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Dehydrin-dehydrin interactions: evidence of homo and heterodimeric associations among acidic and basic dehydrins from Cactus pear and Arabidopsis

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Dr. Juan Francisco Jiménez Bremont

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Constancia de aprobación de la tesis

La tesis ***"Dehydrin-dehydrin interactions: evidence of homo and heterodimeric associations among acidic and basic dehydrins from Cactus pear and Arabidopsis"***, presentada para obtener el Grado de Doctora en Ciencias en Biología Molecular fue elaborada por Itzell Eurídice Hernández Sánchez y aprobada el **primer día de septiembre de dos mil diecisiete** por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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La presente investigación se realizó en el laboratorio de Biología Molecular de Hongos y Plantas de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C., bajo la dirección del Dr. Juan Francisco Jiménez Bremont.

Durante la realización del trabajo la autora recibió una beca académica del Consejo Nacional de Ciencia y Tecnología (No. de registro 423770) y del Instituto Potosino de Investigación Científica y Tecnológica, A. C

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DOCTORA EN CIENCIAS EN BIOLOGÍA MOLECULAR

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Itzell Eurídice Hernández Sánchez

sobre la Tesis intitulada:

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Dr. Juan Francisco Jiménez Bremont

El Jurado, después de deliberar, determinó

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Dedicatorias

A mis padres por darme el ejemplo más grande de amor, compromiso y entrega, a Lili por ser mi segunda madre, a mis hermanas por ser siempre un ejemplo de valor y fuerza, y a mi compañero de vida Israel por transmitir pasión a mi vida.

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Index

List of Figures

Resumen

Dehydrin-dehydrin interactions: evidence of homo and heterodimeric associations among acidic and basic dehydrins from Cactus pear and Arabidopsis

Las dehidrinas (DHNs) son proteínas específicas de plantas que pertenecen al grupo 2 de las LEA (Late Embriogenesis Abundant proteins), una familia grande de proteínas desordenadas, altamente hidrofílicas, que se acumulan durante las últimas etapas de la embriogénesis y en respuesta al estrés. A pesar de la importancia de estas proteínas en la respuesta al estrés, su mecanismo de acción molecular aún es desconocido. Se requieren estudios *in vivo* para esclarecer los mecanismos de acción de estas proteínas. Previamente, en nuestro laboratorio se aisló una DHN de nopal (OpsDHN1) a partir de una biblioteca de cDNA. Posteriormente se demostró que líneas transgénicas de *Arabidopsis thaliana* que expresan la OpsDHN1 son tolerantes a congelamiento. En el presente trabajo se realizó una caracterización bioquímica de la proteína OpsDHN1, en donde se determinó el estado desordenado de esta DHN y se estableció su actividad crioprotectora *in vitro*. Además, se reportó la formación de homodímeros de la OpsDHN1 utilizando el sistema de dos híbridos "split-ubiquitin" en levadura (Y2H) y cromatografía de exclusión de tamaño (SEC). La homodimerización fue confirmada *in planta* mediante complementación bimolecular de Fluorescencia (BiFC). Nuestros resultados revelaron la formación de dímeros *in situ* con una localización dual citoplasmática/nuclear, en células de tabaco. Además, se demostró que la ausencia del motivo rico en histidina y el segmento S excluyen a la proteína OpsDHN1 del núcleo de las células vegetales. Finalmente, proporcionamos pruebas sólidas de que la interacción DHN/DHN no es exclusiva de la OpsDHN1. Nosotros demostramos la homo- y heterodimerización entre tres DHNs de *A. thaliana* RAB18, COR47 y ERD10, y a su vez con su ortólogo en *O. streptacantha* la OpsDHN1. Estos datos constituyen el primer reporte *in vivo* de la interacción entre DHNs dirigiendo el campo de la investigación DHNs hacia un nuevo escenario en donde las asociaciones entre estas proteínas pudieran ser exploradas como parte de su mecanismo de acción molecular. Palabras clave: *Arabidopsis thaliana, Opuntia streptacantha, dehidrinas, dimerización, localización subcelular.*

Abstract

Dehydrin-Dehydrin interactions: evidence of homo and heterodimeric associations among acidic and basic dehydrins from Cactus pear and Arabidopsis

Dehydrins (DHNs) are plant-specific proteins belonging to group 2 of the *Late Embryogenesis Abundant* proteins (LEAs), a large family of highly hydrophilic disordered proteins which accumulate during the later stages of embryogenesis and in response to abiotic stress. Despite the importance of these proteins in stress response, their molecular mechanism of action is still unknown, so *in vivo* studies will be determinant to establishing and clarifying their mechanisms. Previously we isolated, from a cDNA library, a DHN from Cactus pear (OpsDHN1). Subsequently, we reported that transgenic *Arabidopsis thaliana* plants overexpressing *Opuntia streptacantha* SK3 DHN OpsDHN1 show enhanced freezing tolerance. In this thesis project, a biochemical characterization of the OpsDHN1 protein was conducted. We determined that the OpsDHN1 protein is disordered and also established its cryoprotective activity. The dimeric state of OpsDHN1 was demonstrated using the two-hybrid system "split-ubiquitin" in yeast (Y2H) and size exclusion chromatography (SEC). Additionally, the homodimer formation was confirmed *in planta* by the bimolecular Fluorescence (BiFC) complementation approach. These data revealed *in vivo* dimer formation with dual cytoplasmic/nuclear localization in tobacco cells. In this regard, we showed that the histidine rich motif and to some degree the S-segment are involved in OpsDHN1 nuclear localization. Finally, we provide strong evidence that the DHN/DHN interaction is not exclusive to OpsDHN1. We demonstrated homo- and heterodimerization between three DHNs of *A. thaliana* RAB18, COR47 and ERD10, and with their OpsDHN1 orthologue from *O. streptacantha*. These data constitute the first *in vivo* report on the interaction among DHNs, and direct the field of DHN research towards a new scenario where the associations between these proteins could be explored as part of their molecular mechanism of action. Key words: *Arabidopsis thaliana, Opuntia streptacantha, dehydrins, dimerization, sub cellular localization*

I. Introduction

1.1 Abiotic Stress

The concept of stress in plants is defined by the occurrence of external factors or conditions that influence in a negative way their growth and development (Gerszberg and Konka, 2017). In particular, abiotic stress is the principal cause of yield loss in crops worldwide (Silva and Oliveira, 2014). In this regard, the FAO reports (2007) that 96.5% of the global land areas are affected by an abiotic stress factors. The abiotic stresses include low and high temperatures, drought, waterlogging, salinity, acidic conditions, light intensity, anaerobiosis and nutrient starvation (Meena et al., 2017). Additionally, in field conditions these stress factors are often associated with each other.

Plants as organisms of sessile nature have developed sophisticated physiological and molecular mechanisms to respond to stress conditions, which in some cases allow them to tolerate stress, avoid it or even recover after unfavorable conditions (Pandey et al., 2016). When plants sense any sign of stress, the membrane is the first layer where the signaling cascade is activated, from here, the signal transduction will activate the transcription factors and a set of protective genes, which are involved in damage repair, cellular protection, osmotic homeostasis, and/or ionic homeostasis (Da Silva and De Oliveira, 2014).

1.2 Intrinsically disordered proteins

Proteins that completely or partially lack a well-defined secondary structure in their native state are named intrinsically disordered proteins (IDPs), or proteins that contain intrinsically disordered regions (IDRs) (Uversky, 2013). These proteins have been gained interest since it was pointed out that more than 30% of the eukaryotic proteomes contain IDPs or have proteins with IDRs (IDPRs). The IDPs/IDRs display a low proportion of hydrophobic amino acids (Trp, Cys, Phe, Ile, Tyr, Val, Leu, His, Thr, and Asn), and a high content of charged hydrophilic residues that promotes disorder (Lys, Glu, Pro, Ser, Gln, Arg, Asp, and Met) (Uversky, 2013). This singular amino acid composition provides them with conformational plasticity to recognize and interact with

multiple partners (Pietrosemoli et al., 2013). The IDPs/IDRs are implicated in fundamental cellular

processes such as signaling cascades, transcription regulation, cell cycle control, chaperone activity, and stress response (Pazos et al., 2013).

In plants, the genetic plasticity is essential since as sessile organism they are not able to escape or avoid stress conditions. This plasticity is tightly related to IDPs/IDRs. In the model plant *Arabidopsis thaliana,* approximately 30% of its proteome has disordered proteins. However, other plant species have around 50% of their proteomes being disordered, such as *Zea mays* and *Glycine max* (Covarrubias et al., 2017). It has been shown that the plant IDPs/IDRs are particularly abundant in processes involved in stress response, such as DNA metabolism, RNA splicing and regulation of gene expression (Pietrosemoli et al., 2013; Covarrubias et al., 2017).

Some protein families contain a high number of IDPs that have evolved in order to maintain its disordered state as a functional characteristic, such as the transcription factors (TFs), signal transduction proteins (GRAS and CRY families) and Late Embryogenesis Abundant proteins (LEAs) (Sun et al., 2013).

1.3 Late Embryogenesis Abundant proteins

The maturation process of plant seed implies the loss of almost all the water content of the embryos (~90%). This process leads to water stress for the seed and at this stage, it is well known that Late Embryogenesis Abundant (LEA) proteins play a major role in the acquisition of desiccation tolerance, where LEA proteins could attain up to 4% of total cellular proteins (Gao and Lan, 2016). LEA proteins were described for the first time in wheat (*Triticum aestivum*) and cotton (*Gossypium hirsutum*) seeds during the late phase of embryogenesis. Later, these proteins were found in other plant species accumulating in vegetative tissues subjected to water deficit such as drought, salinity, osmotic, and extreme temperatures. These observations suggest that LEA proteins are involved in the plant stress response (Battaglia and Covarrubias, 2013). Abscisic acid (ABA) is a phytohormone that plays an important role in integration of stress signals and control downstream stress responses, in this sense it is reported that ABA can also

induce the expression of LEA genes in seeds and vegetative tissues (Ingram and Bartels, 1996; Tuteja, 2007).

Nevertheless, of the many LEA proteins that have been identified in plants and that at the beginning were considered to be exclusive to plants, particularly to the *Viridiplantae* clade (angiosperms, gymnosperms, nonvascular plants and algae), LEA-like proteins have been detected in anhydrobiotic invertebrates, protozoa, rotifers, nematodes and in bacteria (Shih et al., 2008). The presence of LEA proteins in these quite different organisms is always related to stress conditions but particularly with water scarcity (Covarrubias et al., 2017).

Most of the LEA proteins are rich in charged and uncharged polar amino acid residues, but hydrophobic residues are underrepresented in their sequences. This particular amino acid composition makes LEA proteins a highly hydrophilic and unstructured group. In fact, the LEA family has the largest number of IDP members reported (Battaglia and Covarrubias, 2013; Shih et al., 2008; Sun et al., 2013). Nonetheless, LEA proteins are not always disordered. Alternatively, these proteins can adopt some kind of structure under conditions that evoke dehydration. In some cases it has been observed that LEA proteins may form α-helices in the presence of membranes (Hincha and Thalhammer, 2012). The tendency to gain structure in presence of certain stimuli and/or their binding targets could be a common characteristic of the LEA group and indeed of IDPs (Tompa et al., 2005). The transition to an unfolded-folded state of LEA protein is intimately linked with their function and enables them to perform more than one function; this property is related to those classical “moonlighting" proteins (Hernández et al., 2015; Tompa et al., 2005). In addition the disordered nature of these proteins also facilitates the interaction with multiple partners (hub proteins), making them essential for protein interaction networks, and they also could function as integrators (molecular scaffolds) of environmental and developmental cues in plants (Cino et al., 2012; Cino et al., 2013).

1.3.1 *In vitro* and *in vivo* functions of LEA family

The multifunctional role of the LEA group arises mainly from *in vitro* experiments, where some functions are widespread through all groups, and others have been reported to be exclusive to a single group. The protection of a variety of enzymes, such as lysozyme, alcohol dehydrogenase, luciferase, citrate synthase and lactate dehydrogenase against freezing or dehydration treatments, and also prevention of protein aggregation and thus protein inactivation under these conditions have been demonstrated for almost all LEA groups (Group 1, 2, 3, 4, 5, and 7) of plant and invertebrate. (Goyal et al., 2005; Kovacs et al., 2008; Reyes et al. 2008; Zhang et al., 2014 Boucher et al., 2010;). Additionally, using circular dichroism (CD), nuclear magnetism resonance (NMR) and Fourier-transform IR (FTIR) it has been demonstrated that some LEA proteins can form amphipathic α-helices in the presence of membranes and stabilize them under stress conditions (Popova et al., 2011; Thalhammer et al., 2010; Hincha and Thalhammer, 2012); however, for some cases the membrane lipid composition is crucial for LEA protein binding (Clarke et al., 2015).

Other well-characterized functions for some LEA proteins include metal ion binding (Ca^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{2+}); it has been demonstrated that some of these ions are catalyzer of the formation of reactive oxygen species (ROS) (Hara et al., 2003; Hara et al., 2004). In this case, the LEA proteins could work as antioxidant proteins, preventing lipid peroxidation and a reduction electrolyte leakage induced by cold temperatures; these roles have been reported in the LEA group 2, group 4 and by analogy in the group 3 (Amara et al., 2014; Sun et al., 2013; Tunnacliffe and wise, 2007; Liu et al., 2011).

The *in vitro* studies from several genetic experiments reinforced the idea that LEA proteins function in stress tolerance protection; some LEA proteins (groups 2 and 3) from tomato, wheat, and barley have been demonstrated to confer increased tolerance to osmotic or freeze stresses when these were introduced into yeast cells (Zhang et al., 2000). Also, the barley LEA 3 group protein enhanced the tolerance to dehydration in transgenic rice and wheat (Sivamani et al., 2000). Additionally, Liu and Zheng (2005) have shown that PM2, a group 3 LEA protein from soybean, conferred tolerance to salt stress in *Escherichia coli* cells; also PM11, a LEA group 1 from soybean, enhanced the protection against both salt stress and cold stress in *E. coli* (Lan et al., 2005). However,

not all LEA protein overexpression lead to protection or enhanced tolerance, such as the case of Em a wheat LEA protein from group 1 whose expression in yeast did not lead to any tolerance against freezing (Swire-Clark and Marcotte, 1999). This suggests that maybe not all LEA proteins make a noteworthy contribution to enhance plant stress tolerance, or it could be that they need a particular background or partners to carry out their function inside cells (Hundertmark and Hincha, 2008).

1.3.2 Subcellular localization of LEA proteins

Our understanding of LEA biological function is intimately related to their subcellular localization; nevertheless, LEA groups are highly diverse both in protein sequence and in functions. For this reason, several *in vivo* and *in silico* attempts have been made in order to investigate the subcellular localization of a large number of currently known LEA proteins. In general LEA proteins are widely distributed in the cell, including the nucleus, cytoplasm, endoplasmic reticulum, chloroplast, vacuole, mitochondria, plastids, and indeed some of them have been reported to be secreted proteins (Tunnacliffe and wise, 2007; Candat et al., 2014). Nonetheless, some LEA proteins are confined to a cellular compartment such as LEA group 1, where all the members are predicted to be nuclear proteins, however, just a few cases have been demonstrated (Nair and Rost, 2004; Amara, 2012). The ubiquitous localization of these families gives evidence of their functional versatility.

