# LEA proteins and the evolution of the WHy domain

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## Abstract

The LEA family is composed of a diverse collection of multi-domain and multi-functional proteins, found in all three Domains of the Tree of Life, but particularly common in plants. Most members of the family are known to play an important role in abiotic stress response and stress tolerance in plants, but are also part of the plant hypersensitive response to pathogen infection. The mechanistic basis for LEA protein functionality is still poorly understood. The group of LEA 2 proteins harbour one or more copies of a unique domain, the Water stress and Hypersensitive response (WHy) domain. This domain sequence has recently been identified as a unique ORF in some bacterial genomes (mostly in the phylum Firmicutes), and the recombinant bacterial WHy protein has been shown to exhibit a stress tolerance phenotype in *E. coli* and an *in vitro* protein denaturation protective function. Multi-domain phylogenetic analyses suggest that the WHy protein gene sequence may have ancestral origins in the Domain Archaea, with subsequent acquisition in Bacteria and Eukaryotes via endosymbiont or Horizontal Gene Transfer mechanisms.

## Introduction – abiotic stress response in different organisms

Liquid water loss is one of the most life-threatening abiotic stress conditions, as it negatively affects all biological functions. It may lead to dsDNA breaks and oxidative lesions, damage to RNA, protein aggregation, cell shrinkage and various other deleterious molecular and metabolic changes (Harb et al., 2010, Kriško et al., 2010, Seki et al., 2007). However, organisms in all kingdoms of life have developed mechanisms for resisting or compensating for the effects of desiccation, by either prevention of intracellular water-loss or repair of desiccation-linked damage (Hanin et al., 2011, Kriško et al., 2010). Interestingly, both freezing and hypersaline stresses are related to dehydration stress, in that each leads to a state of low intracellular water potential ($a_w$) (Verslues et al., 2006), despite the fact that physical cause of the low intracellular $a_w$ status differs for each imposed condition: i.e.,

external low %RH atmosphere (aridity-induced desiccation), osmotic imbalance (hypersalinity), or an intracellular water phase transition (freezing). In the latter case, not only is the remaining intracellular water incapable of supporting normal physiological processes (Pearce, 2001), but the formation of ice crystals in intracellular spaces can physically damage the integrity of cell vessel (Kosová et al., 2014).

One of the freeze-tolerance mechanisms evolved in plants is the synthesis of intracellular antifreeze proteins (AFPs) which inhibit ice crystal growth and recrystallization. AFPs are found in a wide range of overwintering plants, including economically important crop species such as winter rye, *Secale cereal* (Griffith & Yaish, 2004). Plant AFPs have multiple, hydrophilic protein domains which bind irreversibly to ice surfaces and inhibit further ice growth by decreasing the freezing temperature of the surrounding solution (Atıcı & Nalbantoğlu, 2003, Griffith & Yaish, 2004).

The synthesis of the osmotically active Late Embryogenesis Abundant (LEA) proteins is one of the better-known mechanisms involved in organismal protection against abiotic stresses, including cold- and desiccation-stresses (Beck et al., 2007, Cao & Li, 2015, Hanin et al., 2011, Sharma & Laxmi, 2015, Verslues et al., 2006). LEA proteins were first identified in cotton (*Gossypium hirsutum*) as proteins that accumulated during the late maturation stages of seed development (Dure III et al., 1981). Extensive research for over three decades has demonstrated that the accumulation of the hydrophilic LEA proteins is not only restricted to embryonic tissues, but is prevalent in vegetative plant tissues under water deficit conditions (Cao & Li, 2015, Honjoh et al., 1995, Liang et al., 2013, Saavedra et al., 2006, Sharma et al., 2016, Singh et al., 2005, Stacy & Aalen, 1998, Wang et al., 2007). Although the function of many LEA proteins are not fully understood, the consensus is that members of this protein family play an important role in organismal stress tolerance, particularly in dehydration and cold stress, by acting as chaperones to protect other cell proteins and membrane structures (Bravo et al., 2003, Hara et al., 2001). LEA proteins have been found in numerous organisms across a wide taxonomic and evolutionary spectrum, including bacteria, yeasts,

plants and vertebrates (Battaglia et al., 2008, Hanin et al., 2011, Sharma et al., 2016, Tunnacliffe & Wise, 2007).

