Minireview

Structural characterisation of late embryogenesis abundant proteins in *Ramonda serbica* Panč.

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Summary. Ressurection plants are extraordinary because of their ability to withstand long periods without water, enter a state of anhydrobiosis, and fully recover upon water arrival. *Ramonda serbica* Panč. is a relic and endemic species that belongs to a very small group of desiccation-tolerant plants in Europe. Underlying physiological, molecular and morphological mechanisms that enable these plants to survive harsh environmental conditions have been an appealing subject of many researchers. Most of the genes responsible for this amazing ability are present in other plants, and research of those genes which could be activated in crops is growing much more attention because of the imminent crisis regarding food supplies in the near future. Key components involved in the response to dehydration in *R. serbica* plants were analysed through a comprehensive transcriptomic, proteomic, metabolite and photosynthetic study. Late embryogenesis abundant proteins play a significant role in the complex defence processes involved in desiccation tolerance. Defining LEAPs physicochemical characteristics and specific physiological functions may lead us to their applicability in other areas of research.

Keywords: dessication-tolerant, intrinsically disordered proteins, LEA proteins, *Ramonda serbica*, secondary structure prediction.

INTRODUCTION

The group of plants called resurrection plants are known for their tolerance to desiccation, i.e. extreme dehydration. They can lose up to 95% of their cellular water, remain in a state without water for prolonged periods that can last for months to years, and fully recover their entire physiological activity upon water arrival (Hilhorst and Farrant 2018; Oliver et el. 2020; Gechev et el. 2021). Resurrection plants are equipped to withstand extreme adverse environmental conditions, and they are very rare, only about 300 flowering species belong to this special group (Gechev et al. 2021). These plants are exceptionally good models for investigating vegetative desiccation tolerance. Despite numerous studies addressing this phenomenon, underlying mechanisms of desiccation tolerance are yet not fully elucidated (Tebele et al. 2021). It is very important to note the fundamental difference between desiccation-tolerant and drought-tolerant, and these two concepts should not be misinterpreted. Desiccation is represented by such low water potential that is in equilibrium with low water air potential, while drought is induced by slighter water loss which leads to deficit induced stress (Oliver et al. 2020).

The drop of water potential caused by lack of water sets in motion a wide array of processes which have consequences such as the pressure of oxidative stress with the escalating production of reactive oxygen species (ROS). Two opposing strategies in terms of defence from ROS, which are generated more during dehydration and subsequent rehydration, are found among these plants while dealing with water deficit. Poikilochlorophyllous species are able to dismantle their pho-

tosynthetic apparatus during desiccation and degrade chlorophyll, thus possibly reducing the effects of photooxidative destruction. Homoiochlorophyllous species instead conserve the photosynthetic machinery and preserve chlorophyll during water deficit. They rely on the production of antioxidants, but also on the morphological modifications, such as leaf folding, to neutralize excess ROS production (Hilhorst and Farrant 2018). The knowledge gathered regarding desiccation-tolerant species and mechanisms of avoiding permanent damage is used in strategies to overcome difficulties with crop yields following significant climate changes, such as more frequent drought episodes (Farrant and Hilhorst 2022).

The main aim of researchers, puzzled by the phenomenon of resurrection plants, was to determine which factors of the complete protective mechanism were responsible for this ability to survive such long periods of water scarcity. The protection system involved in desiccation tolerance in resurrection plants include accumulation of different metabolites, these can differ between species (Dirk et al. 2020). The changes induced by desiccation are characterised by processes consisting of interconnected parts on a cellular, physiological, biochemical and metabolic level used for keeping the key cell components protected, but they are also intended for easier transition back to homeostasis once water becomes available (Farrant et al. 2015; Oliver et al. 2020). Dehydration in desiccation-tolerant plants is associated with a spectrum of various defence mechanisms, including protection of the photosynthetic machinery by the expression of early light-induced proteins (ELIPs) (Xiao et al. 2015). ELIPs seem to contribute to minimizing the photooxidation damage of chloroplasts caused by elevated ROS production by binding to chlorophyll during the decline in water potential (Xiao et al. 2015; VanBuren et al. 2019). Another ubiquitous contributing factor involved in desiccation tolerance is the synthesis of low-molecular-weight osmolytes, e.g. non-reducing sugars, predominantly sucrose, but also raffinose and other oligosaccharides (Peters et al. 2007; Zhang and Bartels 2016; Liu et al. 2019). Their role is dual: firstly, they can maintain turgor pressure as osmolytes in the initial water potential drop. Additionally, they are linked to the process of vitrification, when the cytoplasm is transformed from a gel-like state to a glass state in order to conserve cell membranes in the desiccated state (Oliver et al. 2020). Defence against uninterrupted ROS production during the drop of water potential is mitigated through enzymatic and non-enzymatic antioxidants (Veljović-Jovanović et al. 2006; Challabathula et al. 2018). In plants, non-enzymatic antioxidants include ascorbate, glutathione, α-tocopherol, and enzymes that neutralize ROS are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), class III peroxidases (POD), glutathione reductase (GR) (Challabathula et al. 2018). Be-

