Functional characterization of the Arabidopsis heat shock factor A4A, identified by a novel genetic screen

PhD thesis

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LIST OF ABBREVIATIONS

6xHis - hexa histidine-tag

ABA - abscisic acid

AD - activator domain of GAL4 yeast transcription factor

Ade - adenine

AHA -HSF activation motifs

Ala- alanine

APX1- Ascorbate peroxidase 1

BD - DNA binding domain of GAL4 yeast transcription factor

BIFC- bimolecular fluorescence complementation

Col-0 - Arabidopsis wild type, Columbia ecotype

cYFP- c-terminal of YFP

Cys- cysteine

DAB- 3,3'-diaminobenzidine

DBD- HSF DNA binding domain

DTT- dithiothreitol

GSH- reduced glutathione

GUS- β-glucuronidase

His- histidine

HSF - Heat shock transcription factor

HSFA4m - HSFA4A modified protein with Ser309Ala replacement

HSFA4ox - HSFA4A-overexpressing transgenic lines

HSP- Heat shock protein

HSP17.6A - Heat shock protein 17.6A

LEA- Late embryogenesis abundant protein

Ler - Landsberg *erecta*

Leu- leucine

LPO - lipid peroxidation

LUC- firefly luciferase

MAPK (MPK) - Mitogen Activated Protein Kinase

MAP2K – MAPK kinase

MAP3K - MAPK kinase kinase

MBP- maltose binding protein

mHSFA4A - HSFA4A modified protein with 3 Cys to Ala replacements

MS - Murashige and Skoog medium

MyelineBP -Myeline basic protein

NES- nuclear export signal

Ni-NTA- nickel-nitrilotriacetic acid agarose

NLS- nuclear localization signal

nYFP- N-terminal of YFP

OD- HSF oligomerization domain

PET- photosynthetic electron transport

qRT-PCR - real-time quantitative PCR

ROS- reactive oxygen species

RT-PCR - reverse transcription polymerase chain reaction

SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Ser- serine

Thr- threonine

Trp- tryptophan

Tyr- tyrosine

YFP- yellow fluorescent protein

1 INTRODUCTION

1.1 Plant abiotic stress

1.1.1 Plant stress response

Plants, through the history of evolution, have adjusted their physiology to the terrain they inhabit. Abiotic factors such temperature, light intensity, water availability and soil composition integrate the complex environment that determines plant growth, development and reproduction. Optimal levels of each abiotic factor support plants to achieve their maximal physiological performance, although perfect conditions are rarely found in natural habitats. In changing circumstances, plants respond flexibly to fluctuations in the abiotic environment with fast metabolic readjustments or changes in transpiration rates (Lichtenthaler, 1998) to maintain homeostasis. However, variations in environmental parameters beyond the normal levels have a negative impact on plant fitness, provoking a situation of abiotic stress. All agents can act as stressors since stress is regarded as a dose-dependent matter (Lichtenthaler, 1988). Environmental factors become stress factors when the dosage deviates from the optimal rates (Schulze, 2005), leading to an imbalance that challenges plant adaptation capacity.

As plants are bound to their habitat, they have developed several mechanisms to endure the adverse environmental conditions. Stress resistance mechanisms are based on two main strategies: avoidance and tolerance (Levitt, 1980).

Stress avoidance mechanisms reduce the potential damage by preventing the exposure to stressors, as for example the accumulation of salt inspecialized salt glands on the epidermis of halophytic species such the mangrove tree *Avicennia officinalis* (Tan, 2013); these organs contribute to salt resistance by excreting excess salts. Avoidance mechanisms are result of stable and inheritable genetic modifications in a population (Krasensky, 2012), therefore, the evolutionary changes are constitutive and resistance features are expressed even in the absence of environmental stress. These genotipically-driven characteristics are known as stress adaptations.

On the contrary, stress tolerance mechanisms allow individual plants to resist and combat stress by changing their physiology as long as stress prevails (Bray, 2000; Taiz, 2010) as in the case of regulation of stomatal aperture and synthesis of osmolites during water deficit stress (Hoekstra, 2001). Plastic responses to stress are an adaptive trait in that they increase plant fitness in adverse conditions. Phenotypical plasticity has been suggested to have evolved as a requirement of the dynamic ancestral environment (Price, 2003).

Abiotic stress responses are initiated at molecular level and they reach a global effect throughout the whole plant. Key molecular mechanisms involve expressional regulation of a set of genes that function in stress tolerance (Shinozaki, 2003) and exhibit transcriptional activation at different stages of the abiotic stress response, depending on *de novo* protein or signalling molecules synthesis required for their activation (Mahajan, 2005; Hirayama, 2010). Products of stress responsive genes can be classified as functional or regulatory proteins. Functional proteins act as stress protectors, and include detoxification enzyme systems, molecular chaperones, osmolite biosynthesis enzymes or ion transporters which are part of the protein functions involved in direct stress protection. Regulatory proteins are essential in the regulation of gene expression and signal transduction pathways and such proteins function for example as transcription factors, protein kinases and phosphatases and phospholipid metabolic enzymes. Therefore, products of stress response genes function in both stress tolerance and response (Wang, 2003). Proteins involved in abiotic stress response contribute to the protection of cellular environment, and in turn cells respond to stress with metabolic and membrane adjustments, modifications of the cell wall architecture and changes in cell cycle (Krasensky, 2012). Cellular responses to stress have an impact at the whole plant level, and determine plant growth, development, morphology and life cycle.

1.1.2 Types of abiotic stress

The major environmental factors that affect plant growth and physiology are water, the physical and chemical soil properties, temperature and light. Fluctuations in these environmental conditions are common in natural habitats, subjecting plants to a combination of abiotic stresses (Mittler, 2006).

1.1.2.1 Water imbalance

Water stress emerges as a consequence of excess or deficit of water above- and underground. During a situation of water excess, as for instance during flooding, a decrease in oxygen availability in roots and other submerged tissues results in hypoxic or anoxic conditions which diminish cellular respiration. ATP production and cytosolic pH decrease as a result of anaerobic fermentation; the acidic environment impairs an appropriate cellular metabolism and finally causes cell death. Lack of oxygen increases intracellular Ca^{2+}, alters the redox state and reduces the membrane barrier function (Blokhina, 2003), whereas reactive oxygen species (ROS) accumulation under hypoxic conditions has been extensively documented (Blokhina, 2010; Pucciariello, 2012). On the other hand, water deficit involves a decrease in environmental water potentials (ψ_w); water flows out of plant cells driven by the ψ_w gradient and cellular dehydration arises as result of osmotic stress. Intracellular water loss damages membrane bilayer structure, disrupts cellular metabolism (Mahajan, 2005), inhibits photosynthesis and leads to ROS accumulation. In addition, cellular water loss involves an increase in the intracellular concentration of solutes, leading to their cytotoxicity. Plant growth is inhibited during drought, as it is substantially determined by water availability. Cell enlargement involves biochemical cell wall loosening, which is driven by a positive turgor pressure (Neumann, 1995). In addition, drought-induced stomatal closure prevents further water loss by transpiration, but at the same time decreases CO_2 uptake and photosynthetic activity (Kusumi, 2012).

1.1.2.2 Salt stress

Salinity arises as result of the accumulation of soluble salts in the soil. Soil salinization is a natural process, associated with weathering of rocks and minerals and the consequent release of salts to the environment. In addition, rain water and wind carry oceanic salts that are deposited on the soil surface and the intrusion of sea water in coastal areas also increases salt levels (Munns, 2008). The climate in a given land will determine its salinity, as factors such the amount of precipitation and evaporation

influence the final concentration of salt in the soil (Mahajan, 2005). On the other hand, anthropogenic factors such as inappropriate agricultural practices contribute to soil salinity, as for example the usage of poor quality, high-salt-content water for irrigation, large amounts of chemical fertilizers and the absence of drainage systems (Qadir, 2000). In dry and warm seasons or climates, capillary movements carry water and salts to the soil surface where water is lost by evaporation and plant transpiration and salts are accumulated (Rengasamy, 2006). Saline soils contain predominantly sodium chloride (NaCl) and sodium sulphate (Na_2SO_4), other salts such calcium and magnesium chlorides and sulphates ($CaCl_2$, $MgCl_2$, $CaSO_4$, $MgSO_4$) are also present but to a lesser extent (Szabolcs, 1974). NaCl, $MgCl_2$ and $MgSO_4$ are potentially toxic to plants, as they are very soluble and can reach high concentrations in the soil (Porta, 2003). In saline soils, NaCl is most often the dominant soluble salt (Rengasamy, 2002) and it has been the focus of much of stress research to date. Salinity causes both an osmotic and a toxic effect to plants. High concentration of NaCl in the soil solution lowers its ψ_w, which makes water becoming less available to plants due to the high osmotic potential in the soil. This salt-induced water deficit stress is known as *physiological drought*, and is a common damage from salt to plants (Pessarakli, 2007); as with drought-affected plants, cell expansion is impaired by the restriction of water uptake. In addition to the osmotic effect, Na^+ and Cl^- from the soil solution enter root cells and reach the photosynthetic tissues, main sites of ionic toxicity, by the transpiration stream (Munns, 2008). Inside the plant, Na^+ and Cl^- decrease the water potential of the apoplast and accumulate in the cytosol, resulting in hyperosmotic stress and ionic imbalance (Niu, 1995). High levels of Na^+ and Cl^- accumulated in leaves during transpiration over time eventually cause senescence of older leaves, reducing the photosynthetic capacity of the plant (Munns, 2005). Na^+ and Cl^- are carried by water flow inside roots, specially through the root hair zone. Na^+ enters root epidermal cells passively, down the electrochemical gradient across the plasma membrane but a negative membrane potential functions as a thermodynamic barrier to Cl^- influx (Niu, 1995) though Cl^- can still be actively uptaken via $Cl^-/2H^+$ symporter (Felle, 1994). Na^+ uptake occurs via nonselective cation channels (NSCC) and high-affinity K^+ transporters (HKT), which are estimated to have equal permeability to Na^+ and to K^+ (Demidchik, 2002). Most research has been focused in Na^+ toxicity since, in general, is more harmful than Cl^- and plants have developed effective mechanisms for Na^+ tolerance (Byrt, 2008). As Na^+ and K^+ have similar physicochemical properties, Na^+ competes with K^+ for intracellular transport, which

leads to a reduction in K^+ uptake (Pardo, 2011). Unlike Na^+, K^+ is an essential macronutrient involved in numerous cellular processes (Dreyer, 2011). Na^+ acts as a competitive inhibitor of K^+-binding enzymes, which severely affects cellular metabolism and leads to ROS production. Moreover, Na^+ accumulation disrupts membrane potential, which further facilitates passive cellular influx of Cl^- ions (Roberts, 2006).

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1.1.2.3 Heavy metal toxicity

An imbalance in the mineral content of the soil is also associated with ion toxicity to plants. When in excess, essential metals heavily interact with biological molecules, damaging and inactivating them; those involved in redox reactions, such iron (Fe), can contribute to ROS formation through the Fenton and Haber-Weiss reactions (Halliwell, 1990). Non-essential metals and metalloids such cadmium (Cd), arsenic (As) or selenium (Se) are chemically similar to essential elements and therefore highly toxic to plants. Heavy metal ion Cd^{2+} enters plant cells through Fe^{2+} or Zn^{2+} transporters (Clemens, 2006) and competes for molecular Zn-specific binding sites making Zn-binding proteins candidate targets for Cd^{2+} toxicity (Stohs, 1995). The two forms of arsenic found in soils, arsenate and arsenite, enter plant cells by different mechanisms. Arsenate is taken up by the phosphate transport system, with phosphate transporters having lower affinity for arsenate. Arsenate uptake leads to phosphate deficiency, and due to its chemical analogy, competes with phosphate during phosphorylation reactions, destabilizing target molecules (Finnegan, 2012). Arsenite enters root cells through aquaporin family of major intrinsic proteins (Bienert, 2008) and it inhibits enzyme activity by binding to the thiol groups (Finnegan, 2012).

1.1.2.4 Cold and heat stress

Temperature has a critical effect on plant growth and development. Consequently, plants are very sensitive to minor variations in temperature and can perceive changes as little as 1ºC (Lee, 2012). Although physiological processes function normally within a

temperature range of 0ºC to 40ºC (Went, 1953), temperatures below 13ºC are associated with *chilling injury* (Buta, 1993), a decrease of membrane fluidity which disturbs the function of membrane-associated proteins (Chinnusamy, 2007). Under 0ºC, apoplastic water freeze before intracellular water does, which causes a decrease in apoplastic $\psi_{w.}$ Cytosolic water flows out down the gradient and results in cellular dehydration. In addition, ice crystals cause a mechanical strain that physically damage membranes and organelles (Uemura, 1995). On the other hand, high temperatures are more harmful to plants, since minor increases in temperature greatly affect crop cycle (Mittler, 2010). In the field, heat stress is specially deleterious when combined with drought, as closed stomata (and consequently reduced evaporation) cause leaf temperature to increase (Rizhsky, 2004). At cellular level, high temperatures destabilize membranes by increasing its fluidity, which affects the function of integral membrane proteins; cellular respiration and photosynthesis are affected as consequence of membrane damage (Taiz, 2010). Heat stress also affects RNA and protein stability, promotes protein misfolding, metabolic imbalance due to the activity of defective enzymes and ROS generation (Larkindale, 2002; Lee, 2012).

1.1.2.5 Oxidative stress

In aerobic organisms, reactive oxygen species (ROS) are naturally generated as by-products of metabolic reactions. In photoautotrophs, ROS are continuously produced in chloroplast, mitochondria and peroxisomes by metabolic pathways localized in these cellular compartments. Reaction centres PSI and PSII in the thylakoid membrane are the principal source of ROS (Asada, 2006), followed by the peroxisomes. In non-photosynthetic tissues or in the absence of light, ROS are mainly produced in mitochondria (Koh, 2007). ROS are highly reactive molecules and their accumulation causes protein oxidation, lipid peroxidation (LPO) and nucleic acid damage, leading eventually to cell death (Driscoll, 2006). Aerobic organisms have developed antioxidant mechanisms to maintain ROS homeostasis, by which ROS production and ROS scavenging are effectively balanced. However, when the equilibrium is disturbed, ROS build-up and oxidative stress arises. Environmental stress greatly promotes ROS accumulation, as directly increases ROS production and affects the antioxidant capacity

of plants (Banti, 2011). In chloroplast, light energy absorbed by pigments is used for CO_2 assimilation, but in circumstances in which photon intensity is in excess or CO_2 availability decreases, the components of the photosynthetic electron transport (PET) chain are over-reduced (Mano, 2000). Part of the electron flow is transferred to O_2 at the level of PSI, reducing O_2 to superoxide anion radical (O_2^-) by the Mehler reaction; O_2^- is dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). At PSII level, over-reduction of PET promotes the formation of chlorophyll triplet state, which can react with O_2 and generate singlet oxygen ($^1O_2$) (Gill, 2010). ROS accumulation in thylakoids contributes to photoinhibition as it directly affects plant photosynthetic capacity. In conditions in which O_2 concentration exceeds the concentration of CO_2, ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) can utilize O_2 as substrate instead of CO_2, in a process called *photorespiration*. Photorespiration prevents photoinhibition, as dissipates the excess of photon energy and provides the limiting CO_2 (Mano, 2000). On the other hand, the photorespiration pathway is the major source of H_2O_2 in photosynthetic cells (Noctor, 2002). In the chloroplast, RuBisCO produces glycolate, which is transported to the peroxisome where is oxidized to glyoxylate in a reaction that generates H_2O_2. Peroxisomes produce H_2O_2 at high rates as result of photorespiration, which is the principal source of this ROS in these organelles; processes such β-oxidation of fatty acids and the dismutation of O_2^- also contribute to peroxisomal H_2O_2 production (Miller, 2010). In plant cells, another source of ROS are the mitochondria, although in comparison with chloroplast ROS production by these organelles in light is less (Foyer, 2003). ROS are also generated in the apoplast by the activity of plasma membrane-bound NADPH oxidases (*rboh*, respiratory burst oxidase homologue), which reduce O_2 to O_2^-, which is afterwards converted by SOD to H_2O_2 (Slesak, 2007).

All different environmental stresses converge towards ROS generation and oxidative stress. Plants exposed to direct sunlight can absorb excess light (EL) that will eventually exceed their photosynthetic capacity (Li, 2009). Under drought or salinity conditions, plants close their stomata to prevent further water loss through transpiration; however, this response compromises CO_2 uptake; the reduction of ATP synthesis in chloroplast enhance respiration rate because mitochondria compensate for the photosynthetic ATP decline, which leads to ROS production during respiration (Atkin, 2009). Low or high temperatures also trigger ROS generation and oxidative stress. Photosynthetic and

respiration rates directly correlate with increasing temperatures. However, above 40ºC, heat inactivates the enzymatic components of the electron transport systems. At high temperatures decreases both the affinity of RuBisCO for CO_2 and the solubility of CO_2, which promotes photorespiration and H_2O_2 production (Taiz, 2010). Cold temperatures decrease the enzymatic activity of the components of PET and, as under EL, increase PSII excitation pressure, which, unless the excess energy is dissipated as heat, leads to the damage of PSII and photoinhibition (Huner, 1998). ROS accumulated in response to biotic and abiotic stress represent important signalling molecules in the coordination of stress-acclimation pathways (Foyer, 2005; Suzuki, 2012).

1.2 Defence and detoxification mechanisms

Under an unfavourable environment, plants follow several acclimation strategies that are based on physiological and biochemical adjustments. At whole-plant level, plants embody phenotypical changes in order to cope with a hostile environment. For example, plants tend to reduce leaf area under water deficit or heat stress to minimize water loss by transpiration and prevent overheating. With the same end, plants accelerate senescence and leaf abscission of older leaves, resulting in a reduction of shoot growth; in contrast, a faster response to water deficit stress is stomatal closure, regulated by the water status of guard cells. At cellular level, plants count with a wide range of molecular mechanisms, which can be grouped into three major categories: (i) re-establishment of cellular homeostasis, (ii) detoxification and (iii) direct protection of proteins and membranes.

1.2.1 Molecular mechanisms of stress tolerance

1.2.1.1 Mechanisms to re-establish cellular homeostasis

Abiotic stresses such as drought, salinity and low temperatures are associated with cellular dehydration and osmotic stress. To endure water deficit stress, plants lower their ψ_w increasing the concentration of solutes in the cytosol, through osmotic

adjustment. A decrease of cellular solute potential (ψ_S) creates a gradient, which favours water uptake and prevents further water loss, therefore maintains cell turgor (Wang, 2003). The solutes synthesized during osmotic adjustment are known as compatible solutes or osmolytes. These molecules can reach high concentrations in the cytosol without interfering with the cellular metabolism (Mahajan, 2005). Compatible solutes are organic compounds and uncharged at physiological pH, this feature provokes their exclusion from the hydration shells of proteins, therefore their presence does not disturb protein stability (Bray, 2000). Compatible solutes as biomolecules can be distributed into three major groups: amino acids, such as the proline, quaternary amines and sugars including polyhydric alcohols. Proline is synthesized from glutamate in the cytosol, and its levels are under biosynthetic and catabolic controls that are coordinated by signalling events in response to water deficit stress (Szabados, 2010). An important quaternary amine is the glycine betaine that synthesized in the chloroplast from choline; and one of the enzymes involved in its biosynthesis, the choline monooxygenase, is transcriptionally regulated by osmotic stress (Wang, 2003). The hydroxyl group of sugar alcohols such as sorbitol and mannitol can serve to maintain hydrophilic interactions within membrane components during water deficit stress, besides mannitol is known as a scavenger of hydroxyl radicals. Therefore, osmolytes can function as osmoprotectants by protecting protein structure but also participate in ROS detoxification (Mahajan, 2005).

In response to salinity, plants exhibit the capacity for re-establishing ionic equilibrium in the cell by ion exclusion and compartmentalization in the vacuole. Stress tolerance mechanisms are inclined to avoid toxic ionic levels in the cytosol, especially in leaf mesophyll cells. Plant root morphology greatly contributes to control the salt load to the xylem. Additionally, the ions delivered to the transpiration stream can be absorbed by the adjacent cells along the transpiration pathway, which reduces the ionic load that eventually arrives to the photosynthetic tissues (Taiz, 2006). Na^+ enters cells passively, but active transport mechanisms tend to exclude it from the cytosol across the plasma membrane and the tonoplast. Two Na^+/H^+ antiporters mediate Na^+ extrusion in response to salinity stress: SOS1 transports Na^+ through the plasma membrane to the apoplast, and NHX1 mediates Na^+ transfer to the vacuole. Both transporters have been reported to confer salt tolerance upon overexpression (Shi, 2000; Zhang, 2001). The driving force for Na^+ active transport is provided by a pH gradient created by several H^+ pumps that

actively transport H^+ outside the cell and inside the vacuole: a P-type ATPase in the plasma membrane and a V-type ATPase H^+ and a H^+-pyrophosphatase in the tonoplast (Niu, 1995). The accumulation of Na^+ in the vacuole leads to lower ψ_w, which activates the synthesis of compatible solutes in order to achieve osmotic balance and maintain cell turgor (Munns, 2008).

Similarly to salinity stress responses, plants have two major strategies to cope with high concentrations of heavy metals: these are the exclusion of heavy metals in the soil and the internal tolerance. Both are achieved by several mechanisms: root exudates prevent metal uptake, active transport carry heavy metal ions through the plasma membrane and the tonoplast, and accumulation of metal chelators and ROS-scavenging molecules contribute to metal tolerance (Lin, 2012).

