UNIVERSITÀ DELLA CALABRIA

UNIVERSITA' DELLA CALABRIA

Dipartimento di Biologia, Ecologia e Scienze della terra

Scuola di Dottorato

LIFE SCIENCES

in co-tutela di tesi con

PLANT SYSTEMS BIOLOGY

Indirizzo

BIOLOGIA VEGETALE

Con il contributo di

MINISTERO DELL'ISTRUZIONE, DELL'UNIVERSITA' E DELLA RICERCA

CICLO

XXVI

TITOLO TESI

Identification of a feral olive dehydrin gene and its development as a tool for drought tolerance in *Arabidopsis thaliana*

Settore Scientifico Disciplinare BIO/01

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Abstract

Stress abiotici, quali deficit idrico e salinizzazione del suolo influenzano negativamente la crescita delle piante e la produttività delle colture (Liu *et al*., 2004; Wu *et al*., 2007).

In campo vegetale, tra le strategie sperimentali messe in atto per incrementare la tolleranza a varie tipologie di stress tra cui siccità, salinità e congelamento, l'approccio più efficace è risultato essere l'introduzione, in piante di interesse, di geni codificanti per fattori di trascrizione stress-inducibili o, più in generale, di geni corrrelati alla risposta agli stress, di genotipi vegetali naturalmente stress-tolleranti (Beck *et al*, 2007).

In tale contesto, *Olea europaea* L. *subsp. europaea* var. *sylvestris*, nota comunemente come oleastro, una pianta tipica ed ampiamente diffusa nel bacino del Mediterraneo, presenta molti tratti quali la resistenza al vento ed alla siccità, la capacità di recuperare dopo un incendio, che da una parte potrebbero essere traslati a specie vegetali di importanza agronoma ed economica rilevante, dall'altra ne fanno un candidato eccellente per le pratiche di rimboschimento e della gestione delle zone erose della Macchia Mediterranea (Mulas *et* Deidda, 1998) .

Tra i meccanismi messi in atto dalle piante per fronteggiare stress vari tra cui quello idrico ed osmotico rientra la sintesi di una classe di proteine note come deidrine. Un membro della famiglia genica delle deidrine, denominato *OesDHN* è stato precedentemente identificato da una libreria a cDNA ottenuta da foglie di piante di *Olea europaea* subsp. *europeae* var. *sylvestris* ed interessantemente i suoi livelli di espressione sono risultati essere up-regolati in piante di oleastro esposte a condizioni di stress idrico e da freddo (Bruno *et al*., 2010).

Le analisi volte a definirne l'omologia di sequenza e l'origine filogenetica hanno dimostrato che *OesDHN* codifica per una deidrina acida (pI 5.14) costituita da 211 aminoacidi di 23,846 kDa. *Oes*DHN presenta due segmenti K ricchi in lisina ed un segmento S, ricco in serina, caratteristiche tipiche di una deidrina di tipo SK2. Inoltre, l'analisi Southern blot, condotta al fine di analizzare l'organizzazione genomica, ha dimostra che *OesDHN* è presente in duplice copia nel genoma aploide di oleastro.

Al fine di chiarire il ruolo di *OesDHN* nei meccanismi messi in atto dalle piante in risposta allo stress idrico, abbiamo generato piante transgeniche di *Arabidopsis thaliana* overesprimenti il gene *OesDHN*. I risultati ottenuti hanno messo in evidenza che, in

condizioni di stress osmotico medio, indotto sperimentalmente aggiungendo una concentrazione 25mM di mannitolo nel mezzo di coltura, l'overespressione del gene eterologo, incrementa la tolleranza delle piante a questa specifica condizione di stress.

A conferma di tali risultati, l'analisi in silico condotta ha messo in evidenza la presenza di putativi elementi regolatori stress-inducibili di tipo ABRE e MYB, localizzati nella regione del promotore di *OesDHN*.

Infine, l'analisi confocale sulle linee transgeniche 35S::*OesDHN*:GFP e 35S::GFP:*OesDHN* di *Arabidopsis thaliana*, ha messo in evidenza che la proteina *Oes*DHN è localizzata principalmente a livello nucleare.

Nel loro insieme i risultati ottenuti sulla pianta modello *Arabidopsis thaliana* hanno permesso di chiarire alcuni degli aspetti molecolari chiamati in causa nella tolleranza a svariate condizioni di stress, nelle piante. La prospettiva a lunga scadenza della ricerca affrontata è quella di ampliare le conoscenze utili a definire possibili strategie per incrementare caratteri di tolleranza/resistenza in importanti specie coltivate e non.

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Aim of the work

Currently, global climate change and the unsustainable use of the environment have greatly contributed to the depletion of natural resources. This will soon lead to even more severe risk of desertification in the world, not only as a water shortage but also as the occurrence of extreme climatic and environmental conditions, posing serious questions about the future of agricultural productivity. This risk has been clearly explained in the report "Limiting Global Climate Change to 2 degrees Celsius. The way ahead for 2020 and beyond", of the European Commission of 10 January 2007 and especially in the chapter 4" Cost of action of Europe "(Antonioli *et al*., 2007).

Many researchers believe that global climate changing will begin to have a deeper effect on global food production as early as the twenty-first century (Parry *et al*., 2004). In this context, it is important understand the molecular basis of stress resistance in plants, in order to fully implement agricoltural and plant biotechnologies aimed at overcoming severe environmental stresses. Therefore comprehensive profiling of stress-associated metabolites is most relevant to the successful molecular breeding of stress tolerant plants that, in the future, could be used to increase the font of tolerance/resistance in important crop species (Vinocure *et al*., 2005).

Olive (*Olea europaea* L.) is a typical and widespread tree of the Mediterranean region and plays a central role in eating habits and in the characterization of the rural landscape. The *Olea europaea* species comprises six subspecies, defined on the basis of morphological characters and geographical distribution. *Olea europaea* subsp. *cerasiformis*; *Olea europaea* subsp. *cuspidate*; *Olea europaea* subsp. *europaea*, which includes two botanical varieties: the variety *sativa* (olive of cultivation) and the variety *sylvestris* (wild olive); *Olea europaea* subsp. *guanchica*; *Olea europaea* subsp. *laperrinei*; *Olea europaea* subsp. *maroccana*.

It is interesting to emphasize that the variety *sylvestris*, is considred as the best bioindicator of the Mediterranean Floristic Region (Rubio *et al*., 2002). *Oleaster* plants have, in fact, colonized many Mediterranean environments characterized by semiarid climatic conditions at different altitudes, plant communities and soils, including those with extreme levels of salinity (Zohary and Spiegel-Roy, 1975).

From an ecological point of view, *oleaster* exhibits many relevant features. First of all, due their prevalence within "*Macchia*" association, wild olive populations play a major role in

protecting soil against erosion, by consolidating land through the wide root system. Moreover, on account of *oleaster* resistance to wind and drought, they contributes to the survival of natural plant communities, while their ability to recover after a fire through new shoot formation assures a rapid rebuilding of the original vegetation cover (Mulas *et* Deidda, 1998). Thus oleaster features many traits that would be highly valuable to introduce into agricultural importance species, such as *Zea mays*.

Crucially in the face of a global scarcity of water resources and the increased salinization of soil and water, abiotic stress represent the major limiting factor productivity of many crop species. They negatively affect growth in almost all stages, including the seedling stage, pre-flowering and flowering period, and grain-filling stage (Liu *et al*., 2004; Wu *et al*., 2007).

A lot of mechanisms, often redundant, are involved in growth regulation under stress conditions, therefore, the simultaneous engineering of superior alleles and multiple genes under the control of appropriate, artificial, promoters will allow the fine tuning of growth responses under stress, thus enabling the plant to cope with a range of abiotic stresses (Kasuga *et al*., 1999).

Finally, developing new stress-tolerant crop varieties will require further understanding and modulation of the molecular processes underlying plant stress responses.

Among activate stress-responsive mechanisms, a number of regulatory as well as protective proteins that are involved in the plant tolerance of different stress factors, have been identified (Choi *et al*., 1999; Gulik *et* Kostesha, 2004; Skinner *et al*., 2005; Svensson *et al*., 2006; Stutte *et al*., 2006; Yamaguchi-Shinozaki *et* Shinozaki, 2006). Dehydrin proteins (DHNs) belong to these common protectants. They are part of a large group of highly hydrophilic proteins, known as LEA (Late Embryogenesis Abundant) and have been observed in several independent studies on drought stress, cold acclimation and salinity stress (Close *et al*., 1989; Rorat *et al*., 2006). The DHN amino acid composition is characterized by high content of charged and polar residues, and this determines their biochemical properties, including thermo stability. This aspect may promote their specific protective functions under conditions of cell dehydration; dehydrins may prevent coagulation of macromolecules and maintain integrity of crucial cell structure (Kruger *et al*., 2002; Koag *et al*., 2003; Goyal *et al*., 2005; Rorat *et al*., 2006; Kovacs *et al*., 2008).

Starting from these assumptions, this project wanted to contribute to the elucidate the molecular aspects involved in resistance to specific stress conditions (osmotic, drought, water, cold) in *oleaster* plants. In this context, the research group has previously identified, in *Olea europaea* subsp. *europaea*, variety *sylvestris*, a member of the dehydrin family, called *OesDHN*. This gene encodes for a SK2 DEHYDRIN protein and interestingly transcripts levels of *OesDHN* has been demonstrated up-regulated in *oleaster* plants exposed to drought and cold stressful conditions.

The aim of the project carried out in these three years of PhD, was, therefore, to complete the gene characterization and to verify, through a transgenic approach, whether and how *OesDHN*, isolated from wild plants of olive trees could increase tolerance to water stress, using *Arabidopsis thaliana* as a model plant. The obtained results are encouraging and might be shift in the future in important crop plants, such as *Zea mays*.

CHAPTER 1

Stress and response mechanisms in plants

1.1 Stress concept

Unlike to other pluricellular organisms, plants are sessile and they must adapt to continuous environmental changes.

In particular, an external environmental factor, that exert a detrimental influence on the plant, reducing physiological processes, such as growth and photosynthesis, is called a stress factor (Vernieri *et al.*, 2006).

Generally, stresses are classified as "biotic", if they are determined by other living organisms, and "abiotic", if they are caused by an excess or a deficiency of physical and chemical factors that determine the characteristics of the environment where the plant lives.

Plants, however, thanks to their remarkable genome plasticity, are able to perceive and respond to external stimuli, to process them and generate responses adapted to the various needs, through the production of new and specific proteins or through an increase of those that already exist, allowing adaptation to adverse conditions (Buchanan *et al.*, 2002). The ability of the plant to survive, grow and reproduce under stress, involves mechanisms of resistance to different levels such as:

a)-*escape* = avoid adversity: plant reacts to stresses by changing its life cycle so as to avoid environmental adversity (e.g., early plants escape the summer drought ending their life cycle before the onset of the dry season);

b) *avoidance* = avoid stress: plant reacts to stress by creating morphological or physiological barriers (e.g., plants close stomata to prevent excessive transpiration during periods of drought;

c) *tolerance* = tolerate stress: plant actives mechanisms at the molecular level to resist and adapt or to repair damage caused by stress (Levitt, 1980).

In this context, having regard to the response of individual processes in a setting more pre*ci*se, we can summarize three-point scale of events:

1) the response to stress is the immediate negative impact of stress on a plant process. This generally happens in a time scale that ranges from a few seconds up to several days, resulting in a decline in the *performance* of the process;

2) acclimation is the morphological and physiological bedding of plants, in order to compensate for the decline that follows the initial response to stress. Acclimatization occurs in response to environmental changes and causes changes in cellular metabolism that leads to the synthesis of new biochemical constituents, as well as enzymes, often associated with the production of new tissues. These biochemical processes leads to a cascade of effects at various levels, such as changes in the activity of photosynthesis, in the growth of plant, in the morphology of organs or of the whole plant. Stress acclimation affects every individual and can be demonstrated by comparing genetically similar plants, grown in different environments;

3) adaptation is the evolutionary response resulting from genetic changes in the population and typically requires many generations, although the physiological response mechanisms induced, are often similar to those of acclimatization (Lambers *et al.*, 2008).

1.2 Abiotic stresses

At present, in a global shortage of water resources and increased salinization either of soil or of water, the abiotic stresses, which are already important factor limiting plant growth, will become even more serious by bringing the process of desertification to cover more and more land (Ashraf *et al.*, 1994). Furthermore, in front of a continuous world population growth whose food demand exceeds supply, we understand how development and innovations in the field of agricultural and plant biotechnology, aimed at overcoming such environmental stresses, will be further implemented (Vinocur *et* Altman, 2005).

So in recent years there has been a remarkable and rapid progress in understanding of the molecular mechanisms that the plants put in place to defend themselves against abiotic stresses (Vinocur *et* Altman, 2005).

The adaptation of plants to environmental stress is controlled by a cascade of molecular *networks*, very complex and far from complete understanding. However, while the resistance of plants to biotic stress is regulated mainly by monogenic traits, responses to abiotic stresses are rather complex because multigenic and, therefore, more difficult to monitor and engineer (Zhu *et al*., 2001; Vinocur *et* Altman, 2005). Several studies on plant response to abiotic stresses, such as water scarcity and/or salinity indicate how the response mechanisms are often similar and linked together. In particular, stresses such as water scarcity, high salinity, cold or excessive heat and pollution, considered primary factors of stress, can be attributed to two types of secondary stress: hyperosmotic and oxidative (Wang *et al.*, 2003; Vinocur *et* Altman, 2005) (Fig. 1.1).

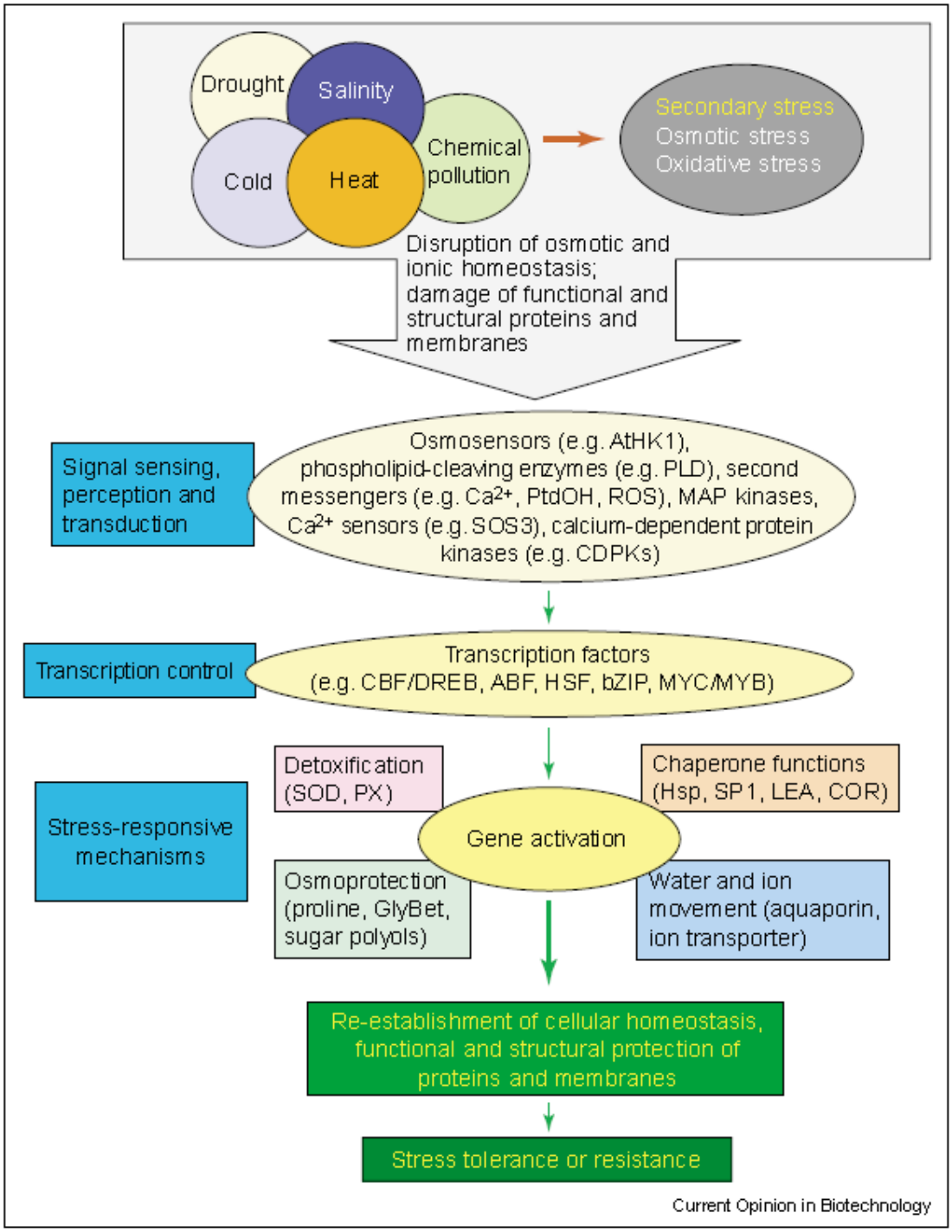


Fig.1.1 The complexity of the plant response to abiotic stress. Primary stresses, such as drought, salinity,cold, heat and chemical pollution, are often interconnected and cause cellular damage and secondary stresses, such as osmotic and oxidative stress (Wang *et al*., 2003, Vinocur *et* Altman, 2005)

Due to the close correlation between the different abiotic stresses, response mechanisms put in place by the plants will all be aimed at restoring cellular homeostasis and to defense and/or shelter of proteins and damaged membranes (Wang *et al*., 2003) (Fig.1.1).

Specific stress-tolerant plants can activate appropriate adaptation mechanisms; when this does not occur, the plant will die (Wang *et al*., 2003).

In response to different environmental stresses, plants often activate *signaling* in cellular pathway (Shinozaki *et* Yamaguchi- Shinozaki, 2000; Knight *et* Knight, 2001, Zhu, 2001) and similar responses such as the production of stress proteins, the up-regulation of antioxidants, the accumulation of compatible solutes (Vierling *et* Kimpal, 1992, Zhu *et al.*, 1997; Cuhsman *et* Bohnert, 2000), which we can summarize in three broad gene categories (Wang *et al.*, 2003):

I. genes which products are involved in *signaling cascade* and in transcriptional control, as well as Myc, MAP kinase and SOS kinase (Shinozaki *et* Yamaguchi- Shinozaki, 1997; Munnik *et al*., 1999; Zhu, 2001), phospholipase and transcription factors such as HSF, the CBF/DREB and ABF/ABAE (Shinozaki *et* Yamaguchi-Shinozaki, 2000; Stockinger *et al.*, 1997);

II. genes whose products are directly involved in protecting cell membranes as the *heat shock proteins* (Hsp), the chaperonin, the LEA proteins (Late embryogenesis abundant) (Vierling 1991; Ingram *et* Bartels, 1996; Tomashow 1998, 1999; Bray *et al* 2000), osmoprotectants and antioxidants proteins (Bohnert *et* Sheveleva, 1998);

III. genes which products are involved in uptake, removal and transport of ions and water as the aquaporins and ion transporters (Maurel, 1997; Serrano *et al*., 1999; Tyeman *et al*., 1999; Zimmermann *et* Sentenac, 1999, Blumwald, 2000).

1.3 *Cross-talk* and specificity in the *signaling* of stress in plants

The term *cross-talk* indicates situations where several *signaling pathway* share intermediate and/or components or have some common output (Chinusamy *et al*., 2003). This terminology describes exactly the set of responses implemented by plants to defend themselves against stress, when multiple perceptions and *signaling-pathways* are involved, some of which specific and others that could intersect at various levels.

In this context, in recent years, considerable progress has been made in order to identify components of *signaling-pathway* involved in different types of stress such as cold, salinity, and drought which are reported below (Chinusamy *et al*., 2003).

-Sensory kinases

Given the specificity of *signaling* resulting from specific stress conditions, it would be easy to assume that for each stress should be present a *sensor*, which could transduce the signal until the *target*. However, at present, only few "stress" sensors have been identified for which there are not sufficient data to confirm if the *cross-talk* occurs at the level of sensors.

Data in literature, show the presence of a stress "sensor" in bacteria and in yeast, consisting of two components, a sensory histidine-kinase and a response regulator (Chinnusamy *et al.* 2003). Among the identified temperature sensors, there are the HIK33 of cyanobacteria, (*Proffer bacterium*), a transmembrane Histidine kinase with two components and the Desk of *Bacillus subtilis* (Suzuki *et al.,* 2000; Aguilar *et al.,* 2001). A Histidine kinase osmosensitiva protein (SLN1) has been identified in yeast and it would seem to activate HOG1 (High-Osmolarity Glycerol 1 response) and a protein to cascade mitogenic activity (MAPK) (Maeda *et al.,* 1994). Also, the histidine kinase of *Arabidopsis thaliana At*HK1, would seem to belong to the category of osmotic sensors. This, in fact, is able to activate the HOG1/MAPK pathway and complement the yeast mutant *Sln1-ts,* showing defects in osmotic sensitivity (Urao *et al.,* 1999).

-Calcium as ubiquitous messenger

Calcium, as secondary messenger is elicited by multiple factors, such as those related to abiotic and biotic stress, to development, as well as to hormonal factors (Sanders *et al.,* 1999; Knight, 2000). It is known, for example, that changes in the cytosolic Ca^{2+} concentration regulate the opening and closing of the stomata (Chinnusamy *et al.,* 2003) or accompany specific stresses differently, depending on the intensity and duration of the stress, on the type of tissue and even on a previous exposure (Knight *et al.,* 1997; Plieth *et al.,* 1999).

Concerning the molecular mechanisms involved in the change of cytosolic Ca^{2+}, concentration, recent studies have introduced the presence of specific channels (Chinnusamy *et al.,* 2003). In *Brassica napus,* for example, it has been demonstrated that changes in membrane fluidity with the reorganisation of the cytoskeleton, caused by drug treatments, affect activities of mechano-sensitive calcium channel and cause oscillations in cytosolic Ca^{2+} under cold stress conditions (Orvar *et al.,* 2000). Also the Inositol 1,4,5-triphosphate channels (IP3), seems to be implicated in the increase of cytosolic calcium during water and saline stress conditions (De Wald *et al.,* 2001; Takanashi *et al.,* 2001). Calcium channels and ADPR cyclase (AplysiaADP-rybosyl cyclase) regulate in turn the ABA-responsive genes expression, elicitated by low temperature stress in *Solanum aurea* (Wu *et al.,*1997) and *Brassica napus* (Sangwan *et al.,* 2001).

-The SOS pathway regulates ion homeostasis

Regulation of calcium homeostasis is essential to induce tolerance to different stresses in plants, including salt stress, and it involves several components. In *Arabidopsis*, for

example, to restore cellular ionic homeostasis, affected by salt stress, is involved the *pathway* SOS (Salt-Overly-Sensitive) (Zhu, 2002). This is a complex consisting of several components such as SOS1, SOS2 and SOS3. In particular, the SOS3 component appears to encode a protein capable of binding ions Ca^{2+}simultaneously, through an N-myristoylation motif, and the component SOS2. The latter, in turn, appears to encode a protein serine/threonine kinase, provided with the kinase catalytic domain, located near the N-terminal (Liu *et al*., 2000). In control conditions, the interaction between the catalytic and regulator domain would prevent the phosphorylation of the substrate, presumably by blocking access to the same substrate. On the contrary, in the presence of Ca^{2+} the SOS3 component would activate SOS2 kinase (Halfter *et al*., 2000; Guo *et al*., 2001). Furthermore, genetic analysis confirmed that SOS1, SOS2 and SOS3 working in a common *pathway* that gives plants the acquisition of mechanisms of salt stress tolerance (Zhu *et al*.,1998). In particular, the component SOS1 coding for an antiporter membrane protein Na */H *, which has a foretold cytoplasmic tail very long (Shi *et al*., 2000), and the SOS2-SOS3 kinase complex which likely checks the expression and activity of SOS1 (Shi *et al.*, 2000). Further, studies on *Saccharomyces cerevisiae* confirm that the calcium signal induced by salt stress is transduced from the SOS3-SOS2 kinase complex, which in turn activates SOS1 and restores cellular ionic homeostasis (Quintero *et al*., 2002).

Starting from these assumptions, Shi *et al.*, (2002, 2003) have suggested that under saline stress conditions, the activation of SOS1 would encourage excessive accumulation of sodium ions coming from the roots, in the xylem, preventing, therefore, their translocation to the vegetative apex.

-Calcium dependent protein kinase-(CDPKs)

Another prominent family involved in the signaling pathways in response to different types of stress, such as water, *wounding* and cold stress are the Calcium Dependent Protein Kinase (CDPKs). Studies of gene *stress-responsive* expression nd analysis conducted on transgenic plants, suggest that some calcium signals induced by stress are perceived and transduced by CDPKs. In particular, studies *in vitro* have shown that when *Mesembryanthemum crystallinum* is subject to conditions of salt and water stress, the substrate of the McCDPK1 phosphorylates the CSP1 (calcium-dependent protein kinase substrate protein 1) in a Ca^{2+}-dependent way, inducing a co-localization of McCDPK1 and CSP2 in their core (Patharkar *et* Cushman, 2000).

-Different signals converge to MAPK cascades

In all eukaryotes, MPK (Mitogen Protein Kinase), which activate relative mitogen kinase dependent cascade MAPK/MPK (mitogen-activated protein kinase), are highly conserved regulators. The MAPK are involved in several *signaling* ranging from development to that hormonal, as well as in response to biotic and abiotic stresses (Ligterink *et* Hirt, 2000).

In the *Arabidopsis thaliana* genome were identified about 60 MAPKKK (mitogen-activated protein kinase kinase kinase), 10 MAPKKs (mitogen-activated protein kinase kinase) and 20 MAPK (Chinnusamy *et al*., 2003). The signals received by the MAPKK must in fact be transduced through MAPK, MAPKK, which act as cross-talk between the different stress signaling (Chinnusamy *et al*., 2003). The MEKK1-MAPK cascade kinases 2 (MKK2)-MPK4/MPK6, for example, appears to work as part of *signaling* activated under stress conditions from cold or salt (Ichimura *et al.,* 2000; Teige *et al*., 2004). Two-hybrid analysis and use of kinases of *Arabidopsis thaliana* for complementation studies in yeast, have led to the identification of a putative MAPK cascade, consisting of *At*MEKK1, *At*MEK1/*At*MKK2 and *At*MP4 (Ichimura *et al.,* 1998). During saline stress the transcriptional activity of *AtMEKK1* actives *At*MP4, through a mechanism that allows to respond to low humidity conditions, osmotic stress, *touch* and *wound*ing (Ichimura *et al*., 2000). MEKK1-MKK4/MKK5 MPK3/MPK6 cascade seems to regulate a *pathway* of answers, which induce resistance mechanisms to pathogens, through the expression of *WRKY22* and *WRKY29* (Nuhse *et al*., 2000; Asai *et al.,* 2002). However MPK6 and MPK3 are also activated by abiotic stresses (Ichimura *et al.*, 2000; Droillard *et al.,* 2002) and are involved in the signaling of hormonal pathway. In particular, MPK3 seems to work as a *signaling* of ABA (Abscisic Acid) during the post-germination phase (Lu *et al.,* 2002), while MPK6 seems to be involved in the production of ethylene (ET), through the phosphorylation of key enzymes of ethylene biosynthesis such as 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, ACS2 and ACS6 (Liu *et al*., 2004). In *Oryza OsMPK5*, ortholog of *MPK3*, appears also to play kinase activity in the presence of ABA or in the presence of abiotic stresses and pathogen attacks (Xiong *et al*., 2003). Furthemore, MPK6 is active under oxidative stress conditions, induced in *Arabidopsis thaliana* cell cultures (Yuasa *et al*., 2001).

The activity of MPK3 and MPK6 in the signaling for ROS seem influenced by serine/threonine kinase of *Arabidopsisthaliana,* OXI1, which kinase activity is induced by hydrogen peroxide (Rentel *et al.*,2004). The latter seems to activate a MAPKKK of *Arabidopsis thaliana,* the ANP1, which again activate as cascade, MPK3 and MPK6. Tobacco plants that constitutively overexpress *NPK1* (Nicotiana protein kinase 1), an homolog of

ANP1, exhibit good tolerance to freezing, heat, drought stresses and to high salinity conditions (Kovtun *et al*., 2000). Analysis *in vitro*, also showed that overexpression of *NUCLEOSIDE-DIPHOSPHATE KINASE 2* (*AtNDPK2*) of *Arabidopsis thaliana* increases the tolerance of plants to several stresses such as cold, salinity and oxidation (Fujita *et al*., 2006).

-Roles of ROS at points of convergencebetween biotic and abiotic stress response pathways

The role of *scavenging enzymes* is to detoxify cells from the cytotoxic effects of ROS (Reactive Oxygen Species), under various stress conditions (Mittler *et al*., 2004; Apel *et al.*, 2004). Several enzymes belonging to this category, such as superoxide dismutase, Glutathione peroxidase, and ascorbate peroxide, were identified through the transcriptome analysis of plants subjected to abiotic and biotic stresses (Seki *et al*., 2002; Shenk *et al.*, 2000; Mittler *et al*., 2004). Furthemore, microarray analysis, using cell cultures of *Arabidopsis thaliana* reveal that, many ABA-inducible genes are induced by oxidative stress (Takahashi *et al*., 2004).

Functional analysis conducted throught transgenic approaches have highlighted that the overexpression of *Zat12*, that encodes the transcription factor C_2H_2-type *zinc finger*, is likely involved in the mechanism of ROS removal. In fact it induces the up-regulation of genes involved in the response to oxidative stresses and strong irradiance, increasing plant tolerance to critical condition of high brightness, freezing and oxidative stress (Davletovs *et al.*, 2005). Then, ROS could mediate the cross-talk between genes expression networks involved in biotic and abiotic stresses (Fujita *et al*., 2006).

-Key role of ABA in the expression of stress responsive genes

The ABA is a plant hormone that plays a key role in various stages of development including germination, dehydration tolerance and dormancy of the seeds, but also plays a crucial role in the response to abiotic (salinity, cold water, and hypoxia) and biotic stresses (Chinnusamy *et al.*, 2003).

Given the wide range of functions in which ABA is involved, it has been proposed that the transcriptional activation of many stress-responsive genes can be adjusted, either in a dependent or independent manner, by ABA *signaling* pathway (Shinozaki *et* Yamaguchi-Shinozachi, 2000; Zhu, 2002). However, little is known about the genes up-regulated by ABA, although it has been demonstrated that genes, responsible for the biosynthesis of this hormone, are activated by a signaling Ca^{2+} dependent (Chinnusamy *et al.*, 2003). Genetic analysis of the mutants ABA-deficient, *aba3/los5* and *los5/aba1*, showed that ABA plays a key role in osmotic stress response, inducing the expression of stress-specific genes. For

example in the mutants *los5* the expression of genes induced by stress, as *RD29A*, *RD22*, *COR15A*, *COR47* and *P5CS*, were found to be reduced or even completely silenced (Xiong *et al.*, 2001); while in mutant *los6*, the expression level of *RD29A*, *RD19* and *CO15A*, *COR47*, *KIN1* and *ADH*, was lower than in the *wild-type* (Xiong *et al*., 2002a). These data confirm that the ABA-dependent signaling plays an essential role in gene evoked response in plants under osmotic stress conditions.

Under stress conditions the ABA activates transcription factors, rich in Leucine zipper type such as ABFs/AREBs, that binding to specific *cis* regulatory sequences, are able to induce the expression of stress-inducible genes (Xiong *et al.*, 2002b). In particular, the heterologous expression in *Arabidopsis thaliana* of the soybean transcription factor *SCOF1*, induced by cold and ABA, increases the constitutive expression of stress responsive genes and improves freezing tolerance in transgenic plants (Kim *et al.*, 2001).

Interestingly, SCOF1, not seems to bind directly to the ABRE or DRE/CRT motifs, so its function might be to facilitate DNA binding of another bZIP transcription factor (SGBF1) and specifically to *cis* ABRE elements of stress responsive genes (Kim *et al*., 2001). Then, the SCOK1-SGBF1 complex would appear to regulate the expression pathway ABA-dependent of stress-responsive genes through ABRE, during cold stress conditions (Kim *et al*., 2001).

The *signaling* of stress responsive genes activation ABA-independent, instead, would seem to be regulated more through *cis*-DRE regulatory elements. In particular, in osmotic stress conditions, the transcriptional factors DREB2A and DREB2B bind directly to *cis* stress inducible promoters (Liu *et al*., 1998), or through the zinc finger transcriptional repressor, STZ/ZAT10 (Lee *et al.*, 2002).

1.4 Role of transcription factors in plant stress tolerance

A transcription factor (TF) is a protein that binds specifically to a DNA sequence and regulates gene transcription (Latchman,1997). Plant genomes contain large numbers of TFs which fall in few multi-genetic families, including for example, MYB, AP2/EREBP, bZIP and WRKY (Wang *et al.*, 2003). It is amply demonstrated that specific members of the same TFs family respond differently to various stresses as well as some stress-responsive genes share the same TFs, as indicated by significant overlap gene expression profiles induced by different stresses (Bohnert *et al*., 2001; Seki *et al.*, 2001; Cheng *et al.*, 2002; Fowler *et* Thomashow, 2002; Krep *et al*., 2002). The DREB (Dehydratation-Responsive Transcription Factors) transcription factors and the CBF (C-repeat Binding Factors) bind to DRE and CRT *cis* elements that contain the same sequence motif (CCGAC) and, at the same time, these

factors are themselves stress inducible (for example, CBF1 CBF2 and CBF3 or DREB1B, DREB1C and DREB1A, respectively). DREB/CBF proteins belong to multigenic family of AP2/EREBP transcription factors (Apetala2/ethylene-responsive element binding protein) and mediate transcription of several genes as well as *RD29A, RD17, COR 6-6, COR15A, ERD10, KIN1* and *KIN2* involved in cold and water stress response (Ingram *et* Bartels, 1996; Stockinger *et al*., 1997; Gilmour *et al.*, 1998; Liu *et al,* 1998; Seki et al., 2001; Thomashow et al., 2001).

