

Invited Synthesis Paper - Artículo de Síntesis por Invitación

The use of sunflower transcription factors as biotechnological tools to improve yield and stress tolerance in crops

El uso de factores de transcripción en girasol como herramienta biotecnológica para incrementar el rendimiento y la tolerancia al estrés de los cultivos

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Abstract. Transcription factors (TFs) are proteins able to specifically recognize DNA sequences in the regulatory regions of their target genes. They bind these specific sequences, an event that leads to the activation or repression of whole signal transduction pathways. In plants about 1500 TFs were informatically identified; identification was mainly based in the presence of DNA-binding domains in the translated sequences. They were classified in families and subfamilies according to several features, including the conservation of the DNA binding domain, the genes structures and the functions they exert. Among transcription factors, several seem to be potential powerful biotechnological tools to improve crops via obtaining transgenic plants. Assigned purposes include: yield improvements, abiotic and biotic stress tolerances, and a combination of them. None of them is up to date a product market, since from the gene discovery to the regulation process (which differs in each country) there is a long pipeline to run.

Since a few years ago, our research group is devoted to the structural and functional characterization of sunflower transcription factors, especially those belonging to the HD-Zip family. Members of this family exhibit in their structure a homeodomain (HD) associated to a leucine zipper (LZ). This association is unique to plants, being these two domains common to transcription factors from several kingdoms. It was proposed by other authors, and also by us, that this unique association is probably due to specific plant responses as plant development, which depends on environmental conditions.

In this work we show an overview and new insights of the sunflower HD-Zip proteins, demonstrating to be useful biotechnological tools to confer drought, salt, herbicide, and herbivory (e.g. insect) tolerance, and other abiotic and biotic tolerance to stress-generating factors.

Like other TFs, sunflower HD-Zip transcription factors confer a complex phenotype to transgenic plants due to a combination of different regulated pathways. It is likely that TFs become the choice for breeders in the near future due to the demonstrated efficiency

Resumen. Los factores de transcripción (FTs) son proteínas capaces de reconocer y unir en forma específica secuencias de ADN presentes en las regiones regulatorias de los genes que resultan ser sus blancos. Cuando los FTs unen estas secuencias se produce la activación o la represión, según el caso, de vías de transducción de señales completas. En plantas cuyo genoma se conoce se han identificado con la ayuda de herramientas informáticas y en base a la presencia de dominios conservados de unión a ADN unos 1500 FTs. Estos fueron clasificados en familias y subfamilias de acuerdo a una serie de características estructurales y funcionales. Algunos de estos FTs podrían convertirse en poderosas herramientas biotecnológicas para el mejoramiento de cultivos de interés agronómico vía transgénesis. Los usos posibles serían, entre otros, el aumento de los rendimientos y la tolerancia a distintos tipos de estrés tanto de origen abiótico como biológico. Sin embargo, ninguno de estos FTs es hasta ahora un producto de mercado y esto es debido a que desde el descubrimiento de la funcionalidad de un gen hasta llegar a los procesos de regulación de transgénicos, que varían en los diferentes países, existen muchas etapas y pruebas a realizar que toman su correspondiente tiempo. Nuestro grupo de investigación se dedica desde hace años a la caracterización funcional de FTs de girasol, especialmente a aquellos que pertenecen a la familia HD-Zip. Los FTs de esta familia se caracterizan por la presencia en su estructura de un homeodominio (HD) asociado a un cierre de leucinas (LZ, del inglés "leucine zipper"). Si bien estos dos dominios existen en FTs pertenecientes a organismos de otros reinos, la asociación de ambos en una única proteína es una particularidad del reino vegetal y por eso se ha postulado que este tipo de FTs intervendría en procesos o eventos exclusivos de las plantas como el desarrollo dependiente de las condiciones ambientales. En este trabajo se muestra un panorama general, así como algunos nuevos aspectos novedosos inherentes a las funciones de las proteínas HD-Zip de girasol. Estas proteínas presentan un potencial biotecnológico relevante para conferir a los cultivos tolerancia a estrés hídrico, altas concentraciones de sales, herbicidas, ataque de insectos y otros factores generadores de estrés. En forma similar a lo que sucede con otros FTs que se expresan como transgenes, las proteínas HD-Zip confieren fenotipos complejos. Estos fenotipos complejos se dan por la

in conferring desired traits to transgenic plants. Additionally, from the public perception standpoint, the over or ectopic expression of a plant gene should be more accepted than the use of molecules from foreign organisms.