1.2.1.2 ROS detoxification

Accumulation of ROS has a detrimental effect on cells, thus plants count with a strong defence system that comprises both enzymatic and non-enzymatic antioxidants. Antioxidant proteins include superoxide dismutase (SOD), catalase (CAT) and the the four enzymes of the ascorbate–glutathione cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). SODs provide the first line of defence in cells and remove O_2^- by catalysing its dismutation to H_2O_2 in reaction with a proton. The enzyme CAT catalyses the dismutation of H_2O_2 in a reaction $H_2O_2 \rightarrow H_2O + ½O_2$ with a high turnover rate. The enzyme APX catalyses the reduction of H_2O_2 using ascorbate as an electron donor and the end products of this reaction are the monodehydroascorbate (MDHA) and H_2O. APX exhibits an order of magnitude higher affinity for H_2O_2 than CAT does (Mhamdi, 2010), therefore it is proposed to have a crucial role in H_2O_2 degradation under stress conditions (Gill, 2010). In chloroplasts, at the level of PSI, a Cu/Zn SOD dismutates the O_2^- into H_2O_2, which is reduced to H_2O by the thylakoidal APX (this known as the water-water cycle, (Asada, 1999)). Ascorbate is regenerated from MDHA in a reaction catalysed by MDHAR or, alternatively, is regenerated from dehydroascorbate (DHA) by the enzyme DHAR. Oxidized MDHA is spontaneously converted to DHA, which is reduced to ascorbate using reduced glutathione (GSH) as

electron donor. The last enzyme of the ascorbate-glutathione cycle, GR, plays an essential role in the antioxidant defence system, by maintaining the pool of GSH (Gill, 2010).

The most abundant non-enzymatic antioxidants are the ascorbate and GSH. Ascorbate accumulation correlates with high chlorophyll levels, and is especially notable in leaf tissues. At subcellular level, ascorbate is mainly concentrated in the stroma of chloroplasts in millimolar concentrations and greatly contributes to the redox state and photosynthesis maintenance (Gallie, 2013). GSH is a highly abundant tripeptide localized in all cellular compartments, reaching millimolar concentrations in the chloroplast (Creissen, 1999). The antioxidant capacity of GSH depends on the redox state of the cysteine residue: in the reduced form, thiol groups donate electrons to ROS or to the disulfide bonds of oxidized proteins; in the oxidized form, two molecules of glutathione are linked by a disulphide bond generating glutathione disulphide (GSSG). Besides scavenging $O_2^{\cdot-}$, H_2O and $OH^{\cdot}$, GSH plays a key role in regenerating ascorbate in the ascorbate-glutathione cycle (Foyer, 2011).

Other important non-enzymatic, low molecular mass antioxidants are the carotenoids, tocopherols and flavonoids. In photosynthetic systems, carotenoids and tocopherols (vitamin E) play a key role in photoprotection of membranes, and are lipophilic antioxidants localized in chloroplasts (Munne-Bosch, 2005). Flavonoids are phenolic compounds accumulated in vacuoles and are suggested to constitute a secondary ROS-scavenging system in plants (Fini, 2011). Flavonoids include coloured pigments, such as anthocyanins, which provide purple and red colours to fruits, flowers and other plant organs. In addition, flavonoids accumulate in the epidermal layers of shoots in response to UV-B light, where protect cells by absorbing UV-B radiation.

1.2.1.3 Protection of proteins and membranes

In response to stress, plants accumulate two major types of proteins with protective role under challenging environmental conditions: heat shock proteins (Hsps) and late embryogenesis abundant (LEA) proteins (Wang, 2003). Hsps are molecular chaperones, accumulated in all organisms in response to elevated temperatures, in a conserved

process known as heat shock response. Heat stress causes extensive protein damage, as the proteins get denatured and aggregated; moreover, all kinds of stress compromise protein stability and function. In fact, gene transcription and accumulation of Hsps occurs in a wide variety of environmental stress conditions (Swindell, 2007). During stress, Hsps have a protective role, as through their ATP-dependent chaperone activity contribute to the proper refolding of denatured proteins and the prevention of protein aggregation. In addition, Hsps are associated with the acquirability of thermotolerance after exposure to heat stress (Hirt, 2004). However, even in the absence of stress, Hsps are expressed at a basal level and contribute to protein folding, trafficking, maturation, degradation and to signal transduction (Ahn, 2003). Hsps comprise five evolutionary conserved protein families classified according to their size: Hsp100, Hsp90, Hsp70, Hsp60, and small Hsps with a size range of 12-40 KDa (Baniwal, 2004). Small Hsps (sHSPs) are the most dominant proteins in heat response (Hirt, 2004). Unlike the other Hsps, chaperone activity of sHsps is independent of ATP, and they cannot refold non-native proteins. However, sHSPs bind and hold denatured or partially unfolded proteins to prevent them from further damage, and subsequently other Hsps are able to refold these substrate proteins (Al-Whaibi, 2011).

LEA proteins are abundant during the late stage of embryogenesis, when the embryo is tolerant to dehydration; LEAs are also accumulated in several plant organs in response to abiotic stresses such drought, cold and osmotic stress (Wang, 2003). In Arabidopsis, 51 LEA proteins have been identified and classified into 9 groups according to their peptide sequence. The most well studied groups are the Pfam LEA_4 family and the dehydrins (Hincha, 2012). LEA proteins are highly hydrophilic, and are unstructured in the hydrated form but fold into amphipathic α-helices during drying (Popova, 2011). Based on this structural feature, LEA proteins are predicted to function as water-binding molecules to maintain cellular hydration, and also in stabilization of membranes and proteins (Wang, 2003). Several LEA or LEA-like proteins are encoded by cold and drought responsive genes such *COR15A*, *COR78/RD29*, *COR47* and *COR6.6*, which are essential for cold tolerance (Mahajan, 2005). In addition, the LEA proteins COR15A and COR85 were shown to be responsible for cryoprotection of membranes and enzymes respectively (Wang, 2003).

1.3 Stress response regulation

Plants have evolved complex signalling pathways to regulate biochemical and physiological processes associated with stress acclimation. These signal transduction pathways lead to the transcriptional regulation of genes that control molecular mechanisms integrating stress responses. Activation of stress signalling pathways starts with the perception of abiotic stresses by specific sensors (**Figure 1.2.1**). Following stress sensing, second messengers such as phospholipids, cyclic nucleotides and ROS are generated, which in turn, amplify the signal by increasing the concentration of cytosolic calcium. Calcium functions as a major messenger in plants, but other second messengers, such as ROS, can regulate signal transduction independently. The signal is further transduced by post-translational modifications targeted to proteins that function in direct stress defence or transcription factors that control the expression of stress response genes. The products of these genes can participate in direct stress defence or perform a regulatory role. These regulators enter transcription cascades or can be involved in the generation of other signalling molecules, such as hormones, which will re-initiate the signalling cascade (Hirayama, 2010).

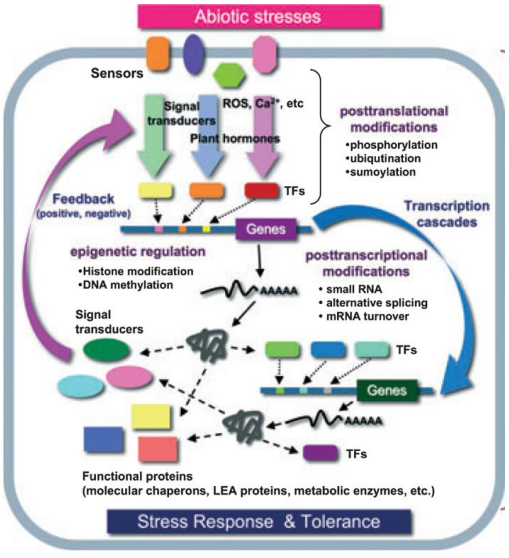

Figure 1.2.1. **Model of the plant response to abiotic stress (Hirayama, 2010).** Abiotic stresses trigger the downstream signalling pathways, in which participate second messengers, hormones and several regulatory proteins involved in phosphorylation/dephosphorylation, ubiquitination or sumoylation of transcription factors. Gene products can function in direct stress defence or as signal transducers. Besides transcriptional regulation, gene expression and protein functions are regulated by post-transcriptional, post-translational modifications and epigenetics.

1.3.1 Stress perception

Plant stress sensors are mostly unknown so far. However, several histidine kinases have been connected with environmental stress perception; in fact, the Arabidopsis ATHK1/AHK1 is a putative osmosensor, that functions as a positive regulator of drought and salt stress responses (Osakabe, 2013).

1.3.2 Second messengers

1.3.2.1 Phospholipids

Membrane phospholipids are hydrolyzed by several phospholipases during stress, generating molecules that function as second messengers. The activation of the phosphatidylinositol-specific phospholipase C phospholipase C (PI-PLC) results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). In plants, IP3 regulates the aperture of intracellular Ca^{2+} channels, which provokes an increase in cytosolic [Ca^{2+}]. Transcriptional activation of PI-PLC upon dehydration and salinity was observed in Arabidopsis (Hirayama, 1995), as well as the accumulation of IP3 in the cytosol upon salt stress (DeWald, 2001).

1.3.2.2 Cyclic nucleotides

Cyclic nucleotides are important second messengers that have been demonstrated to mediate abiotic stress signalling pathways in plants. The cyclic nucleotides cAMP and cGMP activate the cyclic nucleotide-gated ion (CNG) channels, which results in a cation influx across the plasma membrane, one of these cations is Ca^{2+} (Walker, 2013). In addition, cyclic nucleotides were shown to negatively regulate Na^{+} influx via voltage independent channels, therefore contributing to salinity tolerance in Arabidopsis (Maathuis, 2001).

1.3.2.3 Hormones

Abiotic and biotic stresses induce the accumulation of various plant hormones, which trigger specific signalling pathways that can change the expression of stress-response genes. Major hormones involved in abiotic stress responses are abscisic acid (ABA), ethylene and salicylic acid. Besides, other growth regulators like gibberellins, cytokinins, auxins, jasmonates and brassinosteroids greatly contribute to the crosstalk that is important in the stress-related molecular processes (Kumar, 2013). ABA is accumulated in response to drought, salt and cold (Hirt, 2004); it mediates the activation of ABA-dependent signalling pathways that control genes involved in dehydration tolerance. Many of the ABA-inducible genes contain the *cis*-acting ABA-responsive element (ABRE) in their promoter. ABA accumulation results in the phosphorylation of ABRE-binding transcription factors (ABFs) by the SnRK2 kinases. The phosphorylated ABFs can bind to the ABRE elements of target genes and activate their transcription. Some examples for stress responses mediated by ABA are the regulation of stomatal closure during water deficit stress and the inhibition of seed germination (Nakashima, 2013).

1.3.2.4 Calcium

Cytosolic [Ca^{2+}] plays a key role in signal transduction and participates in multiple processes like thigmotropism, gravitropism, cell division, expansion and differentiation, cell polarity, photomorphogenesis, plant defence and abiotic stress responses (Song, 2008). In plant cells, cytosolic [Ca^{2+}] is maintained under normal conditions in the range of 100–200 nM, whereas in the apoplast and cellular compartments such as vacuole, endoplasmic reticulum and plastids it is kept at millimolar level (Tuteja, 2008). Several abiotic stresses like cold, drought and salinity have been reported to induce, within seconds upon sensing, a transient increase in cytosolic [Ca^{2+}], which is required for the activation of downstream signal transduction pathways (Kader, 2010). This increase results in either from the activation of channels mediating Ca^{2+} influx from the apoplast or by ligand-sensitive Ca^{2+} channels, which control internal Ca^{2+} release

(Xiong, 2002); as described above, ligands such as IP3 and cyclic nucleotides bind and open intracellular Ca^{2+} channels. To interrupt Ca^{2+} signalling and re-establish cellular homeostasis, a group of P-type Ca^{2+}-ATPases translocate Ca^{2+} back to the apoplast or intracellular storage compartments (Huda, 2013).

1.3.2.5 ROS

ROS are highly reactive molecules that cause oxidative damage to a wide range of molecules such as membrane lipids, proteins and nucleic acids. However, ROS also regulate gene expression and induce post-translational modifications in regulatory proteins, which demonstrate that cells use ROS as signal molecules. The dual function of ROS is tightly controlled by the antioxidant system, which balances ROS production and ROS scavenging; ROS homeostasis allows plants to accumulate ROS in intracellular compartments where they function as signals (Pucciariello, 2012). ROS such as $^1O_2$, $O_2^{\cdot-}$ and $OH^{\cdot}$ have very short half-lives and diffuse over short distances, which suggest they may participate in intracellular signalling (Pitzschke, 2006). In fact, $^1O_2$, is involved in the regulation of chloroplast retrograde signalling; using the Arabidopsis *flu* mutant, $^1O_2$ was demonstrated to activate a set of genes and a genetic program leading to growth inhibition and cell death (Suzuki, 2012). Along with $^1O_2$, H_2O_2 is considered as the major ROS that can act as a second messenger in plants cells (Choudhury, 2013). H_2O_2 is the most stable ROS, has the longest half-life and can cross plant membranes through aquaporins, as it shares many physical features with water (Bienert, 2006). Based on these qualities, H_2O_2 is a key molecule that can function in both intracellular and intercellular signalling. Abiotic and biotic stresses trigger the accumulation of H_2O_2, referred as the *oxidative burst*, which suggests a central role of H_2O_2 in controlling a wide range of stress responses, physiological changes and cellular hormone and ROS homeostasis (Slesak, 2007). A relation between H_2O_2 and calcium signalling has been suggested, although H_2O_2 has been located both upstream and downstream of calcium in signalling pathways (Hirt, 2004). H_2O_2 participates in multiple cellular processes; for example, H_2O_2 is an essential signal in ABA-mediated stomatal closure, in which an ABA-activated kinase, OST1, regulates H_2O_2 production via plasma membrane NADPH oxidases. H_2O_2 accumulation activates Ca^{2+} channels,

causing loss of turgor in guard cells and stomatal closure (Pitzschke, 2006). H_2O_2 mediates other processes such as root hair development and differentiation of the cellulose-rich cell wall (Foreman, 2003). ROS accumulation in response to pathogen attack promotes local defence, and induces the hypersensitive response (HR), a form of programmed cell death (PCD). During HR, the plant's antioxidant system is repressed to promote ROS accumulation, therefore demonstrating that during biotic stress, ROS accumulation is coordinated with the action of other signalling molecules such as salicylic acid and nitric oxide (Klessig, 2000).

Although H_2O_2 regulates gene expression and the activity of molecules like kinases and phosphatases, no H_2O_2 receptor has been identified in plants so far (Slesak, 2007). However, it has been suggested that, similarly to other organisms, oxidative modifications of molecules might be the key for H_2O_2 perception. The best-studied ROS-mediated protein modification is the reversible oxidation of cysteine residues. The cysteine thiol group (-SH) can be oxidized to different degrees, generating sulphenic acid (-SOH), which can be further oxidized to sulphinic acid ($-SO_2H$) and to sulphonic acid ($-SO_3H$). On the other hand, the reduced state can be restored by the activity of disulphide reductases like thioredoxins or glutaredoxins. The degree of oxidation depends on the redox potential of the thiol group and its availability to H_2O_2. This feature is suggested to allow *in vivo* the specific regulation of different proteins by the intracellular redox state (Hancock, 2006).

1.3.3 Signal transduction

1.3.3.1 Phosphoprotein cascades

The downstream signalling process triggered by stress signals involves the activation of kinase cascades that eventually regulate gene expression. Under abiotic stress, specific phosphorylation cascades are activated depending on the upstream signalling molecules. Transient increases in cytosolic [Ca^{2+}] are sensed by two kinds of proteins: sensor relays and sensor responders (Boudsocq, 2005). The sensor relays calcineurin B-like proteins (CBLs), undergo conformational changes upon Ca^{2+} binding, which leads to the activation of their interacting kinase partners, the CBL-interacting protein kinases

(CIPKs). An example of CBL-CIPK mediated signalling is the well-characterized SOS pathway. An increase of cytosolic [Ca^{2+}] following salt stress is perceived by the Ca^{2+} sensor SOS3 (CBL4), which interacts and activates SOS2 protein kinase (CIPK24). The complex SOS3-SOS2 activate the Na^{+}/H^{+} antiporter SOS1 by phosphorylation, which functions in re-establishing ion homeostasis (Xiong, 2002). Sensor responders on the other hand, are kinases regulated by calcium, such as the calcium-dependent protein kinases (CDPKs). CDPKs are essential factors in abiotic stress tolerance and have been shown to mediate ABA signalling and ROS detoxification (Asano, 2012).

1.3.3.2 MAP kinase signalling

Mitogen-activated protein kinase (MAPK) cascades are signalling modules conserved among eukaryotic organisms. MAPK signalling involves the sequential phosphorylation of three classes of kinases: MAP kinase kinase kinases (MAP3Ks), MAP kinase kinases (MAP2Ks) and MAP kinases (MAPKs). Upon activation, MAP3Ks trigger a transmission cascade, in which MAP3Ks phosphorylate and activate MAP2Ks, which in turn phosphorylate MAPKs, which function controlling the expression of stress-responsive genes (Ahlfors, 2004). MAP3Ks are serine (Ser) or threonine (Thr) kinases and phosphorylate MAP2Ks at S/T-X_{3-5}-S/T motif, and MAP2Ks phosphorylate MAPKs on Thr and tyrosine (Tyr) residues at the conserved T-X-Y motif (Rodriguez, 2010). Phosphorylation of MAPKs can be reversed by the activity of Ser/Thr phosphatases of PP2C family, Tyr phosphatases (PTPs) and MAPK-specific phosphatases (MKPs) (Lumbreras, 2010). A serine or threonine residue followed by a proline (S/T-P) is the minimal consensus motif for protein phosphorylation by MAPK (Ishihama, 2011). The Arabidopsis genome encodes for approximately 80 MAP3Ks, 10 MAP2Ks and 20 MAPKs. MAP3Ks constitute a large family of kinases, which is divided into three subfamilies: MEKK-like kinases, which participate in MAPK cascades, and RAF-like and ZIK-like kinases, whose function is unknown in plants (Colcombet, 2008). MAP2Ks are classified into four groups (A to D) based on phylogenetic analysis (Hamel, 2006). The MPK3, MPK4 and MPK6 are the main activated MAPK kinases, and so far the most characterized plant MAPKs (Colcombet, 2008).

1.3.4 Transcription Regulation

Stress signalling pathways eventually head towards gene expression regulation. In Arabidopsis, about 6% of the genome encodes for more than 1500 transcription factors (Wang, 2003). Transcription factors involved in mediating abiotic stress tolerance can be classified into several multigene families: AP2/ERF, MYB, Leucine zippers, zinc-fingers, WRKY and HSF (Santos, 2011). The AP2/ERF family is the major transcription factor family in Arabidopsis (Feng, 2005). AP2/ERF transcription factors share a conserved AP2-DNA binding domain of 60 aminoacids. The subfamilies DREB and ERF are the most related to abiotic stress tolerance. DREB (dehydration-responsive element binding) proteins bind to DRE/CRT *cis*-elements in the promoters of osmotic-stress response genes, and mostly function in ABA-independent signalling pathways (Yamaguchi-Shinozaki, 2006). ERF (ethylene-responsive factor) interact with GCC box elements, and are associated with biotic and abiotic stress tolerance. MYB proteins are characterized by the highly conserved DNA-binding MYB domain. MYBs are involved in multiple cellular processes and, alike Leucine zipper (bZIP) proteins, regulate abiotic stress responses, mostly through ABA signalling cascades (Santos, 2011). C2H2-type zinc finger proteins are divided into several groups, based on their sequence and position of the zinc-finger domain (Englbrecht, 2004). Several members of the subclass C1– 2i contain a repressor EAR-motif, and are involved in the regulation of stress tolerance: Zat10 is required for tolerance to osmotic stress, and is suggested to function as both an activator and a repressor of stress-response genes (Ciftci-Yilmaz, 2008); Zat12 was characterized as a key regulator of ROS and abiotic stress signalling (Davletova, 2005). Besides, Zat12 was shown to be required for ascorbate peroxidase 1 (APX1) expression during oxidative stress, and was placed downstream of HSFA4A in a signal transduction pathway triggered by mild light stress (Davletova, 2005). WRKY proteins share a highly conserved 60 amino acid long WRKY domain, which contain a conserved amino acid sequence motif WRKYGQK at N-termini and a novel zinc-finger-like motif at C-termini. The WRKY function in responses to biotic stress has been extensively documented, but recently, more experimental data is connecting WRKY and abiotic stress responses (Chen, 2012).

1.3.4.1 Heat shock transcription factors

Heat shock transcription factors (HSFs) regulate the transcription of a large battery of stress-induced genes, and are important regulators of stress responses in eukaryotic cells (Scharf, 2012). HSFs recognize the heat shock sequence elements (HSEs), which are conserved palindromic binding motifs (5'-AGAAnnTTCT-3') found in promoters of heat-induced genes, as for example in promoters of heat shock protein (HSP) genes (Nover, 2001). Yeast and *Drosophila* have one single HSF, whereas vertebrates have three HSFs. On the contrary, plants count with several groups of HSFs, with more than 20 genes in Arabidopsis and more than 50 in soybean. Plant HSFs exhibit great differences in their transcriptional activation, which suggests an important diversification of HSFs function (von Koskull-Doring, 2007).