In recent years, overexpression of stress inducible transcription factors has allowed not only to expand the knowledge about the mechanisms of plant adaptation to stress conditions, but also to open future prospects to engineering plants to increase tolerant stress. From these assumptions, 5.9% of *Arabidopsis thaliana* genome encodes more than 1500 TFs (Riechmann *et al.,* 2000), many of which have been functionally analyzed, using transgenic approaches. Interestingly, overexpression of these transcription factors in *Arabidopsis thaliana* increases the stress tolerance to freezing, water and/or saline in transgenes (Liu *et al*., 1998; Kasuga *et al.*, 1999; Gilmour *et al*., 2000, 2004). In addition, high levels of metabolites such as proline and soluble sugars (glucose, fructose, sugar and raffinose) accumulate in overexpressing plants CBF/DREB1, grown under normal conditions and during cold acclimation (Gilmour *et al*., 2000, 2004; Cook *et al.*, 2004; Achard *et al*, 2008). Significant improvements were obtained also for only one of these transcription factors (Thomashow, 1998).

In *Arabidopsis thaliana*, for example, cold acclimation induced by *cis*-regulatory elements CRT/DRE (Thomashow, 1998), increases the expression of CBF1, which in turn induces the expression of genes elicited by the low temperatures such as *COR6-6*, *COR115A*, *COR47* and *COR78*, finally increasing the freezing tolerance in plants not acclimatized (Jaglo-Ottsen *et al*., 1998). Again in *Arabidopsis thaliana*, the constitutive overexpression of the factor DREB1A seems to increase stress tolerance to freezing, water and salt in plants (Kasuga *et al*., 1999). In particular, the constitutive expression of DREB1A increases the expression of downstream target genes, as well as *RD29A, RD17, COR 6.6, COR15A, ERD10* and *KIN1* (Kasuga *et al*., 1999). Likewise, the overexpression of *CBF3* increases the levels of delta-pyridine-5-carboxylate sintetatasi (P5CS), the key enzyme of proline biosynthesis (Gilmour *et al*., 2000). In *Arabidopsis,* the transcription factor CBF4, a homolog of CBF/DREB, regulated by 35SCaMV constitutive promoter, increases the expression of genes induced by cold and water stress under control conditions (Haake *et al.,* 2002).

In addition, the heterologous expression of *Arabidopsis thaliana* CBF/DREB in other systems have shown satisfactory results. In *Brassica napus*, for example the constitutive overexpression of the *Arabidopsis thaliana CBF* genes, induces the expression of *CBF*

orthologs, increasing tolerance to freezing in plants both non-acclimatized and cold-acclimatized (Jaglo *et al*., 2001). Interestingly, Hsieh *et al*, (2002) also showed that the expression of *CBF1* of *Arabidopsis thaliana* in tomato plants gives a high tolerance to freezing and oxidative stress.

As previously reported, while some *cis* elements respond to a specific environmental stress, others are less selective, like the *cis*-regulatory element ABRE that responds to ABA. The *signaling* of the ABA plays a vital role in plant stress response. In fact many of the genes induced by water stress, are also elicited by ABA. Among *cis* regulatory families that belong to this category it must be remember the bZIP and MYB elements, responsible for the activation of its genes (Wang *et al*., 2003). In particular ABRE presents a region in consensus sequence, ACGT, with a G-box that responds to various stress conditions, such as osmotic stress, UV and strong irradiance (Loake *et al*., 1992). It is thought that G-box and ABRE might share a similar function under the same stress conditions, as demonstrated by the fact that, when it is overexpressed, a single ABRE is not sufficient to activate the gene expression (Guiltinan *et al*., 1990; Skriver *et al.,* 1991), raising the central question if ABRE is a partial component, able to give the activity of promoter and at the same time requires the presence of another component in order to be able to distinguish itself from the G-box. That question was partly clarified with the identification of the EC (coupling element) (Shen *et* Ho, 1995). In transient expression assays, promoter sequences of the genes encoding two LEA proteins, *HVA1* and *HVA22*, increased in barley aleurone layers in the presence of ABA (Shen *et Ho* I, 1995). Other proteins that bind ABRE, were isolated, including TRAB, AREB/ABF and AB15 of *Arabidopsis* (Hobo *et al*., 1999; Choi *et al*., 2000; Finkelstein *et* Lynch, 2000; Lopez-Molina *et* Chua, 2000; Kong *et al*., 2002).

Other *cis* regulatory elements that act as transcriptional activators of genes induced by ABA are *cis* MYBR and MYCR elements (Abe *et al.,* 2003). It was shows how the constitutive expression of ABF3 and ABF4 factors lead to increased drought stress tolerance in *Arabidopsis* plants, inducing an altered expression of ABA/stress responsive genes e.g. *RD29B, RAB18, AB11 and AB12* (Kang *et al.,* 2002). The relationship between the MYB factors and ABA, it also proved by the presence of a saline hypersensitivity in trangenic lines of *Arabidopsis* overexpressing the TFs ABF3 and ABF4 during germination and seedlings. Saline hypersensitivity is usually characteristic of the ABA associated phenotypes, including ABA and sugar hypersensitivity, indicating the possible involvement of ABF3 and ABF4 in the salt response, in these specific developmental stage.

The heat-shock response is primarily regulated at the transcriptional level and in particular attributed to *cis*-regulatory elements found in the promoter region, binding sites for HSFs (trans-active heat-shock factors) (Schoffler *et al*., 1998). About 21 HSFs have been identified in *Arabidopsis*, more than 16 in 15 tomato and soybean (Nover *et al.*, 2001) grouped into three classes A, B, and C and all having a common consensus sequence "nGAAnnTTCnnGAAn". The HSFs are activated during the cellular growth under stress conditions, like chaperones (Morimoto, 1998, Schooffler *et al*., 1998, Mishra *et al*., 2002). Studies in the literature show that 4 HSFs in transgenic plants increases the tolerance under strong irradiance, as observed in mutants of tomato which overexpress the HsfA1 factor (Mishra *et al.,* 2002). On the contrary the antisense HsfA1 expression causes an extreme sensitivity to high temperatures both in plants and fruits (Mishra *et al*., 2002). This response has been attributed to *SPL* genes (spotted leaf), also belong to the HSFs category that acts controlling cell death caused by environmental stress such as high temperatures,. The expression of the *SPL7* gene in transgenic plants of rice under high temperature, slows the formation of lesions (spotted leaf) (Yamanouchi *et al.,* 2002).

In conclusion we can say that, in the context of an improvement of agricultural and environmental practices, if there are obvious benefits of a proven acquisition of stress tolerance in plants overexpressing TF, on the other hand they are also showed negative effects. Studies in the literature show that the transformation of plants with TF, can also activate additional genes unrelated to stress that affect certain stages of plant development. For example, a common adverse effect of plants overexpressing TF is the delayed in growth (Kasuga *et al.,* 1999; Hsiek *et al.,* 2002; Koag *et al.,* 2002). The last effect might be partially overcome by the use of stress inducible promoters that control the TF expression (Kasuga *et al.,* 1999).

1.5 Growth inhibition as the active response of the plant

The reduction and subsequent growth inhibition is currently considered an active response that the plant use when subjected to stressful conditions. Despite this, few information are known about the mechanisms that are involved (Skirycz *et* Inzé, 2010). Many current studies are focused on real *set-up* experiments in order to investigate how stress can induce such inhibition (Skirycz *et al*.., 2011). The growth of an organ is led by two tightly controlled and dynamic processes: proliferation and subsequent cellular expansion. For example, the coordination of these two processes during leaf growth determines its size and shape. In dicotyledons as the model plant *Arabidopsis thaliana*, the leaves protrude from the

sides of the meristem and the two phenomena occur sequentially in time (Claeys *et al*., 2012). Early on, their growth is driven exclusively by cellular proliferation (Donnelly *et al*., 1999). Initially the cells of the leave primodium divide actively, then decrease and stop during development, but starting from the tip to the base, moving through the leaf (Donnelly *et al*., 1999). This transition from the stage of cell proliferation to expansion is indeed accompanied by a switch of the cell cycle to a phenomenon of endoreduplication, during which the genome is replicated, but not mitosis take place, bringing the cells at a high rate of ploidy as well as 4c, 8c, etc.. (Beemster *et al.,* 2005). In the environments with low water availability, plants respond to this drought stress through an initial rapid reduction of growth followed by an adaptation of the growth, which gives rise to the leaves composed of fewer cells and even smaller size (Schuppler *et al*., 1998; Granier *et* Tardieu, 1999; Aguirrezabal *et al.,* 2006). Cyto-histological analysis showed that as soon as osmotic stress is perceived from the plant, it's possible observe a quick arrest of the cell cycle progression with a cell maintenance in a latent stat, followed by an immediate recovery as soon as the conditions become favourable (Skirycz *et al*., 2011). Interestingly the cell cycle arrest coincides with an increase in 1-aminocyclopropane-1-carboxylate and with the activation of the ethylene signaling (Skirycz *et al.*, 2011). In particular, ethylene acts on cell cycle progression by inhibiting the activity of cyclin-dependent kinase (CDKA). When stress persists, cells leaving the mitotic cycle and begin the differentiation process (stop). This stop is accompanied by an initial DNA endoreduplication, in a process independent of the ethylene signaling. Nevertheless, the decrease in the number of cells remains "covered" thanks to meristemoids activities. Together these data present a conceptual framework of how environmental stress reduces plant growth. The inhibition or exiting the mitotic cycle is currently attributed to several factors. For example, a first cause is related to a decrease in the activity of cyclin-dependent kinase (CDK) (De Veylder *et al.,* 2001; Breuer *et al.,* 2010), or contrary to an up-regulation of cell cycle inhibitors as well as KRP2 (Kip-related Protein2) and SIM (SIAMESE). These, in fact, control the endoreduplication cycles in *Arabidopsis* trichomes and interact with CDKB complexes/cyclins (Van Leene *et al*., 2007). Yet Lammens *et al.,* (2008), confirmed as an important pathway in the regulation of endoreduplication, the APC/C complex (anaphase-promoting complex/cyclosome) as activator of CELL CYCLE SWITCH PROTEIN52A subunit (CCS53A). The latter are positive regulators of endoreduplication cycles in which act directly on cyclins gene and often also destroying them. A concluding hypothesis about the function of endoreduplication is that it can keep the fate of a cell and the dedifferentiation (Bramsiepe *et al.*, 2010).

The main activities of the CDKA, in cell cycle progression, can also be reduced through direct degradation of cyclins and/or by the inhibition of phosphorylation, as shown in plants of *Triticum aestivum* subject to drought stress (Schuppler *et al.,* 1998). The hormone abs*cis*ic acid (ABA), acting upstream cell cycle machinery influences the expression of ICK/KRPe/SIAMESE (Wang *et a*l., 1998; Pettkò-Szandtner *et al.,* 2006). Similarly to the ABA, the precursor of ethylene (ACC) is known to be carried by the vegetative apex to the root (Sobeih *et al.,* 2004). In this contest ABA and ethylene are considered to be two good candidates in the communication of changes in ground water concentration at meristems (Sobeih *et al*., 2004).

There are many examples in the literature on positive and negative effects of ethylene and ABA (Sharp *et* LeNoble, 2002; Pierik *et al.,* 2006), though their exact role in regulating the cell cycle remains largely unknown. However, data in the literature have clearly that shown the arrest of the cell cycle is a rapid response to stress and it is mediated by post-transcriptional mechanisms rather than by a transcriptional cascade, with ethylene which acts upstream of arrest (though reversible) of the cell cycle. While thus ethylene represents a primary signal for the stunted, the subsequent exit from the cell cycle is ethylene-independent and takes place relatively late (Skirycz *et al.,* 2011).

CHAPTER 2

Dehydrins and stress tolerance

2.1 Structure and multiple funtions

In plants and mostly in the model plant *Arabidopsis thaliana*, transcriptome analysis, together with the phenotyping of mutated or transgenic plants, a number of regulatory as well as protective proteins that are involved in the tolerance of plants to different stress factors have been identified (Choi *et al*., 1999; Gulik and Kostesha, 2004; Skinner *et al*., 2005; Svensson *et al*., 2006; Stutte *et al*., 2006; Yamaguchi-Shinozaki *et* Shinozaki, 2006). For some related stresses, such as cold/dehydration, salt/drought stress but also dehydration/oxidative stress, oxidative stress/biotic stress, common response mechanisms have been identified. These mechanisms encompass the production of protective compounds such as specific proteins, antioxidant, detoxifiers, compatibile solutes, the regulation and signal transduction i.e. the involvement of transcription factors, hormones, kinases and also structural and metabolic remodelling (Zhu *et al*., 2000; Buchanan *et al*., 2002; Svenson *et al*., 2002; Gechev *et al*., 2006).

Dehydrin proteins (DHNs) belong to these common protective compounds, in fact they have been observed in several independent studies on drought stress, cold acclimation and salinity stress (Close *et al*., 1989; Rorat *et al*., 2006). DHNs are a member of a large group of highly hydrophilic protein, known as protein LEA (Late Embryogenesis Abundant), and initially, have been identified as group II of the LEA proteins (Dure *et al*., 1989; Dure *et* Close, 1993; Battaglia *et al*. , 2008). The distinctive feature of all DHNs is the presence of a highly conserved lysine-rich 15-amino acid motif (EKKGIMDKIKEKLPG), named K-segment, usually located near the C-terminal end of the protein. Other typical characteristics of dehydrins are: a serine-rich domain (S-segment), a consensus motif, T/VDEYGNP (Y-segment), located near the N-terminus and a less conserved regions, rich in polar amino acids (φ segments). On the basis of the number and the order of segments Y, S, and K, DHNs can be divided into five subclasses: Kn, SKn, KnS, YnKn and YnSKn (Close, 1996).

According to Svenson *et al*. (2002), within the Swiss Protein database were found in plants, 5 distinct groups of DHNs; specifically 35 YnSnKn- type, 21 SnKn- type, 17 Kn-type, 10 YnKn- type and 7 of KnSn-type. Typically, one plant species has several dehydrins, representing different subgroup. For example, *Arabidopsis thaliana* has ten different

dehydrins divided into the following subgroups: 2Kn, 3SKn, 3YnSKn, 1YnKn and 1KnS (Eriksson *et al*., 2011).

Interestingly, although most of them present a wide tissue distribution related to stress conditions, a smaller number of DHNs seem to be constitutively expressed (Houde *et al*., 1992; Danyluk *et al*., 1994; Rorat *et al*., 2004). They localize in different types of tissues and organs, such as seeds, buds, roots, root tips, stems, vascular tissues, leaves and flowers (Rorat *et al*., 2004). On the other hand some DHNs activity was restricted to specific domains, or cells within the organs, as in guard cells, meristematic cells, at the level of plasmodesmata or in pollen sacs (Nylander *et al*., 2001; Karlson *et al*., 2003). For example, in *Arabidopsis thaliana* in which are identified 10 DHNs, named *At*DHNs, four have been identified in specific organs such as seeds, leaves, roots, buds, stems, and flowers, 3 in both plant organs and seeds, while the remaining show a specific tissue localization, since two restricted only to seeds and another is instead limited to roots (Hundertmark *et* Hincha, 2008). Subcellular localization analysis showed DHNs in connection with the plasma membrane, in the vacuole, the mitochondria, in the membranes and in the bodies proplastidiali protein (Danyluk *et al*., 1998; Rinne *et al*., 1999; Borovskii *et al*., 2002; Heyen *et al*., 2002; Mehta *et al*., 2009) and some also in chloroplasts and peroxisomes (Tunnacliffe *et* Wise, 2007).

Althoug dehydrins are found exclusively in plants, members of groups of other LEAs are also present in organisms such as bacteria and nematodes (Goyal *et al*., 2005; Tunacliffe *et* Wise, 2007). In plants, they were found in both angiosperms and gymnosperms, but also in mosses (Saavedra *et al*., 2006), lycopods (Iturriaga *et al*., 2006), ferns (Reynolds *et* Bewley, 1993) and liver (Hellwege *et al*., 1994). Immunological studies also show the presence of DHNs even in cyanobacteria (Close *et* Lammers, 1993) and algae (Li *et al*., 1997).

With a molecular mass between 9 and 200 kD (Wisniewski *et al*., 1999), in aqueous solution the DHNs assume a random coil conformation due to the ability to form inter-and intramolecular hydrogen bonds with water molecules (Tompa, 2002, Tompa *et al*., 2005) (Fig.2.1). Due to an unbalanced proportion in favor of the former, they appear unstructured, thus showing typical features of proteins IDP/IUPS (unstructured proteins) (Tompa, 2002, Tompa *et al*., 2005). The DHNs seem to be able to change their conformation according to the type of the microenvironment in which they are located, as also supported by analysis of far-UV circular dichroism conducted in aqueous solution (Lisse *et al*., 1996; Mouillon *et al*., 1998).

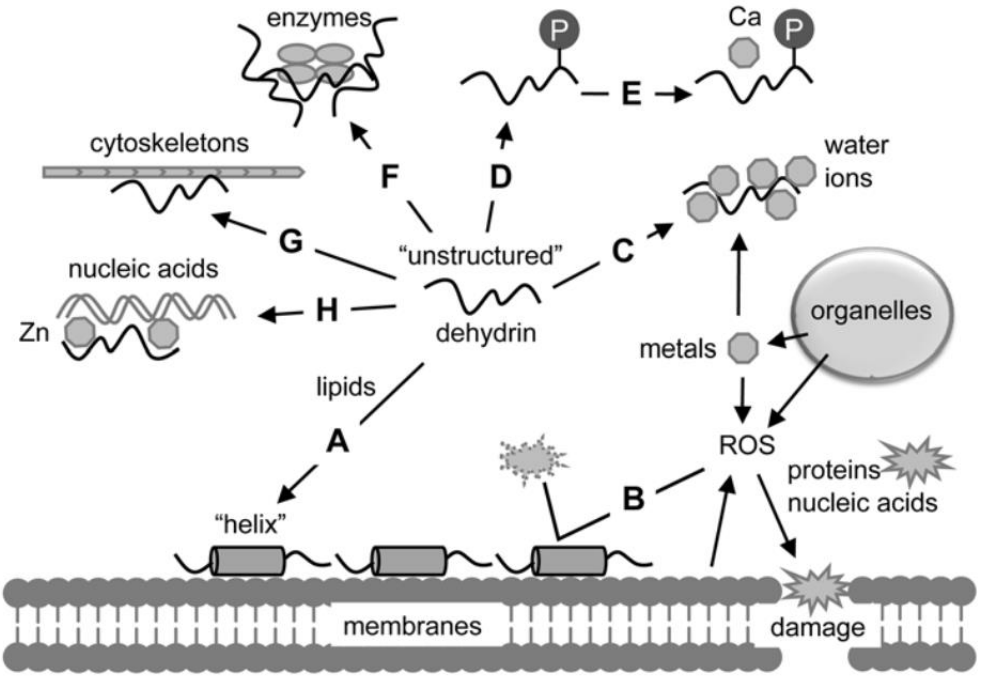


Fig. 2.1 Functions of dehydrins with experimental evidence. Functions are represented by A–H. A, binding to phospholipids; B, radical scavenging; C,binding to water and ions; D, phosphorylation; E, binding to calcium; F, protection of enzymes; G, binding to cytoskeletons; H, binding to nucleic acids.This scheme is produced by combining data from individual studies. Some dehydrins do not have all of the functions shown in the scheme (Hara *et al.*, 2010)

The structural changes seem to be linked essentially to K-segments, which are able to adopt the α-helical conformation similar to class A2 amphipathic α- helices present in apolipoproteins and in α-synucleins (Rorat, 2006).

Based on amino acids (aa) dislocation, the DHNs structure seem to assume a conformation called *moon lighting*: the amino acids negatively charged with acid pI such as D (aspartic acid,Asp) and E (glutamic acid, Glu) lie on one side of the helix, hydrophobic aa, non-polar, e.g., I (isoleucine, Ile) and L (leucine, Leu) lie on the opposite side of the helix and positively loads aa ,with basic pI, for example, K (lysine, Lys) and R (arginine, Arg) lie on the polar-non-polar interface (Svensson *et al.*, 2002; Savaedra *et al.*, 2006), assuming (Tompa, 2002; Tompa *et al.*, 2005).

Since it has been seen that the amphipathic alpha helices can interact with partially dehydrated surfaces of other proteins and, even with surfaces of biomembranes, Ingram *et* Bartels (1996), proposed that different K-segments in a DHN molecule might form bundles when present in the alpha helix conformation, in order to increase their amphipathic character in protein-protein interactions and protein-biomebrane. Assuming this conformation, DHNs can constitute a *molecular shield*, to protect the other proteins, preventing further loss of

water (Hanin *et al*., 2011). Protective function against the thermal aggregation of citrate synthase, firefly luciferase, inactivation of lysozyme and thermal inactivation of alcohol dehydrogenase, were observed in ERD10 and ERD4 dehydrins of *Arabidopsis thaliana* (Kovacs *et al*., 2008). Cryoprotective activity has also been reported for several DHNs, as well as the spinach COR85 (Kazuoka *et al*., 1994), wheat WCS120 (Houde *et al*., 1995) and the PCA60 from peach (Wisniewski *et al*., 1999). As shown by Reyes *et al*., (2008) the presence of the K segment is essential for the cryoprotective activity of the dhydrins. On the other hand, it also plausible to imagine that the DHNs might also act as chaperones, preventing in some cases *heat inactivation* of proteins and helping them to fold properly and/or preventing their aggregation under heat or freezing stress conditions (Hanin *et al*., 2011). However, the classical chaperones, not only preventthe inappropriate protein aggregation but also form complexes with specific target proteins, through hydrophobic interactions of patches (Ellis *et al*., 2004). Recently, Brini *et al*., (2010), showed that *in vitro*, the dehydrin of wheat DHN-5, improved the activity and/or thermostability of the enzymes fungal β-glucusidase (bglG) and glucose oxidase (GOD/POD). For this reason, many authors have described the protective functions of these proteins based on non-specific protein-protein interactions, given the difficulty to establish specific interactions with other proteins, particularly under conditions of water deficit (Tunnacliffe *et* Wise, 2007). Recently it was also associated a *space filler* function to DHNs. Thanks to their unstructured status and to high storage capacity" of water molecules, the DHNs, might keep the original cell volume under a dehydration state, in order to prevent cellular collapse. As they can accumulate to fill the spaces that have occurred and, helping therefore, the maintenance of the original distance between the intracellular complexes (Hanin *et al*., 2011).

DHNs exert an antioxidant function, against the reactive oxigen species (ROS), they have scavenging and metal ion binding properties, thanks to the high presence of H and R and other aa residues on the surface (Hanin *et al*., 2011). Both functions are mediated by direct interactions between aa residues and ROS species (superoxide anion radical O_2, singlet oxygen O_2, hydroxyl radicals HO, hydrogen peroxide H_2O_2) or metal ions (Co2+; Cu2+; Fe2+; Fe3+; Ni^2+;Zn^2+). These interactions lead to oxidation of aa residues, while the interaction with metal ions cause the formation of covalent bonds. For example, the *Citrus Unshu* dehydrins, CuCOR15 and CuCOR19, it was shown to be ions sequestrant (Hara *et al*., 2001, 2005), or still VCaB45 in *Apium graveolens* is able to bind Ca^2+ in the vacuole and in some case it can also capture and transport metal ions such as Fe2 + and Fe^3+, in the phloem sap, as reported for the protein ITP of *Ricinus communis* (Krüger *et al*, 2002). On the basis of

the behavior of these dehydrins, we can affirm, that seem not to be a correlation between the subgroup and the type of stress related (Close, 1997; Nylander *et al*., 2001). Moreover, considering the wide range of functions and the massive presence in the proteome plant, it's clear as these proteins might play an important physiological role in the adaptive responses of plants to various environmental conditions.

2.2 Functional studies of dehydrins

Although much effort has been made to improve plants resistance to abiotic stresses through traditional breeding, success has been limited, especially in woody plants. Thus, engineering approach in order to select new genotypes which present resistance characters to several stress factors might play an important role in economic field. Genetic modification by transferring resistant genes is considered to be one of the most effective experimental approach (Li *et al*., 2008; Harfouce *et al*., 2010). Plant engineering strategies therefore, is an excellent *key study* to functional analysis of these genes, but it can also be a possible resource to counter the environmental problems caused by different stress. In particular, the construction of transgenic plants overexpressing dehydrin genes could give valuable informations about their possible role in plants growing under abiotic and/or biotic stresses (Erikkson *et al*., 2011). On the other hand heterologous expression of *DHNs*genes, especially in dicotyledonous species, showed a positive correlation between the accumulation of transcripts/proteins or proteins and tolerance to freezing stress, dry and salinity (Hara *et al*., 2003; Rorat *et al*., 2006; Choudhury *et al*., 2007). For example, cold tolerance in tobacco transgenic plants, has been increased through the expression of a *Citrus unshiu* Marcov gene encoding a LEA protein, the CuCOR19 (Hara *et al*., 2003), and in *Arabidopsis thaliana* by the overexpression of COR15A (Artus *et al*., 1996), a chloroplast dehydrin, either through co-expression of chimeric constructs with dehydrins RAB18 & COR47, XERO2 &ERD10 (Nylander, 2001). In wheat and rice, the overexpression of the dehydrin gene of barley, HVA1, confers increased tolerance under water stress conditions int plants (Xu *et al*., 1996), while the expression of heterologous PMA80 and PMA1959 of wheat, increases the tolerance in a state of water scarcity and salinity in transgenic plants of rice (Cheng *et al*., 2002).

A powerful method to study the effect of dehydrin expression is by *knockout* mutants, i.e. by silencing the gene expression, however, at present, there are few reports of *knockout* mutants for LEA proteins, and only one known in a moss dehydrin, the DHNA of *Physcomitrella patens* (Saavedra *et al*., 2006). Compared to the *wild type* moss, the *knockout* showed no phenotypic change or any change in growth respons, but the transgenic plants were

seriously compromised in their ability to recover after undergoing saline or osmotic stresses , suggesting a gene stress-tolerance contribution (Saavedra *et al*., 2006). Studies showed by Wang *et al*., (2011) confirmed that the overexpression of a dehydrin SK2-type, isolated from *Populus euphratica*, plant particularly resistant to drought conditions, in transgenic plants of *P. tremulax P.alba* confered high capacity to retain water under drought stress. As well as, the ectopic expression of DHN-5 gene of wheat in *Arabidopsis*, seem to improve tolerance in plants subjected to drought and salt stresses (Brini *et al*., 2007). Still, transgenic *Arabidopsis* plants, overexpressing RcDHN5 n acid dehydrin of *Rhododendrom catawbiense*, a stress tolerant plantincreased tolerance to freezing (Peng *et al*., 2008) similarly to dehydrin DHN24 of *Solanum sogarandinum*, when overexpressed in melon (*Cucumis sativus*) (Yin *et al*., 2006). On the other hand, the constitutive expression of DHN1 (ABA2) of barley, in transgenic *Arabidopsis* plants, increased the germination ratio under osmotic and saline stress conditions, but no significant effects were detected on germination in conditions of low temperatures or frost damage in adult plants (Calestani *et al*., 1998).

Moreover, even if most of the data in literature show a positive effect of dehydrins in response to stress, there are also examples in which dehydrin overexpression has no effect at all. For istance, overexpression of Rab18 gene of *Arabidopsis* has not positive effects on drought and freezing tolerance. But, interestingly, when the same gene was co-expressed with *Arabidopsis* dehydrin Cor47, it was observed an increased freezing tolerance, but not to drought stress (Puhakainen *et al*, 2004). On the other hand, *DHNs* overexpression in transgenic tobacco plants, overexpressing both dehydrin of spinach Kn-type,specifically, CAP85, K11, (Kaye *et al*, 1998) and three *DHNs* of *C. plantagineum* (Iturriaga *et al*, 1992), did not induce significant changes neither in tolerance to freezing in the first case, neither in drought tolerance in the latter.

In general, this result might indicate that not all LEA proteins give a significant contribution to stress tolerance in plants, or, as suggested in strawberry transgenic plants, they need of a specific background (Houde *et al*., 2004) or, in some case, DHNs can act in synergistic way to improve tolerance to stress (Puhakainen *et al*., 2004; Hanin *et al*., 2011).

2.3 Regulatory mechanisms involved in dehydrin expression during stress conditions

In 1993, Nordin *et al*., (1993) identified at the promoter region of some genes, elements that seemed to be involved in the response to low temperatures and named LTRE elements (Low Temperature Response Elements). The function of these short sequences was

subsequently determined by deletion analysis, showing that an 9bp-element, TACCGACAT, activated gene transcription in response to low temperatures, drought and high salinity, but not to ABA (Yamaguchi-Shinozaki *et* Shinozaki, 1994). These elements, known as LTRE/DRE (low/drought Temperature Response Elements), which respond to cold and dehydration stress, are present in different *DHN* promoters as well as in other promoters, belong to cold and drought related genes, which are named C-repeat (CRT) sequence (Baker *et al*., 1994) (Fig.2.2). A small family of proteins that act as transcriptional activators, have been identified in the *Arabidopsis* genome. These factors called CBF1, CBF2 and CBF3 (CRT binding factor) or DREB1B, DREB1C and DREB1A (DRE binding protein), activate the expression of stress related genes by exposure to low temperatures, binding element DRE/CRT/LTRE (Shinozaki *et* Yamaguchi-Shinozaki, 2006). In particular three *Arabidopsis* responsive transcription factors, CBF1, CBF2 and CBF3 were induced by cold (Gilmour *et al*., 2000; Gilmour *et al*., 2004) while the CBF4 seems to be induced by ABA and drought stress but not by cold (Yang *et al*., 2005) (Fig.2.2).

Among the experimental strategies to increase stress tolerance (drought, salinity, freezing) in plants, the most efficient is to introduce genes encoding stress-inducible transcription factors. Many plants, such as *Arabidopsis*, show increased resistance to freezing after being exposed to low temperatures. This response, known as *cold-acclimatation,* is associated with the induction of COR (COLD REGULATED) genes and involves the activation of elements CRT/DRE, which are then present in them regulatory sequences (promoters) (Beck *et al*., 2007). The over-expression of a transcription factor or transcriptional activator of a multigene-sequence whose products directly or indirectly confer improved stress tolerance, represent an excellent stategy to cope with the fact of a strain syndrome (Beck *et al*., 2007). For example, overexpression of stress responsive-element binding factor CBP1 (CRT/DRE binding protein), isolated in *Arabidopsis*,significantly improves plant survivability under cold stress (Jaglo-Ottosen *et al*., 1998). In *Arabidopsis*the CBF1 overexpression , under the control of the strong promoter 35SCaMV, (cauliflower mosaic virus), induce the expression of the four COR genes respectively *cor6.6*, *cor15a*, *cor47* and *cor78* (Jaglo-Ottosen *et al*., 1998) and, similar results were obtained with factor CBF3 (Gilmour *et al*., 2000). However, in plants the overexpression of this factor also led to a delay in the normal growth (Jaglo-Ottosen *et al*, 1998). This problem has been exceeded using the stress-inducible RD29A promoter , to drive the expression of DREB1A, a CBF1 homologue. In this case it was possible to minimize the negative effect on growth plant under normal conditions, and also improve resistance to various types of stress (Kasuga *et al*, 1999).

In fact,in this study were used target genes stress-related such as RD29A, KIN1, COR6/KIN2, COR47RD17, COR 15A AND ERD10, containing DRE elements or motifs related to it (Kasuga *et al.*, 1999). These data demonstrate, therefore, as DREB1A and CBF1 transcription factors, encode proteins involved in protection against stressors (Kasuga *et al.*, 1999; Yamaguchi-Shinozaki *et* Shinozaki, 2006). Interestingly, among stress-related target genes, many belong to dehydrin group, such as COR47 and ERD10.

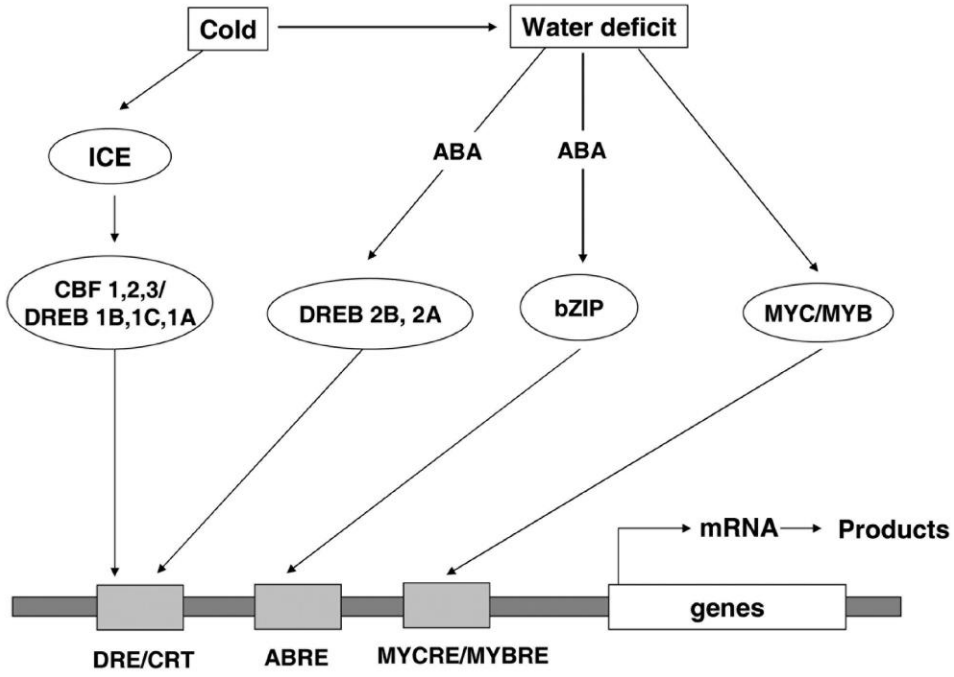


Fig.2.2 Involvement of various signal transduction elements in the adaptation response to cold and drought. ICE, transcription induction factor; ICE; transcription induction factor; MYC, MYB, transcription activators and MYCRE, MYBRE, MYC- and MYB-responsive elements in the promoter (Beck *et al.*, 2007)

In 2007, this approach was also shifted on “no model plants", belonging to the genus *Citrus*, where, once again, it was observed the involvement of the factors CBFs (C-repeat-binding factors), in the stress tolerance response (Champ *et al.*, 2007). *Poncirus trifoliata* is a genus of *Citrus*, extremely cold tolerant that can survive in extreme conditions, until -20°C, when fully cold-acclimated. Interestingly, once isolated and characterized, a factor CBF of this plant, (*Pt*CBF1), has been shown to be implicated in the response to cold tolerance. In particular the CBF element (C-repeat/dehydration-responsive element) of the promoter dehydrin CORc115, is a site recognized by *Pt*CBF1, thus indicating as the related stress gene, CORC115, is also a target for the *Pt*CBF1transcription factor (Champ *et al.*, 2007). The DRE/CRT/LTRE element is also recognized by a family of related transcription factors (DREB2A and DREB2B) responding to drought stress (Liu *et al.*, 1998).

