Diversity of Dehydrins in *Oleae europaea* Plants Exposed to Stress

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Abstract: Dehydrins (DHNs) belong to a large family of proteins whose expression is associated with dehydration during seed maturation, pollen grain development and bud dormancy during winter, as well as plant adaptive response to various stressors. However, the exact roles played by different members of this protein family have not been fully defined. To gain a better understanding of DHN functions in olive plants, we used Western blot analyses to investigate their expression in the leaves of olive plants subjected to wounding, water-deprivation and salt-treatment. Two prominent bands having molecular masses of approximately 40 kD and 42 kD were constitutively expressed, however, their levels increased when the leaves were exposed to stress. Dehydration and salt stress also resulted in the accumulation of two additional proteins, which had molecular masses of approximately 16 kDa and 18 kD. These additional proteins were not detected following wounding. Our results suggest a physiological function for DHNs in olive plants during normal growth conditions and specialized functions during responses to certain types of stress.

Keywords: Leaf, stress factor, Dehydrin, *Olea europaea* cv Leccino.

INTRODUCTION

Environmental conditions, especially water availability, strongly influence plant productivity and health [1-3]. Therefore, to counteract the stress caused by water deficiency, land plants have commonly acquired adaptive mechanisms at the morphological, cellular and molecular levels that confer a selective advantage during times of drought [4-7]. These adaptive mechanisms include enhanced expression of a large set of genes that encode hydrophilic proteins [8, 9]. Phylogenetically widespread, these proteins may confer a high hydration capacity. Chief among these proteins are the highly hydrophilic LEA polypeptides [9-12]. First described in plants, LEA-related proteins have since been identified in bacteria as well as invertebrates [8, 13-15]. The large group two LEA proteins, originally designated as the D-11 family, are also known as the Dehydrins (DHNs) [10], and are the best studied of the drought-induced water-soluble plant proteins [9, 16-20].

DHNs have molecular masses that range from 9 to 200 kilo dalton (kD) and belong to five different subgroups, with each subgroup suggested to exhibit distinct functions [9- 10, 16-17, 21-23]. Overall, DHNs are intrinsically unstructured proteins, and, as such, may exhibit high flexibility, structural adaptability and extended conformations [24-27]. Consistent with these characteristics, recent studies suggest numerous activities for DHNs, including buffering water, sequestering ions, stabilizing membranes, or acting as chaperones or molecular shields [9, 19, 28-31].

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In unstressed plants, DHNs accumulate in organs that undergo programmed functional dehydration, primarily in embryos during the late phase of embryogenesis, but also in the pollen grains [21, 32, 33]. DHNs also accumulate in the apices of some trees during bud dormancy, a period during which buds undergo wintertime dehydration [33]. In addition, DHNs are expressed in nearly all vegetative tissues during normal growth conditions and/or accumulate to high levels following exposure to stress, as observed in numerous independent studies on responses to drought, cold acclimation, salinity and ABA as well as during overexposure to light [9-10, 34-42]. In accordance with these findings, data from *in vitro* experiments have shown that DHNs are involved in stress responses that range from protection against water deprivation and extreme cold to detoxification and hydroxyl radical scavenging [9, 10, 43]. Based on this broad range of functions, DHNs may play a significant physiological role in the adaptive responses of plants. In this context, it is notable that, due in part to their sessile nature, plants are commonly subjected to various types of abiotic environmental stresses as well as to physical damage, commonly referred to as wounding, caused by, among other things, insects and severe weather.

Olea europaea is a member of the *Oleaceae* family and represents one of the emblematic and widespread crop species in the Mediterranean basin. As crops, these plants have a strong impact on both economy and on human health [44]. The olive plant is an evergreen, schlerophyll tree that is a drought-resistant and moderately salt-tolerant species [45, 46]. The tolerance of olive plants to mild-to-moderate water stress involves active and passive osmotic adjustments, which assure the maintenance of an adequate leaf turgor and gas-exchange rate. However, severe water stress, which in the field is often coupled to high levels of irradiation, strongly impairs photosynthetic activity through both the

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reduction of stomatic conductance and photo-inhibition [47]. In order to add further insights into the molecular mechanisms underlying olive drought resistance, the present work was carried out to investigate whether DHNs play a role in the stress response of olive plants to water deprivation, salt stress and wounding. The results generated during this study might aid in the development of more successful growth strategies for this unique oil tree species in geographic areas at risk of desertification due to both climate change and intensive land utilization.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Two-year-old plants of *Olea europaea* L. cv Leccino were purchased from Santa Cruz Olive Tree Nursery (Watsonville, CA 95077). Plants were acclimated for 30 days in the University of Pennsylvania Greenhouse at a temperature of 28° C, at 100 μmol m^{-2} s^{-1} PAR, under a 16/18 hr light/dark regime and 50% relative humidity. Plants were irrigated daily.