HSFs have a modular structure, conserved among eukaryotes (**Figure 1.2.2)**. The DNA-binding domain (DBD), conserved among eukaryotic HSFs, is found at the N-terminal end, and is required for the binding of HSE in the promoters of HSFs target genes. Downstream of the DBD, a flexible linker peptide separates the DBD from the oligomerization domain (OD), which consists in two adjacent heptad repeats of hydrophobic amino acids (HR-A/B) that mediate oligomerization, which is required for the transcription factor activity. Nuclear localization/export signal sequences (NLS, NES) are located downstream of the OD, and determine the subcellular distribution of HSFs (Miller, 2006). HSFs are classified into 3 different classes based on features of the flexible peptide linker and the OD (Nover, 2001). In Arabidopsis, class A is the largest group, and comprises 15 members, which function as transcription activators. The transcriptional activity is provided by the presence of activation motifs (AHA) at the C-terminal domain, which interact with the components of the transcription machinery (von Koskull-Doring, 2007). On the other hand, class B members are thought to function as transcription repressors, due to the absence of AHA motifs and the presence of a conserved –LFGV- repressor motif (Czarnecka-Verner, 2004). Only one member belongs to class C HSFs, and there is currently no information available on its function (Soares-Cavalcanti, 2012). On the contrary to the N-terminal half of HSFs, the C-terminal activation domains are the least conserved (Nover, 2001).

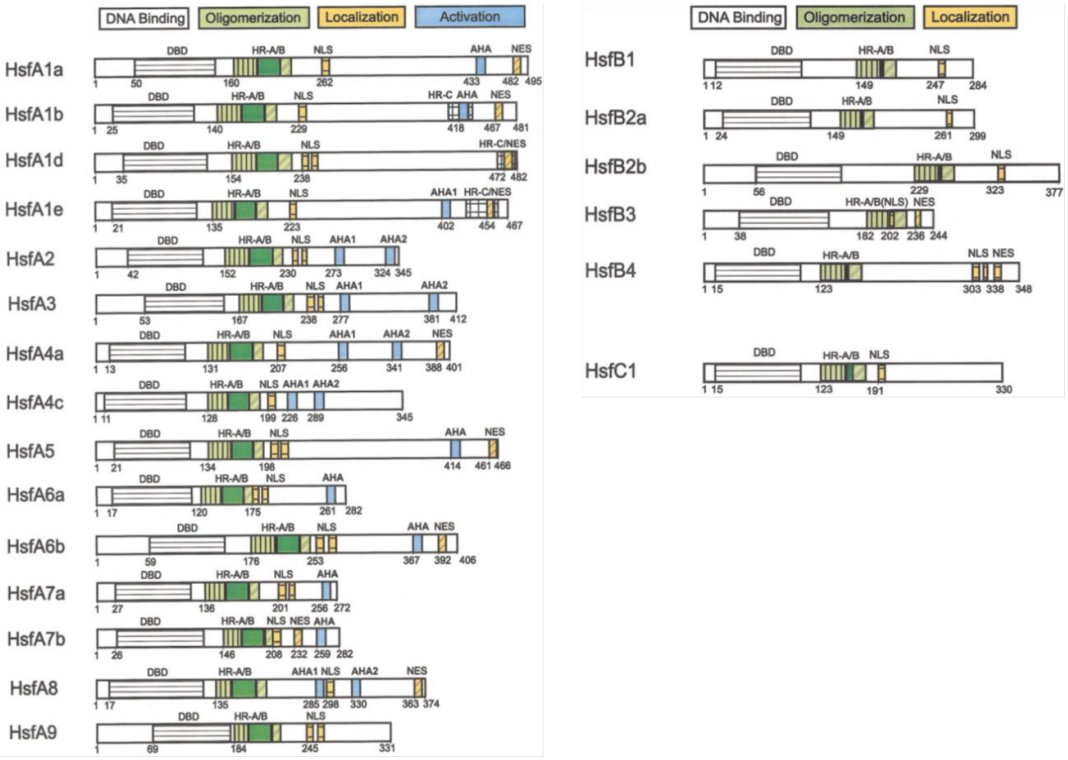

Figure 1.2.2. Arabidopsis HSFs (Nover, 2001)
Block diagrams represent HSFs with their functional domains indicated. **DBD**, DNA-binding domain, **HR-A/B,** heptad repeats of hydrophobic amino acids (oligomerization domain), **NLS**, nuclear localization signal, **AHA**, activator motifs, **NES**, nuclear export signal.

Plant HSFs present high functional diversification (**Figure 1.2.3)**. As exists a high gene redundancy among Arabidopsis HSFs, most of the available data on HSF function is based on the generation of multiple gene knockouts. The HSFA1 subgroup functions as a master regulator of the heat shock response, *HSFA2*, activated by heat and high light, is suggested to be involved in several stress pathways, and its overexpression was shown to confer tolerance to several abiotic stresses. In tomato, heterodimer formation of HSFA1 and HSFA2 leads to a synergistic transcriptional activation of HSF-regulated genes. HSFA3 participates in drought responses in a DREB2A-dependent manner (Scharf, 2012). Wheat and rice HSFA4A were shown to be involved in cadmium tolerance (Shim, 2009). In tomato, heteroligomers of HSFA4 and HSFA5 resulted in HSFA4 inactivation, pointing to a role of HSFA5 as a repressor of HSFA4 (Baniwal, 2007). In fact, in spite of the presence of AHA motifs, no activator function of HSFA5 has been observed in plants. Indeed, HSFA4 and HSFA5 belong to a group of class A HSFs that share similarities in the oligomerization domain and in C-terminal conserved sequence motifs (von Koskull-Doring, 2007). The overexpression of a dominant

negative form of HSFA4A in Arabidopsis compromised the response to oxidative stress and decreased levels of APX1 (Davletova, 2005). In the rice *spl7* mutant, one base substitution in the DBD of HSFA4D caused the appearance of spontaneous necrotic lesions in mature leaves as consequence of stress hypersensitivity (Yamanouchi, 2002). Based on these observations, HSFA4 members have been suggested to function as antiapoptotic factors by regulating plant ROS homeostasis (von Koskull-Doring, 2007). HSFA9 participates in seed maturation in Arabidopsis and sunflower (*Helianthus annuus*), probably by cooperation with transcription factors such ABI3 and DREB2 and hormones like ABA and auxins (Scharf, 2012).

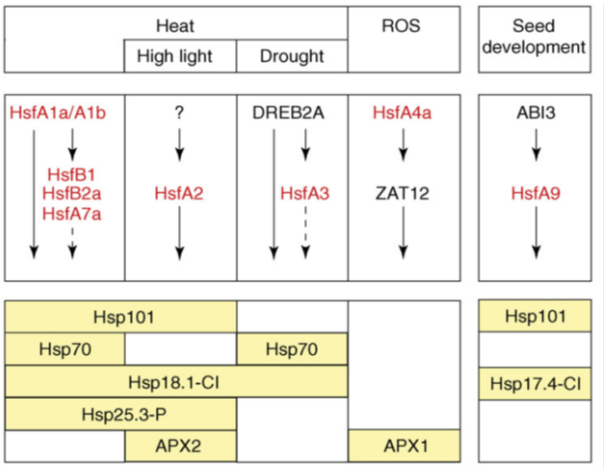

Figure 1.2.3. HSFs involved in different stress signalling pathways (von Koskull-Doring, 2007)
HSFs exhibit strong differences of expression in response to environmental stresses, which in turn place individual HSFs in distinct signal transduction pathways, leading to specific gene activation in response to each stimuli.

Plant HSFs were suggested to function as redox sensors, based on studies of mammalian, *Drosophila* and yeast (Miller, 2006). Mammalian and *Drosophila* HSFs are in a monomeric, inactive state in the absence of stress but, in response to H_2O_2, HSFs are activated and oligomerize. Afterwards, are phosphorylated and translocated to the nucleus where they activate H_2O_2-response. In yeast, ROS activate HSFs by promoting the interaction of two inactive HSF homotrimers with Skn7 co-activator. In plants, certain HSFs are thought to function as molecular peroxide sensors. It has been proposed that plant HSFs respond to alterations in levels of ROS during stress by conformational change and homodimer or heterodimer formation, leading to subsequent transcriptional activation of their target genes (Miller, 2006).

Generation of ROS signals, in particular H_2O_2, during heat and oxidative stress mediates the activation of several HSFs and induction of their downstream target genes (Kotak, 2007). Various HSFs are implicated in signalling crosstalk with key components of ROS-signalling, including the MAP kinases MPK3 and MPK6, and members of the ZAT transcription factor families, thus also contribute to transcriptional regulation of a large battery of oxidative stress responsive genes (Davletova, 2005; Evrard, 2013).

1.4 Genetic approaches to dissect regulation of plant abiotic stress responses

Adaptation to detrimental environmental conditions is controlled by a well-balanced, genetically determined signalling network. Identification and characterization of genes controlling these responses is an essential step to elucidate its complex regulation. Genetic and genomic approaches have been developed and successfully used to identify plant genes that can be connected to stress signalling. Albeit, most of our knowledge about the molecular basis of plant stress responses has derived from studies of model plants such as Arabidopsis and rice, the gained information can evidently be used to improve tolerance of crop plants to salinity, drought or extreme temperatures.

A few decades ago, Arabidopsis turned into a model of plant biology because of its advantageous features that makes suitable for genetic and molecular analysis (Redei, 1975). Among plants the Arabidopsis genome was the first that has been sequenced (AGI, 2000) and this opened the door to develop genomic tools and extend existing approaches such as system biology to this species.

For genetic analysis of a species, genotypic variants are needed that can be used for comparison studies. This variability can derive from the natural diversity or can be generated by mutagenesis approaches such as physical, chemical, insertional mutagenesis or other DNA transfers. To identify genetic variability that affects stress responses, certain distinguishable stress-related traits need to be gained. A type of gene identification approach that is usually referred as reverse genetics, based on the search for allelic variations of genes of interest. Although many different technologies have

been used to identify stress related genes, each of these methods have their own limitations and inconveniences that needs to be considered.

1.4.1 Forward genetics

The concept of forward genetics is to identify the genotype that can be connected to a phenotype. In plant genetics, several mutagenesis methods are used to generate variability for gene identification. These methods frequently rely on the physical or chemical damage of DNA, which result in deletion or substitution of nucleotides with relatively modest effort. These alterations of the DNA frequently result in so called loss-of-function mutations. However, the subsequent genetic screens for altered phenotypes are very laborious, moreover, to identify the mutated loci with gene mapping-based techniques can be utterly challenging.

Physical mutagenesis using fast neutrons or gamma irradiation causes small deletions in DNA, but the doses have to be carefully tuned. Low mutagen doses lead to low frequency of mutagenesis, therefore a huge amount of the progenies has to be screened for altered phenotypes. To choose a higher mutagen dose is apparently more effective, but can result in multiple mutations, therefore repeated back-crossing with the parental lines is required to eliminate the undesired alleles (Koncz, 1992).

The most frequently used mutagenic chemical compound is the ethyl-methanesulfonate (EMS) that is convenient to generate point mutations (Kim, 2006). EMS is widely used in Arabidopsis research for mutagenesis and therefore studies of EMS-mutagenized Arabidopsis lines have also been greatly contributed to our present knowledge of plant stress signalling. Characterization of the EMS-mutant Arabidopsis lines *sos1*, *sos2* and *sos3* was served to identify the main components of the *S*alt *O*verly *S*ensitive (SOS) signalling pathway (Zhu, 1998). The SOS pathway was proposed to be a key regulator of salt tolerance maintaining Na+/K+ homeostasis in plant species (Martinez-Atienza, 2007).

Insertional mutagenesis is another widely used technique, feasible to identify mutant genes by PCR-based methods amplifying the insertion junctions. Insertions can be generated by transposable elements (i.e. maize Ac/Ds transposons) or using the transfer

DNA (T-DNA) of Agrobacterium species. *Agrobacterium tumefaciens* is frequently used to mutagenize Arabidopsis and rice, since it has the ability to transfer and covalently insert a usually several thousand base pairs of T-DNA into the host genomic DNA (Caplan, 1983). These T-DNA insertions can disrupt a promoter-gene structure, and the generated lines are mostly considered as loss-of function mutants. The T-DNA integration into the plant genome is not site specific, it occurs with a process the so called illegitime recombination, and is nearly random (Szabados, 2002). The Agrobacterium-mediated transformation of Arabidopsis has been gained ground in the last few decades, since it is a relatively easy way to gain genetic variants. The transformation procedure is usually carried out by dipping the Arabidopsis floral buds in Agrobacterium suspension. The transformant plants can be selected in the following generation (T1), taking advantage of the antibiotic or herbicide resistance that determined by a marker gene on the T-DNA (Bent, 2000). Many studies presented efficient identification of stress related genes from collections of T-DNA mutagenized Arabidopsis lines. In a genetic screen of more than 250 thousand T-DNA mutants resulted in the identification of approximately. 200 genes with salt and osmotic stress related phenotype (Koiwa, 2006).

T-DNA insertions usually result in loss-of-function phenotypes due to the inactivation of genes, however gain-of function mutations can also be generated if the inserted DNA contains promoters or transcriptional enhancers. The techniques based on these methods are called activation tagging. With activation tagging, genes surrounding of the insertion site can be induced and cause altered phenotypes (Weigel, 2000).

Using the advantage of Agrobacterium or transposon based DNA transfer, further efficient gene identification methods have been developed. The promoter and enhancer trap technologies are based on genomic insertions of promoterless reporter genes such as *β-glucuronidase (GUS),* bacterial *luciferase (LUX)* and firefly *luciferase (LUC).* Position of these insertions can bring reporters under the control of a gene transcriptional regulation, and this can be identified by monitoring the reporter gene activity (Koncz, 1989). A luciferase reporter based system has been used in our laboratory for a large-scale random promoter and gene trapping experiment in Arabidopsis, and many stress related genes have been identified (Alvarado, 2004).

Another way to generate variability is based on cDNA overexpression that can result in gain-of function phenotypes. A random cDNA library from Arabidopsis under the control of the constitutive CaMV35S promoter has been used to generate transgenic Arabidopsis lines, and screened for altered phenotypes (LeClere, 2001). In the Full-length cDNA Over-expressing gene hunting system (FOX), a normalized full-length cDNA collection has been used to identify hormone signalling and stress regulatory genes in Arabidopsis and rice (Ichikawa, 2006), (Nakamura, 2007). The Controlled cDNA Overexpression System (COS) was established in our laboratory to overcome the possible developmental and growth defects caused by the constitutive stress gene activation (Kasuga, 1999). The COS library was inserted into the estradiol-inducible pER8-GW vector, therefore expression of single cDNAs is strictly controlled by the addition of the estradiol as chemical inducer (Zuo, 2000). The COS system has been used to identify a set of cDNAs conferring dominant stress-related phenotypes in different different genetic screens: tolerance to salt stress, insensitivity to ABA and activation of the stress-induced *ADH1-LUC* reporter gene (Papdi, 2008).

2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

Arabidopsis thaliana (Col-0) plants were grown in sterile conditions using half-strength Murashige and Skoog culture medium (0.5 MS (Duchefa), 0.5 % sucrose (Molar Chemicals Kft) and 0.8 % phytoagar (Duchefa)) under controlled conditions with 8 h light/16 h dark light cycle at 22 ºC, and 100 μE m^{-2} sec^{-1} light intensity.

To generate HSF4ox lines, the estradiol-inducible pER8GW-HSFA4A construct was introduced into Arabidopsis Col-0 plants by Agrobacterium–mediated transformation as follows: *Agrobacterium tumefaciens* GV3101 (pMP90) carrying the pER8GW-HSFA4A construct were inoculated in liquid YEB media (5 g/l beef-extract, 1 g/l yeast-extract, 5 g/l peptone (casein-hydrolysate), 5 g/l sucrose and 2 mM $MgSO_4$) containing 100 μg/ml spectinomycin, 50 μg/ml rifampicin and 25 μg/ml gentamicin and grown over night at 28ºC. Cells were collected by centrifugation and ressuspended on destilled water supplemented with 5% sucrose and 0,05% SILWET L-77. Arabidopsis inflorescences were dipped into the Agrobacterium solution and covered with a plastic foil for 24 hours. Plant transformation was repeated two times allowing 4-5 days between each infiltration. Seeds were collected and germinated on 0.5 MS medium supplemented with 20 μg/ml hygromycin (Duchefa), and after 2 weeks antibiotic-resistent plants were transferred to soil for setting seeds. Expression of inserted cDNA was tested by RT-PCR, and two overexpressing lines (HSF4ox1 and HSF4ox2) were selected for further phenotypic analysis.

The knockout Arabidopsis line (FLAG_571A11) containing a T-DNA insertion in the promoter region of the *HSFA4A* gene was obtained from the INRA Versailles T-DNA collection (Wassilewskija background) (http://urgv.evry.inra.fr/FLAGdb). Segregation of T-DNA insertion lines was tested on 50 mg/L phosphinotricin and homozygous *hsfa4a* mutant lines were generated by PCR-based genotyping using the following gene-specific and T-DNA-specific primers: hsfa4a_gen_F, hsfa4a_gen_R and Lb4 (sequences in supplementary primer list). Genetic complementation of *hsfa4a* T-DNA

insertion mutant was performed by *Agrobaterium*-mediated transformation with the 35S-HSFA4A construct. Expression of the insert was tested by qRT-PCR, and one complemented mutant line (*hsfa4a*/C) exhibiting a constitutive *HSFA4A* expression level similar to wild type plants was selected for further phenotypic analysis.

For *HSFA4A* transcription activation studies, *in vitro* grown wild type plants were incubated in liquid 0.5 MS medium (without phytoagar) as control and one of the following additives: 1 mM H_2O_2 (Sigma-Aldrich), 1 μM paraquat (N,N-dimethyl-4,4-bipyridinium dichloride, Sigma-Aldrich), 150 mM NaCl or 300 mM mannitol (Molar Chemicals Kft.). Heat treatments were performed at 37 ºC in a growth chamber (70% relative humidity (RH), 100 $\mu E\ m^{-2}\ sec^{-1}$).

2.1 Analysis of stress tolerance

Tolerance to stress conditions such as salinity, osmotic and oxidative stress was tested *in vitro* conditions as described (Verslues, 2006), (Papdi, 2010). For analysis of stress tolerance traits, 5-day-old seedlings were transferred to agar-solidified 0.5 MS medium containing 5 μM estradiol ((17β)-estra-1,3,5(10)-triene-3,17-diol, Sigma-Aldrich) and one of the following supplements: 100 mM NaCl, 5 mM H_2O_2, 0.1 mM $CdCl_2$ (Molar Chemicals Kft.), 0.1 or 0.3 μM paraquat or 200 or 300 mM mannitol. Plant growth was monitored by capturing images every 3 days as described (Ruibal, 2012). Rosette size and root elongation of 30 plants were measured at each time point using the ImageJ software (rsb.info.nih.gov/ij), and average values were determined. Rosette growth rates were calculated from at least four time points and normalized to the average rosette size of each line at the beginning of the treatment (day 0). To compare paraquat tolerance of Arabidopsis lines, seedlings were grown in the presence of 0.3 μM paraquat as indicated. After two weeks, the anthocyanin-accumulating plants were scored, counting those seedlings that were unambiguously purple-red or showed large colored leaf sections as described by (Kortstee, 2011), and percentage values were calculated. Anoxia tolerance assay was performed on one-week-old HSF4ox and wild type plants

grown on 0.5 MS media in vertical position. Plants were sprayed with 5 μM estradiol solution and kept under high-purity nitrogen gas flow for 6 and 12 hours in a dark container. Low oxygen conditions were confirmed by testing the induction of two anaerobic marker genes: *ALCOHOL DEHYDROGENASE 1* (*ADH1*, *AT1G77120*) and *PYRUVATE DECARBOXYLASE 1* (*PDC1*, *AT4G33070*) in wild type plants. Control plants (air control) were kept in the dark for 6 or 12 hours. Subsequently, plants were transferred to standard growth conditions and survival rates were scored after 7 days. All experiments were performed with two technical replicates and repeated at least 3 times.

2.2 Analysis of biochemical parameters

H_2O_2 content and lipid peroxidation were determined in 2 weeks old plants treated with liquid 0.5 MS medium supplemented with 150 mM NaCl for 6 and 24 hours. A 24 hours treatment with 5 μM estradiol was included prior salt treatments when testing HSF4ox plants. Hydrogen peroxide content was measured using Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Molecular Probes, A22188), following the manufacturer instructions. The H_2O_2 assay was performed in a total volume of 100 μL per microplate well. In each reaction were included 50 μL of each sample (prepared using 50 mg of plant material homogenized in 1XReaction Buffer) and 50 μL of 100 μM Amplex® Red reagent and 0.2 U/mL HRP (Horseradish peroxidase). After 30 minutes incubation in dark, the accumulation of resorufin was determined spectrophotometrically at 560 nm (Thermo Labsystems, Multiskan Spectrum). Triplicates and 3 different dilutions of each experimental sample (1, 1/2 and 1/5) were included. H_2O_2 concentrations were determined using a H_2O_2 standard curve.

Lipid peroxidation was measured using the thiobarbituric acid (TBA) test, which determines malondialdehyde (MDA) as end-product of lipid peroxidation. 100 mg plant material were homogenized in 0.5 ml 20% trichloroacetic acid (TCA) solution, in triplicates. The homogenate was centrifuged at 13.000 rpm for 5 minutes at 4ºC and the supernatant was collected. 0.5 ml 1% TBA were added and the mixture boiled for 30

minutes, and quickly placed in an ice bath. After 10 minutes centrifugation at 13000 rpm, the absorbance of the supernatant was measured at 535 and 730. The amount of MDA was calculated using the difference of A_{535} and A_{730}, and its extinction coefficient 155 mM^{-1} cm^{-1}.