However it has been observed that many of the DHN genes that respond to low temperatures and drought, are also induced by exogenous ABA. Consequently, we can expect that the promoters of these genes, contain *cis* elements, mediators of these responses. Instead, sequences with high similarity to the responsive elements ABA (ABREs) have been identified in these promoters, such as the consensus sequence C/TACGTGGC. Many dehydrins and other cold-regulated genes contain elements ABRE in their promoters, responsible for inducing ABA-regulated expression of many genes (Guiltinan *et al*., 1990; Leung *et* Giraudat, 1998). It were found motifs zipper leucine (bZIP), that can specifically bind to ABREs element (Guiltinan *et al*., 1990). In particular, two bZIP proteins have recently been characterized in *Arabidopsis* (Uno *et al*., 2000). These genes AREB1 and AREB2 (ABA-Responsive Element Binding Protein) respond to ABA, drought and salinity. In another study, a small family of ABRE binding factor (ABFs), has also been characterized in barley (Choi *et al*., 2002).ABFs respond differently to various environmental stresses including low temperature, suggesting that they act in different stress-responsive pathways.

During seed development, ABA plays an important role in embryonic maturation, in the acquisition of tolerance to dehydration, as well as induction and maintenance of dormancy. The ABRE elements are also implicated in the control of the expression of *DHN* genes in the seeds (Busk *et al.*, 1997). Different combinations of the ABRE elements, are active in vegetative tissues and seeds, as shown by the analysis of themaize RAB17 promoter (Busk *et al.*, 1997). Interestingly the expression of the RAB17 dehydrin, containing ABRE elements in its promoter, increased in cold treatment (Busk *et al*., 1997). On the other hand ABA-deficient (*aba-1*) and ABA-insensitive (*ab1-1*) mutants are not able to develop a sufficient level of freezing tolerance compared to *wild-tipe*, under cold stress conditions (Lang *et al*., 1994). The transcriptional activator AB13 in *Arabidopsis* (relative to VP1 in corn) is the key regulator of this response in the seeds (Parcy *et al.*, 1994) and *abi3* mutants remain non-dormant, not acquire tolerance to dehydration and fail in the accumulation of different transcripts related to seed maturation. Moreover the expression of *AB13* in transgenic *Arabidopsis* lines, induced ABA expression of seeds specific genes in vegetative tissues and influences stress responses in these tissues (Parcy *et* Giraudat, 1997).

In summary, *DHN* genes are controlled by a number of distinct but interacting signal pathways that may also exhibit tissue specificity (Svensson *et al.*, 2002).

CHAPTER III

Study models*:*

Olea europaea L. subsp *europaea* var. *sylvestris*

and

Arabidopsis thaliana

3.1 *Olea europaea* L. *subsp europaea* var. *sylvestris*

3.1.1 Classification and geographical distribution

Olive is an economically important woody fruit crop, oil, in fact, is obtained from the fruit of olives tress and it is a genuine fruit juice with excellent nutritional, sensorial and functional properties. On the other hand its biological, nutritional and healthful effects are universally acknowledge (Servilli *et al*., 2004; Morello *et al*., 2005).

Olive tree (*Olea europaea* L.) belongs to the *Oleaceae* family which includes the genus *Olea*, *Fraxinus*, *Forsythia*, *Forestiera*, *Ligustrum* and *Syringa*. The genus *Olea* include approximatively 30 species, distributed in Europe, Asia and Africa (Green, 2002). All the species have the same chromosome number (2n = 2x = 46) (Barone *et* Di marco, 2003; Loureiro *et al.,* 2007).

The *Olea europaea* species comprises six subspecies, defined on the basis of morphological characters and geographical distribution. *Olea europaea* subsp. *cerasiformis* is present on the island of Madeira; *Olea europaea* subsp. *cuspidata* is spread from Iran to China; *Olea europaea* subsp. *europaea*, *Olea europaea* subsp. *guanchica* is present in the Canary Islands; *Olea europaea* subsp. *laperrinei* is located in the Sahara region; *Olea europaea* subsp. *maroccana* is present in Marocco (Green, 2002).

According to Rugini (1995) the Mediterranean basin provides the ideal conditions for olive growing, and supplies more than 97% of the world production of olive, where the tree olive (*Olea europaea* L subsp. *europaea)* are distinguishedby botanists namely var. *sativa*, which is the cultivated form and var. *sylvestris*, which represent the wild olive tree, also known as oleaster (Green 2002; Hannachi *et al*., 2010) (Fig.3.1).

According to Simmonds (1979) three species outside the Mediterranean, have been contributed mainly the evolution of *O. europaea*: the presahariana *O. laperrinii*, the South African *O. chrysophylla* (= O. African Miller) and the Asian *O. ferruginea* (= cuspidata).

Some authors suggest the species *O. europaea* derives by hybridization between *O. ferruginea* or *O. chrysophylla* and another probably extinct. Zohary (1994) suggests that it is not possible identify the *O. europaea* progenitor in any of the related Mediterranean species. On the other hand, more recent research conducted at the chromosomal level, have strengthened the hypothesis that the species has evolved from phenomena of interspecific hybridization, followed by chromosome duplication (Falistocco *et* Tosti, 1996).

Fig.3.1 *Olea europaea* L. subsp. *europaea* var. *sylvestris* plants

3.1.2 Morphology, phenology and reproductive biology

Olea europaea L. subsp. *europaea. Sylvestris* and *Olea europaea* L. subsp. *europaea* var. *sativa*, are both evergreen trees, oleaster, tends to take a shrubby form, whereas *sativa* is a tree which can reach over 15 meter height (Mulas, 2005).

Fertile individuals can be obtained by different approach, i.e. crossing between the two subspecies, with a process of genetic replacement or increased spontaneous pollination, causing a phenomenon of substitution of oleaster, called drowning (Barone *et* Di marco, 2003).

On the other hand, it was shown a low intraspecific variation at least among the studied cultivars and between them and wild olive in fact, the nuclear DNA content of *O. europaea* cultivars ranged between 2.90 ± 0.020 pg/2C and 3.07 ± 0.018 pg/2C and the genome size of wild olive was estimated as 3.19 ± 0.047 pg/2C DNA (Barone *et* Di marco, 2003).

Despite of difficulty in determining actual age, it seems that in many cases they can exceed 1000 years of life (Cervelli, 2005). The olive trunk is often twisted and hollow, and can reach also consistent size in the monumental specimens. In young trees the bark appears gray and quite smooth, becoming wrinkled in the adult specimens. The branches are numerous and thorny in young age, assuming kind appearance in adult plants, with erect, intermediate or pendulous aspect. In general, the leaf is a simple type and persistent, and can survive even three years on the plant (about 30 months). In the wild form it assume, a round shape with a darker green in the upper surface than on the bottom. The flap is normally plan and usually ends with a beak (Cervelli, 2005).

The flowers (Fig.3.2) are white and very numerous, they born on axillary inflorescence, named olive blossom. The corolla consists of four petals (2-4 mm long) with rounded apex, developed more in length than width; there are two stamens for flower; the stigma is bifid and the ovary exhibits four niches. The fruit (Fig.3.2) is an oval drupe whose mesocarp springs initially green then blackish brown at maturity; the endocarp is hard and woody and includes one, rarely two seeds (Mulas, 2005).

Fig. 3.2 Flower and fruit (drupe) of *Olea europaea L. subsp. europaea* var. *sylvestris*

During the vegetative phase the germination start between mid-February and March (depending on the area), with an intense growth between March and June, stopping in summer because of dryness condition and recovering in October (especially in wet years).

Flowering, which appears on the branches formed in the previous year, starts in May until the begin of June, following by fecundation, between late September and early October. The fruits mature between November and early February, with a lot of variability, persisting on the plant until the end of May, depending on the genotype.

3.1.3 *Oleaster* in landscape management, renaturalization and stress tolerance

It is interesting to emphasize that the variety *sylvestris*, is considered as the best bioindicator of the Mediterranean Floristic Region (Rubio *et al*., 2002).

From an ecological point of view, *Oleaster* exhibits many relevant features. First of all, due their prevalence within "*Macchia*" association, wild olive populations play a major role in protecting soil against erosion, by consolidating land through the wide root system. Moreover, on account of *Oleaster* resistance to wind and drought, they contributes to the survival of natural plant communities, while their ability to recover after a fire through new shoot formation assures a rapid rebuilding of the original vegetation cover (Mulas *et* Deidda, 1998).

Thus, many traits of oleaster would be highly valuable to introduce into agricultural importance species, such as *mays* (*Zea mays*).

Biochemical studies on frost tolerance of olive mostly focused on soluble sugars (Lavee, 1989; Bartolozzi *et* Fontanazza, 1999; Bartolozzi *et al*., 1999, 2001) rather than antioxidative enzymes, proteins or lipid metabolism. On the other hand changing in annual patterns of total soluble sugar (TSS) and proteins related to the cold-acclimation process were observed in the olive cv. *Gemlik* (Eris *et al*., (2007). Moreover, alterations in protein composition including dehydrins and antioxidative enzyme activities related to cold hardiness of nine olive cultivars were documented by Cansev *et al*. (2009).

From these data it was shown that some cultivars exhibited more cold tolerant than other, such as cvs *Domat* and *Lecquest*, some were moderate as *Ascolona*, *Gemlik* and *Hojoblanca* and, some sensitive, as observed for cvs *Samanli*, *Meski*, *Uslu* and *Manzanilla* (Gulen *et al*., 2009).

It is clear that to be successful in re-naturalizing highly degraded lands or, simply, for ensuring some degree of soil cover, the species/varieties of plants, which have to be used, must be selected on the basis of several independent features such as: high attitude to reproduction by seed and/or shooting; high attitude to vegetative propagation; high stress tolerance (Mulas, 2005). Certainly, varieties that either possess a high germination rate or easily undergo to vegetative propagation can assure a successful colonization and full expression in the territory. On the other hand, shooting faculty and stress tolerance are intrinsically vital to the single plant that is facing a climatically hardy environment, difficult to became colonized and where a strong capacity to persist and resist to both climate and human pressure (grazing, fire, etc.) is required. For all these reasons wild olive appears

largely adapted to be used in reforestation and management of eroded areas of Mediterranean *"Macchia"*.

3.2 *Arabidopsis thaliana*

3.2.1Classification and geographical distribution

Arabidopsis thaliana is a small flowering plant belongs to the Cruciferae (family Brassicaceae, Capparales) (Mitchell-Olds, 2001).

The genus *Arabidopsis* contains about ten species that are native to Eurasia, North Africa and North America. The closest wild relatives of *A. thaliana* include *A. lyrata* and *A. hallery*, which are self-incompatible plants, they are diploids with eight chromosome pairs. On the other hand in *A. thaliana* chromosome number is reduced to N=5, so diploid mappings crosses with wild relatives are impossible (Nasrallah *et al*., 2000).

Two major seed stock centers, ABRC and NASC, contain over 750 natural accessions (ecotypes) of *Arabidopsis thaliana*, collected around the world. These accessions are quite variable in terms of form and development (e.g. leaf shape, hairiness) and physiology (e.g. flowering time, disease resistance) (Fig.3.3).

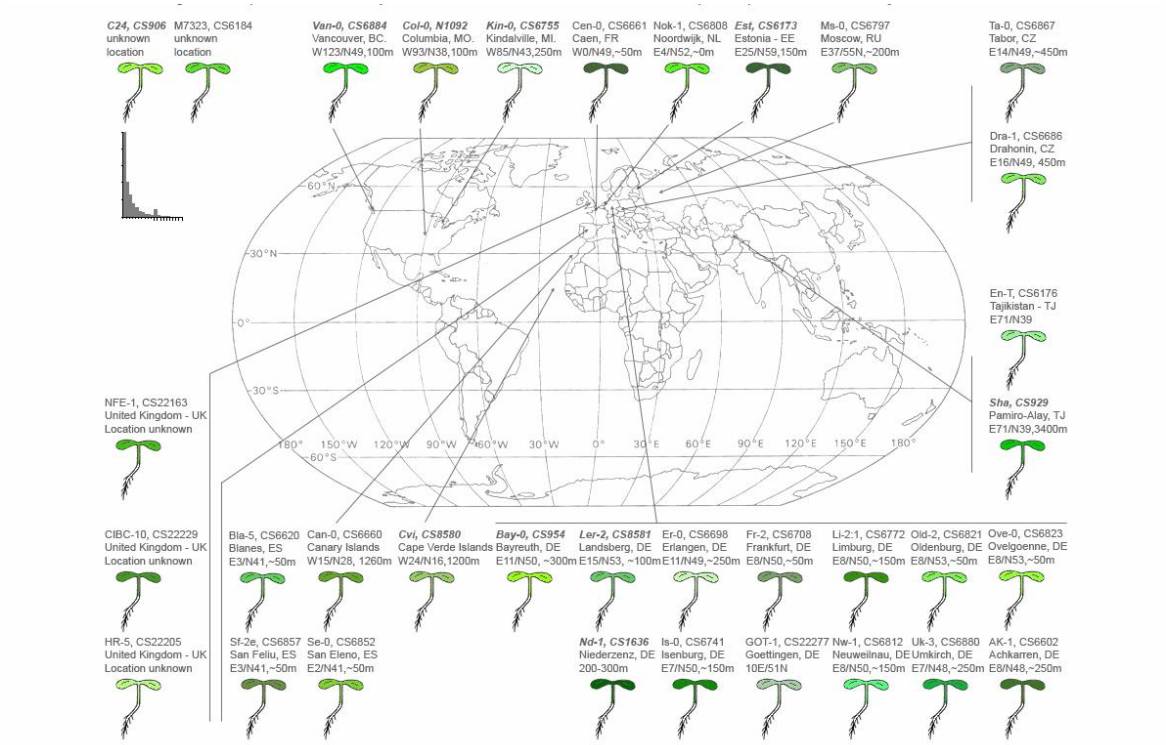


Fig.3.3 World map showing the geographical distribution (longitude, latitude, elevation) of more than 30 *Arabidopsis* ecotypes(This image is available as interactive map on website http://www.bar.utoronto.ca/

3.2.2 The development of *Arabidopsis thaliana* as a model

Because of the recent radiation of flowering plants from a common ancestor in the past 150 million years, *Arabidopsis* is closely related to several hundred thousand plant species and, although is not of major agronomic significance, offers important advantages for basic research in genetics and molecular biology (Somerville *et* Koornneef, 2002).

Arabidodpsis is considered to be an excellent biological model for many aspects of plant biology. First of all for the short life cycle: the entire life cycle, including seed germination, formation of a rosette plant, bolting of the main stem, flowering and maturation of the first seeds, is in fact completed in 6 weeks (Meinke *et al*., 1998). Furthermore the small size, the seedlings develop into rosette plants that range from 2 to 10cm in diameter, and the mature plants reach 15 to 20 cm in height depending on growth conditions. Flowers are composed of an outer whorl of four green sepals and inner whorls containing four white petals, six stamens bearing pollen, and a central gynoecium that forms the siliques. Thus, the plant is a self–fertilizing and can be crossed by applying pollen to the stigma surface. The hermaphrodite flowers, once self-pollinated, produce fruits (siliques) which when mature release thousand of seeds (also more than 5000) of 0.5 mm in length, and this of course facilitate the rapid production of many progeny from single mutants of transgenic plants.

On the other hand, *Arabidopsis* is easily to transformed with a gene of interest, cloned in a plasmid and subsequently transformed by bacteria which can infect the plant, thereby selected for the presence of the transgene in the succeeding generation.

Leaves are useful for studying morphogenesis and cellular differentiation, because covered with small unicellular hairs known as trichomes. It is also known how it is easy to growth *Arabidopsisin in vitro* condition.

Plant can be grown in petri plates or maintaneid in pots located either in a greenhouse or under fluorescent lights in the laboratory. Bolting starts about 3 weeks after planting, and the resulting inflorescence forms a linear progression of flowers and siliques for several weeks before the onset of senescence. The root, with a simple structure, is easy to study in culture, and it is not be able to establish symbiotic relationships with nitrogen-fixing bacteria. Natural pathogens include different types of insects, bacteria, fungi, and viruses (Meinke *et al*., 1998).

The small genome of *Arabidopsis* is organized into 5 chromosomes (125 Mb) and contains an estimated 27.000 genes encoding about 35.000 proteins. Many different ecotypes with more than 50.000 DNA polymorphisms have been collected from their natural set up and readily available to the research community (Bevan *et al*., 2001; Somerville *et* Koornneef, 2002).

Such advantages have made *Arabidopsis* a model organism for studies of the cellular and molecular biology of flowering plants and many information are collects and makes available by the *Arabidopsis* Information Resource (TAIR).

On the other hand, the importance of a model organism as *Arabidopsis thaliana* has been documented several times in the past, because not sudden (Koornneef *et* Meinke, 2010).

Arabidopis thaliana was proposed as suitable model from plant genetic only in 1930s by a young student, Friedrich Laibach, during his PhD, whose described for the first time the correct chromosome number (Laibach, 1907). In the 1930s, this research returned on working on *Arabidopsis*, because of the interesting in natural variation and of the light effect on physiological traits such as flowering time (Laibach, 1951) and seed dormancy (Kugler, 1951).

An important step in the history of *Arabidopsis* research, it was when Laibach and his student, Erna Reinholz, begun to mutagenesis experiment with X-rays, thus discovering the first induced *Arabidopsis* mutants.

Unfortunatelly, when the molecular era appears in the 1970s, the plant were still far to be chosen for model organisms experimentation, for several reasons. First of all for limited availability at that time of shared resources compared with other model organisms, and latter because it was difficult to gain support for elevating an outsider to special research status by agricultural foundation (Koornneef *et* Meinke, 2010). Moreover in the 1975, it was published an interesting review in which it was empathized the genetic potential of *Arabidopsis* to characterize plant-specific processes, (Rédei, 1975).

The potentiality of *Arabidopsis* as a model plant intensified in 1980, when the research begun to understand the potentiality of a small genome size, supported with the cloning of the first *Arabidopsis* gene by Chang and Meyerowitz (Koornneef *et* Meinke, 2010).

Thus, *Arabidopsis* research began in 1987 with the opening of the Third International *Arabidopsis* Conferences at Michigan State University and the subsequent formation of an electronic *Arabidopsis* newsgroup. (Meinke *et al*., 1998)

The increased role of genetics in discipline integration (Pruitt *et al*., 2003) and the availability of powerful tools in molecular biology resulted in the gradual realization that plant biologists needed to focus attention on a single organism most amenable to detailed analysis (Koornneef *et* Meinke, 2010).

3.2.3 Engineering stress tolerant crop plants based on *Arabidopsis* model

The knowledge gained from studies in *Arabidopsis* serves to advance our understanding of other plant species, particularly crop species, and thus translate into new or improved plant products and increased agricultural productivity. Importantly, basic research in *Arabidopsis* provides the foundation for applied studies (Zhang *et al*., 2004).

For example, is already known that adaptation process are under transcriptional control, so many data in literature showed as engineering plant with transcription factor might be an excellent strategy to achieve enhanced stress tolerance.

In the last years, many important crop specied were transformed with CBF (*Cis* Binding Factor). Constitutive overexpression of *Arabidopsis* CBF genes in *Canola* increased freezing and drought tolerance (Jaglo *et al*., 2001). On the other hand *Arabidopsis* transgenic plants showed higher salt, cold and drought tolerance when *OsDREB1* of rise was overexpressed (Dubouzet *et al*., 2003). Also the monocotyledon *Zeamays*, exhibited an increasing in the cold tolerance when the *ZmCBF* gene was overexpressed (Chaiappetta, 2002).

Unfortunatelly these data showed also negative effects of the transgenic transformation such as dark-green, dwarfed plants with higher levels of soluble sugars and proline (Liu *et al*., 1998; Gilmour *et al*., 2000). Thus in order to overcome these problematic the researchers decided to use stress-inducible promoter, obtaining excellent results (Kasuga *et al*., 1999; Lee *et al*., 2003).

Thus, *Arabidopsis* can be considered an excellent model plant for studying abiotic stress responses for biotechnology applications.

CHAPTER 4

Materials and methods

4.1 Molecular analysis

4.1.1 Target Gene

The investigated gene was present in acDNA library made available by research group of Prof. M.B. Bitonti. This cDNA library was generated from 50-100 µg of total RNA, extracted from leaves of wild olive *Olea europaea* L. subsp. *europaeavar. sylvestris* plants,growing in open field in the Mediterranean area, near Cetraro (CS - Italy) (39° 30' 52. 77"N; 15° 56' 28. 83'E. Plants are located 117 metres above sea level and grown under condition of high salinity, high temperature and intense solar irradiance.

Leaves were collected in the summer 2007, after a prolonged (2 weeks period) incidence of very high temperature (35°C±2°C). The cDNA library was generated using SMART system and cloning the sequence (around 1.2kb) in the pSPORT1 vector.The sequencing analysis was performed from 5'end. Generation and sequencing of the library was performed by Eurofins MWG GmbH cDNA Laboratory Fraunhoferstr (De) service.Among the isolated cDNAs, *OesDHN*gene selected for the present study, encodes a protein that belongs to the D-11 family of the large group two LATE EMBRYOGENESIS ABUNDANT (LEA) proteins (Bruno *et al*., 2010) that can detoxify and alleviate cellular damage during dehydration (Vicient *et al*., 2001; Ulrich, 2005).

Interestingly, the expression levels of the *OesDHN* gene, monitored in wild olive plants exposed to cold conditions and water stress were found significantly overexpressed (Bruno *et al*., 2010).

4.1.2 Isolation and purification RNA

The gene was isolated from total RNA according to the protocol described by Giannino *et al*., (2004). Finally, the samples were resuspended in 50 µl of RNase-free water and the RNA concentration was measured by NanoDrop (ND-1000 Spectrophotometer).

The Genomic contamination was eliminated using the protocol of DNase suggested by Roche. 3 µg of each sample were treated with 5µl of DNase (Roche) and 77µl and diethylpyrocarbonate (DEPC)-water, followed by an incubation at 37° C for 50 minutes . In this way is digested the DNA that could be coprecipitated with the RNA.

In order to precipitate the RNA, the reaction was stopped by adding 1/10 volume of sodium acetate (NaAc) 3M, pH 5.2 and two volumes of cold absolute ethanol, overnight at -20°C and then the mixture was centrifuged at 14000 rpm for 20 minutes at 4°C. Then, the pellet was washed with 1ml of 70% ethanol. At these conditions the nucleic acids are insoluble, while salts dissolved in water, can be removed as a supernatant spilling the solution. Finally, the pellet was dried under a sterile hood and resuspended in 40µl of DEPC-water. The integrity of the isolated RNA was checked by electrophoresis on agarose gel 0.8% in 0.5X TBE (Tris 1M, boric acid 0.9M, 0,5M EDTA stock solution, pH 8) at 80V/cm.

4.1.3 cDNA synthesis

For the cDNA synthesis 1µg of total RNA (see 4.1.2) was used according to the protocol of SuperScript III Reverse reagent (Invitrogen).

The reaction was performed by preparing an initial master mix made by: 1µl of 50µM oligo dT (primer mix), 1µl of 10mM of dNTP mix , 1µg of RNA, and DEPC- water until a final volume of 10µl and then incubated at 65°C for 5 minutes to denature the RNA, which was placed on ice for 1 minute. The second master mix was prepared for cDNA synthesis, by adding the following components in the indicatedorder: 2µl of 10X RT buffer , 4µl of 25 mM $MgCl_2$, 2µl of 0.1M DTT, 1µl of RNase OUT and 1µl of SuperScript III Reverse.

10µl of this mix were added to each combination RNA/primer, mixed gently and then subjected to a brief centrifugation. The samples were incubated at 50°C for 50 minutes after which they were briefly centrifuged. the reaction was terminated at 85°C for 5 min and chilled on ice. Finally 1µl of RNase H was added to each sample and then incubated at 37° C for 20 minutes. The concentration of cDNA was determined by NanoDrop (ND 1000).

4.1.4 Extraction and purification of genomicDNA

Total genomic DNA was isolated from leaves of *Olea europaea* L. subsp. *europaeavar. sylvestris*r according to CTAB extraction method (ctyltrimethylammonium bromide) (Murray *et* Thompson, 1980). Subsequently, in order to eliminate the total RNA, the DNA was purified by adding 1µl of RNase (10mg/ml stock) in 100µL of DNA sample and incubated for 1 hour at 37°C.

4.1.5 PCR and purification of DNA

For general PCR the*Taq* DNA polymerase (Promega) was used. The following components were mixed: 2.5µl 10X PCR buffer, 1.25 mM $MgCl_2$, 0.5 µl 10mM dNTP, 1µl of

each 10µM primers, 0.125 µl of Taq polymerase. Template and dH_2O were added to a final volume of 25µl. PCR was performed on a Bio-Rad machine (GeneAmp PCR System 9600 (PE Applied Biosystems, Foster City, California) ed using the program shown in Table 4.1

	Step 1	Step 2			Step 3	Step 4
Temperature	95°C	95°C	58-60°C	72°	72°	4°C
Time	3’	1’	30”	1’-3’	7’	
cycles	1X	35X			1X	

Table 4.1 Thermal cycling program used for general PCR performed in a Bio-Rad iCycler or a Biometra T3000 machine

For cloning purposes polymerase with 3’-5’ exonuclease (proofreading) activity was used. For PCR, using *Platinum® Pfx DNA Polymerase* (Invitrogen) the following components were mixed: 1µl of 50mM $MgSO_4$, 1.5µl of 10mM dNTP mix, 1.5 µl (150ng each) of gene-specific primers (10µM each), 5–10 µl of 10X Pfx Amplification Buffer, 1.25 U Platinum® Pfx DNA and sterile water was added to reach a final volume of 50µl. The template (10pg – 200ng) was added individually to each tube. PCR was performed on Bio-Rad machine using the program shown in Table 4.2.

	Step 1	Step 2			Step 4
Temperature	94°C	94°C	58-60°C	68°	4°C
Time	2-5 min’	15 s	30 s	1’-3’	
cycles	1X	35X			

Table 4.2 Thermal cycling program used for cloning PCR performed in a Bio-Rad iCycler or a Biometra T3000 machine

*OesDHN*ORF was cloned by using Platinum® Pfx DNA Polymerase and cDNA and genomic DNA as template. Tthe genomic DNA was used in order to evaluate the putative presence of introns. In Table 4.3 are listed the thegene-specific primers utilized for PCR amplification.

FwORF*OesDHN*	*5’-ATGGCGGAGGAGGGACCCGTC-3’*
BwORF*OesDHN*	*5’-TTAGTGGCATGCCCCCTCCTT-3’*

Table 4.3 Gene-specific primers designed on the sequences of the ORF gene

Purification was performed by the QIAquick Gel Extraction Kit (QIAGENE), according to the QIAquick handbook (QIAGENE) and products were prepared for sequencing (laboratory GENELab ENEA, Rome).

4.1.6 Isolation of the 5'-flanking region of *OesDHN*

The OesDHN promoter region was obtained by "Universal GenomeWalker™ kit (Clontech Laboratories) with some modifications.

Genomic DNA extracted and purified (see Section 4.1.4) was completely digested with fourdifferent blunt-ended restriction enzymes (DraI , EcoRV , Pvu II , Stu I). The digested products were purified by gel purification kit and each digested genomic DNA was ligated, separately, to Genome Walker adapter to produce four " Genome Walker libraries".

After the libraries construction, the protocol involved the construction of two new PCR library. For the primary PCR were used an AP1 (outer Adapter Primer), provided by the kit,a GSP1 (Gene Specific Primer 1) (Table 4.4) and the ligated products, as template.

The thermal cycling program was 94°C for 25 s and 72°C for 3 min with 7 cycles followed by 32 cycles of 94°C for 25sec and 67°C for 3 min, with a final extension at 67°C for 3 min.

The products of the primary PCR (PCRI) were then diluted and used as template for the secondary PCR (PCRII), together with an AP2 (nested Adapter Primer 2) provided by the kit and a GSP2 (Gene Specific Primer 2) (Table 4.4). For this PCRII parameters used were 5 cycles of 94°C for 25s and 72°C for 3 min, followed by 20 cycles of 94°C for 25sec and 67°C for 3 min, with a final extension at 67°C for 7 min.

As a positive controls were used templates and primers, provided by the kit. In particular for the primary PCR, the primers were PCP1&AP1 (Table 4.4) and, for the PCRII, primers were PCP2&AP2 (Table 4.4). During thesecond PCR we just have a productwhich, once loaded on a 2% agarose gel, prepared in 1X TAE buffer (40mM Tris, 20mM acetic acid, 1 mM EDTA) has been extracted and purified according to the manual of the kit QIAquick Gel Extraction Kit (QIAGENE). The PCR products were prepared for sequencing (laboratory GENELab ENEA, Rome).

PCP1 (tPA1):	*5'-AGA ACCCGACCTACCACGGCTTGCTCCTT-3'*
PCP2 (tPA2):	*5'-CCCTTTCCTCGCAGAGGTTTTCTCTCCAGC-3'*
AP1(Adaptor Primer 1)	*5'–GTAATACGACTCACTATAGGGC–3'*
AP2 (Nested Adaptor Primer 2)	*5'–ACTATAGGGCACGCGTGGT–3*
GSP1 (C)	*5'-TTT CTT CTT CCT TGT GCT CCG GTT CAC A-3'*
GSP2 (B)	*5'-CCC ATG AAA TCA AAC AAC CCA CGG TCC T-3'*

Table.4.4 List of primers used for promoter *OesDHN* isolation respectively for PCRI (GSP1 & AP1 primers),PCRII (GSP2 & AP2) and positive controls (PCRI: PCP1&AP1 and PCRII: PCP2&AP2

4.1.7 Bioinformatics analysis

Amino sequence of *Oes*DHN and its related protein was obtained using the ExPASy Proteomic Tools (http://www.expasy.org/tools/dna.html). The analysis and comparison of the deduced amino acid sequence with published sequence of dehydrins were performed with blastp (Standard Protein-Protein BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

Plant dehydrins with the highest sequence homology to *Oes*DHN protein were used for the alignments, performed using ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

The neighbor-joining tree was generated with published sequence of dehydrins, using the MEGA4 software (Molecular Evolutionary Genetics Analysis) (Tamura *et al.*, 2007) with bootstrap values obtained from 1,000 replications. Protein sequence comparisons were made using the following:*Arabidopsis thaliana* COR47 (P31168), *Arabidopsis thaliana* ERD10 (P42759), *Arabidopsis thaliana* ERD14 (P42763), *Arabidopsis thaliana* DHLEA or PAP310, (Q96261), *Arabidopsis* thaliana Q9SLJ2 (At1g54410), *Arabidopsis thaliana* Q9SVE4 (75313932), *Arabidopsis thaliana* Q9T022, *Arabidopsis thaliana* Rab18 (P30185), *Arabidopsis thaliana* XERO1 (P42758), *Arabidopsis thaliana* XERO2 (P42758), *Avicennia marina* AmDHN1 (A8CVF3), *Brassica juncea* DHN2 (ABD95986), *Brassica oleracea* BOPC34 (CAA64428), *Citrustrifoliata* COR11 (AAA99963.1), *Craterostigma* plantagineum (P22238), *Glycine max* MAT9 (AAA33992), *Hordeum vulgare subsp. vulgare* DHN1(P12951), *Hordeum vulgare subsp. vulgare* DHN5 (AAF01693), *Hordeum vulgare subsp. vulgare* PAF93 (CAA58875), *Jatropha curcas Jc*DHN-1 (ADT65200), *Medicago sativa* G2 (AEV52291), *Medicago sativa subsp. falcata* CAS18 (AAA21185), *Opuntia streptacantha* DHN1 (AEI52546), *Oryza sativa Japonica* WSI724 (BAA05539), *Oryza sativa*

Japonica DH16D or RAB16D (P22913), *Pisum sativum* DHN2 (CAA44788), *Pisum sativum* dhn-cog (CAA78515), *Populus alba x Populus glandulosa Po*DHN (ABH11546), *Prunus persica* DHN1 (P28639), *Prunus persica* PCA60 (AAC49657), *Rhododendron catawbiense* Dhn-5 (ACB41781), *Solanum sogarandinum* DHN24 (AAP44575), *Triticum aestivum* COR410 (P46524), *Triticum aestivum* CS120(P46525), *Triticum aestivum* CS66 (P46526), *Triticum aestivum* RAB15 or DHR15 (Q00742), *Vitis vinifera* DHN1b (526118232), *Zea mays* COR410 Zea mays (226532837), *Zea mays* DEHYDRIN 13 (EU962627.1), *Zea mays* DHN1 (18963), *Zea mays* lipase DHN2 (L35913.1), *Zea mays* Put. unch. protein (219363418), *Zea mays* RAB17 (18963).

Analysis of targeting sequences was performed using PSORT (http://psort.nibb.ac.jp/) and Wolf PSORT (http://wolfpsort.seq.cbrc.jp/).