Key words: sunflower, transcription factors, HD-Zip, homeodomain, leucine zipper, abiotic stress, biotic stress.

Plants respond to environmental stresses by triggering complex molecular mechanisms

Plants are sessile organisms subjected to a variety of environmental factors that continuously influence their development and production. They evolved to trigger different and complex defence mechanisms which allow them to survive under adverse conditions during variable periods of time. External factors may be either abiotic (i.e., drought, wind, extreme temperatures, soil salinity and toxicity) or biotic (i.e., pathogen infections, herbivory). Plant responses involve the activation and repression of certain genes following a fine regulation program that is ultimately written in the linear DNA sequence. Such activation and repression of genes generate the synthesis of specialized proteins, enzymes and metabolites that together constitute the defence response. Stress tolerance, and thus resistance, seems to be controlled mostly at the transcriptional level (Chen & Zhu, 2004), where the main players are proteins called transcription factors (also called *trans*-acting elements). Transcription factors are able to enhance or reduce the rate of transcription of their target genes. They specifically recognize and interact with DNA sequences (*cis*-acting elements) located in the regulatory regions of their targets.

Plant Transcription Factors

It has been estimated that *Arabidopsis* and rice have between 1300 and 1500 transcription factor encoding genes (Riechmann et al., 2000; Goff et al., 2002). Some of them have been identified as stress responsive. Each of these stress-related transcription factor families exhibit a distinctive DNA binding domain, such as NAC, ERF/AP2, Zn-finger, DOF, Myb, WRKY, b-Zip and HD-Zip (Riechmann & Ratcliffe, 2000). The transcriptome comparison of plants under alternative stress treatments, and even those including the combination of different stresses, shed light on the functional basis of multiple stress tolerance (Mittler, 2006; Weiste et al., 2007).

Although transcriptome and additional analyses indicate that the expression of a certain transcription factor is regulated by one or more external conditions, this does not imply that the TF is able to confer tolerance (and thus, resistance) to these conditions. It must be considered that TFs (1) compose numerous gene families, and (2) could be involved in plant responses without necessarily conferring stress tolerance. A series of functional genomics experiments must be performed

regulación positiva o negativa de varias vías de transducción de señales al unísono. Creemos que en un futuro cercano los FTs van a ser los transgenes de elección para los mejoradores ya que han demostrado ser muy eficientes para generar características deseables en plantas. Además, para la percepción pública sería más aceptable sobre-expresar un gen vegetal que introducir en las plantas un gen proveniente de otros reinos.

Palabras clave: girasol, factores de transcripción, HD-Zip, homeodominio, cierre de leucinas, estrés abiótico, estrés biótico.

to test and demonstrate such effect. Functional genomics experiments include (1) obtaining transgenic plants where the tested TF is ectopically or over-expressed, and (2) a deep analysis of these plants under different environmental conditions. This functional and individual analysis of each TF revealed that some of them could be powerful biotechnological tools to improve agronomic crops via obtaining transgenic plants or as molecular markers. None of these TFs became up to date a market product. This is because time-consuming experiments and regulation permits are required to arrive to such point (Arce et al., 2008).

The HD-Zip family

Homeodomain-leucine zipper (HD-Zip) proteins constitute one of the homeodomain-containing transcription factor families. These proteins are characterized by the presence of a specific DNA-binding domain, the HD, associated to an adjacent dimerization motif, the leucine zipper (LZ). Both, HDs and LZs are common motifs present by themselves in other eukaryotic kingdoms, such as animals or fungi. However, the association of these two motifs in a single transcription factor is apparently a unique characteristic to plants. HD-Zip proteins can be classified into four subfamilies according to the conservation within the conforming domains, their target DNA sequences, gene structures and the roles they exert (Chan et al., 1998).

Studies in which HD-Zip I and II genes were over or ectopically expressed in transgenic plants support the role of these proteins as developmental regulators that are responsive to environmental conditions (Ariel et al., 2007).

Since a few years, our research group is devoted to the functional characterization of sunflower members of the HD-Zip family and other transcription factors from different species, including those where genomic tools are rather limited.