Differences between means were determined by Duncan's multiple range test and labelled in all diagrams by different letters. All experiments were repeated twice.

The generation of H_2O_2 was detected by *in situ* histochemical staining procedure with 3,3-diaminobenzidine (DAB, Sigma). Water infiltrated control *Nicotiana benthamiana* leaves and 150 mM NaCl-treated leaves were placed in DAB solution (2 mg/ml DAB pH: 5.5) and incubated in dark for 2 hours. Leaves were boiled in 96% ethanol for 15 minutes, and stored in fresh 96% ethanol. H_2O_2 production was visualized as brown coloration.

Histochemical detection of GUS enzyme activity in pHSFA4A-GUS expressing plants was performed as follows: 10 days old Arabidopsis seedlings were immersed in pre-chilled 90% acetone for 20 minutes, rinsed with water and incubated in GUS stain solution (50 mM sodium phosphate buffer pH 7.2, 0.2% triton X-100 and 2mM X-GlcA-t (5-bromo-4-chloro-3-indolyl-β-glucoronic acid (Duchefa)) at 37ºC overnight. Plants were stored in 96% ethanol and pHSFA4A-GUS expression pattern visualized as blue coloration. Ten independent transgenic lines were assayed.

2.3 Transformation and screening of Arabidopsis cell cultures

Root-derived Arabidopsis cell suspension culture (Mathur, 1995) was used for large-scale transformation and screening. Cell suspension culture was transformed with the COS cDNA library as described (Papdi, 2008), (Rigo, 2012). For the transformation, 25 mL of cell suspension was infected with *Agrobacterium* culture of the COS cDNA library, and co-cultivated for 2 days under continuous shaking. Subsequently, cells were collected by centrifugation, washed with and subcultured in liquid culture medium supplemented by 400 mg/L Claforan and 15 mg/L hygromycin (Duchefa). Transformed cell cultures were subcultured at weekly intervals with 10x volume of fresh culture

medium. To screen for salt tolerance, the hygromycin resistant cell culture was plated onto 100 Petri dishes (150 mm diameter) containing agar-solidified callus culture medium (Mathur, 1995) supplemented with 400 mg/L Claforan, 5 μM estradiol and 175 mM NaCl. Transformation efficiency was estimated by plating an aliquot of transformed cell suspension on medium supplemented with 400 mg/L Claforan and 15 mg/L hygromycin. After 3 weeks of culture, growing microcalli were split and moved to fresh agar plates containing 175 mM NaCl, with or without 5 μM estradiol. Calli growing on estradiol-containing plates were used for rescuing cDNAs carried by the T-DNA inserts.

2.4 Molecular methods

2.4.1 Gene cloning, vector construction

Gene cloning was performed using the Gateway cloning System (Invitrogene) or FastDigest restriction enzymes and T4 DNA ligase (Thermo Scientific) following the manufacturer instructions. cDNA inserts from transgenic COS lines were PCR-amplified using genomic DNA template with the ER8A and ER8B primers and Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Identity of the cDNA inserts was determined by sequence homology searches in the TAIR database (www.arabidopsis.org). PCR fragments were cloned into pDONR201 using the Gateway BP clonase reaction, sequenced and moved into the binary vector pER8GW (Papdi, 2008) or into pH2GW7.0 (Karimi, 2007) by Gateway LR clonase reaction generating the estradiol-inducible pER8GW-HSFA4A and constitutive 35S-HSFA4A constructs respectively. The *HSFA4A* (AT4G18880) promoter was cloned by amplifying the *HSFA4A* promoter region of 2 kb and cloning it into the pENTRY-BS vector as a *EcoRI-BamHI* fragment (pENTRY-BS vector was constructed using backbone of pENTR/D-TOPO (Invitrogene) in which MCS of pBluescript II (Stratagene) was cloned (kindly donated by T.Sarnowski, Laboratory of Plant Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw). The pHSFA4A-GUS vector was generated by moving the promoter

fragment into the binary vector pHGWFS7 (Karimi, 2007) using Gateway LR clonase reaction.

pPCV-YFP-H vector was constructed from the modified pPCV812 vector containing the 35S promoter:mGFP4:NOS cassette (Kircher, 2002). The *YFP* cDNA was PCR-amplified from the pEYFP plasmid template (Clontech) with unique *SmaI* and *SacI* sites at the 5' and 3' termini, respectively. pPCV-YFP-H was produced by replacing the *mGFP4* gene with the amplified YFP fragment in the modified pPCV812 vector via *SmaI* and *SacI* sites. To generate the 35S-HSFA4A-YFP construct, the full-length *HSF4A4* cDNA without the stop codon was cloned into the expression vector pPCV-YFP-H as a *BamHI-SmaI* fragment, generating a *HSFA4A* gene 3' fusion with the YFP coding sequence.

BiFC constructs were prepared by cloning the *HSFA4A* cDNA into the *HindIII* and *SmaI* sites of pSAT1A-nEYFP-N1 and pSAT1A-cEYFP-N1 vectors (http://www.bio.purdue.edu/people/faculty/gelvin/nsf/index.htm). For *Agrobacterium*-mediated transient expression, BiFC constructs were subcloned as *HindIII-NotI* fragments into pENTRY-BS and moved into pH2GW7.0 and pK2GW7.0 (Karimi, 2007) by LR clonase reaction.

Site directed mutagenesis of *HSFA4A* cDNA was performed using a two-step PCR amplification method as described (Brons-Poulsen, 1998). To modify conserved Cys residues, a single point mutation into position C229A was introduced using a combination of HsfA4A-5'(Hind III) and 3'C229_Hsf_R primers; the resulting mega primer was used with HSFA4A-3' (SmaI)_ noSTOP primer to perform the second step PCR. The mutagenized DNA fragment was cloned into the *HindIII* and *SmaI* sites of pENTRY-BS and sequenced. Further point mutations were consecutively introduced into positions C267A and C295A using the generated constructs as PCR templates. Finally, a PCR product containing all three point mutations in the *HSFA4A* coding sequence (mHSFA4A) was cloned into BiFC vectors pSAT1A-nEYFP-N1 and pSAT1A-cEYFP-N1 as a *HindIII-SmaI* fragment. To generate the mutant version Ser309Ala-HSFA4A, a single point mutation was introduced in this position, and the PCR product was cloned into the *HindIII* and *SmaI* sites of pSAT1A-cEYFP-N1.
Mass spectrometry-identified phosphorylation sites were confirmed by introducing point mutations into S198A, T238A, S239A, S309A and T396A following two-step

PCR amplification method. Six individual constructs were generated and cloned into the *BamHI* and *HindIII* sites of pMAL-C2 (NEB), including all combinations of quadruple point mutations and a quintuple mutant.

A PCR fragment of 1100 bp containing the promoter region of *HSP17.6A* (*AT5G12030*) was cloned into pPCV-LUC+ (Toth, 2001) vector as an *EcoRI-SalI* fragment to generate the pHSP17.6A–LUC reporter gene construct.

MPK3 (AT3G45640*)* and *MPK6* (AT2G43790*)* cDNAs were PCR amplified using Arabidopsis (Col-0) cDNA as template, cloned into the bacterial expression vector pET-28c(+) (Novagen) as *BamHI-SalI* fragments and sequenced, generating the 6xHis-MPK3/6 constructs.

To generate the 6xHis-HSFA4A construct, the corresponding cDNA was cloned into the bacterial expression vectors pET-28a(+) as *BamHI-HindIII* fragment. MBP-HSFA4A construct was prepared cloning *HSFA4A* cDNA into the *BamHI* and *HindIII* sites of pMAL-C2 plasmid . To generate yeast two hybrid constructs, *MPK3*, *MPK6*, *HSFA4A* and m*HSFA4A* coding sequences were cloned into both pGAD424 and pGBT9 vectors (Clontech) as *BamHI-SalI* fragments.

2.4.2 Analysis of gene expression

Total RNA was isolated from plant tissues using the Tri-reagent method (Chomczynski, 1987). 100 mg plant material were ground in liquid nitrogen, homogenized with 1 ml Tri-reagent (Sigma) and centrifuged at 13000 rpm 4ºC for 10 minutes. 0.2 ml chlorophorm:isoamyl alcohol (24:1) were added to the supernatant and organic and aqueous phases separated by centrifugation at 13000 rpm 4ºC for 15 minutes. The RNA contained into the aqueous phase was precipitated with 0.5 volume of isopropanol and 0.5 volume of HSPB (0.8M sodium citrate, 1.2M NaCl) the precipitate was collected by centrifugation at 13000 rpm 4ºC for 15 minutes, washed with 70% ethanol and resuspended in 20 µl water. RNA quality was assessed by agarose gel electrophoresis and RNA concentration was determined on the basis of absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific). Contaminating genomic DNA was eliminated by DNAse I treatment (Ambion).

cDNA templates were generated from DNase-treated total RNA (1 μg) samples by reverse transcription using The High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). The cDNA sysnthesis protocol was performed using a thermal cycle following the protocol: 10 minutes at 25ºC, 120 minutes at 37ºC and 5 minutes at 85ºC. The resulting cDNA templates were diluted 1/20 before expression analysis.

Transcript levels were monitored by real time (qRT-PCR) and semi-quantitative RT-PCR analyses. Real time RT-PCR reactions were prepared with 10 μL diluted cDNA, 12.5 μL Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) and 0.5 μL of each forward and reverse primer, and ran employing the following protocol: denaturation 95 ^{o}C/10 min, 40 cycles of 95 ^{o}C/10 sec and 60 ^{o}C/1 min, with ABI PRISM 7700 sequence detection system (Applied Biosystems).

Semi-quantitative PCR reactions were performed in 50 μL volume, using 2 μL diluted cDNA template and Taq DNA Polymerase (Thermo Scientific) employing the following protocol: denaturation 94 ^{o}C/2 min, 30 to 40 cycles of 94 ^{o}C/30 sec, 57 to 62 ^{o}C/30 sec, and 72 ^{o}C/30 min. *TUBULIN2* (*AT5G62690*) and *GAPC2* (*AT1G13440*) genes were used as internal references.

2.4.2.1 RNA-Seq analysis

For transcript profiling and gene expression analysis 2 weeks old Col-0 and HSF4ox2 plants were treated with 5 μM estradiol in the absence or presence of 1 mM H_2O_2 for 6 hours in liquid 0.5 MS medium. Total RNA was isolated using RNeasy kit (Qiagen). Total RNA (10 μg) was DNase I-treated with Turbo DNase (Ambion), precipitated with sodium acetate/ethanol and resuspended in nuclease-free water. RNA-Seq analysis was performed by the sequence laboratory service from BRC (www.brc.hu/services_sequencing_laboratory.php).

Gene ontology analysis was performed with GO Annotation Search tool of the TAIR database (www.arabidopsis.org/tools/bulk/go/index.jsp) and g:Profiler (www.biit.cs.ut.ee/gprofiler/gconvert.cgi) (Reimand, 2011). Co-expression analysis and

clustering was made with Genevestigator (www.genevestigator.com/gv) (Zimmermann, 2004). Identification of predicted promoter elements was done with AthaMap (www.athamap.de) (Steffens, 2005) and Promomer (bar.utoronto.ca) (Toufighi, 2005) tools.

2.5 Yeast two-hybrid assay

For yeast two-hybrid (Y2H) analyses, *Saccharomyces cerevisiae* PJ69-4a (James, 1996) was co-transformed with pGAD424 and pGBT9 vectors carrying the cloned inserts as described (James, 1996). Transformants were grown on appropriate drop-out media to select for transformed cells (-Leu, -Trp) and then transferred to selective media to monitor the activation of the *ADE2* and/or the *HIS3* reporter genes in the presence of 1 mM 3-aminotriazol (-Leu, -Trp, -His, +3AT and -Leu, -Trp,-His, -Ade, +3AT selection plates).

2.6 Protein localization and detection

In vivo microscopic observations were made with Olympus FV1000 confocal laser-scanning microscope. For transient expression, Arabidopsis protoplasts were isolated from cell suspension and PEG-mediated transformation was performed as described (Mathur, 1995). Typically, 10^5 protoplasts were transformed with 20 μg plasmid DNA of 35S-HSFA4A-nYFP and 35S-HSFA4A-cYFP constructs or their mutant versions, and cultured for 24 hours prior fluorescence observation or western blot analysis. The average value of YFP intensity/area of mHSFA4A-n/cYFP expressing protoplasts was normalized to HSFA4A-n/cYFP expressing cells, measuring at least 20 cells for each BiFC combination. Every experiment was repeated 3 times.

For western blot analysis, protoplasts transformed with 35S-HSFA4A-cYFP or 35S-mHSFA4A-cYFP were harvested and boiled with 1 x SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 2.5 % SDS, 0.002 % Bromophenol Blue, 0.7135 M (5%) β-

mercaptoethanol, 10 % glycerol). Protein samples were separated by 10% SDS-PAGE and western blotting was performed as described (Zsigmond, 2008), using anti-GFP mouse monoclonal antibody (Roche) for the detection of cYFP-tagged proteins.

BiFC assays in *N. benthamiana* were performed as described (Lumbreras, 2010). Tobacco leaves were transfected with equal volumes of combined *Agrobacterium* solutions carrying 35S-HSFA4A-n/cYFP and 35S-MPK6-n/cYFP or 35S-MPK3-n/cYFP or 35S-n/cYFP (control) constructs. YFP-derived fluorescence was monitored 24 hours after infiltration. Stress treatments were performed by application of saline solution (100 mM NaCl in 0.5 MS medium) on immobilized infiltrated leaves. Fluorescence was monitored in 20 minutes intervals in the same microscopic field. Reconstituted YFP signal intensities were measured with the ImageJ software.

2.7 *In vitro* and in-gel kinase assays

The kinases (6xHis-MPK3 and 6xHis-MPK6) and the substrate proteins (6xHis-HSFA4A, MBP-HSFA4A, and MBP-tagged mutant versions of HSFA4A) were expressed in *E. coli* strain BL21(DE3) Rosetta (Novagen), and purified by affinity chromatography on Ni-NTA affinity (Qiagen) or amylose resin (NEB) following the manufacturer's instructions. *In vitro* phosphorylation assays with purified 6xHis-MPK3 or 6xHis-MPK6 and MBP-HSFA4A, MBP-tagged mutant versions of HSFA4A, MyelinBP (Sigma), or the maltose-binding protein tag (MBP) were carried out with 1 µg 6xHis-MPK3/6 in 20 µl kinase buffer (25 mM Tris-HCl [pH 7.5], 20 mM $MgCl_2$, 1 mM DTT and 185 kBq [γ-32P]ATP) containing 2 µg MBP-HSFA4A constructs, 2 µg MyelinBP or 2 µg MBP as substrate at 24°C for 30 min. Reactions were boiled with SDS sample buffer, and proteins were size separated in 12% SDS-PAGE. Gels were stained with PageBlue Protein Staining Solution (Thermo Scientific), dried and subjected to autoradiography (Nguyen, 2012). In-gel kinase assay and MPK3/6 protein immunoprecipitation were performed as previously described (Lumbreras, 2010). 50 µg of crude plant proteins and MPK3 and MPK6 precipitates were extracted from wild type plants (Ler) and loaded onto an SDS-10% polyacrylamide gel containing 0.5 mg/mL 6xHis-HSFA4A. The control polyacrylamide gel was assayed without substrate.

2.8 Identification of HSFA4A Phosphorylation Sites

MBP-HSFA4A was phosphorylated by 6xHis-MPK3 in a kinase reaction performed in the presence of 200 µM cold ATP. As control, a parallel reaction without kinase was included. Samples were resolved by SDS-PAGE as described and MBP-HSFA4A protein bands were excised after gel staining with PageBlue Protein Staining Solution. Mass spectrometry was performed by the proteomics service of the BRC (www.szbk.u-szeged.hu/services_proteomics_research.php).

2.9 Transient Expression Assays

Protoplasts were transformed with 10 µg plasmid DNA of pHSP17.6A–LUC construct and 20 µg plasmid DNA of 35S-HSFA4A-cYFP or 35S-S309AHSFA4A-cYFP. When indicated, 10 µg of pK2GW7-mycMKK4^{EE} were included. All transformations were performed in triplicates. After 16 hours culture, protoplasts were transferred to a dark 96-well microtiter plate, sharing each single transformation into two wells containing 3 mM d-luciferin (Biosynth AG) solution. Luciferase activity was determined with an automated luminometer (TopCount NXT; Perkin-Elmer) for 24 hours as described (Palagyi, 2010).

AIMS

The aim of this project was establish a gene discovery system that is suitable for identification of novel regulators of plant stress responses and characterize one of the identified genes.

- Transformation of Arabidopsis cell suspension with an estradiol-inducible random cDNA library.
- Screening of the transformed cell suspension-derived microcolonies for enhanced stress tolerance in the presence of estradiol, i.g. growth on media supplemented by salt.
- Identification of the cDNAs carried by the stress-tolerant microcolonies.
- Confirmation of stress tolerant growth by re-transforming cell-suspension with the identified cDNA.
- Functional characterization of one of the identified genes.
    - Generating Arabidopsis lines overexpressing the identified cDNA and testing their tolerance to stress conditions (salt, osmotic, oxidative sress).
    - Characterization of Arabidopsis mutant lines, in which the identified gene is inactivated.
    - Identify molecular function of the encoded protein, using "*omics"* approaches (transcriptome analysis, metabolomics and proteomics).
    - Describe new signalling pathway regulating stress responses.

3 RESULTS

3.1 Arabidopsis cDNA library screening in cell cultures

An estradiol-inducible Arabidopsis cDNA expression library was previously created in our laboratory (Papdi, 2008). As indicated in **Figure 3.1.1**, the cDNA library was cloned into the plant transformation vector pER8GW using Gateway technology (inserted between attR1 and attR2 sites). The T-DNA sequence contained the XVE inducible system (Zuo, 2000), in which the *XVE* fusion gene was constitutively expressed, and encoded a chimeric transcription factor containing the DNA-binding domain of the bacterial repressor LexA and the C-terminus region of the human estrogen receptor (hER). Separated by a hygromycin resistance marker, a second transcription unit contained several copies of the LexA operator sequence fused to a 35S minimal promoter, which controlled the expression of the cDNA library. The 3'end of the cDNA insert was followed by a transcription terminator sequence 3'A (the RuBisCO small subunit poly(A**)** adenylation sequence (*rbcsS3A*)). In the previous work the Arabidopsis cDNA library was introduced into *Arabidopsis thaliana* wild type plants by Agrobacterium-mediated DNA transfer. Using the *Controlled cDNA Overexpression System* (COS) in these genetic screens, several candidate genes were identified which were involved in plant stress responses (Papdi, 2008).

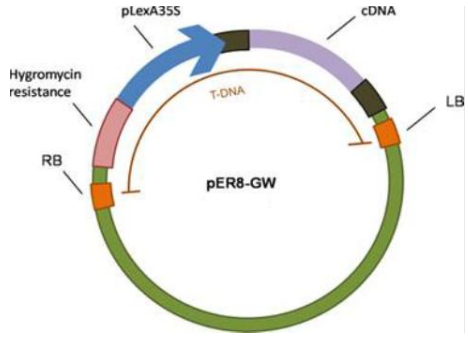

Figure 3.1.1.Schematic map of the cDNA library in pER8GW vector.
Hygromycin resistance: *hygromycin phosphotransferase* (*hpt*) gene, pLexA: LexA operator fused to a minimal promoter of *Cauliflower Mosaic Virus 35S* gene, cDNA: randomly inserted cDNA, RB and LB: T-DNA left and right border sequences, respectively. After (Papdi, 2008).

In the present work, the same genetic tool was utilised, however, an alternative screening strategy was employed. As the performance of genetic screens in plants is usually costly and time-consuming, a system that would reduce these disadvantages associated with making transgenic plants was developed. With this purpose, a root-derived Arabidopsis cell culture was transformed with the COS library, and employed to run the screen. A schematic representation of the screening strategy is shown in **Figure 3.1.2**.

