Heterochromatim organization	$1.52^{n.s.}$	$0.20^{n.s.}$	16.17^{*}	$3.44^{n.s.}$	57.96^{*}	19.48^{*}	$6.12^{n.s.}$	$0.06^{n.s.}$
Bradi1g19940	Protein Methylation (N-methyltransferase)	5.14^{*}	2.26^{*}	$2.21^{n.s.}$	4.16^{*}	$0.71^{n.s.}$	4.25^{*}	8.18^{**}	$0.51^{n.s.}$
Bradi1g52230	Helicase	$1.27^{n.s.}$	317.91^{**}	0.04^{*}	$0.62^{n.s.}$	$0.69^{n.s.}$	20.34^{*}	$0.13^{n.s.}$	$1.38^{n.s.}$
Bradi1g52740	Aldehyde oxidase	$0.52^{n.s.}$	2.43^{*}	$1.06^{n.s.}$	1.10^{*}	3.37^{*}	3.47^{*}	2.91^{*}	$0.67^{n.s.}$
Flowering time and vernalization genes									
Bradi1g48830	Flowering locus T	$9.65e-5^{**}$	$4.21^{n.s.}$	0.04^{*}	$0.62^{n.s.}$	0.69^{*}	20.34^{*}	$0.13^{n.s.}$	$1.38^{n.s.}$
Bradi1g08340	VRN1	$0.02^{n.s.}$	$7.37^{n.s.}$	115.85^{**}	$4.03^{n.s.}$	0.006^{**}	$0.46^{n.s.}$	126.06^{**}	$0.13^{n.s.}$
Bradi3g10010	VRN2	$0.37^{n.s.}$	$2.45^{n.s.}$	$0.33^{n.s.}$	$0.29^{n.s.}$	$0.22^{n.s.}$	$0.16^{n.s.}$	1821.1^{***}	$0.11^{n.s.}$
BdVRN2L	Vernalization (VRN2-like)	$0.26^{n.s.}$	$1.96^{n.s.}$	6.45^{*}	$0.60^{n.s.}$	$0.19^{n.s.}$	$0.64^{n.s.}$	$5.21^{n.s.}$	0.1^{*}

ELD in the allotetraploid *B. hybridum* transcriptome

Constitutively, the total expression level of homologous genes pair in *B. hybridum* was different to that in both diploid parents for 21.55% of the genes, in contrast with those that did not change in relation to its ancestors, 78.45 % (Fig. 6). In water stress, the different total expression level of homologous genes pair between *B. hybridum* and its species ancestors was much higher, 50.63% of the expressed genes (Fig. 6). Overall, the fraction of genes observed for most of categories increased under stress conditions (categories I, XII, IV, IX, III, VII, X, V, VI, VIII in Fig. 6), but for the proportion of genes in the Bs expression-level dominance (categories IV and V; Fig. 6). Specifically, there was a substantial increase in gene pairs that showed Bd-expression level dominance (categories IV and IX 21.25%) and in genes that were transgressively regulated under drought stress (categories III, VII, X, V, VI and VIII 19.56%). Only 111 and 60 (1.03 and 0.57%, categories I and XII) gene pairs exhibited additive expression under drought and control conditions respectively. Moreover, 2048 (19.56%) genes pairs showed transgressive expression under drought stress whereas only 170 (1.6%) were displayed under control conditions (categories III, VII, X, V, VI and VIII 19.56%). Under both conditions, the percentage of genes pairs that exhibited transgressive upregulation was higher compared with downregulated genes. Relationship between homoeolog-specific expression and ELD is given in full in Supplementary Material, Appendix 1 and Fig. S5.

Validation of RNA-seq analysis by qRT-PCR on 8 orthologous gene indicated that for all 8 orthologous gene pairs, the qRT-PCR analysis revealed a similar expression patterns as the RNA-seq data (Fig. S6), demonstrating the reliability of the data produced by RNA-seq.

Although more proportion of genes showed ELD expression than transgressive pattern, we found that transgressive genes were enriched with more GO terms compared with ELD genes (Additional File 1). 624 categories were significantly enriched (FDR<0.05) in transgressive genes (572 in transgressive downregulated and 52 in upregulated genes) and 338 in ELD genes (116 in ELD-Bd and 222 in ELD-Bs). For transgressive up-regulated genes we found significantly enriched terms belonging to lipid oxidation and catabolic process, organonitrogen compound catabolic process, defense response by callose deposition and protein localization. In contrast, for transgressive down-regulation genes we found terms associated with organonitrogen compound process, amide and peptide biosynthesis and metabolic process and translation, gene expression and plastid organization (Table S6). In the top 10 significantly enriched biological process terms for ELD-Bd genes we found terms belonging to acid and carbohydrate metabolic process and isoprenoid metabolic and biosynthesis process. In contrast, ELD-Bs genes were enriched in terms related with peptide and amide metabolic and biosynthesis process, organonitrogen compound biosynthetic process and nuclear transport (Table S6).

Homoeolog expression bias in the allotetraploid *B. hybridum*

In total, 59.57% of all expressed homoeolog pairs (6236 of 10468 homoeolog pairs) in *B. hybridum* retained the ancestral expression patterns and the remaining 40.43 % (4232 of 10468 homoeolog pairs) showed nonancestral expression (Fig. 7). In the homoeolog groups showing ancestral expression patterns, we found that the 20.84% (2184) of the homoeolog pairs displayed novel bias in the *B. hybridum*, while 19.56% (2048) of the homoeolog pairs with preexisting expression bias in the parent reverted to non-differential expression in the *B. hybridum* (Fig. 7). Overall, 78.01% of the homoeolog pairs displayed no bias in the derived *B. hybridum*, and the remaining 21.98 % showed biased expression (Fig. 7). Moreover, the allotetraploid showed balanced biased expression without a preference toward the ancestor transcriptomes (Bd-bias vs Bs-bias = 1159 (11.07%) vs 1142 (10.91%), Fig. 7). In the top 10 significantly enriched biological process terms for novel bias in progeny we found terms belong to carbohydrate biosynthesis and metabolic process, acid and lipid metabolic process and chemical response (Table S7).

Expression patterns	Additivity		Bs expression-level dominance		Bd expression-level dominance		Transgressive upregulation	Transgressive downregulation	No change	Total
	I	XII	II	XI	IV	IX	III,VII,X	V,VI,VIII		
	Bd Bh Bs	Bd Bh Bs	Bd Bh Bs	Bd Bh Bs	Bd Bh Bs	Bd Bh Bs	Bd Bh Bs	Bd Bh Bs		
										
Control	36	24	800	870	184	204	107	63	8331	10619
% by category	0.34	0.23	7.53	8.19	1.73	1.92	1.01	0.59	78.45	-
	0.57		**15.72**		**3.65**		**1.6**		**78.45**	-
Stress	55	56	606	738	1075	1151	1094	954	5168	10468
% by category	0.52	0.53	5.79	7.05	10.26	10.99	10.45	9.11	49.37	-
	1.05		**12.84**		**21.25**		**19.56**		**49.37**	-

Fig. 6. Global patterns of differential expression in the allopolyploid *B. hybridum*. Patterns are evaluated by comparing the total expression of the homoeologs in the allotetraploid (*B. hybridum*) relative to the expression levels found in the parental species *B. distachyon* (Bd) and *B. stacei* (Bs). For pairwise comparisons, control and stress samples were analyzed separately.

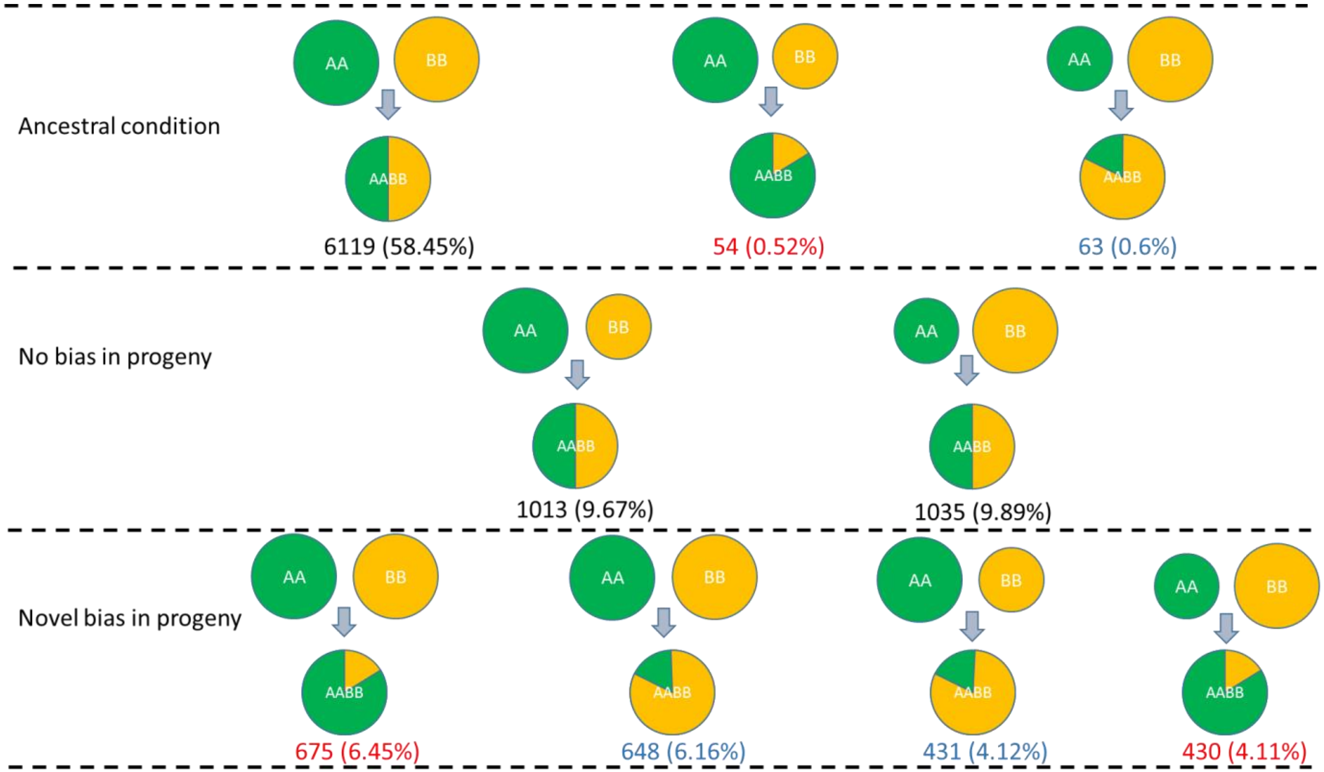


Fig. 7. Homoeolog expression bias in the allotetraploid *B. hybridum*. The relative expression levels of the homoeologs are modeled by the size of the circles in the ancestral species *B. distachyon* (AA) and *B. stacei* (BB) or the area ratio of the circles in the progeny *B. hybridum* (AABB). The number of homoeolog pairs and its proportion to the total number of expressed homoeolog pairs are listed. In total, 1159 (11.07%) homoeolog pairs are A-biased in the progeny (red numbers), 1142 (10.91%) homoeolog pairs are B-biases in the progeny (blue numbers), and 8167 (78.01%) homoeolog pairs have no bias in the progeny (black numbers).

DISCUSSION

This study has addressed for first time a comparative transcriptome analysis in the *Brachypodium distachyon* complex in the frame of drought response and parental legacy. First, our results indicate that *B. hybridum* exhibited a stronger transcriptional response to drought compared to its diploid ancestors and that an important fraction of DEGs *in B. hybridum* were unique. *B. hybridum* expression shared a higher number of genes with *B. distachyon* compared to *B. stacei*, whereas the common number of DEGs between *B. distachyon* and *B. stacei* was relatively low. Interestingly, shared drought up-regulated genes among the three species are involved in the proline biosynthesis, an important osmoprotectan metabolite (Verbruggen & Hermans, 2008). Secondly, we found a significant variation in the drought gene expression among *B. hybridum* genotypes, although, overall, a strong drought induction was recorded for late embryogenesis abundant proteins DHN3 and LEA3, and heat shock proteins genes, which are typical drought-responsive genes (Trejo-Calzada *et al.*, 2019). Third, regarding parental legacy, Bd expression-level dominance and transgressive expression were the most common expression patterns of the homoeolog genes in *B. hybridum* under water stress. Although most of all expressed homoeolog pairs in *B. hybridum* were maintained in the parental condition, we detected significant expression novel bias in the *B. hybridum* for ca. 21 % of the expressed genes under drought. Finally, contrary to our predictions, the quantitative analysis of drought transcriptome and parental legacy conducted here do not support that homoeologs inherited from *B. stacei* may have contributed in a large extent to the transcriptional level drought-response in *B. hybridum.*

The drought-transcriptome in the *Brachypodium distachyon* species complex

Previous studies have already analyzed the drought transcriptome in the diploid *B. distachyon* (e.g., Verelst *et al.*, 2013; Gordon *et al.*, 2014; Priest *et al.*, 2014; Lenk *et al.*, 2019). These studies have identified up-regulation of genes involved in many biological and functional processes as membrane fluidity (e.g., lipid and sterol biosynthesis), osmoprotectan synthesis (e.g., trehalose), biosynthesis of storage carbohydrates (e.g., starch and sucrose biosynthesis), and wax synthesis. Contrarily, photosynthetic, cell cycle, and cell wall expression genes appear usually down-regulated by drought in *B. distachyon* (Priest *et al.*, 2014). In any case, natural variation between *B. distachyon* genotypes in the gene drought-expression is often reported (Gordon *et al.*, 2014). Gene ontology enrichment analyses from Bd30-1 from this study confirm the down-regulation of cell wall genes (Fig. 2; Additonal File 1) detected previously in other *B. distachyon* genotypes (Gordon *et al.*, 2014). Interestingly, cell wall-associated gene expression has been recently pointed out as an important mechanism for conferring drought tolerance in this species (Lenk *et al.*, 2019). In contrast, intracellular and nuclear protein transport, and RNA methylation genes were significantly up-regulated (Fig. 2). Protein import to nucleus is critical for mediating the reprogramming of gene expression that leads to drought tolerance in plants (Luo *et al.*, 2013). Similarly, methylation on mRNAs is a post-transcriptional RNA modification essential in abiotic stress response (Hu *et al.*, 2019).

Unlike *B.distachyon*, no previous study have conducted drought transcriptomes in *B. stacei* and *B. hybridum*. Because these species grow in warm and arid habitats (Manzaneda *et al.*, 2012; López-Alvarez *et al.*, 2015), it would be expected that the drought transcriptome reflects important expression of drought-responsive genes. However, for *B. stacei*, we observed a lower drought-response gene expression than *B. distachyon* one (1386 DEGs vs. 4929 DEGS, for *B. stacei* and *B. distachyon* respectively under water stress; see Fig. 1). Thus, GO analyses for *B. stacei* ALSUR-1 line show the down-regulation of genes involved in protein modification as phosphorylation, whereas lipidic metabolic processes were significantly up-regulated. Interestingly, previous studies have proposed a putative pathway of drought stress response in *Brachypodium* seedlings through phosphorylation modification (Yuan *et al.*, 2016). As mentioned above, for *B. distachyon*, the induction of lipid related genes by drought stress has been previously reported (Verelst *et al.*, 2013), playing an important role in the production of complex lipid polymers of cuticle, a mechanism that hamper the water loss (Kunst & Samuels, 2009; Buschhaus & Jetter, 2011).

B. hybridum exhibited a two-fold gene expression in response to drought compared to their diploid progenitors, and an important fraction of DEGs *in B. hybridum* were unique. Part of these differences could be explained by the larger number of genes and complexity that this species harbors due to its hybrid nature. Little information is currently available on comparative transcriptome-wide response of polyploids to drought stress. We found only two studies that report a stronger drought response of polyploids respects their diploid relatives (Del Pozo & Ramirez-Parra, 2014; Xu *et al.*, 2014). GO analyses of unique down-regulated DEGs genes in *B. hybridum* showed that were enriched in plastid organization processes, whereas unique up-regulated gene were enriched in molecule transport related functions. Previous studies have detected a reduction of transcript accumulation of chloroplast genes encoding subunits of Plastid-Encoded RNA Polymerase (PEP) in *Solanum lycopersicum* and in *A. thaliana* (Kilian *et al.*, 2007; Tamburino *et al.*, 2017). In the case of molecule transport, it plays a significant role when adjusting to water scarcity participating in transmembrane allocations of various molecules in the context of stomatal, cuticular, and root responses (Jarzyniak & Jasiski, 2014).

Finally, our results showed that the three species share the expression of 5.4% of drought-responsive genes (Fig.1). Interstingly, genes enriched in proline and amino acid biosynthesis and metabolism were up-regulated in the whole species complex. Proline acts primarily as osmoprotectant, and also may function as a molecular chaperone and as part of the stress signal influencing adaptive responses (Verbruggen and Hermans 2008). We have shown previously strong drought-induced proline accumulation in *B. hybridum* and *B. stacei* plants (Martínez et al., 2018). Similarly, drought-induced proline has been found significantly elevated in drought-tolerant or intermediate *B. distachyon* ecotypes (Fisher *et al.*, 2016), which together indicates that proline accumulation is probably a key mechanism to cope water stress in the *B. distachyon* species complex. Contrastingly, genes involved in photosynthesis and generation of precursor metabolites and energy were down-regulated. Photosynthesis is one of the main processes affected by water restriction, via diminished CO_2 diffusion to the chloroplast and other metabolic constraints (Pinheiro & Chaves, 2011). At the gene transcription level, concomitantly, most photosynthetic genes are also down-regulated in response to drought (Pinheiro & Chaves, 2011), fact previously documented in *Brachypodium* (e.g., Priest et al., 2014). Down-regulation of photosynthesis is thougth to be a powerful defence mechanism in C3 plants when decreased stomatal conductance is combined with sustained high irradiance (Pinheiro & Chaves, 2011), a situation that is typically found during the dry periods across the natural range of the *Brachypodium* species (López-Álvarez et al., 2015).

Differentiation in expression of drought-response candidate genes among *B. hybridum* genotypes

A crucial question on evolution of gene expression in polyploids is whether changes in transcriptional activity is the same between different populations of polyploids (Soltis et al., 2016). Here, qRT-PCR analyses show notable variation among *B.*

hybridum genotypes from different locations in drought gene expression (Table 1), which is concordant with the polytopic origin of Iberian *B. hybridum* allopolyploids (López-Álvarez et al., 2012).

The magnitude of expression to drought was dependent upon the specific gene and genotype and was apparently independent on the specific susceptibility to drought tolerance of each line. We recorded strong drought-induction for late embryogenesis abundant proteins DHN3 (Bradi1g37410) and LEA3 (Bradi2g18090), the proline biosynthesis related-genes Pyrroline-5-Carboxylate Synthetases PC5S1 and P5CS2 (Bradi2g23507, Bradi2g54920), the arginine transporter BAC2 (Bradi2g07420) and the heat-shock proteins HSP70 and HSC70 (Bradi2g23250, Bradi1g68440; Table 1). LEA proteins appear frequently largely up-regulated under water stress in the Pooideae subfamily (e.g., Suprunova *et al.*, 2004; Park *et al.*, 2006; Shaar-Moshe *et al.*, 2015), acting essentially as molecular chaperones (Hanin *et al.*, 2011). In our experiment, drought induction of LEA proteins and dehydrins was particularly strong in LARVA-20, FARO-3, RUI-11 and FARO-5 (Table 1) and null or much more modest in the rest of genotypes. Similarly, the expression of heat-shock proteins under abiotic stress has been previously observed in many species including *B. distachyon*, and is thought to have also a protective role during multiple abiotic stress including drought (Augustine, 2016; Wen *et al.*, 2017). In our samples, drought-induction of heat-shock proteins was significantly high for FARO-3, JCIM-20 and JHIN-8, whereas were down-regulated in LARVA 20 and MONE-3.

In the case of proline biosynthesis related-genes PC5S1 and P5CS2 were moderately up-regulated in FARO-3, FARO-5, LARVA-20 and RUI-11, whereas the arginine transporter BAC2 only was induced significantly in FARO-3 and LARVA-20 (Table 1). Drought-induction proline accumulation is mainly due to increased synthesis and reduced degradation (Verbruggen & Hermans, 2008; Bhaskara *et al.*, 2015). Proline synthesis arises from two independently pathways, one from glutamate and another one from ornithine (Abdelgawad *et al.*, 2015). In the first case, glutamate is converted to proline by two successive reductions catalyzed by P5CS and pyrroline-5-carboxylate reductase (P5CR), respectively (Verbruggen & Hermans, 2008). In most plant species, P5CS is encoded by two genes, P5CS1 and P5CS2 (Abdelgawad *et al.*, 2015). While P5CS1 may be a rate limiting enzyme for proline accumulation during stress, it is thought that P5CS2 gene is not required for proline accumulation during stress (Verbruggen & Hermans, 2008). Alternatively, proline can be synthesized from ornithine which is generated from arginine by arginase (Abdelgawad *et al.*, 2015). In this case, induction of the arginine transporter BAC2 under drought may be interpreted as an activation of ornithine pathway. Although it has been shown recently that in grasses the glutamate pathway predominates over the ornithine one (Abdelgawad *et al.*, 2015) our results suggest that both pathways are drought-activated in *B. hybridum*. Interestingly, P5CS1 transcript variation was positively correlated to proline leaf accumulation in our samples ($r = 0.81$, $P = 0.015$, $N = 8$).

Contrarily, with the exception of Bradi4g08800 (an ortholog of RBCS gene family that encodes the small subunit of the enzyme RUBISCO), photosynthetic and plastidic genes were significantly down-regulated by drought (Table 1), which is consistent with the overall depression of photosynthesis-related genes observed in an aforementioned drought transcriptome analysis conducted on *B. distachyon* and other Pooideae. Bradi4g08800 appears significantly up-regulated under drought in JCIM-20, FARO-5, JHIN-8 and ROS-9, which is concordant with the capacity of *B. hybridum* to hold photosynthetic rates under water limitation and its drought-escape strategy to cope with water stress (Manzaneda *et al.*, 2015).

Little is known about the drought-response of genes controlling flowering time in *Brachypodium,* despite that it is a key trait for coping with water stress in this species complex (Manzaneda *et al.*, 2015). *B. hybridum* genotypes do not require vernalization to flower rapidly under long days conditions (i.e., they are essentially spring annuals). In *Brachypodium*, spring genotypes have dominant alleles of flowering promoters VRN1 and FT orthologs that are constitutively activated (Woods & Amasino, 2015) and generally correlate with flowering time among accessions (Ream *et al.*, 2014). Our results confirm that this is the case for *B. hybridum*. Constitutively, *B. hybridum* showed intermediate levels of VRN1 and FT transcripts (Fig. S7). Interestingly, such variation among genotypes in VRN1 and FT constitutive levels are negatively and significantly associated to flowering time ($r = -0.56$, $P = 0.0018$, $N = 28$, $r = -0.48$, $P = 0.01$, $N = 28$, pairwise correlation for VRN1 and FT respectively), indicating that a high constitutive level of VRN1 and FT correspond to shorter flowering times. Contrarily, drought repressed FT expression in most of the *B. hybridum* genotypes but JCIM-20 and ROS-9. In this case, induced FT levels were unrelated to flowering time variation ($P>0.05$). Such repression may be best interpreted as an effect of plant senescence once it has flowered. The flowering repressor VRN2 remained overall unresponsive to drought between *B. hybridum* genotypes, with the notable exception of ROS-9 which expression was 1821-fold higher under drought, although such expression did not prevent the early flowering in this genotype.

Despite inter-genotypic variation in gene expression was the rule, such variation was not overall associated to any geographic factor which could question the adaptive significance of such variation (Table S5). Interestingly, for one third of the genes analyzed variation in gene expression was explained by genetic differentiation, suggesting that for some genes, variation in their expression is associated to their genetic relatedness. Together, these results indicate that regulation of these drought-responsive genes here analyzed could be better explained by historical demographic factors than by environmental ones. However, we have shown previously that aridity explained significantly *B. hybridum* genetic differentiation

(Martínez *et al.*, 2018), and that drought-responsive genes could in turn be influenced, indirectly, by aridity variations.

Non-additivity and parental legacy in the *B. hybridum* drought-transcriptome

Patterns of nonadditive expression at whole genome and constitutive level in allopolyploids haven been described in numerous systems (reviewed in Yoo *et al.*, 2014). Thus, the number and level in which homoeologous copies are expressed in allopolyploids may cause variation in expression respect to parental species and can lead to modification of biological functions and novelty in plant traits (Yoo *et al.*, 2013, 2014; Buggs *et al.*, 2014). However, there are growing evidences that indicate that environment may largely determine homoeologous gene expression in allopolyploids (e.g., (Bardil *et al.*, 2011; Akama *et al.*, 2014; Bertrand *et al.*, 2015; Paape *et al.*, 2016; Takahagi *et al.*, 2018). Our results from the analysis of parental legacy and ELD of *B. hybridum* support this view. First, the fraction of change of DEG varied depending on the water stress treatment; only 21.55 % of the genes were differentially expressed in the allopolyploid under control conditions, whereas such fraction increased to 50.63 % in drought, suggesting that extensive changes in the overall patterns of parental gene expression were modulated by drought conditions. Second, in both experimental situations, additivity in gene expression was much less frequent than nonadditivity, yet nonadditivity patterns depended upon treatment. Constitutively, Bs-expression level dominance (ELD-Bs) was the most common nonadditive pattern, whereas Bd expression-level dominance (ELD-Bd) and transgressive expression were the most common expression patterns of the homoeologs genes in *B. hybridum* under water stress. Until our knowledge only a previous study has comparatively analyzed *B. distachyon* species complex transcriptomes in the context of abiotic stress (Takahagi *et al.*, 2018). Our results agree with findings from that study in the fact that nonadditivity is general, and the existence of Bs-expression level dominance at constitutive level, which suggest a concordance between these species in the constitutive expression pattern of those genes that may confer specific pre-adaptations to the dry and warm ecological niches that these species share in nature (López-Álvarez et al., 2015). Conversely, our results diverge from previous studies that indicated that *B. hybridum* and *B. stacei* maintained transcriptional states under abiotic stress similar to those under normal conditions (Takahagi *et al.*, 2018). Non-exclusive arguments for such discrepancy are that nonadditivity is stress-specific and/or genotype dependent. Supporting the first argument, co-expression network analyses performed on *Brachypodium* has revealed distinct gene modules showing specific profiles of expression in response to specific abiotic stress (Priest et al., 2014).

Gene ontology enrichment analyses did not differentiate groups of genes that discriminate clearly between ELD-Bs and ELD-Bd, since we found important biological process related with drought tolerance in both cases. Thus, for ELD-Bd genes we found terms belong to acid and carbohydrate metabolic process and isoprenoid metabolic and biosynthesis process. By contrast, ELD-Bs genes were enriched in terms related with peptide and amide metabolic and biosynthesis process, organonitrogen compound biosynthetic process and nuclear transport (Table S6). The importance of plants metabolome dominated by sugars, organic acids and amino acids to enhance the drought tolerance of shoots have been reported in wheat (Guo *et al.*, 2018). Similarly, internal concentration of isoprene is an important feature for stress protection by preserving the integrity of photosynthetic membranes (Fini *et al.*, 2017). Protein import to nucleus is critical for mediating the reprogramming of gene expression that leads to drought tolerance in plants (Luo *et al.*, 2013). ELD-Bd under water stress may be explained partially by the hierarchy of nucleolar dominance described in the complex (Idziak & Hasterok, 2008; Borowska-zuchowska *et al.*, 2016; Borowska-Zuchowska & Hasterok, 2017), showing that *B. hybridum* expressed rRNA gene loci from *B. distachyon* ancestor due to the inactivation of *B. stacei*-inherited at the epigenetic level. However, this expression pattern contrasts with the drought-functional response of *B. hybridum* which is correlated to *B. stacei* response, or shows a transgressive pattern for some other traits (Martínez *et al.*, 2018) and suggests the existence of post-transcription regulation (miRNAs, cis and trans regulation, etc.). This discordance between transcriptome and proteome has been also observed in other polyploids as *B. napus* (Marmagne *et al.*, 2010), cotton (Hu *et al.*, 2013) and the synthetic *A. suecica* (Ng *et al.*, 2012).

Several studies have revealed that homoeolog pairs may display homoeolog expression bias in allotetraploids (Grover *et al.*, 2012b; Combes *et al.*, 2013; Yoo *et al.*, 2013; Li *et al.*, 2014). Contrarily, our results indicate that expression patterns of more than half of all expressed homoeolog pairs in the diploid parents were conserved in the allopolyploid *B. hybridum* at constitutive level and under drought. This coincides with results obtained by Takahagi *et al.* (2018) that suggests that the 60% of homoeolog pairs in *B. hybridum* inherited preesxisting parental expression. However, homeolog expression bias do not seem to be a static feature under various abiotic stresses offering a great plasticity to polyploids. For example, a number of genes in the allopolyploid *Coffea arabica cv. Java* showed similar expression patterns compared with the parental *C. canephora* under hot conditions, while no parental preference was exhibited under cool conditions (Bardil *et al*., 2011). On the other hand, we found that approximately 21.98% of the 10468 expressed homoeolog pairs displayed expression novel bias, and this expression did not show any preference toward *B. distachyon* or *B. stacei* genome (Figure 7). Gene ontology enrichment analysis of biased homoeologous genes did not distinguish genes with specific functions preferentially expressed by one or other of the two subgenomes (Table S7).

CONCLUSION

The polyploid plasticity hypothesis states that allopolyploid species might utilize the expression patterns of both parents depending on environments (Shimizu-Inatsugi et al., 2017). Our results from the drought-transcriptome conducted on *B. hybridum* give some support to this hypothesis. Thus, in non-stress conditions, Bs-expression level dominance was the most common pattern, whereas Bd expression-level dominance and transgressive expression were the most common in water stress conditions. Contrarily to our expectations, *B. hybridum* ability to tolerate a broader range of aridity did not result from massively differential use of *B. stacei*-inherited homeologs, but differential gene expression is probably mainly dependent on a small number of regulators (e.g., transcription factors, epigenetic mechanisms, transposons and post- transcriptional regulation), which are themselves probably dependent on aridity. On the other hand, expression patterns of more than half of all expressed homoeolog pairs in the diploid parents were conserved in the allopolyploid both at constitutive level and under drought suggesting overall cis-regulatory divergence between the diploid progenitors (*sensu* Buggs et al., 2014). In any case, findings of this study come from the analysis from a single *B. hybridum* allopolyploid line, that given the polytopic origin of *B. hybridum* allopolyploids (López-Álvarez et al., 2012) it results insufficient for generalizations. Future studies must investigate consistency of parental legacy and expression pattern between *B. hybridum* genotypes differing in the ages of formation during the last 1 Mya, genetic background and reciprocity of parentage.

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AUTHOR CONTRIBUTION

AJMA, PJR, LMM planned and design the research; LMM, TS, AFO, performed the experiments, AB, FA and LMM analyzed the data and conducted the bioinformatic analyses, AJMA and LMM wrote the manuscript. All authors checked the submitted version and make critical comments.