Methodology used to functionally characterize sunflower transcription factors

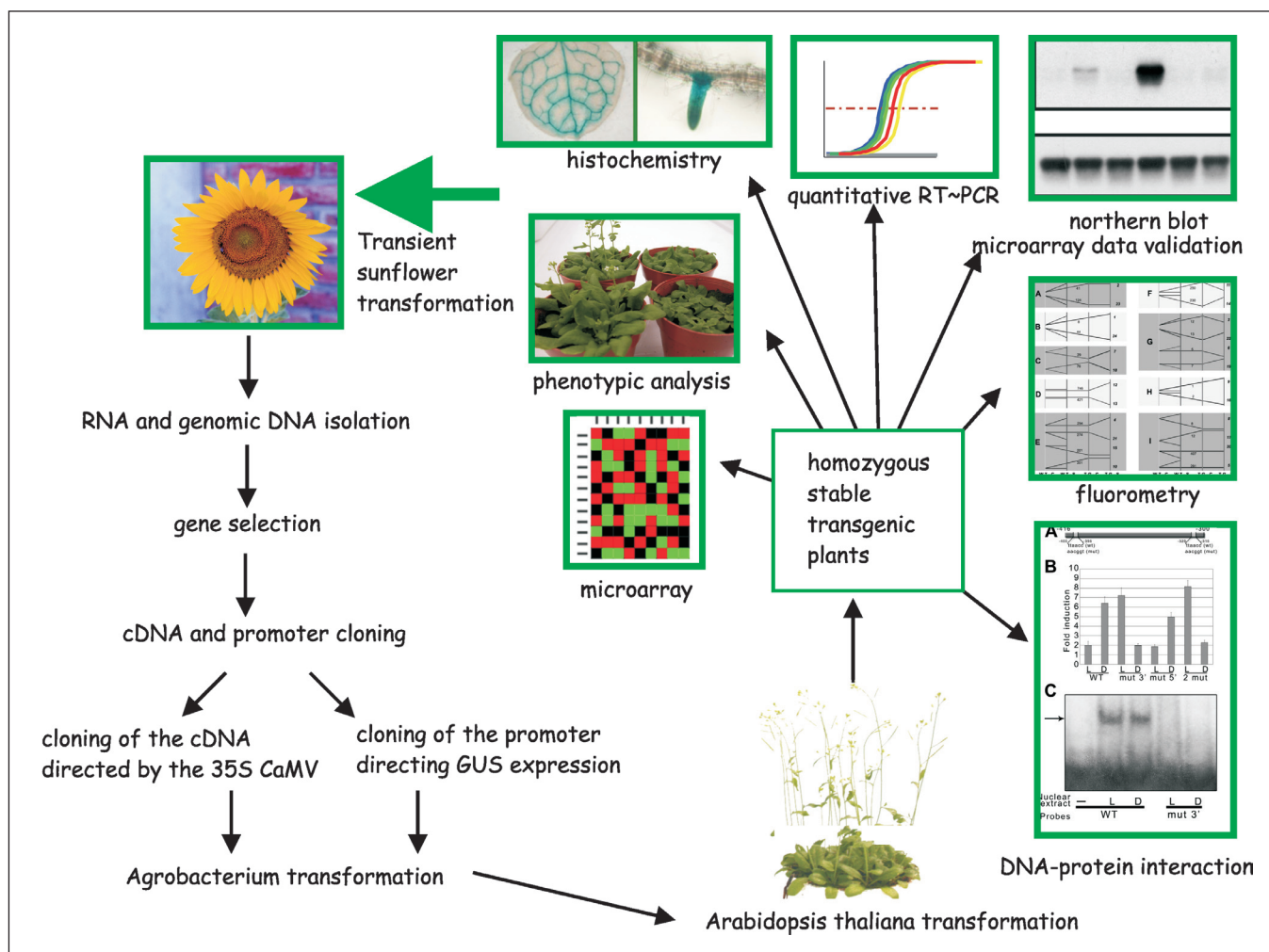
Sunflower, like many crops, is not easily manipulated. Hence, the use of sunflower genes and heterologous systems can be particularly useful to provide clues to the mechanisms where these genes operate. We succeeded in using the information obtained from *Arabidopsis* to demonstrate the existence of conserved mechanisms in sunflower. The methodology used is schematized in Figure 1. It briefly shows that the