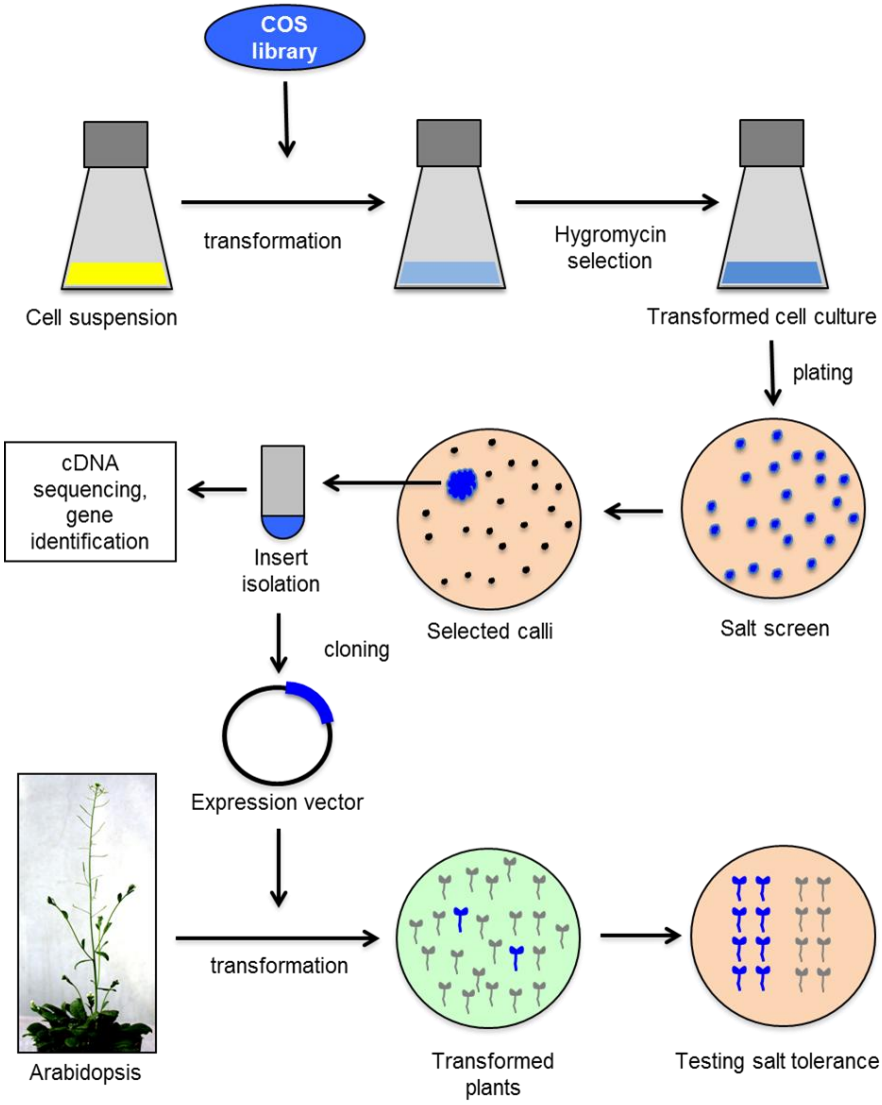

Figure 3.1.2. Schematic illustration of COS screening and cloning strategy of the present work. Arabidopsis cell suspension was transformed with the COS cDNA library. Salt tolerant calli were selected in the presence of 5 μM estradiol and used for DNA isolation and identification of cDNAs carried by the COS expression vector T-DNA insertions. The identified cDNA was cloned into the plant expression vector pER8GW, and transformed into Arabidopsis to verify its capacity to improve salt tolerance in plants.

As **Figure 3.1.2** shows, the estradiol-inducible cDNA library was introduced into root-derived cell suspension via Agrobacterium-mediated infection. Subsequently, the transformed cell culture was transferred onto agar-solidified plant media containing 180 mM NaCl and 5 μM estradiol. Growing microcalli were transferred to fresh media supplemented with salt and with or without estradiol, to test the estradiol-dependent salt tolerance (step not shown in **Figure 3.1.2**). Selected calli were used for rescuing the inserted cDNAs, which were sequenced and re-cloned into the pER8GW vector. The final purpose was to confirm at the whole plant level the salt tolerance phenotype observed on the selected calli. With this intention, cloned cDNAs were introduced into *Arabidopsis thaliana* plants that were subjected to further stress tolerance assays.

The transformation experiment yielded $5x10^5$/L transformed microcolonies and three independent screens were performed to identify salt tolerant microcalli. The results of the transformation with the COS library are shown in **Table 3.1.1**. All together, 1.2 million hygromycin resistant transformed cell colonies were screened for salt tolerance. Of these, 291 microcalli showed various extent of viability and growth in the presence of 180 mM NaCl and 5 μM estradiol, and were subcultured on salt-containing medium with and without estradiol. **Figure 3.1.3A** shows an image of a typical microcolony identified on the salt screen. Finally, four transformant colonies were identified as strictly estradiol-dependent salt tolerant, as they continued to grow in the presence of estradiol but decayed on salt medium without estradiol (**Table 3.1.1**). **Figure 3.1.3B** and **3.1.3C** illustrate the growth of the identified colony 1 in the absence (**B**) and in the presence (**C**) of estradiol, and in **3.1.3D** and **3.1.3E** growth of wild type cell culture in the same conditions can be observed**.**

Experiment	No. of colonies	Selected microcalli	Calli with sustained growth
1	500000	93	2
2	540000	119	2
3	190000	79	0
all	**1230000**	**291**	**4**

Table 3.1.1. Summary of results of cell suspension transformation with the COS library.

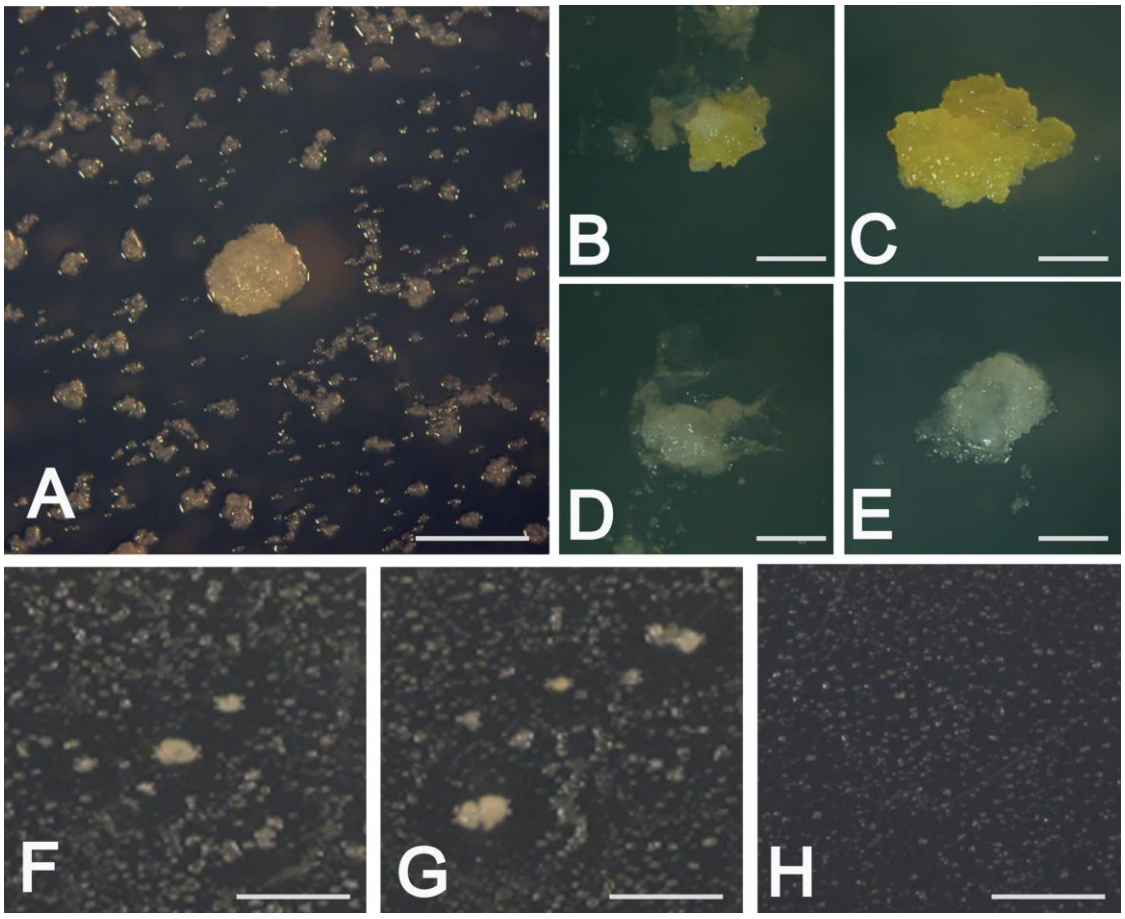

Figure 3.1.3. Identification of salt tolerant Arabidopsis calli by transformation of cell culture with the COS library and subsequent screening on high salt medium.

(A) Screening of COS-transformed cell culture on 0.5 MS medium containing 180 mM NaCl and 5 µM estradiol. **(B, C)** Growth of identified colony 1 on salt medium without (B) and with estradiol (C). **(D, E)** Wild-type Arabidopsis calli are unable to grow on salt medium in the absence (D) or presence (E) of estradiol. **(F, G)** *HSFA4A*-transformed Arabidopsis colonies growing on salt medium with 5 µM estradiol. **(H)** *HSFA4A*-transformed colonies are unable to grow on salt medium in the absence of estradiol. Bar indicates 5mm.

Next, the cDNAinserts from the COS expression vector T-DNAs were isolated by PCR amplification using DNA samples prepared from these salt tolerant transformants. Two transformants (colonies 1 and 7) carried two T-DNA inserts with full-length or truncated cDNAs encoding *NIT2* (*NITRILASE 2*), *HSFA4A* (*HEAT STRESS TRANSCRIPTION FACTOR A-4A*), *CB5-D* (*CYTOCHROME B5 ISOFORM D*) and *MCM2* (*MINICHROMOSOME MAINTENANCE 2*), whereas cDNAs of single COS vector inserts in the other two lines derived from the *XTH11* (*XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 11*) and *GSTF9* (*GLUTATHIONE S-TRANSFERASE PHI 9*) genes. In **Table 3.1.2** the identified cDNA inserts are described.

Callus no.	Fragment size	Accession no.	Encoded protein	Inserted cDNA
1.1	1,9kb	*AT4G18880*	Heat shock transcription factor A4A	Full length
1.2	1,4kb	*AT3G44300*	Nitrilase 2	Full length
7.1	0,8kb	*AT5G48810*	Cytocrome b5 D	Full length
7.2	1,0kb	*AT1G44900*	Minichromosome maintenance 2	Truncated
9	0,5kb	*AT3G48580*	Xyloglucan endotransglucosylase/hydrolase 11	Truncated
14	0,8kb	*AT2G30860*	Glutathione S-transferase PHI 9	Full length

Table 3.1.2. Arabidopsis cDNAs identified in COS-transformed cell cultures showing enhanced growth on high salt medium in the presence of estradiol.

As we were primarily interested in the identification of stress related transcription factors, we focused on studying *HSFA4A*, which was a yet uncharacterized member of the Arabidopsis HSF family. The cDNA sequence of *HSFA4A* is shown at **Figure 3.1.4**. The original cDNA identified in the cell suspension screen was full length and had no errors in its sequence according to a query performed by the Arabidopsis Information Resource (TAIR).

GCTGCTTGAAATTCTCTTGTCTGATTAAAATATATATACCCAAGTCCAGAAGCTGTTTAGGTTCGAGAAGCAGAGAGGGTTCGAGAAG
CTAATAAGGGTTTCTTCTTTTTGATTTTAATGCTAAAAGGGTTCTAGATTCGTTGAATTTTACAAGGGTTTTAGGGGTTCTTAGAAGC
TTTTGCTTGATTGTCTTTTATTTAGAAACAGTGGTGAGTTTTTAGTCTTTCACTTTGTTCAAGTTCGAAGCTTTTTTTGGAGGGAATT
TTGGGCTTCTGATTTTGATCGAAACTTACTGATAGTAAGTTCTTTGAGTCCTCCTTAACTGTAGTTTCTGTGTACTGAAGTTATTGAA
TTGAAAGTTTTTATCTTTTTTGGTTATTGAAACTTTCATAGTTTGATCAAAAGAGTCTCTTGCTCTGTTTTTGGCTCTGTTTTTGTGA
GTGTGATTGTAAGCTTTGTTGTGAGTAGATTGAATCAAGGAGTGTGAGAGTTGTTAAAAGTGTTTTCAGAGATGGATGAGAATAATCA
TGGAGTTTCATCAAGCTCACTTGCACCTTTCCTCACCAAAACATATGAGATGGTTGATGATTCTTCATCCGATTCTATCGTCTCTTGG
AGTCAGAGCAATAAGAGTTTCATCGTTTGGAATCCGCCGGAGTTTTCTAGAGATCTTCTTCCGAGATTCTTCAAGCACAATAACTTCT
CTAGCTTTATCCGCCAGCTTAACACATATGGTTTTAGAAAAGCTGATCCTGAGCAATGGGAATTTGCGAATGATGATTTTGTGAGAGG
TCAACCTCATCTTATGAAGAACATTCATAGACGCAAACCAGTTCATAGCCACTCTTTACCGAATCTTCAAGCTCAGTTAAACCCGTTG
ACGGATTCAGAACGAGTGAGAATGAATAATCAGATTGAGAGATTGACAAAAGAGAAAGAAGGATTGCTTGAAGAGTTACATAAACAAG
ACGAGGAACGAGAAGTGTTTGAGATGCAAGTGAAAGAACTTAAAGAACGATTACAACACATGGAGAAGCGTCAGAAAACAATGGTTTC
GTTTGTTTCTCAAGTATTGGAAAAGCCAGGGCTTGCTTTGAACCTATCGCCGTGTGTTCCCGAAACAAACGAGAGGAAAAGAAGGTTC
CCTAGGATCGAGTTCTTTCCCGATGAACCGATGTTGGAAGAGAACAAAACTTGTGTTGTTGTGAGAGAGGAAGGTTCTACAAGCCCTT
CTTCACACACAAGAGAGCATCAAGTGGAACAGTTAGAGTCATCGATAGCGATTTGGCAGAATCTTGTATCCGATTCTTGTGAGAGTAT
GTTACAATCAAGAAGTATGATGACACTTGATGTGGATGAATCATCTACTTTTCCAGAGAGCCCTCCTCTTTCTTGCATACAGTTAAGT
GTCGATTCACGTCTCAAATCTCCTCCTTCTCCAAGGATCATCGATATGAACTGTGAGCCCGATGGTTCGAAAGAACAGAACACTGTTG
CTGCTCCTCCTCCTCCTCCAGTAGCAGGAGCGAATCATGGCTTCTGGCAGCAGTTTTTCTCAGACAATCCTGGCTCAACCGAGCAACG
GGAAGTTCAATTAGAGAGGAAAGACGATAAAGATAAAGCCGGAGTACGTACTGAGAAATGTTGGTGGAATTCGAGAAATGTTAATGCA
ATTACAGAACAGCTTGGACATCTGACTTCTTCAGAGAGAAGTTGATATGTCAAAGATTAAATTTCTAGTCTGTTTTAGTTACTTGTAA
AATAGGGTTTCTCAGTTTTATTGTTTTCGATTACAGTACTTAGGTATGGTTCAGCTGTTTATTTATCACTTGTATGATCTTTCACAGT
TCATTGTAGCAGACTTCAATGGTAATGATAAGCTAGAGCTTATGGATAGTATTCAT

Figure 3.1.4. Full length cDNA sequence of *HSFA4A (At4g18880)*
Indicated in blue 5' and 3' utr, in yellow the CDS, in red the first methionine and the STOP codon (ATG and TGA). The functional modules are indicated: in lilac HSF DNA-binding domain, in green HSF oligomerization domain (OD) and in dark blue HSF activation domains.

To confirm that the overexpression of *HSFA4A* could improve the salt tolerance at the cellular level, the cDNA in the estradiol-inducible pER8GW vector was re-introduced into Arabidopsis cells. Estradiol-induced expression of *HSFA4A* was sufficient to confer survival and growth of colonies in the presence of salt (**Figures 3.1.3F** and **3.1.3G**), on the contrary, cells failed to proliferate in the absence of estradiol (**Figure 3.1.3H**). These results demonstrated that the overexpression of *HSFA4A* enhances salt tolerance in Arabidopsis cell cultures.

3.2 *HSFA4A* overexpression in Arabidopsis plants

3.2.1 Generation of HSF4ox lines

To test whether *HSFA4A* overexpression could enhance stress tolerance not only at cellular level but also at whole plant level, the estradiol-inducible pER8GW-HSFA4A construct was introduced into Arabidopsis Col-0 plants by Agrobacterium–mediated transformation. Seeds were selected in 20 mg/L hygromycin-containing medium, and the positive transformants were transferred to soil for setting seeds. T2 generation seeds were collected and the presence of the antibiotic-resistance gene was re-tested in selective medium and the segregation was scored. Those lines containing one single insert showing typical 3:1 Mendelian segregation ratio were chosen for expression analysis.

The transcript level of the transgene was checked by semiquantitative RT-PCR. Two-week-old plants were transferred to liquid 0.5 MS media supplemented with 5 μM estradiol, and samples were harvested after 24 hours treatment. Plants kept in liquid medium without estradiol were included as control. The *HSFA4A* expression level was determined by using cDNA-specific primers designed to match sequences located 140 bp upstream and 97 bp downstream of the STOP codon (see **Figure 3.4.1B**). The differential *HSFA4A* gene expression was detected in all the six lines analysed, although transcript levels varied between the different *HSFA4A* overexpressing lines (HSF4ox), (**Figure 3.2.1**.). Lines HSF4ox1 and HSF4ox2 showed the highest level of

overexpression in the presence of estradiol, being the strongest transcript level detected in HSF4ox2. Lines HSF4ox10, 11, 12 and 16 showed a moderate *HSFA4A* transgene induction upon estradiol treatment. No *HSFA4A* transcript was detected in the absence of estradiol after 32 cycles of amplification, indicating that the overexpression of *HSFA4A* in the transgenic lines was strictly estradiol-dependent.

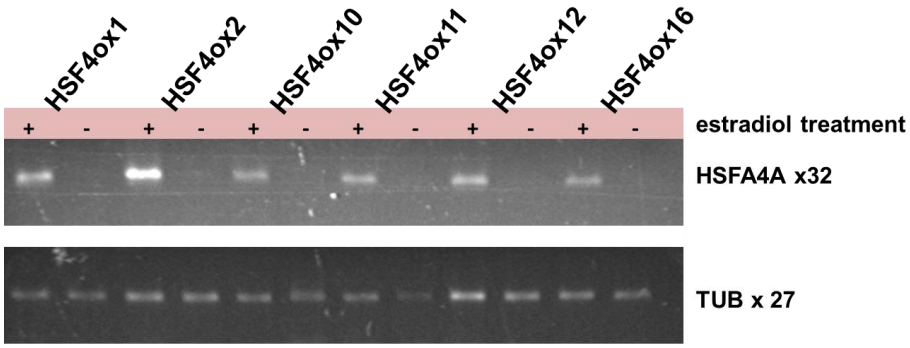

Figure 3.2.1. Estradiol-dependent *HSFA4A* gene expression in HSF4ox lines
Semi-quantitative RT-PCR analysis of transcription of *HSFA4A* in two-week-old plants. Plants were treated with or without 5 μM estradiol for 24 hours prior RNA isolation. As can been observed, *HSFA4A* expression is estradiol-dependent. *TUBULINE2* (TUB) was used as an internal control.

3.2.2 *HSFA4A* overexpression can improve stress tolerance in Arabidopsis

Lines HSF4ox1 and 2 showed the highest *HSFA4A* transcription induction upon estradiol treatment and were therefore selected for further phenotypical characterization. The effect of *HSFA4A* overexpression on growth and stress tolerance of the transgenic Arabidopsis lines was tested in various conditions. Stress assays were performed on Columbia wild type (Col-0), HSF4ox1 and 2 plants. Four-day-old seedlings, germinated on agar-solidified 0.5 MS medium, were sprayed with 5 μM estradiol, and 24 hours later transferred in square petri dishes containing solid medium supplemented with 5 μM estradiol and 100mM NaCl or only with estradiol (control medium). Plates were kept horizontally for rosette growth experiments and vertically, allowing roots to grow on the surface of the medium. Plant growth was monitored during the following two weeks. Overexpression of *HSFA4A* in transgenic lines resulted in 20% reduction of plant growth compared to wild type plants on 0.5 MS media supplemented with 5 μM

estradiol (**Figure 3.2.2A**). However, when grown in the presence of salt and estradiol, the average rosette growth of wild type was more inhibited by salt than the growth of HSF4ox lines (**Figure 3.2.2B**). Similarly, root growth was inhibited by 60% in wild type but only a reduction of 10 to 20% was detected in HSF4ox plants (**Figure 3.2.2C**).