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SUPPLEMENTARY MATERIAL

FIGURES

Fig. S1. Geographical distribution of the *Brachypodium distachyon* complex populations across the Iberian Peninsula used in transcriptome analysis. Triangles, populations used in parental legacy study. Circles, population used in variation in gene expression between *B. hybridum* genotypes. Green, orange and red dots map, respectively, the localities of origin of the *B. distachyon*, *B. stacei* and *B. hybridum.*

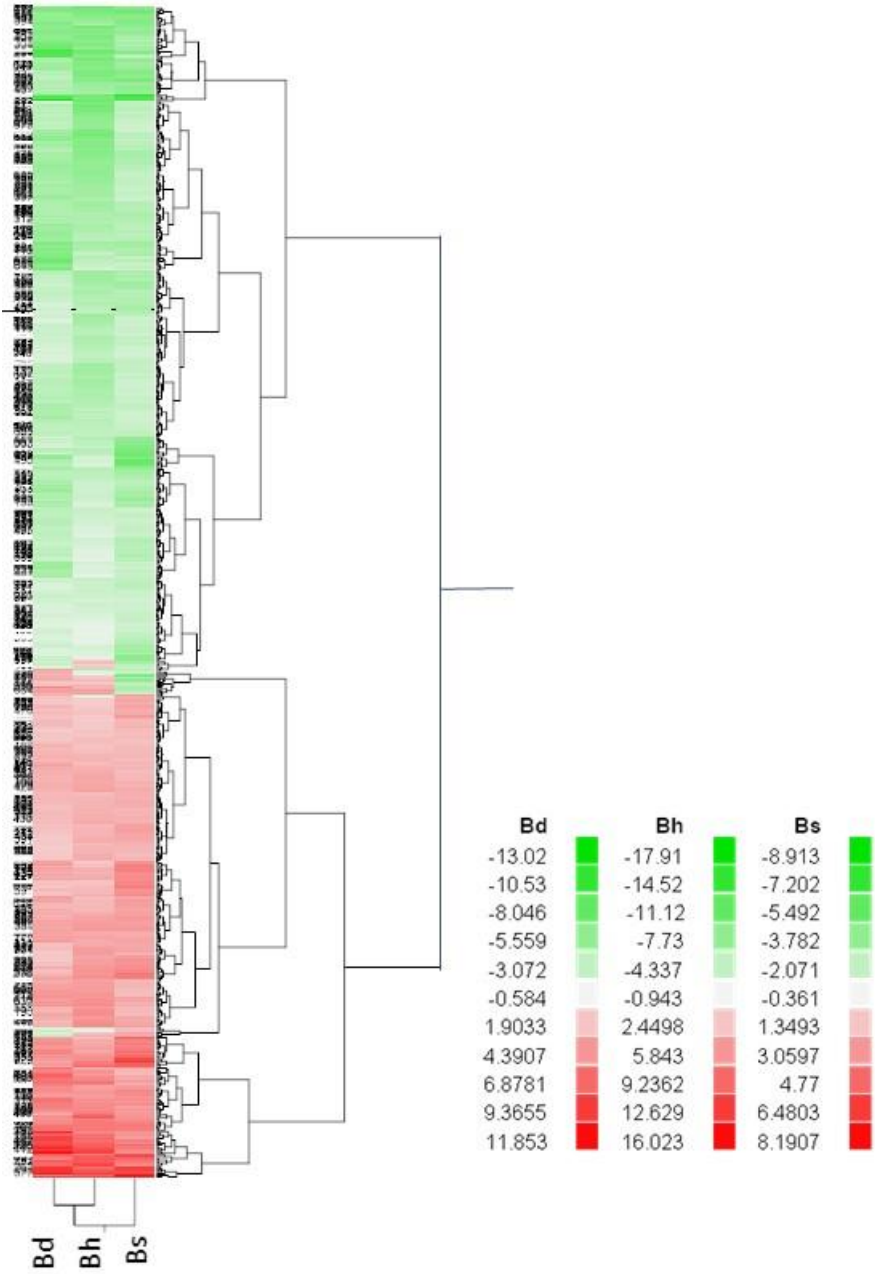
Bd
Bh
Bs
Bd
-13.02
-10.53
-8.046
-5.559
-3.072
-0.584
1.9033
4.3907
6.8781
9.3655
11.853
Bh
-17.91
-14.52
-11.12
-7.73
-4.337
-0.943
2.4498
5.843
9.2362
12.629
16.023
Bs
-8.913
-7.202
-5.492
-3.782
-2.071
-0.361
1.3493
3.0597
4.77
6.4803
8.1907

Fig. S2. Hierarchical clustering of genes with statistically significant changes in expression levels produced by water stress treatment. The 710 shared genes with significant expression-level change were analysed using Ward’s hierarchical clustering method with the Euclid distance.

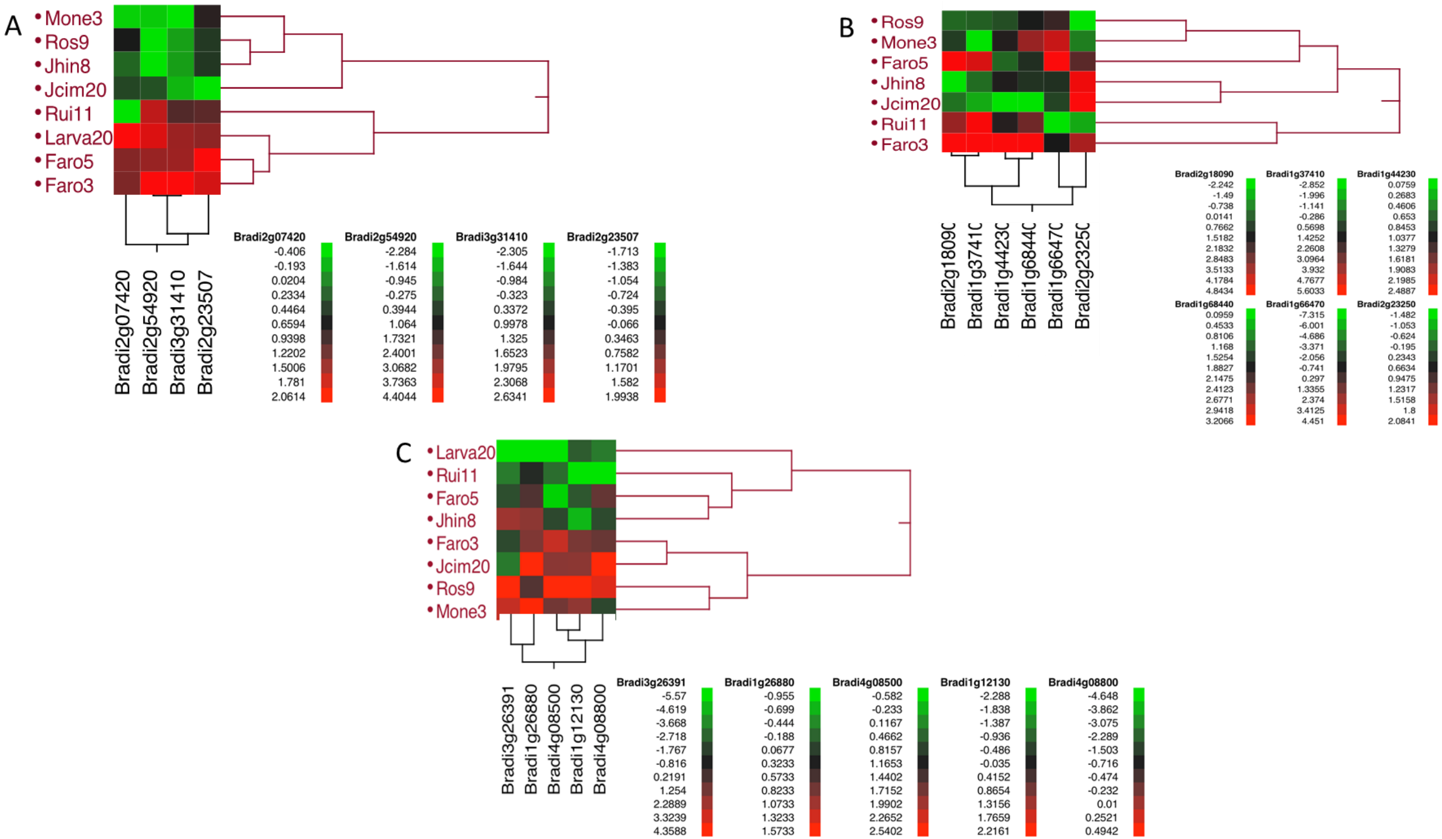

Fig. S3. Transcript level variation of four osmoprotectans genes (A), six chaperones genes (B) and five photosynthetic and plastidic genes (C) between 8 *B. hybridum* genotypes. Hierarchical clustering by Euclidean distance of the normalized gene expression after 21 days of experimental water restriction.

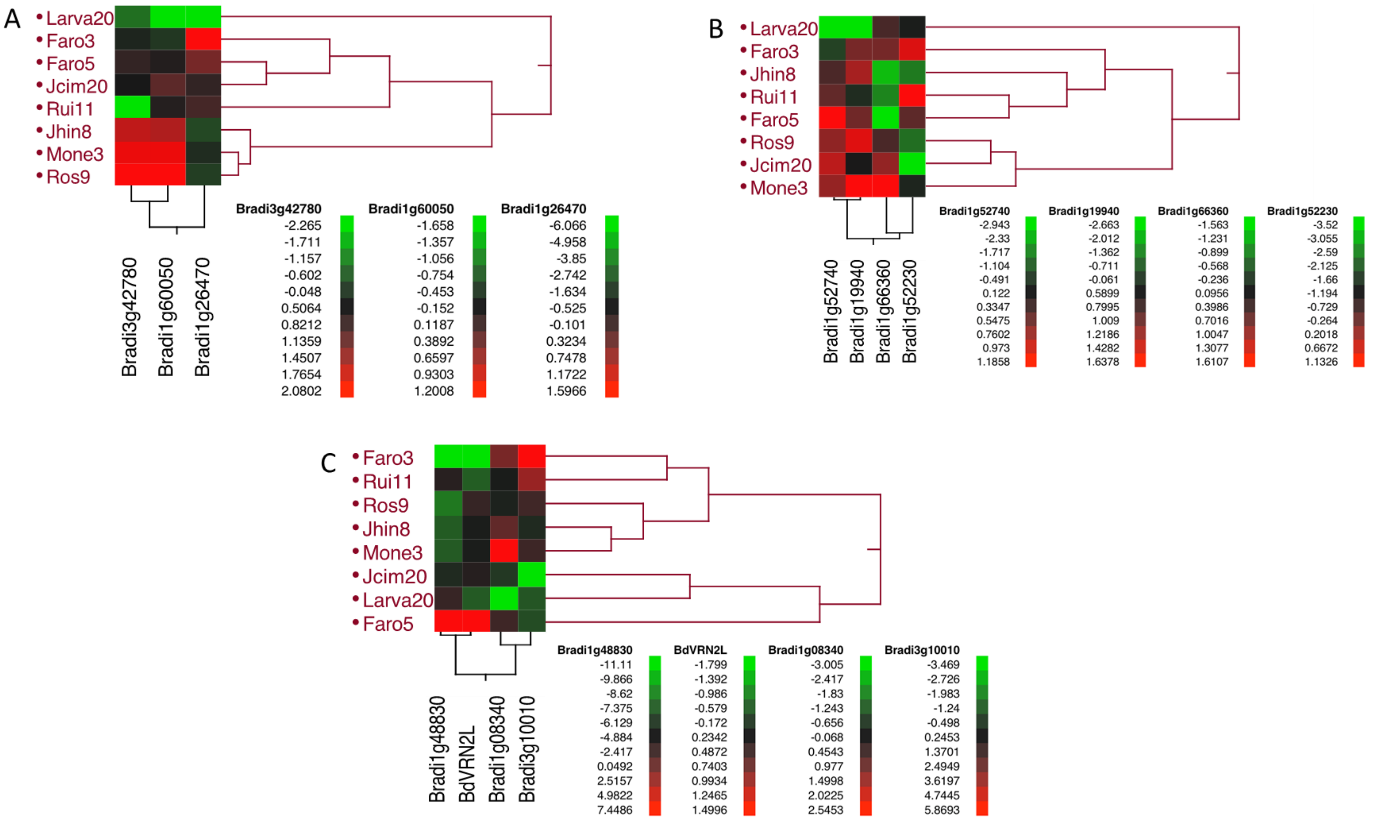

Fig. S4. Transcript level variation of three ROS scavenging and metabolic genes (A), four regulatory genes (B) and four flowering time-related genes (C) between 8 *B. hybridum* genotypes. Hierarchical clustering by Euclidean distance of the normalized gene expression after 21 days of experimental water restriction.

Homoeolog regulation in the progeny	II  Bd Bh Bs	XI  Bd Bh Bs	IV  Bd Bh Bs	IX  Bd Bh Bs	**Total**
Both homeologs upregulated	16	0	2	7	25
Only Bd homeologs upregulated	256	0	121	0	377
Only Bs homeologs upregulated	0	79	3	728	810
Bd up- and Bs downregulated	32	0	88	0	120
Both homeologs downregulated	1	5	13	0	19
Only Bd homeologs downregulated	4	389	0	47	440
Only Bs homeologs downregulated	112	0	524	0	636
Bd down and Bs upregulated	1	41	0	73	115
No change	184	224	324	296	1028
Total number of homeolog pairs	606	738	1075	1151	3570

Fig. S5. Homoeolog expression levels relative to the levels of its diploid parents. The figures shown at the top are parallel to those shown in Fig. 6 and represent the 4 differential expression states of ELD.

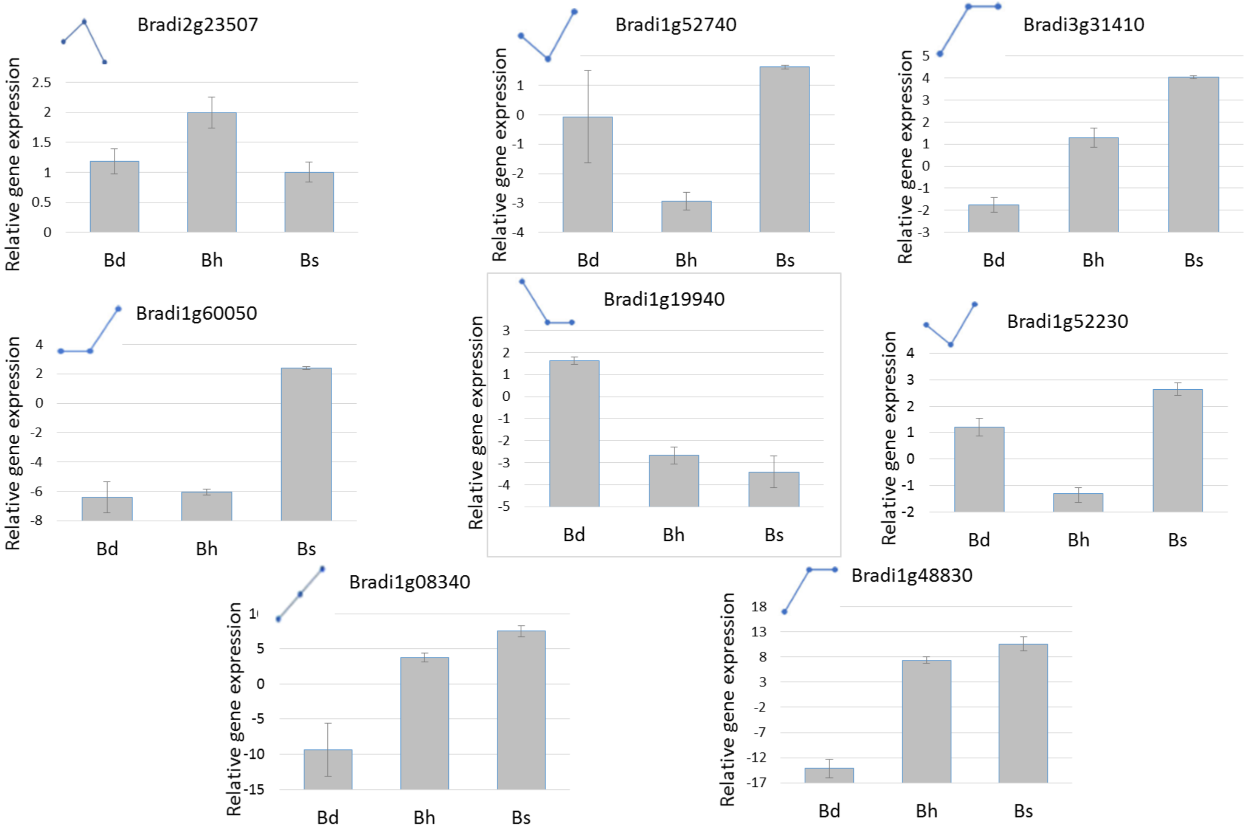

Fig. S6. qRT-PCR confirmation of ELD in the allopolyploid B. hybridum. Error bars represent the standard deviation from two biological replicates. The expression patterns of each categories derived from the RNA-seq data are shown above each histogram. (Bd-ELD, Bs-ELD, additivity, transgressive upregulation and transgressive upregulation.

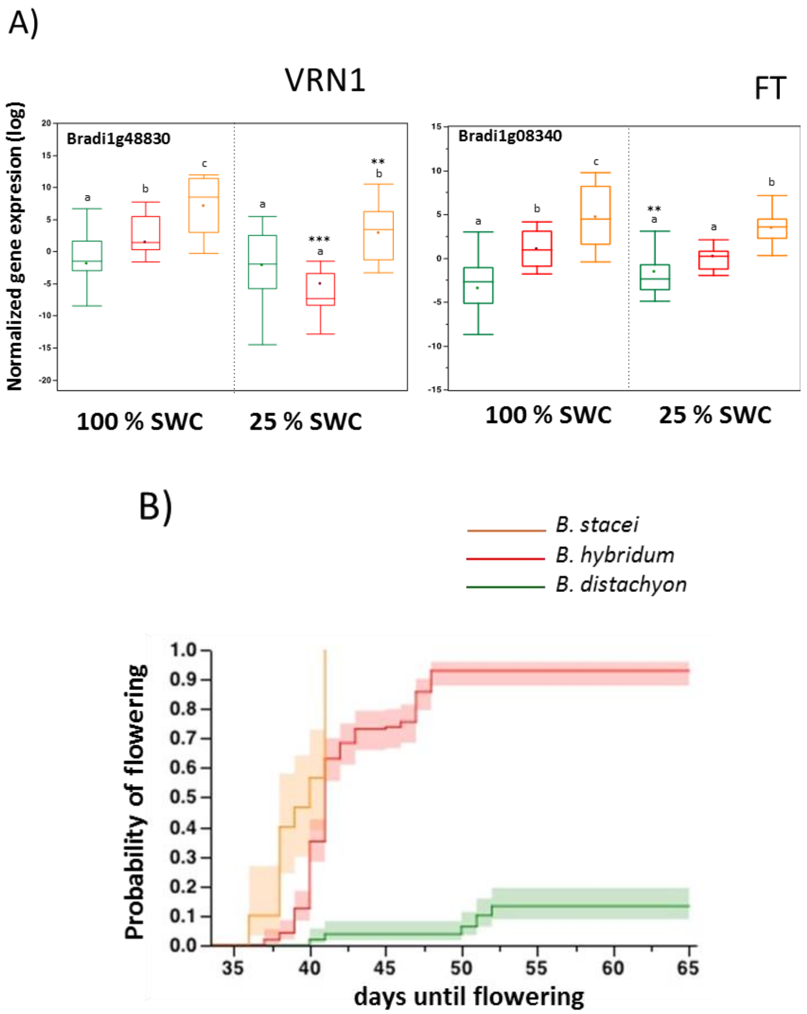

Fig. S7. A) Box-plot diagrams depicting variation in the normalized transcription levels of 2 flowering promotors candidate genes between the three species *(B. distachyon* = green boxes*; B. hybridum* = red boxes; *B. stacei* = yellow boxes) and the two watering treatments (within each graph, left panel corresponds to well-watered 100 % of soil water content, right panel corresponds to water stress treatment 25 % of soil water content). Point within each box is the mean of the distribution, and the horizontal line depicts the median. Within each treatment different letters indicate statistically significant differences at P<0.05 from post-hoc contrast tests. Asterisks above boxes in the water stress treatment indicate statistically significant differences between treatments in the transcription level at P<0.05(*), P<0.01(**), P<0.0001(***) respectively, inferred from tests of the main effects of univariate General Lineal Mixed Models performed on each gene. B) The probability of flowering for the three *Brachypodium* species along the study period. Shading indicates 95% confidence intervals. No vernalization treatment was applied in the experiment.

TABLES

Table S1. *Brachypodium* ID accessions, geographical origin of the lines included in the study. It is also shown the drought-response physiological measurements (from drought treatment; see Martínez et al., 2018 for details). EL (electrolyte leakage), SLA (specific leaf area), WC (water content). Values in the table are the genotypic ranges.

Species	Accession	Population	Geographical coordinates	EL (%)	SLA (m2/kg)	WC (%)	Proline (µmol g^{-1})
B. distachyon	Bd30-1	Sierra Nevada	36º 59' 43" N 3º 34' 18 W	32.50-37.28	31.35-35.5	60.15-61.82	1.81-2.89
B. stacei	ALSUR-1	Sorbas	37º 05' 79" N 2º 06' 35" W	41.38-45.21	20.43-31.90	55.14-63.08	2.42-27.07
B. hybridum	LARVA-20	Larva	37º 48' 38" N 3º 12' 53" W	11.34-22.48	29.89-30.54	44.52-52.66	5.28-22.08
	FARO3	Faro	37º 0' 52" N 7º 58' 35" W	10.23-11.18	68.5-77.14	78.42-83	3.28-3.29
	FARO5	Faro	37º 0' 52" N 7º 58' 35" W	10.09-15.69	51.74-66.8	77.67-79.34	3.31-3.35
	JCIM20	Cimbarra	38º 23' 28" N 3º 22' 13" W	7.57-12.94	57.09-81.54	73.03-78.11	3.39-7.08
	JHIN8	Hinojares	37º 43' 41" N 2º 58' 52" W	5.67-6.94	37.65-50	51.8-74.41	10.18-40.05
	MONE3	Monegros	41º 30' 50" N 0º 30' 30" W	17.81-19.70	42.46-55.22	57.95-71.04	30.02-43.52
	ROS9	Rosas de Mar	42º 14' 45" N 3º 11' 1" E	29.10-43.43	30.16-35.41	28.29-44.34	9.07-34.68
	RUI11	Ruidera	38º 57' 42 N 2º 52' 17" W	7.53-21.07	39.44-57.12	54.24-76.03	12.84-34.48

Table S2. Information of candidate genes selected in this study for qRT-PCR validation.

Gene ID	*B. distachyon* orthologous ID	*B. stacei* orthologous ID	*A. thaliana* orthologous ID	Sequence (5´- 3´)	Primer melting temperature (Cº)	Product size (bp)
Cell- stress osmoprotection genes						
P5CS1	Bradi2g23507	Brast08G099000	AT3G55610.1	F: TGATGACATATTGTTAGCACTCAAAAC R: GCTTTCGGGTAGCCTAATACTTTGT	58.9 61.3	87
P5CS2	Bradi2g54920	Brast01G089500	AT3G55610.1	F: AAGGAAGTGACAACCCGTGAGA R: GCTTCTAGAGCGTCAGCAATGTCTAG	60.3 64.8	119
BAC2	Bradi2g07420	Brast01G334000	AT1G79900.1	F: TCGCCTTCCAGAATGCAATGGTGTT R: TGACGAGCTCTACGGGGGACAGGAT	63 67.9	153
TREHALASE 1	Bradi3g31410	Brast03G176300	AT4G24040.1	F: TACTGGGACTCCTACTGGGTCATC R: GCACCGTTGAGAACAAAACCATAT	64.4 59.3	122
Chaperones genes						
LEA3	Bradi2g18090	Brast08G040500	AT3G15670.1	F: CTTGTTTTCCCAGCGAGACACTGA R: TGGAGGCCATCTCTCACGTACTACTG	62.7 66.4	119
Dehydrin DHN3	Bradi1g37410	Brast07G152200	AT5G66400.2	F: GGTCAGGCGACAAACCGCGT R: GAGCTGCCGGAGCGGTGAAG	63.5 65.5	158
HSC70	Bradi1g66470	Brast02G133500	AT5G02500.1	F: GGTCGAATAATGCAAGTGCTTGTGTA R: TCCAGAGCATAGGGCAAGGAATTAC	61.6 63	125
HSP70	Bradi2g23250	Brast08G095800	AT3G12580.1	F: AATTTAATGGTGTGCCTCTTCTATTC R: GCGTGCACATCAAGAGTTCTAAG	58.5 60.6	131
HSP20	Bradi1g44230	Brast07G092600	AT5G37670.1	F: TGGCAGAGCTGTTCTTTGGGA R: AGGCCCGGGACGTTGATC	59.8 60.5	145
HSP21	Bradi1g68440	Brast02G116300	AT4G27670.1	F: ATGCGGCAGATGCTGGACACGATG R: GCCCCGGCATGTCGAACCTCATC	66.1 67.8	157

Photosynthetic and plastidic genes						
RBCS1A	Bradi3g26391	Brast03G114700	AT1G67090.1	F: GGGTCACACCGATTTTATGTACCTTT R: CCCCTCTCCCAACGATAGTTGTAGT	61.6 64.6	142
RBCS-family	Bradi4g08500	Brast10G096300	AT1G67090.1	F: CTTGTGTCGGATAGGCCAGGATTAG R: CGCGCACGTCTGTATAGTGTGGTA	64.6 64.4	138
RBCS-family	Bradi4g08800	N/A	AT1G67090.1	F: GCCGTCAATTCCAGGCTCGTCGTATG R: CCAAGCTTGGCTCTCCACCCAGTCCT	68 69.5	150
CAB1	Bradi1g12130	Brast02G267200	AT1G29930.1	F: TACTTGTACCACCCTGTTGTAAATTACG R: TACTATCACTGTCACCTCCATTGCTTA	62.2 61.9	153
Not defined	Bradi1g26880	Brast06G119700	AT4G24930.1	F: CGTGAGCTTCAAGGCTGACCAAC R: ATTGCTGTGGTGAACGGGTAGATG	64.2 62.7	177
ROS scavenging and metabolic genes						
SKU5	Bradi1g26470	Brast06G114400	AT1G76160.1	F: CACACCCTGCAGAGCATCTACTCT R: GACGGCGGTTGTGGATATGAT	64.4 59.8	147
G6PD2	Bradi1g60050	Brast02G212000	AT5G13110.1	F: GGAACACCGTCAGCATCACTGTC R: ATCGCTCATCTTGCTACGTGCATAA	64.2 61.3	146
FAR3	Bradi3g42780	Brast03G305800	AT4G33790.1	F: AGTCCAAGATGAGGTGACGGACACTG R: CTCCTCCACGAATAGCTCGAATCCA	66.4 64.6	88
Regulatory control and hormone metabolism genes						
HTA6	Bradi1g66360	Brast02G134800	AT5G59870.1	F: TGAGATGTTGGTGTTCCTCTAGCTAT R: CACGGTAACTAGGCAGATCAGAAC	61.6 62.7	105
PRMT3	Bradi1g19940	Brast06G036300	AT3G12270.1	F: TGGGAGAGGTGGAACTAGCTT R: CAGTATCTGTCACGACATCCTGA	59.8 60.6	143
MTR4	Bradi1g52230	Brast07G003100	AT1G59760.1	F: TGATGCTAAAAGGAGGTGCTGATAGTT R: TCCTCACAGCGCATTTGATTTAATAA	61.9 62.4	85
AAO2	Bradi1g52740	Brast06G171000	AT3G43600.1	F: CTTTCGCACTGGGACAGTTATGGCCT R: GCCTGAAGGGTTGCTGCACAACTAGATTC	66.4 59.3	151

Flowering time and vernalization genes						
FT	Bradi1g48830	Brast07G042400	AT1G65480.1	F: TTCGGGAACAGGAACGTGTCCAAC R: AGCATCTGGGTCTACCATCACGAG	64.4 64.4	129
VRN1	Bradi1g08340	Brast02G311100	AT1G69120.1	F: GTCGCGCTCATCATCTTCTC R: TGCATAGGAGTAGCGCTCATAG	62.1 62.4	102
VRN2	Bradi3g10010	Brast04G226800	AT5G24930.1	F: GTTGAACCGGCCACTCAT R: CATGGTGTCGCCAAATGT	56 53.7	97
VRN2-like	BdVRN2L	N/A	N/A	F: ATGCATGAGAGAGAGGCGAAGG R: TCGTAGCGGATCTGCTTCTCGTAG	62.1 64.4	80
Normalization genes						
UBC18	Bradi4g00660.1			F: GGAGGCACCTCAGGTCATTT R: ATAGCGGTCATTGTCTTGCG	59.4 57.3	193
UBI10	Bradi1g32860.1			F: TGGACTTGCTTCTGTCTGGGTTCA R: TGGTACACAGGCATAACACTGACG	62.7 62.7	138

Table S3. Statistics of RNA-seq reads and mapped reads (against *Brachypodium distachyon* genome). R1 and R2 are the two biological replicates; 100 depicts the well-watered treatment and 25 is the drought treatment. Bd lines (*B. distachyon* diploid parent); Bs lines (*B. stacei* diploid parent); Bh lines (*B. hybridum* derived allotetraplid).

Samples	Total raw reads	Total processed reads	Total processed nucleotides (bp)	Mapped reads (%)	Nº reads aligned 1 time (%)	Nº reads aligned > 1 times (%)
Bd30 100 R1	10249308	9582727	1214673427	89	59	30
Bd30 100 R2	9360186	8809654	1111429832	89	58	31
Bd30 25 R1	8391285	7987770	1022318813	87	59	28
Bd30 25 R2	10139481	9476227	1207589101	83	58	25
BsAlsur1 100 R1	8669114	7477260	967667910	61	39	22
BsAlsur1 100 R2	8392513	7773503	1025975355	62	40	22
BsAlsur1 25 R1	11281188	9847292	1267505631	59	38	21
BsAlsur1 25 R2	12213500	11575862	1552369106	59	39	20
BhLarva20 100 R1	9311443	8957252	1196352040	75	50	25
BhLarva20 100 R2	8400628	8094756	1081073986	74	50	24
BhLarva20 25 R1	15771250	14810863	1956026831	66	46	20
BhLarva20 25 R2	15184976	14294533	1892210773	68	46	22
Total	127364872	118687699	15495192805	-	-	
Average	10613739.33	9890641.58	1291266067	72.6	48.8	24.2

Table S4. Statistics of RNA-seq reads and mapped reads (against *Brachypodium distachyon, Brachypodium stacei and Brachypodium hybridum* reference genomes). R1 and R2 are the two biological replicates; 100 depicts the well-watered treatment and 25 is the drought treatment. Bd lines (*B. distachyon* diploid parent); Bs lines (*B. stacei* diploid parent); Bh lines (*B. hybridum* derived allotetraplid).

Samples	Total raw reads	Total processed reads	Total processed nucleotides (bp)	Mapped reads (%)	Nº reads aligned 1 time (%)	Nº reads aligned > 1 times (%)
Bd30 100 R1	10249308	9582727	1214673427	89	56	33
Bd30 100 R2	9360186	8809654	1111429832	89	55	34
Bd30 25 R1	8391285	7987770	1022318813	87	56	31
Bd30 25 R2	10139481	9476227	1207589101	84	55	29
BsAlsur1 100 R1	8669114	7477260	967667910	86	70	16
BsAlsur1 100 R2	8392513	7773503	1025975355	88	72	16
BsAlsur1 25 R1	11281188	9847292	1267505631	85	69	16
BsAlsur1 25 R2	12213500	11575862	1552369106	86	71	15
BhLarva20 100 R1	9311443	8957252	1196352040	88	24	64
BhLarva20 100 R2	8400628	8094756	1081073986	87	25	62
BhLarva20 25 R1	15771250	14810863	1956026831	79	26	53
BhLarva20 25 R2	15184976	14294533	1892210773	81	25	56
Total	127364872	118687699	15495192805	-	-	
Average	10613739.33	9890641.58	1291266067	85.9	50.3	35.4

Table S5. Results of distance-based redundancy analyses (dbRDA) testing the effects of geography (latitude and longitude) and genetic differentiation (principal components from pairwise F_{ST} matrix) on pairwise gene expression distance of 26 drought-tolerance related traits between 7 *B. hybridum* populations distributed across the Iberian Peninsula. The proportion of multivariate genetic variation explained (% var) by a given predictor is indicated.