1.3.3 Proposed mechanism of action for LEA proteins

Although LEA proteins were described a long time ago, nowadays we know that these are the multifunctional group of disordered proteins whose expression, accumulation and localization are intimately related to stress response and tolerance not only in plant species but also in other phyla; however, the molecular mechanism that defines how LEA proteins attain their biological functions is still an open question. Over years of investigation two proposed model of action for LEA proteins have emerged: the disordered chaperone (Tompa and Kovacs, 2010) and molecular shield (Tunnacliffe and Wise, 2007), both models are based on the hydrophilic nature of LEAs.

In the disordered chaperone model, unlike the classical chaperones, LEA can not folding a protein after it has been unfolded, the main function of these proteins is prevent denaturation. LEA proteins may protect their client proteins through an indirect interaction, here the hydrophilic nature of LEA proteins could act establishing hydrogen bonds between water bonded to LEA proteins and their target molecules, favoring folded state, also in this model LEA proteins could interact in a direct way with hydrophobic exposed patches of partially unfolded proteins to stabilize them (Tompa and Kovacs, 2010; Sun et al., 2013). The molecular shield model proposes that LEA proteins act as space fillers and as a water buffers allowing the establishment of a highly hydrated microenvironment around their targets, preventing denaturation and aggregation by crowding with other denatured proteins during stress conditions. Additionally electrostatic forces between the surfaces of LEA proteins and their clients could mediate these interactions (Tunnacliffe and Wise, 2007; Hughes and Graether, 2011). Along with other hydrophilic proteins and compatible solutes, LEA proteins might also serve as “space fillers” to prevent cellular collapse at low water activities.

1.4 LEA classification

Although LEA proteins have minimal sequence identity and do not share sequence or motif similarity with any other proteins of known function, they contain conserved motifs. Based on this conserved motif present in LEA sequences, these proteins have been classified into seven groups LEA 1-7 (Dure et al., 1989); however, this is not the only classification for this family reported in the literature (Dure et al., 1989; Battaglia et al., 2008). The Dure’s classification allows the recognition of characteristic motifs in each family. Typically, groups 1, 2, 3, 4, 6, and 7 comprise those hydrophilic LEA proteins, while those atypical or hydrophobic LEA proteins are grouped into class 5. Nevertheless, groups 1, 2 and 3 are considered the major or true LEA groups containing most members of the protein family. Here I will provide a brief description of each group.

1.4.1 Group 1

LEA-1 proteins (or also known as D-19 in Dure's classification) are highly hydrophilic and are predicted to be unstructured in aqueous solutions. These proteins share in common a hydrophilic 20-amino-acid motif (TRKEQ[L/M]G[T/E]EGY[Q/K]EMGRKGG[L/E]) that could be present up to four times in plant species and up to eight times in non-plant species such as in bacteria and crustaceans; two additional motifs have been described in plants: an N-terminal motif (TVVPGGTGGKSLEAQE[H/N]LAE) located upstream of the 20-mer, and a C-terminal motif (D[K/E]SGGERA[A/E][E/R]EGI[E/D]IDESK[F/Y] (Amara et al., 2014; Battaglia et al., 2008).

Group 1 also is rich in glycine (Gly, ~18-20%) and also shows highly significant overrepresentation of arginine (Arg) and glutamate (Glu). Indeed Glu and Gly amino acids can be found every ~20 residues; on the other hand, phenylalanine (Phe), tryptophan (Trp), isoleucine (Ile), leucine (Leu) and asparagine (Asn) are significantly underrepresented in this group (Tunnacliffe and Wise, 2007; Shih et al., 2015). Finally, this group of LEA proteins has been found to be principally accumulated in embryonic tissues, in dry seeds and in mature pollen grains (Bhardwaj et al., 2013).

1.4.2 Group 2 (dehydrins)

LEA 2 group was originally identified as the D-11 by Close (1996); commonly these proteins are known as dehydrins (DHNs, Close et al., 1989). DHNs sequences are enriched in polar and charged amino acids and display a low proportion of nonpolar, hydrophobic residues, which makes them thermo resistant to denaturation (Tunnacliffe et al. 2010). This is the best characterized group with more than 100 proteins described among angiosperms, gymnosperms, pteridophytes, bryophytes, algae, and cyanobacteria (Shih et al., 2015). DHNs are plant exclusive proteins characterized by the presence of three conserved segments: Y-segment, S-segment, and K-segment (Bhardwaj et al., 2013); these segments can be present in a variable number depending on the protein, however by rule at least one K-segment per sequence must be present. The presence of these segments are used to classify DHNs into 5 subgroups: Kn, KnS, SKn, YnSKn, YnKn (Campbell and Close, 1997). DHNs accumulate in vegetative tissues under stress conditions that evoke cellular dehydration; however, some of them

are constitutively expressed during all stages of plant development (Hundertmark and Hincha, 2008; Bhardwaj et al., 2013). Since this work is centered on DHNs the main characteristics and functions of this group will be described in detail below.

1.4.3 Group 3

Group 3 is the most diverse of the LEA protein family that could be distinguished by the presence of a repeating motif of 11 amino acids (ΦΦE/QXΦKE/QKΦXE/D/Q, where Φ represents hydrophobic residues). This motif has been proposed to be linked by salt bridges that could play important physiological roles (Dure 2001; Dure et al., 1989). *In silico* secondary structure

predictions of the 11-mer motif suggest that this segment exists principally as amphipathic α-helices, and could exist as dimers (Battaglia et al., 2008); however, the high variability in this motif leads to a subdivide the group 3 LEA proteins into two subgroups: 3A, represented by the cotton D-7 LEA protein; and 3B, represented by the cotton D-29 LEA protein (Bhardwaj et al., 2013). Group 3 LEA proteins have been described across all kingdoms (Shih et al., 2015) and their members have been found accumulating in mature seeds, and also in response to dehydration, salinity or low temperature (Bhardwaj et al., 2013).

1.4.4 Group 4

This LEA group is also recognized as D-113, their members are conserved in its N-terminal length (~80 residues long), while the C-terminal portion is more variable in size; within the N-terminal region a characteristic conserved motif is present (AQEKAEKMTA[R/H]DPXKEMAHERK[E/K][A/E][K/R]) (Battaglia et al., 2008). These proteins are subdivided into two subgroups according to their length: the first subgroup 4A contains small proteins (80-124 aa), the other subgroup 4B has longer representatives (108-180 aa). Additionally, other conserved motif could be found in each subgroup (Amara et al., 2014). The Group 4 proteins has been detected in vascular and non vascular plants species in response to in response to water deficiency and ABA (Olvera-Carrillo et al., 2010). This group contains high proportions of small

amino acids such as Gly or Ala and charged ones such as Asp, Gly, Lys, or Arg, making them highly hydrophilic (Shih et al., 2015).

1.4.5 Group 5 (hydrophobic LEA proteins)

This LEA sub group comprises those atypical LEA proteins with a high proportion of hydrophobic residues (they adopt a globular conformation). In contrast to all other LEA groups the members of this particular group are insoluble after boiling (Amara et al., 2014). Group 5 is considered as part of the LEA family since they accumulate during the late stage of seed development and under stress conditions like drought, salinity, cold, wounding, and UV light (Battaglia et al., 2008). Since the first proteins described in cotton were D-34, D-73 and D-95, this group is subdivided among 5A, 5B and 5C subgroups (Bhardwaj et al., 2013).

1.4.6 Group 6

The proteins of group 6 are very conserved among them, and also are characterized by their small size (approximately 7–14 kD) (Battaglia et al., 2008). Four motifs are described for this group, two of which (motifs 1 and 2) are highly conserved. The PvLEA18 from common bean (*Phaseolus vulgaris*) was the first protein described for this group; thus PvLEA18 is the representative member and the most characterized protein of this LEA group, however, more than 36 members belongs to this group have been reported until now (Colmenero-Flores et al. 1997), *PvLEA18* expression is detected during development in normal growth conditions, and also under water deficiency and ABA treatments (Colmenero-Flores et al. 1999).

1.4.7 Group 7 (ASR proteins)

Proteins that belong to LEA group 7 are also known as Abscisic-acid Stress Ripening (ASR) proteins and these have been found in angiosperm and gymnosperm (Battaglia et al., 2008). As their name suggests these proteins are involved in stress response (water deficit, salinity, cold and limited light conditions), fruit ripening, senescence, seed and pollen maturation and also can be induced by ABA and during late embryogenesis (Bhardwaj et al., 2013; Amara et al., 2014). Members of this group contain three conserved motifs with stretches of His residues, and one of them encodes a specific

sequence Zinc-dependent DNA-binding, these are cytoplasmic but predominantly nuclear proteins (Battaglia et al., 2008).

1.5 Molecular properties and functions of dehydrin group

Dehydrin (DHNs) are modular proteins assembled like a beads on a string (Graether and Boddington, 2014; Mouillon et al., 2006). Strictly, DHNs have at least one region capable of forming an amphipathic α-helix, called the K-segment (EKKGIMDKIKEKLPG), that can interact with macromolecules and specific regions of the membrane to protect them against damage by stress like ZmDHN1 from maize and Lti30, ERD10, and ERD14 from *A. thaliana* (Koag et al., 2009; Kovacs et al., 2008; Eriksson et al., 2011; Hara, 2010). In addition, DHNs may contain other segments, such as a repeat of serine residues named the S-segment which is part of a long conserved sequence LHRSGS4–10(E/D). This segment is predicted as a strong phosphorylation site. In some cases phosphorylation of this site is related to protein nuclear localization such as the case of RAB17 from maize. Also, this posttranslational modification also can activate the DHN calcium-binding activity of ERD10, ERD14 and COR47 *A. thaliana* DHNs (Alsheikh et al., 2003; Alsheikh et al., 2005). The Y-segment (VDEYGNP) is another conserved motif that can be present in DHN sequences and which putatively could function as a nucleotide-binding domain (Close, 1996).

Finally, φ-segments are less defined, comprising the polar and charged residues located between the conserved segments, and their principal function is to provide the necessary flexibility for proper structuring and orientation to the K-segments, so that they can interact with their objectives (Hughes and Graether, 2011; Graether and Boddington, 2014) (Figure 1). In addition to the conserved Y-, S- and K-segments, the presence of histidine-rich regions (H-X3-H, HH, and Hn) and poly-lysine tracks EEKKKKKKEKKK (charge-peptide ChP segment) have also been reported. These regions may participate in the formation of complexes with metal ions, acting as ion chelators and in DNA binding, as reported for CuCOR15 from citrus (Hara et al., 2005; Hara, 2010) (Figure 1).

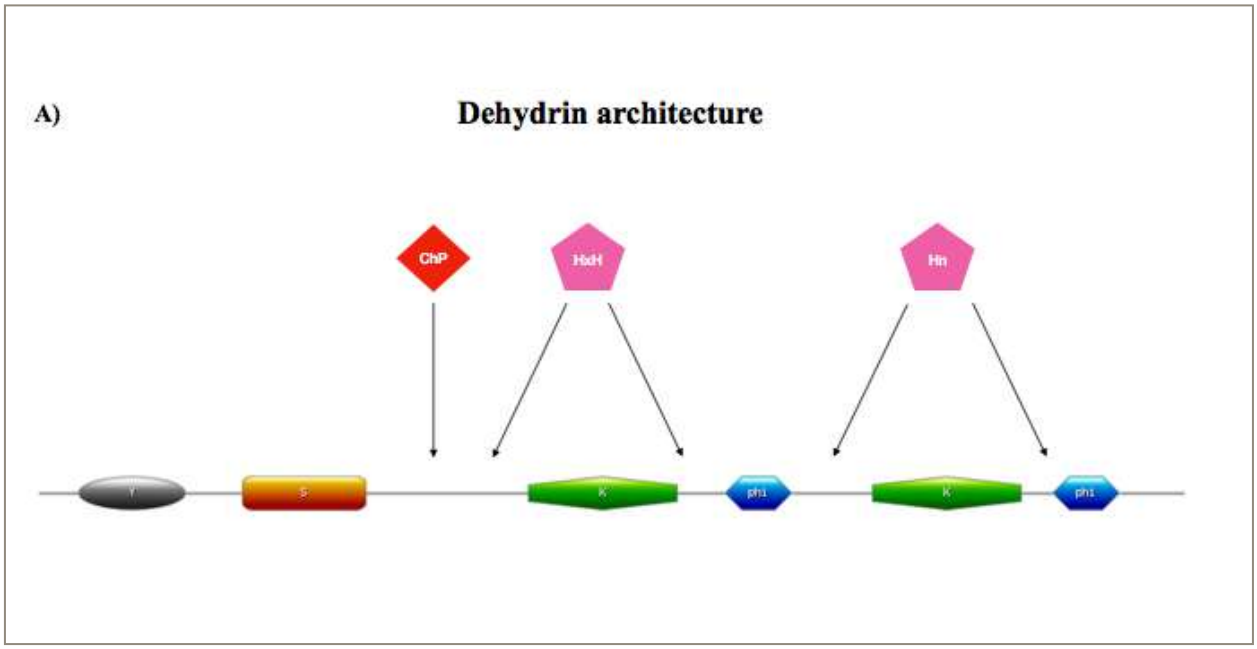

Figure 1. Schematic representation of dehydrin segments. From right to left, in gray Y-segment, orange S-segment, in green K-segments and blue represent phi-segments (φ-segments), at the top of the figure are depicted the non common segments: in red ChP-segment and in pink histidine-rich motifs The schematic representations were performed using MyDomains tool (Hulo et al., 2008). Modified from Battaglia et al., 2008.

In some cases the Y segment could be present up to 35 copies in the same polypeptide chain but always located at the N-terminus. The S-segment usually is present just one time per protein, and the K-segment is mostly present in the C-terminus and can be found in one to 11 copies within the same protein. According to the presence and arrange of these motifs, DHNs are classified into 5 subgroups YnSKn, Kn, KnS, SKn, and YnKn (Campbell and Close, 1997) (Figure 2); based on DHNs physicochemical features (isoelectric point, pI) they also can be divided as acidic, basic or neutral proteins (Amara et al., 2014). The compiled information about sequences comparison with functions and expression patterns known so far suggested a new DHN classification into two subgroups: named 2a and 2b (Wise, 2003; Tunnacliffe and Wise, 2007). According to this new categorization, proteins with Y-segments are contained in the group 2a, and are proteins preferentially expressed late in embryogenesis (as originally defined for LEA proteins) typically 2a DHN members are neutral or basic proteins in its overall charge. The group 2b comprise those DHNs without the Y-segment and are associated with stress response, particularly with cold tolerance, 2b members are mostly acidic proteins (Tunnacliffe and Wise, 2007) (Figure 2).