sides ELIPs and antioxidant enzymes, proteins related to the intricate survival mechanism of desiccation tolerant plants are late embryogenesis abundant (LEA) and small heat-shock proteins (sHSPs). sHSPs belong to a large class of molecular chaperons that protect misfolded proteins by refolding their native state (Zhang et al. 2013; Giarola et al. 2017). LEA proteins (LEAPs) are the main topic of this review, so they will be discussed in greater detail later on.

RAMONDA SERBICA AS A MODEL PLANT FOR DESICCATION TOLERANCE

Ramonda serbica Panč., one of those aforementioned resurrection plants, belongs to the family of Gesneriaceae. It is an endemic and relic species, with its habitat located on the Balkan Peninsula, mainly in northern rocky slopes of gorges with very small amounts of direct sunlight (Dražić et al. 1999) (Fig. 1). *Ramonda serbica* is a part of a quite small group of the poikilohydric flowering plants in the northern hemisphere counting 5-6 species in total (Dražić et al. 1999; Živković et al. 2005).

Besides *R. serbica*, two other resurrection plant species belong to the genus *Ramonda* Rich. (Gesneriaceae), such as *R. myconi* (L.) Rchb. and *R. nathaliae* Pančić & Petrović (Lazarević et al. 2022). Interestingly, the habitat *of R. myconi* is located in the Iberian Peninsula, while the other two reside in the Balkan Peninsula. *Ramonda serbica* is largely distributed in the areas of the Adriatic watershed in Montenegro, south-west Serbia (Kosovo and Metohija province), north west Greece, Macedonia and western parts of Republic of North Macedonia, while few habitats were found in the Black Sea watershed (Serbia, Bulgaria) and the Aegean watersheds (North Macedonia) (Stevanović et al. 1991; Lazarević et al. 2022).

Two different periods during the whole process of dehydration, which takes about 15 days to onset, can be distinguished based on the plants' response. The first one lasts around 7-10 days, when the relative water content (RWC) reduces to values around 40-50%, while the latter phase is much briefer and characterized by extreme water deficit by bringing RWC down to below 10% (Rakić et al. 2014). Different parameters contributing to the molecular and physiological adaptations that take place during various stages of dehydration and rehydration of *R. serbica* were analysed in several studies (Stevanović et al. 1992; Dražic et al. 1999; Quartacci et al. 2001; Augusti et al. 2002; Sgherri et al. 2003; Živković et al. 2005; Veljović-Jovanović et al. 2006, 2008). Since *R. serbica* belongs to homoiochlorophyllous species which conserve the photosynthetic apparatus during water deficit, one of the main strategies comprises morphological changes such as leaf folding, in order to mitigate the influ-

Fig. 1. *Ramonda serbica* Pančić in natural habitat in the gorge of Sićevo, near the city of Niš in south-eastern Serbia.

ence of excess light and consequently higher production of ROS (Rakić et al. 2014). Most notable changes concerning sugars, free amino acids and in particular proline in *R. serbica* plants during dehydration and later rehydration were the accumulation of sucrose, which is a hallmark of desiccation tolerance in resurrection plants (Živković et al. 2005). Proline, as the most abundant of all free amino acids, exhibited a significant decrease during dehydration, as well as the other amino acids. However, the final fall in RWC triggered an increase in proline content. The fluctuations in proline's content might be linked to altered biosynthesis and catabolism during dehydration and rehydration, but likewise to its role as an antioxidant and osmolyte (Rakić et al. 2014). An efficient and timely antioxidative response is believed to be one of the key advantages for the preventing detrimental effect of water deficit.