## Classification of LEA proteins

LEA proteins are present in many organisms but neither their structures nor their functional mechanisms are fully understood, leading to (rather unhelpful) references to this protein family as a 'continuing conundrum' (Tunnacliffe & Wise, 2007) or 'enigmatic' (Battaglia et al., 2008). The classification of LEA proteins is equally controversial. The original grouping of LEA proteins is based on common structural features, first identified in the prototypical cotton plant *(G. hirsutum*) (Dure III et al., 1981) but subsequent alternative classifications have led to large inconsistencies with respect to the original taxonomy (Shih et al., 2008). LEA proteins have been variously assigned to three major groups, associated with their taxonomic origins; i.e., plants, bacteria and vertebrates (Tunnacliffe & Wise, 2007), while other classifications yield five (Shih et al., 2008) or seven (Battaglia et al., 2008) major groups, with nine to fourteen LEA sub-groups (Anderson et al., 2015, Ciccarelli & Bork, 2005, Jaspard & Hunault, 2014, Singh et al., 2005). The different classification structures have been based on the analysis of transcripts (Galau et al., 1986, Hughes & Galau, 1991), amino acid sequences and conserved motifs (Bies-Etheve et al., 2008, Bray, 1994, Jaspard & Hunault, 2014), three-dimensional protein structures or chemical characteristics, including *in silico* analyses of 'Protein or Oligonucleotide Probability Profiles' (POPP) (Bies-Etheve et al., 2008, Bray, 1994, Ciccarelli & Bork, 2005, Cuming, 1999, Dure et al., 1989, Jaspard & Hunault, 2014, Shih et al., 2008, Singh et al., 2005, Tunnacliffe & Wise, 2007, Wise & Tunnacliffe, 2004). Despite the different classification strategies for LEA proteins, most primary structures share similar biophysical features, most prominently the high levels of hydrophilicity (Baker et al., 1988, Dure III et al., 1981). Using database searching, it has been shown that the criteria of a Gly content greater than 6% and a hydrophilicity index of

greater than 1 includes most LEA proteins in the more widespread group of *hydrophilin* proteins (Battaglia et al., 2008).

The most well characterized group of these highly hydrophilic proteins is the LEA group 2; sometimes referred to as LEA 14 (Battaglia et al., 2008) (also, confusingly, associated with group 4 (Hong-Bo et al., 2005)). However, the LEA group 2 proteins include the functionally important dehydrin proteins (Graether & Boddington, 2014). A range of abiotic stress conditions, including drought, cold and salinity stresses, are known to up-regulate dehydrin gene expression and dehydrin protein levels (Graether & Boddington, 2014). For example, the expression of LEA 5 and LEA 14 (cDNA D95) was highly induced in mature leaves of water-stressed plants (Galau et al., 1993).

**Structure, biochemistry and function of the LEA 14 proteins**

LEA 14 proteins all contain a conservative N-terminal sequence and form amphipathic α-helical structures (Hong-Bo et al., 2005). NMR microscopy of LEA 14 showed the presence of a αβ-fold consisting of one α-helix and seven β-strands that form two antiparallel β-sheets (Singh et al., 2005). The first high resolution three-dimensional structure of a LEA protein LEA 14 isolated from *Arabidopsis thaliana* was described in Singh et al., 2005 (Singh et al., 2005). This structure was later confirmed for the LEA 14 protein from the rubber tree (*Hevea brasiliensis*), that also showed a single α-helix and seven β-sheet configuration (Zou et al., 2013).

In plant tissues, LEA proteins are expressed constitutively, at low but varying levels, through all developmental stages but with no obvious tissue specificity (Hong-Bo et al., 2005). However, these levels may be greatly up-regulated in response of imposed stresses. For example, LEA 14 expression was found to be strongly induced by dehydration and NaCl and abscisic acid treatments, in sweet potato (*Ipomoea batatas)* plants (Park et al., 2011). Quantitative RT-PCR also revealed a variety of different *I. batatas* LEA14 expression

patterns under various abiotic stress conditions. Stress-induced up-regulated expression of LEA 14 also induced secondary phenotypic changes in fibrous sweet potato roots, particularly by enhanced lignification (Park et al., 2011). LEA proteins expression is also upregulated as part of the plant hypersensitive response, activated by microbial infections (Ciccarelli & Bork, 2005). After infection by *Aspergillus flavus* and *Aspergillus parasiticus*, maize (*Zea mays L.*) showed, amongst others, up-regulated expression of LEA 3 and LEA 14 proteins (Chen et al., 2002).