Stress Treatments

After acclimation, plants were divided into subgroups and exposed to different stresses. For drought stress, plants (n=5) were progressively deprived of water as described by Tommasini *et al.* [48]. Leaf samples were collected from primary and secondary shoots at the beginning of the stress treatment (T0) and after 5, 10 and 15 days (T5, T10, T15).

For salt stress, plants (n=5) were supplied with 50 mM NaCl per day to a final concentration of 200 mM NaCl. Final salt concentrations were reached gradually to avoid salt shock. Leaf samples were collected from primary and secondary shoots at the beginning of the stress treatment (T0) and after 5, 10 and 15 days (T5, T10, T15) of stress exposure.

Wounding stress was imposed on plants (n=5) by cutting a small apical portion from the leaves of primary and secondary shoots. Wounded leaves were then collected after 5 min, 30 min, 1 hr and 24 hr after cutting.

Protein Extraction and Western Blot Analysis

Total protein was extracted as described by Wang *et al.* [49]. Briefly, olive leaves were pulverized in liquid nitrogen, 0.2 g of powered tissue was washed extensively with acetone and tricholoroacetic acid and then extracted using a phenol-based method. Protein concentrations were quantified using the Bio-Rad ™ protein assay.

All protein samples were stored in NuPAGE lithium dodecyl sulfate sample buffer supplemented with 50 mM dithiothreitol. Samples were run on Bis-Tris NuPAGE gels (Invitrogen®) under denaturing conditions, using morpholinepropanesulfonic acid (MOPS). Finally, proteins were transferred to polyvinylidene difluoride membranes by a Transblot-SD semidry transfer cell (Bio-Rad) at 15 V for 30 min using three different buffers: anode I (300 mM Tris, 10% [vol/vol] methanol, pH 10.4), anode II (25 mM Tris, 10% [vol/vol] methanol, pH 10.4), and cathode (25 mM Tris, 40 mM glycine, and 10% [vol/vol] methanol, pH 9.4). Polyvinylidene difluoride membranes were incubated with the primary anti-plant dehydrin polyclonal antibody raised against the conserved dehydrin K-segment (TGEKKGIMDKIKEKLPGQH), (StressGen™, San Diego, CA) (1:1,000), and then with anti-rabbit secondary antibodies (1:10,000). The K-segment synthetic peptide, kindly provided by Dr. T.J. Close, was used during pre-incubation of the antiserum to confirm that the bands detected by Western blots were specifically recognized by the antigen-binding site of the anti-peptide antibody [50].

RESULTS

Macroscopic Effects of Water Stress on Olive Leaves

Under standard conditions, the leaves of olive plants are fully expanded to allow optimal light harvesting (Fig. **1A**). Conversely, leaves of olive plants subjected to water deprivation exhibited an evident leaf rolling after 7 days (Fig. **1B**) and showed the most pronounced leaf rolling after 15 days of water deprivation (Fig. **1C**).

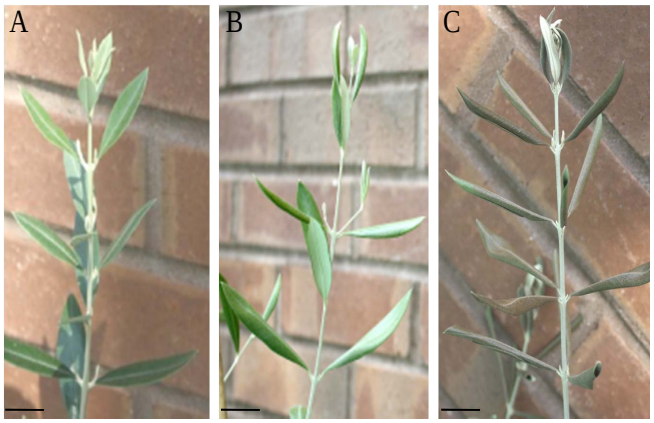

Fig. (1). Macroscopic leaf rolling of Olea europaea upon water-deprivation. Two-year-old *Olea europaea* L. cv Leccino plants were grown under standard conditions (**A**) and deprived of water for 7 (**B**) and 15 (**C**) days. A macroscopic leaf rolling is evident in the drought stressed plant (**B**, **C**). Bars: 2cm.