In that manner, the pools of ascorbic acid and glutathione play important roles, as their content is substantially increased during desiccation, while the enzyme activities of SOD, APX, and GR were quite high during the early stages of desiccation (Augusti et al. 2001; Sgherri et al. 2004). Other studies showed that enzymes related to antioxidative defence such as POD, APX, and SOD were affected in leaves of different age and relative water content, and different isoforms were detected during dehydration (Veljović-Jovanović et al. 2006). In addition, phenolics were proven as important factors for the adaptation process to desiccation as another optional antioxidant resource, by (i) noticeably high amounts determined both constitutively compared to other plants, and during dehydration and; (ii) by increased activity of enzyme involved in removal of O_2 followed by the oxidation of phenols - polyphenol oxidase (PPO) (Veljović-Jovanović et al. 2008). Constitutively, cholesterol is the most abundant free sterol in plasma membranes, with increased content and the plasma membrane lipid content dropped by 75% following dehydration (Quartacci et al. 2002). Initially,

photosystem II photochemical efficiency reduces slowly, but when RWC values fall under 40% the values plummet. The maximum accumulation of zeaxanthin during the first stages of desiccation and its conservation in the fully dehydrated inactive state suggests its crucial influence on non-radiative energy dissipation, which protects against photoinhibition (Augusti et al. 2001; Rakić et al. 2014).

Proteomics data from hydrated and desiccated leaf tissues of *R. serbica* shed some additional light on complex processes intertwined in the desiccation tolerance mechanism. However, the challenge arose with the extraction of high-quality and high yield of protein samples for further shotgun proteomic analysis. *Ramonda serbica* leaf tissue proved to be rather complicated for this purpose, because of the severe damage caused by water deficit, a high abundance of phenolics and polysaccharides, and also potential aggregations of proteins during the extraction and purification steps (Vidović et al. 2020). The optimised phenol-based protocol has been proven to be the most efficient for the best protein-band resolution, band number, and intensity and also showed an expanded number of identified peptides and protein groups by LC-MS/MS. Moreover, membrane-associated proteins were more effectively solubilized with the addition of dodecyl-β-D-maltoside (Vidović et al. 2020).

A comprehensive transcriptomic, proteomic, metabolite and photosynthetic study of *R. serbica*'s traits that provide it with the exceptional feature of desiccation tolerance has been done (Vidović et al. 2022). Data for this study was provided by complex extractions of RNA and proteome (Vidović and Ćuković 2020; Vidović et al. 2020). Transcriptomic analysis revealed 23,935 upregulated and 26,169 downregulated genes in desiccated leaves (DL) compared to hydrated leaves (HL), out of a total of 68,694 differentially expressed genes (DEGs) (Vidović et al. 2022). The proteomics approach was used to identify and quantify differentially abundant proteins (DAPs), using the *R. serbica* transcriptome data. A total of 1192 different protein groups were determined, out of which 229 protein groups were more abundant in HL and 179 in DL (Vidović et al. 2022).

Proteins groups linked to the photosynthetic apparatus were markedly less abundant in DAPs and DEGs in DL, which is in accordance with previous studies regarding the response of resurrection plants to dehydration (Xu et al. 2021). Chlorophyll fluorescence measurements indicated the ceasing of linear photosynthetic electron transport (PET) and switching on cyclic electron transport (CET) at PS I, which may be related to the alleviating the $H_2O_2/O_2^{\bullet-}$ formation (Vidović et al. 2022). The activity reduction of antioxidant enzymes scavenging H_2O_2, e.g. ascorbate-glutathione cycle components, catalases, peroxiredoxins, Fe- and Mn SOD isoforms was observed in DL compared with HL of

R. serbica. On the other hand, four Cu/ZnSOD isoforms, six germin-like proteins (GLPs), three PPOs, and 22 LEAPs were induced by dehydration. Moreover, cell wall remodelling showed to be an important factor in ameliorating the harmful effect of water deprivation through the increased amounts of three pectin methylesterases (PMEs) upon desiccation in the leaves of *R. serbica* (Vidović et al. 2022). Furthermore, the components of cell wall in *R. serbica* (pectin, cellulose, hemicellulose, and xyloglucans) were reduced in DL. The osmolites protection during desiccation was proven yet again by the three times higher accumulation of total soluble sugars (Vidović et al. 2022).