## The WHy domain - a LEA 14 family member

### Structure and function of the WHy domain

A number of protein families, particularly the Hin1, LEA 8 and LEA 14 proteins, contain a unique domain: the Water stress and Hypersensitive response (WHy) domain. The WHy domain was so named simply because it was detectable in proteins expressed during the response to desiccation (Ciccarelli & Bork, 2005). Public databases (NCBI, emble, etc.) show long protein sequences (300 - 615 aa) with multiple WHy domains (each of 92 - 140 aa) for many plants (e.g. *Arabidopsis thaliana* NP_181934.1, *Malus domestica* XP_008394249.1) and archaea (e.g. *Methanotorris igneus* WP_013799711.1, *Archaeoglobus veneficus* WP_013683559.1).

The WHy domain is typically 100-165 amino acid (aa) long and approximately 18.6 kDa in size (Anderson et al., 2015, Ciccarelli & Bork, 2005, Jaspard & Hunault, 2014). The domain sequence is composed of alternating hydrophobic and hydrophilic residues with an invariant NPN motif near the N-terminus with a secondary structure, typical for members of the LEA 14 family, which mostly consists of a β-strand with a C-terminal α-helix (Singh et al., 2005, Zou et al., 2013).

It has been shown that the hydrophilins, LEA proteins, dehydrins and the WHy domain all confer protection against dehydration, possibly through similar mechanisms. In all cases,

these proteins appear to bind to cellular structures (such as proteins) and to reduce denaturation and inactivation by acting as ‘molecular shields’: either by direct binding to protein surfaces and replacement of coordinated water (Close, 1997, Hoekstra et al., 2001, Reyes et al., 2005) or by ordering water molecules around the associated macromolecules (Reyes et al., 2005). The former represents a well-established mechanism of water-driven entropic stabilisation (Rodriguez-Ropero & van der Vegt, 2014) where the entropy of the protein-protein system ($S_{(protein)}$ + $S_{(H_2O)}$) is greater than that of the free protein and/or the denatured protein. The direct binding of hydrophilins to target proteins has been demonstrated by protein-protein cross-linking studies (Reyes et al., 2005).

Interestingly, LEA class 2 proteins and hydrophilins, which exhibit intrinsic structural disorder in solution, also show a cryoprotective effect in freeze-thaw cycles *in vitro*. The degree of protection appears to rely on both the flexible protein structure and the hydrophilic characteristics of the conserved domains (Hughes et al., 2013, Reyes et al., 2008, Reyes et al., 2005).

It has also been shown that dehydrins are able to bind strongly to negatively charged membranes (Graether & Boddington, 2014). This is thought to be due to the α-helical structure of dehydrins and the exposure of ionic side chains, which interact electrostatically with the negatively charged membrane lipids (Koag et al., 2003, Soulages et al., 2003). However, dehydrins also bind both water and ions, acting as buffers during desiccation (Tompa et al., 2006).

The up-regulated expression of this the WHy protein, and proteins containing this domain, during both abiotic stress and pathogen infection argues for a shared mechanism to these two different stress conditions (Ciccarelli & Bork, 2005). A recent *in vitro* study, demonstrating that the recombinant WHy domain protein conferred protection to *E. coli* against freeze-thaw cycle damage (Anderson et al., 2015), suggesting that this ‘domain’ has a very broad stress-response function.

**The WHy protein in Prokaryotes**

Genes encoding a WHy domain protein homologue have recently been identified in both bacteria (e.g. *Pseudomonas, Burkholderia*) and archaea (e.g., *Haladaptatus, Halosimplex*) (see Figure 1) (Anderson et al., 2015, Ciccarelli & Bork, 2005). In bacteria, these genes usually encode a single WHy domain homologous to the LEA 2 superfamily sequence, typically of around 100 aa.

While the primary structure of the bacterial WHy protein structure typically includes non-homologous N-terminal (20 to 38 aa) and C-terminal (26 to 44 aa) sequences, the protein structures of the multi-domain constructs in plants and archaea show inconsistent numbers of amino acids at the flanking termini (Figure 2). An analysis of up- and down-stream sequences (data not shown) for multiple *WHy* homologues suggest that WHy-containing protein genes do not appear be part of any obvious functional island, but are randomly located within the bacterial genomes. A similar random location of the WHy protein gene is evident in both plant and archaeal genomes.