Fig 1. Schematic representation of the methodology used to perform the functional characterization of sunflower transcription factors. The schema shows that the pipeline to characterize sunflower transcription factors, a species whose genome is practically unknown, includes (1) the separate isolation of the cDNA and the promoter region of the selected gene, (2) the cloning of the cDNA in a suitable vector to transform *Arabidopsis* plants (under the control of a constitutive promoter such as the 35S of CaMV) and cloning the promoter region directing the expression of a reporter gene (such as *GUS*), (3) obtaining homozygous transgenic *Arabidopsis* plants and (4) a detailed analysis of these plants using a series of techniques (phenotypic analysis, expression analysis, microarrays, histochemistry, EMSAs, etc.). Finally, the information obtained in the heterologous system (*Arabidopsis*) helps to investigate the homologous one by transient transformation of sunflower leaves and molecular analysis of transformed leaf disks. This final analysis allows determining if certain pathways are conserved at the molecular level.

Fig 1. Representación esquemática de la metodología utilizada para caracterizar funcionalmente factores de transcripción de girasol.

El esquema muestra los pasos a seguir para caracterizar factores de transcripción de girasol, una especie cuyo genoma es prácticamente desconocido. Estos pasos incluyen: 1) el aislamiento del ADN copia y de la región promotora del gen elegido; 2) el clonado del ADN copia en un vector adecuado para la transformación de plantas de *Arabidopsis* bajo el control de un promotor constitutivo como el 35S del virus del mosaico de la coliflor y el clonado de la región promotora dirigiendo la expresión de un gen reportero como el *GUS*; 3) la obtención de plantas transgénicas de *Arabidopsis* homocigotas; 4) un análisis detallado de esas plantas utilizando una serie de técnicas que incluyen la caracterización fenotípica, estudios de expresión, ensayos de microarreglos, histoquímica, geles de retardo, etc. Finalmente, la obtención obtenida en el sistema heterólogo (*Arabidopsis*) ayuda a investigar el homólogo aplicando transformación transitoria de hojas de girasol seguida del análisis molecular de los discos transformados. Este análisis final permite determinar si ciertas vías de transducción de señales están conservadas entre especies a nivel molecular.

Adaptado de Manavella, P. A. (2008). "El factor de transcripción de HAHB4 modula la comunicación entre distintas vías de respuesta a factores bióticos y abióticos en plantas de girasol". Tesis Doctoral, Universidad Nacional del Litoral.



pipeline to characterize transcription factors includes (1) the isolation of the cDNA and the promoter region of the selected gene, (2) the cloning of the cDNA in a suitable vector to transform *Arabidopsis* plants (under the control of a constitutive promoter such as the 35S of CaMV) and cloning the pro-

motor region directing the expression of a reporter gene (such as *GUS*), (3) obtaining homozygous transgenic plants, and (4) a detailed analysis of these plants using a series of techniques (phenotypic analysis, expression analysis, microarrays, histochemistry, EMSAs, etc.). Finally, the information obtained in

the heterologous system helps to investigate the homologous one by transient transformation of sunflower leaves (Manavella & Chan, 2009). This methodology allowed us to characterize a set of sunflower transcription factors belonging to the HD-Zip family. The obtained results were already published, and we continue our research work with several transcription factors from this and other families from sunflower, *Arabidopsis* and *Medicago truncatula* (Dezar et al., 2005a; Dezar et al., 2005b; Rueda et al., 2005; Manavella et al., 2006; Cabello et al., 2007; Manavella et al., 2008a; Manavella et al., 2008b; Manavella et al., 2008c).

Summary of results

Applying these techniques, we determined that the sunflower *HAHB4* HD-Zip protein conferred drought tolerance to transgenic plants, and that it was a component of the ethylene signaling pathway which induced a marked delay in senescence (Dezar et al., 2005a; Manavella et al., 2006). Transgenic plants ectopically expressing *HAHB4* were less sensitive to external ethylene, entering the senescence pathway later, and did not show the typical ethylene-induced triple response of dark-grown seedlings. These effects are illustrated in Figure 2.

Fig. 2. Transgenic plants bearing the construct *35S:HAHB4* are more tolerant to water stress and enter the senescence step later.

A: four-week-old *Arabidopsis* plants subjected to severe water deficit. On the left: WT plants, and on the right, transgenic plants. The photograph was taken 2 days after re-watering.

B: seven-day-old *Arabidopsis* plants. From left to right: WT plants, transgenic plants bearing the constitutive construct *35S:HAHB4*; transgenic plants bearing the inducible construct *promHAHB4:HAHB4*.