CHARACTERISATION OF LEA PROTEINS IN *R. SERBICA* DURING DESSICATION

LEA proteins are considered to be a significant part of the machinery that is responsible for the resurrection plants' outstanding response to extreme lack of water (Olvera-Carrillo et al. 2011; Gechev et al. 2021). Most of them fall in the category of intrinsically disordered proteins (IDPs). IDPs are characterised as proteins that natively contain intrinsically disordered regions (IDRs) or they are devoid of a stable secondary and tertiary structure under physiological conditions (Mao et al. 2010). It was firmly believed that a protein three-dimensional (3D) structure is utterly necessary for it to perform its function. Nevertheless, IDPs have now been recognized as complementary as it comes to functions to well-established ordered protein structures (Uversky 2019). Their flexibility and the ability to change conformations depending on microenvironment allows them to perform specific physiological functions such as linkage between structured domains, regulatory tails and signal transducers, that can be easily degraded upon turning off the signal (Vidović and Milić Komić 2020). IDPs also have a characteristic composition and order of amino acids present in their sequence that define their ability to form these flexible, but yet functional structures, rather than ordered proteins (Romero et al. 2004). They are mainly comprised of charged, hydrophilic residues and structure-breaking residues, namely Arg, Lys, Glu, Pro, Gly, Ala, Asp, Ser, Gln and Asn (Habchi et al. 2014; Vidović and Milić Komić 2020). The relatively high net charge with low mean hydropathy can often help us differentiate between IDPs and structured proteins (Uversky 2019).

LEA proteins were originally described 40 years ago in the seeds of cotton and shown to accumulate late in cotton seed development, coinciding with the embryo becoming desiccation-tolerant (Dure et al. 1981). Later on, they were found in vegetative plant tissues and associated with the plants' response to different abiotic stress, mainly drought, salinity, and cold stress (Tunnacliffe and Wise 2007). Also,

they were depicted in other types of organism, including desiccation-tolerant invertebrates (rotifers, nematodes, and brine shrimps) and microorganisms (Hand et al. 2011). LEAPs have been frequently found to alter their predicted random structure under stress conditions to form stable secondary structures (Hincha and Thalhammer 2012; Gechev et al. 2021). One of LEAPs proposed function is to serve as water replacements during desiccation when proteins and cellular structures lose their hydration shell. It is assumed that LEAPs bind to proteins and other molecules in order to prevent molecular crowding by avoiding aggregation and thus stabilizing its structure. Other function includes direct attachment of water to LEAPs which promotes the conservation of hydration shells (Gechev et al. 2021). Further suggested functions that are attributed to LEAPs include antioxidant activity and sequestering of metal ions (Tunnacliffe and Wise 2007; Olvera-Carrillo et al. 2011). Although all these putative roles are linked to LEAPs no specific physiological function has been ascribed yet (Gechev et al. 2021).

As previously mentioned, a comprehensive transcriptomic and proteomic study has been performed and also the *de novo* transcriptomic analysis of DEGs encoding LEAPs in HL and DL (Pantelić et al. 2022). The obtained results showed that 318 proteins meet the set threshold criteria. Throughout the literature, there have been different approaches to classify LEAPs based on their physicochemical characteristics, including 3D structure, distinct conserved motifs (Dure et al. 1989; Bray 1993; Tunnaclife and Wise 2007; Battaglia et al. 2008; Bies-Etheve et al. 2008, Hundertmark and Hincha 2008). There is still no real consistency in applying certain classification, although efforts have made been made to standardize these categories (Hunault and Jaspard 2010; Amara et al. 2014).