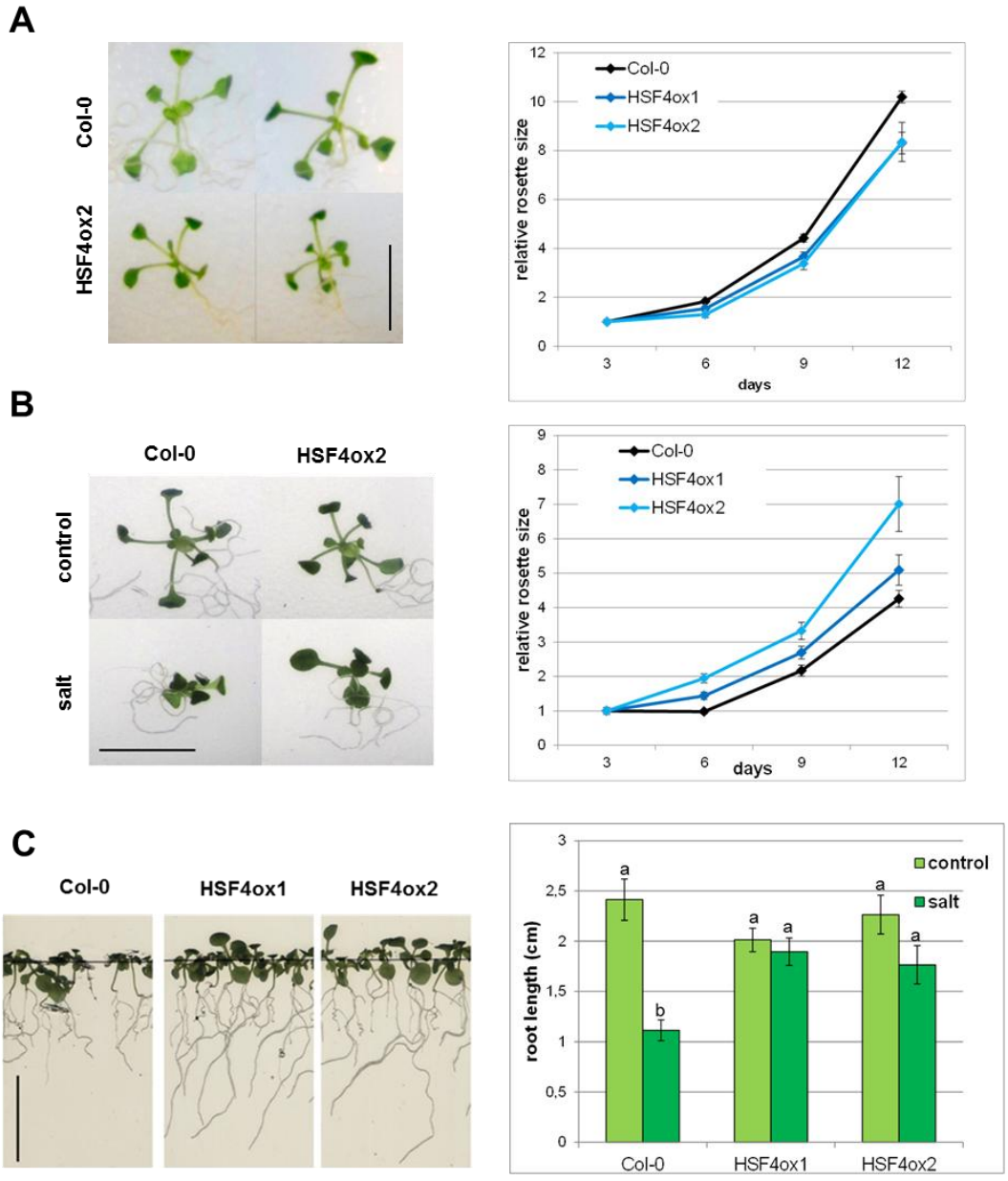

Figure 3.2.2. *HSFA4A* overexpression confers stress tolerance to Arabidopsis
(A) Growth of wild type (Col-0)and *HSFA4A* overexpressing (HSF4ox) plants on 0.5 MS media with 5 µM estradiol. Graph shows growth of relative rosette sizes, calculated from the increase of rosette size in a given time point, relative to the average rosette size of each line at the beginning of the treatment (day 0). **(B)** Image: Rosette growth of Col-0 and HSF4ox plants on control (0.5 MS media with 5 µM estradiol) and salt medium (100 mM NaCl with 5 µM estradiol) for 12 days. Graph shows growth of relative rosette sizes on salt medium. **(C)** Root growth of Col-0 and HSF4ox plants on salt medium for 12 days. Diagram indicates average root lengths after 12 days of growth. Size marker shows 1 cm. Bars indicate standard errors, different letters show significant differences at $p<0.05$ (Duncan-test).

As ROS production and signalling are important components of salt stress acclimation (Miller, 2010), the effects of H_2O_2, paraquat and anoxia on the growth of HSF4ox plants were examined. HSF4ox seedlings grew better and accumulated less anthocyanin compared to wild type on media containing estradiol and either 0.1 or 0.3 µM paraquat, a herbicide that generates superoxide radical anions and promotes anthocyanin accumulation (**Figure 3.2.3A**)(Kytridis, 2006). Similarly, HSF4ox seedlings developed larger rosettes and showed less root damage in the presence of 5 mM H_2O_2 than the wild type (**Figure 3.2.3B**).

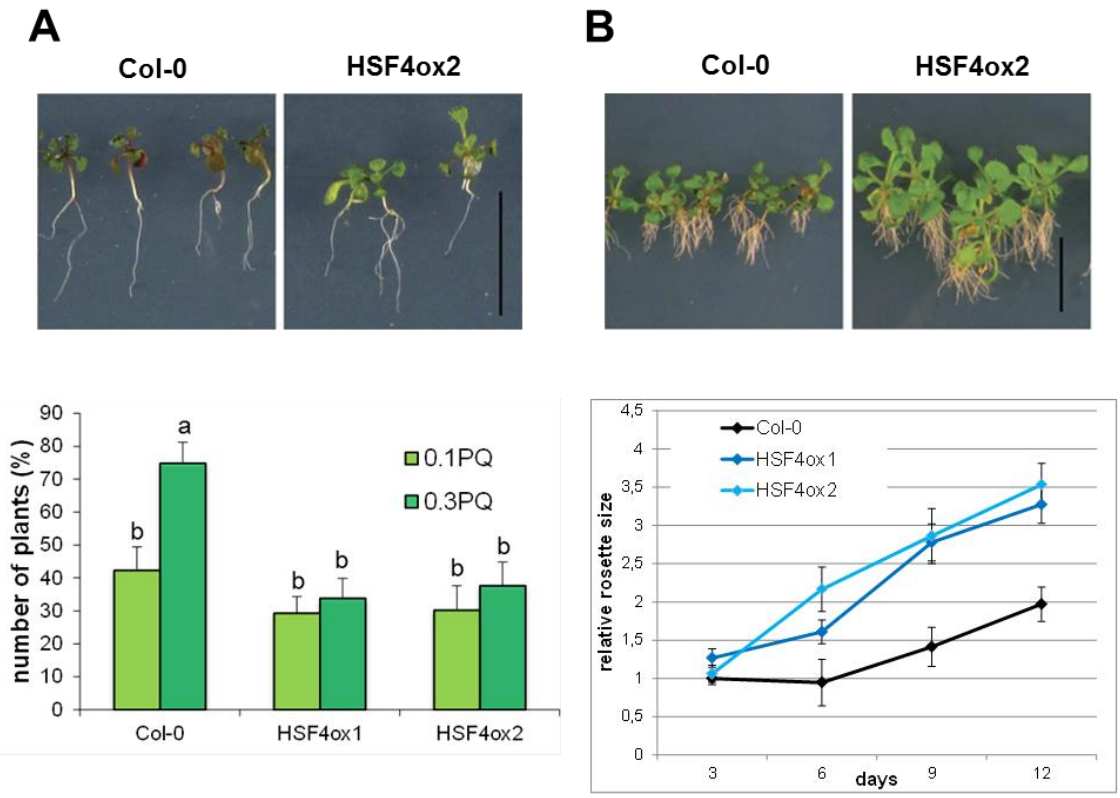

Figure 3.2.3. *HSFA4A* overexpression confers tolerance to direct oxidative stress to Arabidopsis
(A) Paraquat-triggered anthocyanin accumulation in Col-0 and HSF4ox2 plants (12 days on 0.3 µM paraquat with 5 µM estradiol). Diagram indicates the percentage of anthocyanin accumulating plants treated with 0.1 µM or 0.3 µM paraquat for 12 days in the presence of 5 µM estradiol. **(B)** Effect of H_2O_2 on seedling development of Col-0 and HSF4ox2 plants (12 days on 5 mM H_2O_2 with 5 µM estradiol). Relative rosette size graph indicates growth of Col-0 and HSF4ox plants. Size marker shows 1 cm. Bars indicate standard errors, different letters show significant differences at $p<0.05$ (Duncan-test).

Moreover, *HSFA4A* overexpression improved the survival rate under anoxic conditions. Following 8 and 10 hours of anoxia, only 8 and 3% of wild type survived compared to 20 to 25% and 12 to 17% of HSF4ox plants, respectively (**Figure 3.2.4**). To confirm

low oxygen conditions, expression levels of two anoxia-responsive marker genes (*ADH1* and *PDC1*) were tested in wild type plants .

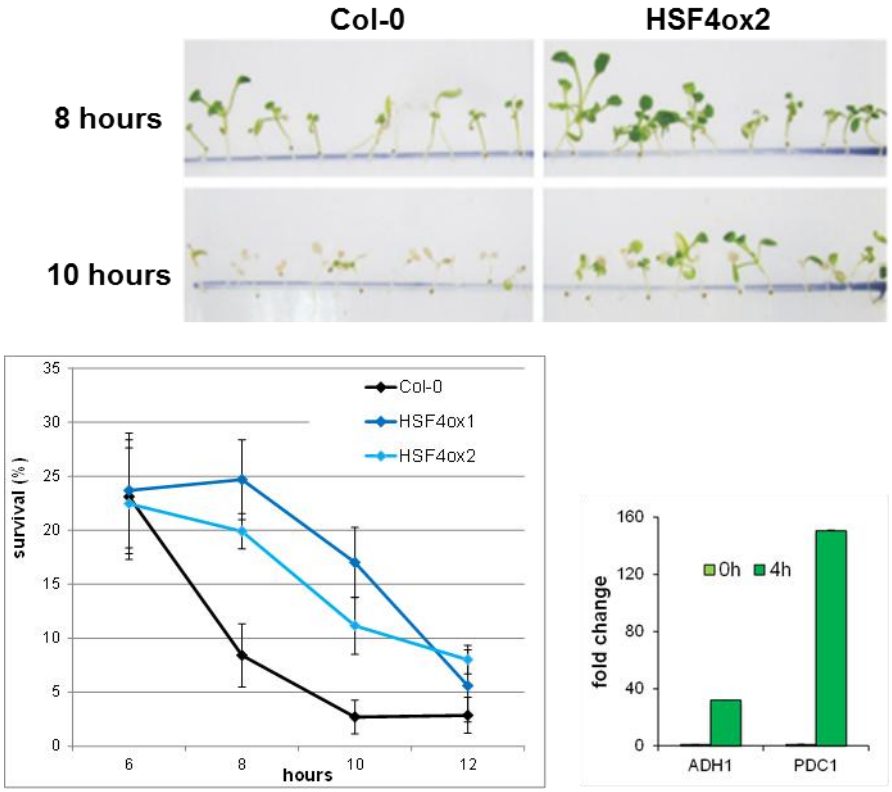

Figure 3.2.4. *HSFA4A* overexpression confers anoxia tolerance
Image shows effect of anoxia on Col-0 and HSF4ox2 plantlets. Seedlings were treated with estradiol, subjected to anoxia, and survival was scored 7 days after recovery. Average survival rates of plantlets after 6, 8, 10 and 12 hours of anoxia is shown on left graph. Expression of anoxia-induced marker genes *ADH1* (*AT1G77120*) and *PDC1* (*AT4G33070*) in anoxia-treated Col-0 seedlings (right graph) in control (0 h) and anoxic (4 h) conditions.

In another set of experiments, Col-0 and HSF4ox plants were grown in the presence of 5 µM estradiol and the following stressors: salt, paraquat, mannitol and cadmium. After 20 days, the fresh weight (FW) was measured, and the relative fresh weight (RFW) was calculated in relation to the FW of plants grown in control medium (**Figure 3.2.5**). In the presence of 100 mM NaCl, the RFW of HSF4ox1 and 2 plants was superior than the RFW of Col-0, and remarkably, the HSF4ox2 line exhibited a lower growth inhibition in comparison to HSF4ox1. In low concentrations of paraquat, RFW of Col-0 was only slightly reduced compared to the growth of HSF4ox lines; nevertheless in the presence

of 0.3 μM paraquat the RFW of wild type plants dropped to 0.2, whereas the RFW levels of HSF4ox plants were around 0.4. Heavy metal toxicity and osmotic conditions caused severe growth inhibition in wild type plants as indicated its RFW value below 0.2, however HSF4ox lines exhibited two times higher RFW in the same conditions.

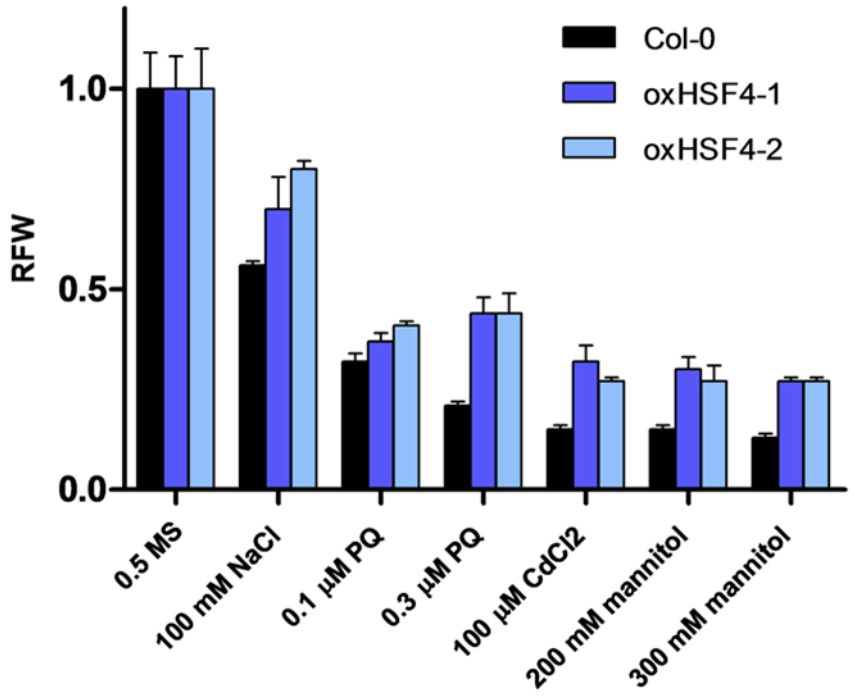

Figure 3.2.5. HSFA4A overexpression confers stress tolerance to Arabidopsis
Relative fresh weight (RFW) of Col-0 and HSF4ox plants grown in the presence of the indicated concentrations of NaCl, paraquat, $CdCl_2$ and mannitol. Diagram shows relative growth rates where 1 equals growth in control media. Bars indicate standard errors.

3.2.3 *HSFA4A* overexpression reduces oxidative damage

Enhanced tolerance of HSF4ox plants to H_2O_2 and paraquat suggested a potential increase of their antioxidant capacity. To assess the degree of oxidative stress and damage, accumulation of H_2O_2 and lipid peroxidation rates were compared in salt-stressed transgenic and wild type plants. Two-week-old plants were sprayed with 5 μM estradiol and 24 hours later were transferred to salt-containing media (0.5 MS media

supplemented with 150 mM NaCl). Control samples were taken before transferring to salt, and stress samples were harvested after 6 and 24 hours NaCl treatment.

In control conditions, hydrogen peroxide levels were similar in wild type and HSF4ox plants (**Figure 3.2.6**) with concentrations of approximately 12 μM H_2O_2. On the contrary, salt treatment greatly induced hydrogen peroxide accumulation in wild type plants, resulting in concentrations of 19 and 21 μM H_2O_2 after 6 and 24 hours treatment respectively. In *HSFA4A* overexpressing plants, salt stimulated H_2O_2 accumulation too, but it only reached maximum concentrations of 16 μM H_2O_2, leading to 20 to 30% lower H_2O_2 content in the transgenic plants.

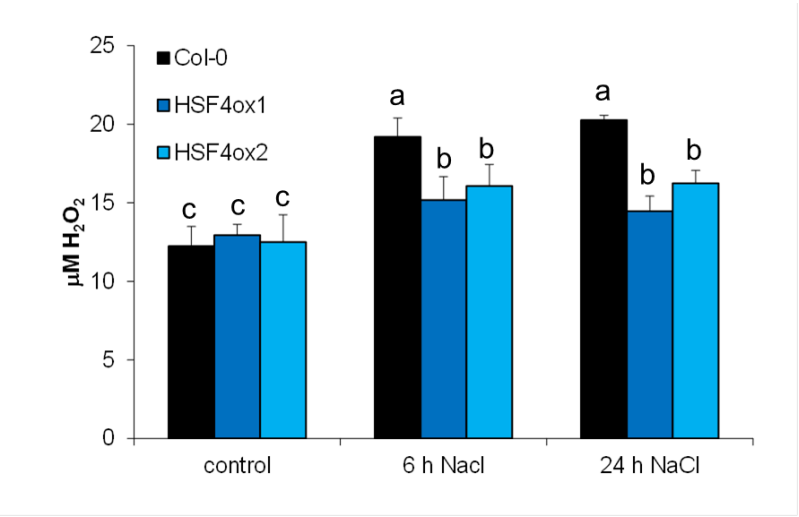

Figure 3.2.6. *HSFA4A* overexpression reduces hydrogen peroxide accumulation during salt stress
H_2O_2 accumulation in Col-0and HSF4ox plants treated with 150 mM NaCl for 6 and 24 h. Two-week-old *in vitro* grown seedlings were used for the experiments. Bars indicate standard errors, different letters show significant differences at $p<0.05$ (Duncan-test). 5 μM estradiol was included in all treatments.

Membrane lipid peroxidation was measured by the accumulation of the end product malondialdehyde (MDA) in stressed plants. After 6 hours salt treatment, MDA levels in wild type plants were elevated by 60%, and by 75% after 24 hours. However, in HSF4ox plants, lipid peroxidation rates increased by 30 to 40% after salt treatment (**Figure 3.2.7**), therefore, analogously to the H_2O_2 content, the stress-induced lipid peroxidation was significantly reduced in *HSFA4A* overexpressing plants.

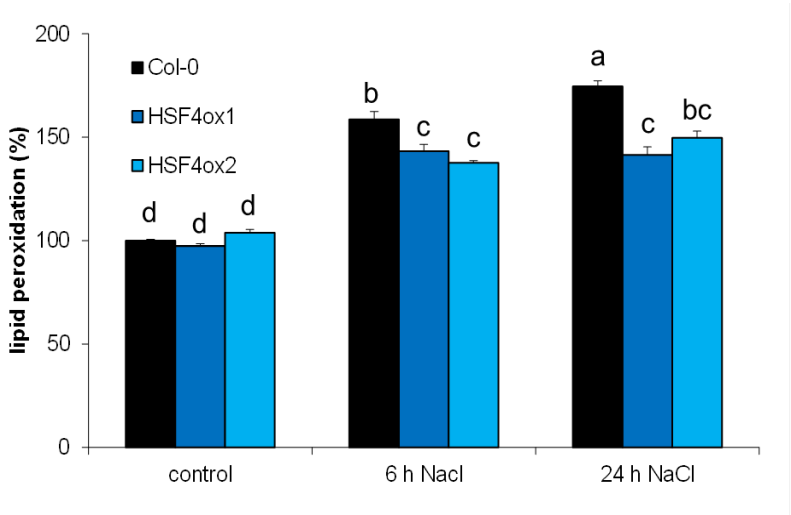

Figure 3.2.7. *HSFA4A* overexpression reduces lipid peroxidation during salt stress
Lipid peroxidation in salt-stressed Col-0 and HSF4ox plants calculated by MDA accumulation. 100% corresponds to lipid damage in Col-0 non-stressed plants. Bars indicate standard errors, different letters show significant differences at $p<0.05$ (Duncan-test). 5 µM estradiol was included in all treatments.

3.3 Transcriptional regulation of HSFA4A

3.3.1 Stress activation of HSFA4A gene expression

Next, I studied how transcriptional regulation of *HSFA4A* was connected to the stress-tolerance phenotype observed in HSF4ox lines. The transcript levels of *HSFA4A* were compared in two-week-old wild type (Col-0) seedlings subjected to different stress treatments: heat stress (37ºC), salt (150 mM NaCl), oxidative stress (1 mM H_2O_2 and 1 mM paraquat) and osmotic stress (300 mM mannitol), and to 0.5 MS plant media as control. All treatments were performed for 1, 6 and 24 hours and *HSFA4A* gene expression was analysed by qRT-PCR, using *GAPDH2* gene as internal reference. Results showed that *HSFA4A* transcription was stimulated by all stress conditions within the first hour, indicating a rapid response (**Figure 3.3.1**). In response to heat-treatment, *HSFA4A* mRNA levels increased to 4-fold within 1 hour and peaked at 6.5-fold higher level at 24 hours. In the other stress conditions, transcript levels peaked at 6 hours reaching the maximum 6 fold change after salt and paraquat treatments. Mannitol-

induced *HSFA4A* expression followed similar expression dynamics, but at lower level. Therefore, transcription of *HSFA4A* was induced by osmotic, salt, oxidative and heat stress, but following different time kinetics.

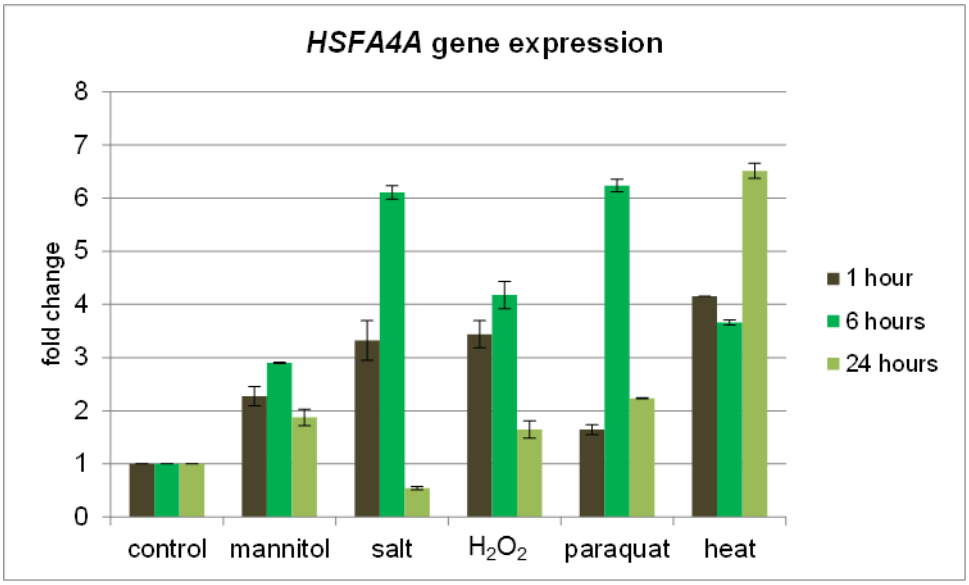

Figure 3.3.1: *HSFA4A* gene expression
Quantitative RT-PCR analysis of *HSFA4A* gene expression regulation by stress in two-week-old Col-0 seedlings. Treatments were performed during 1, 6 and 24 hours, in the presence of control media (0.5 MS), mannitol (300 mM), salt (150 mM NaCl), hydrogen peroxide (1 mM H_2O_2), paraquat (1 mM) or heat (37ºC). Bars show standard deviation.