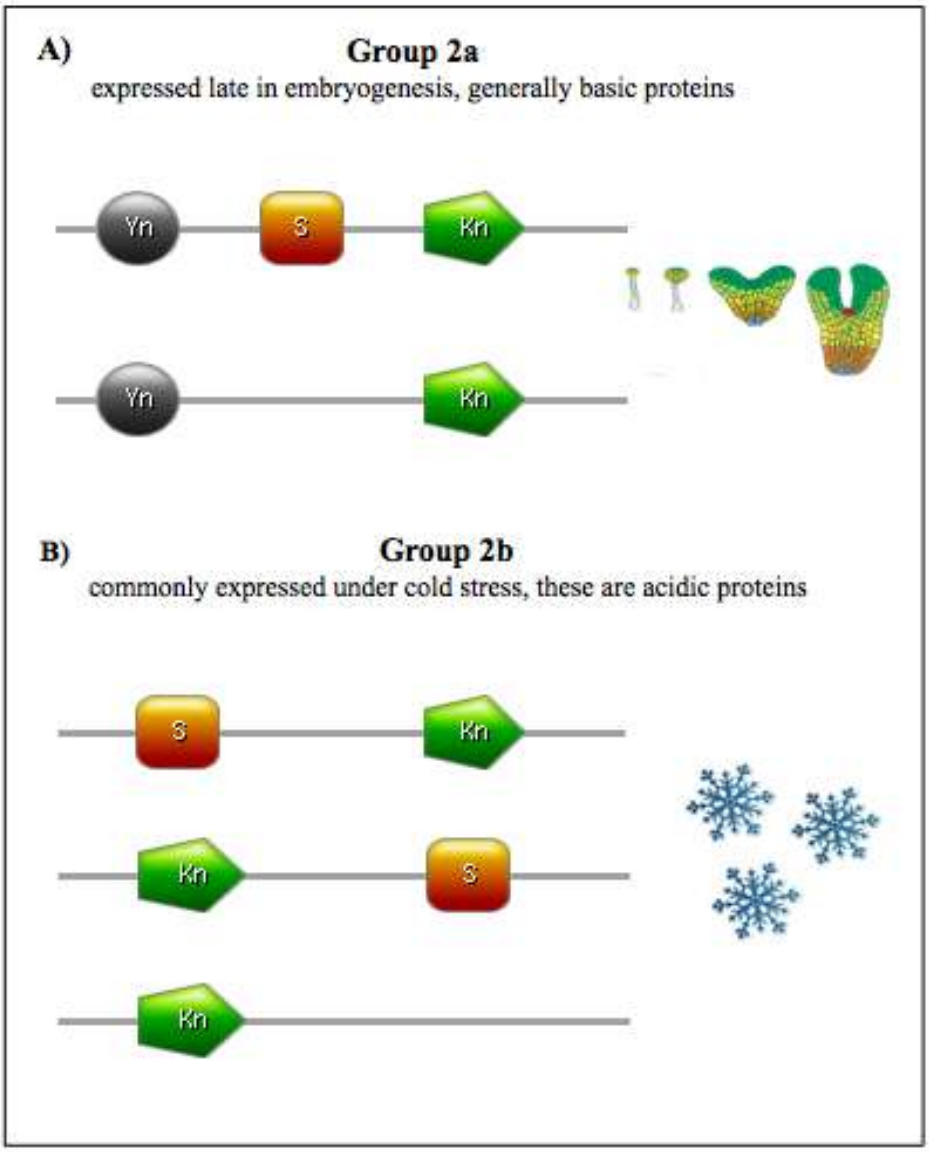

Figure 2. Dehydrin classification based on expression patterns, sequences, and functions. A) The group 2a comprises those proteins with Y-segment such as YnSKn and YnKn DHN types, these basic proteins are predominantly expressed during seed development. B) The group 2b comprise those acidic proteins expressed during cold stress like SKn, KnS, and Kn DHN types. This representation was developed based on Wise (2003) and Tunnacliffe and Wise (2007) proposals.

The expression and accumulation of DHNs under stress conditions that evoke cellular dehydration has been reported in all kind of plant tissues, such as seed, root, stem, leaf, flower, guard cells, and at the plasmodesmata or in pollen sacs among others (Parra et al., 1996; Nylander et al., 2001; Rurek, 2010). At the subcellular level, DHNs can be located in the nucleus, chloroplasts, vacuoles, rough endoplasmic reticulum, mitochondria, cytoplasm and membranes (Heyen et al., 2002; Mueller et al., 2003; Carjuzaa et al. Al., 2008).

Based on *in vitro* experiments, several functions have been proposed for DHNs, for example, cryoprotectants, chaperones, antioxidants, ion quelators and membranes stabilizers (Hara et al., 2010; Graether and Boddington, 2014). The *in vivo* evidence from numerous studies have demonstrated the positive effect of DHN expression on enhancing stress tolerance in a wide variety of plant species and other organisms. For example, the expression of ShDHN a DHN from *Solanum habrochaite*s in tomato enhanced their tolerance to multiple abiotic stresses (Liu et al., 2015) (Figure 3A). Also, the individual expression of five DHNs from *Prunus mume* in tobacco and *Escherichia coli* achieved an enhanced tolerance to cold and drought treatments (Bao et al., 2017)(Figure 3B). The heterologous expression of three *A. thaliana* acidic DHNs COR47, ERD10 and At2g21490 in yeast led to an improved desiccation tolerance (Dang et al., 2014). On the other hand, using knock-outs lines of two dehydrins of the moss *Physcomitrella patens,* a reduced ability of the plants to recover after salt or osmotic stress was observed (Saavedra et al., 2006; Ruibal et al., 2012) (Figure 3C).

The list of evidence keeps on growing, but the main questions still awaits an answer: what is the molecular mechanism through these enigmatic proteins exert their protective function? What is the role of K-, Y- and S-segments in plants, and is there specific *in planta* targets for these DHNs? In order to unveil new aspects of DHNs function and mechanism, we need to redirect the investigation in the DHN field; it is critical focus the effort on the study of the protein-protein interactions and protein localization *in planta*, since stress response involves more than the expression of a single gene, instead other proteins such as LEAs, heat shock proteins, even several DHNs, osmolytes (e.g. trehalose and sucrose) and large protein complexes such as expansins and aquaporins are involved in the defense or repair mechanisms against abiotic stress effects (Oliveira

et al., 2015). For this reason, we have to consider this overall picture for tolerance achievement.

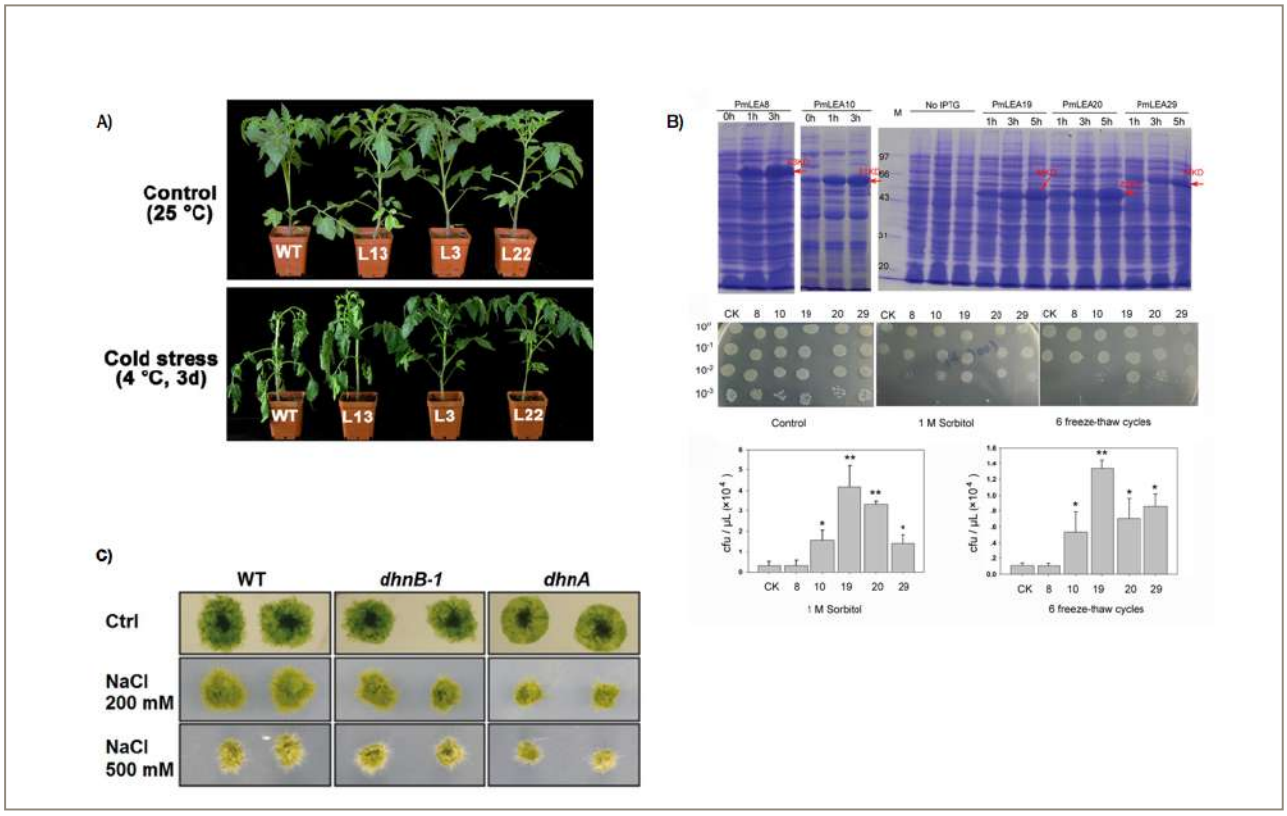

Figure 3. Effect of dehydrin overexpression and knockout mutants. A) The expression of ShDHN enhances cold tolerance in tomato. Phenotypes of seedlings from three transgenic lines and wild-type under normal conditions and after cold stress treatment (4 or 25°C control for 3 d) (Liu et al., 2015). B) The heterologous expression of four DHNs from *P. mume* PmLEA10, PmLEA19, PmLEA20 and PmLEA29 confer improved osmotic and freezing-resistance to *Escherichia coli* (Bao et al., 2017). C) Deletion of two DHN *dhnB-1* and *dhnA* genes in *P. patens* impair plant recover after salt and osmotic stress treatments(Saavedra et al., 2006).

II. Background

Cactus pears are succulent plants of the Cactaceae family adapted not only to grow, but to thrive in extremely arid, hot and cold environments recognized as stressful for most plant species. These characteristic making them an excellent source for isolation of stress tolerant genes, as well as an attractive model for the study of the molecular mechanisms underlying abiotic stress tolerance and for the improvement of other economically important crops. In our research group, a directional cDNA library from 12-month-old cladodes of *Opuntia streptacantha* plants was subjected to six different abiotic stress treatments: salinity, heat-salinity, cold, heat-cold, heat-drought, and drought-cold; under these conditions the most abundant EST identified was the unigene 33, a bioinformatic analysis revealed that this cDNA (905 pb) encodes the OpsDHN1 gene that codifies for an acid DHN of 248 residues, which contains three K- and one S-canonical segments (SK3), a chP-segment and a histidine-rich region (Figure 4A). The OpsDHN1 gene contains an intron inserted within the sequence encoding the S-motif. The qRT-PCR analysis shows that the OpsDHN1 transcript is specifically accumulated in response to cold stress, and also induced by ABA (Ochoa-Alfaro et al., 2012) (Figure 4B). Additionally, we demonstrated that *A. thaliana* transgenic lines overexpressing OpsDHN1 are freezing tolerant, suggesting that this DHN participates in the response to cold stress (Ochoa-Alfaro et al., 2012) (Figure 4C).

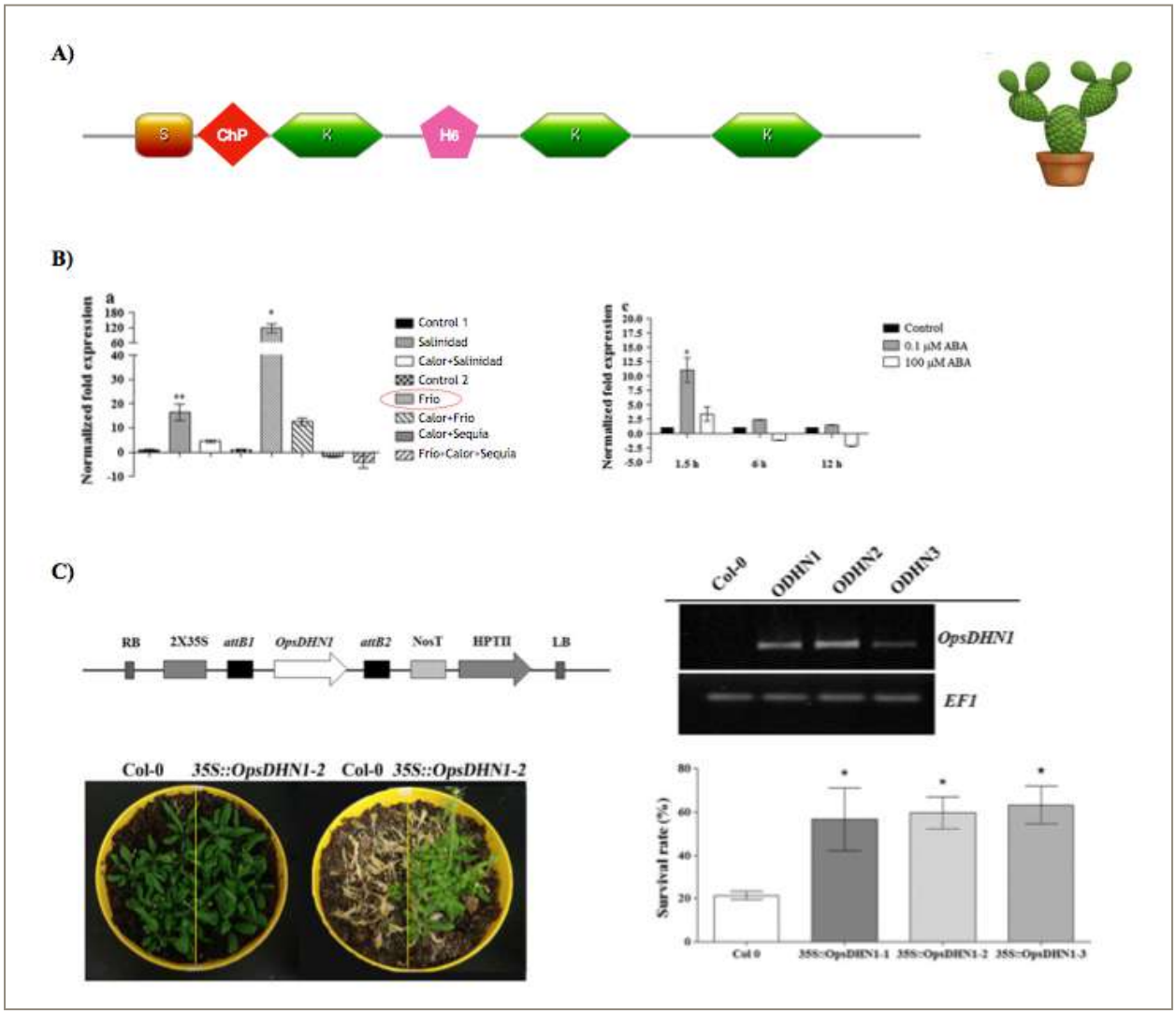

Figure 4. Functional characterization of an acidic SK3 dehydrin isolated from a *Opuntia streptacantha* cDNA library. A) Schematic representation of OpsDHN1 segments. B) qRT-PCR analysis of the OpsDHN1 transcript under abiotic stress and ABA treatment. C) Functional characterization of the OpsDHN1 *A. thaliana* transgenic lines under freezing treatment. The images were taken from Ochoa-Alfaro et al. (2012).