Marginal tests								Conditional tests							
Chaperones															
Variable	*F*	*P*	*%var*	Variable	*F*	*P*	*%var*	Variable	*F*	*P*	*%var*	Variable	*F*	*P*	*%var*
Bradi2g18090 ***Control***				***Bradi2g18090*** ***stress***				***Bradi2g18090*** ***Control***				***Bradi2g18090*** ***stress***			
FstPCoA1	0.31	0.552	10.8	FstPCoA1	0.31	0.667	10.8								

FstPCoA2	0.52	0.501	7.3	FstPCoA2	1.96	0.198	12.1								
Latitude	0.001	0.931	16.5	Latitude	1.70	0.226	9.2	FstPCoA1	0.36	0.601	15.5	FstPCoA1	0.89	0.423	2.4
Longitude	0.71	0.451	4.4	Longitude	1.94	0.22	11.9	FstPCoA2	0.08	0.756	24.1	FstPCoA2	1.82	0.234	14.6
Bradi1g37410 ***Control***				***Bradi1g37410*** ***stress***				***Bradi1g37410*** ***Control***				***Bradi1g37410*** ***stress***			
FstPCoA1	0.085	0.735	15.1	FstPCoA1	0.99	0.423	0.2								
FstPCoA2	0.054	0.854	15.6	FstPCoA2	3.33	0.122	25								
Latitude	0.27	0.618	11.6	Latitude	2.12	0.191	13.7	FstPCoA1	0.17	0.716	24.1	FstPCoA1	2.56	0.183	23.8
Longitude	0.85	0.384	2.2	Longitude	0.67	0.469	4.9	FstPCoA2	0.14	0.720	25	FstPCoA2	1.27	0.275	5.2
Bradi1g44230 ***Control***				***Bradi1g44230*** ***stress***				***Bradi1g44230*** ***Control***				***Bradi1g44230*** ***stress***			
FstPCoA1	0.018	0.884	16.3	FstPCoA1	1.13	0.275	1.8								

FstPCoA2	1.46	0.256	6.1	FstPCoA2	0.080	0.791	15.1								
Latitude	0.027	0.851	16.1	Latitude	0.22	0.625	12.5	FstPCoA1	0.005	0.952	32.6	FstPCoA1	1.56	0.299	11.2
Longitude	0.24	0.649	12.1	Longitude	1.10	0.309	1.4	FstPCoA2	1.06	0.401	1.7	FstPCoA2	0.003	0.964	27.9
Bradi1g66470 ***Control***				***Bradi1g66470*** ***stress***				***Bradi1g66470*** ***Control***				***Bradi1g66470*** ***stress***			
FstPCoA1	3.16	0.137	26.6	FstPCoA1	0.093	0.811	14.8								
FstPCoA2	1.69	0.227	8.9	FstPCoA2	1.52	0.256	6.8								
Latitude	0	0.995	16.6	Latitude	0.025	0.869	16.2	FstPCoA1	4.29	**0.098**	44.3	FstPCoA1	0.050	0.852	27.8
Longitude	0.48	0.524	7.9	Longitude	0.53	0.491	7.2	FstPCoA2	0.70	0.419	7.1	FstPCoA2	5.37	**0.07**	55.3
Bradi2g23250 ***Control***				***Bradi2g23250*** ***stress***				***Bradi2g23250*** ***Control***				***Bradi2g23250*** ***stress***			
FstPCoA1	0.078	0.958	22.5	FstPCoA1	3.24	**0.093**	27.1								

FstPCoA2	2.90	0.203	27.5	FstPCoA2	1.14	0.320	2.26								
Latitude	0.34	0.619	15.3	Latitude	8.85	**0.02**	56.7	FstPCoA1	0.023	0.927	73.2	FstPCoA1	6.17	0.110	23.7
Longitude	0.002	0.950	24.9	Longitude	1.69	0.236	10.3	FstPCoA2	3.10	0.200	62.5	FstPCoA2	0.11	0.790	12.1
Bradi1g68440 ***Control***				***Bradi1g68440*** ***stress***				***Bradi1g68440*** ***Control***				***Bradi1g68440*** ***stress***			
FstPCoA1	1.007	0.364	0.1	FstPCoA1	5.60	**0.070**	39.7								
FstPCoA2	4.77	**0.094**	38.6	FstPCoA2	0.11	0.720	14.5								
Latitude	0.001	0.975	19.9	Latitude	0.018	0.88	16.3	FstPCoA1	85.17	**0.004**	94.9	FstPCoA1	10.70	**0.049**	65.3
Longitude	0.74	0.438	4.47	Longitude	0.52	0.466	7.4	FstPCoA2	2.54	0.187	27.6	FstPCoA2	0.087	0.784	22.1

FT ***Control***				***FT*** ***stress***				***FT*** ***Control***				***FT*** ***stress***			
FstPCoA1	0.74	0.423	3.8	FstPCoA1	0.001	0.99	16.6								

FstPCoA2	0.98	0.349	0.2	FstPCoA2	0.42	0.563	9.1								
Latitude	1.09	0.339	1.3	Latitude	0.88	0.346	1.6	FstPCoA1	0.30	0.522	17.2	FstPCoA1	0.057	0.776	28.2
Longitude	1.96	0.205	12.1	Longitude	0.64	0.416	5.4	FstPCoA2	1.23	0.358	4.7	FstPCoA2	0.13	0.748	25.7
VRN2L				***VRN2L***				***VRN2L***				***VRN2L***			
Control				***stress***				***Control***				***stress***			
FstPCoA1	2.45	0.164	17.1	FstPCoA1	0.15	0.623	13.7								
FstPCoA2	0.65	0.445	5.2	FstPCoA2	2.88	0.146	21.2								
Latitude	0.60	0.50	6	Latitude	0.016	0.876	16.3	FstPCoA1	5.28	**0.066**	48.2	FstPCoA1	0.21	0.513	14.3
Longitude	1.83	0.24	10.6	Longitude	0.92	0.346	1.1	FstPCoA2	0.61	0.432	8.7	FstPCoA2	1.02	0.471	0.3
Bradi3g1001				***Bradi3g1001***				***Bradi3g1001***				***Bradi3g1001***			
Control				***stress***				***Control***				***stress***			
FstPCoA1	4.76	**0.040**	38.5	FstPCoA1	6.76	**0.045**	45.1								

FstPCoA2	0.015	0.900	19.6	FstPCoA2	0.14	0.753	14.1								
Latitude	0.34	0.602	12.3	Latitude	0.14	0.709	14	FstPCoA1	2.55	0.223	38.9	FstPCoA1	6.18	**0.059**	61.1
Longitude	0.36	0.595	11.9	Longitude	0.049	0.844	15.7	FstPCoA2	0.001	0.994	46.4	FstPCoA2	0.015	0.919	29.4
VRN1				***VRN1***				***VRN1***				***VRN1***			
Control				***stress***				***Control***				***stress***			
FstPCoA1	0.030	0.840	16.1	FstPCoA1	0.073	0.752	15.2								
FstPCoA2	2.68	0.158	19.4	FstPCoA2	0.022	0.904	16.2								
Latitude	0.013	0.906	16.4	Latitude	0.013	0.879	16.4	FstPCoA1	0.032	0.868	33.3	FstPCoA1	0.064	0.766	31.7
Longitude	0.047	0.844	15.7	Longitude	0.005	0.948	16.5	FstPCoA2	3.62	0.127	47.6	FstPCoA2	0.091	0.803	30.6
Osmogenes															
Bradi2g07420				***Bradi2g07420***				***Bradi2g07420Control***				***Bradi2g07420stress***			
Control				***stress***											

FstPCoA1	0.16	0.591	13.5	FstPCoA1	0.053	0.847	15.6								
FstPCoA2	0.83	0.468	2.5	FstPCoA2	70.25	**0.002**	90.8								
Latitude	0.69	0.386	4.6	Latitude	1.34	0.275	4.6	FstPCoA1	0.33	0.523	19.5	FstPCoA1	0.002	0.974	27.3
Longitude	0.37	0.490	9.9	Longitude	0.32	0.601	10.7	FstPCoA2	0.33	0.622	19.3	FstPCoA2	99.91	**0.003**	99
Bradi2g54920 ***Control***				***Bradi2g54920*** ***stress***				***Bradi2g54920*** ***Control***				***Bradi2g54920*** ***stress***			
FstPCoA1	0.008	0.934	16.49	FstPCoA1	0.10	0.858	14.7								
FstPCoA2	0.28	0.593	11.43	FstPCoA2	1.60	0.247	7.9								
Latitude	5.14	**0.059**	37.17	Latitude	3.64	**0.084**	27.4	FstPCoA1	0.22	0.724	12	FstPCoA1	0.70	0.443	5.5
Longitude	6.02	**0.055**	41.74	Longitude	2.05	0.195	13.1	FstPCoA2	0.061	0.817	14.9	FstPCoA2	0.62	0.472	7.3
Bradi2g23507				***Bradi2g23507***				***Bradi2g23507***				***Bradi2g23507***			

Control				***stress***				***Control***				***stress***			
FstPCoA1	0.036	0.84	15.95	FstPCoA1	4.20	0.114	31.4								
FstPCoA2	2.51	0.174	17.7	FstPCoA2	0.89	0.362	1.5								
Latitude	0.002	0.972	16.6	Latitude	0.77	0.387	3.4	FstPCoA1	0.038	0.821	26.2	FstPCoA1	13.55	**0.058**	76.4
Longitude	0.45	0.524	8.5	Longitude	1.80	0.251	10.3	FstPCoA2	1.16	0.357	3.3	FstPCoA2	1.45	0.323	8.8
Bradi3g31410				***Bradi3g31410***				***Bradi3g31410***				***Bradi3g31410***			
Control				***stress***				***Control***				***stress***			
FstPCoA1	10.45	**0.006**	57.45	FstPCoA1	0.46	0.589	8.4								
FstPCoA2	0.25	0.628	11.9	FstPCoA2	3.59	0.113	27.1								
Latitude	0.17	0.725	13.49	Latitude	3.64	**0.098**	27.4	FstPCoA1	7.24	**0.05**	72.9	FstPCoA1	1.91	0.266	13.4
Longitude	0.37	0.566	9.8	Longitude	2.08	0.167	13.3	FstPCoA2	0.30	0.596	21.3	FstPCoA2	2.25	0.173	17.4

Photosyntesis genes															
Bradi3g26391 Control				***Bradi3g26391 stress***				***Bradi3g26391 Control***				***Bradi3g26391 stress***			
FstPCoA1	69.73	**0.004**	90.7	FstPCoA1	0.54	0.549	7.1								
FstPCoA2	0.27	0.652	11.6	FstPCoA2	0.99	0.342	0.1								
Latitude	0.25	0.587	11.9	Latitude	4.36	**0.086**	32.5	FstPCoA1	52.12	**0.007**	98.1	FstPCoA1	0.12	0.743	15.9
Longitude	0.085	0.721	15.1	Longitude	1.03	0.352	0.4	FstPCoA2	0.68	0.563	9.2	FstPCoA2	0.022	0.885	18.1
Bradi4g08500 Control				***Bradi4g08500 stress***				***Bradi4g08500 Control***				***Bradi4g08500 stress***			
FstPCoA1	3.06	0.122	22.7	FstPCoA1	0.17	0.728	13.4								
FstPCoA2	0.013	0.913	16.4	FstPCoA2	0.65	0.441	5.3								
Latitude	0.48	0.518	8.1	Latitude	2.57	0.162	18.3	FstPCoA1	1.88	0.265	17.8	FstPCoA1	0.76	0.466	4.6

Longitude	1.01	0.349	0.2	Longitude	0.59	0.502	6.1	FstPCoA2	0.01	0.923	29.5	FstPCoA2	0.003	0.953	22.6
Bradi4g08800 ***Control***				***Bradi4g08800*** ***stress***				***Bradi4g08800*** ***Control***				***Bradi4g08800*** ***stress***			
FstPCoA1	2.75	0.133	20	FstPCoA1	2.61	0.146	18.7								
FstPCoA2	0.22	0.675	12.6	FstPCoA2	0.30	0.611	11.1								
Latitude	0.16	0.662	13.7	Latitude	0.21	0.696	12.6	FstPCoA1	1.69	0.217	16.6	FstPCoA1	2.69	0.175	34.1
Longitude	0.073	0.76	15.2	Longitude	0.085	0.805	15.1	FstPCoA2	0.081	0.805	30.7	FstPCoA2	0.66	0.46	9.8
Bradi1g12130 ***Control***				***Bradi1g12130*** ***stress***				***Bradi1g12130*** ***Control***				***Bradi1g12130*** ***stress***			
FstPCoA1	16.92	**0.019**	69.5	FstPCoA1	0.87	0.451	1.8								
FstPCoA2	0.39	0.548	9.4	FstPCoA2	0.001	0.961	16.6								
Latitude	0.001	0.98	16.5	Latitude	2.31	0.189	15.7	FstPCoA1	16.25	**0.029**	98	FstPCoA1	3.04	0.171	24.3

Longitude	0.005	0.96	16.5	Longitude	0.26	0.649	11.7	FstPCoA2	0.43	0.586	17.9	FstPCoA2	1.40	0.318	6.3
Bradi1g26880 Control				***Bradi1g26880 stress***				***Bradi1g26880 Control***				***Bradi1g26880 stress***			
FstPCoA1	10.23	**0.001**	56.8	FstPCoA1	0.24	0.433	12.1								
FstPCoA2	0.10	0.768	14.7	FstPCoA2	3.67	**0.09**	27.6								
Latitude	0.70	0.43	43.6	Latitude	0.18	0.637	13.3	FstPCoA1	6.32	**0.054**	63.8	FstPCoA1	0.33	0.505	16.8
Longitude	0.25	0.647	12.1	Longitude	0.11	0.675	14.6	FstPCoA2	0.006	0.953	30.7	FstPCoA2	1.40	0.315	7.9
Regulation genes															
Bradi1g52740 Control				***Bradi1g52740 stress***				***Bradi1g52740 Control***				***Bradi1g52740 stress***			

FstPCoA1	0.033	0.826	16.1	FstPCoA1	0.013	0.845	16.4								
FstPCoA2	3.53	0.102	26.6	FstPCoA2	4.17	**0.085**	31.2								
Latitude	0.087	0.741	14.9	Latitude	0.60	0.405	6	FstPCoA1	0.049	0.784	29.1	FstPCoA1	0.081	0.798	25.4
Longitude	0.056	0.792	15.6	Longitude	0.014	0.873	16.4	FstPCoA2	1.78	0.278	16.6	FstPCoA2	1.57	0.286	11.6
Bradi1g19940 ***Control***				***Bradi1g19940*** ***stress***				***Bradi1g19940*** ***Control***				***Bradi1g19940*** ***stress***			
FstPCoA1	0.14	0.648	13.9	FstPCoA1	0.017	0.857	16.3								
FstPCoA2	7.23	**0.035**	47.1	FstPCoA2	1.86	0.22	11								
Latitude	0.027	0.871	16.1	Latitude	0.49	0.457	7.7	FstPCoA1	0.11	0.724	24.8	FstPCoA1	0.001	0.97	25
Longitude	0.25	0.581	12	Longitude	0.018	0.831	16.3	FstPCoA2	4.30	0.108	45.6	FstPCoA2	0.35	0.626	15.1
Bradi1g66360 ***Control***				***Bradi1g66360*** ***stress***				***Bradi1g66360*** ***Control***				***Bradi1g66360*** ***stress***			

FstPCoA1	1.09	0.355	1.3	FstPCoA1	1.40	0.33	5.4								
FstPCoA2	0.82	0.389	2.6	FstPCoA2	0.14	0.721	13.9								
Latitude	0.009	0.939	16.5	Latitude	0.88	0.406	1.7	FstPCoA1	1.12	0.335	2.7	FstPCoA1	2.64	0.204	25.7
Longitude	0.49	0.53	7.8	Longitude	0.028	0.883	16.1	FstPCoA2	0.29	0.655	19.4	FstPCoA2	0.13	0.74	21.9
Bradi1g52230 ***Control***				***Bradi1g52230*** ***stress***				***Bradi1g52230*** ***Control***				***Bradi1g52230*** ***stress***			
FstPCoA1	0.018	0.905	16.3	FstPCoA1	3	0.138	22.2								
FstPCoA2	0.006	0.931	16.5	FstPCoA2	0.014	0.903	16.4								
Latitude	0.17	707	13.5	Latitude	0.77	0.432	3.3	FstPCoA1	0	1	25.2	FstPCoA1	7.51	**0.056**	59.7
Longitude	1.40	0.264	5.4	Longitude	1.88	0.209	11.3	FstPCoA2	0.097	0.764	22.2	FstPCoA2	0.037	0.852	25.1
ROS genes															

Bradi3g42780 ***Control***				***Bradi3g42780*** ***stress***				***Bradi3g42780*** ***Control***				***Bradi3g42780*** ***stress***			
FstPCoA1	0.74	0.41	3.8	FstPCoA1	0.16	0.711	13.5								
FstPCoA2	0.59	0.559	6.2	FstPCoA2	0.039	0.872	15.9								
Latitude	0.52	0.502	7.3	Latitude	0.87	0.359	1.8	FstPCoA1	1.93	0.241	12.8	FstPCoA1	0.37	0.467	16.4
Longitude	2.99	0.148	22.2	Longitude	0.12	0.711	14.4	FstPCoA2	0.31	0.605	13.1	FstPCoA2	0.16	0.743	22.9
Bradi1g26470 ***Control***				***Bradi1g26470*** ***stress***				***Bradi1g26470*** ***Control***				***Bradi1g26470*** ***stress***			
FstPCoA1	0.63	0.505	5.6	FstPCoA1	0.011	0.898	16.4								
FstPCoA2	0.018	0.883	16.3	FstPCoA2	0.17	0.661	13.3								
Latitude	1.35	0.282	4.7	Latitude	0.65	0.414	5.2	FstPCoA1	1.95	0.962	13.7	FstPCoA1	0.015	0.91	23.1
Longitude	3.66	**0.097**	27.6	Longitude	2.45	0.171	17.1	FstPCoA2	0.009	0.924	21.1	FstPCoA2	0.075	0.773	21.4

Bradi1g60050				***Bradi1g60050***				***Bradi1g60050***				***Bradi1g60050***			
Control				***stress***				***Control***				***stress***			
FstPCoA1	1.73	0.233	9.5	FstPCoA1	0.009	0.905	16.4								
FstPCoA2	0.99	0.69	0.1	FstPCoA2	5.38	**0.048**	38.5								
Latitude	0.29	0.593	11.3	Latitude	1.84	0.209	10.8	FstPCoA1	0.008	0.92	16.5	FstPCoA1	0.044	0.766	22.6
Longitude				Longitude	0.30	0.529	11.1	FstPCoA2	1.20	0.321	4.5	FstPCoA2	1.91	0.232	14.7

Table S6. Top 10 GO significantly enriched biological process terms for expression-level dominance (ELD) genes in the allopolyploid *B. hybridum*. All significantly enriched GO terms are listed in Additional file 1.

Expression patterns	GO ID	GO Name	FDR
ELD-Bd	GO:0044710	single-organism metabolic process	3.90E-08
	GO:0044711	single-organism biosynthetic process	6.10E-08
	GO:0006082	organic acid metabolic process	7.10E-07
	GO:0043436	oxoacid metabolic process	1.10E-05
	GO:0019752	carboxylic acid metabolic process	3.00E-05
	GO:0044283	small molecule biosynthetic process	4.40E-05
	GO:0005975	carbohydrate metabolic process	0.00019
	GO:0006720	isoprenoid metabolic process	0.00019
	GO:0008299	isoprenoid biosynthetic process	0.0002
	GO:0065008	regulation of biological quality	0.0011
ELD-Bs	GO:0044237	cellular metabolic process	
	GO:1901564	organonitrogen compound metabolic process	8.50E-07
	GO:0006412	translation	2.90E-06
	GO:0006518	peptide metabolic process	3.80E-06
	GO:0043043	peptide biosynthetic process	3.80E-06
	GO:0009058	biosynthetic process	3.80E-06
	GO:0043604	amide biosynthetic process	3.90E-06
	GO:0043603	cellular amide metabolic process	7.60E-06
	GO:1901566	organonitrogen compound biosynthetic process	7.70E-06
	GO:0051169	nuclear transport	5.10E-06
Additive	GO:0005975	carbohydrate metabolic process	0.041
Transgresive up-regulation	GO:0044712	single-organism catabolic process	3.60E-07
	GO:0009056	catabolic process	4.10E-05
	GO:0044710	single-organism metabolic process	7.60E-05
	GO:0006810	transport	9.30E-05
	GO:0051179	localization	0.00017
	GO:0044242	cellular lipid catabolic process	0.00031
	GO:1901565	organonitrogen compound catabolic process	0.00072
	GO:0052542	defense response by callose deposition	0.0016
	GO:0034440	lipid oxidation	0.0019
	GO:0008104	protein localization	0.0019
Transgresive down-regulation	GO:1901566	organonitrogen compound biosynthetic process	3.60E-72
	GO:1901564	organonitrogen compound metabolic process	1.90E-69

	GO:0043604	amide biosynthetic process	1.40E-46
	GO:0006412	translation	3.20E-46
	GO:0043043	peptide biosynthetic process	9.10E-46
	GO:0006518	peptide metabolic process	1.70E-45
	GO:0009058	biosynthetic process	1.20E-43
	GO:0034660	ncRNA metabolic process	2.10E-43
	GO:0010467	gene expression	1.30E-36
	GO:0009657	plastid organization	1.20E-33

Table S7. Top 10 GO significantly enriched biological process terms for homoeolog pairs that displayed novel bias in the allopolyploid *B. hybridum*. All significantly enriched GO terms are listed in Additional file 1.

Expression in parents	Expression in progeny	GO ID	GO Name	FDR
Bd=Bs	BH_D>BH_S	GO:0072657	protein localization to membrane	0.02
		GO:0044281	small molecule metabolic process	0.026
		GO:0010033	response to organic substance	0.034
		GO:0016051	carbohydrate biosynthetic process	0.034
		GO:1902580	single-organism cellular localization	0.034
		GO:0042221	response to chemical	0.041
		GO:0044711	single-organism biosynthetic process	0.042
		GO:0014070	response to organic cyclic compound	0.046
		GO:0005975	carbohydrate metabolic process	0.046
		GO:1901700	response to oxygen-containing compound	0.046
Bd=Bs	BH_D<BH_S	GO:0043436	oxoacid metabolic process	0.0019
		GO:0019752	carboxylic acid metabolic process	0.0019
		GO:0006082	organic acid metabolic process	0.0019
		GO:0032787	monocarboxylic acid metabolic process	0.011
		GO:0006665	sphingolipid metabolic process	0.022
		GO:0006629	lipid metabolic process	0.035

		GO:0051186	cofactor metabolic process	0.039
		GO:0044283	small molecule biosynthetic process	0.045
		GO:0044255	cellular lipid metabolic process	0.045
		GO:0034220	ion transmembrane transport	0.045
Bd>Bs	BH_D<BH_S	-	-	
Bd<Bs	BH_D>BH_S	GO:0005975	carbohydrate metabolic process	0.0039
		GO:0044283	small molecule biosynthetic process	0.005
		GO:0072330	monocarboxylic acid biosynthetic process	0.0061
		GO:0034637	cellular carbohydrate biosynthetic process	0.013
		GO:0008152	metabolic process	0.018
		GO:0016051	carbohydrate biosynthetic process	0.023
		GO:1901565	organonitrogen compound catabolic process	0.023
		GO:0016053	organic acid biosynthetic process	0.025
		GO:0046394	carboxylic acid biosynthetic process	0.025
		GO:0006633	fatty acid biosynthetic process	0.025
		GO:0006082	organic acid metabolic process	0.025
		GO:0000165	MAPK cascade	0.028
		GO:0009250	glucan biosynthetic process	0.029
		GO:0044042	glucan metabolic process	0.031
		GO:0008610	lipid biosynthetic process	0.031
		GO:0071229	cellular response to acid chemical	0.034
		GO:1901606	alpha-amino acid catabolic process	0.037
		GO:1901701	cellular response to oxygen-containing compound	0.037
		GO:0043436	oxoacid metabolic process	0.039
		GO:0070887	cellular response to chemical stimulus	0.041
		GO:0044264	polysaccharide metabolic process	0.043
		GO:0033692	cellular polysaccharide biosynthetic process	0.043

		GO:0019752	carboxylic acid metabolic process	0.043
		GO:0000023	maltose metabolic process	0.043
		GO:0006766	vitamin metabolic process	0.043

APPENDIX 1

RT-qPCR analyses of gene expression

Real-time quantitative PCR amplification was performed in 10 µl of reaction mixture per well containing 1 µl of diluted cDNA, 0.4µM of each gene-specific primer and 5 µl of 2x SsoFast™ EvaGreen® Supermix (Bio-Rad). All reactions were analyzed using CFX96 real-time PCR detection system (Bio-Rad) following this two-step amplification protocol: an inicial step of enzyme activation/DNA denaturation of 95ºC for 1 min, followed by 60 cycles of 95ºC for 10 sec, 60ºC for 10 sec and a final standard dissociation protocol to obtain the melting profiles of 65ºC to 95ºC. Data (quantification cycle values, Cq) were adquired by means of the Bio-Rad CFX Manayer v3 software (Bio-Rad). The ubiquitin genes Bradi4g00660 and Bradi1g32860 were used as internal reference genes to normalize gene expression values (Hong et al., 2008; Chambers et al., 2012).

RT-qPCR analyses of gene expression and relation between gene expression between *B. hybridum* genotypes and gene differentiation

We conducted distance-based redundancy analyses (dbRDA, Legendre and Fortin 2010) using the 'capscale' function in the 'Vegan' package in R (Oksanen *et al.,* 2017) to examine whether *B. hybridum* genetic differentiation explains variation in gene expression between *B. hybridum* populations. We obtained pairwise distance matrices (based on the Manhattan distance, Oksanen *et al*., 2017) for the expression of each gene as response matrices. Gene expression distance matrices were then tested against three predictor variables: population' s PC scores of the first two axes from a PCA (accounting for 65 % of variance) performed on the pairwise FST matrix (Table S5), latitude and longitude. Significance of the predictors was assessed using multivariate F-statistics with 9999 permutations using the 'anova.cca' function in R. In both cases, we firstly analyzed the relationship between the genetic distance matrices and each set of variables separately (namely marginal test), and then we performed a partial dbRDA (namely conditional test) for each set of variables while controlling for the influence of latitude and longitude (included as covariates).

Relationship between homoeolog-specific expression and ELD

Up to 71% ((3570–1028)/3570) of the ELD-Bd and ELD-Bs homoeolog pairs, the main reason for the ELD was that only one of the homoeologs modified their expression under stress conditions (Fig. S5). Most homoeolog expression modifications were upregulations, reflecting the upregulation of the alternative homoeolog (1187 pairs = 377+810). Moreover, we observed more modifications in the Bd homoeolog of the gene pairs (860 genes = 179 + 77 + 4 + 11 + 386 + 23) than in the Bs homoeolog (772 genes = 179 + 40 + 4 + 11 + 515 + 23) (Fig. S5). For the gene pairs in ELD-Bd and ELD-Bs, where the dominant parent had a higher expression than the nondominant parent (ELD higher-expression parent, category II and IV respectively). This ELD can mostly be explained by the upregulation of the homoeolog from the dominant parent 209 (121 + 88) pairs in IV) and downregulation of the homoeolog from the dominant parent 144 (32+112) pairs in II) or the downregulation of the homoeolog from the nondominant parent (612 (88 + 524) pairs in IV, and upregulation of the homoeolog from the nondominant parent 226 (236+32) pairs in II) (Fig. S5). For the gene pairs in ELD-Bd and ELD-Bs, where the dominant parent had a lower expression than did the nondominant parent (ELD lower-expression parent, categories IX and XI respectively), (Fig. S5). Here, ELD can be explained by the downregulation of the homoeolog from the dominant parent (120 (47+73) pairs in IX, and upregulation of the homoeolog from the dominant parent 120 (79+41) pairs in XI) or the upregulation of the homoeolog of the nondominant parent 801 (728+73) pairs in IX, and downregulation of the homoeolog of the nondominant parent 430 (389+41) pairs in XI).

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CAPÍTULO 3

OVER-EXPRESSION OF THE *BRACHYPODIUM* DHN GENE, *BDDHN3*, ENHANCES DROUGHT TOLERANCE IN *BRACHYPODIUM DISTACHYON*

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Manuscrito inédito

ABSTRACT

Drought is the major abiotic stress that affects biomass productivity and grain yield of crops. DHNs (dehydrins), that belong to LEA (Late Embryogenesis Abundant) proteins, a large family of highly hydrophilic proteins, and are classified into Group II LEA protein, are typically upregulated under water limitation conditions playing a fundamental role in plant response and adaptation to stressful environments. However, the specific role of dehydrins and their relationship with functional traits is still poorly known. Here, we analyze the role of *BdDHN3* in the drought response of *Brachypodium distachyon* L, a well-studied monocot model plant for temperate grasses. We first investigate variation in the expression level of *BdDHN3* gene among 11 genetically distinct *B. distachyon* lines to examine genetic-based consistency in gene expression. Second, we generated transgenic *Brachypodium* plants (named 35S:*BdDHN3*) constitutively overexpressing *BdDHN3* under the control of CaMV35S promoter to analyze the drought response at functional and genetic expression level.

We found that *BdDHN3* gene is consistently up-regulated in response to drought stress across natural inbred *B. distachyon* lines. In transgenic 35S:*BdDHN3* plants, over-expression of *BdDHN3* lead to enhanced drought tolerance because these plants preserved more water and showed lower cell damage and higher proline contents under water stress conditions than the wild type (WT) *Brachypodium* plants. These findings suggest that *BdDHN3* may play an important role in the plant response to drought stress. Given its proximity and gene synteny to other important temperate cereal crops our results suggest that DHN3 proteins may be also relevants for temperate grasses with socio-economic importance.

Key words: *Brachypodium distachyon*, Dehydrin, Cloning, Drought.

INTRODUCTION

Plants are incessantly exposed to diverse environmental biotic and abiotic stresses. Drought is the major abiotic stress that affects biomass productivity and grain yield of crops. However, plants respond to water deficit and adapt to drought conditions by complex mechanisms such as physiological biochemical, anatomical, morphological and molecular changes, including transitions in gene expression (Reddy *et al.*, 2004; Hossain *et al.*, 2016). Different genes are induced by drought stress, and functions of such gene products include not only protection of plant cells from dehydration, but also regulation of certain genes for signal transduction in response to drought (Hossain *et al.*, 2016). Thus, the functional study of these genes is one of the important research approaches at the molecular level to know the mechanism underlying plant response to drought stress.

Dehydrins are drought-responsive proteins that belong to Late Embryogenesis Abundant (LEA) group, a large family of highly hydrophilic proteins. They are classified into Group II LEA proteins (Graether & Boddington, 2014) and are typically upregulated under water limitation conditions and other abiotic stresses as salinity or freezing, playing a fundamental role in plant response and adaptation to stressful environments (Hanin *et al.*, 2011; Graether & Boddington, 2014; Yu *et al.*, 2018; Hand *et al.*, 2011). Particularly, they are thought to have an important protective role (acting as molecular chaperones) in plant cell against water deficit by holding water molecules, binding transition metals to scavenge ROS, or binding to nucleic acids, proteins, sugars, and phospholipids preventing protein aggregation during desiccation or water-stress to maintain biological activity (Hanin *et al.*, 2011; Graether & Boddington, 2014; Liu *et al.*, 2017).

In grasses, dehydrins accumulation improves tolerance to water-deficit stress and other abiotic stresses (Graether & Boddington, 2014; Kosová *et al.*, 2014; Yu *et al.*, 2018; Liu *et al.*, 2017 and references therein). For example, a Cross-Species meta-Analysis of progressive drought stress revealed upregulated dehydrin genes in rice, wheat and barley (Suprunova *et al.*, 2004; Park *et al.*, 2006; Shaar-Moshe *et al.*, 2015). Similarly, Karami *et al.*, (2013) showed an induction of several dehydrins in barley under drought and a dehydrin expression was positively assocaited to chlorophyll contents, osmotic adjustment, plant biomass and grain yield. In barley, it has been found a correlation between drought tolerance traits and accumulation of dehydrin proteins (Park *et al.*, 2006). Likewise, evidences from transgenic studies using dehydrins isolated from grasses have also confirmed that dehydrin expression enhances plant stress tolerance. For example, overexpression of a DHN gene (DHN-5) from wheat enhanced the salinity tolerance of transgenic *Arabidopsis* plants (Saibi *et al.*, 2015); A DHN gene, CdDHN4, from bermudagrass (*Cynodon dactylon L.*), also showed an induced expression pattern when challenged by high/low temperatures, drought, salt and ABA (Lv *et al.*, 2017). A DHN gene from the perennial grass *Cleistogenes songorica*, enhances tolerance to drought in transgenic alfalfa (*Medicago Sativa L.*) (Zhang *et al.*, 2016). In addition, a DHN gene, SbDhn1, from *Sorghum bicolor* showed transcript accumulation when subjected to high temperature and osmotic stress. In addition, overexpression of this gene in tobacco plants led to a protective effect under high temperature and osmotic stress treatments (Halder *et al.*, 2017). However, despite such evidences, the specific role of dehydrins and their relationship with functional traits is still poorly known.

Brachypodium distachyon L. (Poaceae) is an acknowledged monocot model plant for temperate grasses due to its close phylogenetic relationships with important cereal crops, including maize, wheat, rice, and barley (Watt *et al.*, 2009; Catalan *et al.*, 2014; Vogel, 2016; Scholthof *et al.*, 2018). Very recently, several transgenic *B. distachyon* plants have been generated to identify the biological characteristics and functions of candidate genes that enhanced abiotic plant stress tolerance, including drought (Ryu *et al.*, 2014; Kang *et al.*, 2016; Yoon *et al.*, 2019). Nevertheless, molecular mechanisms and biological studies of dehydrin genes and its variation among genotypes in *B. distachyon* have not been elucidated yet. Based on our previous transcriptome study by RNAseq in the *B. distachyon* species complex (see Chapter 2), we selected as a drought-responsive candidate gene a dehydrin3 (ID Bradi1g37410, *BdDHN3*) that appeared consistently strongly up-regulated (447.2-fold higher on average) in water stress conditions. Here, in order to prove its role as drought-tolerance candidate gene we first investigate variation in the expression level of *BdDHN3* gene among 11 genetically distinct *B. distachyon* natural inbred lines to examine genetic-based consistency in gene expression using RT-qPCR. Second, we transformed *B. distachyon* Bd21 line with *BdDHN3* gene generating *BdDHN3*-overexpressing transgenic *Brachypodium* plants (named 35S:*BdDHN3*) to analyze the functional and genetic drought response of 35S:*BdDHN3* transgenic plants in comparison to plants with natural expression. This work is the first report that certifies the potentiality of dehydrin *BdDHN3* in rendering tolerance to water stress in *Brachypodium*.