The Genevestigator V3 microarray expression database (Zimmermann *et al.,* 2004) (www.geneinvestigator.com) and electronic Fluorescent Pictograph (eFP) (Winteer *et al.,* 2007) (http://www.bar.utoronto.ca/) were used (with Anatomy tool) to extract the relative expression levels of the *At*DHN genes across all organs, tissue and cells.A research on *Arabidopsis thaliana* genomic database with the *OesDHN* gene sequence was conducted by blastn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=OGP__3702). Global alignment between *Oes*DHN and published dehydrins amino acid sequences of *Arabidopsis thaliana* (AtDHN), COR47-SK3-type: (P31168), ERD10-SK3, (P42759), ERD14-SK2 (P42763), PAP310 or dehydrin LEA- Y2SK2 (Q96261), XERO2 or LTI30 K6 (P42758), XERO1-YSK2 (P25863), Rab18-Y2SK2(P30185), Q9SVE4-Kn(75313932), Q9T022-YnKn (AT4G39130), Q9SLJ2_ARATH KnS (AT1G54410), was performed with EMBOSS (algorithm NEEDLEMANN-WUNSH) (http://www.ebi.ac.uk/Tools/psa/emboss_stretcher/) and amino acid local alignment by Blast2sq program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins&PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&QUERY=&SUBJECTS). Amino a*cis* sequences were then used for the alignments, performed by ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using BioEdit (version 7.1.3.0) (Hall *et al.,* 1999).

4.1.8 Southern Blot analysis

4.1.8.1 Extraction of total DNA and blotting

Genomic DNA used for Southern blot analysis was isolated from 2g of oleaster leaves using cetyltrimethyl ammonium bromide (CTAB) precipitation. After extraction and purification (see section 4.1.4), 10 µg of sample was digested overnight at 37°C with appropriate restriction enzymes, respectively EcoRV and XbaI, which did not cut within the *OesDHN*sequence.

The digested DNA was then precipitated at -20°C by adding1/10 volume of sodium acetate (NaAc) 3M pH 5.2 and two volumes of cold absolute ethanol, and aftercentrifugation at 14000 rpm for 20 minutes at 4°C, resuspended in a final volume of 50 µl of DEPC-water. Finally, the digested DNA fragments were separated on agarose gel 0.8% (w/v) for 20 hours at 45V and blotted onto a nylon membrane Hybond-N + (Amerscham Pharmacia Biotech), using the system Vacuum Blotting System (BioRad, GeneAmp PCR System 9600 (PE Applied Biosystems, Foster City, California).

4.1.8.2 Hybridization

The membrane was subsequently hybridized with a specific probe, obtained by a PCR amplification product and corresponding to the ORF of *OesDHN* gene. The probe was amplified using the primers FW*OesDHN*ORF and BW*OesDHN*ORF (Table 4.3) and a mixture of nucleotides labeled with DIG-dNTPs (PCR Dig Probe Synthesis Kit Roche) according to the protocol shown in Table 4.5.

Components	Volume
cDNA	5µl
Primers DHNFW	5µl
Primers DHNBW	5µl
PCR DIG Probe Synthesis Mix	5µl
Enzime mix	0,75µl
Buffer	5µl
H2O	24,25 µl
Finale volume	

Table 4.5 Components for the probe synthesis reaction

The thermal cycler program used for the probe synthesis is shown in Table 4.6.

	Step 1	Step 2			Step 3	Step 4
Temperature	94°C	94°C	55°C	72°	72°	8°C
Time	4’	2’	55”	1’	5’	∞
cycles	1X	35X			1X	1X

Table 4.6. Thermal cycling program used for probe synthesis and performed in a Bio-Rad iCycler or a Biometra T3000 machine

The membrane was dr ied in a oven at 80° C for 30 minutes. Pre-hybridization and hybridization were performed in a rotary hybridization oven HB- 2D (Techne, Milan, Italy). Pre-hybridizationwas performedat 52°C for 3 hoursin 70 ml of Pre-hybridization Buffer solution, containing 25% SSC20X (3M NaCl, sodium citrate 300mM), 0.01% Sarkosyl, 0.1 % blocking reagent (Roche) 2.5% prepared in malic acid buffer pH 7.5. Hybridization was performed overnightat 52°C by adding 30ml of Probe Solution .In the Probe Solution probehas previously been denatured for 3 minutes at 94°C, immediately transferred on ice for 5 minutes and used for the hybridization reaction. After hybridization the membrane was washedat room temperature with a 2X solution of 20X SSC, 0.1% SDS 20 % and later with 0.5X of the same solution at 52°C. The detection was performed through an enzymatic reaction using anti- DIG-AP (Roche) and dyes such as salt NBT (4-Nitro blue tetrazolium chloride, solution) and BCIP (5-bromo -4- chloro-3-indolyl-phosphate) (Roche). The colorimetric reaction was performed for two days in the dark, at room temperature and then stopped with a pH 8.0 TrisEDTA (0.5M) solution.

4.2 Plant transformation, selection of transgenic lines, and stress treatments

4.2.1 Plant materials and growth conditions

Arabidopsis thaliana Columbia (*Col*) ecotype was used as the experimental plant in this project.The plants, both *in vivo* and *in vitro*, were under 16h light per daily photoperiod (8:00 to 12:00 light; 22°C/18°C and day / night) and for the roots under continuous light (8:00 to 5:00, 22°C/day and 18°C/night), with an light intensity of 100μmol*m^{-1} sec^{-1} and a relative humidity (RH) of 65-70%.

The growth medium (GM) used for this project was composed of Murashige and Skoog medium (MS) plus vitamins (GM+V) supplemented with 0.1g/l of myo-inositol, 0.5g/l

of 2-N-morpholine ethane sulphonic acid (MES), 10g/l sucrose and 8g/lagar. The medium was brought to a pH of 5.7 with KOH 1N.

For the selection medium, appropriate antibiotics were added only when the medium temperature reached 50°C, before pouring out into the plates. In particular for high density plating 50µg/ml of Kanamycin (Km) to select the construct, 250 µg/ml di Carbenecillin (Cb) against *Agrobacterium* overgrowth and 50µg/ml of Nystatin (Ns), an antifungal, were added to autoclaved GM medium, and only 50µg/ml of Kanamycin (Km) was used in the subsequent preparation of selection media.The latter the plates were then sealed and stored in a cold room (4°C) until use. For osmotic stress treatments, a concentration of mannitol 25mM (Sigma) was added before autoclaving the soil to GM+V.

4.2.2 Seed sterilization

The number of seeds to be sown out was determined,wrapped in a miraclotth package and put in a sterile 200ml bottle containing a magnetic stirrer.70% of ethanol (EtOH) was added and left to stir on a shaker for two minutes, then a 5% hypochlorite solution containing some drops of 1% tween20 was added for 10 min.After sterilization, seeds are washed and rinsed several times with distilled water and sawed in 150*25mm petri dishes. Plates are sealed with gas-permeable medical tape and kept two days at 4ºC to vernalized, then were transferred to the phytotron.

4.2.3 Recombinant constructs using GATEWAY technology

4.2.3.1 General introduction

Alongside the traditional cloning systems, based on the series of pET vectors, Invitrogen (Monza, Italy) has introduced a cloning technology based on phage λ site-specific recombination in *Escherichia coli* called Gateway. This technology allows to transfer the gene of interest from a donor vector to a whole series of destination expression vectors in bacterial cells, in insect cells and in plant, making possible the cloning and expression of proteins of interest to a high degree of processivity and relatively short time (Stevens, 2000) (Fig. 4.1). The application of the Gateway method provides the amplification of a selected gene by PCR with a pair of oligonucleotides which contain, in addition to the specific sequence of the gene, the *attB* sites, necessary for recombination. In particular, the PCR product obtained is constituted by the gene of interest, flanked by *attB* sequences of 25bp (*attB1* which is located at the 5' of the gene and *attB2* which is located at the 3’ of the gene) and this construct is

subsequently used to prepare the reaction BP in which a donor vector (eg a pDONR221), containing the *attP* sequences, anneals specifically with the PCR product.

This process, whose theoretical efficiency is estimated at 90%, gives rise to a recombinant vector containing the gene of interest flanked by sequences *attL*. Then, the recombinant vector is used to set up the LR reaction which takes place in a specific recombination between the donor vector (recombinant plasmid) and the destination vector, containing sequences *attR* (Fig. 4.1).

In particular, the entry clone has been generated by a PCR product, containing the *attB* sites. The process requires two overlapping PCR reactions because of the addition of 25bp of the *attB* sites in both ends of the PCR product. The *attB* sequences, long about 21bp and the *attB* and are shorter than four types of *att* sites. The addition of the specific *attB* sequences to each primer confer directionality to the PCR product, while site-specific changes were made in the sequence *attB2* to confer specificity in the recombination reaction. The *attB* sites were also engineered to eliminate stop codons. The four G residues added at 5'-end of each sequence *attB* improved the efficiency of the recombination reaction. On the other hand the *readingframe* is determined by two codons encoding lysine (AAA AAA) in *attB1*. If our clone is *in frame* with these codons then will be *in frame* with the subsequent reactions. A similar situation occurs for the C-terminal fusion proteins, where the sequence needs to be *in frame* with the two TTT GTA codons but without the stop codon.

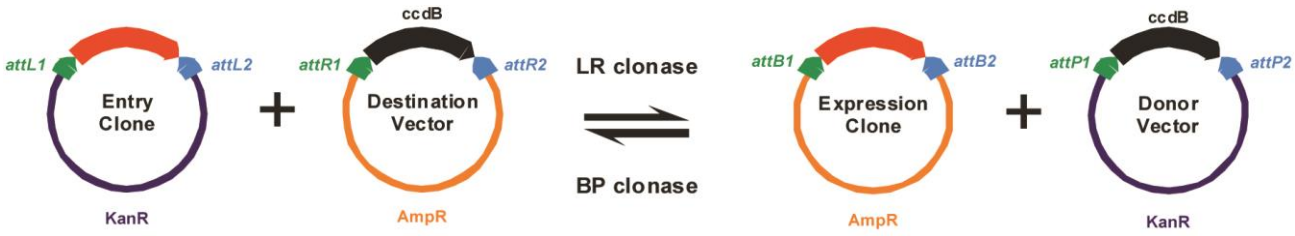


Fig.4.1 Representative scheme of Gatewaytechnology

4.2.3.2 Amplification of *OesDHN* cDNA with attB extensions

In this project, the Gateway method, was used to clone the *OesDHN* (*Olea europaea* DEHYDRIN) gene encoding a deduced dehydrin SK2-type in the overexpression vector pK7WG2 (Karimi *et al*., 2002). We also introduced *OesDHN*into pKF7WG2 and pK7WGF2 overexpression vectors (Karimi *et al*., 2002), to produce constructs N- and C- terminal with GFP (green fluorescent protein). In the first PCR (PCR1) the *OesDHN* open reading frame

(636bp) including stop codon and not, was amplified by polymerase chain reaction (PCR). The cDNA synthesis was obtained according to the SuperScript III manual (Invitrogen)(for more details see paragraph 4.1.3). The amplification reaction of *OesDHN* cDNAwas performed using Pfx polymerase (Invitrogen),preparing a mastermix as follow: 1.5 MgSO4, 2.5µl of dNTP mix, 1.5 µl of gene-specific primers completed with universal adapters (150ng each) (Table 4.7), 10X Buffer, 1.25U of Taq enzyme, and water to reach a final volume of 50µl. The template was added individually to each tube. The PCR program included adenaturation step at 94°C for 4min, followed by 35 cycles of 94°C for 15 s, 55°C for 30s, 68°C for 1min. Aliquots of the PCR1 products were used as template for the second PCR (PCR2), and again used thePfx polymerase (Invitrogen) with attB1 and attB2 primers (Table 4.7)

PCRI	
N-Terminal fusion to protein (c.terminal fusion to fluorescent tag)	
FWattB1ORFnoSTOP	*5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGGAGGAGGGACCCGTC3'*
BWattB2con STOP	*5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGTGGCATGCCCCCTCCTT-3'*
C-Terminal fusion to protein (N.terminal fusion to fluorescent tag)	
FWattB1ORFnoSTOP	*5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGGAGGAGGGACCCGTC-3'*
BWattB2 no STOP	*5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCGTGGCATGCCCCCTCCTT-3'*
PCRII	
FWattB1	*5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'*
BwattB2	*5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'*

Table 4.7 Sequence primers foramplification of *OesDHN* cDNA with attB extensions with Gateway technique. PCR1:sequences primers for the ORF of *OesDHN* gene(with stop codon and not) with extensions sequences; PCR2: primer sequences with only *attB*extensions

4.2.3.3 BP reaction

PCR products with attB1 and attB2 extensions (including and not stop codon), were recombinated into thepDONOR221 (Invitrogen) vector (Fig. 4.2), using the Gateway ® BP Clonase ® II enzyme mix (COD.12535-029).

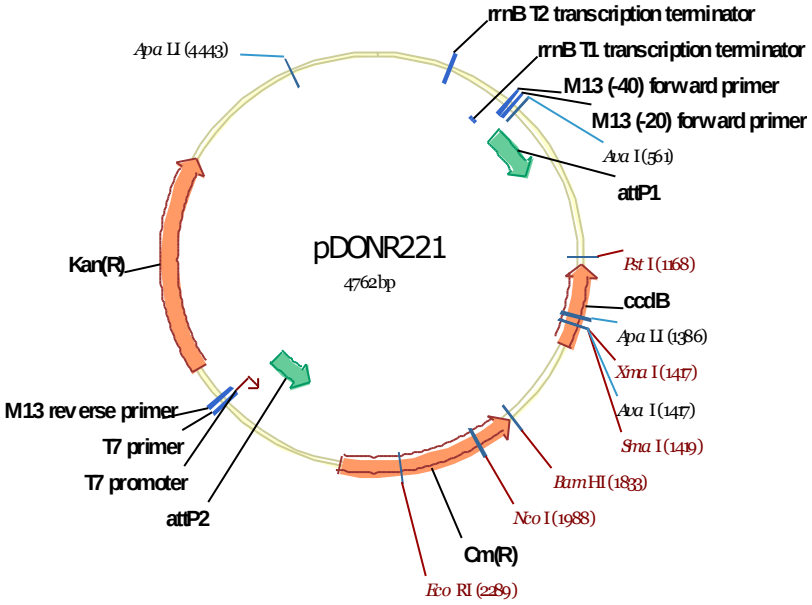


Fig. 4.2 pDONOR221 map (Invitrogen)

The following components (Table 4.8) were added to a 1.5 ml microcentrifuge tube at room temperature:

Components	Sample pDonor221	Negative control	Positive control
attB.product 100fmol	3µl (100fmol)	1-7 µl	-
pDonor vector (150ng/µl)	1 µl	1 µl	1 µl
pMS/GW control plasmi(100ng/µl)	-	-	2 µl
TE Buffer, pH8	4 µl	-	5 µl
Vol finale	8 µl	To 10µl	8 µl

Table4.8 BP reaction components

2 µl of BP Clonase II were added to each sample and the mixture was incubated for 1h at 25°C. Finally, the reaction was stoppedadding1µl of Proteinase K (2µg/µl Proteinase K) into BP Clonase ™ enzyme mix and incubated for 10 minutes at 37°C. Final products obtained were, respectively, the *pENTR221-OESDHN+ stop* and *pENTR221-OESDHN-stop* vectors.

4.2.3.4 *E. coli*transformation with BP reaction

Competent *E. coli* cells (One Shot® Omnimax™ 2 T1 Phage-Resistant Cells, Invitrogen), were used for gene cloning and transformed by *heat shock*.The cells were kept on ice and for each transformation 1µl of each BP reaction was added in 50µl of resistant cells. Cells were placed on ice for 30 minfollowed by a heat shock at 42°C for 30 sec, and again on ice for 2 min.

After addition of 1ml di S.O.C. medium (20g/L tryptone, 5g/L yeast extract, 0.5 g/L NaCl, 100 ml/L 1M MgCl2, 10 mL/L 2M glucose), bacteria were placed in an incubator (Gallenkamp orbital incubator)at 200 rpm, at 37°C for 60 min. Finally, aliquots of 100, 200 and300µl of the transformations were plated on LB-medium(10g/L Bacto-Tryptone, 5g/L Bacto-yeast 5g/L NaCl, 15g/L Bacto agar), containing the appropriate antibiotic (Kan 50µg/ml) and incubated overnight at 37°C. The successful of *E. coli* cells transformation was verified by colony PCR, using gene specific primers (see table 4.3), M13 primers (see table 4.9), and *E.coli* colonies as the template.

M13 Forward	*5'-GTAAAACGACGGCCAG -3'*
M13Reverse	*5'-CAGGAAACAGCTATGAC -3'*

Table 4.9 M13 sequencing Primers

4.2.3.5 LRreaction

After recombination of the matching *attB* and *attP* sites, the DNA fragment was inserted into the donor vector, resulting in an entry clone (pENTR), flanked by two attL sites. Entry clones are a key substrates in the LR reaction,catalyzed by the LR Clonase II enzyme mix (integrase, integration host factor, and the phage ex*cis*ionase.

The LR clonase mix transfers the DNA fragment of interest, flanked by two attL sites (in the entry clone), into a destination vector (pDEST) carrying two *attR* sites. After recombination of the matching *attL* and *attR* sites, the DNA fragment of interest is inserted into a novel expression clone (pEXPR) and again flanked by *attB* sites.

Destination vectors through the LR reaction can be used for different analyses, like protein localization by GFP FUSION, yeast two-hybrid analysis and overexpression.

In our project pENTR221-*OESDHN*+stop were recombined in both destination vectors pK7WG2) (Fig.4.3), and pK7WGF2 (Fig.4.4) (Karimi *et al.*, 2002),and the pENTR221-*OESDHN*-stop, in the pK7FWG2 (Karimi *et al.*, 2002) (Fig.4.4) for overexpression analysis

and for the localization of *OesDHN* in C- and N-terminal GFP fusion,under the control of the cauliflower mosaic virus 35S promoter (Odell *et al*., 1985).

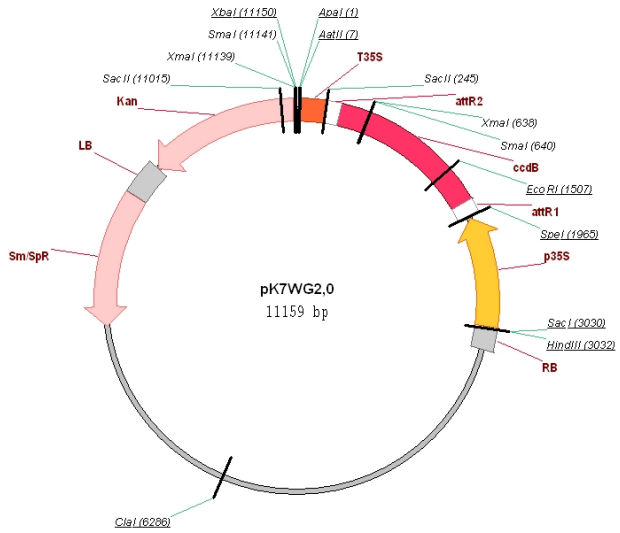


Fig. 4.3 pK7WG2 map (Karimi *et al,* 2002)

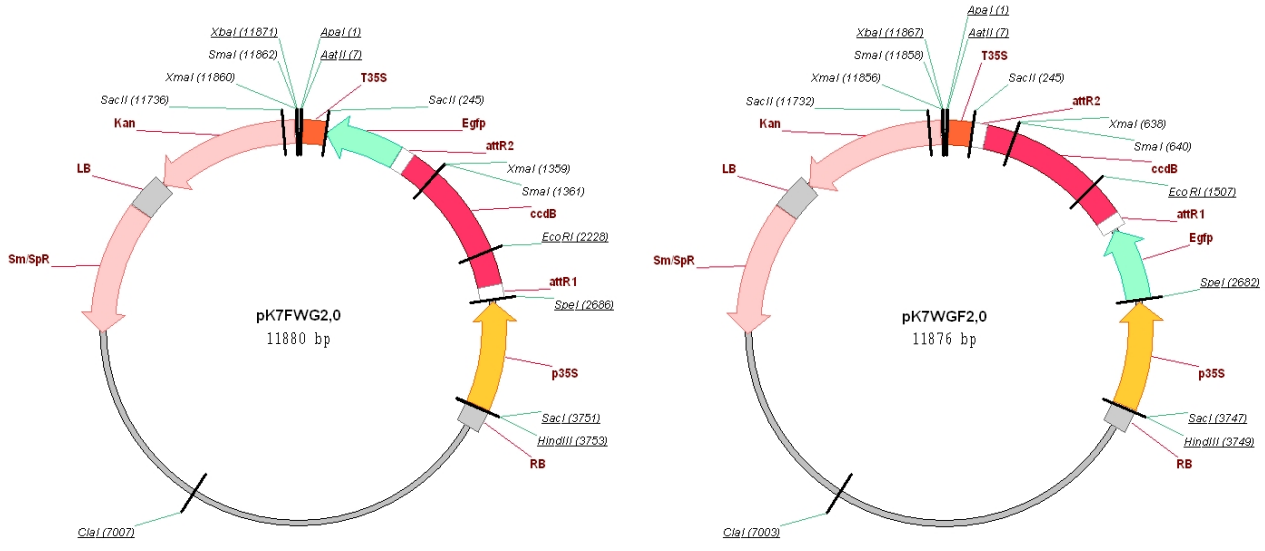


Fig. 4.4 pK7FWG2 and pK7WGF2 maps (Karimi *et al,* 2002)

The following components (Table 4. 10) were added to a 1.5 ml microcentrifuge tube at room temperature:

Components	Samples
Entry clone (50-150 ng)	1-7 µl
Destination vector (150 ng/µl) 1 µl	1 µl
TE buffer, pH 8	0 to 8 µl

Table 4.10 Components for LR reaction

2 µl of LR Clonase™ II enzyme mixwas added to each sample and the mixture was incubated for 1h at 25°C. Finally, the reaction was stoppedadding1µl of Proteinase K (2µg/µl Proteinase K) and incubated for 10 minutes at 37°C. Final products obtained were, respectively, 35S::*OesDHN*, 35S::*OesDHN*:GFP and 35S::GFP:*OesDHN*:

4.2.3.6 *E. coli*transformation with LR reaction

*E. coli*competent cells (One Shot® Omnimax™ 2 T1 Phage-Resistant Cells, Invitrogen),were used for cloning of the gene and transformed by *heat shock*.The cells were thawed on ice and for each transformation 1µl of each BP reaction was added in 50µl of resistant cells. Then cells were placed on ice for 30 min, heat shocked for30 sec at 42°C and put on ice for 2 min. Afterwards, 1ml di S.O.C. medium was added and cells were incubated for 60 min at 37°C and 200 rpm in a Gallenkamp orbital incubator.Finally, aliquots of 100, 200 and300µl of the transformed cells were plated on LB-medium(10g/l Bacto-Tryptone, 5g/l Bacto-yeast 5g/l NaCl, 15g/l Bacto agar), containing the appropriate antibiotic (streptomycin (25µg/ml) and incubated overnight at 37°C. The successful of *E. coli* cells transformation of was verified by colony PCR, using gene specific primers (see table 4.3), *attB* primers (see table 4.7) and *E.coli* colonies as the template.

4.2.3.7 Preparation of *Agrobacterium tumefaciens* competent cells

For plant transformation competent *Agrobacterium tumefaciens* GV3101 (PMP90) cells were used *Agrobacterium* is grown for two days at 28°C in YEB plates (5g/l beef extract, 1g/l yeast extract, 5g/peptone, 5 g/ sucrose,0.5 g/ $MgCl_2$bacterial agar to 1.5%) with appropriate antibiotics (100µg ml-1 Rifampicina, 45µg ml-1 Gentamicina). The competent cells were prepared by inoculating 8ml of YEB-medium with appropriate selection (100 µg ml-1 Rifampicina, 45µg ml-1 Gentamicina) with a freshly streaked single colony of *A. tumefaciens* GV3101 (PMP90) and incubated overnight at 160 rpm, 28°C in a Gallenkamp

orbital incubator. The overnight culture was used to inoculate 192ml YEB medium without antibiotics in a 500ml flask and the culture was incubated overnight at 160 rpm, 28°C until it reached an $O.D._{600}$ of 0.5. The culture was harvested by centrifugation for 15min, at 4000 rpm, 4°C in a Beckman Coulter Avanti. The pellets were then resuspended in 10ml of ice-cold 10mM Tris-HCl (pH 7.5) and again centrifuged for 15min, at 4000 rpm, 4°C. The cells were then resuspended in 20ml cold YEB and aliquots of 200µl of cells were added into pre-chilled microcentrifuge tubes. The cells were frozen in liquid N_2adding to the solution up to 20% glycerol.and stored at -80°C.

4.2.3.8 Transformation of *Agrobacterium tumefaciens*

The competent *Agrobacterium tumefaciens* GV3101 (PMP90) cells were transformed with appropriate plasmids, 35S::*OesDHN* e 35S::*OesDHN*:GFP e 35S::GFP:*OesDHN* by heat-shock. About 200µl of competent cells were thawed on ice and mixed with 1µg of plasmidDNA. The mixture was then placed on ice for 5 minutes and frozen in liquid N_2 for 5min before hear-shocked for 5 min at 37°C. After addition of 800µl of YEB medium,cells were incubated for 4h at 28°C in an incubator, shaking gently (Gallenkamp orbital incubator). Finally, aliquots of 50, 150 and 300µl of the transformations were plated on YEB solid-medium, containing the appropriate antibiotics to select the construct and *Agrobacterium* (100µg ml-1 Rifampicin, 25µg m-1 Gentamicin, Streptomycin 300µg ml-1).Afterwards the plates were incubated for 3 days at 28°C. The DNA of the *Agrobacterium* was checked with colony PCR using gene specific primers (see table 4.3) and *attB* primers (see table 4.7). The positive clones were stored at -80°C and used for the floral dipping transformation of *Arabidopsis thaliana.*

4.2.3.9 Colony PCR

The presence and the size of the inserts in the colonies were verified by colony PCR in which, the template DNA was replaced by a bacterial colony.

In order to test the bacterial colony, the latter were drawn directly from the plate with a pipette tip and then immersed in Microamp containing the reaction mix. In order to store clones, the same tip was smeared on a plate, divided in several regions, and incubated at 37°C for *E.coli* and 28°C for *Agrobacterium*.

For each sample the culture was added to a PCR mix of 10Mm dNTPs (0.5µl; Promega), 10X PCR buffer (2.5µl, containing 500Mm potassium chloride, 100Mm tris-hydrochloridre and 15mM magnesium chloride), 1µl of each appropriate primer (10µM) and

finally Taq polymerase (0,125µl) (Promega). Sterile distilled water was added to bring samples to 25µl. Samples were amplified using the following PCR cycle: 10min at 95°C; 35 cycles of 30 s at 95°C, 30 sec at 55 °C and 1 min at 72°C; and 7 min at 72°C. The PCR product was checked by electrophoresis on agarose gel. Verified the size of the fragment, the positive clones were grown overnight with shaking at 37°C in liquid LB for *E.coli* and at 28°C in YEB for *Agrobacterium*, containing appropriate antibiotic, to proceed with the plasmid DNA purification.

4.2.3.10 Plasmid preparation and analysis

For isolation of the plasmid about 35ml of a bacterial overnight culture were processed using the MIDI MACHEREY-NAGEL Plasmid DNA Purification Kit following the manufacturer's protocol. The generated plasmids were analyzed by agarose gel electrophoresis and products were prepared for sequencing (laboratory GENELab ENEA, Rome). The clones sequence were aligned using ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/), in order to identify the correct sequences.

4.2.4 Floral dipping

Stable *Arabidopsis thaliana* transgenic lines were obtained from *Agrobacterium* mediated floral dip transformation (Clough *et* Bent, 1998; Zhang *et al*., 2006; Davis *et al*., 2009).

This technique was performed during the PhD in the VIB Institute (Department of Plant Systems Biology), University of Ghent, under the supervision of Prof. M. Van Lijsebettens. In the VIB institute is present an*Arabidopsis* transformation platform. In this project for each different *OesDHN* construct were transformed five plants named: A, B, C, D, and E.

4.2.5 Genotyping of *Col*-0 plants with *OesDHN*-constructs

The transgenic lines 35S::*OesDHN*, 35S::*OesDHN*:GFP and 35S::GFP:*OesDHN*, were selected for the T-DNA insert. The screening was started by high density plating of transformation with the seeds of primary transformant lines, T1, obtained from *Agrobacterium* mediated floral dip transformation (Clough *et* Bent, 1998; Zhang *et al*., 2006; Davis *et al*.,2009). T1 seedswere plated and grown *in vitro* on GM+V, containing 50µg/ml of Kanamycin (Km) to select the construct, 250µg/ml of Carbenecillin (Cb) against *Agrobacterium* overgrowth and 50µg/ml of Nystatin (Ns), an antifungal. Once sterilized seeds

(25mg of seeds/plate, 2 plates per line) were spreaded on GMusing sterile water or with 0.1 % sterile agarose to facilitate plating. In addition seeds *Col*-0 were sown on normal GM+V as a positive control and, with antibiotics, as a negative control.

The plates were left on the flow bench to dry before sealing them with micro pore tape. The plates were also labeled, put in the cold room (4ºC) overnight and transferred to the tissue culture chamber with 21ºC under long day night regime.After two weeks on selection medium, green seedlings (T1 plants) were transferred to soil pots and grown to maturity in a growth chamber.

The T1 plants, containing the transgene, were grown to maturity and seeds were collected (T2 seeds).The T2 seeds were germinated on selective medium again.The growing T2 seedlings were scored for resistance and sensitivity to Km by counting the number of resistant and sensitive seedlings.The number of seeds that did not germinated was also monitored. The number of T-DNA loci for each line was determined by chi-square statistical method.

Resistant seedlings were transferred to soil, grown to maturity and T2 seeds were harvested dried, vernalized and then germinated *in vitro* on GM+V containing selective antibiotic.The number of resistant and sensitive seedlings of the lines, as well as the seeds that did not germinate, was counted and determined the homozygosity/heterozygosity. We classified as homozygous lines in which seedling were all resistant (100% resistant) and as heterozygous lines with sensitive seedlings.

Also *Col-0* seeds were germinated *in vitro* on normal GM+V.

4.2.5.1 Chi-squareχ2 test analysis

The chi-square (χ^2) test is statistical hypothesis test in which the sampling distribution of the test statistic is a chi-square distribution when the null hypothesis is true.It has been applied when carrying out hypotheses for categorical data.

The chi-square test was used in this project to determine the T-DNA loci number of T2 seedlings of lines grown in vitro on GM+V containing Km for selection.The growing seedlings were scored for resistance and sensitivity to Km by counting the number of resistant and sensitive seedlings. By the use of chi-square statistical method, the differences between the observed and expected counts were obtained by the formula for the Person chi-squared statistic: the sum of the squares of the differences between observed (O) and expected conts (E) divided by the expected counts:

$$X=\sum \frac{(O-E)2}{E}$$

In our case there were two classe of elements, sensitive (S) and Resistance (R), and the degrees of freedom (df) was defined as n-1, where n represent the number of classes.The null (H0) and alternative (H1) hypothesis were defined and expressed as exemplified in table 4.11.

The null hypothesis is usually an extrinsic hypothesis, one for which the expected proportions are determined before the experiment.

The supposed statistical null hypothesis (H0) was that the number of observations in each line (category) would beenequal to that the predicted by the S:R ratio (theory), and the alternative hypothesis (H1) was that the observed counts would been different from the expected ones.

T-DNA loci	1	2	3
Hypothesis proportions	1S:3R	1S:15R	1S:63R

Table 4.11 The null (H0) and alternative (H1) hypothesis

With the chi-square values obtained (Table 4.12), using the Person chi-squared statistical method as above described, we considered true theH0 hypothesis if the value was $\chi^2 \leq 3{,}84$ or $P \leq 0.05$, hence accepted and the H1 was rejected.Similarly, H0 hypothesis was considered false when the value was $\chi^2 > 3.84$ thus rejected and the H1 was accepted. This principle was followed for all the possible T-DNA loci number and the results were computed.

df	Level of significance									
	0.99	0.90	0.70	0.50	0.30	0.20	0.10	0.05	0.01	0.001
1	0.00016	0.0158	0.148	0.455	1.074	1.642	2.706	3.841	6.635	10.827
2	0.0201	0.211	0.713	1.386	2.408	3.219	4.605	5.991	9.210	13.815
3	0.115	0.584	1.424	2.366	3.665	4.642	6.251	7.815	11.341	16.268
4	0.297	1.064	2.195	3.357	4.878	5.989	7.779	9.488	13.277	18.465
5	0.554	1.610	3.000	4.351	6.064	7.289	9.236	11.070	15.086	20.517
6	0.872	2.204	3.828	5.348	7.231	8.558	10.645	12.592	16.812	22.457
7	1.239	2.833	4.671	6.346	8.383	9.803	12.017	14.067	18.475	24.322
8	1.646	3.490	5.527	7.344	9.524	11.030	13.362	15.507	20.090	26.125
9	2.088	4.168	6.393	8.343	10.656	12.242	14.684	16.919	21.666	27.877
10	2.558	4.865	7.267	9.342	11.781	13.442	15.987	18.307	23.209	29.588
11	3.053	5.578	8.148	10.341	12.899	14.631	17.275	19.675	24.725	31.264
12	3.571	6.304	9.034	11.340	14.011	15.812	18.549	21.026	26.217	32.909
13	4.107	7.042	9.926	12.340	15.119	16.985	19.812	22.362	27.688	34.528
14	4.660	7.790	10.821	13.339	16.222	18.151	21.064	23.685	29.141	36.123
15	5.229	8.547	11.721	14.339	17.322	19.311	22.307	24.996	30.578	37.697
16	5.812	9.312	12.624	15.338	18.418	20.465	23.542	26.296	32.000	39.252
17	6.408	10.085	13.531	16.338	19.511	21.615	24.769	27.587	33.409	40.790
18	7.015	10.865	14.440	17.338	20.601	22.760	25.989	28.869	34.805	42.312
19	7.633	11.651	15.352	18.338	21.689	23.900	27.204	30.144	36.191	43.820
20	8.260	12.443	16.266	19.337	22.775	25.038	28.412	31.410	37.566	45.315
21	8.897	13.240	17.182	20.337	23.858	26.171	29.615	32.671	38.932	46.797
22	9.542	14.041	18.101	21.337	24.939	27.301	30.813	33.924	40.289	48.268
23	10.196	14.848	19.021	22.337	26.018	28.429	32.007	35.172	41.638	49.728
24	10.856	15.659	19.943	23.337	27.096	29.553	33.196	36.415	42.980	51.179
25	11.524	16.473	20.867	24.337	28.172	30.675	34.382	37.652	44.314	52.620
26	12.198	17.292	21.792	25.336	29.246	31.795	35.563	38.885	45.642	54.052
27	12.879	18.114	22.719	26.336	30.319	32.912	36.741	40.113	46.963	55.476
28	13.565	18.939	23.647	27.336	31.391	34.027	37.916	41.337	48.278	56.893
29	14.256	19.768	24.577	28.336	32.461	35.139	39.087	42.557	49.588	58.302
30	14.953	20.599	25.508	29.336	33.530	36.250	40.256	43.773	50.892	59.703

Table 4.12The critical value of the Chi-square distribution. The area given across the top are the areas to the right of the critical value. To look up an area on the left, substract it from one, and then look it up (ie:0,05 on the left or 0,95 on the right)

4.2.6 Selection of transgenic lines using qRT-PCR analysis (qRT-PCR, real time)

The Real-Time PCR (qRT-PCR) is a technique that allows to quantify and monitorthe amplification of a target DNA in real time, through the use of fluorochromes. It is a quantitative method which can beused to quantify the expression of specific gene.