Fig. 2. Plantas transformadas con la construcción *35S:HAHB4* son más tolerantes al estrés generado por sequía y entran tardíamente en la etapa de senescencia.

A: plantas de *Arabidopsis* de 4 semanas de edad sometidas a estrés hídrico severo. A la izquierda: plantas salvajes y a la derecha, plantas transgénicas. La fotografía fue tomada dos días después de regar.

B: plantas de *Arabidopsis* de siete días. Desde la izquierda hacia la derecha: salvajes, transgénicas con la construcción constitutiva *35S:HAHB4*; transgénicas con la construcción inducible *promotorHAHB4:HAHB4*.

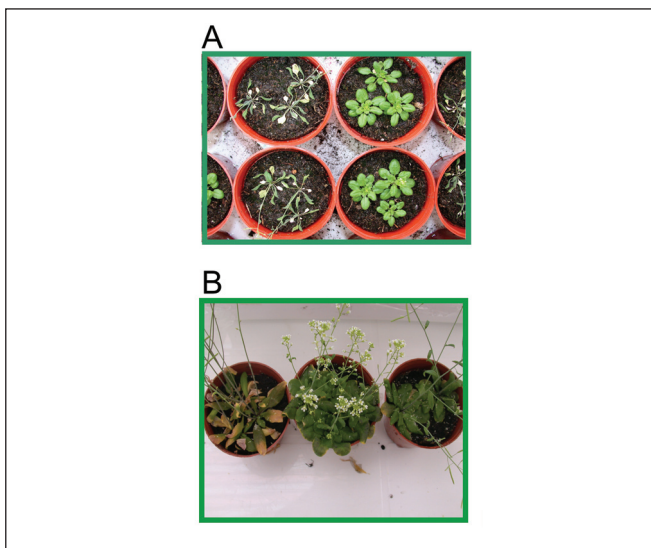


Fig. 3. Transgenic plants bearing the construct *35S:HAHB4* are more tolerant to insect defoliation than their WT counterparts.

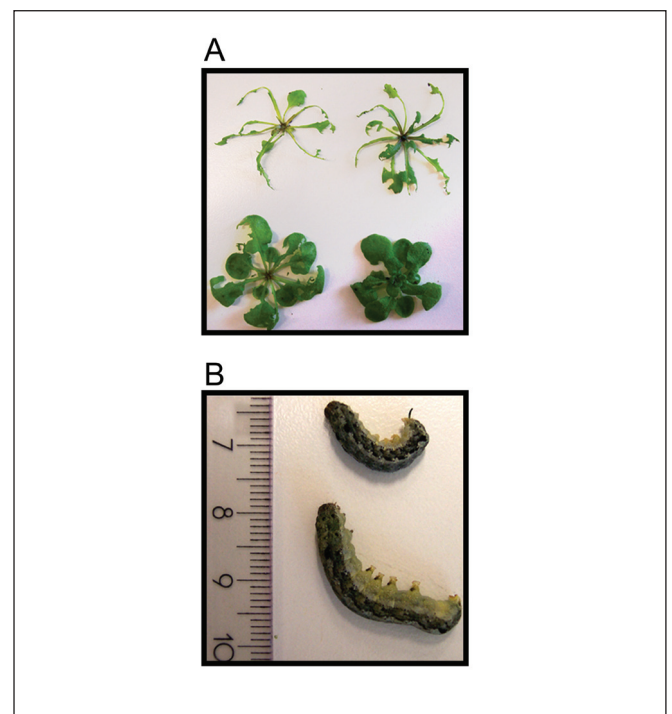
A: upper panel: WT plants defoliated by *Bradisia impatiens*; lower panel: transgenic plants defoliated by the same herbivore for an equal period of time in the same plate.

B: insects fed exclusively with: upper panel: transgenic plants (construct *35S:HAHB4*); lower panel: WT plants.

Fig. 3. Las plantas transformadas con la construcción *35S:HAHB4* son más tolerantes al ataque de insectos que sus pares salvajes.

A: panel superior: plantas salvajes atacadas por *Bradisia impatiens*; panel inferior: plantas transgénicas atacadas por el mismo herbívoro durante un período de tiempo igual en idénticas condiciones.

B: insectos alimentados exclusivamente con plantas transgénicas (panel superior) o plantas salvajes (panel inferior).