**Revisiting the evolutionary history of the Why domain**

Proteins containing the WHy domain have been reported to be widespread in the genomes of archaea, bacteria and plants, but, are apparently absent from fungal and animal genomes. The current hypothesis on the evolutionary origin of this domain postulates that WHy domain-containing proteins originated in plants and that the prokaryotes acquired the Why-encoding gene via horizontal gene transfer in two separate events (i.e., for archaea and bacteria (Ciccarelli & Bork, 2005)). This hypothesis was based on the premise that proteins containing WHy domains are a part of the hypersensitive response system, activated in plants after microbial infection, and that the prokaryotic distribution of the WHy domain is

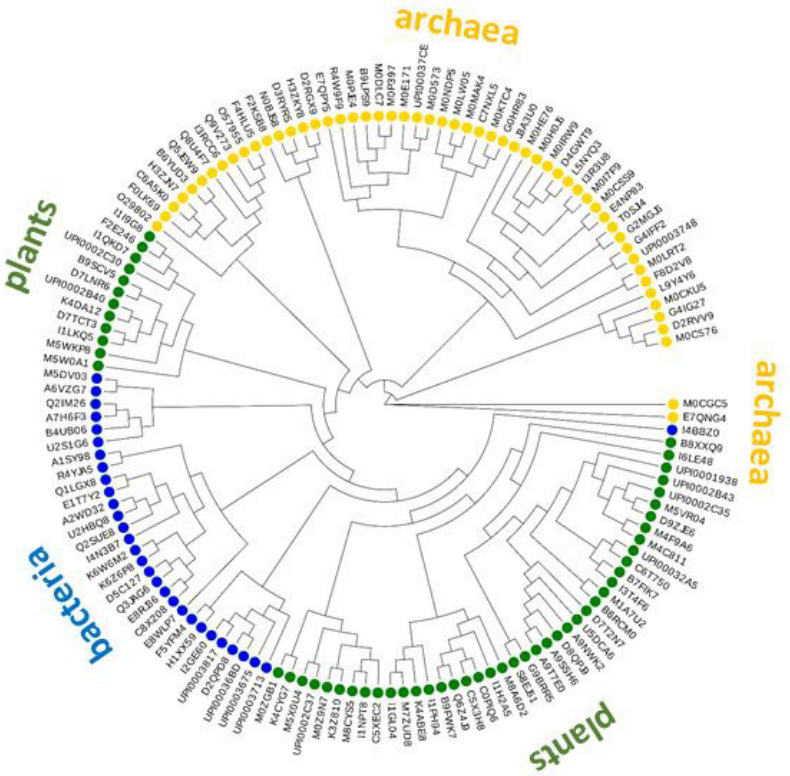


**Figure 1**
Phylogenetic relationship among 138 WHy domain-containing protein sequences from the three domains of life. The blue, gold and green circles represent the sequences of Bacteria, Archaea and Eukaryotes (plants), respectively. The maximum-likelihood tree was generated using RAxML (Stamatakis, 2014) based on LG substitution model predicted using PhyML-SMS (Lefort et al., 2017). The tree was visualised using Evolview v2 (He et al., 2016).