Gene expression studies were contrasted with the publicly available transcript profiling data obtained from the Arabidopsis eFP Browser (http://bar.utoronto.ca/efp_arabidopsis) AtGeneExpress Pathogen and Abiotic Stress Series (Kilian, 2007). Overall, my qRT-PCR analysis showed correlation with the Affimetrix transcript profiling data. According to the information from the database, steady-state transcript levels of *HSFA4A* were induced by cold, osmotic, salt and UV-B stress (**Figure 3.3.2)**, but only moderately increased by heat-stress. Cold stress activated the transcription of *HSFA4A* after 1 hour, and it reached its maximum 5 fold change after 3 hours. Interestingly, heat stress slightly increased *HSFA4A* transcription which did not match with my results. According to my expression data, heat stress dramatically increased *HSFA4A* transcription within 1 hour (4-fold) and peaked at 24

hours (6,5 fold change). These differences could be attributed to the experimental settings in which expression studies were performed. As described in (Kilian, 2007), heat treatments were carried out at 38ºC for 3 hours and plants were afterwards allowed to recover at 25ºC before harvesting the samples. (Cohen-Peer, 2010) showed that the expression of the homolog *HSFA2* was dramatically induced after 3 hours at 37ºC, but there was no *HSFA2* gene product during the recovery period. Therefore, it is possible that no major changes in the expression of *HSFA4A* are scored after the recovery period following heat stress. Osmotic stress stimulated mRNA accumulation gradually, peaking at 24 hours, and salt induced *HSFA4A* transcription following the same time kinetics as in my results. UV-B light irradiation stimulated the transcript accumulation in green tissues, increasing up to 10 fold change after 3 hours.

Available transcript profiling data indicated that another source of environmental stimuli regulated *HSFA4A*. According to the data generated from the Pathogen Stress Series, *HSFA4A* transcription was stimulated by the bacterial-derived elicitors Flagellin22 and harpin protein HrpZ and moderately increased after *Pseudomonas syringae* infection in comparison to mock. Fungal infection with *Hyaloperonospora arabidopsidis* (Hpa) induced a more long-term response, that progressively increased after six days.

Therefore, as a summary, abiotic stress modulated *HSFA4A* gene expression and these alterations occured fast, within the first hour, which indicated a role for *HSFA4A* as a possible regulator of the early stress response. The expression patterns of *HSFA4A* pointed that this gene could act as a coregulator of the plant response against abiotic stress and pathogens.

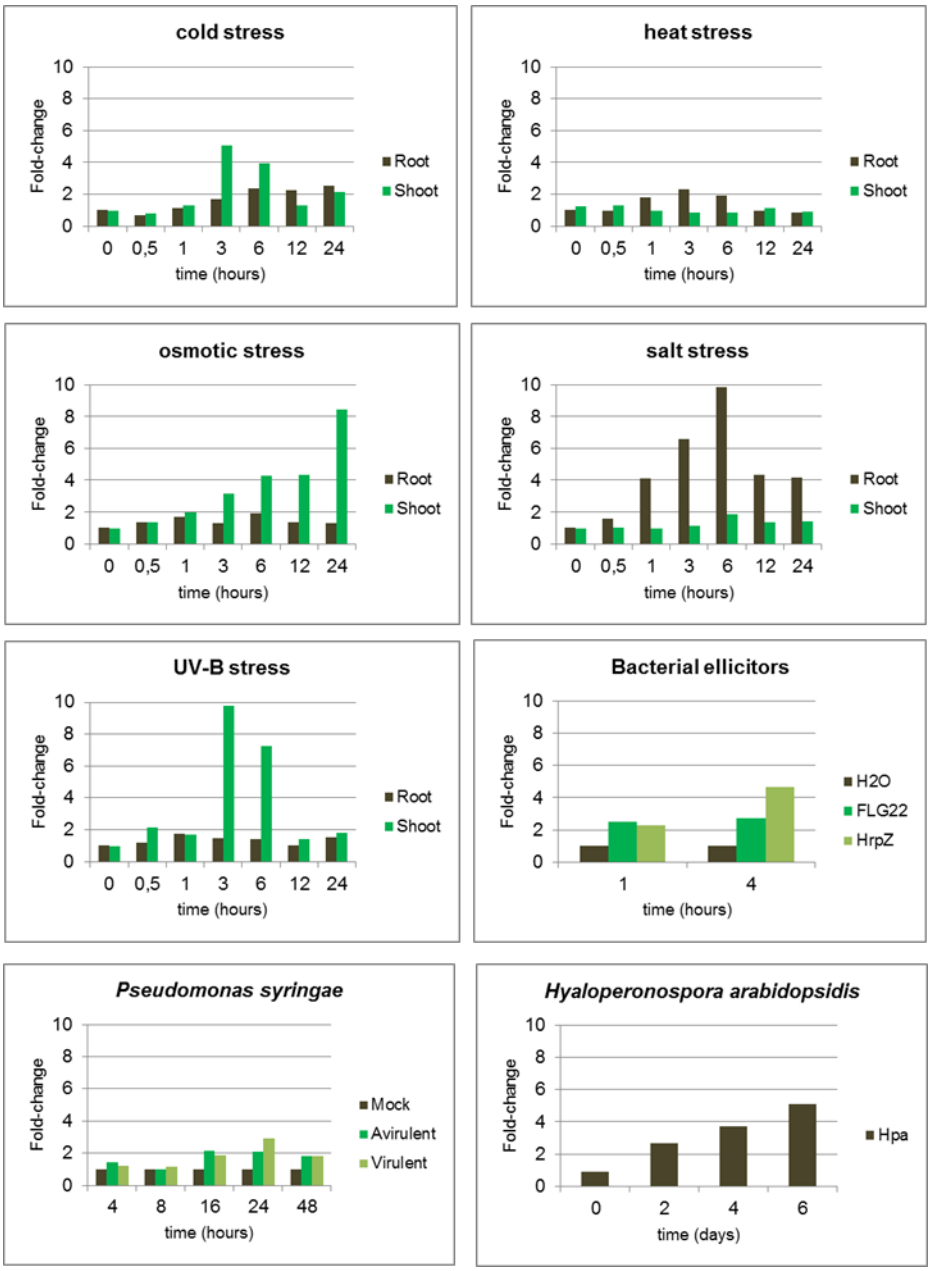

Figure 3.3.2: Transcriptional activation of *HSFA4A* gene by abiotic and biotic stress.
Expression data were extracted from the public database Arabidopsis eFP Browser. The diagrams show both abiotic stress stimulation of *HSFA4A* transcription in shoots and roots, following cold, heat, osmotic, salt and UV-B stress for 24 hours, and the effect of bacterial elicitors (FLG22, HrpZ), bacterial infection (*Pseudomonas syringae*) and fungal infection (*Hyaloperonospora arabidopsidis*) on *HSFA4A* expression.

3.3.2 Spatial expression of *HSFA4A* gene

After analysing how environmental stimuli affect *HSFA4A* gene expression, the spatial expression pattern was analysed during development. For this approach, a genomic fragment from the promoter region of 2039 bp was amplified and fused to the β-

glucuronidase (GUS) reporter gene in the pHGWFS7 vector (Karimi, 2007).Transgenic Arabidopsis plants carrying the pHSFA4A-GUS construct were generated and the reporter gene activity visualized. Histological studies of GUS expression revealed that *HSFA4A* was ubiquitously and constitutively expressed in 12 days-old plants (**Figure 3.3.3**). The p*HSFA4A* promoter was active in the shoot apex and in the hypocotyl-root junction (**Figure 3.3.3B** and **3.3.3C**), as well as in cotyledons and leaves (**Figure 3.3.3D**, **3.3.3E**, **3.3.3F**). In young leaves, pHSFA4A-GUS expression was weak, but it was higher in older leaves. *HSFA4A* promoter activity was detected in the meristematic zones of root tips and in the elongation zones of lateral roots (**Figure 3.3.3G** and **3.3.3H**) and in leaf trichomes (**Figure 3.3.3I**).

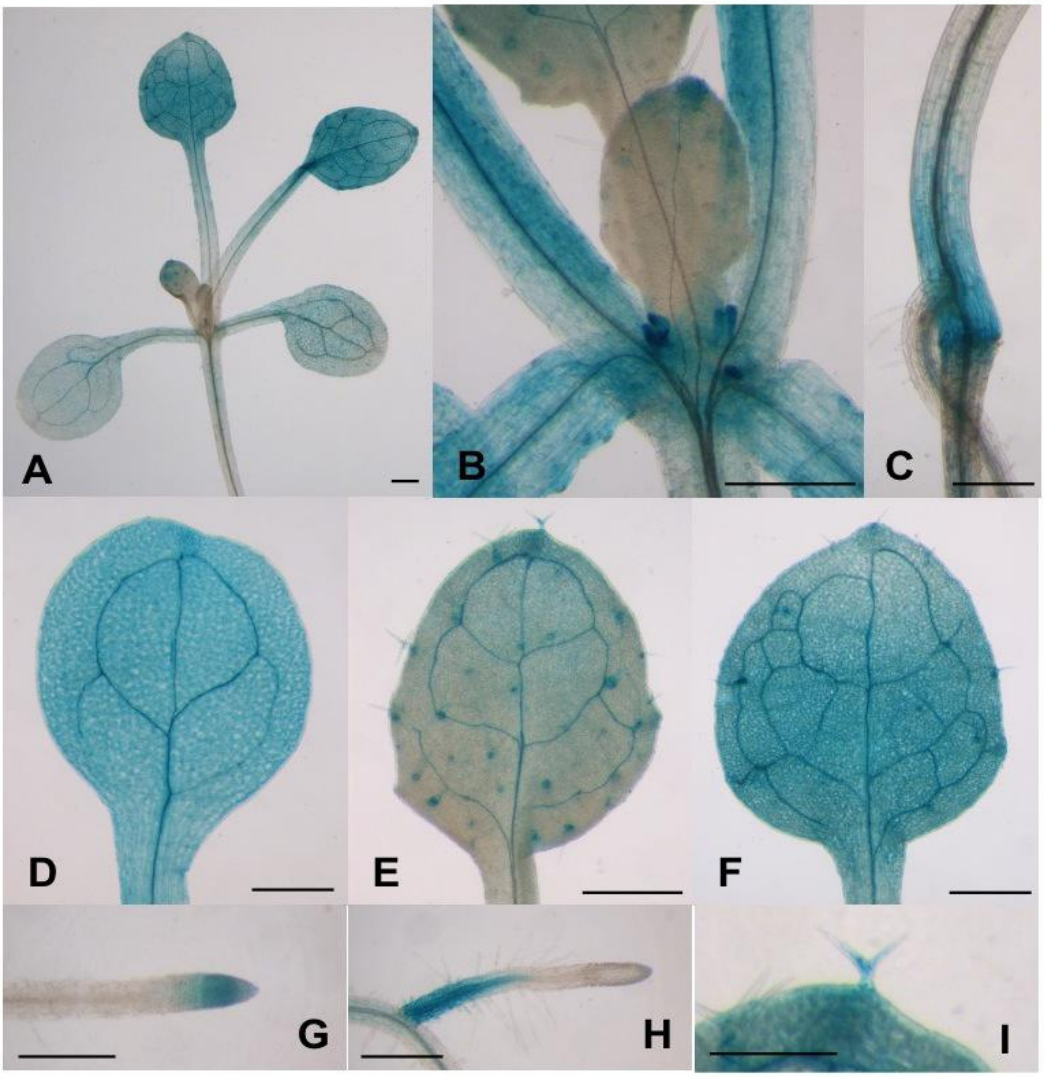

Figure 3.3.3: GUS activity in transgenic Arabidopsis containing pHSFA4A-GUS fusion. Histochemical analysis was performed using 12 days-old plants. Expression in whole seedlings (A), the shoot tip region (B), hypocotyl and root junction (C), cotyledons (D), young leaves (E), mature leaves (F), root tips (G), lateral roots (H) and trichomes (I). Bars indicate 0.5 mm.

Complementary information about spatial *HSFA4A* expression was obtained from an Anatomy dataset of Affymetrix microarrays data stored in the Genevestigator database (www.genevestigator.com). As described in **Figure 3.3.4**, predicted *HSFA4A* expression patterns differed between developmental stages and tissues. In cells, *HSFA4A* was highly expressed, especially in protoplasts. On the contrary, in seedlings, *HSFA4A* was downregulated in cotyledons, imbibed seeds and in the shoot apical meristem, and was only expressed in the hypocotyl. In later developmental stages, *HSFA4A* gene was greatly repressed in the inflorescence, being only expressed in sepals. *HSFA4A* was expressed in the whole rosette, but with different expression patterns. As indicated by my GUS expression studies, *HSFA4A* expression increased from juvenile to adult leaves, and according to the transcript profiling data, it reached the maximum expression level in senescent leaves. In roots, *HSFA4A* expression was more heterogeneous. It was expressed in the root hair zone and in stele and pericycle, but was downregulated in the primary root, root tip and in the meristematic and elongation zones. According to my results, pHSFA4A-GUS was active in the root tip meristem and in the secondary roots, which was supported by the microarray data.

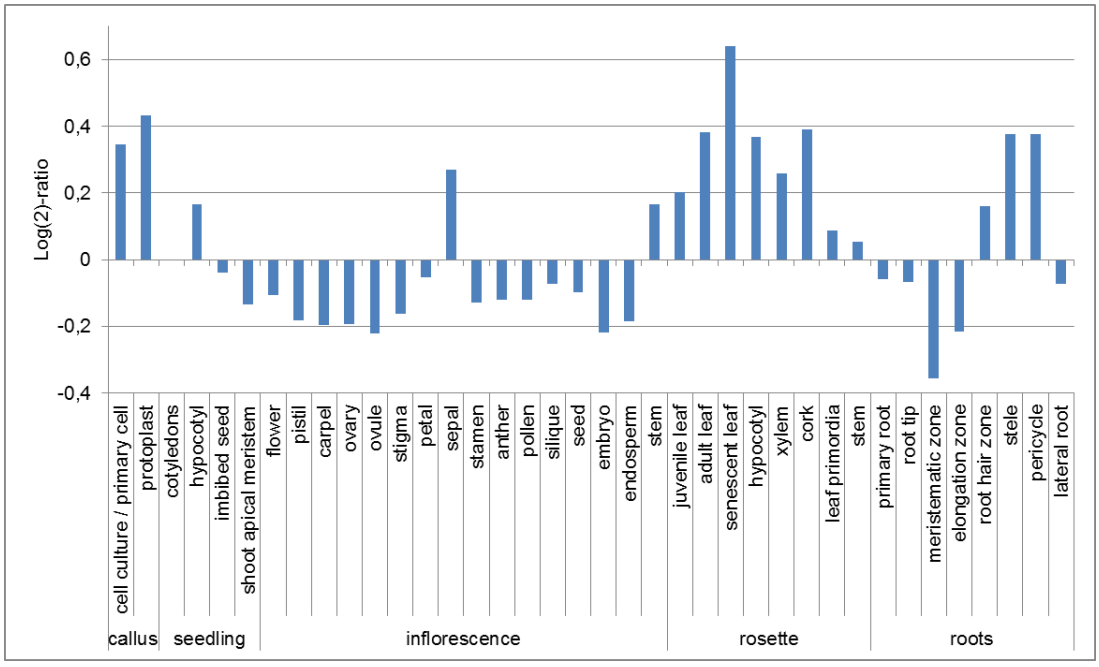

Figure 3.3.4: *HSFA4A* gene expression in different organs (from Genevestigator database)

3.4 Identification of HSFA4A Regulated Genes

3.4.1 Time-kinetics of gene activation

Next, the kinetics of stress-induced *HSFA4A* gene expression and the estradiol-dependent overexpression system were compared. As *HSFA4A* overexpression could confer tolerance to several abiotic stress conditions, and the H_2O_2-dependent *HSFA4A* gene activation was previously demonstrated in wild type plants, I choosed this chemical to perform my studies. Two weeks-old Col-0 and HSF4ox2 plants were transferred to liquid 0.5 MS supplemented with 5 μM estradiol plus/minus 1 mM H_2O_2, having all together four samples: Col-0, Col-0 plus H_2O_2, HSF4ox2, HSF4ox2 plus H_2O_2, all with estradiol. Plant material was harvested in different time intervals, and *HSFA4A* transcript level was determined by semi-quantitative RT-PCR. Expression analysis on Col-0 samples treated with estradiol shown that estradiol did not affect *HSFA4A* gene expression in wild type plants (not shown). **Figure 3.4.1.** shows the time-dependent *HSFA4A* transcript accumulation in three samples: (A) Col-0 plus H_2O_2 (and estradiol), (B) HSF4ox2 plus estradiol and (C) HSF4ox2 plus estradiol and H_2O_2. In all cases, 30 minutes exposition with H_2O_2 or estradiol were sufficient to activate *HSFA4A* gene transcription. Transcriptional activation of endogenous *HSFA4A* gene by H_2O_2 showed a similar time-kinetics as estradiol-induction of *HSFA4A* transcript from the pER8GW overexpression construct but never reached a comparably high level. This confirmed that the expression level of *HSFA4A* transcript in wild-type compared to HSF4ox2 was a limiting factor in mounting tolerance to salt and oxidative stress. Additional activation of *HSFA4A* by H_2O_2 in HSF4ox plants could not be revealed by semiquantitative RT-PCR (**Figure 3.4.1B** and **3.4.1C**).

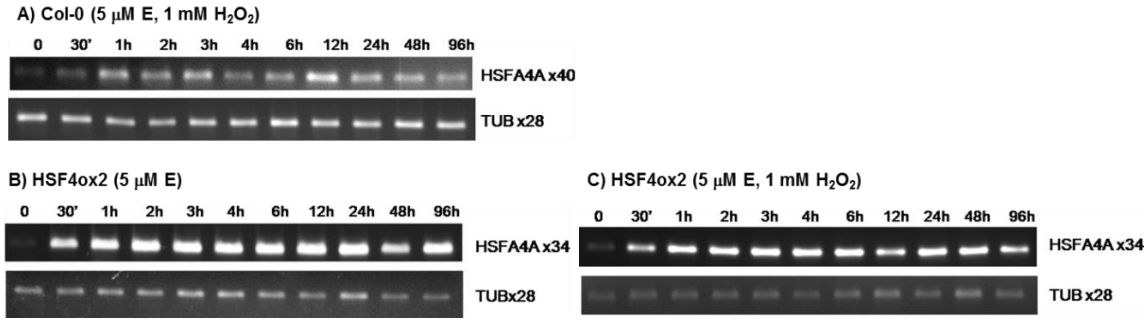

Figure 3.4.1. Time-dependent *HSFA4A* gene transcription activation
(A) Kinetics of H_2O_2 activation of HSFA4A gene expression in wild type (Col-0) plants. (B) Kinetics of estradiol-dependent HSFA4A transcription in HSF4ox2 plants.(C) Kinetics of estradiol-dependent HSFA4A transcription in HSF4ox2 plants treated with H_2O_2. Although time activation is similar, transcript level accumulation in HSF4ox2 plants is superior.

After analysing the induction time-course of *HSFA4A* transcription, I tested, in the same RNA samples, the expression pattern of *ZAT12/RHL41 (AT5G59820),* which functions downstream of HSFA4A and has a Heat Shock Element (HSE) in its promoter sequence (Davletova, 2005). As can be seen in **Figure 3.4.2.**, *ZAT12* transcription activation was observed between 30 minutes and 1 hour after starting the estradiol treatment in both Col-0 and HSF4ox2 samples. Transient induction could be caused by damaging the plants during their transfer to liquid media or when subjecting the plants to the air stream of the sterile hood for a short time during the performance of the treatments. *ZAT12* was shown to respond fast to wounding after 30 minutes and to other stressors such heat, cold, drought, paraquat, H_2O_2 or salinity within less than 1 or 2 hours (Davletova, 2005). Differences between Col-0 and HSF4ox2 samples in *ZAT12* expression were visible after 4 hours estradiol treatment, with *ZAT12* transcript level peaking after 12 hours (**Figure 3.4.2.).**

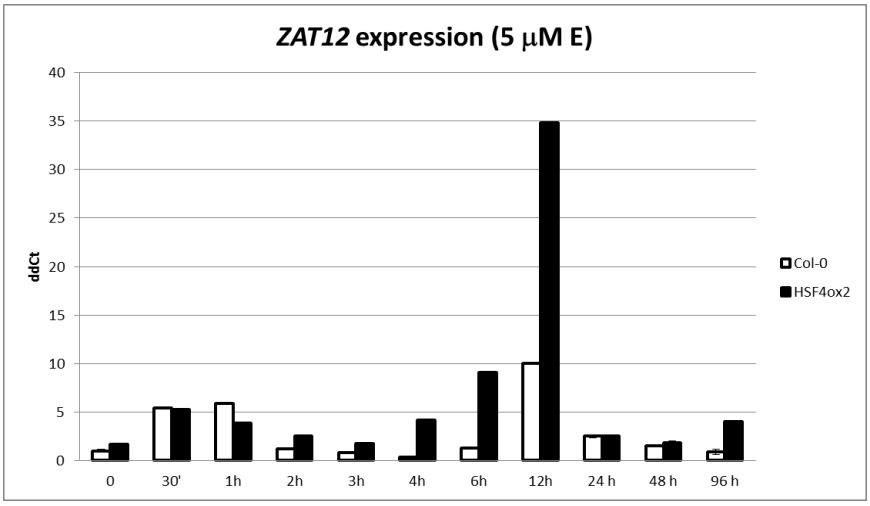

Figure 3.4.2. Transcription regulation of *ZAT12* expression by *HSFA4A* overexpression.
Expression analysis of the putative target of HSFA4A, *ZAT12* inCol-0 and HSF4ox2 treated with 5 μM estradiol at the indicated time points after starting the treatments.