Similar effects were also observed in salt-stressed plants where upon 4 days of exposure leaf rolling became evident and more extensive leaf rolling occurred after 10 days (data not shown).

DHNs are Constitutively Expressed

DHNs belong to a diverse family of hydrophobic proteins that are expressed under dehydration conditions. However, certain proteins belonging to this family are also expressed when no stress is present. Taking advantage of the fact that all proteins within this family contain the conserved K-segment domain, we performed Western blot analyses of olive DHN diversity under stressed and unstressed conditions using an antibody raised against the K-segment.

Two bands, corresponding to polypeptides with molecular weights of approximately 40 kD and 42 kD, were detected in protein extracts from the leaves of plants grown under non-stressed conditions (T0), suggesting low-level constitutive expression of some DHNs (Fig. **2A**, **B**).

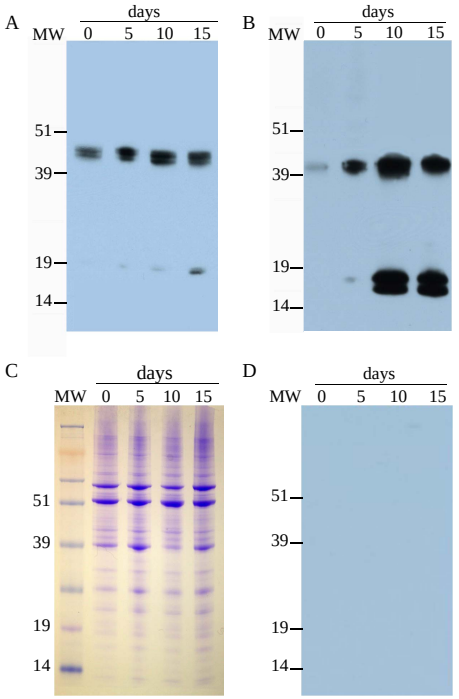

Fig. (2). Expression of olive DHNs under standard conditions and dehydration or salt stress. Immunoblot analysis of soluble leaf proteins from olive plants grown under progressive water deprivation (**A**), or subjected to salt stress treatment by adding NaCl to a final concentration of 200mM (**B**). Equivalent amounts of protein were subjected to LDS-PAGE and visualized by immunoblotting using an anti-DHN K-segment antibody. Gel containing soluble leaf proteins from olive plants subjected to salt stress stained with Coomassie-Blue to ensure quality of the extraction procedure and equal loading of protein samples (**C**). Immunoblot analysis of soluble leaf proteins from olive plants subjected to salt stress performed with antibodies pre-incubated with a synthetic K-segment (**D**).The sizes of protein standards are indicated on the left.

Drought and Salt Stress Appear to Exhibit Distinct DHN Expression Patterns from Non-Stressed Leaves

Western blot analysis of protein extracts from leaves of water-deprived olive plants showed increased abundance of two protein bands that were identified in extracts from the non-stressed leaves (Fig. **2A**). Moreover, two additional bands, corresponding to polypeptides having molecular weights of approximately 16 kD and 18 kD, were detected in olive leave protein extracts 5 days into the drought treatment (Fig. **2A**).

Leaves exposed to salt stress also showed increased abundance of the higher molecular weight DHNs on day 5 of the treatment. However, distinct from dehydration-stress, the leaves from plants exposed to salt contained a considerably larger amount of these proteins at later stages (10 and 15 days). Even more pronounced was the amount of the smaller molecular weight DHNs in salt-stressed compared to water-deprived plants (Fig. **2A**, **B**). We confirmed that similar amounts of total protein were loaded in each experiment using coomassie blue staining (Fig. **2C** and data not shown).

To confirm that the bands detected by Western blots were specifically recognized by the antigen-binding site of the anti-peptide antibody, we performed Western blot analysis using an immune serum pre-incubated with a synthetic K-segment. Consistent with antibody recognition of the K-segment in the leave preparation, no labeled bands were detected under these conditions (Fig. **2D**).

DHN Expression Pattern of Wounded Leaves Appear to be Unique

The abundance and diversity of DHNs were also investigated by Western blot analysis of protein extracts from olive leaves subjected to wounding, as described above (Fig. **3A**). Similar to salt and dehydration stress, the higher molecular weight DHNs were more abundant following stress. Higher levels of these proteins were apparent within 30 minutes of wounding and increased over time, peaking at approximately 24 hours post-wounding. Interestingly, this stress condition did not result in the presence of the lower molecular weight DHNs (Fig. **3B**).