III. Argument

Despite significant progress of functional characterization of DHNs from different plant species, their molecular action mode remains elusive. In this regard, it is well known that members of the group 3 of the LEA family exist and function as dimers both *in vivo* and *in vitro* (Battaglia et al., 2008; Wise, 2003); Interestingly, LEA group 2 (DHNs) and group 3 are suggested to be phylogenetically closely related (Wise, 2003). Just a few reports about a possible *in vitro* dimerization in DHN family have been published at this time. Notwithstanding, the dimerization in DHN group has not been explored *in vivo*. In the genome of the model plant *A. thaliana*, 10 DHNs genes have been reported. It has been reported that ERD10, COR47 and RAB18 DHNs are up-regulated and accumulate at low temperatures, contributing to the cold stress response in Arabidopsis, and being the same case for the OpsDHN1 in cactus pear (Hundertmark and Hincha, 2008; Ochoa-Alfaro et al., 2012). In the present thesis, I attempt to answer the questions about DHN molecular mechanisms and function by studying their interactions and subcellular localization. Here we report for the first time the *in vivo* DHNs associations in two non-related species *Opuntia streptacantha* (OpsDHN1) and *Arabidopsis thaliana* (COR47, ERD10, and RAB18). Using these data, a new scenario where associations among DHNs play a key role in their molecular mechanism is discussed.

IV. Objective

General

Explore the homodimerization of the acidic OpsDHN1 dehydrin from Cactus pear, and the protein associations of three representative dehydrins from both basic (RAB18) and acidic group (COR47 and ERD10) of Arabidopsis.

Specific

1.- Biochemical characterization of the OpsDHN1 *in vitro*.

2.- Investigate the possible OpsDHN1 dimer interaction *in vitro* and *in planta.*

3.- Analyze the subcellular localization of the OpsDHN1 and the segments involved in it.

4.- Explore the homo- and heterodimeric interactions of COR47, ERD10 and RAB18 *A. thaliana* DHNs *in planta.*

5.- Examine the possible heterodimeric associations among COR47, ERD10 and RAB18 and the OpsDHN1 *in planta.*

6.- Determine the subcellular localization of the COR47, ERD10 and RAB18 *in planta.*

Chapter I:

A dehydrin-dehydrin interaction: the case of SK3 from *Opuntia streptacantha*

At this part of the research, we already knew that the OpsDHN1 was an acidic DHN (SK3) involved in freezing tolerance in cactus pear. Taking in mind that at least a few other *in vitro* reports have suggested a potential dimerization state for other DHNs, we decided to continue the characterization of the OpsDHN1 protein centering our effort to find out its molecular mechanism by which it enhances stress tolerance in plants by studying protein dimerization of OpsDHN1 using a split-ubiquitin yeast two-hybrid system and also by size-exclusion chromatography of the recombinant protein. In this regard, we report that the OpsDHN1 protein is able to dimerize in yeast and *in vitro* but only in the presence of ZnCl2, suggesting that its histidine-rich motif could play a role in the OpsDHN1 interaction. Additionally, we found that the deletion of regions that contain the conserved motifs (K-segments and the histidine-rich region) present in OpsDHN1 protein affects dimer formation. Finally, we conducted biochemical and functional *in vitro* characterization. The intrinsically disordered state of the recombinant expressed protein was demonstrated by circular dichroism (CD) and gel filtration; also we determined that OpsDHN1 is highly effective at preventing the denaturation of LDH from freeze/thaw damage (cryoprotective activity) *in vitro*. These data bring new insights into the molecular mechanism of OpsDHN1 SK3-dehydrin, opening a new hypothesis about the function of this protein.

Part of this research work was conducted during my master degree and the first year of my Ph.D. when I finished the last experiments and a draft of the paper. These data were finally published in the *Frontiers in Plant Science* journal as an original research article.

Published Article (open access)

I. E. Hernández-Sánchez, D. M. Martynowicz, A. A. Rodríguez-Hernández, M. B. Pérez-Morales, S. P. Graether,, and J. F. Jiménez-Bremont: A dehydrin-dehydrin interaction: the case of SK3 from Opuntia streptacantha, Front. Plant Sci. 5 (2014) 520. https://doi.org/10.3389/fpls.2014.00520

Chapter II:

Nuclear localization of the dehydrin OpsDHN1 is determined by histidine-rich motif

Previously, we demonstrated that the OpsDHN1 protein is able to dimerize in yeast (heterologous system) using the split-ubiquitin yeast two-hybrid (Y2H) and also *in vitro* using a size-exclusion chromatography approach using a recombinant protein (Hernández-Sánchez et al., 2014). Now, our next challenge was to confirm this OpsDHN/OpsDHN1 protein interaction *in planta.* For this aim, we used the Bimolecular Fluorescence Complementation (BiFC) assay that allowed direct visualization *in planta* of OpsDHN1 dimer formation and its subcellular localization. We demonstrated that *in planta* OpsDHN1 is able to form a homodimer with a dual cytoplasm and nuclear distribution in tobacco epidermal leaf cells; in contrast with the yeast interaction data the OpsDHN1 protein was able to interact with itself in the absence of its histidine tract but dimers were only localized in the cytosol of the tobacco cells, our data are the first to report an *in vivo* DHN-DHN interaction. Since the study of the subcellular localization of the DHNs could help to gain new insights about its role inside the cell and the molecular mechanism through which these proteins attain their function. An *in silico* analysis of the OpsDHN1 sequence revealed a putative NLS overlapped with the S-segment; additionally the histidine-rich region was weakly predicted as another putative NLS we evaluated the subcellular localization of the OpsDHN1 and deleted versions (ΔS and ΔHis) using GFP translational fusions. Our analysis reveals a dual cytoplasmic and nuclear location for the OpsDHN1 protein in tobacco cells. Our data suggest that the OpsDHN1 nuclear localization is mediated by the histidine-rich motif and to some degree by the S-segment.

These data were obtained during the first and second year of my Ph.D. and were published in the *Frontiers in Plant* journal *Science* as an original research article. *Published Article (Open access)*

I. E. Hernández-Sánchez, I. Maruri-López, S. P. Graether,, and J. F. Jiménez-Bremont: Nuclear localization of the dehydrin OpsDHN1 is determined by histidine-rich motif Front. Plant Sci. 6 (2015) 702. https://doi.org/10.3389/fpls.2015.00702

Chapter III:

In vivo evidence for homo- and heterodimeric interactions of *Arabidopsis thaliana* dehydrins COR47, ERD10, and RAB18

In our previous research studies, we reported the homodimerization of an acidic DHN from cactus pear (OpsDHN1) both *in vitro* and *in vivo*. In this sense, other studies have reported the formation of homodimers in other groups of the LEAs family such as PvLEA6 (LEA 6, Riviera-Najera et al., 2014), ASR1 (LEA 7, Ricardi et al., 2012); indeed for the AtLEA4-2 (LEA 4) high molecular mass complexes were isolated from cold acclimated plants (Olvera-Carrillo et al., 2010). With our previous OpsDHN1 results and these previously reported data, the question that arises was: is this DHN/DHN interaction is an isolated event for the Cactus pear OpsDHN1 protein or other DHNs could self-associate or interact with other DHNs in the plant?. In order to answer this question we explored the homo and heterodimerization of three representative DHNs of the model plant Arabidopsis of the basic and acidic groups (AtCOR47, AtERD10, and RAB18) using the bimolecular fluorescence complementation technique (BiFC). Moreover, these proteins were analyzed for heterodimeric interactions with their OpsDHN1 orthologue; as a supporting feature the *in vivo* sub-cellular localization of AtCOR47, AtERD10, and AtRAB18 DHNs was examined. While Arabidopsis DHNs AtCOR47, AtERD10, and AtRAB18 have been widely characterized the potential interaction among them has not been studied. Here, we provide detailed evidence on the *in planta* Arabidopsis DHNs homo- and heterodimers. These data constitute the first report of *A. thaliana* DHN interactions. We found that dimerization is not exclusive to the acidic group since the basic ones can interact with their homologous and also with acidic proteins. We discuss oligomerization as a distinctive characteristic of DHNs that could enhance their functions in plants under stress conditions.

The experimental data and paper draft were obtained during the third and fourth year of my Ph.D. and we have submitted a manuscript to the prestigious journal *Scientific Reports* as an original research article, which is currently undergoing the first round of revision.

Paper draft

***Research paper submitted**

In vivo evidence for homo- and heterodimeric interactions of *Arabidopsis thaliana* dehydrins COR47, ERD10, and RAB18

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ABSTRACT

Dehydrins (DHNs) are intrinsically disordered proteins that play central roles in plant abiotic stress responses; however, how they work remains unclear. Herein, we report the *in planta* sub-cellular localization of *Arabidopsis thaliana* DHNs COR47, ERD10, and RAB18 through GFP translational fusions. To explore the dimerization ability of the Arabidopsis acidic DHNs AtCOR47 and AtERD10, we conducted a direct DHN binding assay using an *in planta* Bimolecular Fluorescence Complementation (BiFC) approach. Our analyses revealed homodimeric interactions for AtCOR47 and AtERD10; interestingly, heterodimeric associations also occurred with these DHNs, and the interactions were observed in the cytosol of tobacco cells. Furthermore, we evaluated whether Arabidopsis basic DHNs, such as AtRAB18, could interact with itself and/or with AtCOR47 and AtERD10 in the BiFC system. Our data reveal homodimeric RAB18 complexes in the nucleus and cytosol; however, heterodimeric associations between AtRAB18 and acidic DHNs occurred only in the cytosol. Finally, we demonstrated the presence of heterodimeric complexes among Arabidopsis AtCOR47, AtERD10, and AtRAB18 DHNs with their *Opuntia streptacantha* acidic ortholog OpsDHN1; in this case, homo- and heterodimeric interactions showed different distributions. Our results direct DHN research to a new scenario where DHN/DHN associations could be explored as a part of their molecular mechanism.

Keywords: Arabidopsis thaliana, dehydrins, AtCOR47, AtERD10, AtRAB18, dimer, localization.

Introduction

Global climate change is a fact; this phenomenon is accompanied by environmental stresses factors like drought, salinity, and extreme temperatures with which plants must deal with more often in order to survive. To withstand stress, plants have evolved physiological and molecular responses involving changes in their transcriptome, proteome, and metabolome [1]. Dehydrins (DHNs) are plant-specific proteins that accumulate during the abiotic factors that cause cellular dehydration, such as drought, salinity, freezing, or by the phytohormone ABA [2]. Transcriptional and proteomic studies of abiotic tolerance in several plant species have demonstrated that DHNs transcripts and proteins levels increase in tolerant varieties [3, 4, 5]. In addition, transgenic studies have widely demonstrated the positive effect of DHNs expression and accumulation to survive to changing conditions [6, 7, 8]. Through *in vitro* experiments it has been hypothesized that this effect relies on cryoprotective and chaperone activity, as well as metal and reactive oxygen species scavenging functions; however, not all DHNs exhibit all of these functions [2].

According to their expression patterns and physicochemical properties, DHNs can be divided into two groups, 2a and 2b [9]. Proteins that belong to group 2a are preferentially expressed during the late embryogenesis stage, and these are proteins with basic or neutral isoelectric point (pI). Members of group 2b are associated with cold tolerance, and do not typically accumulate during the late embryogenesis stage. The 2b members also contain a large proportion of acidic residues [10]. In the model plant *Arabidopsis thaliana* genome, 10 DHNs genes have been annotated [11]; the DHN acidic group contains At1g20440 (COR47), At1g20450 (ERD10), At1g76180 (ERD14), At2g21490, At4g39130, and At4g38410, and the basic or neutral DHN group contains At1g54410 (AtHIRD11), At3g50970 (XERO2), At3g50980 (XERO1) and At5g66400 (RAB18) [11].

In *A. thaliana*, AtERD10 and AtCOR47 are the principal DHNs that accumulate in response to low temperature, contributing to the cold stress response [12]. *In vitro* functions have been established for *A. thaliana* acidic DHNs AtCOR47 and AtERD10 such as ion and water-binding, cryoprotective activity, thylakoid membrane-binding, and

metal-binding [13, 14, 15]. In particular, chaperone activity has been reported for ERD10 [16]. The over-expression of AtCOR47 and AtERD10 correlated with improved *A. thaliana* cold stress tolerance under low-temperature conditions [12]. On the other hand, the *erd10* mutant showed reduced tolerance to drought, cold stress and reduced seed germination [17]. The Arabidopsis basic DHN AtRAB18, is also up-regulated and accumulates under low temperatures, drought, salinity, and ABA, suggesting that it is involved in these abiotic stress responses [18, 19, 20, 21, 11]. Despite significant progress in the Arabidopsis DHN field, their molecular action mode remains elusive.

In our previous research we found that the OpsDHN1 from *Opuntia streptacantha* improved cold tolerance in *A. thaliana* [7] and is also able to interact with itself in both *in vitro* and *in vivo* systems [22, 23]. With these data, the question that arises was: is this DHN/DHN interaction an isolated event or could other DHNs self-associate or interact with other DHNs *in planta*? to answer this question, our main goal in this study was to investigate the homo- and heterodimerization of three representative members of the Arabidopsis basic and acidic groups (AtCOR47, AtERD10, and RAB18) through the bimolecular fluorescence complementation technique (BiFC); as a supporting feature the *in vivo* sub-cellular localization of AtCOR47, AtERD10, and AtRAB18 DHNs was analyzed using GFP translational fusions. Here, we provide detailed evidence on the *in planta* Arabidopsis DHNs oligomeric complexes, and the interaction of AtCOR47, AtERD10 and AtRAB18 proteins with its acidic OpsDHN1 orthologue. We discuss the oligomerization as a distinctive characteristic of DHNs and possibly other members of the LEA family that could enhance their function in plants under stress conditions.

Results

Sub-cellular localization of acidic AtCOR47 and AtERD10 dehydrins

In order to visualize the *in planta* sub-cellular distribution of *A. thaliana* DHNs COR47 and ERD10, we independently fused the DHN coding regions to GFP (Figure 1A and B). The generated plasmids (pMDC43-AtCOR47 and pMDC43-AtERD10) were introduced into *Nicotiana benthamiana* epidermal cells through an agro-infiltration method. To reveal nucleus localization, DAPI stain was used, and as a localization control the *N. benthamiana* leaves were transformed with the pMDC43 vector (Supplementary Figure 1). The images were acquired using a confocal laser scanning microscopy (CLSM). In these assays, only cytosolic areas exhibit fluorescent signal, indicating that AtCOR47 and AtERD10 proteins are excluded from the plant nuclei (Figure 1C and D). These data are in agreement with our *in silico* cytosolic localization data of both DHNs [24] (http://abi.inf.uni-tuebingen.de/Services/YLoc/webloc.cgi), which predicted a probability of 72.5% for cytosolic localization of AtCOR47 and a 96.7% probability for AtERD10 (data not shown).

In planta AtCOR47 homodimer formation

To explore if the COR47 self-association could occurs in plant cells, we analyzed AtCOR47/AtCOR47 direct interactions using the BiFC assay. The AtCOR47 coding region was separately fused to the N- (pYFN43-AtCOR47) and C- (pYFC43-AtCOR47) terminal domains of GFP in BiFC vectors [25] (Figure 2A). The BiFC-COR47 constructs were co-expressed using the agro-infiltration method using *N. benthamiana* epidermal leaf cells. The fluorescent signals were acquired using a CLSM, and DAPI stain was used to detect nuclei. Our fluorescence analyses revealed a strong GFP fluorescent signal in the cytosol of tobacco cells; no signal was detected in the nuclei (Figure 2B). Since possible alterations in the structure of the pYFN43 vector with the fusion protein could lead to autofluorescence, we analyzed the fluorescent signal of the pYFN-AtCOR47 construct alone and no fluorescence signal was detected (Figure 2C). The interaction of the Arabidopsis SnRK kinases subunits AKIN10 and AKINβ2 was used as a positive interaction control; in addition, the interaction of AKIN10 and AKINβ2 with AtCOR47 was used as non-DHN interaction assay (Supplementary Figures 2 and 3).