MATERIAL AND METHODS

Plant materials and growth conditions

For examining genetic variation in the expression profile of *BdDHN3* in response to drought stress, 11 different *B. distachyon* natural inbred lines differing in drought tolerance were selected (Bd21, Bd30, CANU14, CASIB15, CER5, JHIN13, RON11, SCA5, SOBRE7, VILLA13 y VILLA3; see Figure 2 middle cluster based in electrolyte leakage and relative water content in Martínez *et al.*, 2018). Seeds of these lines were placed on moistened filter paper and incubated at 4°C for 2 days. Germinated seeds were transferred into pots (7 × 7 × 8 cm) and grown on sterile soil containing perlite, sand and organic substrate (0.5:0.5:1, v/v). The seedlings were then grown in a growth chamber at 21 ºC, 65 % relative humidity, under long-day conditions (16 h light/8 h dark; 120–200 µEm−2 s−1). Three-week old plants (3 biological replicates per line) were subjected to control conditions – without any watering restriction for 21 d, and watered twice per week – or to drought conditions, by ceasing the watering after 21 d of plant growth (see Martínez et al., 2018 for a similar procedure).

B. distachyon inbred line Bd21 (USDA-ARS, Plant Science Research Unit and Department of Agronomy and Plant Genetics, University of Minnesota, St Paul, MN, USA) was transformed with *BdDHN3* gene to generate 35S:*BdDHN3* transgenic plants. Seeds were germinated and seedlings were grown during nine weeks as described above. *Agrobacterium*-mediated transformation method described earlier was used to introduce *BdDHN3* into Bd21 (Alves *et al.*, 2009). Transgenic (35S:*BdDHN3*) lines constitutively expressing BdDHN3 under the control of CaMV35S promoter were generated. Two T1 35S:*BdDHN3* transgenic lines were used for the confirmation experiment and stress tolerance assay. Seeds were germinated, grown and subjected to control and drought conditions described above.

Variation in expression profiles of *BdDHN3* among *B. distachyon* lines

Total RNA was isolated from the leaves from three weeks old plants (three replicates per line and treatment) using TRIzol® Reagent (Gibco-BRL, Life Technologies, Paisley, UK) according to the manufacturers' protocol. First-Strand cDNA was synthesized from 1 mg of total RNA using the First-Strand Synthesis Kit for RT-PCR (AMV, Roche). Real-time quantitative PCR amplification was performed in 10 µl of reaction mixture per well containing 1 µl of cDNA, 0.4µM of each gene-specific primer and 5 µl of 2x SsoFast™ EvaGreen® Supermix (Bio-Rad). The *BdDHN3* primers were designed using the Oligo Primer Analysis software v. 6.65 and their sequences are listed in Table S1. All reactions were analyzed using CFX96 real-time PCR detection system (Bio-Rad) following this two-step amplification protocol: an inicial step of enzyme activation/DNA denaturation of 95ºC for 1 min, followed by 50 cycles of 95ºC for 10 sec, 60ºC for 10 sec and a final standard dissociation protocol to obtain the melting profiles of 65ºC to 95ºC. Data (quantification cycle values, Cq) were adquired by means of the Bio-Rad CFX Manayer v3 software (Bio-Rad). The ubiquitin genes *BdUBC18* and *BdUBI10* were used as internal reference genes to normalize gene expression values (Hong *et al.*, 2008; Chambers *et al.*, 2012). For each gene, calculation of normalized relative quantities (i.e., relative mRNA expression data) from Cq values were computed following the procedures described in Hellemans *et al.*, (2007). Three technical measurements were conducted.

Isolation of the *BdDHN3* gene for cloning

Coding sequence of *BdDHN3* gene was amplified from *B. distachyon* Bd21 plants complementary cDNA. cDNA was synthetized from three weeks old plants (two plants in total). A highly-specific region of *BdDHN3* was selected to avoid the overexpression of ‘off-targets’. A 492 bp fragment from this specific region was amplified by PCR using designed primers that provide DNA recombination sequences (attB sites) on 5’ and 3’ ends for subsequent utilization with the Gateway cloning technology (Life Technologies, USA) (see primer sequences in Table S1). PCR amplification was performed using Platinum® Taq DNA Polymerese High Fidelity (Sigma-Aldrich) with the following conditions: an initial step of enzyme activation/DNA denaturation of 98ºC for 5 min, followed by 39 cycles of 95ºC for 30 sec, 62.8ºC for 60 sec (annealing), 72ºC for 45 sec and a final dissociation step 72ºC for 10 min. PCR *BdDHN3* attB products were analyzed on 1.5% agarose gel and purified using Wizard SV Gel and PCR Clean-Up System kit (Promega) before cloning.

Vector construction and plant transformation

Following Gateway cloning technology protocol (Life Technologies, USA), *BdDHN3* attB product was inserted into the donor vector pDONR221 (Life Technologies) via BP Clonase mediated recombination. The resulting recombinational donor vector pDONR221-*BdDHN3* was used to cloning *BdDHN3* gene into a monocot-specific gateway binary vector pH7WG2D (DNA Cloning Service, Germany) via LR Clonase mediated recombination to produce the overexpression vector pH7WG2D-*BdDHN3* (Fig. 1). The overexpression of *BdDHN3* in this vector is driven by the constitutive 35S promoter from the cauliflower mosaic virus (35SCaMV). In addition, this vector is provided C-terminal fusion with the fluorescence EgfpER protein after successful expression and resistance to hygromicin, which was conferred by the resistant hygromicin gene under control of the double 35SCaMV promoter (Fig. 1).

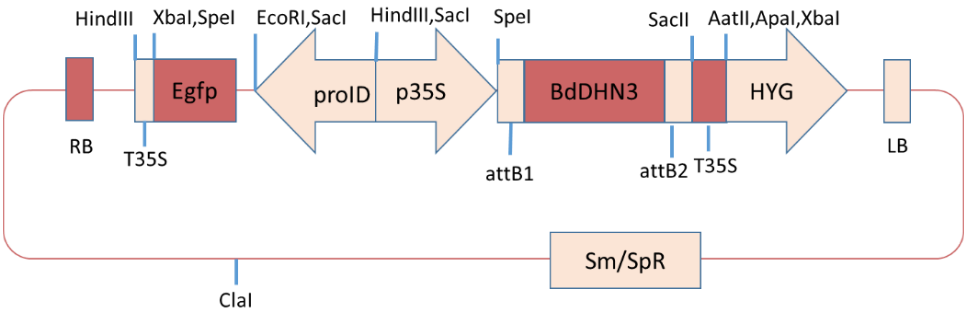

Fig. 1. Diagram of the T-DNA regions of the binary vector (pH7WG2D-*BdDHN3*) used for *B. distachyon* transformation. The plasmid contain two expression units within the T-DNA region bordered by the right and left border sequences, RB and LD respectively. The first unit contain the Enhanced green fluorescent protein (Egfp) driven by rolD root loci D (rolD) promoter and terminated by the T35S CaMV 35S sequence (T35S). The second unit contain the hygromycin phosphotransferase gene (HYG) confering hygromicin resistance driven by 35S cauliflower mosaic virus (CaMV) 35S promoter, attB1 and attB2 LR reaction site and terminated by the T35S CaMV 35S sequence (T35S). A bacterial gene for Spectinomycin resistance (Sm/SpR) is also present in the vector backbone. Examples of single and double cutter restriction sites are indicated.

The overexpression vector pH7WG2D-*BdDHN3* was then transformed into *Agrobacterium tumefaciens* AGL1 strain by the freeze–thaw method and then transformed into Bd21 calli using the *Agrobacterium*-mediated method following the protocol from Alves *et al.*, (2009). Transformed calli were selected on MS agar supplemented with timentin (225 mg L−1), 2,4-D (2.5 mg L−1), and CuSO4 (0.6 mg L−1). After chemical selection with hybromicin B, only fluorescence GFP expressing calli were selected using a stereomicroscope with UV light and biweekly subcultured. Then, calli were placed in regeneration medium containing hygromicin B (20 mg L−1), kinetin (0.20 mg L−1) and timentin (225 mg L−1) during root formation and shoot elongation for 4 weeks. Regenerated and rooted GFP expressing plantlets were transferred to sterile soil containing perlite, sand and organic substrate (2:0.5:1, v/v) and maintained in a growth chamber at 22/18 °C (day/night) under 20h photoperiod for seed production.

Transgenic plants carrying the overexpression plasmid were identified by visual screening of *BdDHN3*-GFP fusion proteins expressed under the control of the 35S promoter. The *BdDHN3*-GFP fusion proteins were detected in calli and then in roots of transgenic plants using a stereomicroscope (Leica MZ6) with a fluorescent module (Leica, cat. No. 10446093) (Fig. 2A). To further identify the transgenic plants, two transgenic lines were confirmed for the integration of the transgene by genomic PCR (Fig. 2B), after an initial selection with hygromicin resistance.

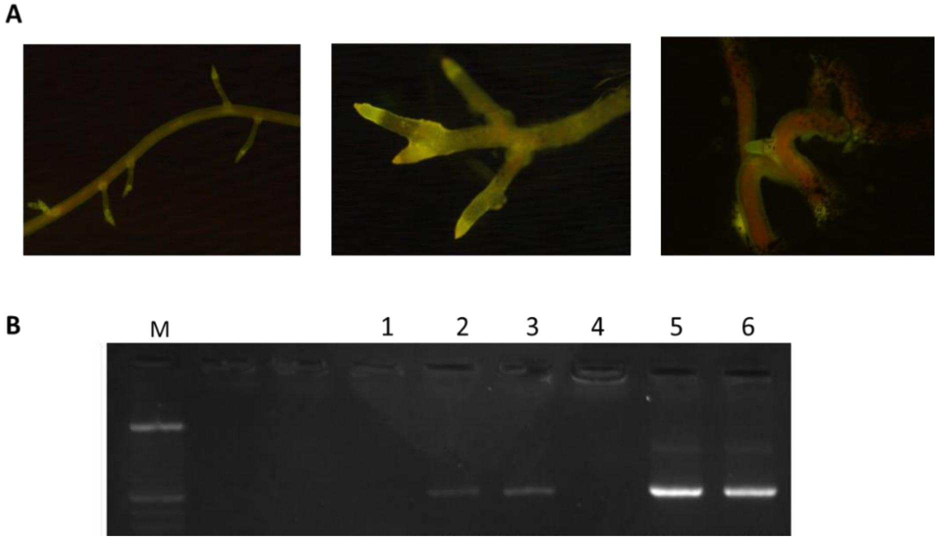

Fig. 2. Identification of transgenic *Brachypodium* plants with *BdDHN3* expression. (A) Identification of constitutively expressing 35s:*BdDHN3* transgenic *Brachypodium by BdDHN3*-GFP fusion proteins visualization. (B) Identification of constitutively expressing 35s:*BdDHN3* transgenic *Brachypodium* by genomic PCR. M represents DL2000 DNA marker,1 empty, 2, 3, 4, 5, 6 represent different transgenic.

Confirmation and gene expression analysis of transgenic *Brachypodium distachyon* plants

To corroborate the integration of the transgene in T1 35s:*BdDHN3* transgenic *Brachypodium* lines OE1 and OE2, leaf tissues of two weeks-old transgenic and WT *Brachypodium* plants were used for the extraction of genomic DNA by CTAB method. PCR was performed using a set of specific primers (Table S1) with extracted genomic DNA. The PCR amplified products were separated by eletrophoresis on a 1% agarose gel and stained with RedSafe Nucleic Acid Stain Solution (iNtRON Biotechnology).

To verify the constitutively overexpression of *BdDHN3* in transgenic 35s:*BdDHN3* lines in response to drought stress, two transgenic lines (OE1 and OE2) and WT plants subjected to control and drought conditions were used. The RNA isolations and the expression level of *BdDHN3* were assessed by real-time PCR following the indicated process above. Three technical measurements were conducted.

Stress tolerance assay on leaf functional traits

For the drought stress tolerance assay on leaf traits, transgenic and WT lines were subjected to control and drought conditions. We selected only OE1 transgenic line for tolerance assays because it presented a high expression level of *BdDHN3* (see in Results). We measured Leaf Water Content (LWC), Electrolyle Leakage (EL), Specific Leaf Area (SLA) and Proline content under normal conditions and drought stress condition. (1) To asses leaf water status LWC was analyzed harvesting the leaf samples under control and drought conditions. The fresh weight (FW) was recorded immediately after harvest and the dry weight (DW) was determined after 16 h incubation at 80ºC. LWC was measured according to previously described method using the following formula: LWC (%) = (FW- DW)/FWx100 (Martínez *et al.*, 2018). (2) EL was determined to evaluate cell membrane integrity by measuring electrical conductivity (Martínez *et al.*, 2018). For this analysis, 0.1 g of leaves was incubated in 20 mL of deionized water and then shaken at 150 rpm and room temperature for 5 h. The initial conductivity (Ci) was measured using a conductimeter (HI9033 Multi-Range Conductivity Meter, Hanna® Instruments). The solution was then boiled for 20 min, cooled at room temperature and the conductivity of the tissues (Cmax) was determined again. We calculated EL (%) according to the equation: EL = (Ci/Cmax) ×100. (3) The specific leaf area (SLA) of the largest leaf was calculated as: leaf area/DW (m2 kg−1). Leaf area was assessed using a leaf area meter (LI-3000C Portable Area Meter from LI-COR® Biosciences). (4) Free proline was extracted by grinding 100 mg of dried plant material with 600 ml of 3% (w/v) sulfosalicylic acid. The extract was centrifuged at 13 000 rpm for 15 min at room temperature. A 0.3 ml aliquot of the extract was mixed with 1.5 ml of a solution of acidic ninhydrin [40%

acidic ninhydrin (8.8 mM nihydrin, 10.5 M glacial acetic acid, 2.4 M orthophosphoric acid), 40% glacial acetic acid and 20% of 3% sulfosalicylic acid]. The samples were then incubated for 60 min at 96 ºC and the reaction was terminated by incubating the samples on ice for 5 min. Samples were then extracted by adding 3 ml of toluene and vortexing for 20 s. Absorbance at 520 nm was measured using toluene as a reference. A standard curve was made using L-proline in a range of 0 – 57.5 mg ml-1. Free proline content was calculated according to (Bates *et al.*, 1973): Free proline = (mg proline ml −1 × ml toluene) /115 x 5 mg mmol −1/ (g sample/5).

Statistical Analysis

ANOVA analysis was conducted to analyze variation in *BdDHN3* gene expression between *B. distachyon* lines. Line, treatment and their interaction were treated as fixed factors in the analysis. The same statistical approach was used to analyze variation in *BdDHN3* gene expression and leaf functional traits between 35s:*BdDHN3* transgenic and WT plants. Analyses were performed in JMP software (SAS Institute Inc).

RESULTS

Expression profiles of *BdDHN3* gene in *B. distachyon* lines

Drought treatment affected significantly the expression of *BdDHN3* gene ($F_{1,10}$ = 26.4, $P < 0.0001$), which was consistently up-regulated in drought stress conditions across *B. distachyon* lines, although variations in the magnitude of such expression was also recorded ($F_{1,10}$ = 2.23, $P = 0.058$; Fig. 3). Thus, drought-expression of *BdDHN3* gene was stronger for Bd30, CASIB15, SCA5 and VILLA13. Contrarily, was weaker for Bd21, CANU14 and SOBRE7. The rest of lines showed intermediate drought gene expression values (Fig. 3).On average, expression of the *BdDHN3* gene was 447.2-fold higher in drought conditions compared to control.

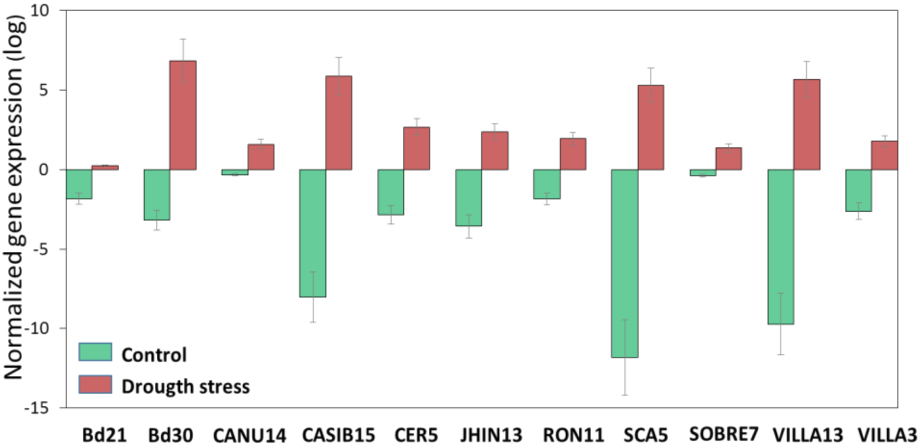

Fig. 3. Variation in the normalize expression levels of *BdDHN3* gene between 11 *B. distaschyon* lines in control (well-watered, in green) and after 21 days of experimental water restriction (25 % of soil water content, in red) estimated by RT-qPCR. Two-week-old *Brachypodium* plants were subjected for treatments , after which the leaves were collected and used for BdDHN3 expression analysis. BdUBC18 and BdUBI10 were used as internal controls.

Generation and confirmation of transgenic *Brachypodium* plants

The expression of *BdDHN3* gene in T1 transgenic 35s:*BdDHN3* plants was confirmed using RT-qPCR. As shown in Fig. 4, under both control and stress conditions, *BdDHN3* mRNA expression was significantly increased in the two transgenic lines assayed compared to WT plants. The OE1 and OE2 transgenic lines displayed 43.6- and 10.8-fold higher expression levels of *BdDHN3* than WT under control conditions, respectively (Fig. 4). Under drought, OE1 and OE2 transgenic lines showed 4.91 and 2.18- fold higher expression levels of *BdDHN3* than WT, respectively (Fig.4). Since *BdDHN3* was highly expressed in the transgenic line OE1, we selected this transgenic line to study its physiological traits.

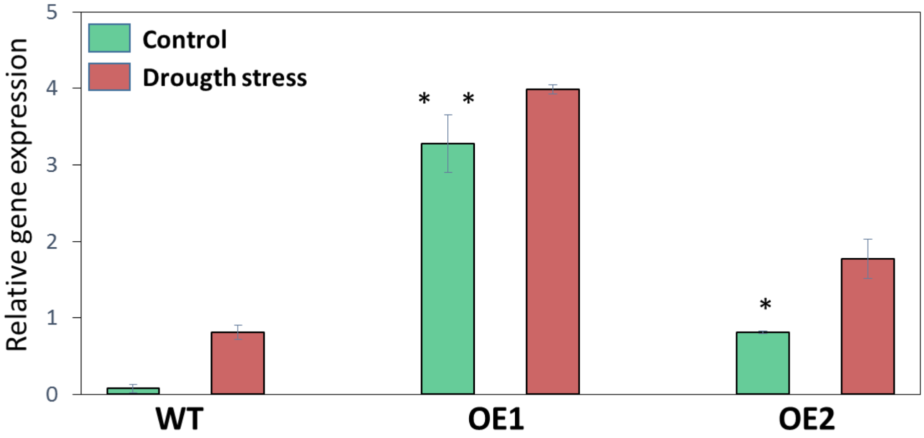

Figure 4. Variation in the normalized *BdDHN3* expression level in 35s:*BdDHN3* transgenic (OE1 and OE2) and WT *Brachypodium* plants under control and drought stress. Total RNA from leaves of WT and transgenic plants were isolated and used for Real-time PCR employing gene-specific primers with actin as an internal control. Values are means ± SE (n = 5 or 3 groups) from three independent experiments. Each group contained 3 plants. Asterisks indicate significant differences between the transgenic and WT plants (*P < 0.05, **P < 0.01).

Over-expression of *BdDHN3* enhances the drought tolerance of transgenic *Brachypodium* plants

The look of T1 transgenic *Brachypodium* plants 35s:*BdDHN3*, specifically the transgenic line OE1, and WT Bd21 plants are shown in Fig. 5. The plants were photographed 5 weeks after sowing. Among plants subjected to a continuous drought stress during 21 days of no watering, we found that WT plants were withered and transgenic plants were less dehydrated and green (Fig. 5). In the case of plants grown in control conditions during 5 weeks without water restrictions, WT plants shown a greater vigor than transgenic plants (Fig. 5). Visually, there were no notable morphological differences between WT and transgenic plants before drought treatment (data not shown).

With the exception of SLA, drought affected expression of traits, although its magnitude depended upon on the specific line (Table 1). Thus, transgenic plants showed higher LWC (7.4 fold) and less EL (2.3 fold) under water stress than WT plants (Figure 6A-B). Similarly, proline drought induction was significant only in transgenic plants compared to WT ones, 3.6 fold (Fig. 6C). Finally, drought did not affect the expression of SLA either in OE and WT plants (Fig. 6D).

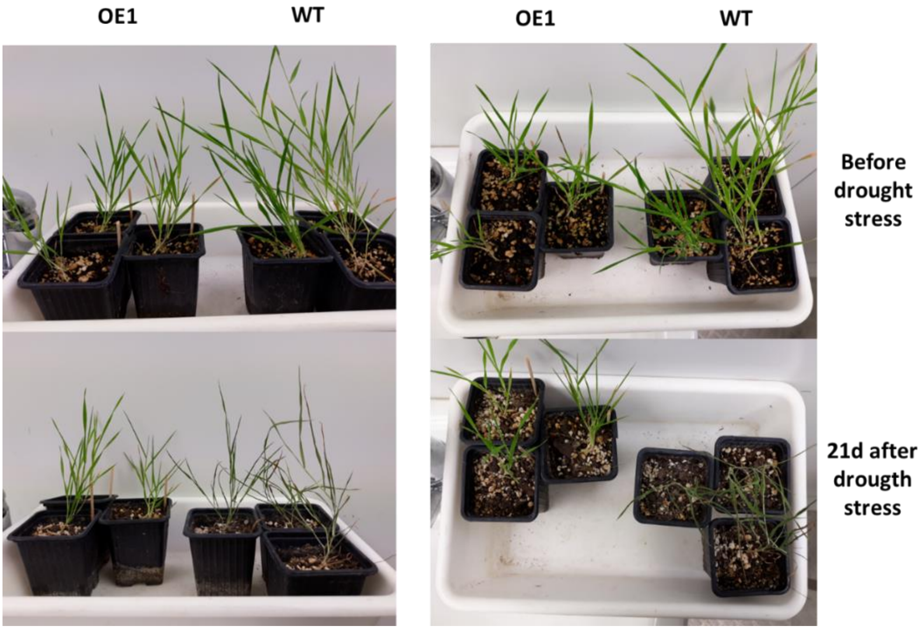

Fig. 5. *BdDHN3* over-expression improves drought stress tolerance in transgenic *Brachypodium* 35s:*BdDHN3* OE1. The phenotypes of the transgenic and WT plants under control and drought stress.

Table 1. Summary results of ANOVA conducted to test the effect of drought treatment, line (WT vs. 35s:*BdDHN3* OE1) and their interaction in the expression of four drought-related-traits. In bold, significant P-values (P<0.05).

Trait	Electrolyte leakage		Water Content		Proline		SLA	
Effect	F	P	F	P	F	P	F	P
Treatment	64.95	**0.0013**	26.7	**0.0021**	1.33	0.317	0.95	0.363
Line	54.82	**0.0018**	49.16	**0.0004**	22.38	**0.0091**	1.65	0.245
Treatment x Line	59.22	**0.0015**	26.7	**0.0021**	14.11	**0.019**	0.07	0.8

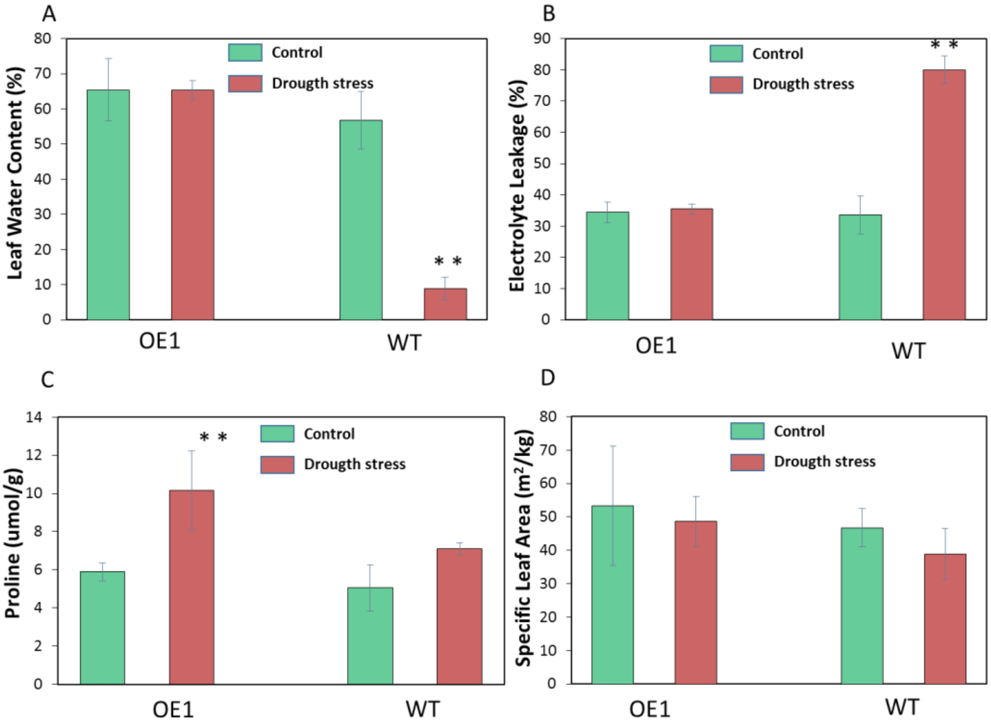

Fig. 6. Analysis of leaf functional traits in transgenic 35s:*BdDHN3* and WT plants under normal and drought conditions. (A) Leaf Water Content (LWC). (B) Electrolyte leakage (EL). (C) Proline content and (D) Specific Leaf Area (SLA). For each trait, data represent mean ± SD (n = 3) from three replicates. Asterisks indicate significant difference between the transgenic and WT plants (*P < 0.05, **P < 0.01).

DISCUSSION

Our results indicate that overexpression of the gene *BdDHN3* in the model grass *B. distachyon* improved the tolerance to drought stress. Similarly, the expression profiles of *BdDHN3* gene across 11 *B. distachyon* lines showed consistently up-regulation under drought stress conditions, although some variation between lines also existed. Previous studies have reported differences in DHN3 gene expression between tolerant and sensitive lines in the annual grass *Hordeum vulgare* (Karami et al., 2013), documenting a stronger drought-induction in the tolerant line. Contrarily, here we have observed that two of the most drought-sensitive *B. distachyon* lines, CASIB-15 and SCA-5, showed stronger *BdDHN3* induction when exposed to water restriction, whereas the most tolerant ones, Sobre-7 and Bd21 showed a relatively lower drought expression of DHN3 (Fig. 3). These differences between drought-tolerance *B. distachyon* lines and drought-sensitive have based in electrolyte leakage and relative water content (see Martínez *et al.*, 2018).

On the other hand, physiological analyses here conducted revealed that 35s:*BdDHN3* transgenic *Brachypodium* plants over-expressed *BdDHN3* gene and showed higher leaf water content (LWC), lower cell damage and higher proline contents under water stress conditions, which are indicators related to better drought tolerance in this system (Martínez *et al.*, 2018). Whereas DHNs have been associated with stress tolerance to water deficit in many plants (Graether & Boddington, 2014; Liu *et al.*, 2017; Yu *et al.*, 2018), including cereals (Kosová *et al.*, 2014), this is the first report of the characterization of a drought stress tolerance dehydrin in *B. distachyon*. Previous in-silico studies revealed the importance of DHNs in *B. distachyon* identifying dehydrin genes and conserved promoter motifs (Filiz *et al.*, 2013; Zolotarov & Strömvik, 2015). Moreover, our previous transcriptome study by RNAseq in *B. distachyon* complex have showed that drought-induction of DHNs, including BdDHN3, was pronounced in the three species of complex (see Chapter 2).

Thus, together, these results indicate the importance of DHNs regulation under drought stress in *B. distachyon*.

LWC reflects the responses of the whole plant, being a relevant tool for the measurement of drought stress tolerance in *B. distachyon* (Luo *et al.*, 2011). The value of LWC in our transgenic *Brachypodium* remained invariant in drought and control conditions and was similar to WT in control but substantially higher than WT when the latter was subjected to drought stress (Figure 6A). This indicates that *BdDHN3* over-expression contributes to maintain the leaf water status of the plant at the same level that was detected in plants without stress treatment, although the specific physiological mechanism is unknown. Given that dehydrins can produce an increase in compatible solutes inside vacuoles, as proline, and given their multifunctional role, binding to molecules such as water, ions, phospholipids, proteins and nucleic acids, this could explain the considerable decrease in water potential inside the cells by accumulating such solutes inside across the tonoplast to the vacuoles (Graether & Boddington, 2014; Liu *et al.*, 2017). The result would be precisely the increase in LWC we are observing in our results, and therefore, the intact maintenance of the turgor in the cells, even in conditions of drought stress, as we can see in the photographs of the plants subjected to severe water restriction (Fig. 5) with respect to control plants.

As a significant indicator of cellular membrane injury (Dexter *et al.*, 1932), the EL value was significantly higher in the WT than in transgenic lines, indicating that the transgenic plants suffered less membrane damage than the WT under drought treatment (Figure 6B). The contribution of LEA proteins and dehydrins to transgenic plants maintaining higher water content and less membrane damage under drought stress has been previously reported in *Nicotiana tabacum* (Sun *et al.*, 2015), *Hordeum vulgare* (Karami *et al.*, 2013) and *Ipomoea pes-caprae* (Zhang *et al.*, 2018), and has been suggested to be related to inhibition of the ROS response and/or improvements of antioxidative activity (Karami et al., 2013; Sun et al., 2015).

Cellular accumulation of free proline is considered a protective cellular mechanism against environmental stresses, including drought (Verbruggen & Hermans, 2008). Proline acts as both an osmoprotectant and a molecular chaperone maintaining cell viability and preventing oxidative damage caused by dehydration (Verbruggen & Hermans, 2008). A higher accumulation of proline was detected in the *BdDHN3* overexpression transgenic lines (Figure 6C), indicating that under drought treatment *BdDHN3* overexpression provided better protection by regulating proline metabolism to maintain the water status and the growth of plants. Studies examining the functional characterization in relation to over-expression of dehydrin genes, and associated improved drought tolerance in transgenic plants, have reported similar results to those found here. Thus, transgenic alfalfa over-expressing a dehydrin, CsLea, from a desert grass *Cleistogenes sogorica* showed higher free proline content, higher relative water content (RWC) and decreased membrane injury during drought and salt stresses (Zhang *et al.*, 2016). Transgenic Arabidopsis over-expressing a dehydrin, IpDHN, from *Ipomoea pes-caprae* showed higher content of proline, higher RWC and a decreased membrane damage when exposed to different abiotic stresses, including drought (Zhang *et al.*, 2018).

Because SLA reflects leaf thickness, reduced SLA is presumed to be a way to improve water-use efficiency in drought stress since a more tightly packed photosynthetically active tissue allows more efficient use of a relatively smaller surface (Wellstein *et al.*, 2017). However, here SLA values did not significantly differ between transgenic and WT plants (Figure 6), indicating that BdDHN3 overexpression did not have an effect on SLA in our constructions.

CONCLUSION

The functional analyses conducted here indicate that the *BdDHN3* gene plays an important role in drought stress tolerance in *B. distachyon*. Similarly, consistency in the drought gene expression pattern across *B. distachyon* lines differing in tolerance to water stress (Martínez et al., 2018) suggests that up-regulation of *BdDHN3* gene is key in the drought-response of the species, which is concordant with observations from transcriptome analysis (Chapter 2). Given its proximity and gene synteny to other important temperate cereal crops (Scholthof *et al.*, 2018) our results suggest that DHN3 gene may be also relevant for temperate grasses with socio-economic importance. This work thus provides experimental basis for further research and use of the DHN3 gene in genetic manipulation in other crops.

In summary, our research highlights the importance of a continued investigation into the function and mechanisms of the *BdDHN3* gene in *B. distachyon* for the further development of stress-tolerant crops in genetic breeding. We are currently working in the transgenic construction for silencing the native *BdDHN3* transcript in *B. distachyon* to investigate possible function cessation in the case that endogenous transcript level was down-regulated enough to cause a loss-of-function phenotype. Once the transgenic lines are obtained (already available), the expression level of *BdDHN3* will be examined by real-time PCR analysis to compare it between transgenic T1 plants and WT plants

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SUPPLEMENTARY MATERIAL

TABLES

Table S1. List of specific primers used in the study. Underlined sequences indicate restriction enzyme sites.