As in a normal PCR reaction, it's possible use a thermal cycler, to set temperature during the various steps of the reaction. In the lid there is an optical module with a laser source that excite fluorophores, then a detector collects the data of fluorescence emitted from each sample and transmits them to a computer. The computer with a specific software allows visualization of the kinetics of the amplification, through the increase of fluorescence.

Different fluorophores that fluoresce when excited by a certain wavelength, can be used, such as intercalating fluorochromes (ethidium bromide and SYBR Green), or probes, labeled with fluorochromes. In the last case there are probes for hybridization with hydrolysis (eg Taqman probes), probes without hydrolysis (eg Molecular Beacons, Fret probes) and Probes incorporated into primers (eg Amplifuor, Scorpions).

In particular the SYBR Green approach, used in this projectis a chemical dye intercalates, that binds to the groove minor of double stranded DNA.

qRT-PCR was used in this project to analyze the overexpression of *OesDHN* gene in T3 homozygous transgenic lines with 1 T-DNA locus. The procedure has involved a step in

which total RNA extraction was performed by using RNasy Plant Mini Kit (Quiagen, Hilden, Germany), followed by the cDNA synthesis and then qRT-PCR reaction.

Whole seedlings of T3 homozygous lines selected were grown *in vitro*for 15 days and used to extract RNA according to Plant Mini kit (Qiagen, Hilden, Germany).

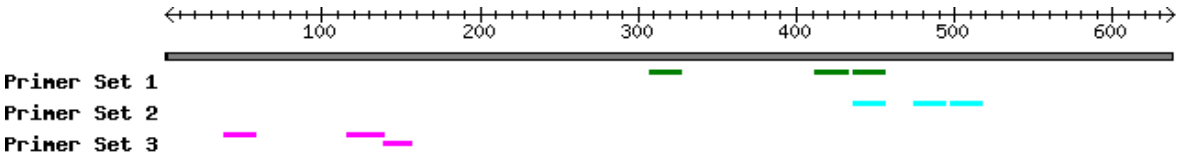
Once harvested, seedlings were put into 2ml eppendorf tubes containing metallic bullets and straight away placed in liquid nitrogen followed by grinding with Retch MM301 machine. The extraction was performed by adding to 100 mg of powered tissue 450 µl of RLT buffer (extraction buffer provided by Qiagen kit) in which it was previously added β-mercaptoethanol in the amount established by the protocol (10µl for each ml of buffer RLT used). The samples were shaken vigorously by vortexing and then incubated at 56°C for 3 min in order to facilitate the lysis of the material. The lysate was then transferred into the QIAshredder spin column (lilac) placed in a 2 ml collection tube and centrifuged for 2 minutes at full speed (14.000 rpm). After the centrifugation, (which induces a mechanical separation of particles of a solution, through the application of a centrifugal field), the supernatant of the flow-through was transferred to a new eppendorf without disturbing the pellet. 0.5 volumes of ethanol (96%-100%) was added to the supernatant to assist the precipitation of nucleic acids.

The supernatants including any precipitate, were then transferred into RNeasy spin column (pink) placed in a 2ml collection tube and centrifuged for about 15 seconds at 10000 rpm. Once discarded the flow-through, 500 µl of washing buffer RPE (supplied byRNasy Plant Mini Kit, Quiagen; and containining ethanol 96–100%) were added in the appropriate eppendorf in order to remove contaminantsand then centrifuged at 8000 rpm for 15 seconds, to wash the membrane of the column. 500 µl of RPE buffer were added to RNeasy spin column, for the second time and then centrifuged for 2 min at 10.000 rpm. Afterwards, the flow-through was discarded andit followed a centrifugation at full speed (14000 rpm) for 1 min. Finally, samples were resuspended in 50µl of RNase-free water and the concentration determined by Nanodrop (ND-1000).

For RNA purification was used the kit RQ1 DNase (Promega). According to the manual, 2µg of RNA were digested, adding into reaction mix respectively 2µl of RQ1 RNase free DNase, 10X reaction buffer, 2µl of enzyme RQ1RNase Free DNase (1u/µg), and RNase free water to reach a final volume of 20 µl. The total mix was then incubated at 37°C for about 30 minutes and the reaction was stopped adding 1µl of RQ1 DNase stop solution, respectively at 65°C for 10minutes.

For the cDNA synthesis 1µg of total RNA was used according to the protocol of SuperScript III Reverse reagent (Invitrogen) (see paragraph 4.1.3 for more details).

The synthesized cDNA was then used to analyze the*OesDHN* gene expression through amplification in real time quantitative PCR. The transcripts expressions were analyzed by testing at least three pairs of gene specific primers (Table. 4.13) located in different points of the ORF *OesDNH*gene and designed by GenScript Real-time Primer Design program (https://www.genscript.com/ssl- bin / app / primer).



FW1*OesDHN*RT	*5'CTGGTGAGCACAAGGAAGAA-3'*
BW1*OesDHN*RT	*5'-TGGTGCAGAAACTTCCTCAG-3'*
FW2*OesDHN*RT	*5'-CTGAGGAAGTTTCTGCACCA-3'*
BW2*OesDHN*RT	*5'-TCTCCTTTGCCTCAACGTC-3'*
FW3*OesDHN*RT	*5'-TGGGTTGTTTGATTTCATGG-3'*
BW3*OesDHN*RT	*5'-CTTCCTTGTGCTCCGGTT-3'*

Table.4.13 *OesDHN* specific primers sequences used for Q-PCR

The qRT-PCR was performed byusing theLightCycler 480 machine (Roche Diagnostics) in 384-well plates with LightCycler 480 SYBR Green Master (Roche) according to the manual and using the parameters of annealing and extension respectively shown in the table (Table 4.14).

	Step 1	Step 2	Step 3
Temperature	95°C	60°C	72°C
Time	10'	50''	50"
cycles	45X		

Table 4.14 Thermal cycling program used forqRT-PCR performed in a LightCycler 480 (Roche Diagnostics)

The reaction mix was prepared by using a robotic platform (JANUS Mini Format, Perkin Elmer) (Fig.4.5). The melting curves were performed to analyze the specificity of the primers. Normalization of values was calculated against the average of the followed

housekeepinggenes:SAND family (AT2G28390), TIP41-like (AT4G34270), UBC (AT5G25760), and PP2A subunits (AT1G13320) (Table 4.15) (Czechowski. *et al.*, 2005) using the LightCycler software.

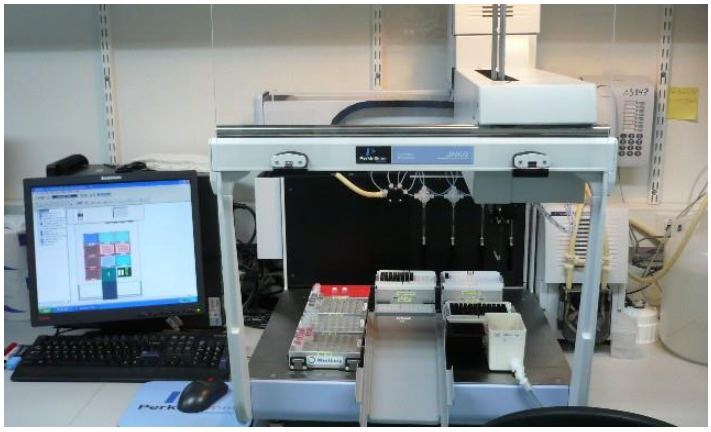


Fig.4.5 robotics platform (JANUS Mini Size, Perkin Elmer)

The method used for statistical analysis was the "Comparative Ct method" where the ΔCt= Ct (gene) - Ct (mean [housekeepinggenes]), ΔΔCT = ΔCT (target) and ΔCT (control). The Ct values were referred to the number of cycles at which the fluorescence of SYBR Green reaches an arbitrary value during the exponential phase of the cDNA amplification.

FWPP2A subunit	*5'-AACTCTATGCAGCATTTGATCCACT-3'*
BWPP2A subunit	*5'-TGATTGCATATCTTTATCGCCATC-3'*
FWUBC	*5'-CTGCGACTCAG^GGAATCTTCTAA-3'*
BWUBC	*5'-TTGTGCCATTGAATTGAACCC-3'*
FWSAND family	*5'-AACTCTATGCAGCATTTGATCCACT-3'*
BWSAND family	*5'-TGATTGCATATCTTTATCGCCATC-3'*
FWTIP41-like	*5'-GTGAAAACTGTTGGAGAGAAGCAA-3'*
BWTIP41-like	*5'-TCAACTGGATACCCTTT^CGCA-3'*

Table 4.15 Primer sequences for housekeeping genes used for qRT-PCR

4.2.7 Phenotypic analysis

4.2.7.1 Leaf analysis

Leaf series were obtained from 16 plants with one T-DNA locus, grown for 22 days in both control (mannitol 0mM) and stress conditions (mannitol 25mM).

Leaves from 16 plants of the respective genotype (35S::*OesDHN*, 35S::*OesDHN*:GFP and 35S::GFP:*OesDHN*and *Col*-0 as controlwere placed on large square plates containing 1% plant tissue agar in distilled water, and then photographed. Furthermore, length and leaf area of 16 seedlings per genotype were measured using ImageJ program (rsbweb.nih.gov / /

ijdownload.html)and the measurements were subjected to statistical analysis of the mean, standard deviation and two-tailed t-test ($p<0,05$).

4.2.7.2 Primary root analysis

Primary root morfology was investigated by growing 35S::*OesDHN*and *Col*-0 plants on normal growth medium (control) and in growth medium enriched with mannitol 25mM, in order to induce an osmotic stress.

For this purpose, seeds of *Col*-0 and 35S::*OesDHNB 1-2* (line1 in the graphic) were placed to germinate *in vitro* on square plates, in vertical position under long day conditions with a temperature of 21°C. The position of the sown seeds was marked and subsequently the primary root tip was marked every 2 days for 13 days.

After 13 days images of the plates have been acquired by scanner (Epson perfection 1670) and the length of the primary root at each time point was measured using the ImageJ Program (rsbweb.nih.gov // ijdownload.html). The obtained data were used for statistical analysis of average, standard deviation and t-test.

The **number of the lateral roots** was investigate sowing seeds of *Col*-0 and 35S::*OesDHNB 1-2* on square plates placed in vertical position on normal growth medium (control) and in growth medium enriched with mannitol 25mM, in order to induce an osmotic stress.The plates were subjected to a regime of continuous light at 21°C. After 11 days, the number of lateral roots was calculated using a stereo microscopy LEICA M80 and the images of the plates were acquired using a scanner (Epson perfection 1670). The total length of the primary root was measured using ImageJ program (rsbweb.nih.gov///ijdownload.html) and statistical analysis of mean, standard deviation, t-test and density of lateral roots (LRN), have been determined.

4.2.8. Fluorescence and Confocal microscopy

T2 seeds with 1 T-DNA locus of lines 35S::*OesDHN*:GFP and 35S::GFP:*OesDHN* and*Col*-0 were placed to germinateon GM+V with Km, for selection, and grown *in vitro* for 6 to 8 days in vertical position under continuous light.The GFP fluorescence was used to detect the presence of the GFP fusion protein (screening) prior to the actual localization of the protein by the confocal microscope.

Transgenic *Arabidopsis* lines were assayed for GFP fluorescence either with Olympus Fluo-View FV1000 microscope equipped with a X63 water-corrected objective (numerical aperture of 1.2) and with a confocal microscope 100M Zeiss equipped with the software

package LSM 510 version 3.2. The GFP fluorescence was excited with a 488-nm laser (emission fluorescence was captured in the frame-scanning mode alternating GFP fluorescence via to 500–550-nm band-pass filter). Chlorophyll autofluorescence was imaged using 488 nm for excitation and 633nm for emission. The green and red autofluorescence were collected in separate channels.

CHAPTER 5

Results

5.1 Isolation and characterization of *OesDHN* sequence and genomic organization

5.1.1 Characterization of *OesDHN* gene

The investigated gene was present in a cDNA library generated by the research group of Prof. M.B. Bitonti. This cDNA library was made from 50-100 μg of total RNA, extracted from leaves of *Olea europaea* (L) subsp. *europaea* var. *sylvestris* plants, using the "SMART" system and cloning the sequences, around 1.2kb, in the pSPORT1 vector.

Generation and sequencing of the library was carried out by Eurofins MWG GmbH cDNA Laboratory Fraunhoferstr (De) service and sequencing analysis were performed from 5'end of obtained fragments. Between the EST clusters produced, a dehydrin sequence was identified and named *OesDHN*.

The cDNA full-length of *OesDHN* was 1207 bp long and showed an open reading frame (ORF) of 636bp. It encode a putative protein of 211 amino acids, with a predicted molecular weight of 23.846 kDa and an isoelectric point of 5.28. Upstream of the start codon ATG and after the stop code TAA, the ORF has a 31bp 5'-UTR and a 540bp 3'-UTR respectively. Furthermore, the amplification reaction, by using the genomic DNA and gene specific primers, revealed the presence of a single product of 73 bp. Sequencing and T BLASTX analysis suggested that the *OesDHN* ORF was interrupted by a single intronic region 95bp long.

The analysis of the deduced amino acid sequence showed that *Oes*DHN is predominantly an hydrophilic protein rich in glycine residues, as reported for the other plant dehydrins. Close to the C-terminal region, *Oes*DHN included two lysine-rich consensus motifs, the K-segments, located respectively between the residues 126-140 and 171-185. It also has a conserved region with serine residues, called S-segment, located between the residues 66 and 80. The last region is probably involved in phosphorylation events, related to the binding of a nuclear localization signal (NLS) peptides (Figure 5.1) and therefore, as reported in literature for other DHNs, involved in the nuclear transport of *Oes*DHN (Jensen *et al*., 1998; Brini *et al*.,

2007). There is also a poly-proline cluster, located at residues 151-156 between K1- and K2 –segments.

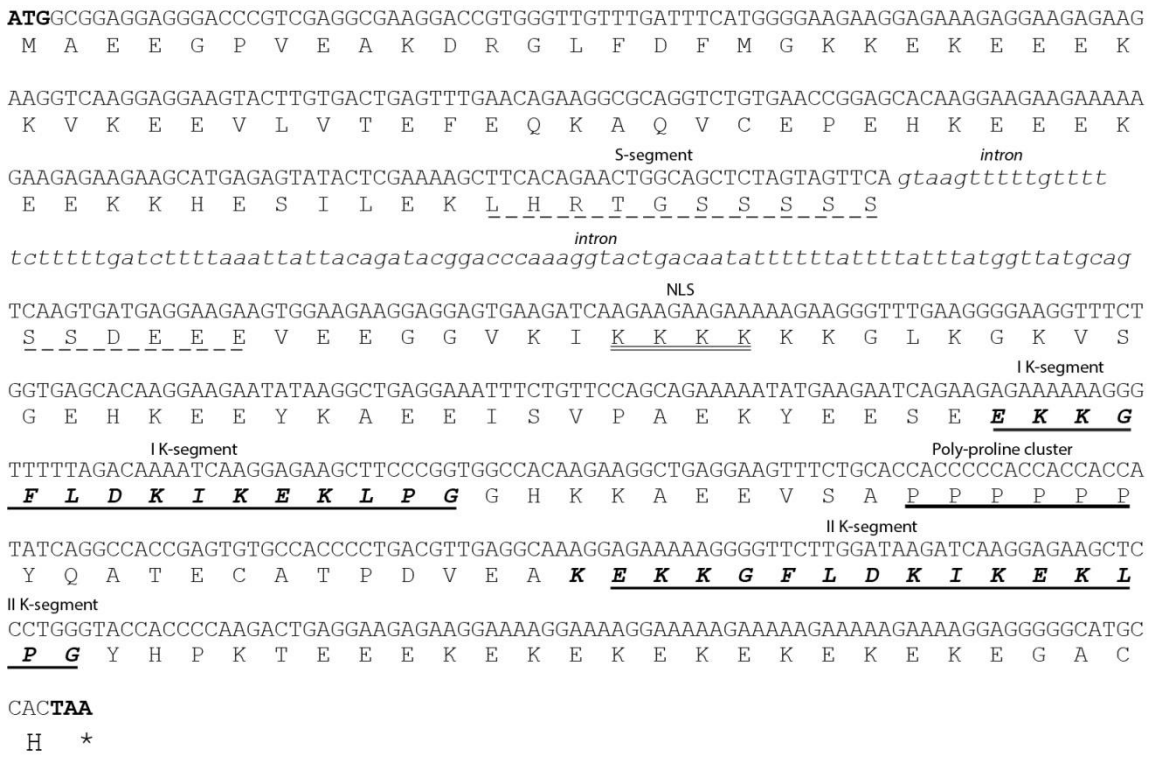


Figure 5.1 Features of of *Oes*DHN protein. Predicted amino acids are shown in one letter code. The S-segment is shown with an interrupted line, and the two K-sements are shown in bold italics and underlined with a single line. The putative NLS (nuclear localization signal) is double underline and the poly-proline cluster is shown with one single line. The intronic region is represented with italic lowercases and the asterisk indicated the stop codon position

The putative NLS of *Oes*DHN was identified by using a bioinformatic prediction program, the Wolf PSORT (Horton *et al*., 205) and PROlocalizer (integrated web service for protein Subcellular localization prediction) (Laurila *et al*., 2011). The NLS, identified by the sequence *KKKK*, was located after the serine tract and just before the first "K" segment, between residues 90-93, as showed for the other plant dehydrins (Mehta *et al*., 2009).

The amino acid alignment of the predicted *Oes*DHN with homologues from other plant species, including the S-tract and the putative NLS sequence, was shown in Figure 5.2.

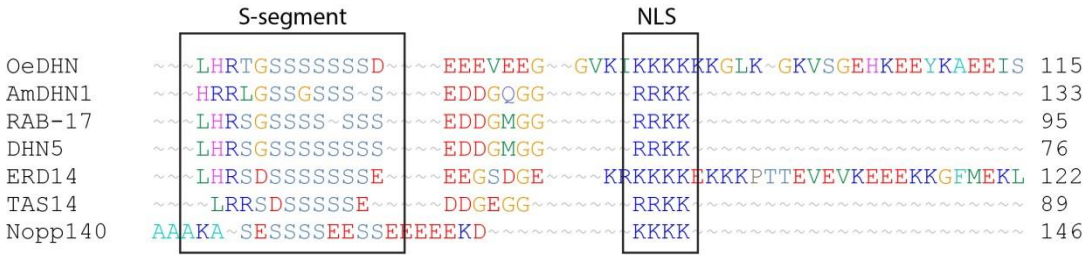


Figure 5.2 Amino acid alignment of the predicted OesDHN protein with homologues from other plant species. The sequences include the S-tracts and the putative NLSs. Accession numbers are given in parentheses:AmDHN1, (A8CVF3.1) RAB-17 (CAA34123.1) DHN5 (AAT40309.1), ERD14 (P42763) TAS14 (P22240) and Nopp140 (AAA41718.1)

The *Oes*DHN protein was blasted in NCBI database and the obtained results indicated that it exhibited a 63% amino acid identity and a 73% similarity with DHN of *Solanum chilense*, followed by a 60% amino acid identity and a 67% similarity with BDN1 of *Paraboea crassifolia,* a 50% amino acid identity and a 70% similarity with *Populus maximowiczii*, a 56% amino acid identity and a 68% similarity with *Coffea canephora*, a 52% amino acid identity and a 66% similarity with DHN in *Citrus x paradise* (50%).

Furthermore, the multiple amino acid sequence alignment reveals that *Oes*DHN share all common features of plant dehydrin family (Figure 5.3).

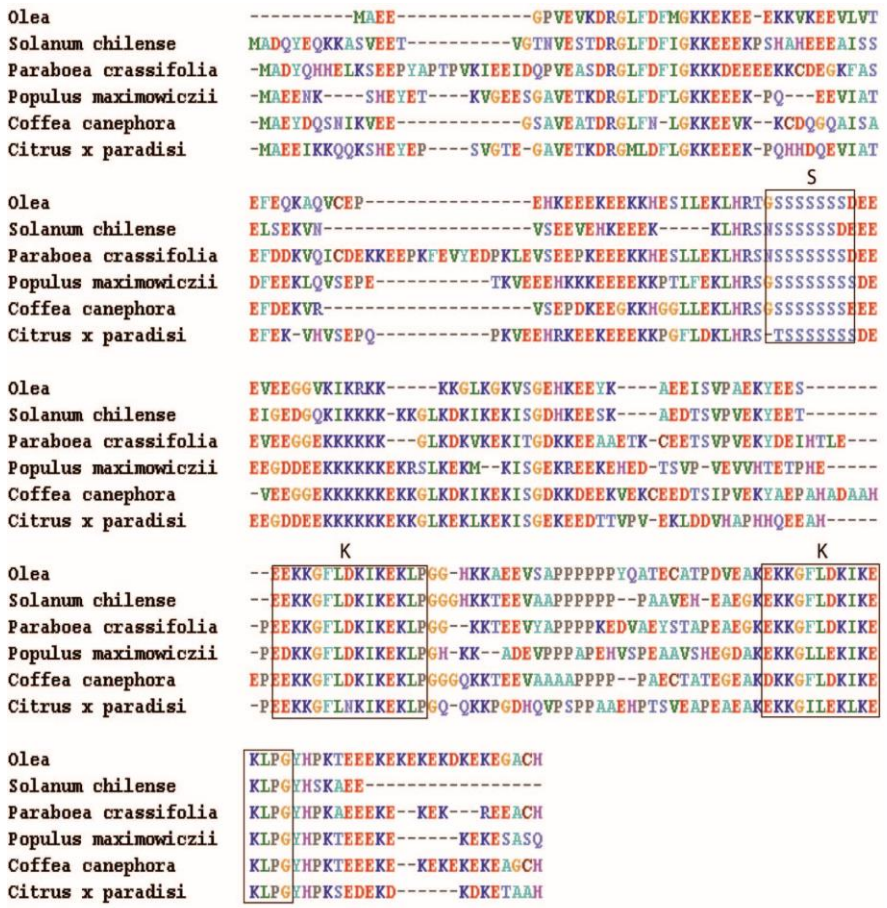


Figure 5.3 Amino acid alignment of predicted protein *Oes*DHN with homologues from other plant species performed with the NCBI database. Accession numbers are given in parentheses: *Solanum chilense* (ADQ73953.1), *Paraboea crassifolia* (AAF014652.2), *Populus maximowiczii* (ABS12346.1), *Coffea canephora* (ABC68275.1) and *Citrus x paradise*(AAN78125.1).The multiple amino acid sequence alignment reveals thecommon features of *OesDHN* with plant dehydrin family, such as the S- and K- segment, that are boxed

Phylogenetic analysis of YnSKn, SKn, Kn, YnKn, and KnS- type deydrins indicated that *Oes*DHN clustered together with SKn-type dehydrins, especially with SK2-types (Figure 5.4). Thus, the results of both the homology and phylogenetic analyses suggest that *Oes*DHN is an SK2-type dehydrin.

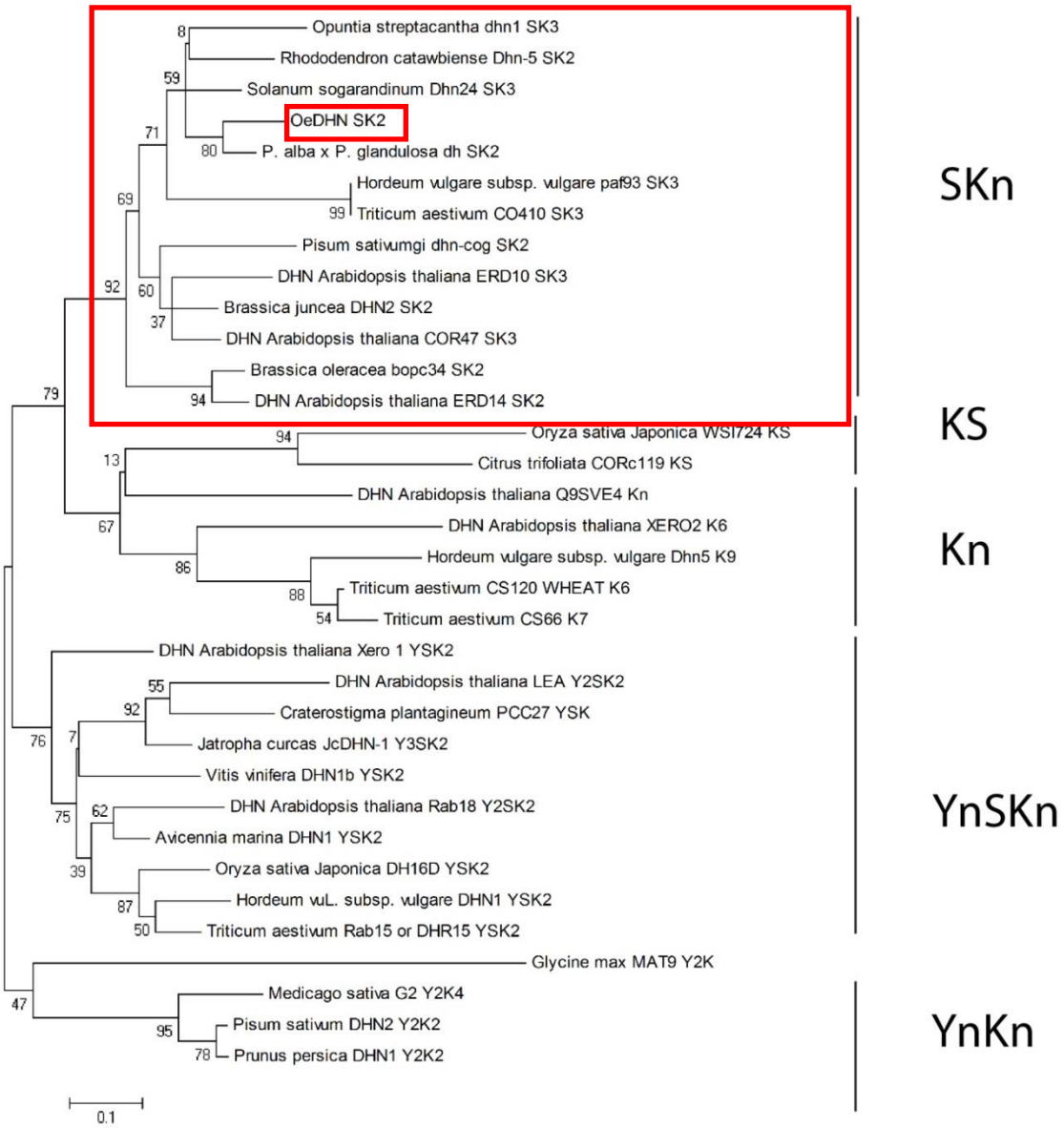


Figure 5.4 Phylogenetic tree of DHN proteins. The neighbor-joining tree was generated using the MEGA4 (www.megasoftware.net) version 5.0 software, with bootstrap value obtained from 1,000 replications

5.1.2. Isolation of the putative *OesDHN* promoter

A putative partial *OesDHN* promoter region was obtained using the "Genome walking method" (Clontech Laboratories). A genomic fragment, located upstream of the *OesDHN* translation initiation start site (TSS), the ATG codon, was sequenced (ENEA GENELab, Roma) and putative *cis*-acting elements were identified using "PLACE" and "PlantCARE" databases.

The analysis of the *OesDHN* promoter region indicated the presence of a putative TATA-box sequence, located 70bp upstream of the ATG codon. Sequence motifs showing hight homology to *cis*-acting elements that confer stress

responsiveness, were also detected. In fact, an ABA-responsive element (ACGT sequences) also named ABRE, with a G-box (CACGTT) which is another type *cis*-acting element, responding to various stressful conditions such osmotic stress, UV light, and intense light, was identified -610 bp upstream the TSS forms.

The regulatory *cis*-acting elements respond to various stressful conditions such as osmotic stress, UV light, and high irradiance, and they might share similar functions under some stressful conditions (Guiltinnan *et al*., 1990; Skriver *et al*., 1991).

In the *OesDHN* promoter region has also been identified elements that can bind to MYB transcription factors (WAACCA), located -479bp and -246 upstream the TSS.

MYB-binding consensus sequences are present in the promoter of many dehydratation-responsive genes (Zhu, 2002).

Finally, two GATA boxes, light regulatory elements, has been detected 478 and 419bp respectively, located upstream of the TSS of *OesDHN*, and also a CAAT-box (or C-box), a common *cis*-enhancer promoter regions (Figure 5.5).

Figure 5.5 The putative partial *OesDHN* promoter sequence. The TATA-box, the ABRE element, G-box, C-boc, GATA-box and MYB-box are underline and indicated with capitol letters

5.1.3 Genomic organization of *OesDHN*

In order to estimate the copy numbers of *OesDHN*, the genomic DNA extracted from mature leaves of *Olea europaea* L. subsp. *europaea* var. *sylvestris* was digested with EcoRI and XbaI endonucleases, which do not cut within the cDNA sequence and in the intron regions. Southern blot analysis was carried out using the ORF of 636-bp of the dehydrin gene as a probe.

The hybridization profile obtained suggested that two *OesDHN* members were harboure per haploid genome (Figure.5.6).

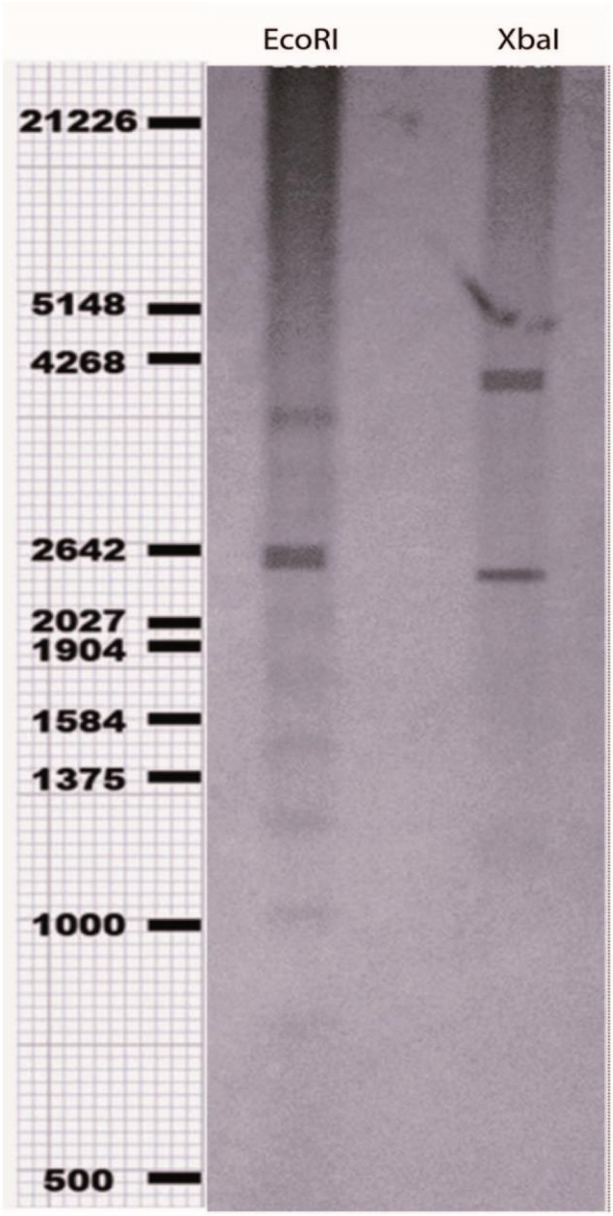


Figure 5.6 Southern blot hybridization of leaf gDNA from *Olea europaea* subsp. *europaea* var .*sylvestris*. Genomic DNA was digested with EcoRI and XbaI enzymes and hybridized with the *OesDHN* probe. DNA size standards in bp are shown on the left

5.2 Functional analysis of *OesDHN* in overexpression *Arabidopsis* lines

5.2.1 Bioinformatics analysis

Comparing the *OesDHN* gene sequence with the *Arabidopsis thaliana* genomic database we did not provide any data about a complete "sequence matches". Currently, in *Arabidopsis thaliana* are identified 10 dehydrins: COR47-SK3-type,(P31168), ERD10-SK3 (P42759), ERD14-SK2 (P42763), PAP310 or dehydrin LEA- Y2SK2 (Q96261), XERO2 or LTI30 K6 (P42758), XERO1-YSK2 (P25863), Rab18-Y2SK2(P30185), Q9SVE4-Kn(75313932), Q9T022-YnKn (AT4G39130), Q9SLJ2_ARATH KnS (AT1G54410). Moreover, the *Oes*DHN deduced protein, compared with the *Arabidopsis thaliana* dehydrins, share higher homology with ERD14 (SK2) (47% identity and 57% similarity), with ERD10 (SK3) (40% identity and 53%similarity), with COR47 (SK3) (44% identity and 53% similarity and RAB18 (Y2SK2) (42% identity and 52% similarity) (Figure 5.7).