These data show that AtCOR47 DHN is able to dimerize *in planta,* but only in the cytosol.

In planta AtERD10 homodimer formation

Our next step was to analyze whether another acidic DHN, such as AtERD10, also has the ability to homodimerize. The ERD10 coding sequence was cloned in frame to the N- (pYFN43-AtERD10) or C- (pYFC43-AtERD10) terminal domains of GFP (Figure 3A). As previously described, we transiently expressed pYFN-AtERD10 and pYFC-AtERD10 constructs in tobacco leaf epidermal cells using an agro-infiltration method; nuclei localization was highlighted using a DAPI stain. As Figure 3B shows, AtERD10/AtERD10 interaction exhibited fluorescent signal in the cytosol but not in the nuclei of the plant cells. To test that self-activation is not occurring, we analyzed the fluorescent signal of the pYFN-AtERD10 construct (Figure 3C). As a positive control the interaction between AKIN10 and AKINβ2 subunits was used (Supplementary Figure 2); additionally, we checked the interaction between AKIN10 and AKINβ2 subunits and AtERD10, and no fluorescence signal was detected (Supplementary Figure 4). These results show that AtERD10 can self-interact in the cytosol of plant cells.

In planta AtRAB18 homodimer formation

We tested whether the *A. thaliana* basic DHN AtRAB18 is capable of interacting in the BiFC system as their acidic paralogues do. The AtRAB18 coding region was individually fused to the N- (pYFN43-AtRAB18) and C- (pYFC43-AtRAB18) terminal domains of GFP (Figure 4A); both constructs were agro-infiltrated into *N. benthamiana* leaf cells and analyzed for fluorescent signal by CLSM. DAPI was used for nuclear staining. In contrast to the acidic DHNs, the reconstitution of fluorescent signal mediated by RAB18 interaction was observed in the cytosol and nuclei of the tobacco epidermal cells as shown in Figure 4C. No fluorescent signals were detected when non-DHNs AKIN10 and AKINβ2 subunits constructs were infiltrated in combination with the AtRAB18 BiFC constructs (Supplementary Figure 5), nor when the pYFN-AtRAB18 has expressed alone (Figure 4D). These data indicate that the basic AtRAB18 DHN interacts specifically with itself *in planta* in the cytosol and in the nucleus.

Sub-cellular localization of basic AtRAB18 dehydrin

To determine the *in vivo* sub-cellular localization of the Arabidopsis basic DHN AtRAB18, we generated a translational fusion between the AtRAB18 coding region and GFP (Figure 4B). The pMDC43-AtRAB18 construct was agro-infiltrated into *N. benthamiana* leaf cells. The nuclear staining, controls, and images acquisition were performed as described in the previous sections. According to our *in silico* prediction, there is a 62% probability that the protein will be in the cytoplasm, and a 38% probability that it will be in the nucleus [24] (http://abi.inf.uni-tuebingen.de/Services/YLoc/webloc.cgi; data not shown). The confocal images revealed a dual nuclear/cytosolic distribution for AtRAB18 *in planta* (Figure 4E), which agrees with the localization of the AtRAB18 dimers.

In planta heterodimeric interactions between the Arabidopsis acidic DHNs AtCOR47 and AtERD10 and the basic AtRAB18 protein

To investigate whether the Arabidopsis DHNs AtCOR47, AtERD10 and AtRAB18 can interact with each other, we performed the BiFC assay. For this analysis AtCOR47, AtERD10, and AtRAB18 BiFC constructs were used (Figure 5-7, panels A and B). All constructs were co-expressed in different combinations in *N. benthamiana* epidermal leaf cells and analyzed for a fluorescent signal. Previously, we demonstrated that there was no-self activation for pYFN-AtCOR47, pYFN-AtERD10 and pYFN-AtRAB18 constructs (Figure 2C, 3C and 4D, respectively). As presented in Figures 5-7, panels C and D, the fluorescent signal is observed in all tested combinations (AtCOR47/AtERD10, AtRAB18/AtCOR47, andAtRAB18/AtERD10, or their swapped versions). The signals were confined to the cytosol of the tobacco plant cells. These results suggest that the formation of these heterodimers come from specific DHN/DHN interactions, and show that the interaction between the Arabidopsis DHNs is not only restricted to acidic group DHNs, also the neutral DHN RAB18 is capable of interacting with itself and with acidic DHNs AtCOR47 and AtERD10 *in planta.*

Heterodimeric interactions of Arabidopsis DHNs AtCOR47, AtERD10, and AtRAB18 with its ortholog OpsDHN1

Previously, we demonstrated the *in planta* dimer formation of the acidic OpsDHN1 protein [23]. We conducted the BiFC assay to determine if the *A. thaliana* DHNs

AtCOR47, AtERD10, and AtRAB18 can interact with its OpsDHN1 cactus pear ortholog. For this, the AtCOR47, AtERD10, AtRAB18 and OpsDHN1 BiFC constructs were employed [23] (Figure 8A-C); the transformations, images acquisition and BiFC controls were performed as previously described. Our confocal analyses revealed fluorescent signal when the combinations AtCOR47/OpsDHN1, AtERD10/OpsDHN1, and AtRAB18/OpsDHN1 (Figure 8D-F) or their swapped versions were analyzed (Supplementary Figure 6). Acidic AtCOR47/OpsDHN1, AtERD10/OpsDHN1 heterodimeric complexes showed only cytosolic distribution (Figure 8D-E), no signal was detected inside the plant nuclei, however, the interaction between AtRAB18 and OpsDHN1 showed a dual nuclear/cytosolic distribution (Figure 8F). Our data demonstrate that the three representative members of the Arabidopsis acidic and neutral groups (AtCOR47, AtERD10, and AtRAB18) are able to interact with their acidic orthologue OpsDHN1 *in planta.*

Discussion

In a cellular context, proteins are often organized into dynamic groups to perform their biological functions [26]. The stress response is orchestrated at different levels through proteins that usually team up into complexes. The accumulation of DHNs during abiotic stress has been reported in herbaceous dicotyledons, woody plants, and cereals [27]. In *Arabidopsis thaliana*, AtERD10, AtCOR47, and AtRAB18 DHNs contribute to the abiotic stress response; however, their molecular mechanism is unknown [27, 18, 28, 21]. In the present work, we report the formation of homodimeric complexes of Arabidopsis acid and basic DHNs, and also discover the ability of DHNs to form heterodimers. To increase our knowledge of these Arabidopsis proteins, we first aimed to determine the sub-cellular localization of the *A. thaliana* DHNs AtCOR47, AtERD10 and AtRAB18 using GFP translational fusions. Our data indicate that both acidic DHNs AtCOR47 and AtERD10 are located in the cytosol of *Nicotiana benthamiana* epidermal leaf cells. In the case of AtRAB18, this basic protein displays a dual nuclear/cytosolic localization; these results are in agreement with our *in silico* sub-cellular localization analysis (YLoc web server, data not shown) [24]. Studies performed by Candat et al. [29] using GFP translational fusions reported that 36 of 51 *A. thaliana* LEA members are cytosolic or cytosolic/nuclear proteins in Arabidopsis leaf protoplasts. Our results and these previous data are in agreement with AtCOR47 and AtERD10 *in vivo* cytosolic localization and with AtRAB18 nuclear/cytosolic localization. Resolving the AtCOR47, AtERD10 and AtRAB18 sub-cellular localizations could help to establish their particular biological functions.

Recently, we reported the first *in planta* DHN association where, the dimeric state of the OpsDHN1 acidic DHN from *Opuntia streptacantha* was discovered with a dual nuclear/cytosolic distribution [23]. To explore the *in vivo* homodimer formation in other plant species, we selected three representative members of the acidic and basic/neutral groups of the model plant *A. thaliana.* We first analyzed the homodimerization between the acidic DHNs AtCOR47 and AtERD10 through a BiFC assay. Our data revealed that both acidic AtCOR47 and AtERD10 proteins assemble into homodimers (AtCOR47/AtCOR47 and AtERD10/AtERD10) in the cytosol of Nicotiana leaves. On the other hand, in order to determine if this characteristic of being able to form homodimers

is exclusive for acidic DHNs, the homodimerization of the basic DHN AtRAB18 was also examined. We demonstrate that the Arabidopsis basic AtRAB18 DHN can also assemble into homodimers (AtRAB18/AtRAB18) in the nucleus and cytosol of the tobacco leaves.

Interestingly, previous *in vitro* studies have suggested the formation of homo-oligomeric complexes for some DHN of other plant species, such as the case of the homotetramer (350 kDa) of COR85 from *Espinacea olaracea* purified from acclimated and non-acclimated plants [30], and the homodimer (42 kDa) of the 20 kDa DHN from *Zea mays* purified from whole plant extracts [31]; the presence of both DHN complexes was proven through gel filtration. Also, Still et al. [32] demonstrated by Western blot the accumulation of two DHN bands of 21 kDa and 38 kDa in *Oriza sativa* embryos 15 days after anthesis; they suggested that this 38 kDa band could be the dimeric form of the 21 kDa DHN. Recently, Rahman et al. [33] reported a potentially dimeric state for the recombinant TsDHN-2 from *Thellungiella salsuginea* using single-molecule force spectroscopy [33]. These data support the idea that Arabidopsis AtCOR47, AtERD10, and AtRAB18 dimerization is not an isolated event, instead pointing out an association as a common feature of DHNs (see Figure 9).

Plant tolerance and survival is attained by the accumulation of a subset of stress-response proteins. In this respect, protein-protein interactions are a central part of this response, so one of the major challenges is to elucidate this network. Here, we tested the capacity of AtCOR47 and AtERD10 to form heterodimers in a BiFC system. Our results reveal that both Arabidopsis acidic DHNs are able to assemble into heterodimeric complexes (AtCOR47/AtERD10) *in planta* with a cytosolic distribution. This finding was extended to the acidic-basic DHN interaction, demonstrating heterodimeric formations between the acidic DHNs AtCOR47 and AtERD10 with the basic DHN AtRAB18. Finally, we showed that these Arabidopsis acidic AtCOR47 and AtERD10 DHNs are capable of interacting with their *O. streptacantha* OpsDHN1 ortholog in the cytosol of Nicotiana leaf cells. Also, the basic DHN AtRAB18 was able to interact with the acidic OpsDHN1; however, this interaction was observed in the nuclei/cytosol of tobacco cells. The ability of DHN to form *in planta* homo- and heterodimeric interaction at specific sub-cellular locations could help to reinforce models

proposed for DHN function. It is worthwhile to mention that the identity between the Arabidopsis acidic paralogues COR47 and ERD10 is 65%, and with their acidic OpsDHN1 orthologue is around 36%. However, the identity of these acidic proteins with the basic Arabidopsis RAB18 is less than 19%, and a regret of this low identity percentage, those proteins are capable of heterodimerizing. In our previous report, we demonstrated in the yeast two-hybrid system that the conserved K-segments of OpsDHN1 are involved in the DHN/DHN interaction [22]. Thus, we propose that, as in the case of OpsDHN1 self-interaction, the conserved K-segments of DHNs could play a role in established DHN-DHN interactions, both in the formation of homo- and heterodimers.

The over-expression of single LEA or DHN gene generally results in an improvement in plant stress tolerance [34, 35]. However, this is not an absolute rule since some single DHNs expression revealed just a slight or even no increase in stress tolerance [36, 37, 38, 39]. Puhakainen et al. [12] generated transgenic Arabidopsis lines over-expressing pairwise combinations of four DHNs. Despite all lines exhibiting similar DHN accumulation levels, pTP9 (expressing AtRAB18 and AtCOR47) and pTP10 (harboring AtERD10 and LTI30) transgenic lines showed significant differences in freezing tolerance, 41% for TP9 lines and 86% for TP10 lines in comparison to 22% in control plants, and no freezing tolerance was obtained when a single DHN was overexpressed. We propose that the dimerization or oligomerization among DHNs could offer advantages since the association of two shorter proteins could be a more effective molecular shields than one, as determined by Hughes et al. [40], where the authors observed that longer DHNs are more efficient at protecting LDH from activity loss *in vitro*. Also, DHNs, as intrinsically disordered proteins, are susceptible to random degradation, so this interaction could favor avoiding proteolysis [41, 42]. The accumulation of different types of DHNs in low temperature or ABA-treated plants suggests that the role of DHNs is accentuated during conditions of stress [21], and also that different DHNs combination could determinate the behavior and function of these proteins in response to determined stress stimuli.

Quaternary associations have also been described, at least *in vitro*, for other LEA proteins. Members of groups 3, 4, 6 and 7 of the LEA family also had reported forming

dimers and oligomers. In fact, members of the LEA 3 group are predicted to exist as dimers, interacting principally through amphipathic α-helices formed by their 11-mer motif [43]. A mass spectrometric approach revealed that the AfrLEA2 LEA 3 group could exist as homodimers and homotrimers in embryos of *Artemia franciscana* [44], and Goyal et al. [45] used immunoblotting and cross-linking experiments to demonstrate that AavLEA1 from *Aphelenchus avenae* (LEA group 3) is present as oligomers in solution. Interestingly, LEA group 2 (DHNs) and group 3 are suggested to be phylogenetically closely related [9].

This observation extends to other LEA proteins. High molecular mass complexes of AtLEA4-2 (LEA group 4) were obtained from cell extracts of *A. thaliana* stressed plants [46]. The same research group reported in Rivera-Najera et al. [47] the *in vivo* dimerization of PvLEA6 (LEA group 6) from *Phaseolus vulgaris* using the BiFC assay; using the same approach, the dimerization of RcLEA (LEA group 7) from *Rosa chinensis* was seen in Nicotiana leaves [48]. Based on previous findings of high molecular mass complexes detected in cell extracts from stressed plants, Olvera-Carrillo et al. [49] proposed that the interaction could occur in the LEA family and that the conserved sequences present in the different LEA groups could play a role in the formation of high order structures between LEA proteins from the same or different groups.

The study of DHN interactions and sub-cellular localization is central to understanding the molecular mechanism by which these proteins works; the molecular shield effect [10, 50, 40] and the disordered chaperone effect [51] are the two models proposed for DHN function with respect to protecting proteins from abiotic stress damage. In both scenarios, DHN assembly into homo- and heterodimers could offer the combinatorial effect of producing multiple complexes with different affinity and specificity to their biological targets, giving the cell an instrument for fine-tuning the stress response.

Material and Methods

Plant material and growth conditions

Nicotiana benthamiana seeds were spread on a mix of 50% vermiculite and 50% soil and grown in a greenhouse with long-day photoperiod cycles (16 h light/8 h dark) at 22 ºC ± 2 ºC for three to four weeks.