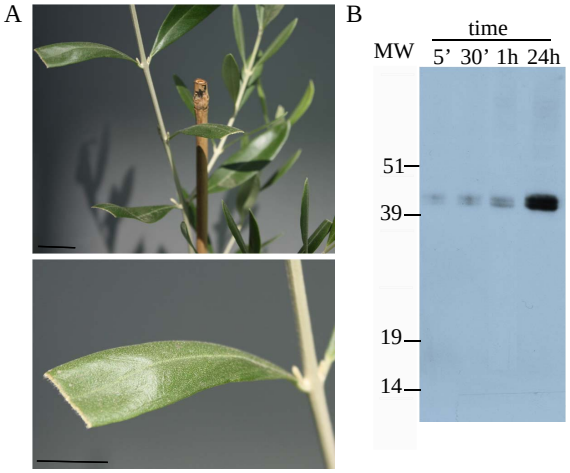

Fig. (3). Expression of olive DHNs upon wounding. Immunoblot analysis of soluble leaf proteins from olive plants subjected to a wounding stress. Equivalent amounts of protein were subjected to SDS-PAGE and visualized by immunoblotting using an anti-DHN K-segment antibody (**B**). The sizes of protein standards are indicated on the left.

DISCUSSION

DHNs are a family of closely related proteins that have been shown to accumulate in vegetative tissues of plants exposed to various stressors [9, 10]. While some DHNs are expressed at basal levels in non-stressed cells, others appear to only be detectable in plant tissues that have been exposed to stress [9, 10, 39]. Western blot analysis of olive leave protein extracts, using anti-K-segment antibodies, strongly suggest the upregulation of two constitutively expressed putative DHNs when the plants are subjected to drought, salt stress or wounding. These results are also in line with the multifunctional role proposed for DHNs spanning from protection against drought and extreme cold to detoxification and hydroxyl radical scavenging [9, 10, 43]. In this respect it is worth noting that wounding, which frequently occurs in plant organs as a consequence of insect feeding, is generally coupled to an immediate oxidative burst in damaged tissues [51-53]. Thus, the very early increase in levels of DHNs that we observed in wounded leaves may be consistent with radical scavenging activity [54]. The increased levels of

these two proteins demonstrates that their abundance is modulated by stress and also supports the notion that the bands detected on Western blots by anti-K-segment antibodies are indeed DHNs.

Moreover, the stress-related increase in DHNs is higher in the leaves of salt-treated compared to both water deprived and wounded leaves. One explanation for such differential accumulation might be related to the primary function of DHNs as regulators of cell osmotic potential as demonstrated in various tissues of *Arabidopsis thaliana* [39]. In this context, it is interesting to note that the wheat LEA (group 1) protein Em demonstrates osmo-protective activity when expressed in yeast [55], and that a shorter lag period is observed in yeast cells expressing the barley LEA (group 3) gene *HVA1* when the yeast are transferred to a medium containing 1.2 M NaCl [56].

Notably, two additional putative DHNs of approximately 16 kD and 18 kD are expressed in the leaves of olive plants following either water deprivation or salt treatment, while they are not detected in either unstressed leaves or wounded leaves. Thus, specific DHN members seem to be involved in the dehydration/osmotic stress response in olive plants and might account for the drought and salt tolerance of such relevant schlerophyll species. This assumption is supported by data showing that under conditions of water deficit, transcription of *DHN* genes is significantly higher in drought-tolerant than in drought-sensitive species. A correlation between plant drought tolerance and DHN accumulation was also found in sorghum and in sunflowers plants [57, 58]. Moreover, in salt-tolerant lines of rice, the level of ABA-induced expression of DHNs is significantly higher than it is in sensitive plants [59]. A direct interrelationship between the level of DHNs and cold tolerance has also been observed [60-62].

In summary, this is the first report presenting data strongly suggesting the involvement of DHNs in the molecular mechanisms of stress response in *Olea europaea*, a traditional tree crop of the Mediterranean basin, which has a world-wide impact as a food product as well as having positive effects on human health. As many as four different members belonging to large DHNs family appear to be expressed in olive leaves and a functional specialization for osmotic stress response was highlighted for two of the detected DHNs. Efforts are underway to clone the genes encoding the DHNs expressed in the olive leaves. Cloning these genes may also lead to identification of additional DHNs that are expressed under other conditions. Further investigation will be necessary to fully define the expression patterns of various DHN members in the different organs of the olive plant, with our focus being on proteins expressed in the fruit, which is frequently exposed to a variety of pathogens [44, 63]. Based on the results of the present work, we show that DHNs may serve as integral components of olive plant responses to stressful conditions that strongly impact both plant productivity and product quality.

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