Potential targets of *HAHB4* were identified by comparing the transcriptome of transformed and wild-type plants using micro arrays and quantitative RT-PCR. Expression of this TF had a major repressive effect on genes related to ethylene synthesis. Accordingly, expression studies in sunflower indicated that transcript levels of this gene were elevated in mature/senescent leaves in relation to ethylene action. Transient transformation of sunflower leaves demonstrated the action of *HAHB4* in the regulation of ethylene-related genes (Manavella et al., 2006).

Recently, we observed that transcript levels of this TF were strongly induced when sunflower plants were defoliated by herbivores, mechanically damaged or treated with methyl-jasmonic acid (MeJA). In *HAHB4* sunflower over-expressing tissue, increased activities of lipoxygenase, hydroperoxide lyase and trypsin inhibitors (TPI) were detected whereas in *HAHB4*-silenced tissue these activities were reduced. Transgenic *Arabidopsis thaliana* and *Zea mays* plants also exhibited higher transcript levels of de-

fense-related genes when (1) ectopically expressing *HABH4* and (2) *Spodoptera littoralis* or *Spodoptera frugiperda* larvae fed on each plant species, respectively; larvae both consumed and gained less mass when feeding on transgenic than control plants. Before and after wounding *Arabidopsis* plants ectopically expressing *HABH4* exhibited higher concentrations of JA, JA-isoleucine and ET compared to controls (Fig. 3 and Manavella et al., 2008c).

We conclude that *HABH4* coordinated phytohormone production during biotic stress responses and mechanical damage, specifically by positively regulating JA and ET production and negatively regulating ET sensitivity and SA accumulation.

HABH10, another transcription factor from this family (subfamily II), conferred tolerance to oxidative stress produced by paraquat to transgenic plants (Fig. 4). Transgenic *Arabidopsis* plants that expressed *HABH10* under the 35S cauliflower mosaic virus promoter showed a pronounced acceleration in development, reducing the life cycle about 25% due to a shortened flowering period. Differences in developmental rate between transgenic and non-transformed individuals increased when the number of plants per pot increased (Rueda et al., 2005). We observed the same phenotype when the constitutive promoter was replaced by the own gene promoter (Dezar et al., personal communication). In this case, the gene is increasingly regulated by SA, determining SA accumulation and a subsequent tolerance to pathogen infections.

Fig. 4. Transgenic plants bearing the construct *35S:HABH10* are more tolerant to paraquat treatments.

Upper panel: four-week-old, three independent transgenic lines (first, third and fourth columns) and WT plants treated with paraquat.

Lower panel: a more detailed picture taken in a similar experiment.

Fig. 4. Las plantas transformadas con la construcción *35S:HABH10* son más tolerantes a los tratamientos con paraquat.

Panel superior: tres líneas independientes de plantas transgénicas de cuatro semanas (primera, tercera y cuarta columna) y plantas salvajes tratadas con paraquat.

Panel inferior: una foto más detallada tomada de un experimento similar. Adaptado de Chan, R.L., D.H. Gonzalez, C.A. Dezar y E.C. Rueda. US20070234439 (2007).



Like other TFs, *HABH4* and *HABH10* conferred complex phenotypes to transgenic plants as a result of a combination of different regulated pathways. It is likely that these and other TFs become the choice for breeders in the near future, due to the efficiency in conferring desired traits to transgenic plants. Additionally, for the public perception standpoint, the over or ectopic expression of a plant gene should be more accepted than the use of molecules from foreign organisms.

Future perspectives

The knowledge about gene expression in response to biotic and abiotic stresses, and the way they participate in each mechanism, would be of great consequences in breeding and genetic manipulation programs. Identification of transcription factors, able to switch defense responses, will contribute to obtain potential biotechnological tools. In this sense, sunflower HD-Zip proteins appear to be good candidates to confer tolerance to a combination of stresses. Preliminary results from our research group indicated that drought and insect tolerance mechanisms mediated by *HABH4* are conserved in species like soybean, wheat and maize. These results were not as surprising for soybean, a C₃ dicot like sunflower and *Arabidopsis*, as they were for the monocots wheat (C₃) and maize (C₄). Physiological studies combined with molecular research will aid to better understand the system as a whole. Besides, regarding use of transgenic crops, it is of great importance to understand which additional mechanisms the transgenes may unchain, so as to guarantee the quality of the obtained product.

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