Purpose	Gene	Forward (5´→3´)	Reverse (5´→3´)
Full-length	attB-BdDHN3	GGGG ACAAGTTTGTACAAAAAAGCAGGCT T A ACC **ATGGAGTACCAGGGCCAGCACGGTCAGG**	GGGG ACCACTTTGTACAAGAAAGCTGGGT C **TCAGTGCTGGCCAGGCAGCTTCTCCTTA**
RT-PCR	*BdDHN3*	GGTCAGGCGACAAACCGCGT	GAGCTGCCGGAGCGGTGAAG
	BdUBC18	GGAGGCACCTCAGGTCATTT	ATAGCGGTCATTGTCTTGCG
	BdUBI10	TGGACTTGCTTCTGTCTGGGTTCA	TGGTACACAGGCATAACACTGACG
***Agrobacterium*-mediated transformation (sequencing primers)**	*pH7WG2D P35S*	ATGACGCACAATCCCACTATCCTTCG	
	pH7WG2D T35S	AACACATGAGCGAAACCCTATAAGA	

CAPÍTULO 4

RESPONSE TO INSECT HERBIVORY IN THE *BRACHYPODIUM DISTACHYON* SPECIES COMPLEX: INFLUENCE OF LEAF FUNCTIONAL TRAITS AND POLYPLOIDY

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ABSTRACT

- Insect herbivory is a major ecological biotic factor impacting on plant populations and a main driver of selection of plant traits. Polyploidy (whole-genome duplication) typically induces dramatic phenotypic and genotypic changes that may impact on species interactions, including herbivory. However, herbivory response of polyploid lineages is still poorly known.
- We conducted here a bioassay to analyze variation in resistance and tolerance to herbivory damage by locusts in multiple diploid and allotetraploid populations of the *Brachypodium distachyon* species complex. We further investigate which functional traits are associated to herbivore response in this system.
- Herbivory had a significant impact on maternal fitness in the species of the *B. distachyon* species complex, yet its magnitude was dependent on species and fitness estimator. Our results do not support that polyploidy enhances herbivory response in this complex. Differences in resistance appeared associated to functional trait differentiation, especially in plant size, silica content and C:N ratio, whereas tolerance variation tended to be linked negatively to SLA variation.
- Because insect herbivory affected in a larger extent to *B. hybridum* allotetraploids, of superior competitive ability, than *B. distachyon* diploids we hypothesize that insect herbivory may act as a stabilizing factor that promotes species coexistence in contact zones

Key words: *Brachypodium*, functional traits, insect herbivory, palatability, plant damage, resistance, tolerance.

INTRODUCTION

Insect herbivory is a major ecological biotic factor impacting on plant populations and productivity in managed and natural vegetation (Strauss and Zangerl 2002). Across all vegetation systems, foliage, sap and root feeding herbivores may remove up to 20% of net plant productivity (Agrawal, 2011). For crops, damage caused by arthropod pests can exceed 15% annually (Mitchell et al. 2016). Beyond its impact on productivity, herbivory is also a main driver of selection of plant traits (Agrawal, 2011).

For plants, the amount of herbivory received depends largely on the balance between their ability to produce defensive traits that deter herbivore feeding (i.e., resistance) and their nutritional quality (Agrawal and Fishbein 2006). Once herbivore damage occurs, its impact on plant fitness is essentially determined by the plant's ability to regrow and reproduce after herbivory, (i.e., tolerance; Strauss and Agrawal 1999). Together, resistance to herbivory, tolerance to damage and plant nutritional status constitute the plant response to herbivory (Nuñez-Farfán et al., 2007), which pivots on a set of several functional traits of complex nature (e.g., specific leaf area, dry matter and water content, C:N ratio, concentration of secondary compounds in tissues, silica content, etc.; Agrawal and Fishbein 2006, Agrawal, 2011; Mitchell et al., 2016). Although intra and interspecific variation in plant response is dependent upon the ecological context, plant response variation to herbivory hinges basically on the natural variation and plasticity of these functional traits (Reese et al., 2016). Likewise, habitat may also influence plant traits (e.g., specific leaf area, trichomes density, etc.) that impact indirectly on herbivores (Agrawal, 2011). Identification of plant defensive traits and their deployment is also essential to improve the future sustainability of crop protection (Mitchell et al., 2016).

Among all the evolutionary processes, polyploidy (whole-genome duplication) typically induces dramatic phenotypic and genotypic changes that may impact on species interactions, including herbivory (Segraves and Anneberg 2016). Polyploidy may affect species interactions directly by altering key phenotypic traits that mediate such interaction (e.g., flower size, scent chemistry, defensive chemicals, etc., reviewed in Segraves and Anneberg 2016), and/or indirectly by natural selection following polyploidization. In the particular case of polyploid plant-herbivory interactions, theory predicts that polyploids may enhance resistance and tolerance to herbivory compared to their diploid ancestors, yet whether polyploid lineages are more resistant and/or tolerant to herbivory than diploid ones is largely unclear (Segraves and Anneberg 2016). This is especially true for tolerance to damage, which virtually there are no studies that have compared the reaction norms of maternal fitness across different levels of damage between the different cytotypes within heteroploid species. Thus, the hypothesis that the existence of a linkage between tolerance and ploidy of endoreduplicated cells, which would allow to higher-level polyploids to exhibit enhanced tolerance, remain untested (Segraves and Anneberg 2016).

In this study, we conduct the first comprehensive analysis of resistance and tolerance to damage by insects in the *Brachypodium distachyon* (Poaceae) species complex. This complex is a suitable system to address plant response to herbivory in the context of ploidy variation because first, is a genetic and cytogenetically tractable system; the natural allotetraploid *Brachypodium hybridum* (2n = 4x = 30) is derived from bidirectional crosses of its parents *Brachypodium distachyon* (2n= 2x= 10) and *Brachypodium stacei* (2n= 2x = 20), that have occurred repeatedly approximately 1 million years ago (Catalán *et al.*, 2016). In this complex, *B. stacei* is the oldest linage that arose ~10 million years ago, while *B. distachyon* emerged ~ 7 million years ago (Scholthof *et al.*, 2018). Second, there is inter-specific and inter-populational variation in functional traits (Martínez et al., 2018) that indirectly may influence herbivory (Agrawal 2011). Thus, in dry conditions, we have shown previously that *B. stacei* and *B. hybridum* show higher specific leaf area and water content than *B. distachyon* (Martínez et al., 2018). Third, inter-specific variation in plant damage in natural sympatric populations exists, indicating that the proportion of plants with any sign of insect damage is higher in *B. hybridum* than in *B.distachyon* in humid populations (Manzaneda and Rey unpublished). If damage impacts on fitness, herbivory could in turn contribute to species differentiation observed in this complex (Manzaneda et al., 2012). However, a proper evaluation of the inter-specific variation in resistance to insect herbivory and tolerance to damage in this system has not been conducted yet. Here, by performing a lab bioassay, we investigate the covariation of leaf traits that may make plants more palatable to insects, such as specific leaf area (SLA), high nitrogen content and water content, and traits that increase resistance to insect damage as silica content (Agrawal 2011; Frew et al., 2018), with insect plant damage and maternal fitness. In particular, we tackle the following questions: 1) do the three species of the complex vary in resistance and tolerance to damage? 2) within each species, is there any variation in resistance and tolerance to damage among populations or genotypes? 3) do the three species vary in functional traits potentially important for resistance and palatability? 4) which traits determine in a larger extent resistance and tolerance to insect herbivory? and 5) are there any genetic tradeoffs between resistance and tolerance in this species complex? By addressing these questions, first we investigate which plant traits drive differences in herbivore response in *Brachypodium*. Second, we answer whether insect herbivory may be an important ecological factor for *Brachypodium* species differentiation across its range of distribution in the Iberian Peninsula (Manzaneda et al., 2012). Given the role of *B. distachyon* species complex as plant model for temperate grasses (Brkljacic et al., 2011) we further discuss the implications of these results for crop breeding.

METHODS

Study system

The *Brachypodium distachyon* (Poaceae) species complex comprises three annual species – *B. distachyon*, *B. stacei* and their allotetraploid derived *B. hybridum* – all native to the Mediterranean Basin, Middle and Near East (Catalán et al. 2012). They inhabit a wide variety of climatic and ecological conditions from sea level to 2000 m elevation, frequently found in forest edges, natural xerophytic meadows, abandoned fields, and along roadsides (Manzaneda et al. 2012). These three species are ecologically differentiated, *B. stacei* and *B. hybridum* are found in drier locations than *B. distachyon* diploids (Manzaneda et al., 2012; López-Alvarez et al., 2015). Most populations include only a single species, although sympatric populations of *B. hybridum* and *B. distachyon* exist (Manzaneda et al., 2012; López-Alvarez et al., 2015).

Flowering takes place between April and June and all species are fundamentally selfing (Vogel et al., 2009). Seed maturation and shedding occur during the summer and germination and seedling emergence occur in October-November with the first autumn rainfalls. Grasshoppers (Orthoptera) typically feed on newly emerged seedlings in natural *Brachypodium* populations in the Iberian Peninsula, yielding important plant damage in some populations (A.J. Manzaneda & P.J. Rey pers. obs.).

Plant material and growth conditions

We selected 53 *B. distachyon* genotypes (inbred lines), 56 *B. hybridum* genotypes and 10 *B. stacei* genotypes from ten, ten and three different Iberian populations, respectively (Table S1). We chose two to six genotypes from each population. Genotypes come from populations genetically differentiated and with contrasting climate conditions (Table S1). Nine mature seeds per genotype were put into Petri dishes at 4ºC during 4 days for cold stratification. Once germinated, they were individually planted and grown on a mix of perlite, sand and standard soil (0.5:0.5:1 v/v) in randomized blocks. Each block consisted in one tray of 48 (7x7x8 cm^3) pots. Plants were grown under controlled conditions in a growth chamber (22ºC, 16 h light and 8 h dark).

Experimental procedure

After 4 weeks of growth, plants were exposed to an herbivore treatment. Each genotype was replicated three times for a total of 357 plants. Insect species used in this bioassay was the generalist herbivore *Locusta migratoria* (Orthoptera: Acrididae). Locusts species are distributed widely from tropical to subarctic regions of Africa, Europe and Asia, typically feeding on various Poaceae species including cereal crops and grasslands (Massey et al., 2006 and references therein) and have been used previously to investigate palatability in grasses (e.g., Massey et al., 2006). Thus, fourth-instar locusts (average weight 600 mg) supplied by *Animalia Co.* were starved for 12 h and weighed before being placed on each plant. To secure a single locust on each plant, each plant–locust pair was enclosed using a cylindrical tube (diameter, 6 cm; height, 21 cm) made of acetate (3M®) with both ends open (Fig. S1). One end was inserted into the soil and the other was covered by organza bags (see a similar procedure in Manzaneda et al., 2010). After 24 h locusts were removed from the plants, locusts were reweighed and plants were checked for damage. Growth performance of herbivores was calculated as the relative growth rate (RGR) by dividing the initial weight by the final weight (Massey et al., 2006). For each plant, we recorded the proportion of leaves with any sign of herbivore damage, and estimated visually the percentage of tissue removed per leaf (taken by the same person and ranging from 1% to 100%) to calculate the percentage of plant damage (PD) as the total number of leaves damaged multiplied by the average of leaf damage / total number of leaves. Resistance to herbivory values for each genotype were obtained from 1 – PD.

Plant traits measurements

Constitutive trait measurements for all plants were taken on undamaged plants (on three biological replicates by genotype). Plant water content (WC) was calculated as 100 × [(FW − DW)/FW], where FW is fresh weight and DW is dry weight. The FW was determined by weighing the leaves on a precision scale immediately just after plant harvest. To estimate DW, leaves were dried for 48 h at 70 ºC and weighed again. The specific leaf area (SLA) of the largest leaf was leaf area/DW (m^2 kg^{-1}). Leaf area was assessed using a leaf area meter (LI-3000C Portable Area Meter from LI-COR® Biosciences). To quantify the amount of leaf nutrients, leaves were dried for 48 h at 70ºC and then ground using a mixer miller MM 200 & MM 400 (Retsch). About 2 mg of dry leaves were weighed and placed in a tin container for measuring the percentages of nitrogen, carbon and hydrogen following elemental analysis by the flash combustion technique using the Thermo Finnigan organic elemental analyzer FlashEA1112 CHNS-O (Thermo Fisher Scientific Inc.). Calibration was based on the synthetic standard sulfanilamide. Leaf silica content was determined using 1% Na2CO3 extraction at 85°C (Meunier et al., 2017). Approximately 33 mg of dried plant material were mixed with 40 ml of 1% Na2CO3 solution in a polypropylene bottle and placed in a shaker bath at 85°C and 100 rpm for 1h. 1 ml aliquot was removed from each sample bottle and placed into pre-labelled Pyrex glass tubes containing 9 ml of a solution of 0.021 N HCl to neutralize Na2CO3. Dissolved Si (DSi) was obtained by the molybdenum blue colorimetric method using Spectroquant® reactants manufactured by Merck (Fontenay sous Bois, France). Absorption was measured at 820 nm and calibration lines (R2 > 0.999) was done using dilute solutions from a standard Si solution CertiPUR® at 1000mg/l (Merck). DSi was calculated by averaging the three values at 3, 4 and 5 h. Chemical analyses were performed in the Center of Technical Scientist Instrumentation (CICT) at the University of Jaén.

For each plant, we recorded the number of spikes and seeds produced and the reproductive biomass (the weight of the total seeds produced per plant) as estimates of maternal fitness.

Statistical analyses

All statistical tests were conducted using 'R' software, version 3.5.3. (R Development Core Team, 2019). To analyze interspecific differences in RGR, plant damage, resistance and leaf functional traits we fitted general linear mixed models with residual maximum likelihood (REML) estimation using the 'lme4' package (Bates et al., 2015). In these models, species was considered always a fixed factor and we included genotype nested within species as a random factor. In addition, in the models exploring insect damage we included the plant leaf number as a covariate accounting for interspecific differences in plant size ($F_{2,106}$ = 5.19, P = 0.007). Statistical significance of fixed and random factors (by using likelihood ratio tests, LRT) was evaluated using the 'lmerTest' package (Kuznetsova et al., 2017). Response variables were transformed when necessary (log or angular transformation in the case of proportion-based data).

To investigate variation in tolerance to damage between species and populations (only for *B. distachyon* and *B. hybridum* populations) we conducted linear mixed models, in which fitness response variables were the number of spikes produced per plant, the total of number of seeds and the total seed biomass produced per plant. Predictors in these models were species or population (treated as a fixed factors), plant damage, their interaction and genotype nested within species (as random factor). Tolerance of herbivore damage was then calculated as the slope of a regression of plant fitness on a continuous damage level (Wise and Carr 2008). To obtain and compare estimates of slopes between species or populations we used the 'emtrends' function implemented in the 'emmeans' package (Lenth 2019). Because when comparing tolerances among groups of plants, damage and performance need to be on the same multiplicative scale (Wise and Carr 2008), all fitness measurements were previously log-transformed.

To analyze interspecific and inter-populational trait variation, we performed multivariate in addition to univariate analyses (see above). Thus, we performed a multivariate analysis of variance (MANOVA) to test the effects of species on overall trait variation. Next, we used a linear discriminant analysis using the 'DiscriMiner' package (Sanchez 2013) to determine which traits best discriminated between *Brachypodium* species or among *B. hybridum* and *B. distachyon* populations.

Finally, to investigate predictors of herbivore resistance and tolerance to damage we carried out stepwise multiple regression analyses using the 'MASS' package with the 'stepAIC' function, which selects the best model by AIC criteria. In these analyses, traits were C:N, WC, plant size, SLA, and silica content and their interactions, which were regressed against resistance and tolerance separately.

RESULTS

Variation in plant damage and resistance to damage

Insect RGR was significantly correlated to plant damage and resistance (N = 318, r = 0.3, p <0.0001; N = 318, r = -0.3, p <0.0001, respectively), indicating that insect growh was higher in more damaged and less resistant *Brachypodium* plants. Insect RGR did not vary significantly among *Locusta* specimens feeding on different species ($F_{2,91}$ = 1.76, P = 0.177; Fig. S2). However, plant damage trended to vary between *Brachypodium* species ($F_{2,112}$ = 2.85, P = 0.06, Fig. 1). Thus, *B. stacei* plants received 1.42 and 1.28 times more damage than *B. hybridum* and *B. distachyon* plants (average proportion of damage ± 1 SE: 0.65 ± 0.06, 0.51 ± 0.03, 0.45 ± 0.03, for *B. stacei*, *B. distachyon* and *B. hybridum* respectively; Fig. 1). Plant damage varied significantly among genotypes within species (LRT = 13.71, d.f. = 1, P=0.0002). Species differed significantly in resistance ($F_{2,112}$ = 3.16, P = 0.046, Fig. 1). *B. stacei* was the less resistant species, whereas resistance of *B. hybridum* and *B. distachyon* was similar (Fig. 1). Similarly, resistance also varied among genotypes within species (LRT = 14.26, d.f. = 1, P>0.0001). Plant damage and resistance were significantly influenced by the number of leaves (estimate ± SE: -0.021 ± 0.003; $F_{2,260}$ = 38.13, P < 0.0001; estimate ± SE: 0.016 ± 0.002; $F_{2,261}$ = 45.4, P < 0.0001), suggesting that bigger plants are more resistant and receive less damage.

Plant damage and resistance varied notably among populations of *B. distachyon* and *B. hybridum* (*B. distachyon*: $F_{11,121}$ = 4.9, P< 0.0001, $F_{11,121}$ = 1.75, P = 0.07; *B. hybridum*: $F_{9,148}$ = 2.54, P= 0.009, $F_{9,148}$ = 2.53, P= 0.01, plant damage and resistance respectively. Fig. S3). Thus, the coefficient of variation (CV) among *B. distachyon* populations was respectively 73.3% and 77.6%, and for *B. hybridum* was 78.7 % and 65.8 %. However, these variations were lower and not significant among *B. stacei* populations (P>0.05, CV = 51.05 %, 93.5 %, plant damage and resistance, respectively; Fig. S3). For *B. hybridum* we found a significant negative association between plant damage and latitude (R = -0.72, P = 0.02, Fig. S4), yet not for the other species or geographical parameters as longitude or elevation (P>0.05).

Variance partition analyses showed that both for plant damage and resistance the variation occurs mainly at inter-genotypic and inter-populational level rather than among species (23.24 %, 6.14 % and 0.17 % for effects of genotype within population, population within species and species, respectively).

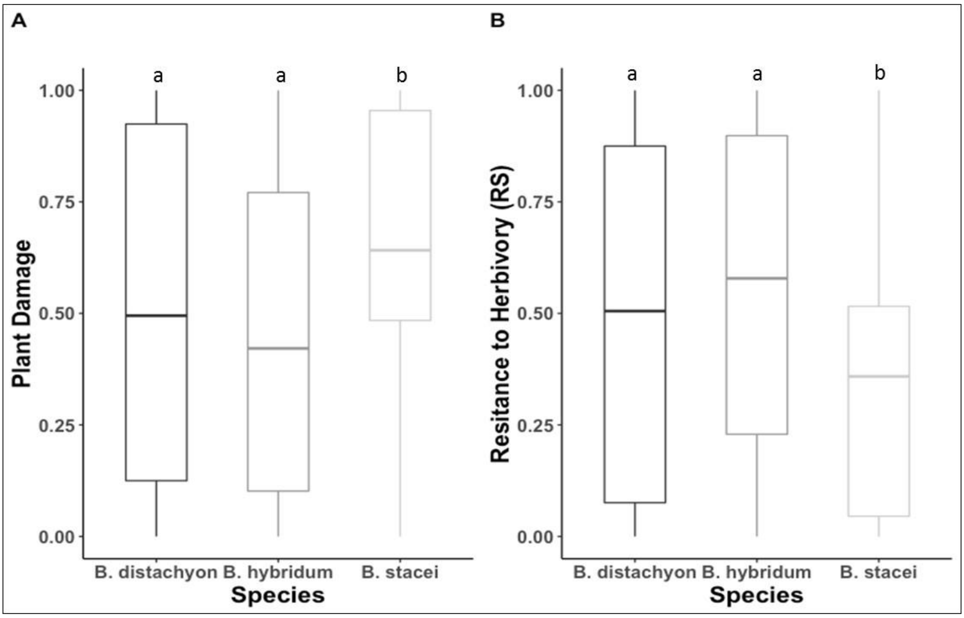


Fig. 1. Variation in (A) plant damage and (B) resistance to insect herbivory (RS) between the three species of the *Brachypodium distachyon* species complex. Different letters on the graphs inidcate significative differences (P<0.05) in post hoc contrasts tests.

Variation in tolerance to herbivory

Herbivory damage affected significantly fitness, although its effect depended on fitness measurement and species (Table 1). Thus, the number of spikes was reduced significantly according to plant damage (estimate ± 1 SE: -0.62 ± 0.11; Fig. 2A) and this was consistent between species as indicated by the lack of significance of the interaction term Plant damage x Species (Table 1). Species also influenced fitness, reflecting interspecific differences in vigor (Fig. 2A). For number of seeds and total biomass, influence of plant damage was dependent on the species (Table 1; Fig. 2), suggesting interspecific variation in tolerance to damage for these later fitness components (Table S2, Fig S5). Thus, *B. distachyon* was significantly more tolerant to damage than *B. hybridum* and *B. stacei* (P>0.05; Table S2, Fig S5), suggesting that despite that *B. distachyon* plants are less vigorous than their relatives, they were able to tolerate in a large extent herbivory damage (Figs. 2, S5).

Table 1. Summary results of the general linear mixed model testing the effects of plant damage, species and their interaction on three different maternal fitness components in *Brachypodium* distachyon species complex. A significant interaction of the plant damage x species term denotes inter-specific variation in tolerance to damage. Significant P values (P < 0.05) are in bold.

	Number of spikes			Seeds (total number)			Seeds (biomass)		
Effects	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Plant damage	1,267	28.4	**<0.0001**	1,299	17.4	**<0.0001**	1,288	21.58	**<0.0001**
Species	2,211	3.87	**0.022**	2,220	30.36	**<0.0001**	2,219	30.47	**<0.0001**
Plant damage x Species	2,279	1.13	0.322	2,290	8.33	**<0.0001**	2,288	9.08	**<0.0001**
Random effect	df	*LRT*	*P*	df	*LRT*	*P*	df	*LRT*	*P*
Genotype	1	34.22	**<0.0001**	1	71.45	**<0.0001**	1	57.63	**<0.0001**

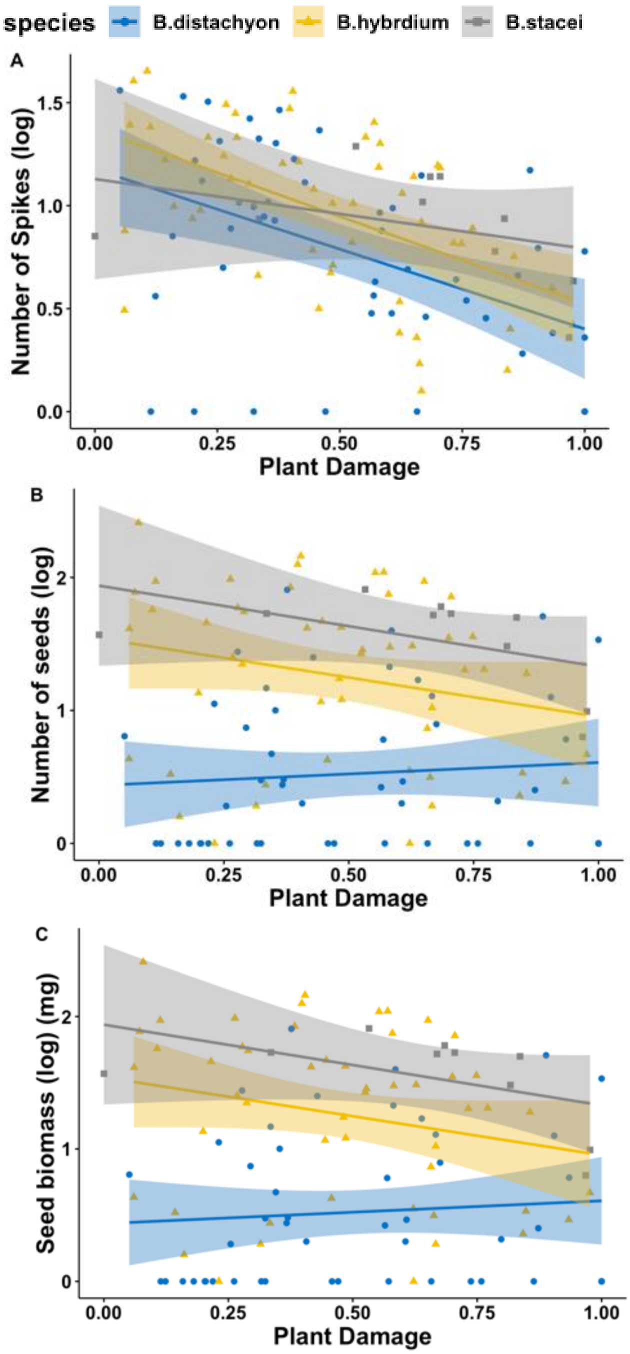


Fig. 2. Variation in fitness across insect herbivory damage in three species of the *Brachypodium distachyon* species complex. A) the number of spikes, B) the number of seeds, and C) the seed biomass. Lines in graphs depict a linear fit between the two variables for each species, and shades the 95 % interval of confidence for each fit.

At population level, tolerance to damage of the number of spikes and seeds did not vary significantly among *B. distachyon* or *B. hybridum* populations (#spikes: $F_{11,82}$ = 0.63, P = 0.81, #seeds: $F_{11,92}$ = 1.13, P = 0.34; #spikes: $F_{9,123}$ = 1.27, P = 0.26, #seeds: $F_{9,116}$ = 1.38, P = 0.2; GLM results for the interaction term plant damage x population for *B. distachyon* and *B. hybridum* respectively). Tolerance of seed biomass was similar for all populations of *B. hybridum* (plant damage x population: $F_{9,119}$ = 0.94, P = 0.49), however, for *B. distachyon* we detected significant variation in tolerance to damage among populations (plant damage x population: $F_{11,87}$ = 8.62, P < 0.0001; Fig. S6). Thus, only Bd-21 genotypes reduced fitness significantly due to plant damage, whereas JHIN genotypes were able to overcompensate for herbivory (Fig. S6).

Variance partition analyses indicate that inter-genotypic and inter-specific variation accounted most for the variation observed in tolerance to damage in late fitness components (21.5 %, 78.5% for seed biomass; 24.3%, 75.7% for number of seeds) while for number of spikes most variation was found at inter-populational and inter-genotypic level (24.2% and 75.8 %, respectively).

Variation in functional traits

For *B. distachyon* and *B. hybridum*, silica content and C:N were negatively correlated, and SLA and WC positively associated (Table S3). In *B. stacei*, traits appeared uncorrelated (Table S4).

Overall, leaf functional traits varied between species (MANOVA result: Wilk's λ = 0.57, $F_{8,606}$ = 24.34, P < 0.0001). Thus, linear discriminant analysis showed that *B. distachyon* plants were significantly differentiated from *B. hybridum* and *B. stacei* in leaf functional traits related to herbivore response (Wilk's λ = 0.64, P<0.0001; Fig. 3). Silica content was the trait with the highest weight for the discriminant variables DF1 and DF2 (Table S5), accounting for a total of 81.25 % and 18.75 % of between-species variation, respectively (Table S5). Univariate analysis performed on each trait confirmed such interspecific variation ($F_{2,103}$ = 13.41, P < 0.0001; $F_{2,104}$ = 28.16, P < 0.0001; $F_{2,104}$ = 3.49, P = 0.034; $F_{2,104}$ = 25.24, P < 0.0001; results for silica content, C:N, SLA and WC respectively; Fig. 4). In particular, *B. distachyon* showed a lower silica content, water content and SLA, and a higher C:N ratio than *B. hybridum* and *B. stacei* (Fig. 4).

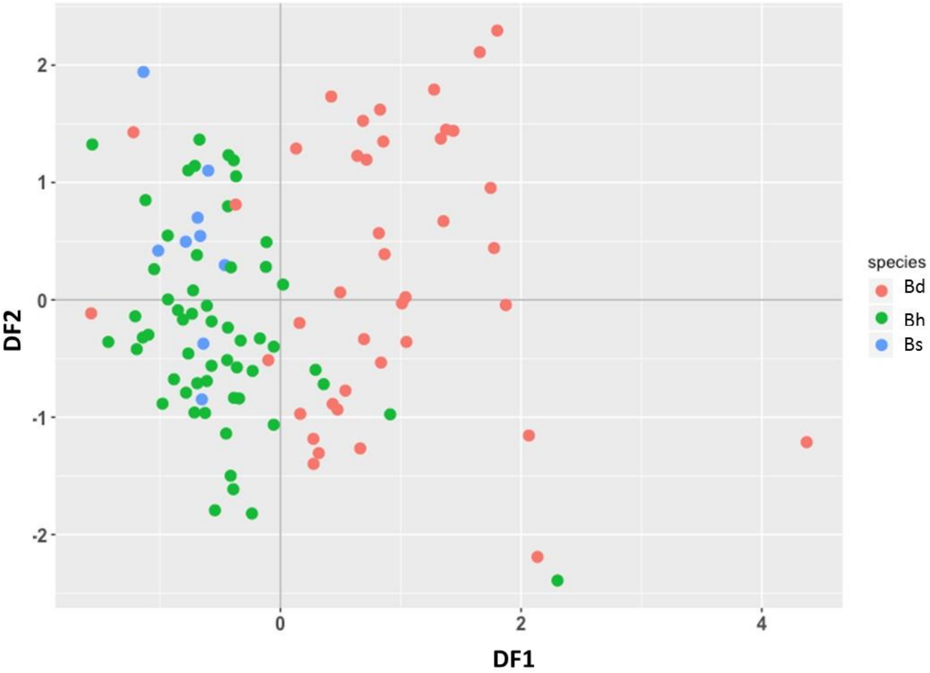


Fig. 3. Position of the three species of the *Brachypodium distachyon* species complex over the plane defined by the first two discriminant variables DF1 y DF2 , obtained from discriminant analyses conducted on four functional traits. Species are B. distachyon (Bd), B. hybridum (Bh) and B. stacei (Bs).

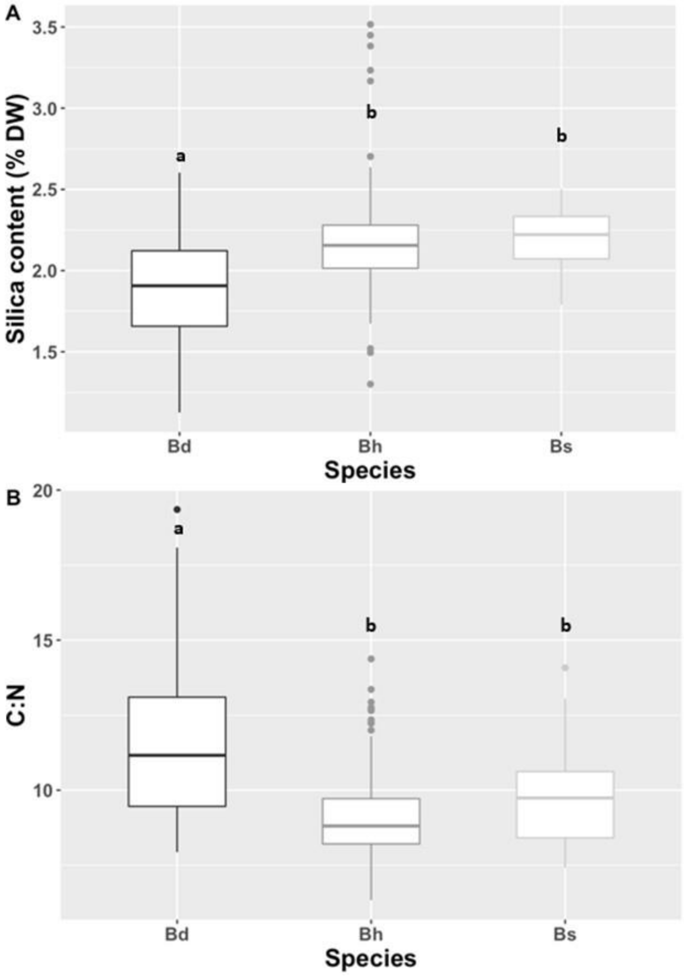


Fig. 4. Variation in (A) Silica content, (B) Carbon nitrogen ratio,(C) Specific leaf área and (D) wáter content between the three species of the *Brachypodium distachyon* species complex. Different letters on the graphs inidcate significative interspecific pairwise differences (P<0.05) in post hoc contrasts tests. Species are B. *distachyon* (Bd), *B. hybridum* (Bh) and *B. stacei* (Bs).