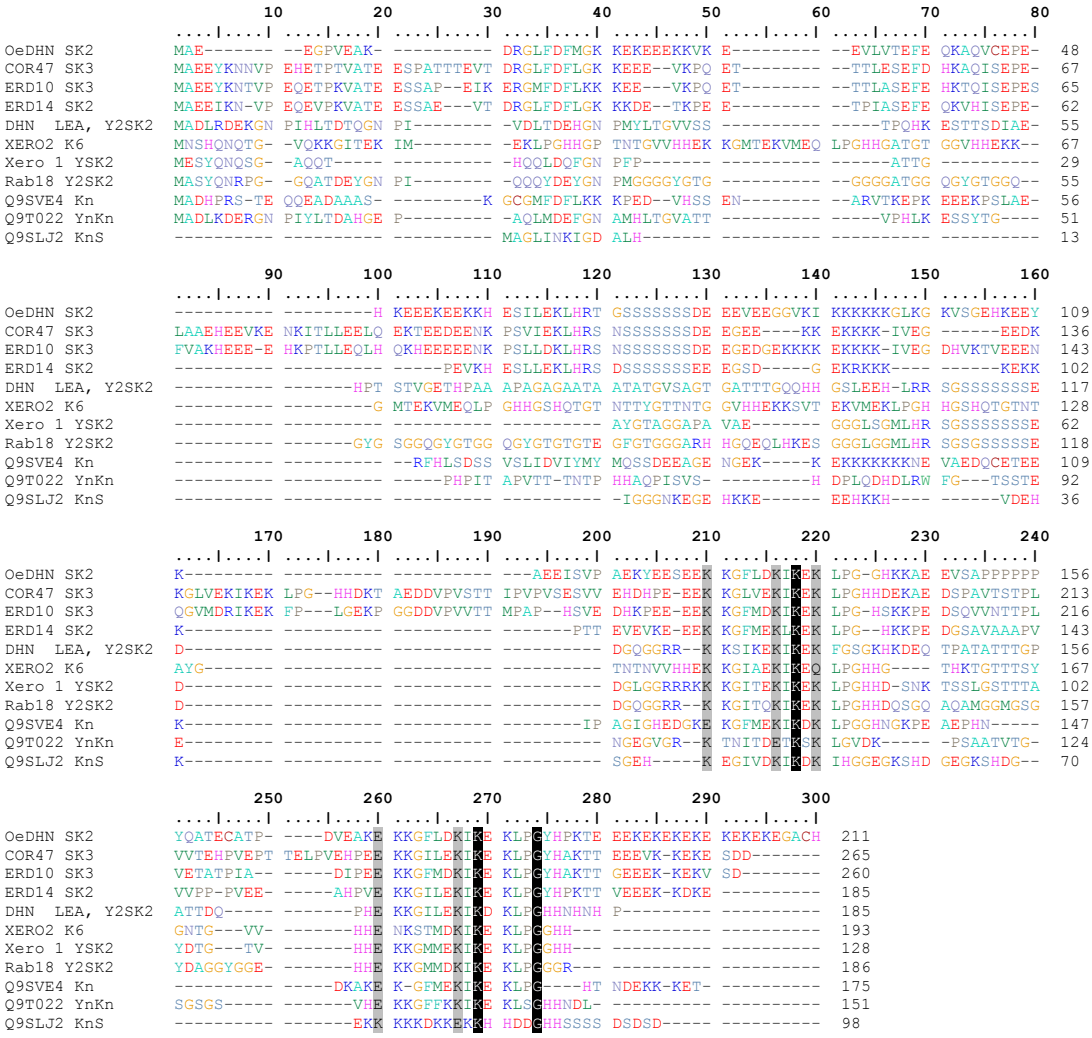


Figure 5.7 Multi-alignment of *OesDHN* with dehydrin proteins from *Arabidopsis thaliana*. The identical amino acids are indicated with black background and the similarity of aminoacids residue is shown in gray background. Gaps are introduced for optimal alignment and maximum similarity between all compared sequences

5.2.2 35S::*OesDHN*, 35S::*OesDHN*:GFPand35S::GFP:*OesDHN* constructs

To obtain *OesDHN* overexpression *Arabidopsis* lines, the ORF of *OesDHN* gene, including or not the stop codon, was first cloned into the donor vector PDONR221, in order to obtain the entry vectors pENTR221-*OesDHN* by GATEWAY technique. Then, the entry vectors were introduced, by recombination, into the destination vector pK7WG2 (Karimi *et al*., 2002) (Figure 5.8).

In order to investigate the subcellular localization of *Oes*DHN, GFP constructs both C- and N-terminal protein fusion, were also generated by GATEWAY, using as destination vectors pK7FWG2 and pK7WGF2 respectively (Karimi *et al*., 2002) (Figure 5.8). Then the constructs were used to transforme

Arabidopsis Col-0 plants by *Agrobacterium tumefaciens*-mediated floral dipping (Clough *et* Bent, 1998; Zhang *et al.*, 2006; Davis *et al.*, 2009) and that work was completed in the VIB Institute, department of Plant System Biology in Ghent (Belgium**).**

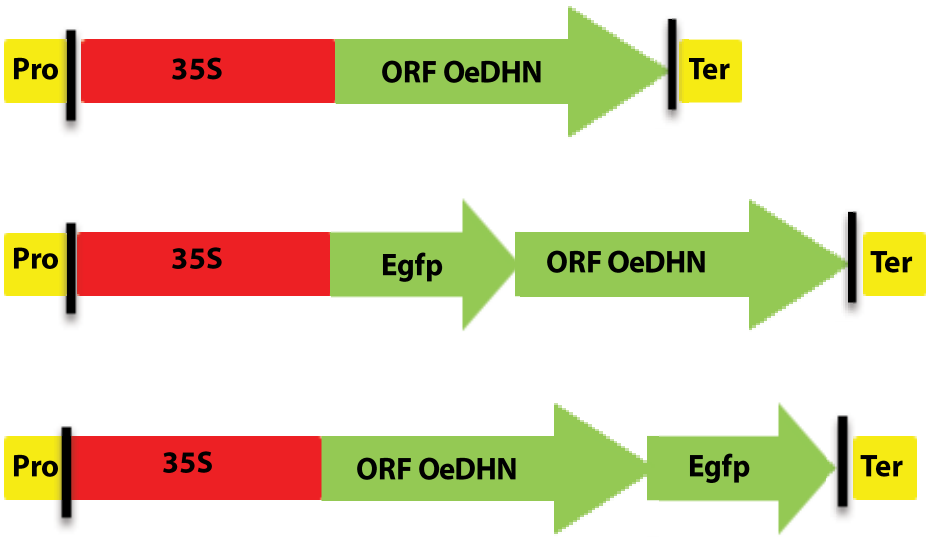


Figure5.8 Schematic representation of the 35S::*OesDHN*, 35S::*OesDHN*:GFP and 35S::GFP:*OesDHN* constructs

5.2.3 Genetic screening 35S::*OesDHN* lines

The transgenic lines selection was started with high density plating of transformation, using the seeds of primary transformant lines, T1.

The 35S::*OesDHN* A and B stock seeds of T1 lines were plated and grown *in vitro* to obtain the T2 generation seeds. Then T2 seeds were harvested, dried, vernalized and germinated *in vitro* in order to select lines with a single insertion locus by segregation analysis in each population. The growing T2 seedlings were scored for resistance and sensitivity to selective medium and the number of T-DNA loci was determined by Chi-square statistical method (Table 5.1). 12 independent 35S::*OesDHN* lines with 1 T-DNA locus were found and used to obtain the T3 generation seeds. The T3 seeds were harvested, dried, vernalized and sown *in vitro* to check the sensitivity of seedlings.

In Table 5.2 are shown both the homozygous lines containing all resistant seedlings and the heterozygous ones in which were present sensitive seedlings. It was also looked phenotype for homozygosity and eterozygosity.

Line	sensitive	resistence	not germinated	Total	1 T-DNA locus (1:3)		2 T-DNA locl (1:15)		3 T-DNA locl (1:63)		T-DNA loci
					—	P value	—	P value	—	P value	
pK7WG2-OeDHN2, A-plt1	2	55	11	68	14,04094	P<0,01	0,73099415	0,50<P>0,30	1,403787246	0,30<P>0,20	2 or 3
pK7WG2-OeDHN2, A-plt2	20	36	11	67	3,428571	0,10<P>0,05	82,9714286	P<<0,01	424,6530612	P<<0,01	1
pK7WG2-OeDHN2, A-plt3	19	32	11	62	4,084967	0,05<P>0,01	83,6718954	P<<0,01	422,4167445	P<<0,01	?
pK7WG2-OeDHN2, A-plt4	9	38	19	66	0,858156	0,50<P>0,30	13,3460993	P<0,01	94,50894968	P<<0,01	1
pK7WG2-OeDHN2, A-plt5	21	38	6	65	3,531073	0,10<P>0,05	86,699435	P<<0,01	444,235943	P<<0,01	1
pK7WG2-OeDHN2, A-plt6	0	52	16	68	17,33333	P<0,01	3,46666667	0,10<P>0,05	0,825396825	0,50<P>0,30	3
pK7WG2-OeDHN2, A-plt7	65	3	4	72	180,7059	P<<0,01	926,258824	P<<0,01	3908,605042	P<<0,01	?
pK7WG2-OeDHN2, A-plt8	27	45	2	69	6,820896	P<0,01	129,328358	P<<0,01	650,0618337	P<<0,01	?
pK7WG2-OeDHN2, A-plt9	16	38	13	67	0,617284	0,50<P>0,30	50,3753086	P<<0,01	276,5726044	P<<0,01	1
pK7WG2-OeDHN2, A-plt10	3	54	11	68	11,84211	P<0,01	0,09473684	0,90<P>0,70	5,07518797	0,05<P>0,01	2
pK7WG2-OeDHN2, A-plt11	2	60	10	72	15,67742	P<0,01	0,96774194	0,50<P>0,30	1,115207373	0,30<P>0,20	2 or 3
pK7WG2-OeDHN2, A-plt12	40	18	6	64	59,7931	P<0,01	389,337931	P<<0,01	1713,192118	P<<0,01	?
pK7WG2-OeDHN2, B-plt1	9	41	13	63	1,306667	0,30<P>0,20	11,7813333	P<0,01	87,83365079	P<<0,01	1
pK7WG2-OeDHN2, B-plt2	9	41	20	70	1,306667	0,30<P>0,20	11,7813333	P<0,01	87,83365079	P<<0,01	1
pK7WG2-OeDHN2, B-plt3	2	58	8	68	15,02222	P<0,01	0,87111111	0,50<P>0,30	1,223280423	0,30<P>0,20	2 or 3
pK7WG2-OeDHN2, B-plt4	19	43	11	73	1,053763	0,50<P>0,30	62,972043	P<0,01	340,9411162	P<<0,01	1
pK7WG2-OeDHN2, B-plt5	11	50	9	70	1,579235	0,30<P>0,20	14,4535519	P<0,01	107,5849597	P<<0,01	1
pK7WG2-OeDHN2, B-plt6	16	37	16	69	0,761006	0,50<P>0,30	51,8352201	P<0,01	282,3722671	P<<0,01	1
pK7WG2-OeDHN2, B-plt7	6	55	10	71	7,480874	0,01<P>0,001	1,33879781	0,30<P>0,20	27,1478012	P<0,01	2
pK7WG2-OeDHN2, B-plt8	3	37	27	67	6,533333	0,05<P>0,01	0,10666667	0,90<P>0,70	9,168253968	0,01<P>0,001	2
pK7WG2-OeDHN2, B-plt9	14	36	21	71	0,24	0,70<P>0,50	40,368	P<0,01	227,2114286	P<<0,01	1
pK7WG2-OeDHN2, B-plt10	12	42	13	67	0,222222	0,70<P>0,50	23,5111111	P<0,01	149,8518519	P<<0,01	1
pK7WG2-OeDHN2, B-plt11	3	60	8	71	13,7619	P<0,01	0,23809524	0,70<P>0,50	4,192743764	0,05<P>0,01	1
pK7WG2-OeDHN2, B-plt12	13	39	16	68	0	P=1	31,2	P<0,01	185,7142857	P<<0,01	1

Table 5.1 Number of T-DNA loci of 35S::*OesDHN Arabidopsis* lines. The seeds were germinated on GM +V. The green background indicates the lines with one insertion locus K "?", indicates that is not easy to tell the number of T-DNA loci since the x2>3,84 in all the possibilities.

Table 5.2 Homozygosity and heterozygosity of T3 lines 35S::*OesDHN*. The homozygous lines are highlighted in green, the red background indicates the undetermined genotypes. The shaded parts indicate seedlings selected for qRT-PCR analysis of *OesDHN*.

Lines 35S::*OesDHN*	Sensitiv	resistant	Not germinate	Total	homozygous /heterozygous	Comments
A-plt4-1	17	41	5	63	*heterozygous*	
A-plt4-2	18	40	7	65	*heterozygous*	
A-plt4-3	0	64		64	*homozygous*	
A-plt4-4	17	46	0	63	*heterozygous*	
A-plt4-5	0	64		64	*homozygous*	
A-plt4-6	17	41	19	77	*heterozygous*	
A-plt4-7	0	64		64	*homozygous*	
A-plt4-8	15	45	1	61	*heterozygous*	
A-plt4-9	16	44	1	61	*heterozygous*	
A-plt4-10	0	64		64	*homozygous*	

A-plt4-11	0	64		64	*homozygous*	
A-plt4-12	13	41	10	64	*heterozygous*	
A-plt9 -1	12	41		53	*heterozygous*	
A-plt9 -2	15	46	2	63	*heterozygous*	
A-plt9 -3	17	37	2	56	*heterozygous*	
A-plt9 -4	0	64		64	*homozygous*	
A-plt9 -5	12	46	4	62	*heterozygous*	
A-plt9 -6	0	64		64	*homozygous*	
A-plt9 -7	10	51	3	64	*heterozygous*	
A-plt9 -8	21	34	6	61	*heterozygous*	
A-plt9 -9	21	38	1	60	*heterozygous*	
A-plt9 -10	12	45	4	61	*heterozygous*	
A-plt9 -11	16	46	2	64	*heterozygous*	
A-plt9 -12	0	64	2	66	*homozygous*	
B-plt1-1	0	64	1	65	*homozygous*	
B-plt1-2	0	64	0	64	*homozygous*	
B-plt1-3	0	64	0	64	*homozygous*	
B-plt1-4	0	64	3	67	*homozygous*	
B-plt1-5	0	64	4	68	*homozygous*	
B-plt1-6	19	38	4	61	*heterozygous*	
B-plt1-7	6	54	3	63	*heterozygous*	
B-plt1-8	4	59	2	65	*heterozygous*	
B-plt1-9	3	60	0	63	*heterozygous*	
B-plt1-10	0	64	1	65	*homozygous*	
B-plt1-11	0	64	2	66	*homozygous*	
B-plt1-12	7	45	5	57	*heterozygous*	
B-plt2-1	13	38	8	59	*heterozygous*	
B-plt2-2	0	64	0	64	*homozygous*	
B-plt2-3	13	40	5	58	*heterozygous*	
B-plt2-4	10	46	5	61	*heterozygous*	
B-plt2-5	14	47	0	61	*heterozygous*	

B-plt2-6	0	64	3	67	*homozygous*	
B-plt2-7	0	64	2	66	*homozygous*	
B-plt2-8	0	64	4	68	*homozygous*	
B-plt2-9	7	53	2	62	*heterozygous*	
B-plt2-10	15	44	5	64	*heterozygous*	
B-plt2-11	14	49	0	63	*heterozygous*	
B-plt2-12	12	49	0	61	*heterozygous*	
B-plt4-1	?	?	?	?	*?*	sensitive/etiolate
B-plt4-2	?	?	?	?	*?*	sensitive/etiolate
B-plt4-3	?	?	?	?	*?*	sensitive/etiolate
B-plt4-4	?	?	?	?	*?*	sensitive/etiolate
B-plt4-5	?	?	?	?	*?*	sensitive/etiolate
B-plt4-6	?	?	?	?	*?*	sensitive/etiolate
B-plt4-7	?	?	?	?	*?*	sensitive/etiolate
B-plt4-8	?	?	?	?	*?*	sensitive/etiolate
B-plt4-9	?	?	?	?	*?*	sensitive/etiolate
B-plt4-10	?	?	?	?	*?*	sensitive/etiolate
B-plt4-11	?	?	?	?	*?*	sensitive/etiolate
B-plt4-12	?	?	?	?	*?*	sensitive/etiolate
B-plt5-1	0	64	3	67	*homozygous*	
B-plt5-2	20	37	4	61	*heterozygous*	
B-plt5-3	11	42	2	55	*heterozygous*	
B-plt5-4	9	44	3	56	*heterozygous*	
B-plt5-5	8	44	10	62	*heterozygous*	
B-plt5-6	16	45	1	62	*heterozygous*	
B-plt5-7	17	40	1	58	*heterozygous*	
B-plt5-8	15	54	10	79	*heterozygous*	
B-plt5-9	2	63	0	65	*heterozygous*	
B-plt5-10	18	47	0	65	*heterozygous*	
B-plt5-11	16	45	1	62	*heterozygous*	
B-plt5-12	19	44	2	65	*heterozygous*	

B-plt6-1	12	44	4	60	*heterozygous*	
B-plt6-2	11	42	2	55	*heterozygous*	
B-plt6-3	0	64	0	64	*homozygous*	
B-plt6-4	0	64	1	65	*homozygous*	
B-plt6-5	2	63	0	65	*homozygous*	
B-plt6-6	11	51	2	64	*heterozygous*	
B-plt6-7	23	45	0	68	*heterozygous*	
B-plt6-8	22	46	3	71	*heterozygous*	
B-plt6-9	13	45	3	61	*heterozygous*	
B-plt6-10	8	52	2	62	*heterozygous*	
B-plt6-11	15	46	2	63	*heterozygous*	
B-plt6-12	0	64	0	64	*homozygous*	
B-plt9-1	16	41	3	60	*heterozygous*	
B-plt9-2	7	54	2	63	*heterozygous*	
B-plt9-3	0	64	1	65	*homozygous*	
B-plt9-4	16	49	0	65	*heterozygous*	
B-plt9-5	14	40	3	57	*heterozygous*	
B-plt9-6	12	40	9	61	*heterozygous*	
B-plt9-7	0	65	2	67	*homozygous*	
B-plt9-8	15	45	6	66	*heterozygous*	
B-plt9-9	0	63	0	63	*homozygous*	
B-plt9-10	23	38	3	64	*heterozygous*	
B-plt9-11	13	52	0	65	*heterozygous*	
B-plt9-12	15	50	1	66	*heterozygous*	
B-plt10-1	8	48	2	58	*heterozygous*	
B-plt10-2	13	50	3	66	*heterozygous*	
B-plt10-3	0	64	1	65	*homozygous*	
B-plt10-4	0	67	5	72	*homozygous*	
B-plt10-5	0	70	2	72	*homozygous*	
B-plt10-6	17	55	4	76	*heterozygous*	
B-plt10-7	0	65	0	65	*homozygous*	

B-plt10-8	12	57	2	71	*heterozygous*	
B-plt10-9	17	60	2	79	*heterozygous*	
B-plt10-10	15	57	3	75	*heterozygous*	
B-plt10-11	o	64	0		*heterozygous*	
B-plt10-12	17	55	6	78	*heterozygous*	
B-plt12-1	64	0	0	64	*?*	
B-plt12-2	64	0	0	64	*?*	
B-plt12-3	64	0	0	64	*?*	
B-plt12-4	64	1	0	65	*?*	
B-plt12-5	20	53	0	73	*heterozygous*	
B-plt12-6	64	0	0	64	*?*	
B-plt12-7	24	62	0	86	*heterozygous*	
B-plt12-8	60	3	0	63	*?*	
B-plt12-9	14	60	9	83	*heterozygous*	
B-plt12-10	64	0	0	64	*?*	
B-plt12-11	64	0	0	64	*?*	
B-plt12-12	16	67	6	89	*heterozygous*	

5.2.4 Genetic screening 35S*OesDHN*:GFP e 35S:GFP:*OesDHN* lines

In order to identify the *OesDHN* cyto-histological localisation, *OesDHN* was fused in a recombinant constructs in two separate orientation: C-terminal fusion of *OesDHN* to GFP (GFP::*OesDHN*) and N-terminal fusion of *OesDHN* to GFP (*OesDHN*::GFP). Each construct was also fused to the CaMV 35S-promoter by Gateway technique.

The selection of the transgenic lines was started with high density plating of transformation using seeds of primary trasformant lines, T1.

35S::GFP:*OesDHN* A and B and 35S::*OesDHN:*GFP C and D T1 stock seeds were plated and grown *in vitro* on selective medium. Resistent seedlings were then transferred to soil and grown to maturity to obtain the T2 generation seeds.Then, the T2 seeds were germinated *in vitro* in order to select, by segregation analysis, lines with a single insertion locus in each population, determined by Chi-square statistical method (Table 5.3).

8 seedlings of 10 35S::*OesDHN*:GFP lines (A-2, A-4, A-9, A-11, A-12, B-1, B-2, B-4, B-6, B-12) and 8 seedlings of 8 35S::GFP:*OesDHN* lines (C-1, C-2, C-3, C-4, C-5, C-7, C-9, C-11) with one insertion locus were transferred to soil and grown to maturity to obtain the T3 generation seeds. T3 seeds were used to check the seedlings sensitivity.

In Table 5.4 are shown both the homozygous lines containing all resistant seedlings and the heterozygous ones in which were present sensitive seedlings. It was also looked phenotype for homozygosity and eterozygosity.

Plate	Line	Sensitive	Resistant	Not germinated	Total	Remark	#loci
1	pK7FWG2-OeDHN.4#8094,A-1	0	60	4			3
2	pK7FWG2-OeDHN.4#8094,A-2	18	42	4			1
3	pK7FWG2-OeDHN.4#8094,A-3	3	60	1			2
4	pK7FWG2-OeDHN.4#8094,A-4	17	40	7			1
5	pK7FWG2-OeDHN.4#8094,A-5	10	54				1
6	pK7FWG2-OeDHN.4#8094,A-6	6	58				2
7	pK7FWG2-OeDHN.4#8094,A-7	5	59				2
8	pK7FWG2-OeDHN.4#8094,A-8	6	*53			*5small/bleached	1or2
9	pK7FWG2-OeDHN.4#8094,A-9	13	48	3			1
10	pK7FWG2-OeDHN.4#8094,A-10	1	*61	1		*1small aberrant	3
11	pK7FWG2-OeDHN.4#8094,A-11	12	57				1
12	pK7FWG2-OeDHN.4#8094,A-12	17	44	3			1
13	pK7FWG2-OeDHN.4#8094,B-1	13	*43	2		*7small bleached	1
14	pK7FWG2-OeDHN.4#8094,B-2	15	*38	4		*5" "	1
15	pK7FWG2-OeDHN.4#8094,B-3	0	63	1			3
16	pK7FWG2-OeDHN.4#8094,B-4	16	*47			*1small	1
17	pK7FWG2-OeDHN.4#8094,B-5	*24	34	2		*17are half green-stuck in cotyl stage	
18	pK7FWG2-OeDHN.4#8094,B-6	18	46				1
19	pK7FWG2-OeDHN.4#8094,B-7	7	*57			*8have phenotype(hub-like)	
20	pK7FWG2-OeDHN.4#8094,B-8	9	55				1or2
21	pK7FWG2-OeDHN.4#8094,B-9	4	59	1			2
22	pK7FWG2-OeDHN.4#8094,B-10	9	52	3			1or2
23	pK7FWG2-OeDHN.4#8094,B-11	3	*57	3		*1small bleached	2
24	pK7FWG2-OeDHN.4#8094,B-12	20	39	3			1
25	pK7WGF2-OeDHN.7(8)#8095,C-1	15	49	1			1
26	pK7WGF2-OeDHN.7(8)#8095,C-2	13	52				1
27	pK7WGF2-OeDHN.7(8)#8095,C-3	19	45				1
28	pK7WGF2-OeDHN.7(8)#8095,C-4	12	49	1			1
29	pK7WGF2-OeDHN.7(8)#8095,C-5	15	46				1
30	pK7WGF2-OeDHN.7(8)#8095,C-6	all					
31	pK7WGF2-OeDHN.7(8)#8095,C-7	16	46	2			1
32	pK7WGF2-OeDHN.7(8)#8095,C-8	10	*53	1		*4small bleached	1
33	pK7WGF2-OeDHN.7(8)#8095,C-9	16	47	1			1
34	pK7WGF2-OeDHN.7(8)#8095,C-10	5	58	1			2
35	pK7WGF2-OeDHN.7(8)#8095,C-11	13	50	1			1
36	pK7WGF2-OeDHN.7(8)#8095,C-12	6	64				2
37	pK7WGF2-OeDHN.7(8)#8095,D-1	4	63	1			2
38	pK7WGF2-OeDHN.7(8)#8095,D-2	3	60	1			2
39	pK7WGF2-OeDHN.7(8)#8095,D-3	1	*60	2		*1small bleached	3
40	pK7WGF2-OeDHN.7(8)#8095,D-4	30	31	3			

Table 5.3 The number of T-DNA loci in 35S::*OesDHN*:GFP and 35S::GFP:*OesDHN Arabidopsis* lines. The seeds were germinated on GM +V, with K"?", indicates that is not easy to tell the number of T-DNA loci since the x2>3,84 in all the possibilities. The green background indicates the lines with one insertion locus

Table 5.4 Homozygosity and heterozygosity of T3 lines 35S::*OesDHN*:GFP and 35S::GFP:*OesDHN* (homozygous lines are highlighted in green, red background indicates the undetermined genotypes

T3 lines 1 T-DNA locus	sensitive	resistant	not germinated	total	*Homozygous / heterozygous*	comment
35S::*OesDHN*:GFP,A-2-1	15	60	0	75	*heterozygous*	
35S::*OesDHN*:GFP,A-2-2	13	43	1	57	*heterozygous*	
35S::*OesDHN*:GFP,A-2-3	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,A-2-4	12	47	2	61	*heterozygous*	
35S::*OesDHN*:GFP,A-2-5	0	62	3	65	*homozygous*	
35S::*OesDHN*:GFP,A-2-6	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,A-2-7	0	64	1	65	*homozygous*	
35S::*OesDHN*:GFP,A-2-8	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,A-4-1	0	64	1	65	*homozygous*	
35S:*O*:GFP,A-4-2	15	41	0	56	*heterozygous*	
35S::*OesDHN*:GFP,A-4-3	11	45	6	62	*heterozygous*	
35S::*OesDHN*:GFP,A-4-4	11	55	4	70	*heterozygous*	
35S::*OesDHN*:GFP,A-4-5	16	47	4	67	*heterozygous*	
35S::*OesDHN*:GFP,A-4-6	14	40	2	56	*heterozygous*	
35S::*OesDHN*:GFP,A-4-7	0	64	2	66	*homozygous*	
35S::*OesDHN*:GFP,A-4-8	14	45	0	59	*heterozygous*	
35S::*OesDHN*:GFP,A-9-1	17	50	2	69	*heterozygous*	
35S::*OesDHN*:GFP,A-9-2	0	64	5	69	*homozygous*	
35S::*OesDHN*:GFP,A-9-3	19	40	2	61	*heterozygous*	
35S::*OesDHN*:GFP,A-9-4	14	45	1	60	*heterozygous*	
35S::*OesDHN*:GFP,A-9-5	13	49	0	62	*heterozygous*	
35S::*OesDHN*:GFP,A-9-6	8	54	4	66	*heterozygous*	
35S::*OesDHN*:GFP,A-9-7	0	64	1	65	*homozygous*	
35S::*OesDHN*:GFP,A-9-8	11	54	4	69	*heterozygous*	
35S::*OesDHN*:GFP,A-11-1	0	64	6	70	*homozygous*	
35S::*OesDHN*:GFP,A-11-2	0	64	2	66	*homozygous*	
35S::*OesDHN*:GFP,A-11-3	?	?	?	0	?	fungi
35S::*OesDHN*:GFP,A-11-4	13	37	5	55	*heterozygous*	

35S::*OesDHN*:GFP,A-11-5	0	64	4	68	*homozygous*	
35S::*OesDHN*:GFP,A-11-6	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,A-11-7	13	47	2	62	*heterozygous*	
35S::*OesDHN*:GFP,A-11-8	16	46	1	63	*heterozygous*	
35S::*OesDHN*:GFP,A-12-1	13	47	2	62	*heterozygous*	
35S::*OesDHN*:GFP,A-12-2	13	48	3	64	*heterozygous*	
35S::*OesDHN*:GFP,A-12-3	12	54	2	68	*heterozygous*	
35S::*OesDHN*:GFP,A-12-4	15	54	5	74	*heterozygous*	
35S::*OesDHN*:GFP,A-12-5	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,A-12-6	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,A-12-7	12	53	3	68	*heterozygous*	
35S::*OesDHN*:GFP,A-12-8	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,B-1-1	21	40	0	61	*heterozygous*	
35S::*OesDHN*:GFP,B-1-2	15	40	2	57	*heterozygous*	
35S::*OesDHN*:GFP,B-1-3	20	44	1	65	*heterozygous*	
35S::*OesDHN*:GFP,B-1-4	12	55	0	67	*homozygous*	
35S::*OesDHN*:GFP,B-1-5	0	64	2	66	*homozygous*	
35S::*OesDHN*:GFP,B-1-6	15	45	1	61	*heterozygous*	
35S::*OesDHN*:GFP,B-1-7	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,B-1-8	2	64	2	68	*heterozygous*	
35S::*OesDHN*:GFP,B-2-1	11	57	0	68	*heterozygous*	
35S::*OesDHN*:GFP,B-2-2	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,B-2-3	21	49	1	71	*heterozygous*	
35S::*OesDHN*:GFP,B-2-4	0	64	2	66	*homozygous*	
35S::*OesDHN*:GFP,B-2-5	19	44	2	65	*heterozygous*	
35S::*OesDHN*:GFP,B-2-6	17	47	2	66	*heterozygous*	
35S::*OesDHN*:GFP,B-2-7	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,B-2-8	4	63	0	67	*heterozygous*	
35S::*OesDHN*:GFP,B-4-1	19	40	2	61	*heterozygous*	
35S::*OesDHN*:GFP,B-4-2	11	44	0	55	*heterozygous*	
35S::*OesDHN*:GFP,B-4-3	0	64	0	64	*homozygous*	
35S::*OesDHN*:GFP,B-4-4	15	48	0	63	*heterozygous*	
35S::*OesDHN*:GFP,B-4-5	?	?	?	?	?	fungi

35S::*OesDHN*:GFP,B-4-6	?	?	?	?	?	fungi
35S::*OesDHN*:GFP,B-4-7	16	42	2	60	*heterozygous*	
35S::*OesDHN*:GFP,B-4-8	1	50	2	53	*heterozygous*	fungi
35S::*OesDHN*:GFP,B-6-1	10	48	5	63	*heterozygous*	
35S::*OesDHN*:GFP,B-6-2	0	64	1	65	*homozygous*	
35S::*OesDHN*:GFP,B-6-3	0	64	2	66	*homozygous*	
35S::*OesDHN*:GFP,B-6-4	11	53	3	67	*heterozygous*	
35S::*OesDHN*:GFP,B-6-5	0	64	7	71	*homozygous*	
35S::*OesDHN*:GFP,B-6-6	12	47		59	*heterozygous*	little
35S::*OesDHN*:GFP,B-6-7	5	33		38	*heterozygous*	fungi
35S::*OesDHN*:GFP,B-6-8	0	64	1	65	*homozygous*	
35S::*OesDHN*:GFP,B-12-1	15	54	1	70	*heterozygous*	
35S::*OesDHN*:GFP,B-12-2	16	40	1	57	*heterozygous*	
35S::*OesDHN*:GFP,B-12-3	4	58	1	63	*heterozygous*	
35S::*OesDHN*:GFP,B-12-4	12	47	4	63	*heterozygous*	
35S::*OesDHN*:GFP,B-12-5	19	46	1	66	*heterozygous*	
35S::*OesDHN*:GFP,B-12-6	20	43	0	63	*heterozygous*	little
35S::*OesDHN*:GFP,B-12-7	19	32	1	52	*heterozygous*	
35S::*OesDHN*:GFP,B-12-8	18	40	0	58	*heterozygous*	little
35SGFP:*OesDHN*C-1-1	16	59	3	78	*heterozygous*	
35SGFP:*OesDHN*C-1-2	7	60	0	67	*heterozygous*	
35SGFP:*OesDHN*C-1-3	2	67	4	73	*heterozygous*	
35SGFP:*OesDHN*C-1-4	21	67	0	88	*heterozygous*	
35SGFP:*OesDHN*C-1-5	20	65	1	86	*heterozygous*	
35SGFP:*OesDHN*C-1-6	14	73	2	89	*heterozygous*	
35SGFP:*OesDHN*C-1-7	18	54	1	73	*heterozygous*	
35SGFP:*OesDHN*C-1-8	23	50	0	73	*heterozygous*	
35SGFP:*OesDHN*C-2-1	6	43	4	53	*heterozygous*	
35SGFP:*OesDHN*C-2-2	13	43	2	58	*heterozygous*	
35SGFP:*OesDHN*C-2-3	0	64	3	67	*homozygous*	
35SGFP:*OesDHN*C-2-4	10	46	3	59	*heterozygous*	
35SGFP:*OesDHN*C-2-5	13	40	9	62	*heterozygous*	
35SGFP:*OesDHN*C-2-6	3	47	5	55	*heterozygous*	

35SGFP:*OesDHN*C-2-7	0	64	3	67	*homozygous*	
35SGFP:*OesDHN*C-2-8	0	64	5	69	*homozygous*	
35SGFP:*OesDHN*C-3-1	12	52	2	66	*heterozygous*	
35SGFP:*OesDHN*C-3-2	9	45	1	55	*heterozygous*	
35SGFP:*OesDHN*C-3-3	0	64	0	64	*homozygous*	
35SGFP:*OesDHN*C-3-4	0	64	1	65	*homozygous*	
35SGFP:*OesDHN*C-3-5	13	46	2	61	*heterozygous*	
35SGFP:*OesDHN*C-3-6	16	47	1	64	*heterozygous*	
35SGFP:*OesDHN*C-3-7	14	48	2	64	*heterozygous*	
35SGFP:*OesDHN*C-3-8	0	64	1	65	*homozygous*	
35SGFP:*OesDHN*C-4-1	11	41	0	52	*heterozygous*	
35SGFP:*OesDHN*C-4-2	0	64	2	66	*homozygous*	
35SGFP:*OesDHN*C-4-3	12	45	2	59	*heterozygous*	
35SGFP:*OesDHN*C-4-4	8	36	7	51	*heterozygous*	
35SGFP:*OesDHN*C-4-5	15	41	4	60	*heterozygous*	
35SGFP:*OesDHN*C-4-6	0	64	2	66	*homozygous*	
35SGFP:*OesDHN*C-4-7	0	64	1	65	*homozygous*	
35SGFP:*OesDHN*C-4-8	14	47	6	67	*heterozygous*	
35SGFP:*OesDHN*C-5-1	13	45	0	58	*heterozygous*	
35SGFP:*OesDHN*C-5-2	16	40	1	57	*heterozygous*	
35SGFP:*OesDHN*C-5-3	11	48	1	60	*heterozygous*	
35SGFP:*OesDHN*C-5-4	6	49	5	60	*heterozygous*	
35SGFP:*OesDHN*C-5-5	14	45	2	61	*heterozygous*	
35SGFP:*OesDHN*C-5-6	15	47	0	62	*heterozygous*	
35SGFP:*OesDHN*C-5-7	11	48	2	61	*heterozygous*	
35SGFP:*OesDHN*C-5-8	11	46	2	59	*heterozygous*	
35SGFP:*OesDHN*C-7-1	11	40	4	55	*heterozygous*	
35SGFP:*OesDHN*C-7-2	16	46	3	65	*heterozygous*	
35SGFP:*OesDHN*C-7-3	12	43	2	57	*heterozygous*	
35SGFP:*OesDHN*C-7-4	15	40	2	57	*heterozygous*	
35SGFP:*OesDHN*C-7-5	12	29	11	52	*heterozygous*	
35SGFP:*OesDHN*C-7-6	0	64	2	66	*homozygous*	
35SGFP:*OesDHN*C-7-7	14	37	4	55	*heterozygous*	

35SGFP:*OesDHN*C-7-8	0	64	5	69	*homozygous*	
35SGFP:*OesDHN*C-9-1	17	37	4	58	*heterozygous*	
35SGFP:*OesDHN*C-9-2	0	64	2	66	*homozygous*	
35SGFP:*OesDHN*C-9-3	14	45	7	66	*heterozygous*	
35SGFP:*OesDHN*C-9-4	12	35	5	52	*heterozygous*	
35SGFP:*OesDHN*C-9-5	14	40	3	57	*heterozygous*	
35SGFP:*OesDHN*C-9-6	0	64	7	71	*homozygous*	
35SGFP:*OesDHN*C-9-7	10	43	3	56	*heterozygous*	
35SGFP:*OesDHN*C-9-8	14	42	2	58	*heterozygous*	
35SGFP:*OesDHN*C-11-1	14	47	3	64	*heterozygous*	
35SGFP:*OesDHN*C-11-2	15	47	3	65	*heterozygous*	
35SGFP:*OesDHN*C-11-3	10	43	0	53	*heterozygous*	
35SGFP:*OesDHN*C-11-4	18	44	0	62	*heterozygous*	
35SGFP:*OesDHN*C-11-5	0	64	0	64	*homozygous*	
35SGFP:*OesDHN*C-11-6	8	41	0	49	*heterozygous*	
35SGFP:*OesDHN*C-11-7	10	46	5	61	*heterozygous*	
35SGFP:*OesDHN*C-11-8	17	40	1	58	*heterozygous*	

5.2.5 Selection of 35S::*OesDHN* lines using qRT-PCR analysis

None of the 35S::*OesDHN* homozygous lines show any notable phenotype differences.