Vector construction

First, the entry clones were generated by PCR amplification of each *AtCOR47* (At1G20440), *AtERD10* (At1G20450), and *AtRAB18* (At5G66400) open reading frame. Next, the PCR products were cloned into the pCR8 entry vector (Invitrogen, Carlsbad, CA). Selected clones were sequenced using an M13 forward primer. Second, for sub-cellular analysis, GFP translational fusions were carried out through a recombination of the pCR8-AtCOR47, pCR8-AtERD10 and pCR8-AtRAB18 entry vectors with the pMDC43 binary destination vector [52]. To perform the Bimolecular Fluorescence Complementation (BiFC) test, the AtCOR47, AtERD10, and AtRAB18 entry clones were shifted into both pYFN43 and pYFC43 BiFC expression vectors [25]. All shift reactions were done by site-specific recombination using Gateway LR Clonase II Enzyme Mix (Invitrogen). Finally, all generated destination vectors were introduced into *Agrobacterium tumefaciens* GV3101/pMP90 strains. The *A. tumefaciens* cells harboring the pYFN43-OpsDHN1 and pYFC43-OpsDHN1 vectors were created previously [23].

Nicotiana benthamiana transient transformation

Abaxial leaf cells from *N. benthamiana* plants were transiently transformed by *A. tumefaciens* GV3101/pMP90 strains containing the generated expression vectors. To inhibit gene silencing during the BiFC tests, an *A. tumefaciens* strain harboring the tomato bushy stunt virus p19 protein was used during co-infiltration [53, 25]. The *A. tumefaciens* cells were collected at OD_{600} of 1.0, and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 200 µM acetosyringone). Then the strains were incubated at room temperature on a rocking platform for 3 h. Afterward, the *N. benthamiana* leaf abaxial space was co-infiltrated using a needleless syringe. Three days after infiltration, two leaves from three independently transformed plants were analyzed for fluorescence under a confocal microscope. The analyses were repeated

three times for all constructs, and gave similar results.

Nuclei staining

For nuclei staining, DAPI reagent (Sigma, St. Louis, MO) was used. Briefly, *N. benthamiana* transformed leaf segments were cut from the plant and then incubated in a water solution containing 5µg/mL reagent for 5 min.

Fluorescence confocal microscopy

The transiently transformed *N. benthamiana* leaves were observed under a Leica TCS SP5 multiphoton confocal microscope (Leica, Wetzlar, Germany). The laser excitation wavelength was 488 nm and the spectral detection was set between 497-537 nm for GFP and 684-758 nm for chlorophyll fluorescence, with a beam splitter MBS 488. For DAPI laser excitation the wavelength was set to 405 nm and detection was made at 410-492 nm. The objective used was 20x Multi-Immersion. The image analysis was performed with Fiji imaging software [54].

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Author Contributions

S.P.G. and J.F.J.B. supervised the project and developed the concepts. I.E.H.S., I.M.L., S.P.G., and J.F.J.B. designed the experiments and wrote the manuscript. I.E.H.S. and I.M.L. performed the experiments and data analyses.

Competing financial interests statement

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

FIGURE LEGENDS

Fig. 1. *In vivo* sub-cellular localization of *A. thaliana* acidic AtCOR47 and AtERD10 dehydrins. Schematic representation of the pMDC43 vectors containing (A) AtCOR47 or (B) AtERD10; both constructs are under the 2x35S promoter and nosT terminator. Sub-cellular localization of the (C) pMDC43-AtCOR47 and (D) pMDC43-AtERD10 protein fusions in tobacco epidermal leaf cells. From left to right: GFP, chlorophyll, DAPI, bright field, and overlay panels. The white arrow indicates the nuclei and yellow one indicates the cytosol. The scale bar corresponds to 23 μm.

Fig. 2. Determination of the AtCOR47 homodimers using a BiFC assay. (A) Schematic diagram of pYFN43-AtCOR47 and pYFC43-AtCOR47 BiFC vectors; both constructs are under the control of the 2x35S promoter and nosT terminator. (B) Confocal images of pYFN43-AtCOR47 and pYFC43-AtCOR47 co-transformations in tobacco epidermal leaf cells. (C) Auto-fluorescence test in the BiFC system of the pYFN43-AtCOR47 construct transformed alone. From left to right: GFP, chlorophyll, DAPI, bright field, and overlay channels, white arrow indicates the nuclei and yellow arrow indicates the cytosol. The scale bar corresponds to 23 μm.

Fig. 3. Visualization of AtERD10 homodimers using BiFC assay in tobacco epidermal leaf cells. (A) Schematic representation of BiFC vectors containing the AtERD10 protein fused to N- or C-terminus of GFP; both constructs are under the control of the 2x35S promoter and nosT terminator. (B) Fluorescence analysis of pYFN43-AtERD10/pYFC43-AtERD10 co-transformation in tobacco epidermal leaf cells. (C) Analysis for no fluorescent auto-activation of the pYFN43-ERD10 construct transformed alone. From left to right: GFP, chlorophyll, DAPI, bright field and overlay channels, white arrow indicates the nuclei and yellow indicates the cytosol. The scale bar corresponds to 23 μm.

Fig. 4. Detection of the Arabidopsis basic AtRAB18 homodimer in BiFC assay and subcellular localization in tobacco epidermal cells. (A) Schematic representation of BiFC vectors containing the AtRAB18 protein fused to N- or C-terminus of GFP; both constructs are under the control of the 2x35S promoter and nosT terminator. (B) Diagram of pMDC43 vector containing AtRAB18 protein fused to the GFP; the construct

is under the 2x35S promoter and NosT terminator. (C) Fluorescence analysis of pYFN43-AtRAB18/pYFC43-AtRAB18 co-transformed vectors in tobacco epidermal leaf cells. (D) Auto-fluorescence test of BiFC system of the AtRAB18 protein cloned into pYFN43 vector (pYFN43-AtRAB18). (E) Sub-cellular localization of the pMDC43-AtRAB18 protein fusion in tobacco epidermal leaf cells. From left to right: GFP, chlorophyll, DAPI, bright field and overlay panels. The white arrow indicates the nuclei and yellow arrow indicates the cytosol. The scale bar corresponds to 23 μm.

Fig. 5. The AtCOR47 and AtERD10 DHNs are able to heterodimerize in BiFC assay. Schematic representation of the (A) AtCOR47 and (B) AtERD10 BiFC constructs used in the heterodimeric interaction. Confocal images of transient transformation containing (C) pYFN43-AtCOR47/pYFC43-AtERD10 and (D) pYFN43-AtERD10/pYFC43-AtCOR47 BiFC constructs in tobacco leaves. From left to right: GFP, chlorophyll, DAPI, bright field and overlay channels, white arrow indicates the nuclei and yellow arrow indicates the cytosol. The scale bar corresponds to 23 μm.

Fig. 6. Heterodimerization between the Arabidopsis dehydrins: AtCOR47 and RAB18 in BiFC assay. (A) Schematic representation of the pYFN43-AtRAB18/pYFC43-AtCOR47 vectors cotransformed in tobacco leaf cells. (B) Schematic representation of pYFN43-AtCOR47/pYFC43-AtRAB18 constructs. Fluorescent analyses of (C) AtRAB18/AtCOR47 and (D) AtCOR47/AtRAB18 interactions in tobacco epidermal leaf cells. From left to right: GFP, chlorophyll, DAPI, bright field and overlay channels, white arrow indicates the nuclei and yellow arrow targets the cytosol. The scale bar corresponds to 23 μm.

Fig. 7. Identification of the AtERD10/AtRAB18 heterodimer interaction using BiFC assay.

Schematic illustration of the BiFC constructs containing the ERD10 and RAB18 proteins assayed for a dimer interaction. (A) The pYFN43-AtRAB18/pYFC43-AtERD10 constructs. (B) The pYFN43-AtERD10/pYFC43-AtRAB18 vectors. (C-D) Fluorescence visualization of RAB18/ERD10 heterodimer formation in *N. benthamiana* leaves. From left to right: GFP, chlorophyll, DAPI, bright field and overlay channels, white arrow

indicates the nuclei and yellow arrows target the cytosol. The scale bar corresponds to 23 μm.

Fig. 8. BiFC analysis of heterodimeric interaction among the Arabidopsis dehydrins: AtCOR47, AtERD10 and AtRAB18 with their orthologue OpsDHN1 in tobacco cells. Diagrammatic illustration of the BiFC constructs AtCOR47, ERD10, RAB18 and OpsDHN1 dehydrins assayed for heterodimeric interactions. (A) The pYFN43-AtCOR47/pYFC43-OpsDHN1 vectors combination. (B) The pYFN43-AtERD10/pYFC43-OpsDHN1 vectors combination. (C) The pYFN43-AtRAB18/pYFC43-OpsDHN1 vectors combination. Confocal interaction analysis of the transient cotransformed of (D) AtCOR47/OpsDHN1constructs, (E) AtERD10/OpsDHN1 constructs, and (F) AtRAB18/OpsDHN1 constructs. From left to right: GFP, chlorophyll, DAPI, bright field and overlay channels, white arrow indicates the nuclei and yellow arrows target the cytosol. The scale bar corresponds to 23 μm.

Fig. 9. Proposed model for DHN dimerization in plant cells. Representation of homo- and heteromeric interaction among Arabidopsis dehydrins, and its interaction with the Opuntia streptacantha OpsDHN1 in tobacco cells. According to their localization results, the interactions AtCOR47/AtCOR47, AtCOR47/OpsDHN1 AtCOR47/AtERD10 and AtERD10/AtERD10, AtERD10/OpsDHN1 proteins only occur in the cytosol, and could be protecting proteins from damage caused by stress. Whereas, AtRAB18/AtRAB18 and AtRAB18/OpsDHN1 were observed in both, cytosol and nucleus, and could be protecting proteins from stress damage in both subcellular sites, or interacting with acid nucleics.

Supplementary Figure 1. GFP sub-cellular localization. (A) Schematic representation of the pMDC43 vector; GFP is under the control of the 2x35S promoter and NosT terminator. (B) GFP sub-cellular localization in tobacco epidermal leaf cells. From left to right: GFP, chlorophyll, DAPI, bright field and overlay panels. The white arrow indicates the nuclei and yellow arrow indicates the cytosol. The scale bar corresponds to 23 μm.

Supplementary Figure 2. Positive BiFC interaction controls. Schematic representation of BiFC vectors containing the *A. thaliana* SnRK kinases AKIN10 and AKINβ2 fused to N- or C-terminus of GFP. (A) The pYFN43-AKINβ2 and pYFC43-AKIN10 vectors. (B) The pYFN43-AKIN10 and pYFC43-AKINβ2 vectors. (C) Fluorescent analysis of pYFN43-AKIN10 and pYFC43-AKINβ2 and their swapped versions. From left to right: GFP, chlorophyll, DAPI, bright field and overlay channels, white arrow indicates the nuclei and yellow arrow indicates the cytosol. The scale bar corresponds to 23 μm.

Supplementary Figure 3. Interaction analysis between AtCOR47 and non-DHNs proteins. Schematic representation of BiFC constructs containing either AtCOR47 or the *A. thaliana* SnRK kinases AKIN10 and AKINβ2 fused to the N- or C-terminus GFP. (A) Co-transformed combination of the pYFN43-AKINβ2/pYFC43-AtCOR47 constructs. (B) Co-transformed pYFN43-AtCOR47/pYFC43-AKIN10 vectors. (C) Fluorescent analysis of AKINβ2/AtCOR47 and AtCOR47/AKIN10. Each combination was transiently co-transformed in *N. benthamiana* epidermal cells. From left to right: GFP, chlorophyll, DAPI, bright field and overlay channels, white arrow indicates the nuclei. The scale bar corresponds to 23 μm.

Supplementary Figure 4. Interaction analysis between ERD10 and non-DHNs proteins. Schematic representation of BiFC vectors containing either ERD10 or the *A. thaliana* SnRK kinases AKIN10 and AKINβ2 fused to the N- or C-terminus of GFP. (A) Co-transformed combination of the pYFN43-AKINβ2/pYFC43-ERD10 constructs. (B) Co-transformed pYFN43-ERD10/pYFC43-AKIN10 vectors. (C) Fluorescent analysis of AKINβ2/AtERD10 and AtERD10/AKIN10, each combination was transient co-transformed in *N. benthamiana* epidermal cells. From left to right: GFP, chlorophyll, DAPI, bright field and overlay channels, white arrow indicates the nuclei. The scale bar corresponds to 23 μm.

Supplementary Figure 5. Interaction analysis between RAB18 and non-DHNs proteins. Schematic representation of BiFC vectors containing either RAB18 or the *A. thaliana* SnRK kinases AKIN10 and AKINβ2 fused to the N- or C-terminus of GFP. (A) Co-transformed combination of the pYFN43-AKINβ2/pYFC43-AtRAB18 constructs. (B)

Co-transformed pYFN43-ERD10/pYFC43-AKIN10 vectors. (C) Fluorescent analysis of AKINβ2/AtRAB18 and AtRAB18/AKIN10, each combination was transient co-transformed in *N. benthamiana* epidermal cells. From left to right: GFP, chlorophyll, DAPI, bright field and overlay channels, white arrow indicates the nuclei. The scale bar corresponds to 23 μm.

Supplementary Figure 6. Heterodimeric interactions between the swapped version of Arabidopsis dehydrins AtCOR47, ERD10, and RAB18 with their orthologue OpsDHN1 from cactus pear. Diagrammatic illustration of the BiFC constructs AtCOR47, ERD10, RAB18 and OpsDHN1 dehydrins assayed for heterodimeric interactions. (A) The pYFN43-OpsDHN1/pYFC43-AtCOR47 vectors combination. (B) The pYFN43-OpsDHN1/pYFC43-AtERD10 vectors combination. (C) The pYFN43-OpsDHN1/pYFC43-AtRAB18 vectors combination. Confocal interaction analysis of the transient cotransformed (D) OpsDHN1/AtCOR47 constructs (E) OpsDHN1/ERD10 constructs and (F) OpsDHN1/RAB18 constructs. From left to right: GFP, chlorophyll, DAPI, bright field and overlay channels, white arrow indicates the nuclei and yellow arrows target the cytosol. The scale bar corresponds to 23 μm.