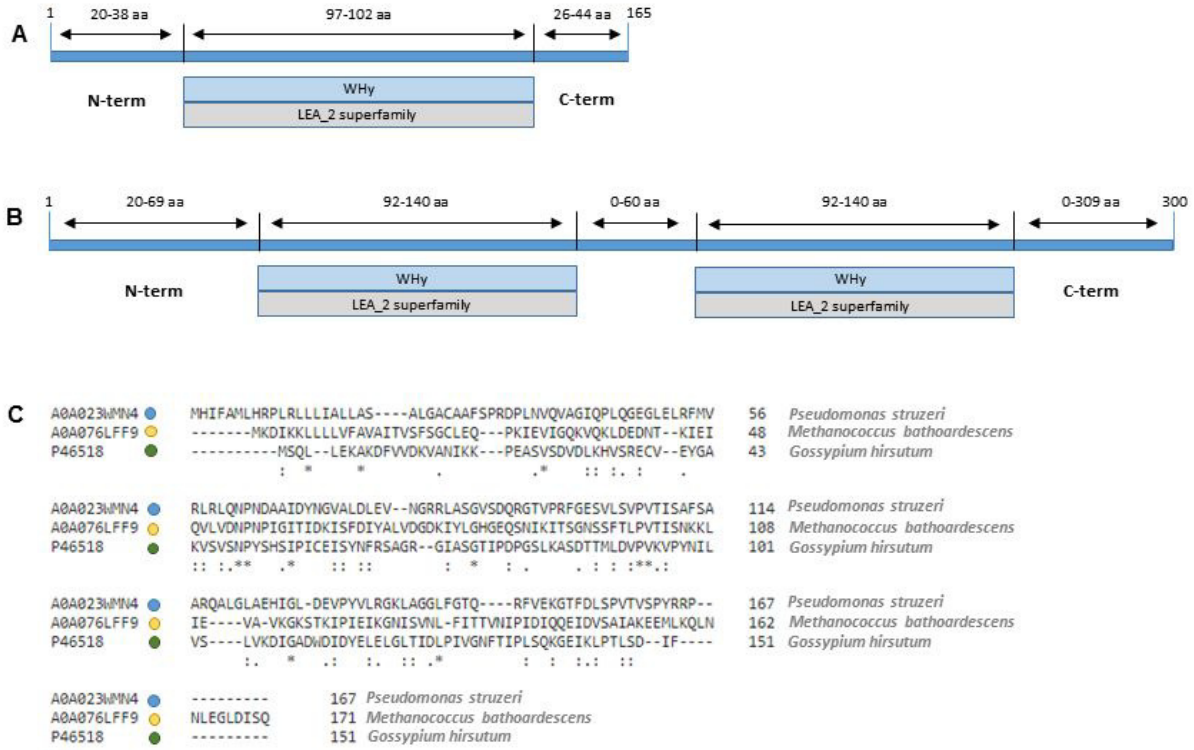


**Figure 2**
**Schematic structure of the WHy domain-containing protein.**
a) Bacterial protein containing one WHy domain and an N- and C-terminus. Multi domain-containing WHy protein in plants and archaea with inconsistent termini sizes and numbers.

dominated by plant pathogenic or symbiotic species, such as *Pseudomonas* and *Burkholderia,* which may have acquired the protein as a mechanism to allow the prokaryotic symbiont to evade the plant hypersensitive response system (Ciccarelli & Bork, 2005). It was also postulated that the presence of WHy-containing Hin1 gene in the green alga *Chlamydomonas reinhardtii* (AV395132) represents further support for plant origin of the domain, in addition to the restriction of the domain to plant pathogenic or symbiotic species (Ciccarelli & Bork, 2005). However, a SMART search (Letunic et al., 2014) of the translated protein (AV395132) above and the putative_Hin1_116192 protein (AT1G32340.1) of *Chlamydomonas reinhardtii* showed that neither of these proteins contain homologues of any known WHy domain.

In order to determine the possible origin of the Why domain within the three domains of life, 709 protein sequences containing the domain were obtained via SMART (Letunic et al., 2014). Of these, 138 non-redundant proteins (Supplementary Table S1), selected based on similarity threshold of 98% using JelView 2 (Waterhouse et al., 2009), were used to reconstruct the possible ancestry of the WHy domain using the FastML web server (Ashkenazy et al., 2012).

Contrary to earlier hypotheses (Ciccarelli & Bork, 2005), our phylogeny suggests that the WHy domain most likely originated among the archaea. The archaeal protein sequences M0CGC5 and E7QNG4 from *Haladaptatus paucihalophilus* and *Halosimplex carlsbadense* (Figure 1, Supplementary Table 2) were predicted as the most ancient of the WHy domain containing sequences included in the analyses. The phylogeny of the WHy domain containing proteins reconstructed using RAxML (Stamatakis, 2014) suggests that plants may have initially acquired the domain from archaea, and subsequently via bacterial lineages. Although the first lateral gene transfer events most likely occurred between archaea and plants, the possibility of subsequent horizontal gene transfers between the three domains is evident from the tree topology (Figure 1).

The mechanism of the horizontal gene transfer (HGT) process, potentially underlying the distribution of the WHy domain within plant and bacterial taxa, might be explained by endosymbiotic theory (Zimorski et al., 2014). We note that endosymbiotic theory suggests that the earliest Eukarya, anaerobic mastigotes, may have originated from permanent whole-cell fusions between archaea (e.g., *Thermoplasma*-like organisms) and eubacteria (e.g., *Spirochaete*-like organisms) (Margulis, 1996). Such a mechanism provides a LGT pathway which is compatible with our suggestion of an ancestral origin of the WHy gene in archaea, and with the subsequent proliferation of the gene product (as a domain in larger protein constructs) in plant proteins.

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