In order to verify the integration as well as the transcriptional activity of the 35S::*OesDHN* we performed qRT-PCR analysis, using different primers and independent homozygous lines chosen randomly.

For this purpose, 15 days old whole seedlings, belonging to different mother plants in order to obtain an higher genetic variability, such as A-plt4-7, B-plt1-2, B-plt2-2, B-plt5-1, B-plt6-3, B-plt6-5, B-plt9-7, B-plt10-3 have provided the initial plant material and the *wild-type Col*-0 plants were used as negative control (Table 5.5).

35S::*OesDHN* homozygous lines
A-plt 4-3, 4-4, 4-5, 4-7, 4-10, 4-11
A-9-4, 9-6, 9-12
B-pl1-1, 1-2, 1-3, 1-4, 1-5, 1-10, 1-11
B-plt2-2, 2-6, 2-7, 2-8
B-plt5-1
B-plt6-3, 6-4, 6-5, 6-12
B-plt9-3, 9-7, 9-9
B-plt10-3, 10-4, 10-5, 10-7

Table 5.5 Homozygous T3 lines 35S::*OesDHN*; lines in red were selected for qRT-PCR analysis

The obtained results showed different variations in the *OesDHN* expression levels. High expression level has been detected for the 35S::*OesDHN* B 1-2 , B 6-3, B 9-7 lines while low transcript levels where detected in A-4-7e B-5-1 lines (Figure 5.9).

Such variability within the lines could be attributed among other factors, to a different insertion of the T-DNA in constitutive or facultative heterochromatin. It is well known that while the heterochromatin regions affect genes expression close to it, by way of a phenomenon known as position effect variegation, facultative heterochromatin could determines the silencing of genes through a mechanism known as histone methylation or even through RNA interference using small interfering RNA (siRNA).

In conclusion, based on qRT-PCR results, the selected lines showing a significant increased expression of the *OesDHN* transgene, sush as B-1-2 and B-9-7, were selected for further investigation.

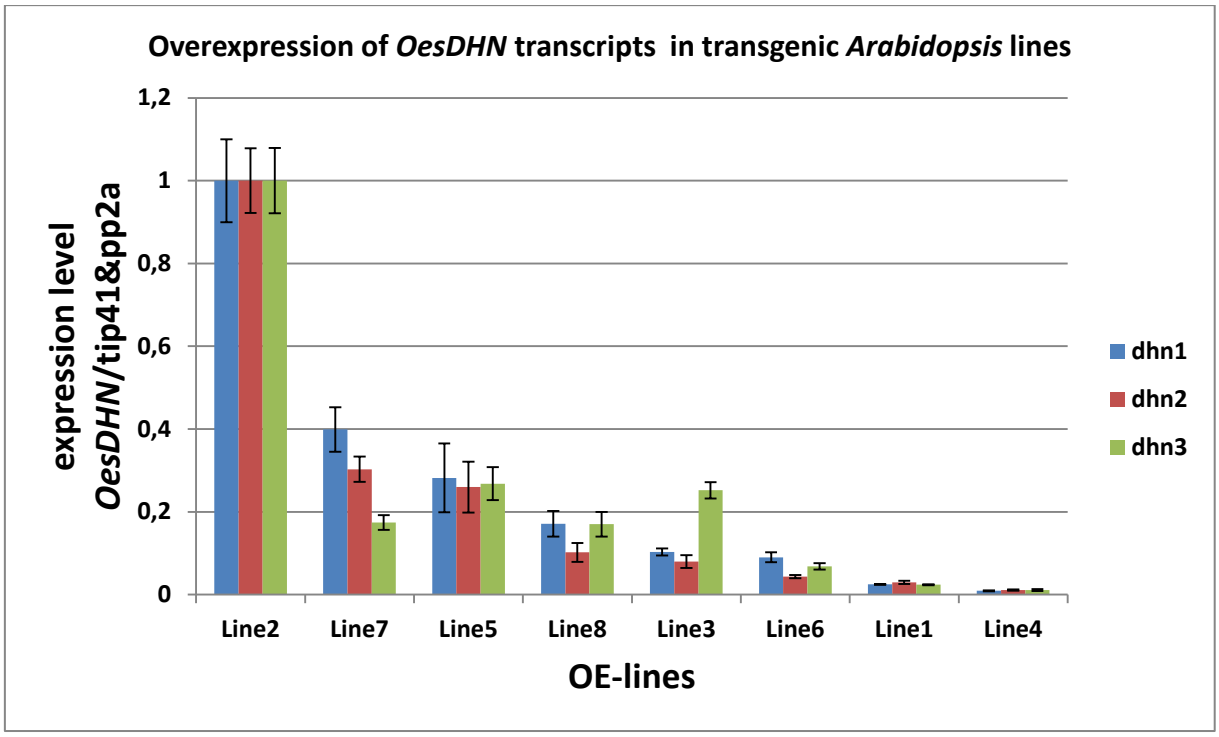


Figure 5.9 qRT-PCR analysis of *OesDHN* overexpression in 8 homozygous *Arabidopsis* seleted lines. Line1: A 4-7; line2: B 1-2, line3: B 2-2; line4: B 5-1; line5: B 6-3; line6: B 6-5; line7: B 9-7; line8: B-10-3). The levels of *OesDHN* (log scale), were normalized to tip41 and pp2a housekeeping genes. The results represent the average of three biological repeats

5.2.6 Selection 35S::*OesDHN*:GFP and 35S:GFP:*OesDHN* lines using qRT-PCR analysis

As well as 35S::*OesDHN* lines, additional evidence for the integration as well as transcriptional activity of both 35S::*OesDHN*:GFP and 35S::GFP:*OesDHN* lines was provided by qRT-PCR analysis, using different pair of primers.

15 day old whole seedlings, belonging to different mother plants in order to obtain an higher genetic variability, such as 35S::*OesDHN*:GFP A-2-3, A-4-1, A-9-2, A-11-6, A-12-5, B-1-7, B-2-7, B-4-3, B-6-8 and 35S::GFP:*OesDHN* C-4-2, C-7-6, C-9-2, C-11-5 have provided the initial plant material (Table 5.6). We used the *wild-type Col*-0 plants as negative control.

Linee T3 35S::*OesDHN*:GFPhomozygous
A-plt2-3, 2-5,2-6, 2-7,2-8,
A-pl4-1, 4-7, 9-12
A-pl9-2, 9-7
A-pl11-1, 11-2, 11-5, 11-6
A-plt12-5, 12-6, 12-8
B-plt1-4, 1-5,1-7
B-plt2-7
B-plt4-3
B-plt6-26-3,6-5, 6-8
T3 35S:GFP:*OesDHN* homozygous
C-pl2-3,2-7,2-8
C-pl3-3, 3-4, 3-8
C-pl4-1, 4-2, 4-6, 4-7
C-pl7-6, 7-8
C-pl9-2, 9-6
C-pl11-5

Table 5.6 Homozygous T3 35S::*OesDHN*:GFP and 35S:GFP:*OesDHN* lines. The lines marked in red were selected for qRT-PCR analysis

Figure 5.9 shows the transcript levels of *OesDHN* evaluated among the different lines with GFP protein fused in N-and C-terminal to *OesDHN* dehydrin. Also in this case, the variability observed within the lines could be attributed among other factors, to a different insertion of the T-DNA in constitutive or facultative heterochromatin.

In conclusion, based on qRT-PCR results, the lines showing increased expression of *OesDHN* transgene, sush as 35S::*OesDHN*:GFP A-4-1, 35S::GFP:*OesDHN* C-11-5, were selected for further investigation.

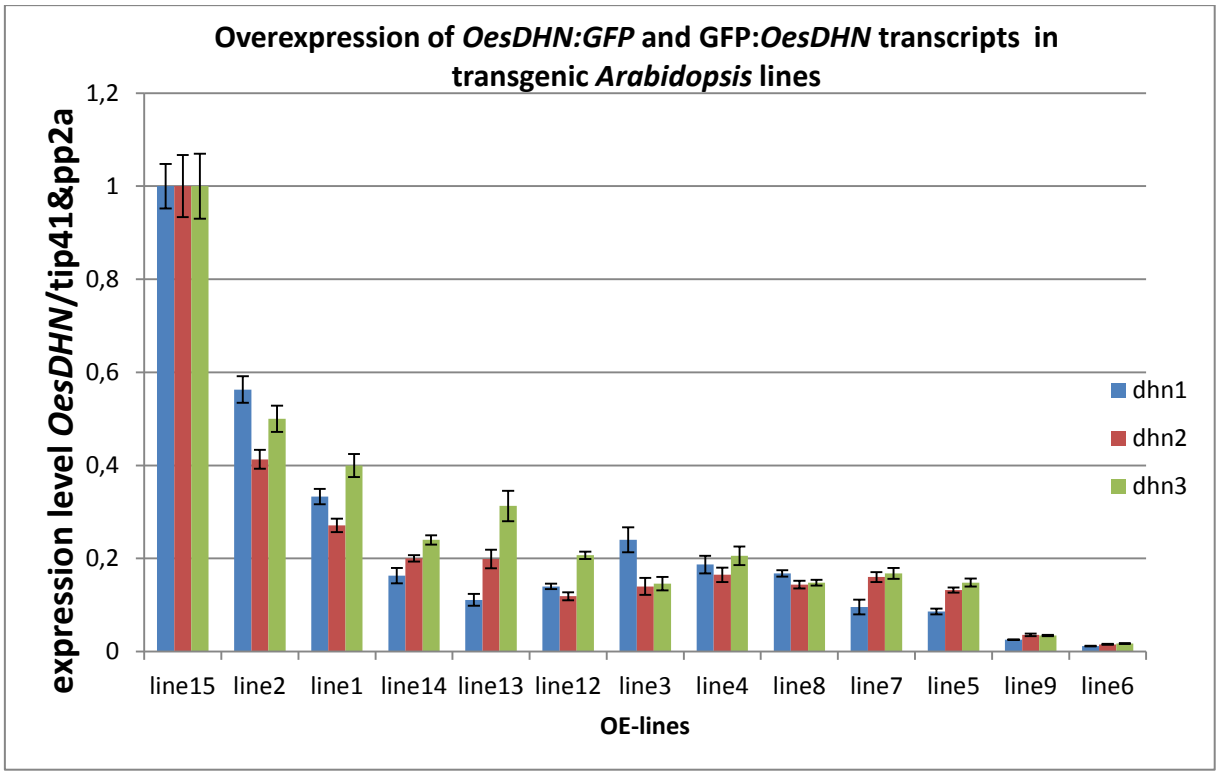


Figure 5.9 qRT-PCR analysis of *OesDHN* overexpression of in 13 homozygous seletedlines. For 35S::*OesDHN*:GFP we select: line1 : A 2-3; line2: A 4-1, line3: A 9-2; line4: A 11-6; line5: A 12-5; line6: B 1-7; line7: B 2-7; line8: B-4-3, line9: B-6-8.For 35S::GFP:*OesDHN* we select: line12: C-4-2, line13: C-7-6, line14 C-9-2, line 15:C-11-5. The levels of *OesDHN* (log scale), were normalized to tip41 and pp2a housekeeping genes.The results represent the average of three biological repeats

5.2.7 Overexpresion of *OesDHN* under mild osmotic stress in *Arabidopsis* transgenic lines

In order to investigate the *OesDHN* function and evaluate its possible role in stress response, the homozygous T3 lines 35S::*OesDHN* B 1-2 and 35S::*OesDHN* B 9-7, were subjected to a water deficit, induced by controlled mild osmotic stress and using a specific experimental set up, developed by Skirycz *et al*., (2010).

The experimental set up chosen allows to decipher the mechanisms by which water deficit inhibits cell proliferation by reduction of the 50% leaf area.

The mild osmotic stress was obtained by addition of 25mM mannitol into growth medium, thereby decreasing the water potential of the medium and, hence, water uptake of the exposed roots (Skirycz *et al*., 2010).

❖ ***Leaf area***

Preliminarly we tested the mannitol 25mM only on *Col-0* genotype.

The Figure 5.10 shows the effects of the chosen mannitol concentration on the vegetative growth and the leaf size of *Col-0* plants. More specifically, plants showed a reduced leaf size by 50% in presence of mannitol, but interestingly, the leaf area size in the *Col-0* plants was significantly smaller then compared to the 35S::*OesDHN* B 1-2 line (Table 5.7; Figure 5.11).

The obtained results indicate that *Arabidopsis* 35S::*OesDHN* plants, under a mild osmotic stress, are able to reprogram their growth better than the *Col-0* plants.

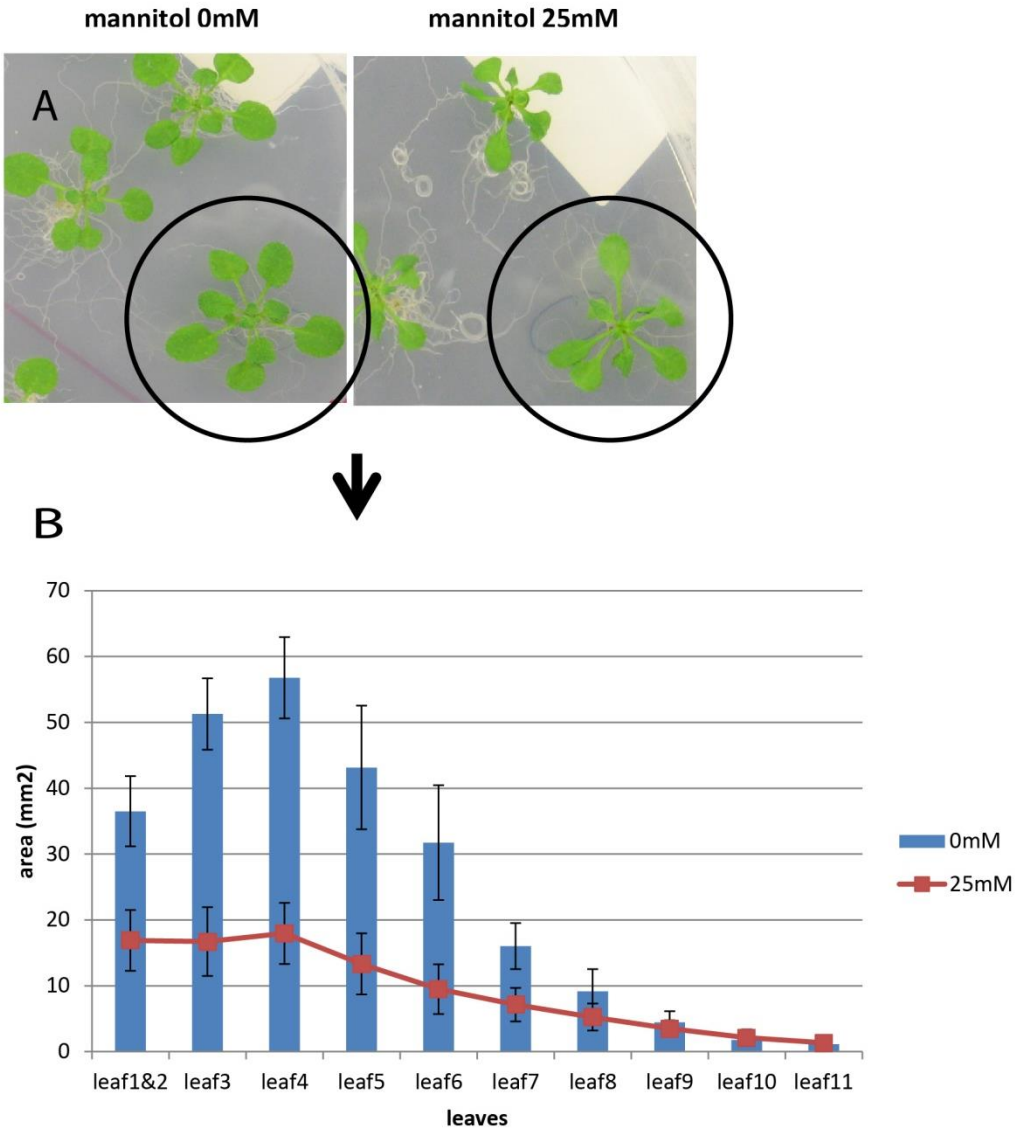


Figure 5.10 Effects of mannitol concentration on the vegetative growth *Col*-0 plants. A: plants were grown in the absence (left) or presence (right) of 25Mm mannitol up to 22 days. B, Leaf series area was measured using ImageJ program (rsbweb.nih.gov / / ijdownload.html) and statistical analysis were performed according to t-test

Table 5.7 Leaf series area (mm^2) of *Col*-0 and transgenic line 35S: *OesDHN* B 1-2 plants , grown under control conditions (0mM mannitol) and controlled osmotic stress (25 mM mannitol). The measurements were carried out using the program Image J. The results represent the average value of two indipendent replicate.Statistical analysis were performed according to t-test p <0.05 (t-test p <0.05 is significant *; p<0,01 is very significant **; p<0,001 is highly significant; n.s: not significant)

Control (0mM mannitol)										
Wt *Col*-0										
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*	*leaf11*
average	40.11	45.87	50.71	43.16	31.54	17.44	10.31	5.15	4.46	5.74
SD	6.15	6.67	7.81	9.10	8.08	6.57	4.07	2.77	5.45	7.924
35S::*OesDHN* B 1-2										
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*	*leaf11*
average	45.38	54.18	63.57	47.65	31.39	21.37	12.67	6.79	3.77	2.57
SD	5.21	6.07	5.68	6.53	8.41	7.15	3.83	3.04	1.31	1.26
T-test (P*) wt /35S::*OesDHN*										
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*	*leaf11*
FTEST	0.36	0.72	0.23	0.21	0.88	0.75	0.83	0.74	0.80	0.55
TTEST	0.0005	0.1347	0.0002	0.116	0.959	0.201	0.101	0.123	0.046	0.421
Conclusions	**	n.s	**	n.s	n.s	n.s	n.s	n.s	*	n.s
Mild osmotic stress (25mM mannitol)										
WT *Col*-0										
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*	*leaf11*
average	19.10	21.48	24.07	16.16	12.95	7.79	5.69	4.23	2.90	2.46
SD	5.32	4.22	5.28	4.29	3.15	2.85	1.42	1.43	0.95	0.55
35S::*OesDHN* B 1-2										
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*	*leaf11*
average	23.37	24.88	27.80	17.87	14.73	9.92	7.83	5.67	2.97	2.79
SD	7.83	5.67	2.97	2.79	7.83	5.67	2.97	2.79	7.83	5.67
T-test (P*) wt /35S::*OesDHN*										

	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*	*leaf11*
FTEST	0.364	0.920	0.521	0.910	0.418	0.548	0.343	0.423	0.219	0.084
TTEST	0.0011	0.0280	0.0389	0.2608	0.1647	0.0303	0.0009	0.0196	0.8841	0.6179
Conclusions	***	*	*	n.s	n.s	*	***	**	n.s	n.s

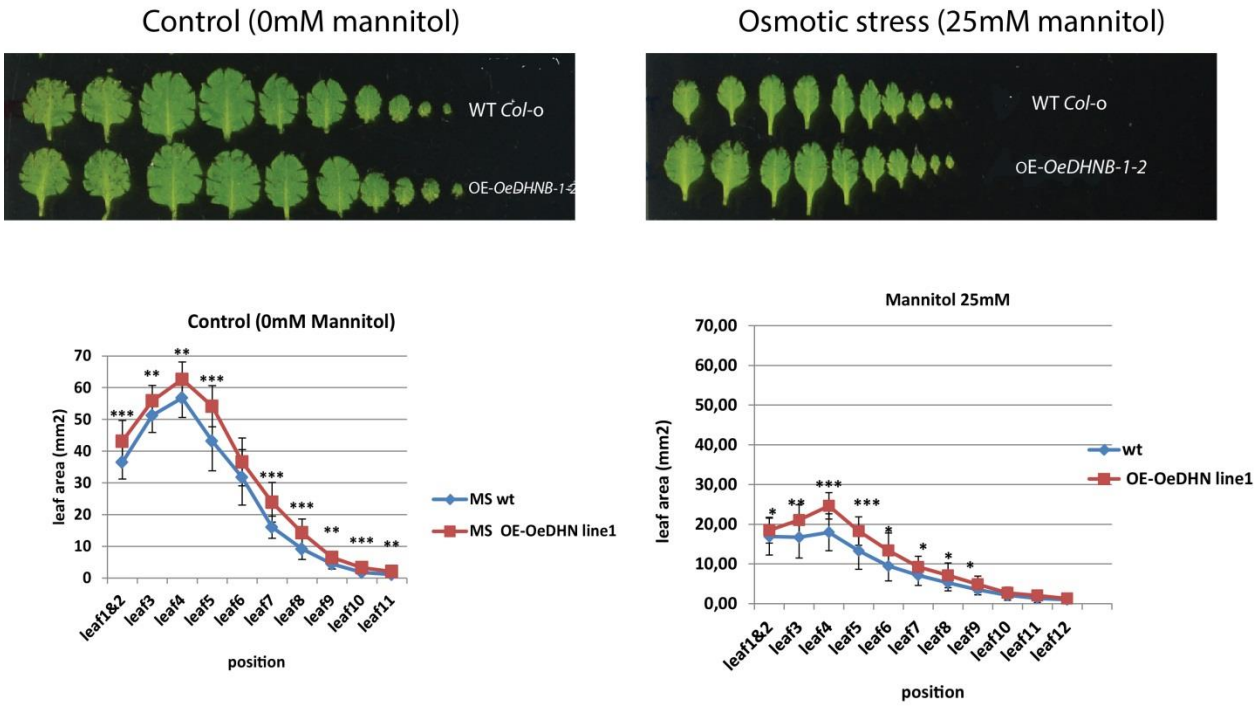


Figure 5.11 Area of the leaves (mm^2) of plants belonging to the transgenic lines 35S::*OesDHN* B 1-2 and WT *Col*-0 grown under control conditions (0mm mannitol) and induced osmotic stress (25 mM mannitol). The measurements were carried out using the program Image J (t-test p <0.05 is significant *;p<0,01 is very significant **; p<0,001 is highly significant; n.s: not significant)

❖ Phenotypic analysis of the root

Funtional analysis of *OesDHN* and its possible role in stress response was also investigated in roots of the homozygous T3 line 35S::*OesDHN* B 1-2. Preliminarly we looked for the effects of the controlled mild osmotic stress on *Col*-0 primary roots.

In Table 5.8. are summarized the length of the primary root after 13 days of culture.

Primary root lenght measurememements in centimetres (cm) under control condition (0mM mannitol)							
Days after sowing	***wt Col-0***		***OE:OesDHN***		**T-test (P*) WT /OE-LINE**		**Conclusions**
	Average	**SD**	**Average**	**SD**	**FTEST**	**TTEST**	
Day5	1.083	0.197	1.159	0.27	0.134	0.251	**n.s**
Day7	1.61	0.22	1.753	0.443	0.001	0.162	**n.s**
Day9	2.25	0.314	2.423	0.484	0.689	0.181	**n.s**
Day11	2.326	0.199	2.4	0.331	0.555	0.388	**n.s**
Day13	2.296	0.620694	2.656	0.39	0.084	0.056	**n.s**
Primary root lenght measurememements in centimetres (cm) under stress condition (25mM mannitol)							
Days after sowing	***wt Col-0***		***OE:OesDHN***		**T-test (P*) WT/OE-LINE**		**Conclusions**
	Average	**SD**	**Average**	**SD**	**FTEST**	**TTEST**	
Day5	1.214	0.195	1.202	0.28	0.087	0.861	**n.s**
Day7	1.494	0.29	1.536	0.245	0.339	0.589	**n.s**
Day9	1.85	0.306	1.97	0.295	0.783	0.123	**n.s**
Day11	1.771	0.313	1.768	0.434	0.161	0.789	**n.s**
Day13	1.92	0.536	1.939	0.386	0.051	0.539	**n.s**

Table 5.8 Primary root length. evaluated at different time points. of *Col*-0 and 35S::*OesDHN* B 1-2 line plants growing *in vitro* under control conditions (0mm mannitol) and induced osmotic stress (25mM mannitol). The measurements were determined using Image J program (t-test p <0.05 is significant *;p<0.01 is very significant **; p<0.001 is highly significant; n.s: not significant)

The results indicated a reduction in the primary root lenght in *Col-0* plants grown under mild osmotic stess with respect to the control condition. The reduction in primary root lenght was time-dependent and reached the value of 13.77% in the roots grown in the presence of osmolite (Figures 5.12 and 5.13).

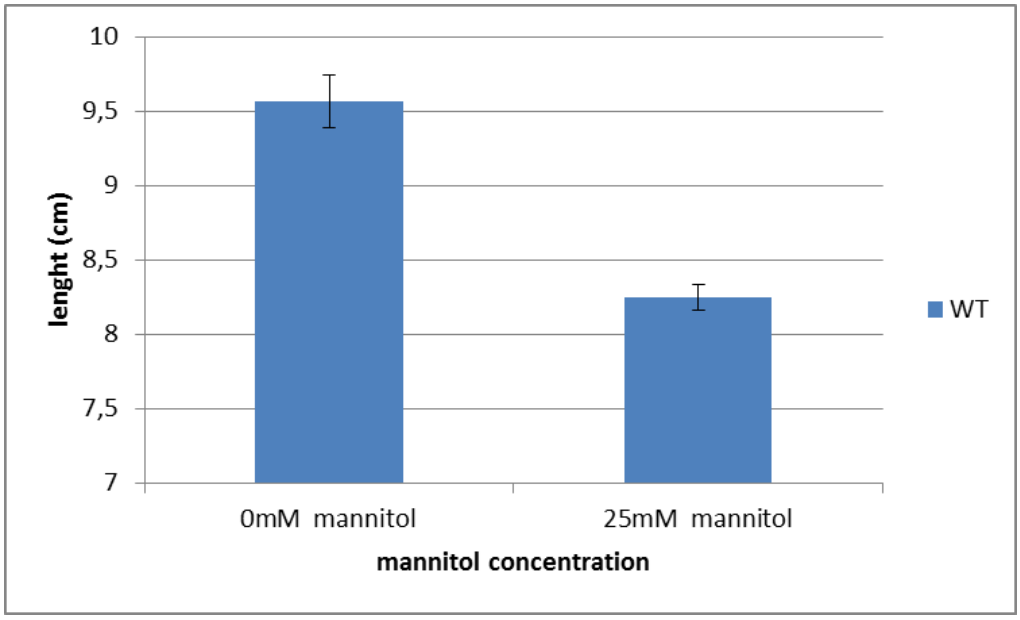


Figure 5.12. Primary root length of *Col*-0 seedlings grown in vitro up to 13 days in the absence (left) or presence (right) of 25Mm mannitol. For statistical evaluation of data, t test was used

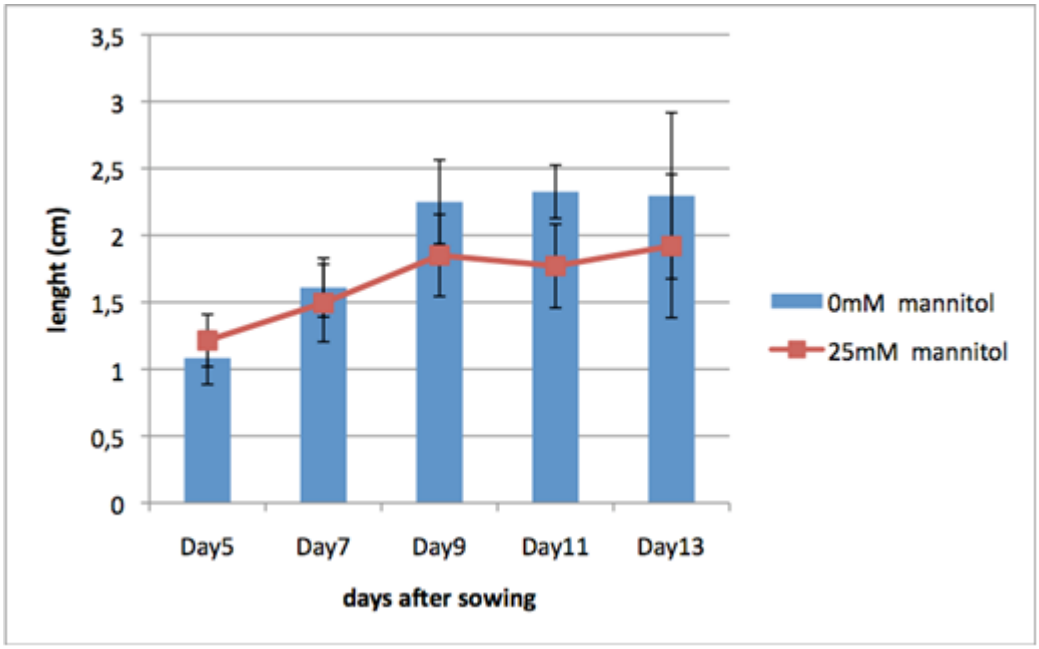


Figure 5.13 Primary root length at different time point of *Col*-0 seedlings grow in vitroup to 13 days in the absence (left) or presence (right) of 25Mm mannitol. For statistical evaluation of data, t test was used

Moreover, no significant difference in the primary root length and in growth times were observed between *Col*-0 and 35S::*OesDHN B 1-2* line plants grown on a medium with or without mannitol (Figures 5.14 and 5.15).