Ramonda serbica LEAPs were annotated according to domains and categorized based on the classification done by Hundertmark and Hincha (2008), into seven protein family groups, ranging from LEA1 to LEA5, dehydrins, and seed maturation proteins (SMPs). The LEA2 family group was the most abundant in proteins, making for almost 40 % of the total pool of defined LEAPs, while the smallest LEA5 group contained only 11 proteins (Pantelić et al. 2022). In order to examine similarities and differences between LEA groups a set of physicochemical characteristics (sequence length, pI, amino acid composition, protein's molecular weight, and grand average hydropathy—GRAVY) was analysed for each group. The average sequence length is the highest in the LEA2 protein family (~226 aa) and the shortest in LEA5 (118 aa). The Gravy index values, being of great importance due to the hydrophilic nature of LEAPs in general, were negative for all LEA groups, with the most negative values found in dehydrins (Pantelić et al. 2022). However, the average GRAVY

value for the most hydrophobic group, LEA2, was -0.09. Dealing with the subject of the composition of amino acids it was verified that *R. serbica* LEAPs lack or have a very low content of cysteine and tryptophan residues, and oppositely a remarkably high content of lysine and glycine residues, concurring with available literature data (Hundermarkt and Hincha 2008; Bhattacharya et al. 2019).

Since LEAPs mostly fall into the category of IDPs, they are rarely deposited in the Protein Data Bank (PDB). Therefore, an *in silico* analysis of the propensity for different secondary structures was evaluated for each group with available predictors. For the LEA4 family group a high percentage of α-helices was predicted, while random coil was prevailing in dehydrins and LEA3 proteins (Pantelić et al. 2022). LEA2 protein group was distinguished by the lowest propensity for disorder and a substantial potential to form β-sheets. Domain architecture analysis was done in order to acquire more information regarding structural diversity and conserved motif divergence of LEAPs from *R. serbica* in seven distinctive LEA protein family groups. Based on the presence of different unique motifs subgroups within each group were established and presented in detail in Pantelić et al. (2022).

Ramonda serbica LEA1 protein family group includes LEAPs similar in lengths that mostly contained two highly conserved motifs, and were clustered into three subgroups (Pantelić et al. 2022). Based on the nine homologous motifs found, LEA2 family was clustered into five major subgroups. According to the same study, LEA3 group contained five distinctive motifs, and was divided to two subgroups. Notably, motifs were rich in proline and glycine residues, and contained almost ten completely preserved charged amino acids. Four distinctive motifs were identified in the LEA4 protein family group and almost all motifs were very polar, rich in charged amino acid residues (>50%), namely lysine (20-33%) and were classified into three subgroups (Pantelić et al. 2022). LEA5 protein family group contained only 11 LEAPs, and three subgroups were formed with one highly conserved motif with glycine and charged residues. Pantelić et al (2022) clustered dehydrins in two subgroups: DEH1 and DEH2 which contained four distinct polar motifs. The motif commonly known as Y-segment, DEYGNP is present in this group, as well as the other K-segment: KKG[_N][MF]M[DE] KIKEK (Pantelić et al. 2022). Additionally, the prevalent S-segment was SGSSSSSSS was determined in this group. Col-
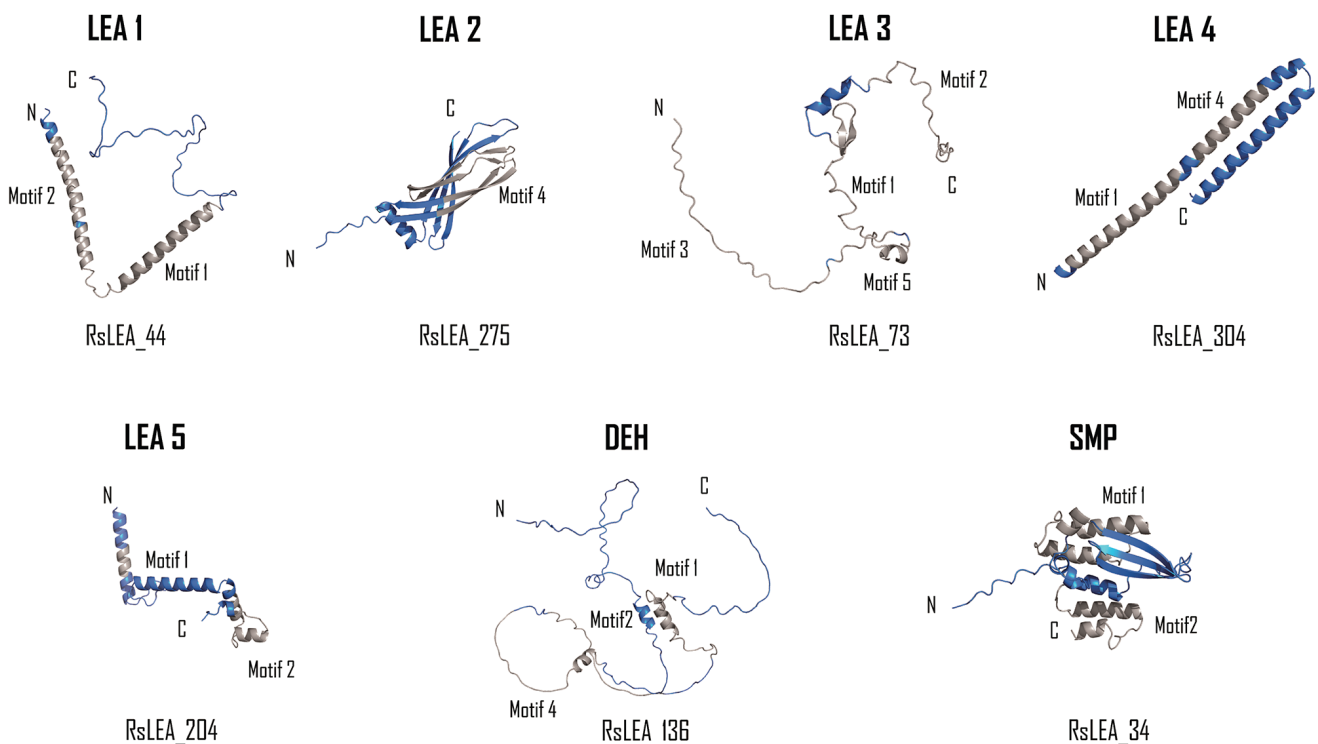