Figures 1-8

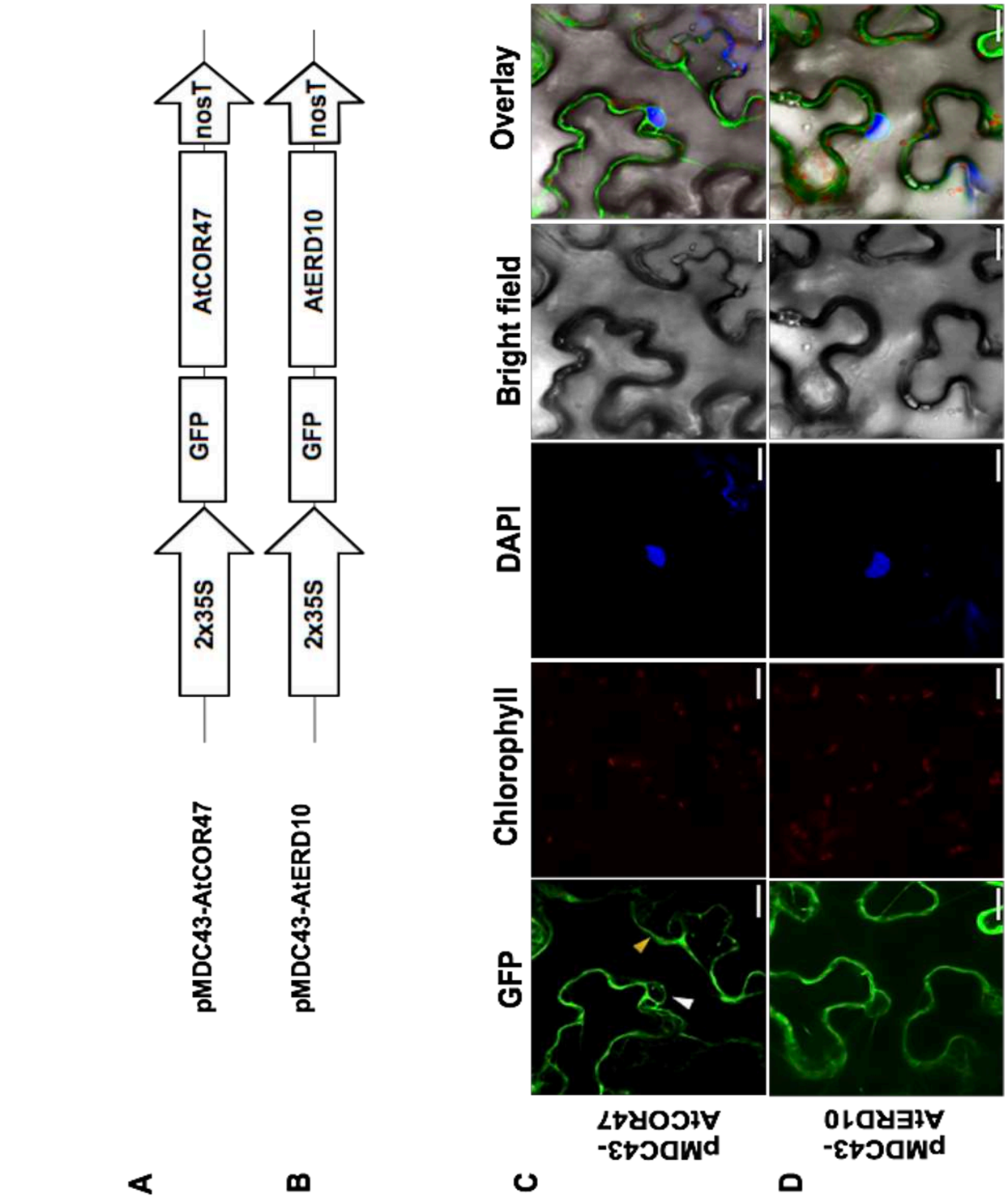

Fig. 1

Fig. 2

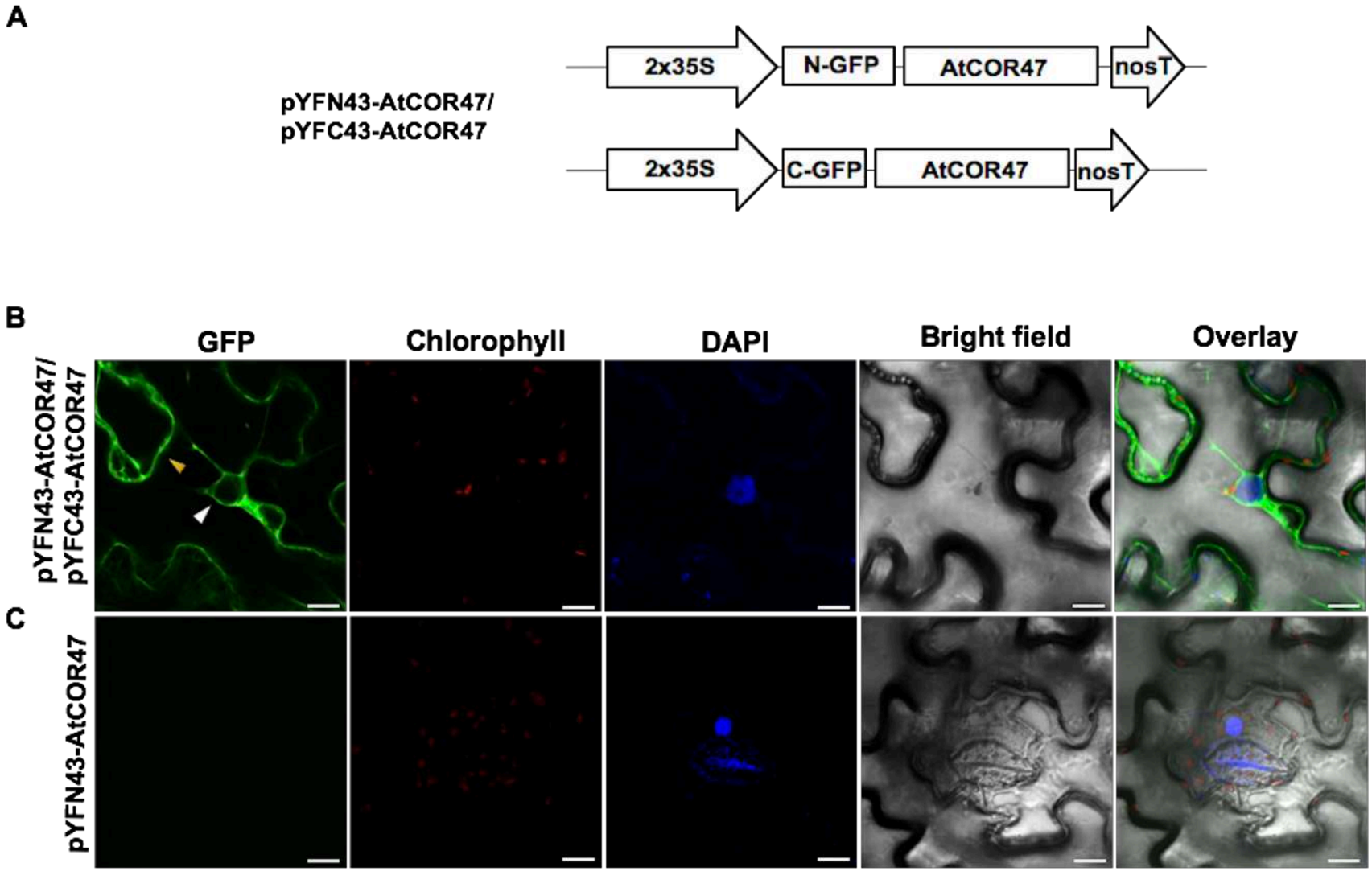
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Fig. 4

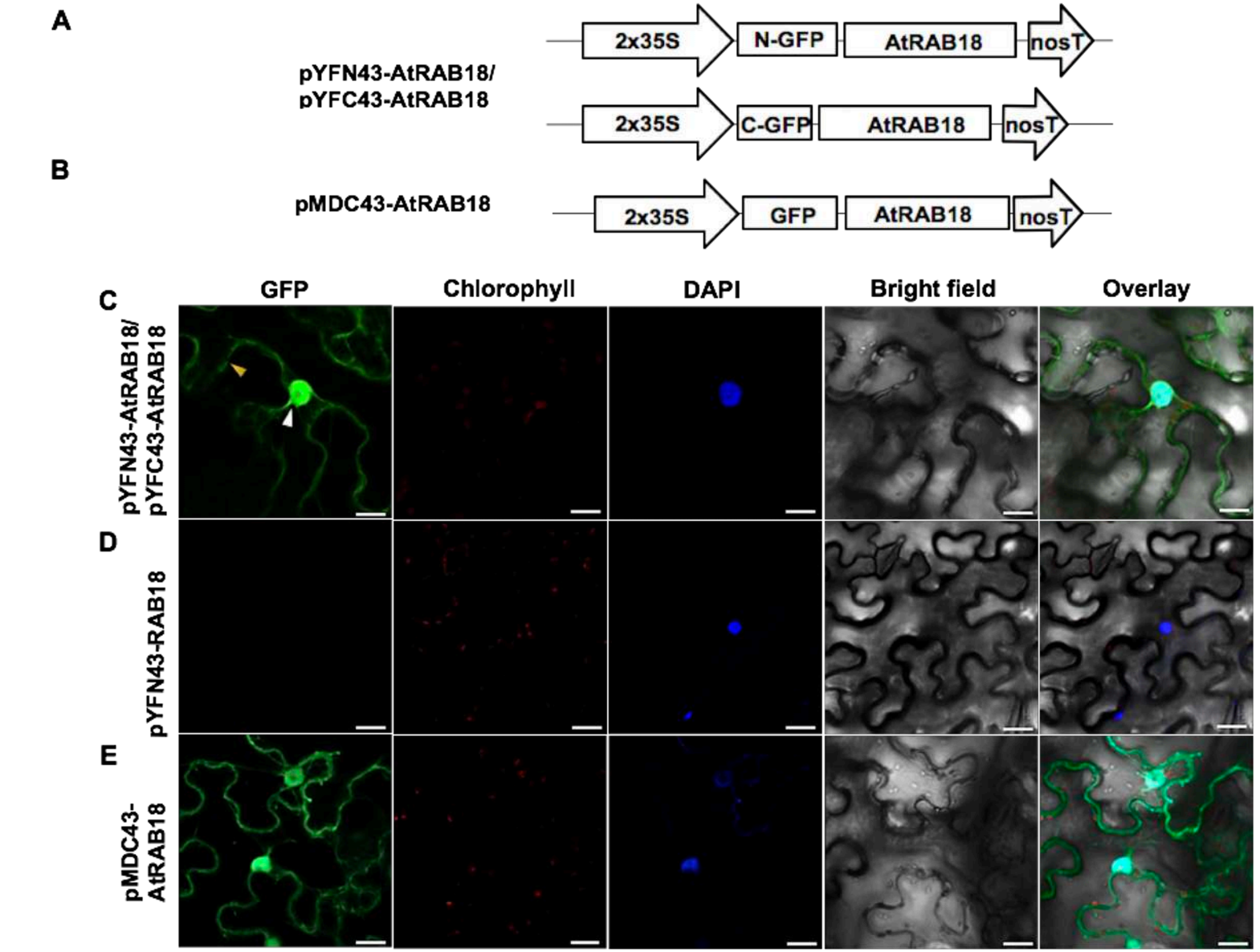
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Fig. 5

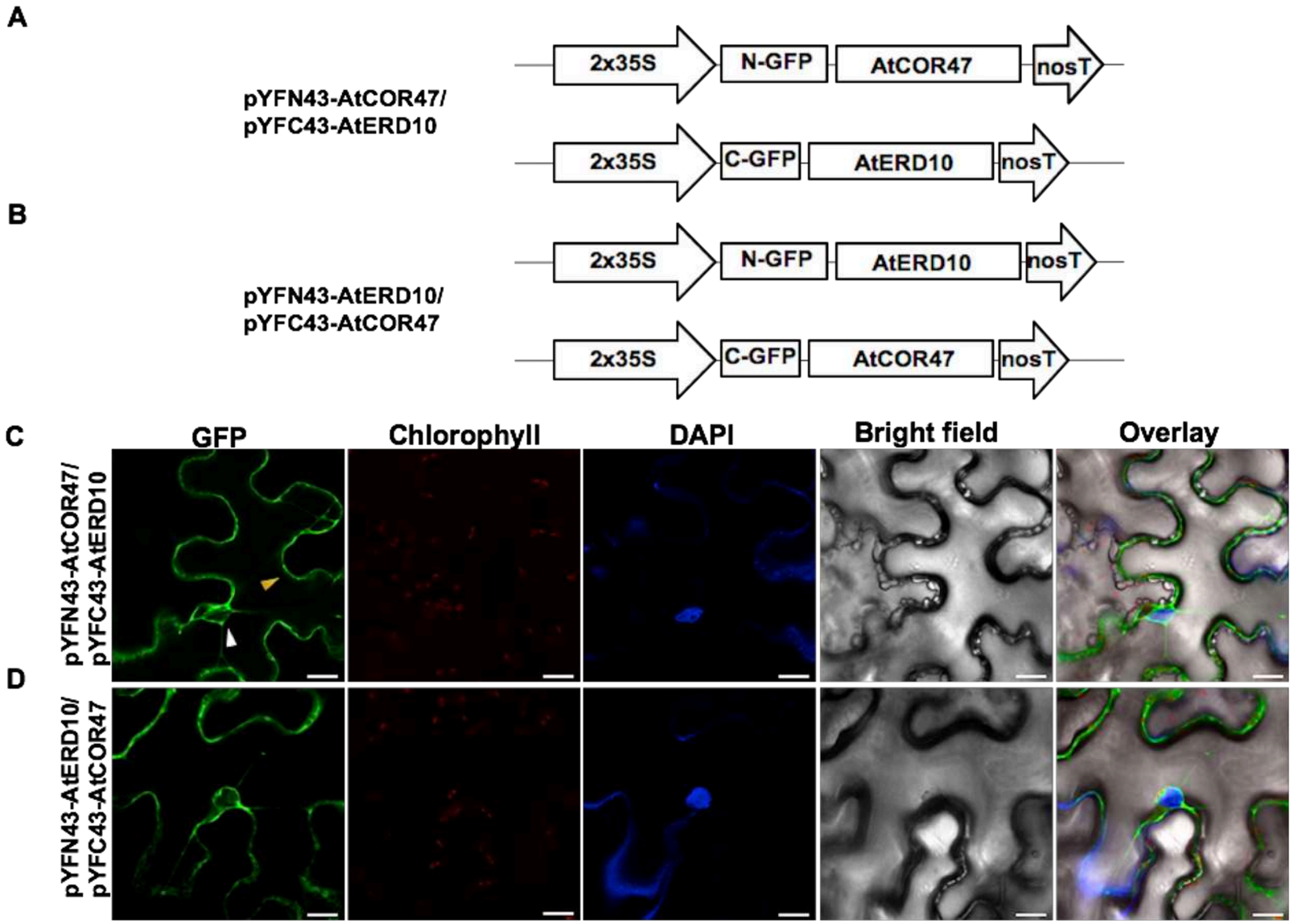
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Fig. 6