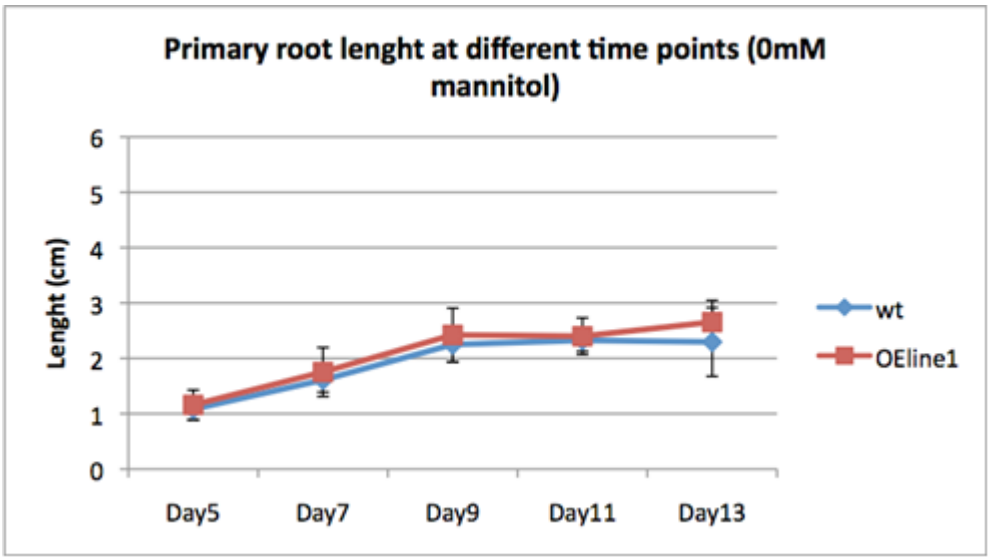


Figure 5.14. Primary root length (cm) at different time point of *Col*-0 and 35S::*OeDHN* B 1-2 lines grown *in vitro*up to 13 days in absence of mannitol

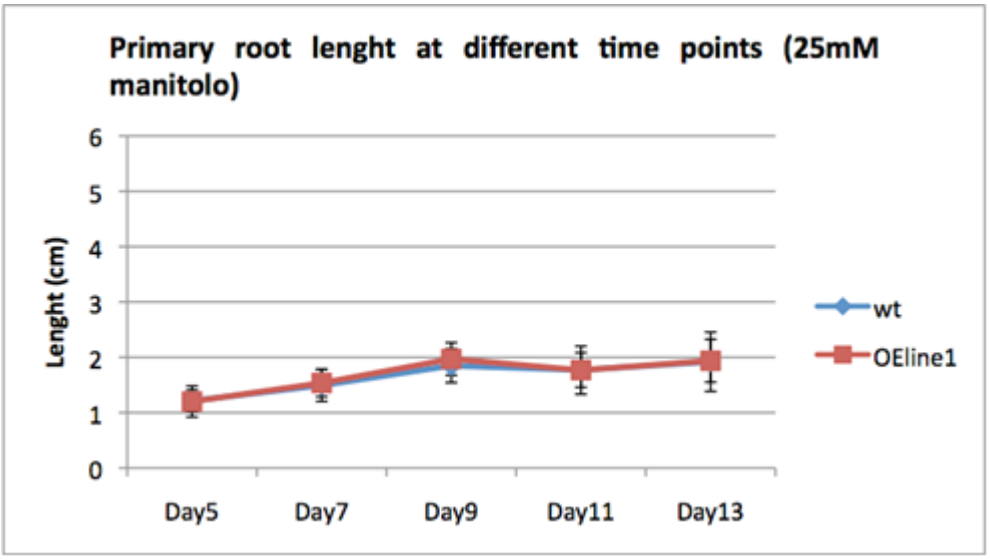


Figure 5.15 Primary root length (cm) at different time point of *Col*-0 and 35S::*OesDHN* B 1-2 line grown in vitro up to 13 days in presence of 25Mm mannitol.

We have also investigated the effects of the mild osmotic stress on the lateral roots of both *Col-0* and 35S::*OesDHNB 1-2* line seedlings.

In Table 5.9 and Figures 5.16 and 5.17 are summarized the results after 11 days of *in vitro* culture. A reduction in the number of lateral roots and lateral root density was observed in both genotypes grown in presence of mannitol. Moreover difference were not significant.

Control (0mM mannitol)			
Items	***WT Col*-0**		
	Length	**LRN**	**LR DENSITY**
average	7.39	26	3.6
SD	1.32	5.73	0.93
	35S::*OesDHN* B 1-2		
	Length	**LRN**	**LR DENSITY**
average	8.21	28.91	3.53
SD	0.9	4.67	0.62
T-test (P*) WT /OELINE1			
FTEST	0.03	0.25	0.02
T TEST	0	0.03	0.73
conclusions	**s.n**	*	s.n
Mild osmotic stress (25mM mannitol)			
Items	**WT *Col*-0**		
	Length	**LRN**	**LR DENSITY**
average	6.42	22.37	3.48
SD	0.92	5.1	0.62
	35S::*OesDHN* B 1-2		
	Length	**LRN**	**LR DENSITY**
average	6.94	23.63	3.37
SD	0.88	5.83	0.59
T-test (P*) WT /OELINE1			
FTEST	0.76	0.46	0.79
T TEST	0.02	0.35	0.45
conclusions	*	s.n	s.n

Table 5.9 Primary root length. number of lateral roots and lateral root density of *Col*-0 and 35S::*OesDHN* B 1-2 line plants grown up to 11 days in in presence of 25Mm mannitol

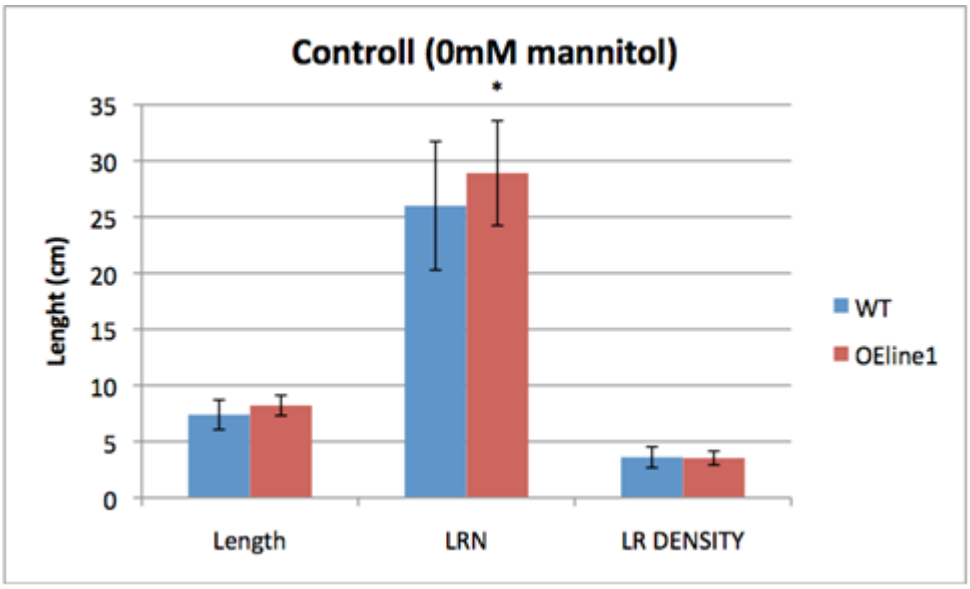


Figure 5.16 Primary root length, number of lateral roots and lateral root density o fCol-0 and 35S::*OeDHN* B 1-2 (ine 1) grown *in vitro* up to 11 days in absence mannitol

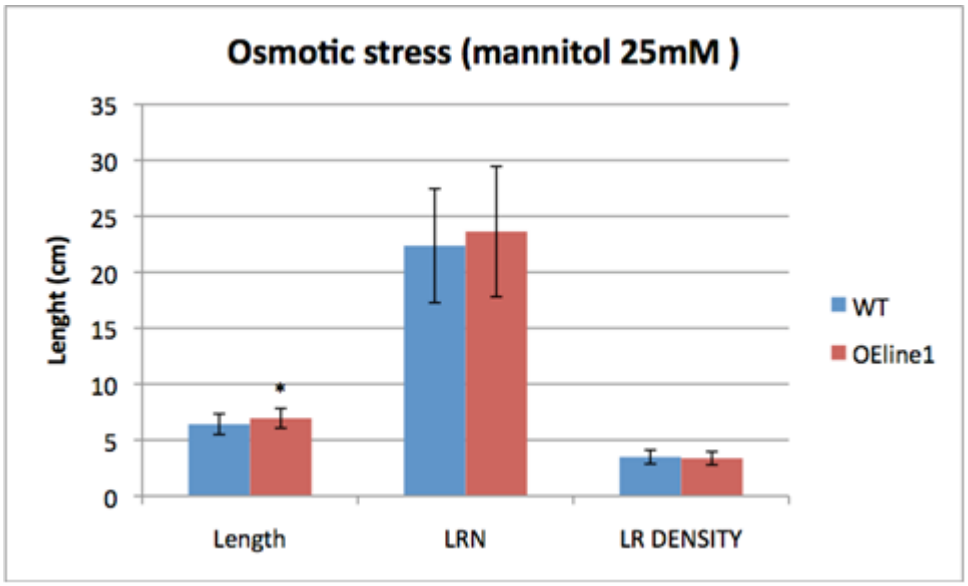


Figure 5.17 Primary root length. number of lateral roots and lateral root density of *Col*-0 and 35S::*OesDHN* B 1-2 (line 1) grown in vitro up to 11 days in presence of 25Mm mannitol.

5.2.8 Overexpresion of *OesDHN*:GFP and GFP:*OesDHN* under mild osmotic stress in *Arabidopsis* transgenic lines

The effects of the mild osmotic stress were also evaluated on the *Arabidopsis* 35S::*OesDHN*:GFP and 35S::GFP:*OesDHN* plants, using the experimental set up as previously reported. On the basis of qRT-PCR results we selected the 35S::*OesDHN*:GFP A-4-1 (line 2) and the 35S::GFP:*OesDHN* C-11-5 (line 15) plants for the treatment.

❖ ***Leaf area***

According to Skirycz *et al*., (2010), preliminarly we tested the proto*Col* on *Col-0* plants and the obtained results confirmed a 50% reduction of the leaf area in plants grown in presence of the controlled mild osmotic stress (Figure 5.18). Our results indicated that also transgenic plants were suffering in the presence of mannitol but interestingly, leaf area size was smaller in *Col-0* plants compared to the 35S::*OesDHN*:GFP *A-4-1* (line 2) (Table 5.10; Figure 5.18).

Control (0mM mannitol)									
	Wt *Col*-0								
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*
average	36.80	45.33	49.94	32.44	21.52	11.57	5.84	2.70	1.67
SD	7.44	7.24	7.31	7.71	7.62	6.29	2.37	1.41	0.95
35S::*OesDHN*:GFP A-4-1(line2)									
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*
average	36.87	44.93	45.96	38.32	22.81	11.93	6.20	2.66	1.03
SD	7.74	5.96	4.17	10.07	4.52	4.02	1.75	0.89	0.28
35S:GFP:*OesDHN* C-11-5 (line 15)									
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*
average	35.91	44.76	49.68	37.22	24.81	13.39	6.80	3.58	1.72
SD	5.48	6.04	10.95	10.10	5.83	6.03	2.17	2.33	0.83

T-test (P*) wt / OE-*OesDHN*:GFP line2									
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*
FTEST	0.87	0.57	0.11	0.44	0.14	0.20	0.38	0.18	0.07
TTEST	0.98	0.89	0.15	0.16	0.65	0.88	0.70	0.95	0.24
Conclusions	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
T-test (P*) wt / OE-*OesDHN*:GFP line15									
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*
FTEST	0.20	0.50	0.28	0.54	0.38	0.81	0.70	0.19	0.86
TTEST	0.68	0.67	0.61	0.22	0.41	0.51	0.32	0.33	0.77
Conclusions	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
Mild osmotic stress (25mM mannitol)									
WT *Col*-0									
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*
average	20.20	19.03	22.72	14.76	9.39	5.77	3.87	2.10	1.28
SD	3.44	4.67	5.13	5.46	3.14	2.95	1.27	1.11	0.45
35S::*OesDHN*:GFP A-4-1(line2)									
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*
average	17.41	17.52	18.92	20.45	15.50	11.42	6.54	4.71	2.53
SD	3.32	3.54	4.81	3.83	4.04	3.55	1.97	1.52	1.23
35S:GFP:*OesDHN* C-11-5 (line 15)									
	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*
average	17.87	17.22	20.02	15.64	10.28	7.59	4.63	2.43	1.64
SD	3.50	4.37	3.11	2.96	3.29	2.40	1.95	0.91	0.94
T-test (P*) wt / OE-*OesDHN*:GFP line2									
FTEST	0.874	0.928	0.396	0.384	0.719	0.244	0.597	0.784	0.732
TTEST	0.013	0.957	0.278	0.734	0.193	0.500	0.193	0.446	0.622
Conclusions	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
T-test (P*) wt / OE-*OesDHN*:GFP line15									

	leaf1&2	*leaf3*	*leaf4*	*leaf5*	*leaf6*	*leaf7*	*leaf8*	*leaf9*	*leaf10*
FTEST	0.945	0.848	0.151	0.083	0.886	0.544	0.214	0.564	0.107
TTEST	0.670	0.382	0.171	0.658	0.545	0.147	0.316	0.487	0.391
Conclusions	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

Table 5.10 **Leaf series area (mm^2) of *Col*-0 and 35S::*OesDHN*:GFP A-4-1(line2) 35S::GFP:*OesDHN* C-11-5 (line 15) transgenic line plants . grown under control conditions (0mM mannitol) and controlled osmotic stress (25 mM mannitol). The measurements were carried out using the program Image J. The results represent the average value of two indipendent replicate.Statistical analysis were performed according to t-test p <0.05 (t-test p <0.05 is significant *; p<0.01 is very significant **; p<0.001 is highly significant; n.s: not significant)**

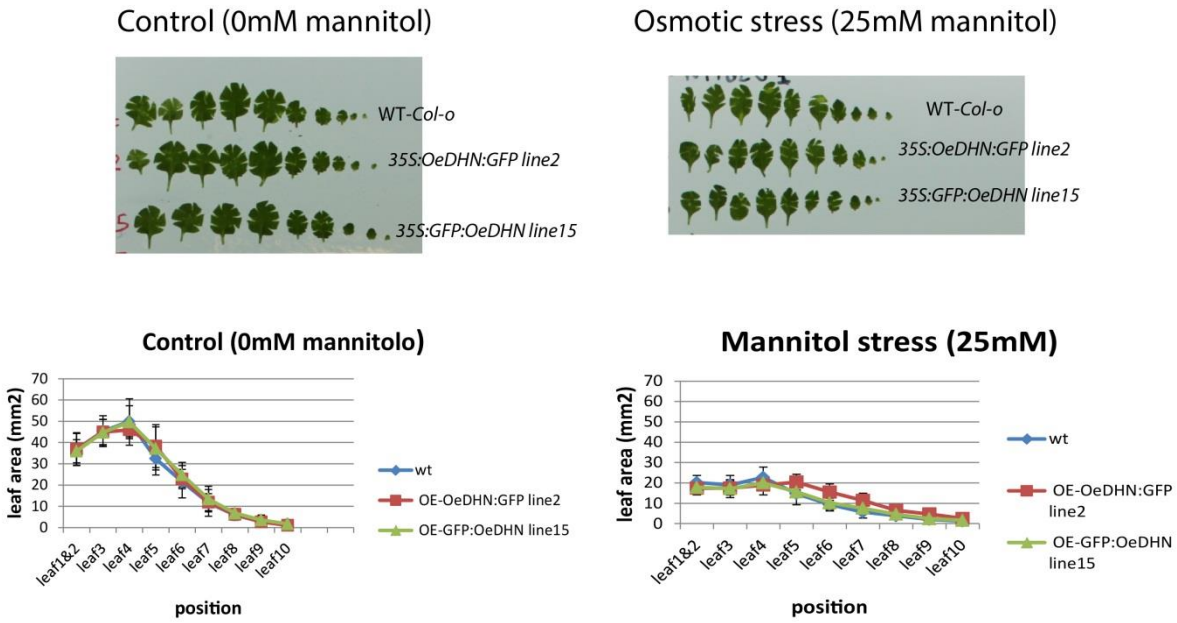


Figure 5.18 Leaf area (mm^2) of plants belonging to the transgenic lines 35S::*OesDHN*:GFP A-4-1 (line 2) 35S::GFP:*OesDHN* C-11-5 (line15) andCol-0 grown in vitro under control conditions (0mM mannitol) and induced osmotic stress (25 mM mannitol). The measurements were carried out using the program Image J (t-test p <0.05 is significant *;p<0,01 is very significant **; p<0,001 is highly significant; n.s: not significant)

5.2.9 Subcellular localization of *OesDHN* protein

To verified the cyto-histological localization of *Oes*DHN, two constructs C- and N-terminal GFP fusion under the control of the cauliflwer mosaic virus 35S promoter. were used to transformed stably *Arabidopsis thaliana* plants.

Preliminarily by using a fluorescence microscope we analyse GFP positive plants (plants expressing GFP fluorescence).

For confocal analysis, attention was paid to the maturation zone of the primary roots and to the abaxial surface leaf of the independent T2 plants, with one insertion locus (see Table 5.3) and grown *in vitro* for 6-8 days in a vertical position under continuous light.

Concerning the maturation zone of the primary root, protein was localized in the nucleus and in a thin portion of cytosol, pressed between the cell wall and vacuole (see Figure 5.19 a'. b'.c').

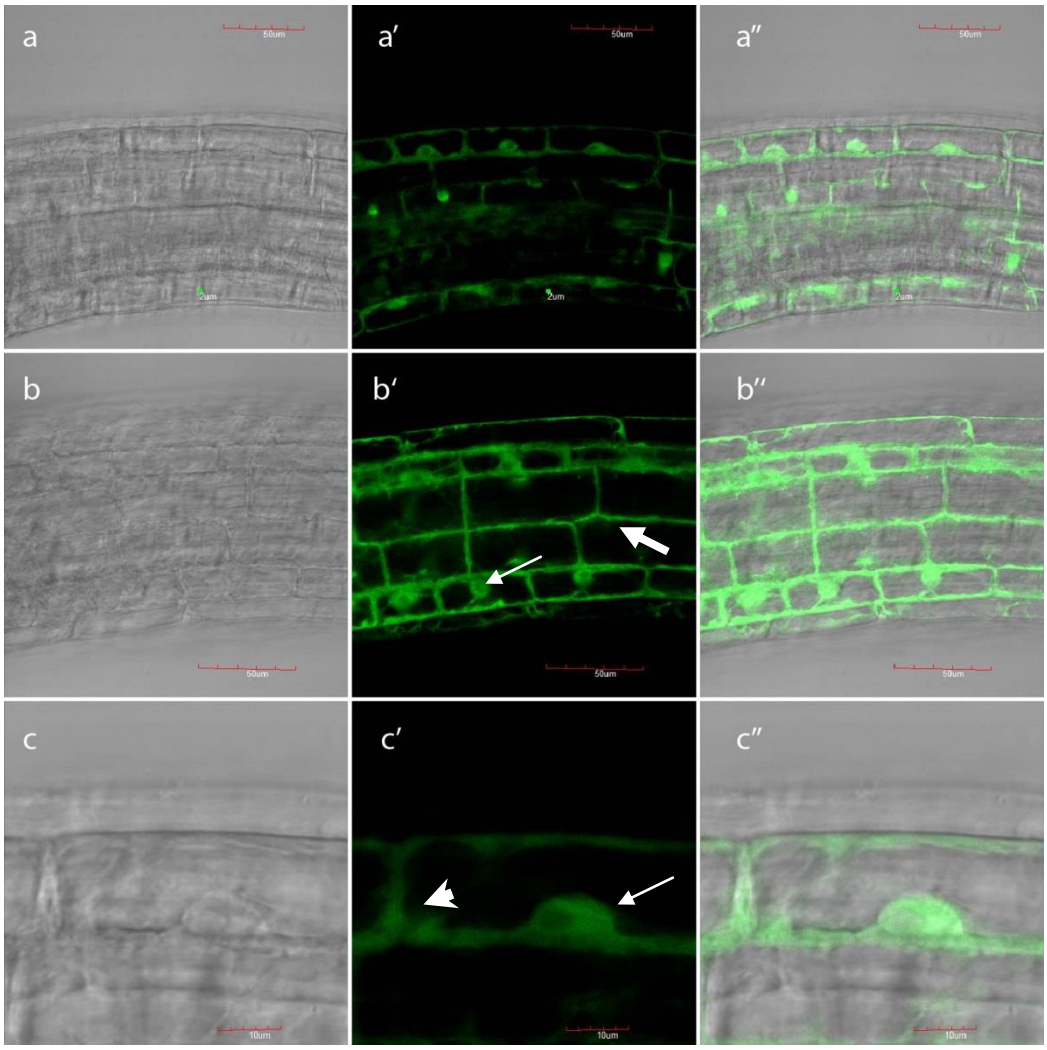


Figure 5.19 Subcellular localization of the *Oes*DHN protein fused in N and C-terminal GFP under the control of the CaMV35S promoter in primary roots of stable T2 lines of *Arabidopsis* with one T-DNA locus. Representative images of maturation zone. (a. b. c) bright field. (a '. b'. c ') GFP signal. (a''. b '' c'') merge nuclear localization; (a. b. a '. b'. a''. b'': scal bar 50µm); (c. c '. c'': scal bar 10 µM). → nuclear localization; ➤ Cytoplasmic signal ; ➡ cell membrane localization

In leaves, fluorescent signal was present in the guard cells of stomata (Figure 5.20).

*Oes*DHN protein is clearly localized in the nucleus, in cell membrane and in the cytosol of the stomata while a week signal was present only in the cytosol of mesophyll cells.

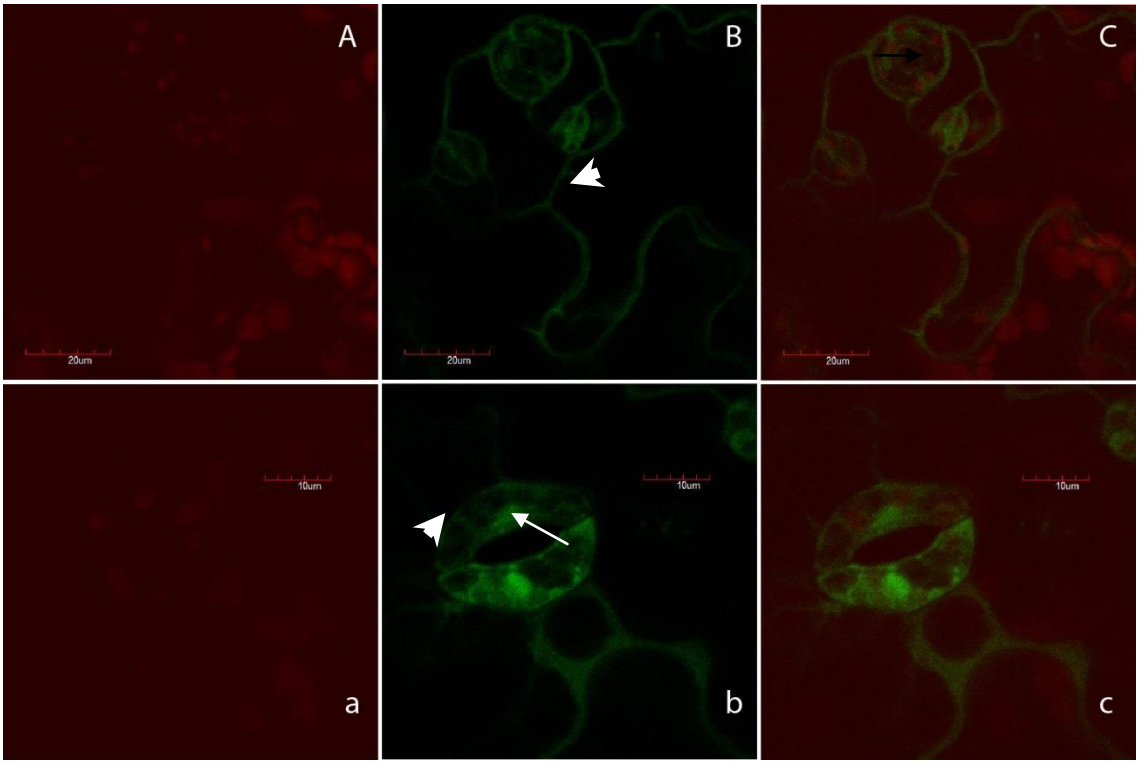


Figure 5.20 Localization of the *Oes*DHN protein fused in N and C-terminal GFP under the control of the CaMV35S promoter in leaves of stable lines T2 of *Arabidopsis* with one T-DNA locus. Guard cells of stomata located on the abaxial side of leaves taken from plants grown in the light nuclear localization ⟶; Cytoplasmic signal ➤; (a. b) chlorophyll autofluorescence (a'. b') GFP signal. (a". b") merge. Scal bar: (A. B.C.) 50µm; (a. b. c.) 10 uM

CONCLUSIONS

Abiotic stress such as drought, high salinity, and cold, have long been known affect or even inhibit plant development, and adversely influence the productivity of many crop species. They negatively affect growth in almost all stages, including germination, seedling stage, pre-flowering and flowering period, and grain-filling stage (Liu *et al*., 2004; Verslues *et al*., 2006; Wu *et al*., 2007).

The seriousness of this issue has prompted political institutions to consider like a priority, both basic and applied research, in order to develop new technologies to be introduced in farming practice to limit the adverse effects.

In the Mediterranean region, and in particular in Italy, cold and drought are the most frequent abiotic stresses: the first is a limiting factor for both winter or summer cycle seasons plants, especially during the early phases of the life, while the water scarcity adversely affect the stability and quality of the production of almost all the crop species.

From an ecological point of view, *oleaster* (*Olea europaea* L. subsp. *europaea* var. *sylvestris*) the wild form of cultivated olive, exhibits many relevant features. First of all, due to their prevalence within "*Macchia*" association, it play a major role in protecting soil against erosion, by consolidating land through the wide root system. Moreover, on account of *oleaster* resistance to wind and drought, it contributes to the survival of natural plant communities, while its ability to recover after a fire, through new shoot formation, assures a rapid rebuilding of the original vegetation cover (Mulas *et* Deidda. 1998). Thus, *oleaster* features many traits that would be highly valuable to introduce into agricultural importance species, such as *Zea mays*.

To induce resistance to stressor, genetic improvement has always been particularly difficult to achieve because of the strong interaction between genotype and environment as well as for the still limited knowledge of the genetic and molecular knowledge that undergo tolerance.

Starting from these assumptions, in order to contribute to elucidate molecular aspects involved in drought resistance in *oleaster* plants, in this project we carried out the characterization and the functional analysis of the *OesDHN* gene, in stable transgenic lines of *Arabidopsis thaliana*, overexpressing this gene.

OesDHN was obtained from a cDNA library induced by drought and high irradiance, and transgenic *Arabidopsis* plant were improved under drought stress.

On the other hand. in a previous study on *oleaster*, transcripts levels of *OesDHN* has been demonstrated up-regulated in plants exposed to drought and cold stressful conditions. The *Oes*DHN protein was supposed to be involved in protecting stressed-cells by water-deficit, including the exclusion of solute from the surface membranes, thereby preventing denaturation and maintaining the integrity of the cell wall (Gilmour *et al*.,1992; Ali-Benali *et al*.. 2005).

It is known that salinity, osmotic and drought stress, can cause a drastic reduction of cellular metabolic activity because of the excesses and imbalances ionic, ionic salts or interference with the absorption of essential macro-and micronutrients (Tester *et* Davenport. 2003).

Most of the drought responses have been studied only in mature tissues and under rather extreme stress conditions with complete growth arrest or even plant death as a result (Seki *et al*., 2002; Catala *et al*., 2007; Kilian *et al*., 2007; Bouchabke-Coussa *et al*., 2008; Giraud *et al*., 2008; Matsui *et al*., 2008; Perera *et al*., 2008; Weston *et al*., 2008; Zhang *et al*., 2008; Zeller *et al*., 2009).

Starting from these assumptions, aim of this PhD project was to investigate the function of *OesDHN* in *Arabidopsis* transgenic plants, under a controlled mild osmotic stress, following the experimental set-up suggested by Skirycz and collaborators (2010), which slowed growth without affecting plant survival.

Our results are encouraging, the *Arabidopsis OesDHN* plants showed lower reduction of leaf area compared to *wild type* plants. These data indicated that *OesDHN Arabidopsis* overexpression lines are more mannitol tolerant than control lines.

The obtained result imply that the *OesDHN* may be involved in the drought and other stress response in *oleaster*. On the other hand, according to literature data, several studies on transgenic plants have revealed a relationship between dehydrins accumulation and increased tolerance to stress conditions (Hara *et al*., 2003; Rorat *et al*., 2006; Choudhury *et al*., 2007).

Furthermore, the analysis conducted on 35S::*OesDHN*-GFP and 35S::GFP:*OesDHN* lines indicates that *OesDHN* is predominantly localized to the nuclear and the plasma membranes. Moreover, the cytological localization confirms the data obtained with the *in silico* analysis and the use of bioinformatics prediction programs which have provided the presence of a putative nuclear localization signal *KKKK* within the sequence, located between the S-segment and the first K domain. In particular the nuclear localization of dehydrins and also of *OesDHN* would seem associated with the phosphorylation status of the S-segment

(Riera *et al*., 2004). The obtained results are consistent with the data in the literature, where the dehydrin distribution seem affected by CK2 phosphorylation (Mentha *et al.,* 2009).

On the other hand, the mays dehydrin, Rab17/DHN5 YSK2, could act as a shuttle between the cytoplasm and the nucleus, probably due to its phosphorylated state, DHN5 showed a differential phosphorylation pattern in two *Tunisiandurum* cultivars, which exhibited a different ability to tolerate drought and salt stress conditions.

Interenstingly, DHN5 was found as a mixture of phosphorilated and unphosphorylated forms in nuclear and cytoplasmic compartments as well as Rab17-GFP (Goday *et al.,* 1994; Brini *et al*., 2007). In other cases, the phosphorylated forms of acidic dehydrins such as COR47, ERD10 and ERD14 of *Arabidopsis* and VCB45 of *Apium graveolens,* localized in the vacuole are able to bind Ca^{2+} much more efficiently than dephosphorylated forms (Heyen *et al.,* 2002 ; Alsheikh *et al.,* 2003; Alsheikh *et al*., 2005). All these data supporte the idea that the dehydrins phosphorylation on S-segment may have a key role in stress tolerance in plants.

In conclusion, we can assert that taken together, the results obtained on the model plant *Arabidopsis thaliana* have allowed us to clarify some of the molecular aspects implicated in tolerance to a variety of stress conditions in plants. The future will be to broaden the knowledge useful to define strategies to increase traits of tolerance/resistence in important crop species or not.

Acknowledgements

This work has been carried out at the University of Calabria and in the Plant Systems Biology Institute in Ghent. Belgium.

Many people need to be thanks, and not only for circumstance in this my journey in the last three years.

I would like to thank Prof.ssa Bitonti MB and my supervisor Dott.ssa Chiappetta A, for giving me the opportunity to perform my PhD thesis in their group and for their support and scientific advice throughout my thesis, but above all to believe in my abilities.

Many thanks to all Chromatin & Growth Control Group lab members, for providing a great working atmosphere as well as for friendships beyond work!

A special thank you goes to Prof. Prof. Mieke Van Lijsebettens, not only for supporting my project but also for her understanding and her optimism in the difficult times.

Of course I can not forget all the component of the Chromatin group: Feng, Tom, Liz, Steen, Pia, Marina, Magdalena, Leen and Greet. I have always enjoyed our chats during the coffee break about work and life. I have never forgotten the amazing holiday spent together in summer, the nice dinner organized and the magic atmosphere of Ghent festival … THANK YOU!

Now, I want to thank all my colleagues from labs and friends in Italy.

A due thanks to my best friend Silvia, always ready to support me even in difficult times and Cinzia, friend of adventures and misadventures in this way whose I want dedicate my last: "*Non è il momento di morire*" and Domenico and Leonardo for support research and for the great fun!!! To my PhD *Col*leagues, Luisa, Lia, and Davide withouth whose this PhD would not have been the same!!! I would like to thank through the depths of my heart my friends Natasha, MariaTeresa, and of course Olimpia a wonderful and proper person whose I was very happy to share an important part of my life!!! Finally I want thanks two most important people in the lab which always making it a really pleasant place to do research: Giovanni and Federico…

Dear Fede, I can't believe that you left us so early…the lab is not the same without you...all the people here miss you and your contagious laugh… THANK YOU for everything!!

A very big THANK YOU goes to my family, especially to my parents Francesco and Rosa, who enabled to do my studies and who always supported me in "quite" all decisions and my wonderful sisters Erminia and Alessandra, toghether with my inseparable cousin Monica, and also Daniel, Federico, Angela e Claudio to made and encouraged me whenever needed! Sorry for my crazy moment!!

Finally this adventure would not have taken place without the immense support of my boyfriend Francesco…thank you for your amazing patient …!!

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APPENDIX

In work…

-Muto A, Chiappetta A, Bruno L, Lijsebettens M, Bitonti B-*Functional characterization of an oleaster (Olea europaea subsp. europaea var sylvestris) SK2-type dehydrin enhances stress osmotic tolerance in overexpressing Arabidopsis lines*

-Wang F, Muto A, Lijsebettens M- *Arabidopsis TETRASPANIN genes:from promoter activity to phenotype*

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Bruno L , Muto A, Spadafora D. N. , Iaria D. L. , Chiappetta A , Lijsebettens M, Bitonti M. B. A.-*Multi-probe in situ hybridization to whole mount Arabidopsis seedlings*". The International Journal of Developmental Biology (2011) Vol. 55:197-203.

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