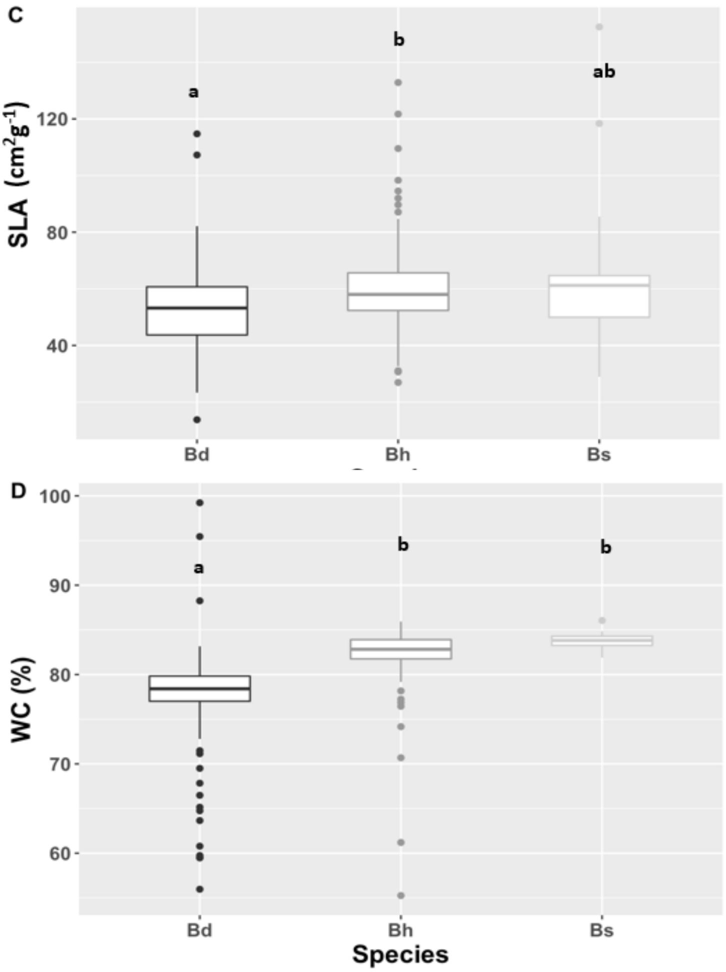


Fig. 4 (continued)

At population level, linear discriminant analysis indicated that *B. hybridum* and *B. distachyon* populations were significantly differentiated in leaf functional traits (Wilk's $\lambda = 0.4$, $P<0.0001$; Wilk's $\lambda = 0.28$, $P<0.0001$ for *B. hybridum* and *B. distachyon* respectively; Fig. S7). For both species, silica content was the trait with the highest weight for the main discriminant variable DF1 accounting for a total of 49 % and 47.1 % of inter-populational variation, respectively for *B. hybridum* and *B. distachyon* (Table S6). For *B. distachyon*, the C:N ratio was also significant for discriminating among populations accounting for a 31 % of inter-populational variation (Table S6).

Thus, JHIN, LARVA and MON were significantly differentiated from the rest, essentially in terms of leaf silica content (Fig. S7), whereas SCA, VILLA and VISC appeared differentiated from the rest of *B. distachyon* populations according to silica content and C:N (Fig. S8).

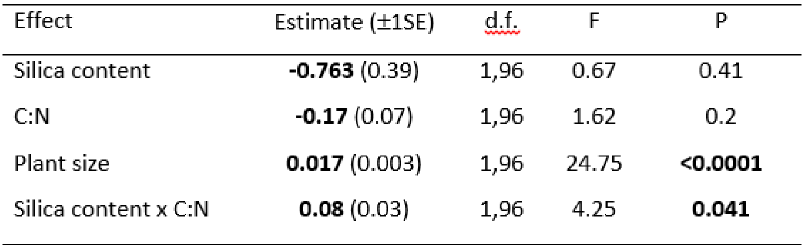
Univariate analyses showed that, for both species, all traits but SLA varied significantly among populations (*B. hybridum*: $F_{9,46} = 13.4$, $P < 0.0001$; $F_{9,46} = 28.16$, $P = 0.001$; $F_{9,46} = 1.39$, $P = 0.219$; $F_{9,46} = 2.09$, $P = 0.049$; *B. distachyon*: $F_{9,31} = 9.56$, $P < 0.0001$; $F_{9,31} = 6.5$, $P < 0.0001$; $F_{9,31} = 1.35$, $P = 0.252$; $F_{9,31} = 2.36$, $P = 0.036$; results for silica content, C:N, SLA and WC respectively; Fig. S8). For both species, inter-populational trait variation was not related to any geographical and/or environmental parameter ($P>0.05$ data not shown).

Plant resistance and tolerance predictors

Plant size, C:N and silica content were significant predictors of resistance to herbivory in *Brachypodium* plants (Table 2). Plant size was associated directly to resistance indicating that bigger plants are more resistant (Fig. 5), whereas the effect of C:N and silica content on resistance was interdependent (Table 2; Fig. 5). Thus, resistance was mainly function of a combination of high silica content and low C:N determining low palatability (Fig. 5).

Regarding tolerance, only SLA was marginally associated to tolerance to damage estimated as the number of spikes or seeds (coefficient estimate $\pm$ 1SE: $- 0.03 \pm 0.02$, $F_{1,90} = 2.89$, $P = 0.09$; $- 0.05 \pm 0.03$, $F_{1,90} = 2.7$, $P = 0.1$), suggesting that plants with lower SLA trend to manifest lesser tolerance (Fig. S9).

Table 2. Summary results of the stepwise multiple regression analysis performed between leaf traits and insect herbivory resistance. Only significant predictors are shown. Significant coefficients ($P<0.05$) are in bold.



Effect	Estimate (±1SE)	d.f.	F	P
Silica content	**-0.763** (0.39)	1,96	0.67	0.41
C:N	**-0.17** (0.07)	1,96	1.62	0.2
Plant size	**0.017** (0.003)	1,96	24.75	**<0.0001**
Silica content x C:N	**0.08** (0.03)	1,96	4.25	**0.041**

Resistance-tolerance tradeoffs

No evidence of genetic tradeoffs between resistance to herbivory and tolerance to damage was detected in our samples in any species, in fact tolerance and resistance trend to be positively associated in *B. distachyon* and *B. stacei* (Table S7, Fig. S10).

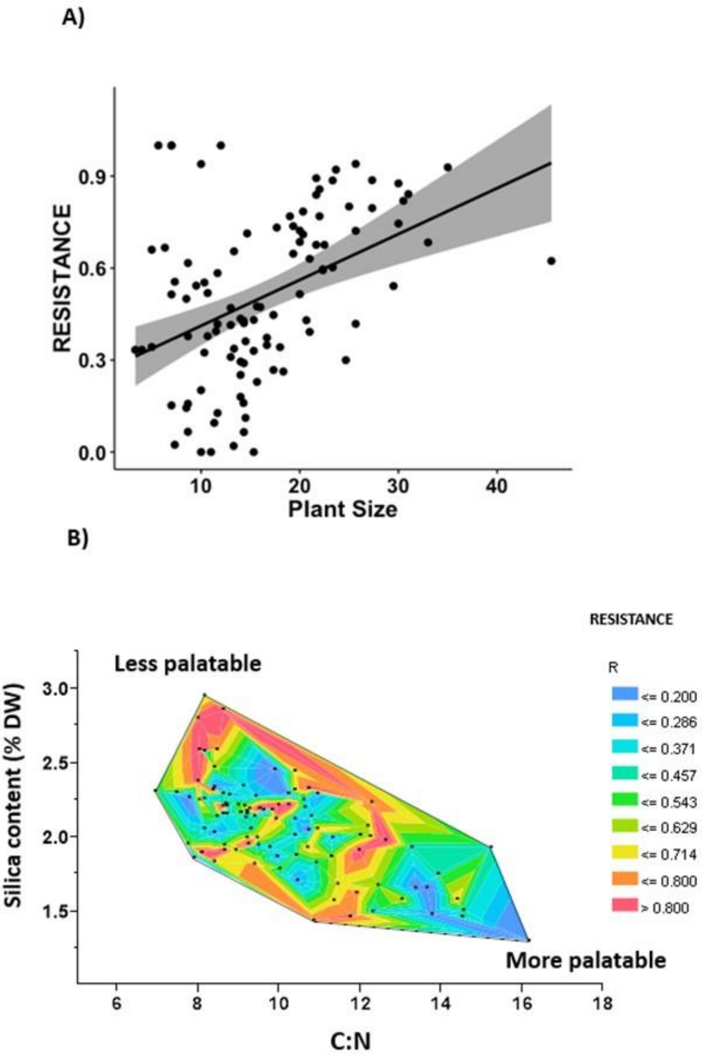


Fig. 5. A) Relationship between plant size (estimated as the total number of leaves) and resitance to herbivore. B) Contour plot depicts the interactive effect of leaf silica content and C:N ratio on resitance to herbivore damage.

DISCUSSION

Results from our bioassay indicate that insect herbivory had a significant impact on maternal fitness in the species of the *B. distachyon* species complex. Insect herbivores may be thus potential selection agents of *Brachypodium* genotypes. However, impact of herbivory on fitness was largely dependent on species (*B. distachyon* is more tolerant to damage than its relatives) and fitness estimator (impact of damage is higher in early fitness estimates). The three species differed in the level of damage received by herbivores suggesting inter-specific natural variation in herbivory resistance within the species complex (*B. stacei* was less resistant to damage than its closest relatives). Nonetheless, inter-genotypic and inter-populational variations within each species were, in relative terms, more important than interspecific differences for explaining variation in plant damage and resistance in this species complex. Overall, differences in resistance appeared associated to functional trait differentiation, especially in plant size and palatability (C:N ratio and silica content). Differences in tolerance to damage trended to be linked negatively to SLA variation. Finally, our results do not support that polyploidy enhances herbivory response in this species complex.

Herbivory response in *B. distachyon* species complex

Evolutionary theory predicts that polyploidy may cause instantaneous changes in physiology, morphology, and gene expression that can impact resistance to herbivores (e.g., Lou and Baldwin, 2003; Hull-Sanders et al., 2009; Segraves and Anneberg 2016). Therefore, if polyploidy confers resistance, lower damage on polyploid populations would be expected. Results from our assay however do not support this expectation since herbivory resistance of the allotetraploid *B. hybridum* was equivalent to one of its diploid parental, *B. distachyon*. Intriguingly, such equivalence in resistance between *B. hybridum* and *B. distachyon* did not arise from a similarity between these two species in functional traits related to herbivore resistance; instead, there was a higher correspondence in such functional traits between *B. hybridum* and the species showing higher damage levels, *B. stacei* (Fig. 4). However, importantly, *B. hybridum* and *B. distachyon* developed a higher number of leaves than *B. stacei* (18.38 ± 7.8, 15.2 ± 8.06, 11.6 ± 4.29, number of leaves developed at the beginning of the experiment for *B. distachyon*, *B. hybridum* and *B. stacei*, respectively), being this trait positively associated to resistance in this complex (Fig. 5A).

For all species, intra-specific variation in resistance was more important than interspecific variation. In particular, genetic variation at intra and inter-populational scale accounted for almost one third of the variation in resistance observed. Inter-individual variation in resistance to herbivores at different spatial scales has been described repeatedly in many systems (e.g., Maddox and Root 1987; Adler 1995; Ivey *et al*., 2009; Schranz *et al*., 2009; Moreria et al., 2018) and it is also the case here. Causes underlying inter-genotypic variation in resistance within populations are beyond the scope of this study but it may be likely related to inter-individual variation in traits that confer resistance to herbivores in this system (i.e., plant size, C:N ratio and silica content). In any case, because herbivory affected fitness, existence of significant inter-genotypic variation in resistance could result in herbivore-mediate trait selection if herbivore pressure is sustained (Agrawal *et al*., 2012), which occurs in several Iberian *Brachypodium* populations (A.J. Manzaneda and P.J. Rey unpublished).

Interestingly, at population level, we found a genetically based latitudinal cline in damage across allotetraploid *B. hybridum* populations, where populations from lower latitudes showed higher levels of leaf herbivory (Fig. S4). Many studies have investigated latitudinal gradients in herbivory in order to verify the latitudinal herbivory defence hypothesis (e.g., Anstett *et al*., 2015; Moreira et al., 2018 and references therein), which predicts that plants growing at lower latitudes should be more resistant to herbivory because experience greater amounts of herbivory than their counterparts at higher latitudes. These studies and others, however, often have found high variability in such expected pattern, and mixed results are frequently reported (Moles *et al*., 2011). Overall, our findings do not support the latitudinal herbivory defence hypothesis because we did not find any latitudinal cline in herbivory resistance for *B. distachyon* plants, and the observed pattern for *B. hybridum* was the opposite, i.e., lower herbivory resistance in *B. hybridum* from low latitudes. In addition, we did not find any geographical or environmental pattern in herbivore-avoidance traits (resistance or nutrient). In any case, if the observed greater herbivory for *B. hybridum* at low latitudes results from latitudinal clines in the evolution of plant defense, or from local adaptation or historical factors (a possibility given the polytopic origin of Iberian *B. hybridum* populations; López-Álvarez *et al*., 2012) still merit further attention.

Beyond resistance, plants may evolve alternative defensive strategies that do not influence the amount of damage received but maintain fitness, such as tolerance (Strauss and Agrawal 1999; Strauss *et al*., 2002). In the context of polyploidy, although largely untested, theory predicts that higher-level polyploids should exhibit enhanced tolerance compared to diploids (Segraves and Anneberg 2016). Our results, however, do not back such expectation since *B. distachyon* diploid plants showed higher tolerance to insect damage than *B. hybridum* allotetraploids in all fitness component here analyzed (Fig. S5). Similarly, when comparing to the other diploid parental, *B. stacei*, tolerance to damage of *B. hybridum* plants was lower or similar to *B. stacei* one (Fig. S5). Our results indicate that tolerance variation depended upon the fitness component analyzed, the species and the specific genotype. Thus, for early fitness component (the number of spikes yielded per plant) herbivory damage affected fitness of all species in a similar manner, i.e., a reduction of the reproductive potential around 60 %. For this fitness component, inter-genotypic variation was more important than inter-specific one, suggesting extensive genetic variation in tolerance to damage. For later fitness components (the number of seeds and seed biomass), *B. distachyon* was the only species that could compensate herbivory damage (Fig. 2), whereas for *B. stacei* and *B. hybridum* herbivory reduced the reproductive potential of plants about 73% and 84% respectively. For these components, inter-specific variation was more important to describe variation in tolerance with ca. 75 % of variance explained. Genetic heterogeneity in response to damage is common at different levels (Strauss and Agrawal 1999; Stevens *et al*., 2007; Manzaneda *et al*., 2010), ranging from mortality to overcompensation. Our results support this view, with *B. distachyon* genotypes, being overall able to overcome plant damage with a variable magnitude (with the exception of Bd21 line), whereas fitness of *B. hybridum* and *B. stacei* genotypes is decreased by insect damage. Traits that modify physiological processes such as photosynthetic activity and growth, phenology, and/or the use of stored nutrient typically underlie variation in tolerance to herbivory damage (Mitchell *et al*., 2016). Here, we have detected a negative trend between tolerance and constitutive SLA, which is a trait directly related to the photosynthetic activity, plant performance and growth (Reese *et al*., 2016), indicating that plants with higher SLA tend to show lower tolerance (see the following section for further discussion on this relationship).

Although our study did not find any evidence that polyploidy may enhance herbivore response in this species complex, altogether, findings from this study may be relevant to understand coexistence of *B. distachyon* diploids and *B. hybridum* allotetraploids in contact zones and the establishment of allotetraploids populations. We have recently shown a higher competitive advantage of *B. hybridum* allotetraploids compared with their parental *B. distachyon* diploids in natural mixed populations situated in southern Spain, forecasting in addition the exclusion of *B. distachyon* diploids, in particular from contact zones at dry localities (Rey *et al*., 2017). However, insect herbivory could reduce the competitive ability of allotetraploids directly by diminishing the reproductive potential of *B. hybridum* allotetraploids and indirectly allowing *B. distachyon* diploids to counterbalance fitness differences between species derived from a higher vigor of polyploids. Consistent with this idea is the fact that (i) mixed populations of *B. hybridum* allotetraploids and *B. distachyon* diploids are frequent in southern of the Iberian Peninsula (21% of sampled Iberian populations; Manzaneda et al., 2012) despite predictions of cytotype competitive exclusion (Rey *et al*., 2017), and (ii) in natural populations *B. hybridum* allotetraploids are more frequently attacked by herbivores than *B. distachyon* diploids (proportion of plants with any sign of leaf damage: 47.5 % vs. 26.5%, N = 819, data observed for *B. hybridum* and *B. distachyon*, respectively; Manzaneda and Rey unpublished). Many studies have already demonstrated the interactive effect of herbivory and plant competition in many systems (e.g., Hambäck and Beckerman 2003; Hanley and Sykes 2009; Agrawal *et al*., 2012). In any case, whether insect herbivory is a stabilizing factor that promotes species coexistence in this system still will require experimental verification in natural populations.

Functional traits underlying herbivory response in *B. distachyon species complex*

Differentiation in ecologically important characters between ploidies in heteroploidy species is common for many functional and physiological traits (see Manzaneda *et al*., 2015; Soltis *et al*., 2016), including herbivory-response traits in auto- and allopolyploids (Lou and Baldwin, 2003; Hull- Sanders *et al*., 2009). Here, we have confirmed that for *B. hybridum* and *B. stacei* traits related to leaf palatability and resistance to herbivory covary, whereas functional traits of *B. distachyon* were clearly differentiated from the other two species, in particular in terms of leaf silica content. A similar result has been described recently for drought-response traits in the same system, suggesting adaptive convergence between *B. hybridum* and *B. stacei* (Martínez *et al.*, 2018). Therefore, again, our results do not support the hypothesis that ploidy mediates differentiation of herbivore-response in this species complex. For *B. distachyon* and *B. hybridum*, we also detected population trait differentiation, although we did not find any link between trait variation, environment and/or geography across our study sites, which contrasts with our previous findings for other functional traits (Manzaneda *et al.*, 2015; Martínez *et al.*, 2018). In any case, as we have discussed above, *B. hybridum* resistance is similar to *B. distachyon* one, despite trait convergence between *B. hybridum* and *B. stacei*. A reason for that may reside in the fact that *B. hybridum* and *B. distachyon* develop a higher number of leaves and have a bigger size. This observation is congruent with the idea that larger plants may produce higher levels of traits, including resistance traits (Agrawal 2011). In fact, plant size appears correlated negatively to SLA and WC ($r = -0.2$, $P = 0.049$; $N = 92$, $r = -0.39$, $P = 0.00013$; $N = 92$, respectively) and positively to C:N ratio ($r = 0.236$, $P = 0.023$; $N = 92$). Thus, more vigorous plants show lower SLA and WC and have a relatively high carbon content in their leaves than smaller plants, which are all traits related to palatability (Agrawal and Fishbein 2006; Cardenas *et al.*, 2014). Leaves with a higher SLA are typically thinner and have greater levels of herbivory (Agrawal and Fishbein 2006), whereas plants with lower leaf WC and nitrogen levels are less palatable. Together, our results suggest that variation in palatability traits underlies the strong association here found between plant size and insect resistance. Nonetheless, leaf silica content (a defensive trait in grasses; Massey *et al.*, 2006) also was related to insect resistance, although such relationship was not independent of C:N ratio (Fig. 5B) due to both traits are negatively correlated each other (Table S3). This interdependence between silica content and C:N ratio makes the interpretation of the influence of silica content and C:N ratio on resistance more complex, although plants with higher levels of silica are overall more resistant and plants with a lower silica content are less resistant regardless that leaf carbon fraction is higher (Fig. 5B). For intermediate values of silica content and C:N ratio resistance values were more variable.

Regarding tolerance to damage, it is expected that plants with high SLA will be more tolerant to damage because SLA is a correlate of growth rate and foliar nutrient content (e.g., Cingolani *et al.*, 2005). Our results contradict this expectation since we found a negative trend between SLA and tolerance to damage. However, such trend is concordant with findings of a study conducted on 26 old-field species including grasses, where SLA was negatively correlated to plant performance after simulated damage (Reese *et al.*, 2016). Authors argued that plants with high SLA may experience higher herbivory costs per unit leaf loss (Reese *et al.*, 2016), which may perhaps be also the case here. SLA also varied among genotypes within and among populations (see also Martínez *et al.*, 2018). Thus, whether SLA might be adaptive in an environment with high herbivore pressure, lower SLA plants could be favored by natural selection, in this case, *B. distachyon* genotypes, which have leaves with lower SLA, could be benefited. An important consideration however is the fact that most of functional traits here analyzed have a plastic nature, by which expression may be affected by multiple environmental conditions, as for example drought (Agrawal *et al.*, 2008; Martínez *et al.*, 2018), that may affect in turn the outcome of plant-herbivore interactions (Grinnan *et al.*, 2013).

Finally, we found that resistance and tolerance were positively correlated in the three species indicating the absence of resistance-tolerance trade-offs that could explain genetic variation in tolerance within species (Manzaneda *et al.*, 2010).

Concluding remarks

Here we report results from the first attempt to understand the variation in the herbivory response in the *Brachypodium distachyon* species complex. Findings from this study are relevant because, first, *Brachypodium* is a model for temperate cereals, and our approach focused on functional traits provide a way of comparing species independent of species identity, comparable across taxa (Reese *et al*., 2016). Second, they allow generalization to others C3 temperate grasses, and are useful for crop breeding of C3 cereals. Thus, we have shown that plant size and silica content are two candidate traits that may enhance resistance to insect herbivory in *Brachypodium*. Similarly, SLA is a promising trait associated to tolerance to damage in this complex. Third, from an evolutionary side, our results point out the potential of locust and grasshoppers (natural enemies of *Brachypodium* in our populations) as agents of selection of *Brachypodium* genotypes and for herbivore-response traits, promoting population differentiation, at least across *B. hybridum* populations.

In the context of the ecological consequences of polyploidy, we have addressed for first time whether higher-level polyploids exhibit enhanced tolerance to insect damage. Our results do not support that polyploidy may enhance herbivore response in this species complex. However, because insect herbivory affected in a larger extent to *B. hybridum* allotetraploids, of superior competitive ability, than *B. distachyon* diploids (Rey *et al*. 2017), we hypothesize that insect herbivory may act as a stabilizing factor that promotes species coexistence in contact zones.

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AUTHOR's CONTRIBUTION

AJMA, PJR, AMO and LMM design and planned this research. LMM and TS conducted the bioassay. AJMA analyzed the data. AJMA and PJR wrote the paper. All authors read the paper and made critical comments on early versions of the manuscript.

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SUPPLEMENTARY MATERIAL

FIGURES

Fig. S1. Details of the method followed to infest Brachypodium plants with locusts. Each plant–locust pair was enclosed using a cylindrical tuve made of acetate (3M®) which upper end is covered by using organza bags.

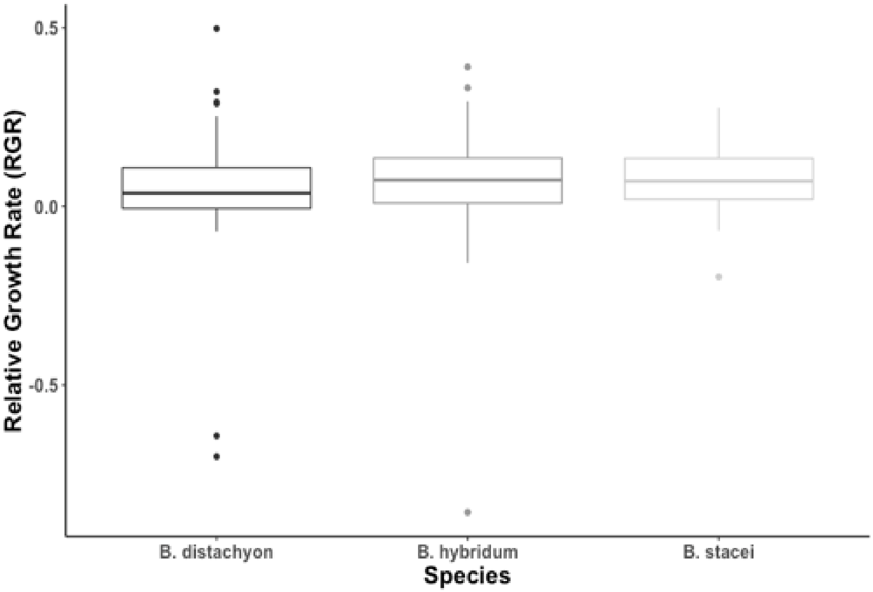


Fig. S2. Variation in insect relative growth rate (RGR) between the three species of the *Brachypodium distachyon* species complex.

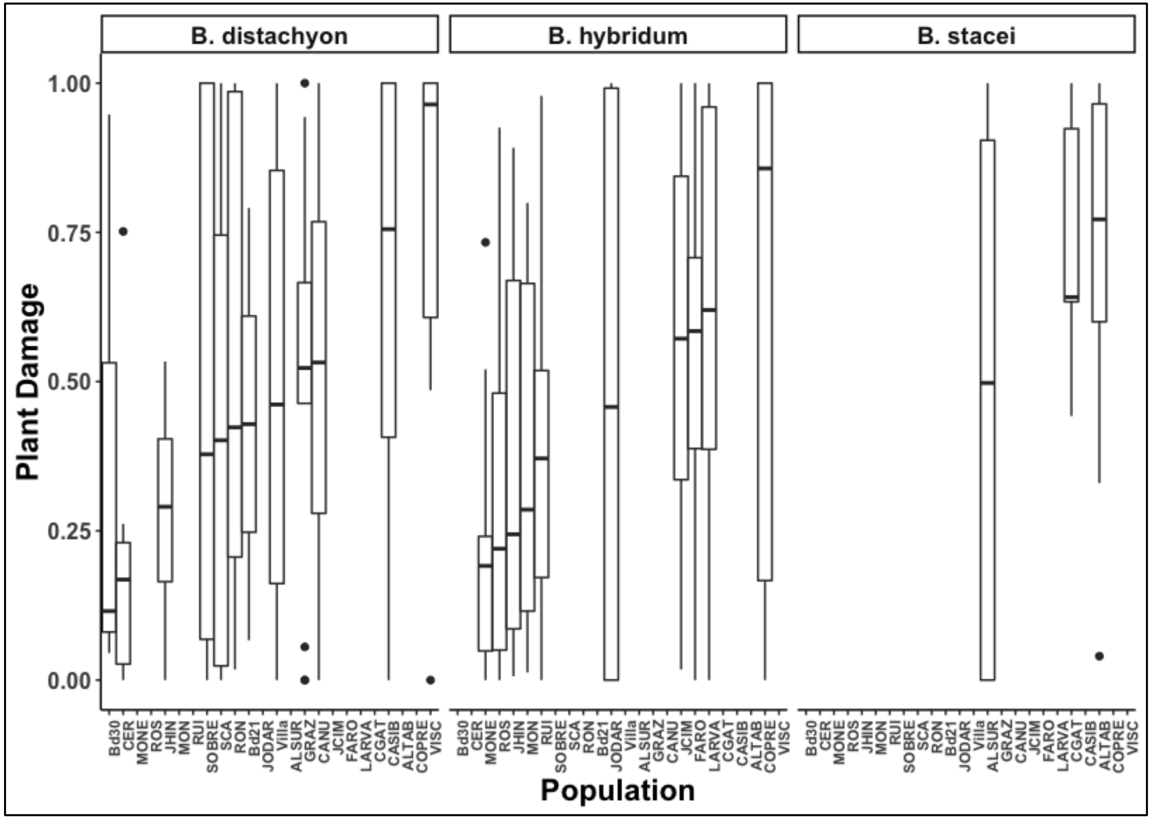


Fig. S3. Variation in plant damage and resistance to insect herbivory (RS) (next page) between Iberian populations of the three species of the *Brachypodium distachyon* species complex.

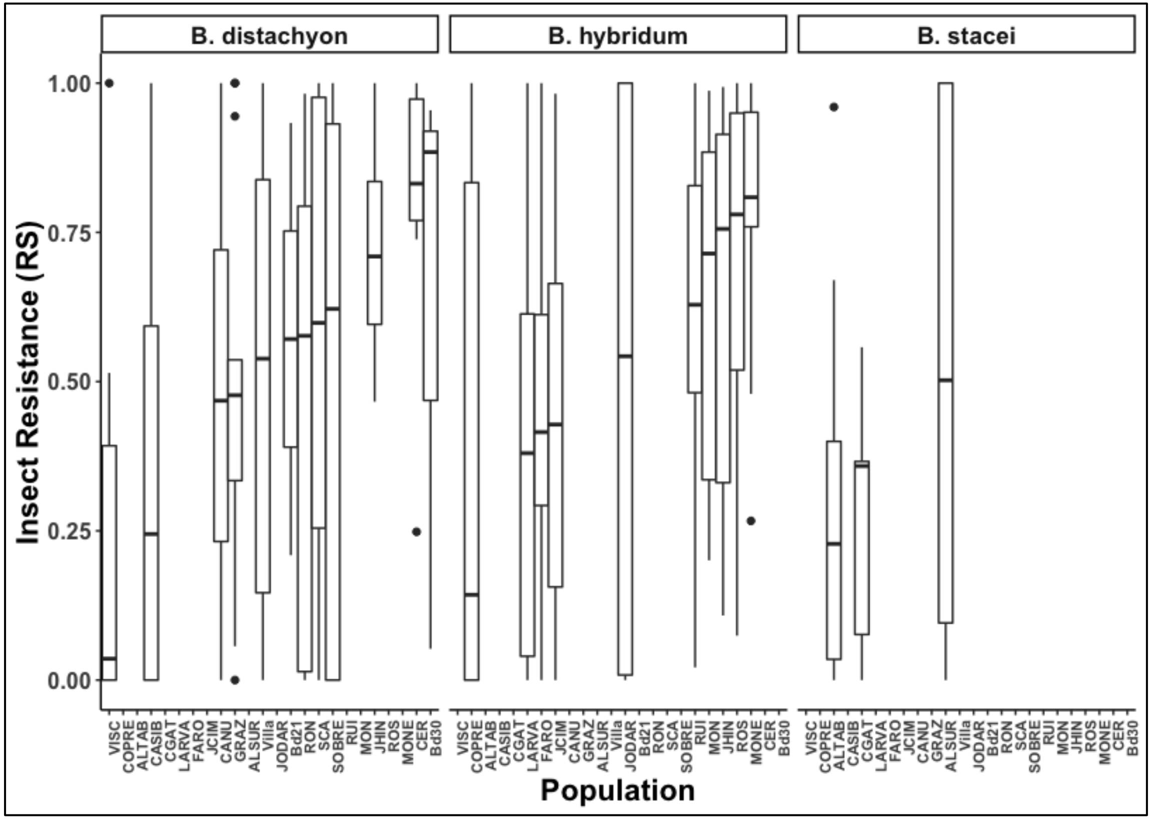


Fig. S3 (continued)

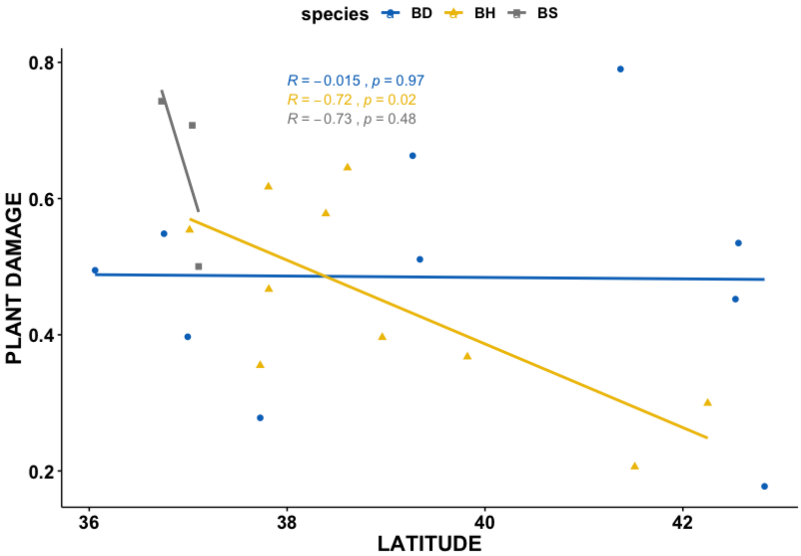


Fig. S4. Relationship between average plant damage (i.e., population mean) and latitude across the Iberian Peninsula.