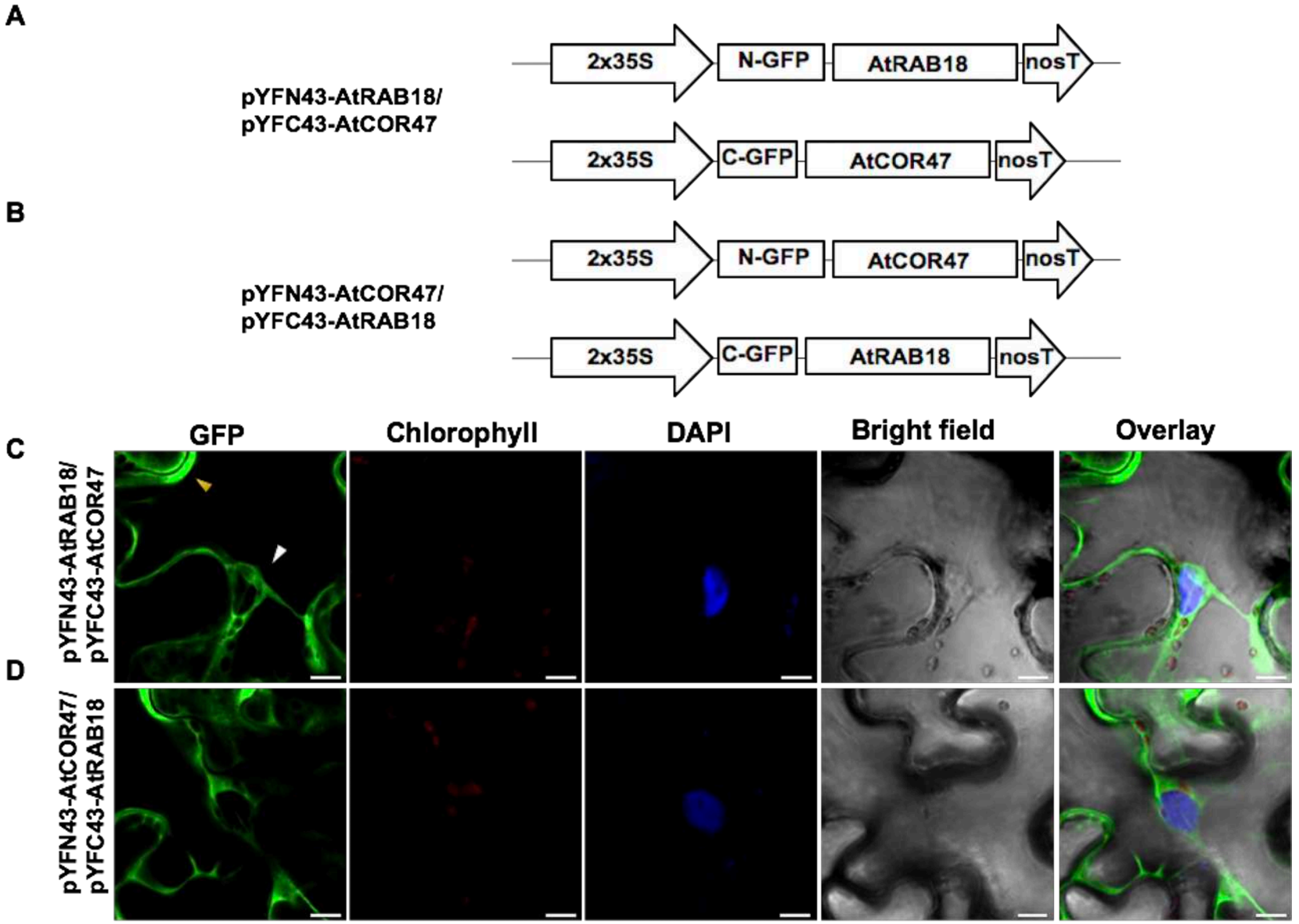
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Fig. 7

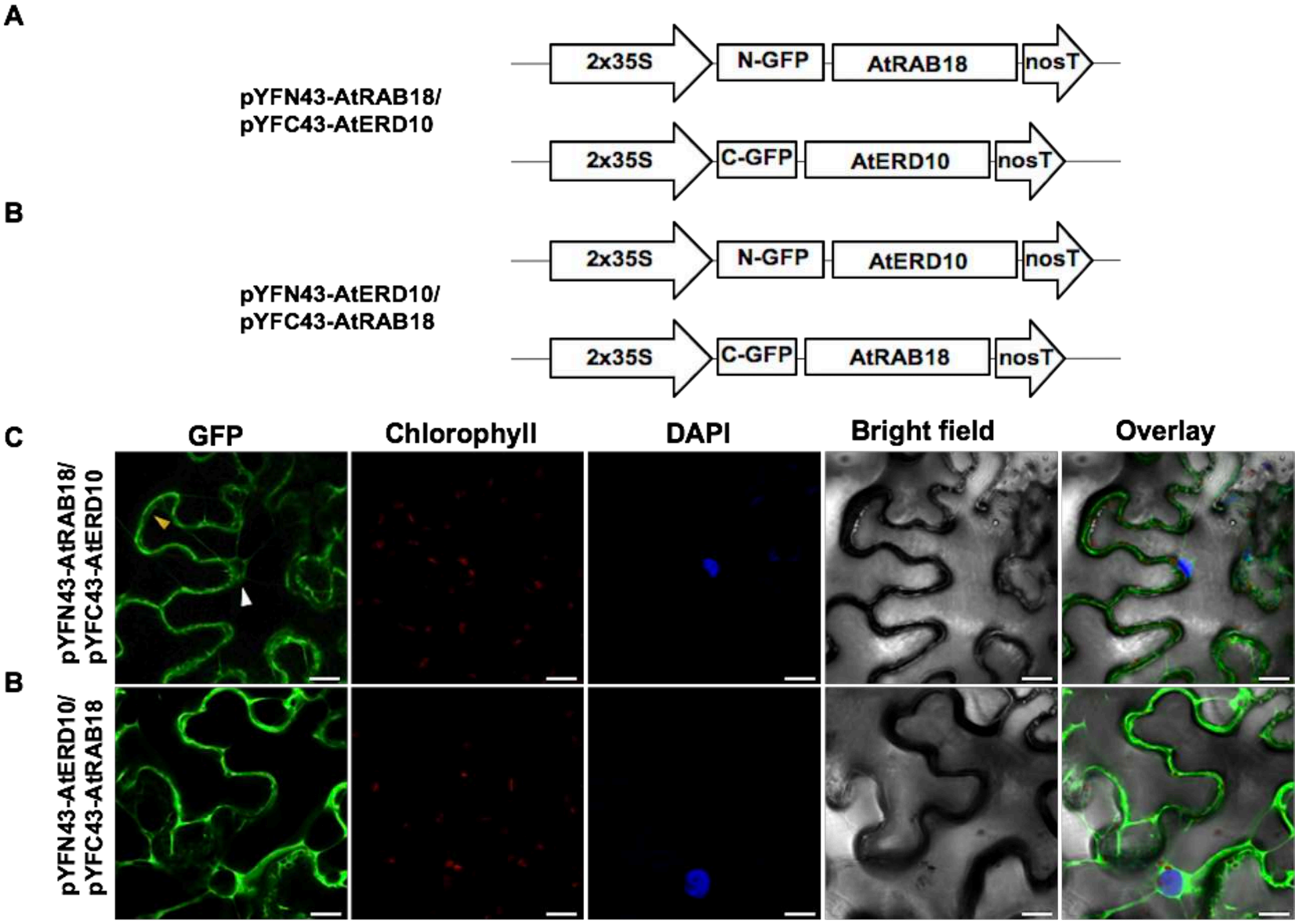
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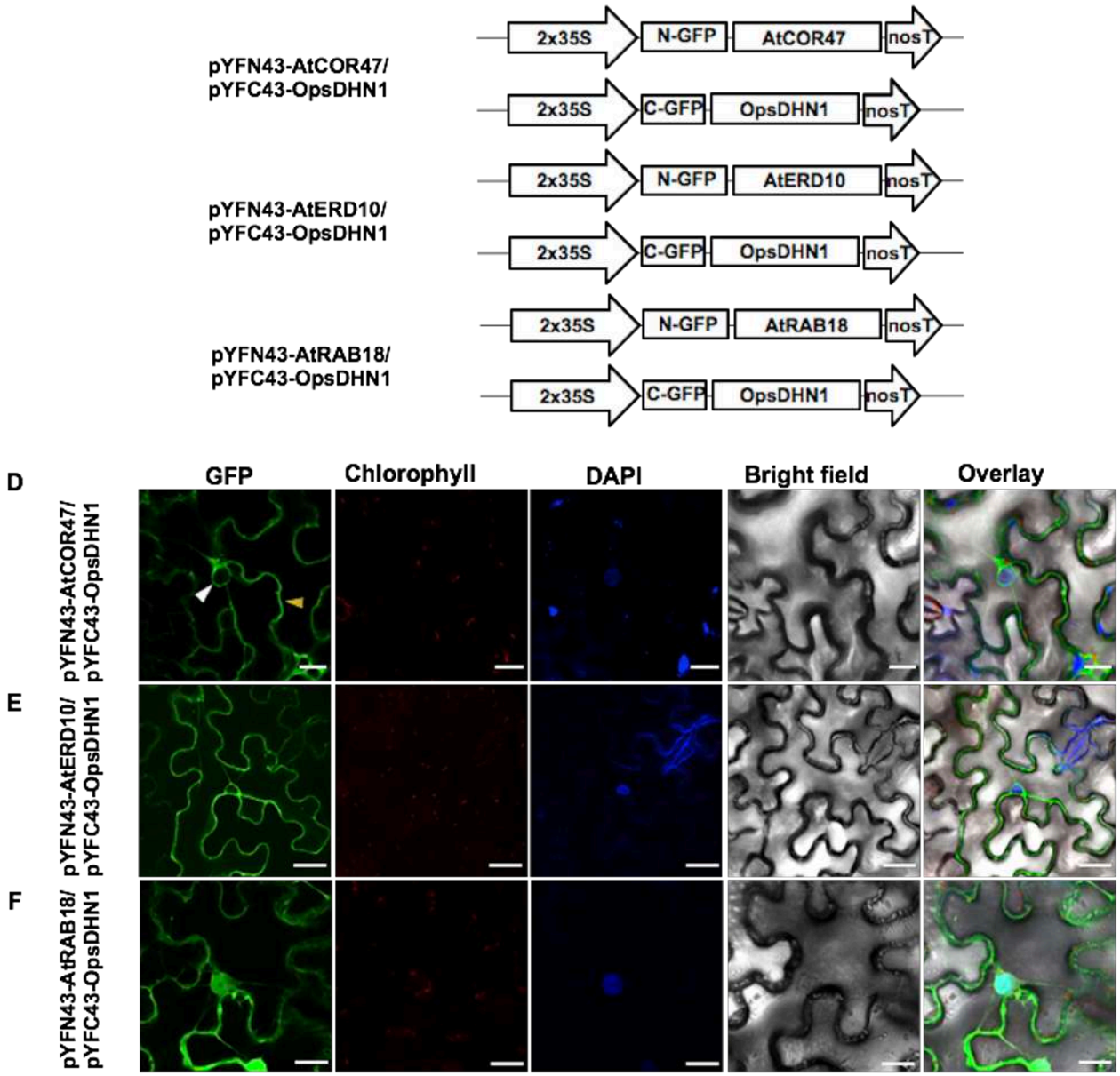
Fig. 8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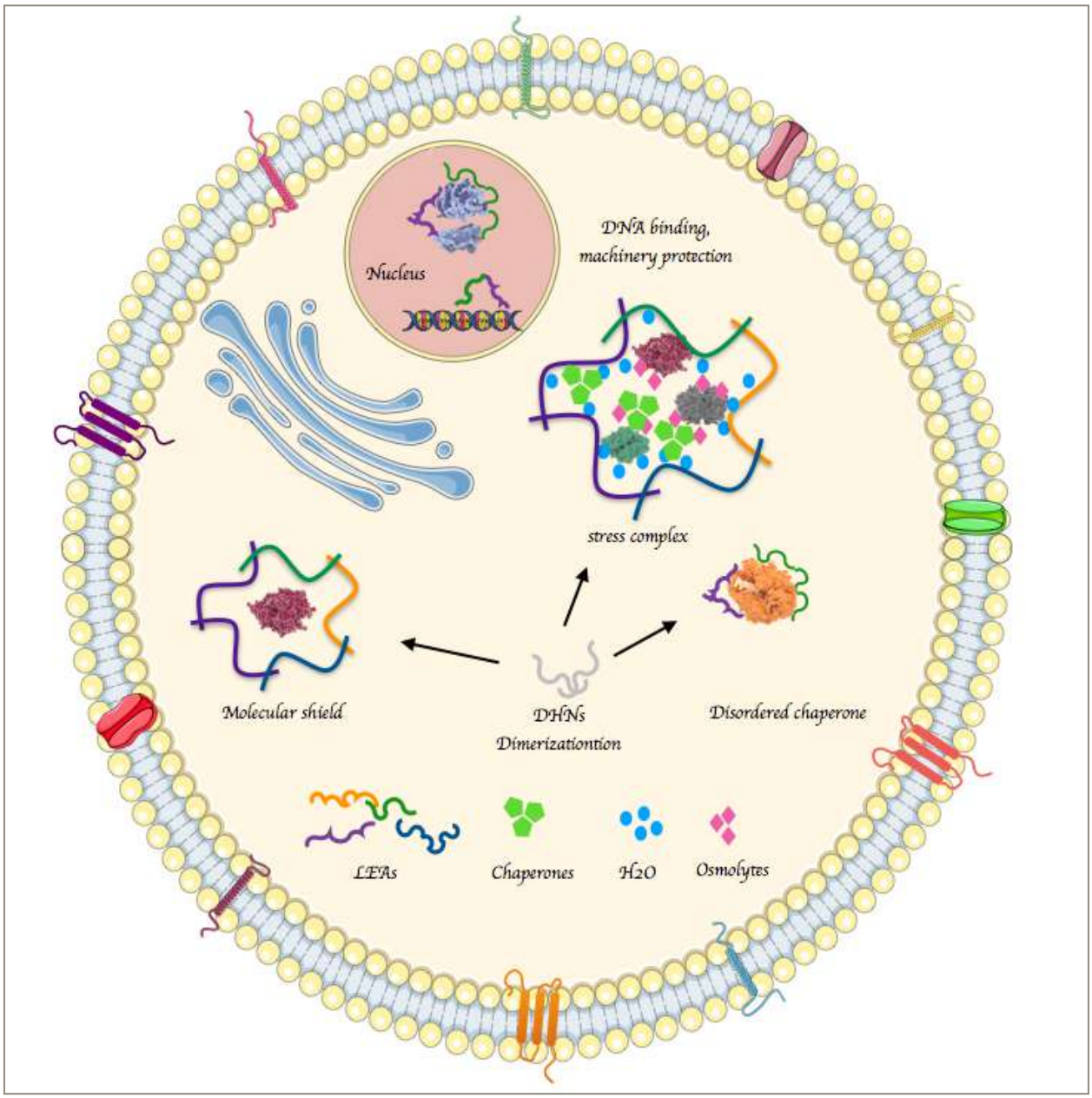
V. Concluding remarks and perspectives

This thesis contributes significantly to the DHN field since our data about the OpsDHN1-OpsDHN1 interaction suggest some clues as to how these proteins function within the plant cell. We propose that DHNs could function through the formation of large multimeric complexes with other DHNs, forming a "molecular shield" around their biological targets, capable of stabilizing partially denatured proteins. Most biological processes depend on the interactions between proteins, so the study of the *in vivo* interaction among DHNs will provide interesting data about its mechanism of action. In this sense, the study of DHNs interactions and subcellular localization is a central part of understanding the molecular mechanism by which these proteins could work. The molecular shield and the disordered chaperone are the two proposed models for DHN function with respect to protecting proteins from abiotic stress damage. In both scenarios, DHN assembly into homo- and heterodimers could offer the a combinatorial effect producing multiple stress complexes with different affinity and specificity to their biological targets, giving the cell an instrument for fine-tuning the stress response. Additionally, the study of the segments present in DHNs could help to elucidate the relationship between domains and their corresponding physiological roles. In spite of several *in vitro* attempts that have been conducted using deletion mutants, a more refined approach is necessary; the use of point mutants in specific residues of the conserved segments could help to establish physiological differences between WT and mutant DHNs. These data will allow DHN optimization for a specific stress response. Also the generation of knowledge about the *in vivo* function of conserved segments present in DHN sequences eventually leads to the development of cold-resistant genotypes using genetically engineered DHNs.

Figure 5. Proposed model of action for DHNs. The interactions between proteins are



fundamental to almost all biological processes. In the molecular shield and the disordered chaperone models the interaction among DHNs, LEA proteins, other stress response proteins and indeed some osmolytes, could offer a combinatorial effect producing stress complexes with different affinity and specificity to their biological targets, giving the cell an instrument for fine-tuning the stress response.

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