Fig. 2. Three-dimensional models of the representative LEAPs of each *R. serbica* LEA protein group. Detected structural motifs, according to Pantelić et al. (2022) are denoted in blue. The RsLEA code for each protein according to Pantelić et al. (2022) is given. DEH- dehydrins; SMP seed maturation proteins.

lectively, all dehydrins annotated in *R. serbica* included at least one dehydrin-determining segment. The SMPs were clustered into three subgroups, based on the occurrence of two detected motifs (Pantelić et al. 2022). Representative 3D model of proteins in each LEA family group with specific motifs, present in its structure are depicted in Fig. 2 (Pantelić et al. 2022).

CONCLUSION

Resurrection plants continue to be the subject of many ongoing studies because of their peculiar and astonishing capacity to withstand extreme environmental conditions of desiccation, with the reestablishment of all cellular functions upon water arrival. Researchers have been trying to pinpoint particular participants that are involved in these elaborate mechanisms. *Ramonda serbica* belongs to a small family of resurrection plants located in Europe. Different biochemical parameters and photosynthetic parameters of *R. serbica* were analyzed during the last 40 years in response to dehydration, including different antioxidants, sugars, amino acids, lipids and enzymes linked to the antioxidative system. *Ramonda serbica*'s response to desiccation was investigated in great detail through a comprehensive transcriptomic, proteomic, metabolite and photosynthetic study by our group. A special part of this research was dedicated to LEA proteins, and deciphering their possible role in the desiccation tolerance of *R. serbica*. A very high number of DEGs encoding LEAPs was revealed. They were categorized to different groups and their characteristic traits were analyzed by using diverse available software packages. The characterisation of LEAPs present in the model desiccation-tolerant plant *R. serbica* may help us to locate particular candidates for insightful studying of the interactions between LEAPs and other proteins or lipid membranes upon desiccation. The knowledge gathered concerning these interactions could lead to other conceivable applications, such as cryopreservation or the protection of other proteins native structure by its anti-aggregation activity. They could prevent the aggregation of proteins involved in different neurological disorders. The prediction of potential interactions between suitable partners could introduce us to a whole new way in the treatment of these disorders by stopping the formation of insoluble, amyloid fibrils.

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