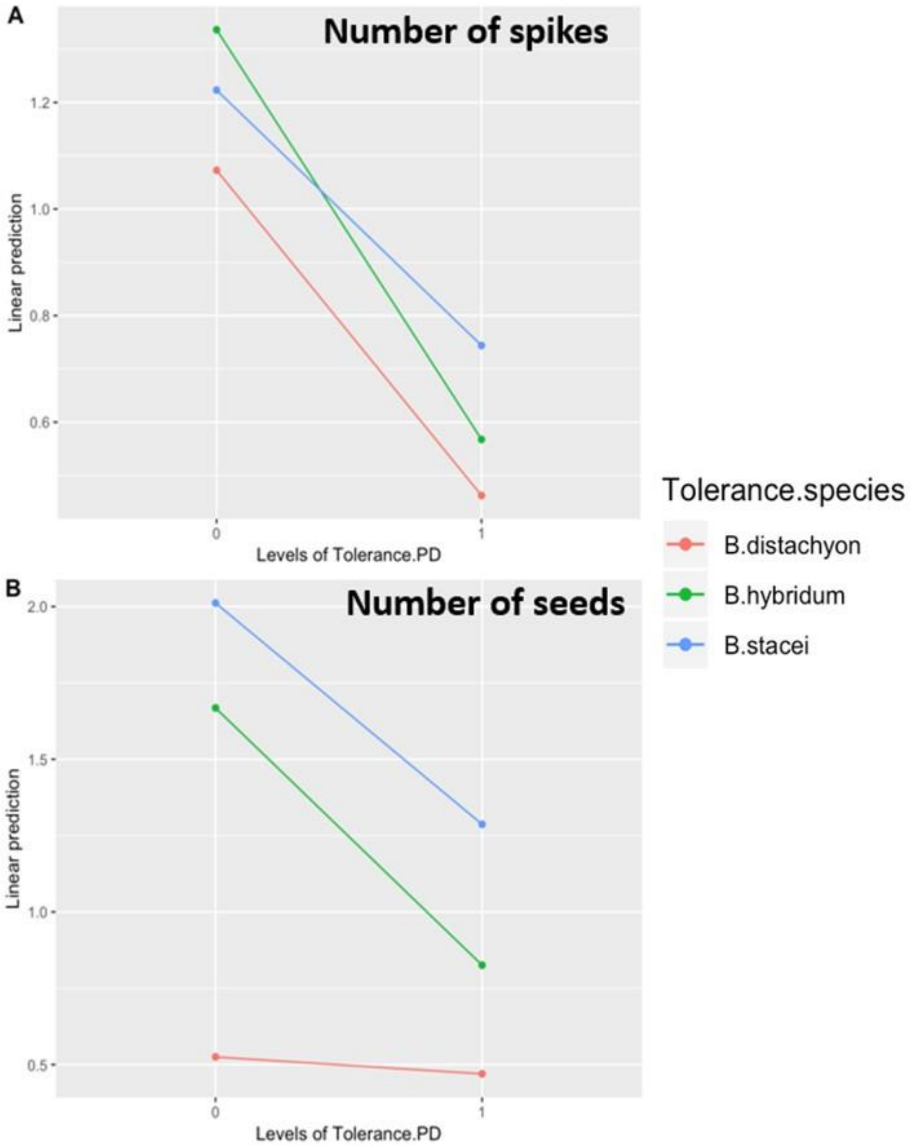


Fig. S5. Tolerance slopes for the three species of the *Brachypodium distachyon* complex. Graphs indicate the linear prediction of fitness variation across the range of plant damage (0-1) for three fitness measuremnts A) the number of spikes, B) the number of seeds c) seed biomass (cont. next page).

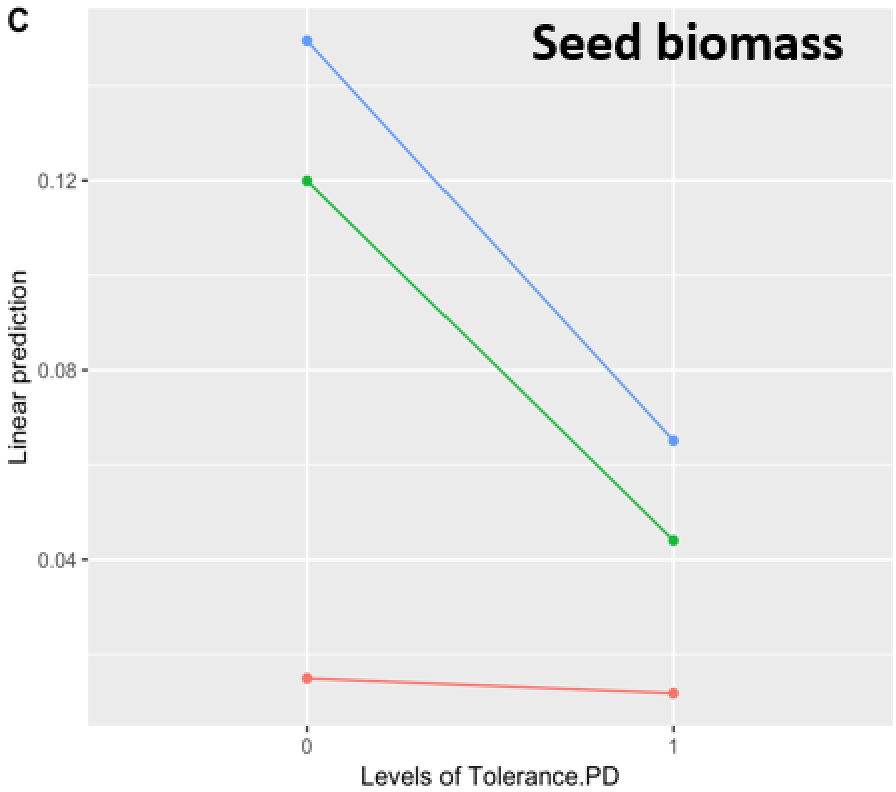


Fig. S5 (continued)

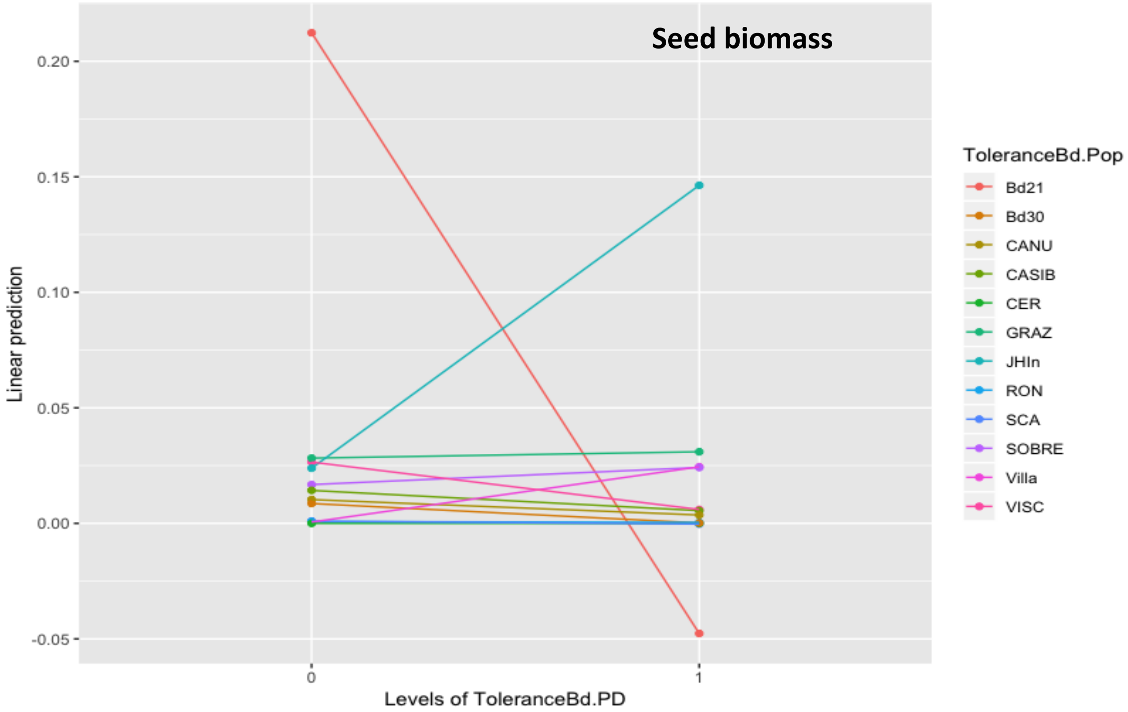


Fig. S6. Tolerance slopes for *B. distachyon* populations. Graphs indicate the linear prediction of fitness variation across the range of plant damage (0-1) for fitness (seed biomass).

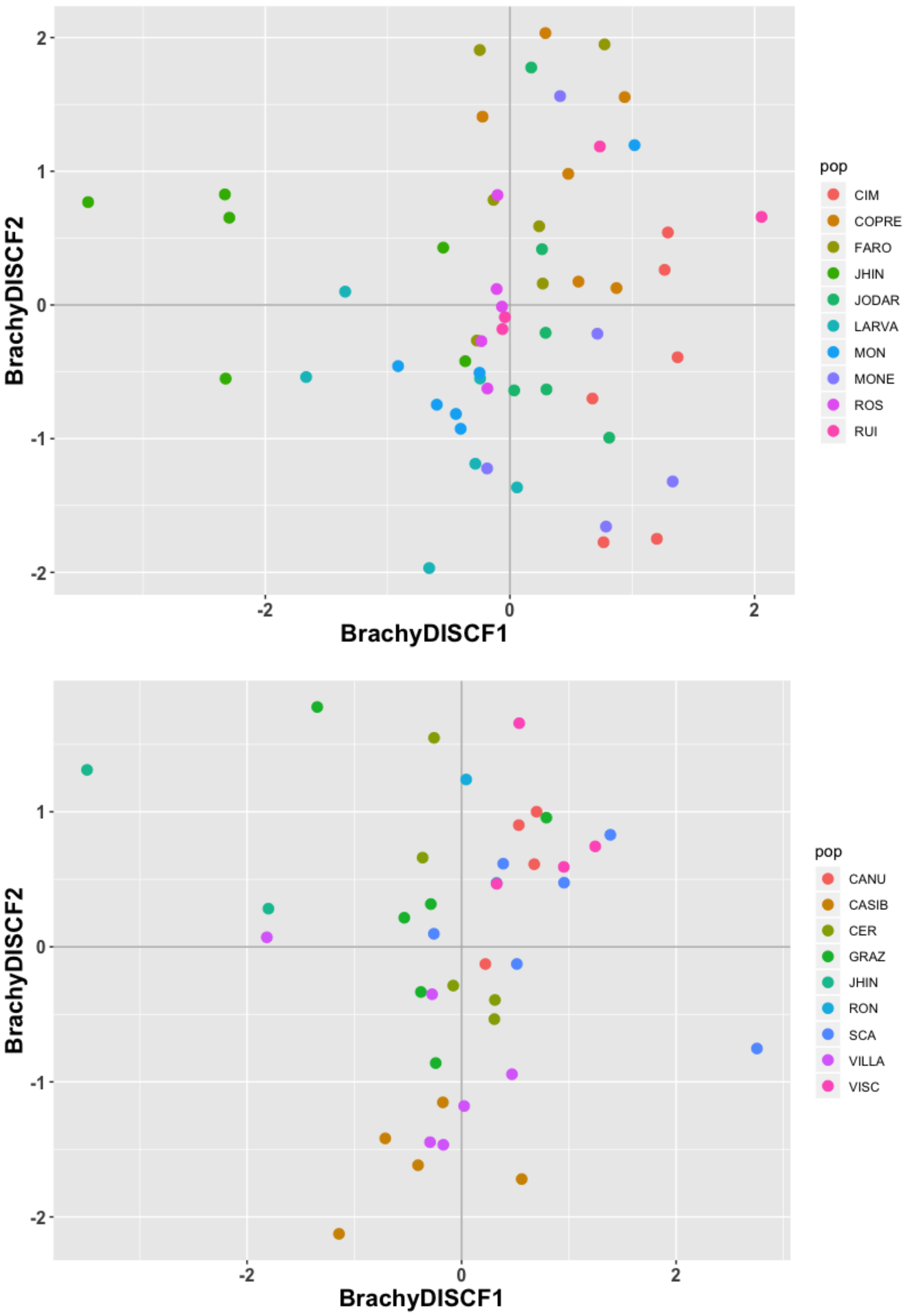


Fig. S7. Position of the *B. hybridum* populations (above) and *B. distachyon* (below) over the plane defined by the first two discriminant variables DF1 y DF2 , obtained from discriminant analyses conducted on four functional traits.

B. hybridum

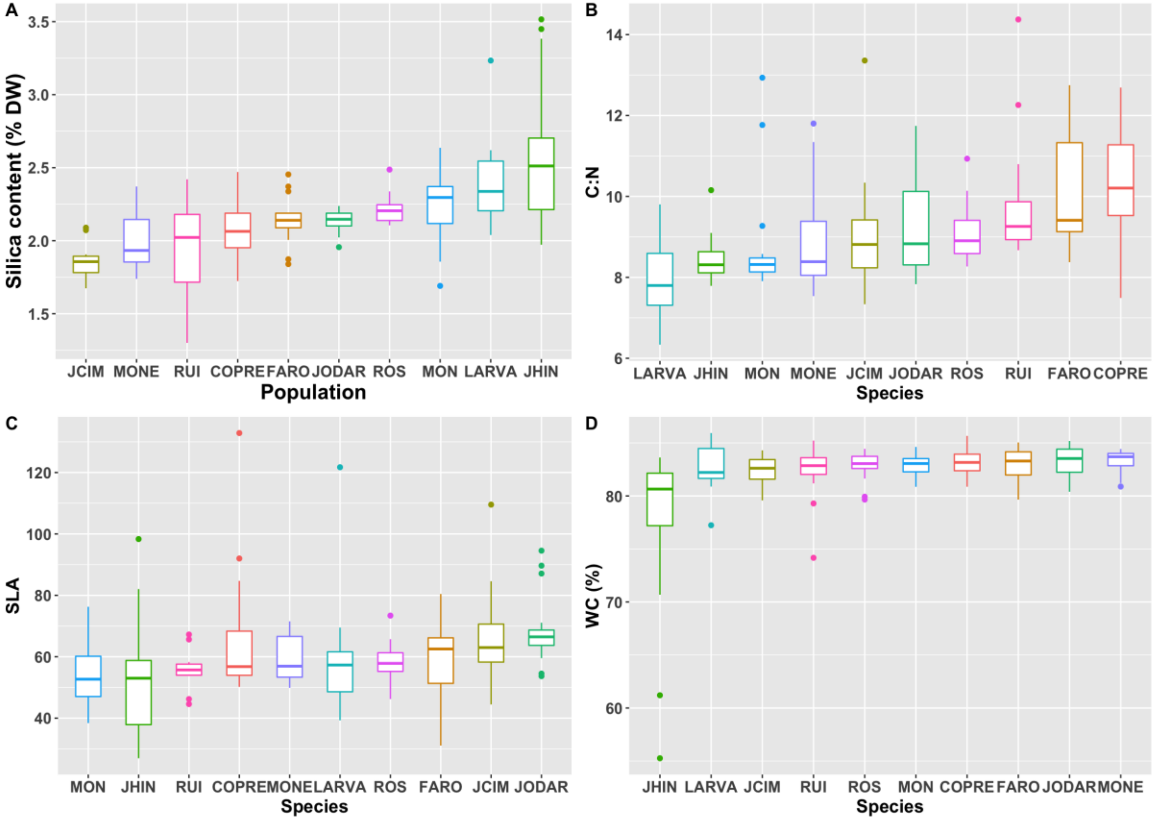


Fig. S8. Variation in four functional traits A) sliica content, B) C:N ratio, C) specific leaf área and D) water content among *B. hybridum and B. distachyon* Iberian populations (cont. next page).

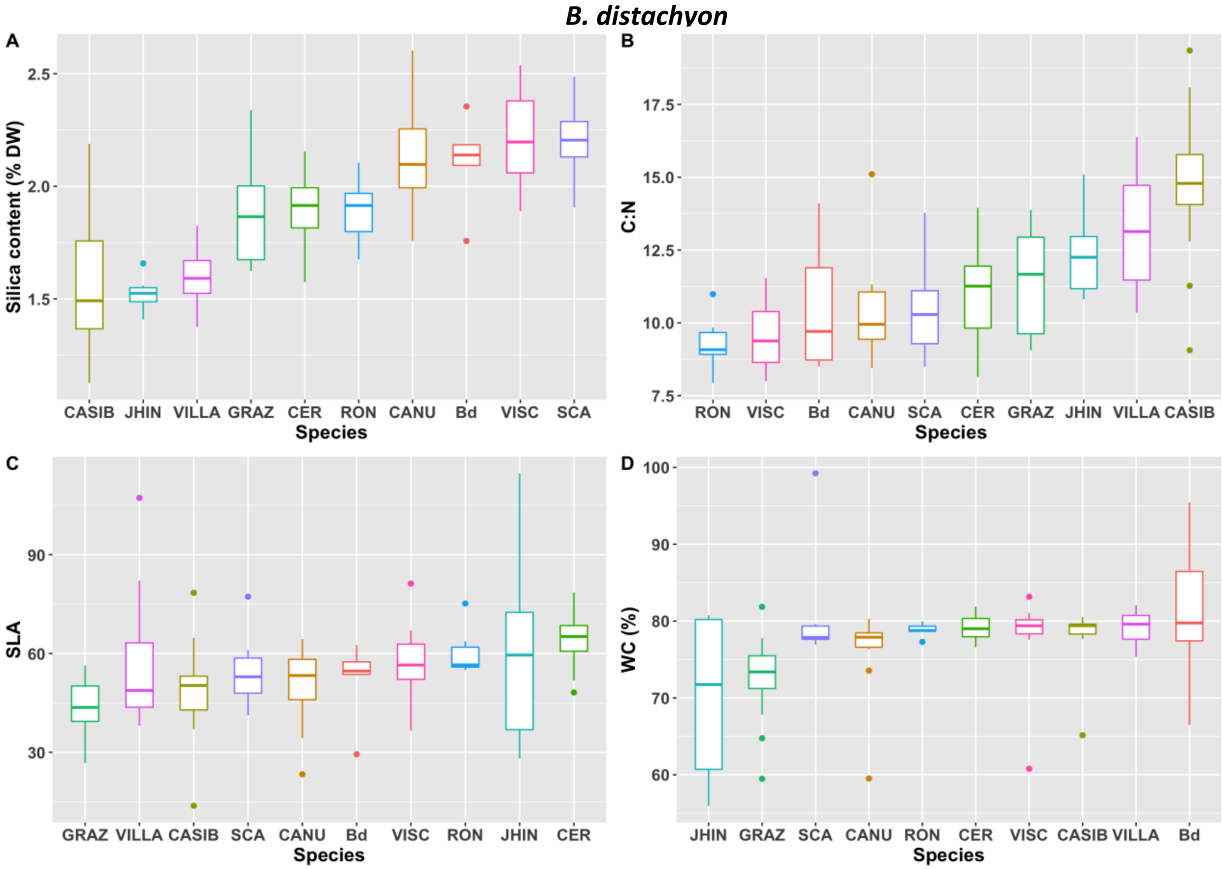
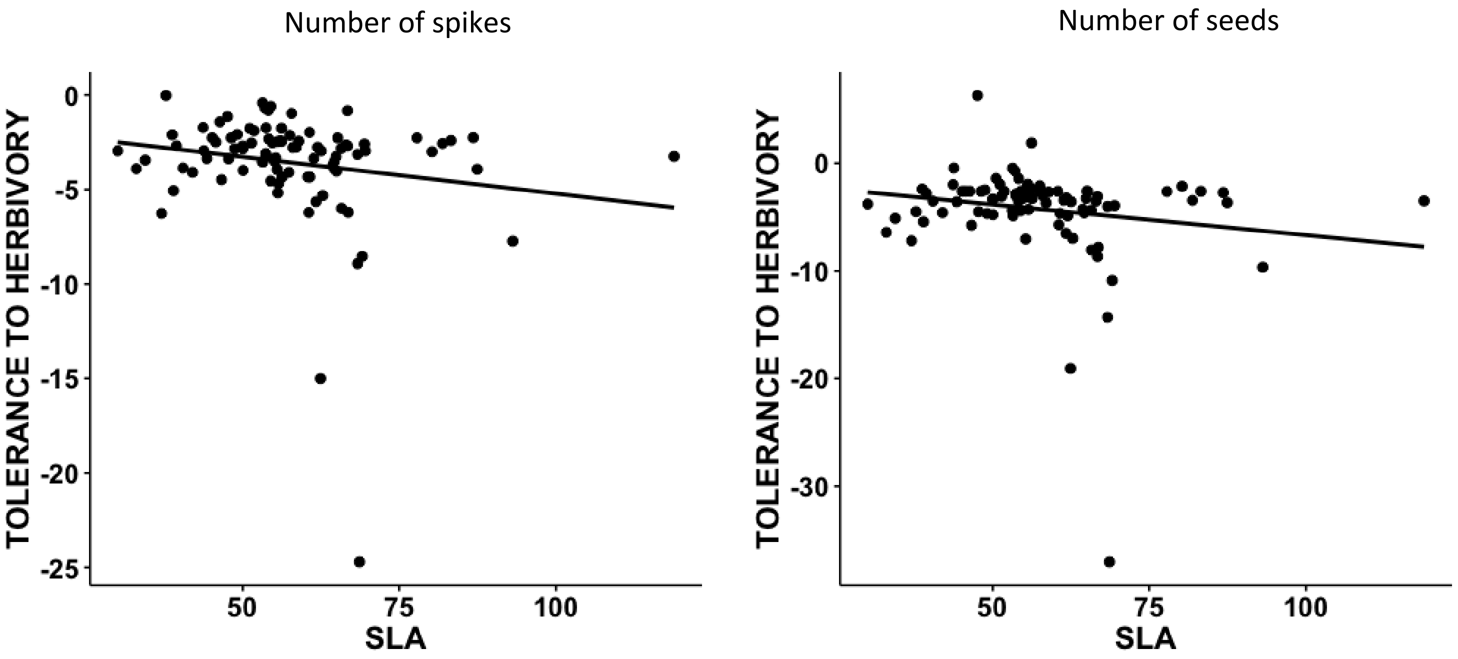


Fig. S8 (continued)


Number of spikes

Number of seeds

Fig. S9. Relationship between SLA and tolerance to insect damage across *Brachypodium* plants. Left graph tolerance comes from slopes on the number of spikes, right graph tolerance comes from slopes on the number of seed produced.

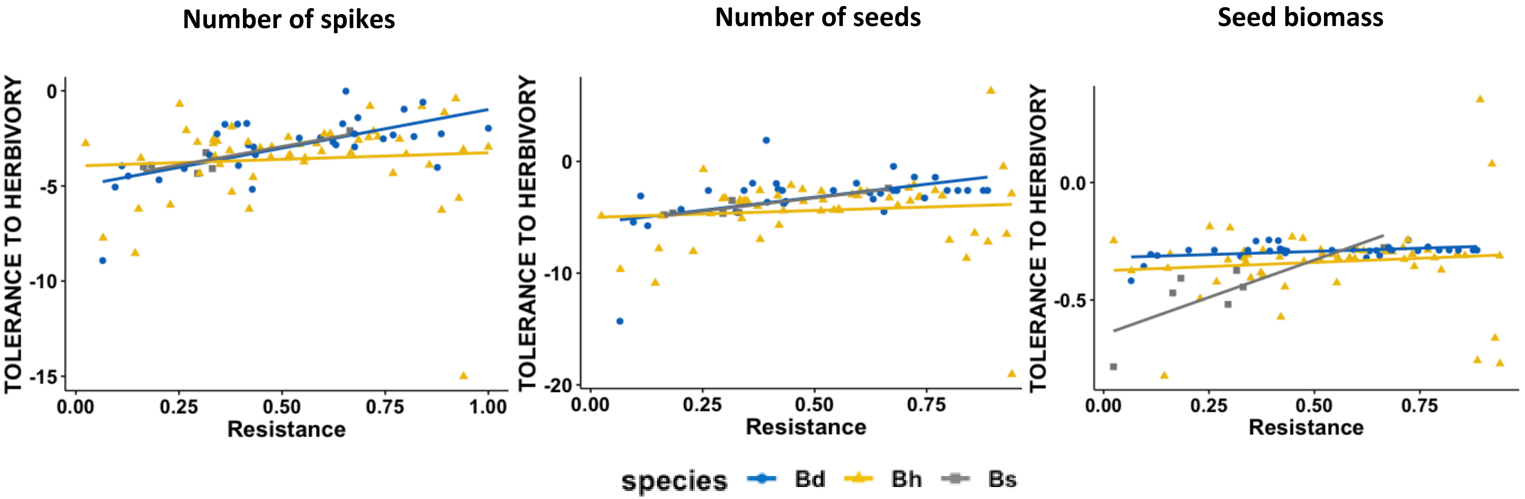


Fig. S10. Relationship between resistance and tolerance to insect damage across *Brachypodium* genotypes. A significant negative correlation between tolerance and resistance would indicate a resistance-tolerance tradeoff. However, no evidence for such costs is detectable.

TABLES

Table S1. *Brachypodium* ID accessions and geographical origin of the plants included in the study.

Species	Accession	Population	Geographical coordinates	Annual rainfall (mm)	Annual temperature (°C)	Origin
B. distachyon	Bd21	Mosul	N/A	622	14	Iraq (USDA)
	Bd30-1	Sierra Nevada	36º 59' 43'' N 3º 34' 18 W	440	10	Spain (USDA)
	SCA-5, SCA-7, SCA-8, SCA-9, SCA-10	Sierra Nevada	36º 59' 43'' N 3º 34' 18 W	440	10	Spain (UJAEN)
	CANU-4, CANU-13 CANU-14, CANU-15	Algeciras	36º 08' 37'' N 5º 30' 43'' W	968	17	Spain (UJAEN)
	CER-2,CER-4.CER-5 CER-6,CER-16,CER-18	Cervera	42º 49' 32'' N 4º 29' 27'' W	930	9	Spain (UJAEN)
	JHIN-10, JHIN-13	Hinojares	37º 43' 41'' N 2º 58' 52'' W	725	11	Spain (UJAEN)
	VILLA-3, VILLA-4, VILLA-5, VILLA-6, VILLA-7, VILLA-13	Villatoya	39º 20' 32'' N 1º 20' 47'' W	397	16	Spain (UJAEN)
	RON-2, RON-3, RON-5 RON-7, RON-11 RON-13	Roncal	42º 46' 50'' N 0º 57' 48'' W	1102	11	Spain (UJAEN)

	GRAZ-1, GRAZ-5, GRAZ-7, GRAZ-10, GRAZ-16, GRAZ-20	Grazalema	36º 45' 21'' N 5º 26' 30'' W	1773	16	Spain (UJAEN)
	CASIB-1,CASIB-8, CASIB-10, CASIB-12, CASIB-14, CASIB-15	Casas Ibañez	39º 16' 09'' N 1º 29' 22'' W	409	13	Spain (UJAEN)
	SOBRE-2, SOBRE-6, SOBRE-7, SOBRE-8, SOBRE-18, SOBRE-19	Sobredo	42º 31' 54'' N 6º 51' 7'' W	1008	12	Spain (UJAEN)
	VISC-4, VISC-8, VISC-13, VISC-14	Íscar	41º 22' 12'' N 4º 32' 18'' W	468	12	Spain (UJAEN)
B. stacei	ALTAB-1, ALTAB-3, ALTAB-5, ALTAB-12, ALTAB-15	Tabernas	37º 02' 28'' N 2º 24' 22'' W	298	16	Spain (UJAEN)
	CGAT-4, CGAT-6	Cabo de Gata	36º 43' 51'' N 2º 12' 4'' W	311	19	Spain (UJAEN)
	ALSUR-1, ALSUR-2, ALSUR-4	Sorbas	37º 05' 79'' N 2º 06' 35'' W	342	18	Spain (UJAEN)
B. hybridum	JHIN-8, JHIN-9, JHIN-11, JHIN-16, JHIN-18, JHIN-19	Hinojares	37º 43' 41'' N 2º 58' 52'' W	725	11	Spain (UJAEN)
	COPRE-4, COPRE-8, COPRE-9, COPRE-12, COPRE-13, COPRE-20	Los Pedroches	38º 36' 38'' N 5º 9' 3'' W	542	16	Spain (UJAEN)
	FARO-1, FARO-2, FARO-3, FARO-4, FARO-5, FARO-6	Faro	37º 0' 52'' N 7º 58' 35'' W	776	16	Portugal (UJAEN)
	MON-1, MON-3, MON-10, MON-13, MON-14	Monfrague	39º 49' 20'' N 6º 02' 52'' W	807	18	Spain (UJAEN)
	RUI-1, RUI-9, RUI-10, RUI-11	Ruidera	38º 57' 42 N 2º 52' 17'' W	433	14	Spain (UJAEN)

	JODAR-3, JODAR-6, JODAR-7, JODAR-8, JODAR-10, JODAR-19	Jodar	37º 48' 53'' N 3º 19' 09" W	302	14	Spain (UJAEN)
	LARVA-1, LARVA-3, LARVA-8, LARVA-15, LARVA-18, LARVA-20	Larva	37º 48' 38'' N 3º 12' 53" W	500	14	Spain (UJAEN)
	MONE-2,MONE-3,MONE-4 MONE-5,MONE-6	Monegros	41º 30' 50'' N 0º 30' 30'' W	349	15	Spain (UJAEN)
	ROS-3,ROS-6,ROS-7,ROS-9 ROS-10	Rosas de Mar	42º 14' 45'' N 3º 11' 1'' E	591	14	Spain (UJAEN)
	JCIM-7, JCIM-9, JCIM-16 JCIM-18, JCIM-19, JCIM-20	Cimbarra	38º 23' 28'' N 3º 22' 13'' W	509	14	Spain (UJAEN)

Table S2. Regression coefficients (± 1SE) of the relationship between plant damage and fitness (several fitness measurements considered) for each *Brachypodium* species. The steeper the slope (the coefficient), the less tolerant the plant.

	Fitness		
Species	Number of spikes	Number of seeds	Seed biomass
B. distachyon	-0.61 (0.114)	-0.05 (0.162)	-0.003 (0.015)
B. hybridum	-0.77 (0.112)	-0.84 (0.159)	-0.07 (0.015)
B. stacei	-0.48 (0.278)	-0.73 (0.396)	-0.08 (0.04)

Table S3. Phenotypic correlations (Pearson's product-moment correlations) between leaf functional traits for *Brachypodium distachyon* (N = 41, above the diagonal) and *Brachypodium hybridum* (N = 56, below the diagonal). Significant correlations (P<0.05) are in bold.

Trait	Silica content (% DW)	C:N	SLA (cm^2g^{-1})	WC
Silica content (% DW)		**-0.646**	-0.002	0.078
C:N	**-0.468**		-0.29	-0.146
SLA (cm^2g^{-1})	-0.13	-0.03		**0.497**
WC	-0.208	0.158	**0.3**	

Table S4. Phenotypic correlations (Pearson's product-moment correlations) between leaf functional traits for *Brachypodium stacei* (N = 9). Significant correlations (P<0.05) are in bold.

Trait	Silica content (% DW)	C:N	SLA (cm^2g^{-1})	WC
Silica content (% DW)		0.139	0.107	-0.4
C:N			-0.547	0.179
SLA (cm^2g^{-1})				-0.199
WC				

Table S5. Coefficients of the linear discriminant functions (DFs) of the four functional traits included in the discriminant analysis conducted at species level. Most important trait values in each discriminant variable are in bold.

Trait	DF1	DF2
Silica content (% DW)	**-1.268**	**2.286**
C:N	0.0737	**0.725**
SLA (cm^2g^{-1})	-0.0038	-0.029
WC	-0.1674	-0.081

Table S6. Coefficients of the linear discriminant functions (DFs) of the four functional traits included in the discriminant analysis conducted at population level. The most important trait value in each discriminant variable is in bold.

B. hybridum		
Trait	DF1	DF2
Silica content (% DW)	**-3.184**	**1.724**
C:N	-0.006	1.047
SLA (cm^2g^{-1})	0.014	0.014
WC	0.090	-0.076

B.distachyon		
Trait	DF1	DF2
Silica content (% DW)	**1.962**	0.247
C:N	-0.081	**-0.383**
SLA (cm^2g^{-1})	-0.054	0.029
WC	0.205	-0.135

Table S7. Correlations (Pearson's product-moment correlations) between tolerance and resistance in the three species of the *Brachypodium* species complex. Significant correlations (P<0.05) are in bold.

Species	Tolerance	R	P
B. distachyon	Number of spikes Number of seeds Reproductive biomass	**0.63** **0.46** **0.42**	**<0.0001** **0.0066** **0.016**
B. stacei	Number of spikes Number of seeds Reproductive biomass	**0.85** **0.9** **0.8**	**0.03** **0.016** **0.031**
B. hybridum	Number of spikes Number of seeds Reproductive biomass	0.076 0.092 0.099	0.59 0.52 0.49

Discusión general

DISCUSIÓN GENERAL

El estrés hídrico y la herbivoría son importantes factores ambientales que limitan la supervivencia y la productividad en las plantas. Además, son importantes agentes de selección que pueden originar procesos adaptativos. Este trabajo de tesis ha puesto de manifiesto la importancia de ambos factores en el complejo de especies de *B. distachyon.*

En relación con el estrés hídrico, nuestro trabajo ha demostrado que la respuesta a dicho estrés a nivel funcional está generalmente bien diferenciada entre las especies del complejo *B. distachyon*, aunque dicha diferenciación depende del nivel de estrés hídrico y del rasgo específico considerado. Considerando el contenido de fitohormonas, detectamos un patrón de expresión transgresivo en respuesta al estrés hídrico de *B. hybridum*, mientras que considerando caracteres fisiológicos a nivel de hoja la respuesta al estrés de *B. hybridum* se mostró correlacionada esencialmente con la de *B. stacei*. Estudios de nicho ambiental han mostrado que *B. distachyon* se encuentra en zonas de mayor altitud, frías y húmedas y que, por el contrario, *B. stacei* crece en zonas de menor altitud, templadas y secas (López-Alvarez *et al*., 2015). *B. hybridum* se puede encontrar en zonas con características climáticas intermedias pero frecuentemente crece en zonas de baja altitud, templadas y secas como su progenitor *B. stacei* (Manzaneda *et al*., 2012; López-Alvarez *et al*., 2012, 2015). De hecho, a lo largo de su rango de distribución nativa, poblaciones simpátricas de *B. stacei* y *B. hybridum* se han encontrado mezcladas en varias localidades (López-Álvarez *et al*., 2015; A. J. Manzaneda pers. obs.). Curiosamente, *B. hybridum* y *B. stacei*, también comparten caracteres asociados con el escape a la sequía como el rápido desarrollo de la planta, la carencia de vernalización y la floración temprana (líneas ibéricas de *B. stacei* florecen a los 31-39 días después de germinar; Martínez y Manzaneda, datos no publicados). En nuestro trabajo también hemos visto que, en condiciones de sequía, las poblaciones ibéricas de *B. hybridum* aparecen fisiológicamente diferenciadas. La aridez ambiental se asoció con la diferenciación de caracteres de *B. hybridum* entre poblaciones a niveles moderados de estrés hídrico. En particular, detectamos un vínculo entre la aridez, la diferenciación fisiológica y la diferenciación genética a escala poblacional. Estos resultados apoyan la hipótesis sugerida por Manzaneda *et al*., (2015) de que la variación clinal existente entre poblaciones de *B. hybridum* en toda la Península Ibérica puede tener su origen en la adaptación de *B. hybridum* a las diferencias de aridez a lo largo de los gradientes climáticos, probablemente como resultado de la selección ejercida por la disponibilidad de agua (Ackerly *et al*., 2000; Donovan *et al*., 2009).

Nuestros resultados a nivel genético obtenidos a partir del análisis comparativo de los transcriptomas de las tres especies del complejo *Brachypodium distachyon*, en el marco de la respuesta a la sequía y el legado parental, indican, en primer lugar, que *B. hybridum* exhibe una respuesta transcripcional a la sequía más fuerte que la de sus ancestros diploides y que una fracción importante de DEG (genes diferencialmente expresados) regulados por la sequía en *B. hybridum* se expresaron únicamente en dicha especie. Parte de estas diferencias podrían explicarse por el gran número de genes y por la complejidad que esta especie posee por su naturaleza híbrida. Curiosamente, la fracción de DEG regulados por la sequía y compartidos por las tres especies están involucrados en la biosíntesis de prolina, uno de los metabolitos más importantes que actúa como osmoprotector y como chaperona preservando la estructura de otras proteínas y la integridad de las membranas en situaciones de estrés (Verbruggen & Hermans, 2008). En segundo lugar, nuestro trabajo indica que hay una variación significativa en la expresión de genes bajo condiciones de sequía entre los genotipos de *B. hybridum*, aunque, en general, se registró una fuerte inducción de genes que codifican proteínas abundantes en la embriogénesis tardía (proteínas LEA), entre las que se encuentran las dehidrinas (DHN3) y genes de proteínas de choque térmico. Ambos son grupos de genes que tienen gran importancia en la respuesta a la sequía (Trejo-Calzada *et al.*, 2019). En tercer lugar, y en el contexto del legado parental, nuestros resultados a nivel transcriptómico muestran que los dos patrones de expresión más comunes de los genes homeólogos en *B. hybridum* bajo condiciones de estrés hídrico fueron: el dominio de expresión Bd, donde la expresión total de los homeólogos se asemejó al parental *B. distachyon*; y la expresión transgresiva, donde los genes se regularon disminuyendo o aumentando su expresión en relación con su expresión en los parentales diploides. Sin embargo en condiciones control, el dominio del nivel de expresión Bs fue el patrón más común, es decir, el patrón de expresión de los homeólogos en *B. hybridum* se asemejó al de *B. stacei*. Estos resultados apoyan la hipótesis de la plasticidad poliploide que establece que las especies alopoliploides podrían utilizar los patrones de expresión de ambos progenitores dependiendo de los ambientes (Shimizu-Inatsugi *et al.*, 2017). Contrariamente a nuestras expectativas, estos resultados contrastan con los resultados obtenidos a nivel funcional, no respaldando que los homeólogos heredados de *B. stacei* puedan haber contribuido en gran medida al nivel de transcripción de la respuesta a la sequía en *B. hybridum*. Luego la capacidad de *B. hybridum* para tolerar un rango más amplio de aridez no resultó del uso masivamente diferencial de los homeólogos heredados de *B. stacei*, sino que la expresión diferencial de genes probablemente depende principalmente de un pequeño número de reguladores (por ejemplo, factores de transcripción, mecanismos epigenéticos, transposones y regulación postranscripcional), que probablemente dependen de la aridez. Luego el que haya un dominio de expresión Bd en condiciones de estrés hídrico puede ser explicado por la teoría de dominancia nucleolar descrita en el complejo (Idziak & Hasterok, 2008; Borowska-zuchowska et al., 2016; Borowska-Zuchowska & Hasterok, 2017), que muestra que *B. hybridum* expresa genes rRNA del parental *B. distachyon* debido a la inactivación de los genes heredados de *B. stacei* a nivel epigenético. Por otro lado, los patrones de expresión de más de la mitad de todos los pares homoeologos expresados en los padres diploides se conservaron en el alopoliploide, tanto a nivel constitutivo como bajo sequía, lo que sugiere una divergencia cis-reguladora general entre los progenitores diploides (sensu Buggs *et al.*, 2014). En cualquier caso, los hallazgos de este estudio provienen del análisis de una sola línea de alopoliploides de *B. hybridum*, que dado el origen politópico de los alopoliploides de *B. hybridum* (López-Álvarez et al., 2012) resulta insuficiente para las generalizaciones. Los estudios futuros deben investigar la consistencia del legado parental y el patrón de expresión entre los genotipos de *B. hybridum* que difieran en las edades de formación durante el último 1 Mya y que difieran en antecedentes genéticos y reciprocidad de parentesco.

En el contexto de la alopoliploidía (hibridación y posterior duplicación del genoma completo), la ventaja principal es que la heterosis resultante de la hibridación ancestral permita a la descendencia alopoliploide mostrar un patrón transgresivo asociado a una mejora en comparación con sus especies progenitoras diploides, lo que mejoraría la respuesta de los alopoliploides a los cambios ambientales y posteriormente contribuiría a la exitosa persistencia de las poblaciones alopoliploides más allá de los rangos ambientales de los progenitores (Hegarty & Hiscock, 2008; Madlung, 2013; Barker *et al.*, 2016). Dicha teoría la hemos podido verificar a nivel funcional en *B. hybridum* en el caso del contenido en fitohormonas, y a nivel transcriptómico en el patrón de expresión transgresivo de parte de los genes homeólogos que se expresan de forma diferencial en *B. hybridum* en respuesta a la sequía. Pero, en general, la respuesta funcional de *B. hybridum* al estrés por sequía está correlacionada esencialmente con *B. stacei*, mientras que en la respuesta a nivel transcriptómico el mayor porcentaje de genes que se expresan en *B. hybridum* con la sequía lo hacen con un patrón similar a como se expresan en *B. stacei* y *B. distachyon*. Por tanto, nuestros resultados, no apoyan dicha teoría de la alopoliploidía en respuesta al estrés hídrico en el complejo *B. distachyon*.

Es destacable el rol del gen que codifica un tipo de dehidrinas, dehidrin3, en *B. distachyon BdDHN3*), cuya expresión fue alta y consistentemente inducida en varias líneas naturales de *B. distachyon* bajo condiciones de estrés hídrico. Nuestros principales resultados con plantas transgénicas de *Brachypodium* generadas de novo (llamadas 35S:*BdDHN3*), que sobreexpresan constitutivamente dicho gen bajo el control del promotor 35S del virus del mosaico de la coliflor (CaMV), indican que la sobreexpresión del gen *BdDHN3* está ligada a un elevado contenido de agua en hoja, bajo daño celular y alto contenido en prolina bajo condiciones de estrés mejorando así la tolerancia a la sequía. Dada la proximidad entre los genomas de *B. distacyon* y los cereales de clima templado, el gen *BdDHN3* es prometedor para iniciar programas de *breeding* o *Marked Assisted selection* (MAS) (Hoisington & Ribaut, 1998; Collard & Mackill, 2007). Nuestros resultados reafirman el papel importante que tienen las proteínas LEA, en general, y las dehidrinas, en particular, en la tolerancia a la sequía en cereales (Graether & Boddington, 2014; Kosová *et al*., 2014; Yu *et al*., 2018; Liu *et al*., 2017). En cuanto a los futuros marcos de trabajo interesantes que se llevarán en esta dirección se analizaran con más detalle otros caracteres fenotípicos que puedan ser importantes para afrontar la sequía en la línea transgénica generada 35S:*BdDHN3*. Además se abordará el estudio de otras líneas transgénicas de *Brachypodium* recientemente creadas en nuestro laboratorio para inhibir el nivel de transcripción endógeno del gen *BdDHN3* y así causar un fenotipo de pérdida de función. Una vez que sea confirmada la inhibición del gen por RTq-PCR, se analizaran los caracteres fenotípicos en las plantas T1. Este punto será esencial para completar la información acerca de la función de este gen en la sequía.

Los resultados de nuestro bioensayo de resistencia y tolerancia a herbivoría son relevantes dado que es muy poco lo conocido acerca de este tema en el sistema de *Brachypodium,* a pesar de que la herbivoría por insectos provoca importantes daños en cereales (Mitchell *et al.*, 2016). Nuestro trabajo indica que la herbivoría por insectos tiene un impacto significativo en la adecuación biológica (*fitness)* de las especies del complejo *B. distachyon*. Las tres especies difirieron en el nivel de daño recibido por los herbívoros, lo que sugiere una variación natural interespecífica en la resistencia a la herbivoría dentro del complejo de especies (*B. stacei* fue menos resistente al daño que sus ancestros más cercanos). No obstante, las variaciones intergenotípicas e interpoblacionales dentro de cada especie fueron, en términos relativos, más importantes que las diferencias interespecíficas para explicar la variación en el daño y la resistencia de las plantas en este complejo de especies. La variación intergenotípica en la resistencia a los herbívoros a diferentes escalas espaciales se ha descrito repetidamente en muchos sistemas (e.g., Maddox and Root 1987; Adler 1995; Ivey et al., 2009; Schranz et al., 2009; Moreria et al., 2018) y este es el caso en nuestro trabajo también. En general, las variaciones intergenotípicas en la resistencia a la herbivoría aparecieron asociadas a la variación de caracteres funcionales que confieren resistencia a los herbívoros, especialmente al tamaño de la planta y a la palatabilidad (relación C: N y contenido de sílice). En cualquier caso, debido a que la herbivoría afecta al fitness, la existencia de una variación intergenotípica significante en la resistencia podría resultar en una selección de caracteres mediada por la herbivoría si la presión de herbivoría se mantiene (Agrawal *et al*., 2012), como ocurre en varias poblaciones ibéricas de *Brachypodium* (A.J. Manzaneda y P.J. Rey datos no publicados). Luego, desde un punto de vista evolutivo, nuestros resultados señalan el potencial de la langosta y los saltamontes (enemigos naturales de *Brachypodium* en nuestras poblaciones) como agentes de selección de genotipos de *Brachypodium* y de caracteres de respuesta a herbívoros, promoviendo la diferenciación de la población, al menos a través de las poblaciones *B. hybridum.* En el contexto de las consecuencias ecológicas de la alopoliploidía, hemos abordado por primera vez si los poliploides de nivel superior exhiben una mayor tolerancia al daño por insectos. Nuestros resultados no respaldan tal expectativa ya que las plantas diploides de *B.*

distachyon mostraron mayor tolerancia al daño por insectos que los alotetraploides de *B. hybridum*. De manera similar, cuando se compara con el otro progenitor diploide, *B. stacei*, la tolerancia al daño de las plantas de *B. hybridum* fue menor o similar a la de *B. stacei*. Sin embargo, debido a que la herbivoría de insectos afecta en mayor medida a los alotetraploides de *B. hybridum*, de capacidad competitiva superior que los diploides de *B. distachyon* según Rey *et al.* (2017), planteamos la hipótesis de que la herbivoría de insectos puede actuar como un factor estabilizador que promueve la coexistencia de especies en zonas de contacto. Muchos estudios han demostrado ya el efecto de interacción de la herbivoría y la capacidad competitiva de las plantas en algunos sistemas (e.g., Hambäck and Beckerman 2003; Hanley and Sykes 2009; Agrawal *et al*., 2012).

En asociación con la tolerancia al daño en este complejo, destacamos como rasgo el SLA constitutivo. El SLA es un rasgo directamente relacionado con la actividad fotosintética, el rendimiento y el crecimiento de la planta (Reese et al., 2016), por lo que cabe esperar que las plantas con un SLA más alto muestren una tolerancia mayor (Cingolani *et al*., 2005). Sin embargo, nuestros resultados no apoyan esta teoría ya que hemos encontrado una relación negativa entre el SLA y la tolerancia al daño que coincide con los resultados publicados por Reese *et al*., (2016). Estos autores argumentaron que las plantas con alto SLA pueden experimentar mayores costos de herbivoría por unidad de hoja perdida (Reese et al., 2016), lo que quizás también sea el caso aquí.

Finalmente, encontramos que la resistencia y la tolerancia a la herbivoría se correlacionaron positivamente en las tres especies, lo que indica la ausencia de *trade-offs* de resistencia-tolerancia que podrían explicar la variación genética en la tolerancia dentro de las especies (Manzaneda et al., 2010).

CONCLUSIONES

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Los principales resultados de este estudio se pueden resumir en las siguientes conclusiones:

1. El análisis de la respuesta funcional a la sequía en el complejo *Brachypodium distachyon* nos muestra que dicha respuesta está bien diferenciada entre las especies que forman el complejo. Considerando el análisis del contenido de fitohormonas, la respuesta funcional a la sequía de *B. hybridum*, comparada con la de sus dos especies ancestrales diploides, es transgresiva, lo que es concordante con los beneficios esperados derivados de la heterosis en el poliploide. Por otro lado, considerando caracteres fisiológicos, se muestra que la respuesta funcional a la sequía de *B. hybridum* está correlacionada con uno de sus ancestros, *B. stacei*, lo que coincide con la evolución adaptativa post-polipoidización del híbrido. También se encontró que las poblaciones ibéricas y naturales de *B. hybridum* han divergido fisiológica y genéticamente en respuesta a variaciones locales de aridez.

2. El análisis comparativo del transcriptoma de las tres especies del complejo *Brachypodium distachyon* nos muestra que *B. hybridum* exhibe una respuesta transcripcional a la sequía más fuerte que la de sus ancestros diploides, y que una fracción importante de DEG (genes diferencialmente expresados) regulados por la sequía se expresaron únicamente en *B. hybridum*. Entre la fracción de DEG compartidos por las tres especies se encuentran genes que codificadores de dehidrinas e involucrados en la biosíntesis de prolina. En el contexto del legado parental, la mayoría de los genes homeólogos en *B. hybridum* muestran un patrón de expresión en

condiciones control semejante al de *B. stacei* (*B. stacei* expression level dominance), mientras que en estrés los dos patrones de expresión más frecuentes en *B. hybridum* son un patrón semejante al observado en el ancestro *B. distachyon* (*B. distachyon* expression-level dominance) y un patrón transgresivo. Por tanto, contrariamente a los resultados obtenidos en la respuesta funcional, la habilidad de *B. hybridum* de tolerar un amplio rango de aridez no es el resultado de los homeólogos heredados de *B. stacei*. Por otra parte, más de la mitad de los pares de genes homeólogos expresados en el híbrido conservaron la misma expresión que en los parentales tanto en condiciones control como en condiciones estrés.

3. Los análisis funcionales del gen *BdDHN3* realizados en plantas transgénicas indican que éstas tiene un papel importante en la tolerancia a la sequía en la especie. Los resultados indican que la sobreexpresión de *BdDHN3* en plantas transgénicas bajo condiciones de sequía está ligada a la expresión de tres caracteres típicamente relacionados con la tolerancia a la sequía como son un elevado contenido en agua de la hoja, bajos niveles de daño celular y alto contenido en prolina bajo condiciones de estrés.

4. Los análisis de la respuesta funcional al estrés por herbivoría en el complejo *B. distachyon* indican que *B. stacei* es la especie menos resistente mientras que *B. distachyon* y *B. hybridum* fueron más resistentes al daño. Caracteres como el tamaño de la planta y el contenido en sílice son caracteres candidatos que mejoran la resistencia a la herbivoría por insectos. Por otro lado, *B. distachyon* mostró una mayor tolerancia a la herbivoría que *B. stacei* y *B. hybridum* siendo el SLA el carácter asociado a la tolerancia al daño. Desde el punto de vista evolutivo, nuestros resultados muestran el potencial de los saltamontes como agentes de selección de genotipos y de caracteres de respuesta a la herbivoría, promoviendo así la diferenciación

en las poblaciones de *B. hybridum*. En el contexto de la poliploidía, nuestros resultados no indican que la poliploidía mejore la resistencia a la herbivoría en el complejo *B. distachyon*. Finalmente, la herbivoría podría promover la coexistencia local de especies del complejo Brachypodium, actuando como un factor estabilizador frente a la exclusión por competencia.

CONCLUSIONS

The main results of this study could be summarized in the following conclusions:

1. The analysis of drought-functional response in the *Brachypodium distachyon* complex shows that this response is well differentiated between the species that form the complex. Considering the analysis of the phytohormones content, the drought-functional response of *B. hybridum,* compared to that of its two ancestral diploid species, is transgressive, which is consistent with the expected benefits derived from heterosis in the polyploid. On the other hand, considering physiological characters, the drought-functional response of *B. hybridum* is correlated with one of its ancestors, *B. stacei*, which coincides with the adaptive post-polypoidization evolution of the hybrid. It also found that the Iberian and natural populations of *B. hybridum* have diverged physiologically and genetically in response to local variations of aridity.

2. The transcriptome comparative analysis in the three species of the *Brachypodium distachyon* complex shows that *B. hybridum* exhibits a stronger drought-transcriptional response compared to its diploid ancestors and that a significant fraction of DEG (differentially expressed genes) regulated by drought were expressed only in *B. hybridum*. Among the fraction of DEG shared by the three species, genes that encode dehydrins and involved in proline biosynthesis were found. In the context of the parental legacy, most of the homoeolog genes in *B. hybridum* show an expression pattern similar to that of *B. stacei* (*B. stacei* expression level dominance) in control conditions, while two most common expression patterns in *B. hybridum* are *B. distachyon* expression-level dominance and a

transgressive pattern under stress. Contrarily to our expectations, *B. hybridum* ability to tolerate a broader range of aridity did not result from massively differential use of *B. stacei*-inherited homeologs. On the other hand, expression patterns of more than half of all expressed homoeolog pairs in the diploid parents were conserved in the allopolyploid both at constitutive level and under drought, suggesting overall cis-regulatory divergence between the diploid progenitors.

3. The functional analysis of *BdDHN3* in transgenic plants indicates that these dehydrins have an important role in drought tolerance in the species. The results indicate that the overexpression of *BdDHN3* in transgenic plants under drought conditions is linked to the expression of three traits typically related to drought tolerance such as high leaf water content, low levels of cell damage, and high content in proline under stress conditions.

4. The functional response analysis to herbivory stress in the *B. distachyon* complex indicates that *B. stacei* is the least resistant species while *B. distachyon* and *B. hybridum* were more resistant to damage. Plant size and silica content are candidate traits that improve resistance to insect herbivory. On the other hand, *B. distachyon* showed a greater tolerance to herbivory than *B. stacei* and *B. hybridum*. Differences in tolerance to damage trended to be linked negatively to SLA variation. From the evolutionary point of view, our results show the potential of grasshoppers as agents of genotype selection and herbivory-response traits, promoting population differentiation in *B. hybridum*. In the context of polyploidy, our results do not indicate that polyploidy improves herbivory resistance in the *B. distachyon* complex. Herbivory could be acting as staying factor against

competitive exclusion, allowing local species coexistence on this species complex.

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OTRAS APORTACIONES DE LA DOCTORANDA

Se ha solicitado la protección de dos líneas vegetales de *Brachypodium hybridum*, “BHJCIM” y “BHJHIN”, como fruto del trabajo de dicha tesis desarrollada en base al proyecto de investigación “Selección natural y significado adaptativo de genes candidatos asociados a la tolerancia al estrés hídrico en *Brachypodium distachyon* (Poaceae)” con referencia CGL2012-30838 y financiado por el Ministerio de Economía y Competitividad. Dichas líneas se proponen patentar para su uso como material vegetal en la restauración y mantenimiento de la cobertura vegetal del olivar ya que fueron seleccionadas por su floración temprana y resistencia a la sequía en los trabajos de esta tesis.

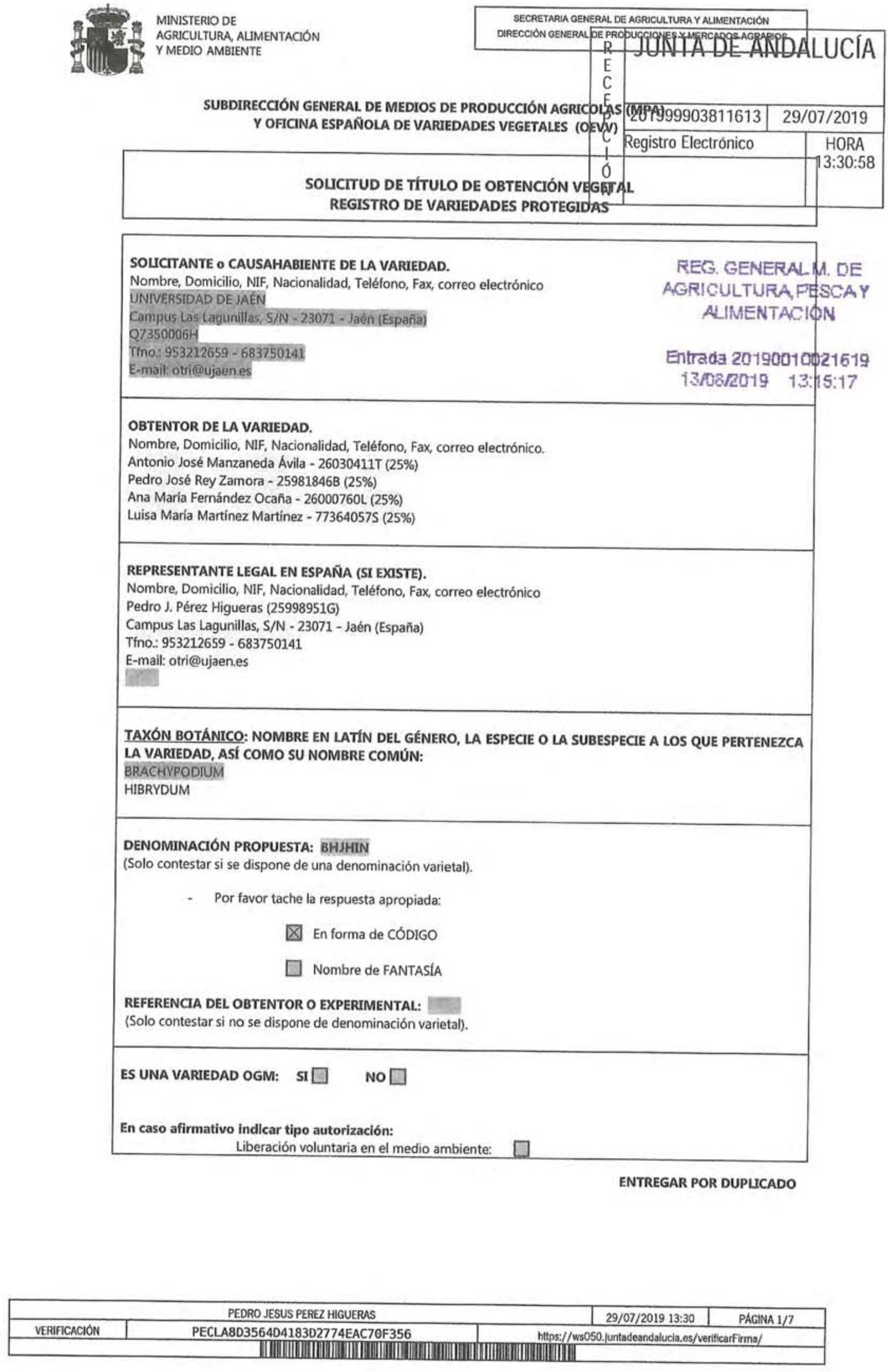
Las solicitudes de patente para ambas líneas han sido presentadas a la Consejería de Agricultura, Ganadería, Pesca y Alimentación de la Junta de Andalucía que se está encargando de gestionar ante la Oficina Española de Variedades Vegetales (OEVV) este primer trámite de la solicitud. Actualmente se están generando en nuestro laboratorio las semillas de ambas líneas que serán entregadas al Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) para que lleven a cabo los diferentes ensayos técnicos en campo. A continuación se muestran los documentos que acreditan la solicitud de la patente de ambas líneas.

INFORMACIÓN SUPLEMENTARIA INTRODUCCIÓN Y METODOLOGÍA GENERAL

Tabla 1. Origen geográfico de las líneas (inbred) usadas en los experimentos y ensayos de este trabajo de tesis doctoral.

Species	Accession	Population	Geographical coordinates	Annual rainfall (mm)	Annual temperature (°C)	Origin
B. distachyon	Bd21	Mosul	36° 20' 24” N 43° 7' 48” E	622	14	Iraq (USDA)
	Bd30-1	Sierra Nevada	36º 59' 43" N 3º 34' 18 W	440	10	Spain (USDA)
	SCA-5, SCA-7, SCA-8, SCA-9, SCA-10	Sierra Nevada	36º 59' 43" N 3º 34' 18 W	440	10	Spain (UJAEN)
	CANU-4, CANU-13 CANU-14, CANU-15	Algeciras	36º 08' 37" N 5º 30' 43" W	968	17	Spain (UJAEN)
	CER-2,CER-4.CER-5 CER-6,CER-16,CER-18	Cervera	42º 49' 32" N 4º 29' 27" W	930	9	Spain (UJAEN)
	JHIN-10, JHIN-13	Hinojares	37º 43' 41" N 2º 58' 52" W	725	11	Spain (UJAEN)
	VILLA-3, VILLA-4, VILLA-5, VILLA-6, VILLA-7, VILLA-13	Villatoya	39º 20' 32" N 1º 20' 47" W	397	16	Spain (UJAEN)
	RON-2, RON-3, RON-5 RON-7, RON-11 RON-13	Roncal	42º 46' 50" N 0º 57' 48" W	1102	11	Spain (UJAEN)
	GRAZ-1, GRAZ-5, GRAZ-7, GRAZ-10, GRAZ-16, GRAZ-20	Grazalema	36º 45' 21" N 5º 26' 30" W	1773	16	Spain (UJAEN)
	CASIB-1,CASIB-8, CASIB-10, CASIB-12, CASIB-14, CASIB-15	Casas Ibañez	39º 16' 09" N 1º 29' 22" W	409	13	Spain (UJAEN)
	SOBRE-2, SOBRE-6, SOBRE-7, SOBRE-8, SOBRE-18, SOBRE-19	Sobrado	42º 31' 54" N 6º 51' 7" W	1008	12	Spain (UJAEN)
	VISC-4, VISC-8,	Íscar	41º 22' 12" N	468	12	Spain

	VISC-13, VISC-14		4º 32' 18" W			(UJAEN)
B. stacei	ALTAB-1, ALTAB-3, ALTAB-5, ALTAB-12, ALTAB-15	Tabernas	37º 02' 28" N 2º 24' 22" W	298	16	Spain (UJAEN)
	CGAT-4, CGAT-6	Cabo de Gata	36º 43' 51" N 2º 12' 4" W	311	19	Spain (UJAEN)
	ALSUR-1, ALSUR-2, ALSUR-4	Sorbas	37º 05' 79" N 2º 06' 35" W	342	18	Spain (UJAEN)
B. hybridum	JHIN-8, JHIN-9, JHIN-11, JHIN-16, JHIN-18, JHIN-19	Hinojares	37º 43' 41" N 2º 58' 52" W	725	11	Spain (UJAEN)
	COPRE-4, COPRE-8, COPRE-9, COPRE-12, COPRE-13, COPRE-20	Los Pedroches	38º 36' 38" N 5º 9' 3" W	542	16	Spain (UJAEN)
	FARO-1, FARO-2, FARO-3, FARO-4, FARO-5, FARO-6	Faro	37º 0' 52" N 7º 58' 35" W	776	16	Portugal (UJAEN)
	MON-1, MON-3, MON-10, MON-13, MON-14	Monfrague	39º 49' 20" N 6º 02' 52" W	807	18	Spain (UJAEN)
	RUI-1, RUI-9, RUI-10, RUI-11	Ruidera	38º 57' 42 N 2º 52' 17" W	433	14	Spain (UJAEN)
	JODAR-3, JODAR-6, JODAR-7, JODAR-8, JODAR-10, JODAR-19	Jodar	37º 48' 53" N 3º 19' 09" W	302	14	Spain (UJAEN)
	LARVA-1, LARVA-3, LARVA-8, LARVA-15, LARVA-18, LARVA-20	Larva	37º 48' 38" N 3º 12' 53" W	500	14	Spain (UJAEN)
	MONE-2,MONE-3,MONE-4 MONE-5,MONE-6	Monegros	41º 30' 50" N 0º 30' 30" W	349	15	Spain (UJAEN)
	ROS-3,ROS-6,ROS-7,ROS-9 ROS-10	Rosas de Mar	42º 14' 45" N 3º 11' 1" E	591	14	Spain (UJAEN)
	JCIM-7, JCIM-9, JCIM-16 JCIM-18, JCIM-19, JCIM-20	Cimbarra	38º 23' 28" N 3º 22' 13" W	509	14	Spain (UJAEN)



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Antonio José Manzaneda Ávila - 26030411T (25%)
Pedro José Rey Zamora - 25981846B (25%)
Ana María Fernández Ocaña - 26000760L (25%)
Luisa María Martínez Martínez - 77364057S (25%)

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TAXÓN BOTÁNICO: NOMBRE EN LATÍN DEL GÉNERO, LA ESPECIE O LA SUBESPECIE A LOS QUE PERTENEZCA LA VARIEDAD, ASÍ COMO SU NOMBRE COMÚN:
BRACHYPODIUM
HIBRYDUM

DENOMINACIÓN PROPUESTA: BHJHIN
(Solo contestar si se dispone de una denominación varietal).

- Por favor tache la respuesta apropiada:

☒ En forma de CÓDIGO

☐ Nombre de FANTASÍA

REFERENCIA DEL OBTENTOR O EXPERIMENTAL:
(Solo contestar si no se dispone de denominación varietal).

ES UNA VARIEDAD OGM: SI ☐ **NO** ☐

En caso afirmativo indicar tipo autorización:
Liberación voluntaria en el medio ambiente: ☐

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TAXÓN BOTÁNICO: NOMBRE EN LATÍN DEL GÉNERO, LA ESPECIE O LA SUBESPECIE A LOS QUE PERTENEZCA LA VARIEDAD, ASÍ COMO SU NOMBRE COMÚN:
BRACHYPODIUM
HIBRYDUM

DENOMINACIÓN PROPUESTA: **BHJCIM**
(Solo contestar si se dispone de una denominación varietal).

- Por favor tache la respuesta apropiada:

☒ En forma de CÓDIGO

☐ Nombre de FANTASÍA

REFERENCIA DEL OBTENTOR O EXPERIMENTAL:
(Solo contestar si no se dispone de denominación varietal).

ES UNA VARIEDAD OGM: **SI** ☐ **NO** ☐

En caso afirmativo indicar tipo autorización:
Liberación voluntaria en el medio ambiente: ☐

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