In the other set of samples (**Figure 3.4.3.)**, *ZAT12* was induced after 30 minutes H_2O_2 and estradiol treatment, reaching a maximum transcript level after 12 hours and decreasing afterwards. During the first 12 hours, expression patterns were similar in both Col-0 and HSF4ox2 plants, but differences could be observed at 6 hours time point, being the transcript level of *ZAT12* more than 2-fold higher in HSF4ox2 plants comparing to the wild type.

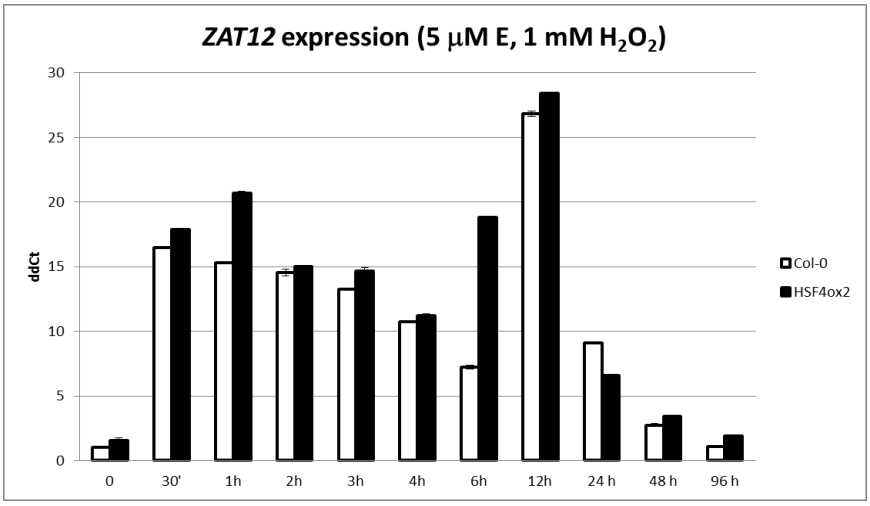

Figure 3.4.3. Transcription regulation of *ZAT12* expression by *HSFA4A* overexpression and H_2O_2. Expression analysis of the putative target of HSFA4A, *ZAT12* in Col-0 and HSF4ox2 treated with 5 μM estradiol and 1 mM H_2O_2 at the indicated time points after starting the treatments

3.4.2 Identification of HSFA4A regulated genes by RNA-Seq

Gene expression data were used to choose a time point to identify HSFA4A-regulated genes by whole genome transcript profiling. I was interested in identifying early targets of HSFA4A therefore I resolved to use the 6 hours treatment, as was the earliest time point in which the highest differences in *ZAT12* expression were detected . To screen for genes showing differential transcription due to *HSFA4A* overexpression and to relate this to ROS responses, an RNA-Seq transcript profiling experiment (GEO accession GSE40735) was performed. I used Col-0 and HSF4ox2 plants that were treated with 5 μM estradiol in the presence or absence of 1 mM H_2O_2 for 6 h. Three pair wise comparisons for differentially expressed genes were done; (i) Col-0 plus / minus H_2O_2, (ii) Col-0 vs HSF4ox2 (iii) Col-0 vs HSF4ox2 in the presence of H_2O_2 (**Figure S.1**). Genes that were common in these three comparisons (**Table S.1 and S.2**) and annotated to stress responses were selected for quantitative RT-PCR (qRT-PCR) analysis and I ended up with a set of 8 HSFA4A regulated genes: small heat shock protein HSP17.6A (*AT5G12030)*, copper transporter CTP1 *(AT5G52760)*, C2H2 zinc finger proteins ZAT6 (*AT5G04340)*, and ZAT12 (*AT5G59820)*, transcription factor WRKY30 (*AT5G24110)*, cystein-rich receptor kinase-like protein CRK13 (*AT4G23210)*, ubiquitin ligase ATL31

(AT5G27420) were all upregulated, while the isopropylmalate isomerase *IPMI1 (AT3G58990)* gene was reduced.

To confirm the differential expression of this set of genes, two-week-old Col-0 and HSF4ox2 plants were treated for 6 hours with 0.5 MS (control), 1 mM H_2O_2, 100 mM NaCl or heat (37ºC) always in the presence of 5 µM estradiol, and RNA levels were detected by qRT-PCR. **Figure 3.4.4** shows qRT-PCR analysis of H_2O_2 and heat-treated samples. As can be seen in **Figure 3.4.4**, in control conditions (0.5 MS plus estradiol) transcript levels of putative HSFA4A target genes were higher (or lower in the case of *IPMI1*) in HSF4ox2 plants comparing to Col-0, which indicated that HSFA4A acts as an upstream regulator of gene expression. Expression of HSF4ox2 upregulated genes was further elevated by H_2O_2 treatment, while heat stress did not modify the induction of some of these genes in HSF4ox2 plants compared to the wild type. The HSF4ox2 downregulated gene, *IPMI1*, was repressed by both H_2O_2 and heat treatments.

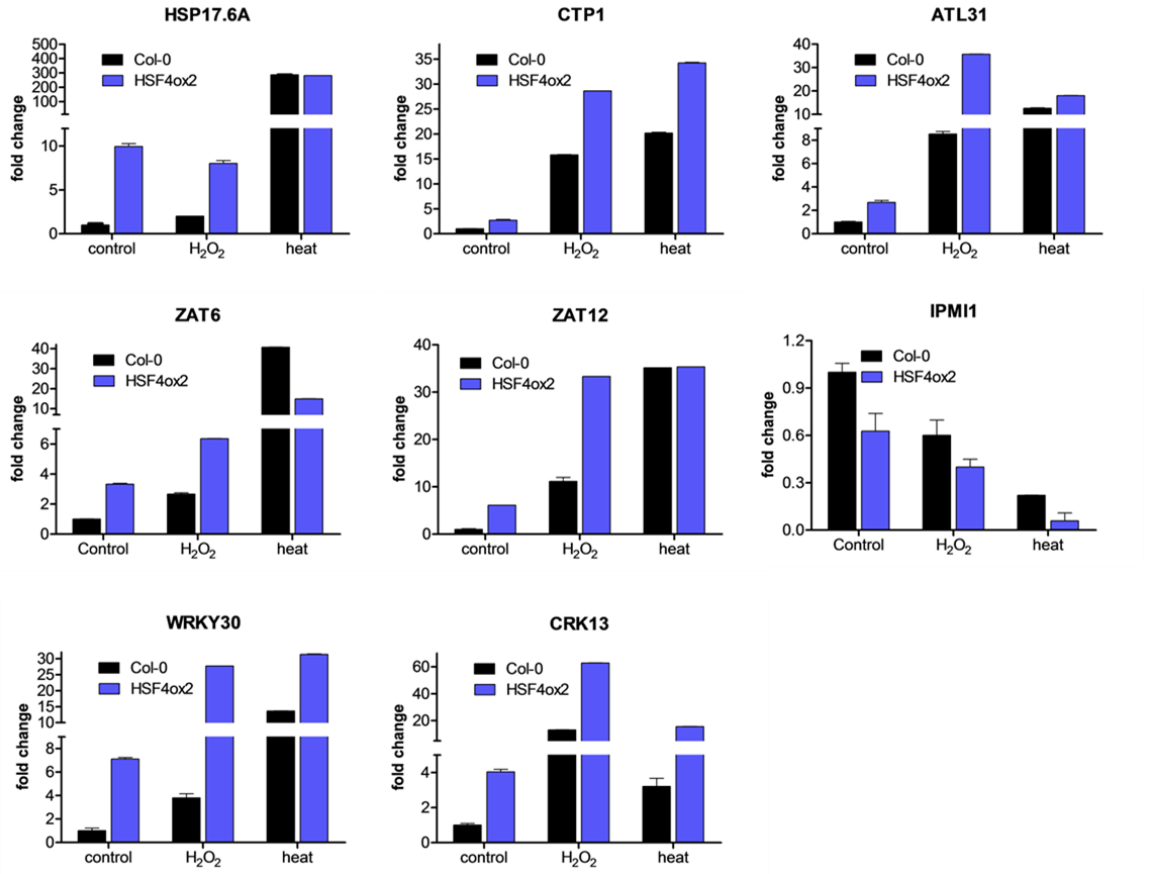

Figure 3.4.4. HSFA4A regulates the expression of a set of stress-related genes
Transcript analysis of eight HSFA4A target genes in HSF4ox2 and Col-0 wild type plants using qRT-PCR: *HSP17.6A* (*AT5G12030)*, *CTP1 (AT5G52760)*, *ATL31 (AT5G27420)*, *ZAT6* (*AT5G04340)*, *ZAT12* (*AT5G59820)*, *IPMI1 (AT3G58990)*, *WRKY30* (*AT5G24110)* and *CRK13* (*AT4G23210*. Plants were treated with 1 mM H_2O_2 or heat stress (37°C) for 6 hours. All plants were treated with 5 µM estradiol.

Expression of HSFA4-activated genes was tested in salt-treated samples as well. As **Figure 3.4.5** shows, the expression of *WRKY30*, *CTP1*, *CRK13, ZAT6, ZAT12* and *HSP17.6A* was induced by salt stress in Col-0 samples. As previously observed, stress had a synergistic effect on HSFA4A-regulated gene expression, as transcription of *CTP1, CRK13 and ZAT12* was further enhanced by salt treatment in HSF4ox2 plants.

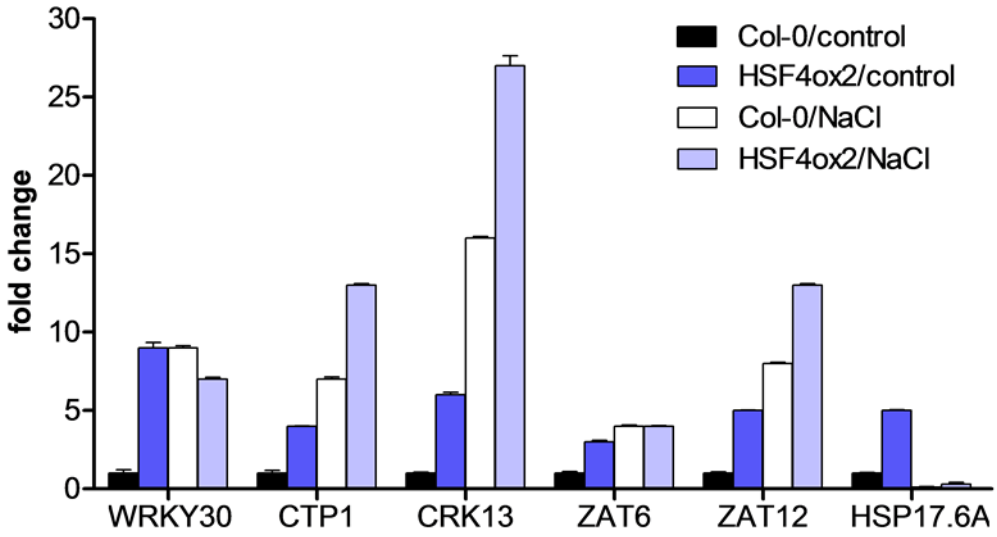

Figure 3.4.5. HSFA4A regulates the expression of a set of stress-related genes
Most of the HSFA4A-regulated genes can be induced by salt. Expression of selected HSFA4A-regulated genes in HSF4ox2 and Col-0 plants in control conditions or treated with 100 mM NaCl, for 6 hours. All plants were treated with 5 μM estradiol.

Next, the presence of consensus heat-shock response elements (HSE) in the promoters of HSFA4A-responsive genes was examined. Among the 57 genes induced by HSFA4A, and by H_2O_2 in both Col-0 and HSF4ox2 background, 33 promoters contained one or more conserved HSE, while putative HSFB-binding sites were found in 12 promoters (Table S1). However, 11 promoters with HSE elements, and 4 with HSFB-binding sites were identified in the 42 genes that showed downregulation by HSFA4A and H_2O_2 (Table S2). HSE elements and putative binding sites for HSFB, Trihelix and MYC_MYB transcription factors were more frequent in the promoters of HSFA4A and H_2O_2 upregulated genes, whereas promoter sequences of downregulated genes showed an enrichment of predicted GRF, C2C2(Zn)GATA, MADS and HD-ZIP transcription factor sites (Table S3).

3.5 Characterization of *hsfa4a* T-DNA insertion mutant

3.5.1 Identification of *hsfa4a* knockout mutant

To obtain further supportive evidence for that the overexpression of *HSFA4A* was indeed responsible for enhancement of stress tolerance, a T-DNA tagged *hsfa4a* mutant allele was identified from the INRA Versailles T-DNA collection. In the mutant line FLAG_571A11 (Wassilewskija ecotype), the single T-DNA insertion was localized in the 5' UTR region of the *HSFA4A* gene, 671 bp upstream of the ATG (**Figure 3.5.1A**). The presence of the BAR gene was tested by scoring herbicide tolerance of M3 generation lines on 50 mg/L phosphinotricin (PPT)-containing medium. The T-DNA insertion mutant lines were analysed by PCR genotyping using combinations of the *hsfa4a*_gen_F primer with the T-DNA left (Lb4) border primer or with the *hsfa4a*_gen_R primer (**Figure 3.5.1A,** in red).

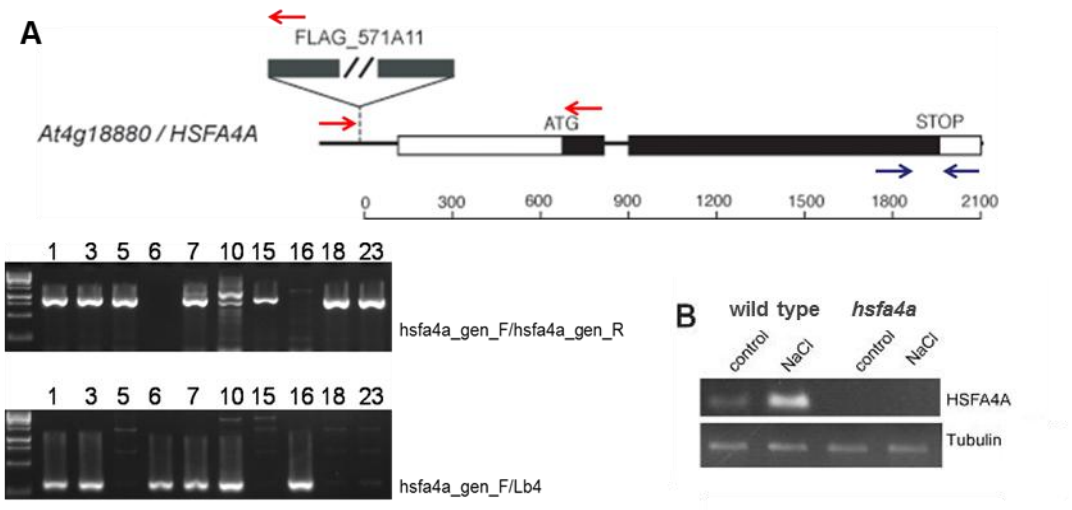

Figure 3.5.1. Identification and characterization of FLAG_571A11 T-DNA insertion mutant
(A)The *hsfa4a* (FLAG_571A11) carries a single T-DNA insertion in the promoter region at position -671. PCR genotyping primers are indicated in red, and semi-quantitative RT-PCR primers in blue. Note that RT-PCR primers were designed nearby the STOP codon, where DNA sequence is more divergent among HSFs. Example of PCR genotyping, where lines 1, 3, 7 and 10 are heterozygous, lines 5 and 15 are wild type and line 6 is homozygous for T-DNA insertion. PCR genotyping for lines 16, 18 and 23 was not clear and lines were not used for further experiments. (B) RT-PCR analysis of *HSFA4A* transcription in wild type and homozygous *hsfa4a* mutant seedlings treated with 100 mM NaCl for 6 hours.

To test the transcription of *HSFA4A* gene in the T-DNA insertion lines, RNA was prepared from wild type and homozygous *hsfa4a* plants subjected to 100 mM NaCl treatment or to 0.5 MS (control). *HSFA4A* transcript analysis was performed by semiquantitative RT-PCR using the *HSFA4A* cDNA specific primers (**Figure 3.5.1B**). Expression analysis revealed that the *HSFA4A* basic expression level rised upon salt treatment in wild type plants. In contrast, no *HSFA4A* transcript was detected with the RNA templates from homozygous mutant lines. Comparison of *HSFA4A* transcription in the FLAG_571A11 insertion mutant and wild type indicated that the T-DNA insertion in the *hsfa4a* mutant completely abolished both basic and salt-inducible expression of the *HSFA4A* gene (**Figure 3.5.1**) demonstrating that the T-DNA insertion knocked out *HSFA4A* gene expression.

3.5.2 The *hsfa4a* mutant is hypersensitive to salt

Soil-grown *hsfa4a* mutant plants were fertile and showed no morphological alteration in comparison to wild type plants. However, phenotypic analysis revealed that *HSFA4A* gene inactivation caused hypersensitivity to salt. Five day-old wild type and *hsfa4a* mutant seedlings were transferred to 0.5 MS medium or to medium supplemented with 100 mM NaCl, and rosette growth was scored after two weeks. In control conditions growth of wild type and *hsfa4a* mutant plants was indistinguishable. On the other hand, upon salt treatment the *hsfa4a* mutant showed 40% reduction of rosette growth compared to wild type (**Figure 3.5.2.**). Similar experiments were performed in the presence of 2 mM H_2O_2 or 75 μM $CdCl_2$, but no differences were observed (data not shown).

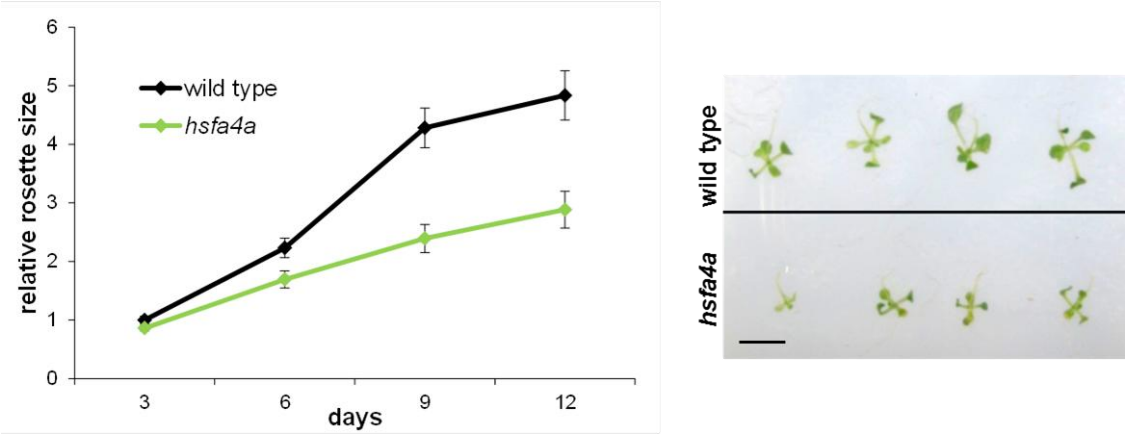

Figure 3.5.2. The *hsfa4a* T-DNA insertion mutant is hypersensitive to salinity
Rosette growth of wild type and *hsfa4a* mutant in salt media. Graph shows relative rosette sizes on 100 mM NaCl, where 1 corresponds to size of size of each line at the beginning of the treatment (day 0). Image shows typical seedlings grown on media supplemented by 100 mM NaCl, confirming that *hsfa4a* plants are hypersensitive to salt. Bars indicate standard errors, size marker shows 1 cm.

To verify that the T-DNA insertion in the *HSFA4A* gene was responsible for the salt-hypersensitivity phenotype, a genetic complementation was performed. The *hsfa4a* mutant was transformed with a pH2GW7-HSFA4A construct, constitutively expressing *HSFA4A* under the control of a CaMV35S promoter. Wild type, *hsfa4a* mutant plants and one complemented line (*hsfa4a*/C) were analysed. First, rosette measurements were performed on plants growing for two weeks in the presence of 100 mM NaCl, 0.3 μM paraquat, 2 mM H_2O_2, 75 μM $CdCl_2$ or 200 mM mannitol. Salt-induced growth reduction of the *hsfa4a* mutant was restored to wild type level by the constitutive expression of wild type *HSFA4A* cDNA in the mutant. Remarkably, the complemented mutant showed 30% larger rosettes compared to wild type in the presence of NaCl (**Figure 3.5.3**).

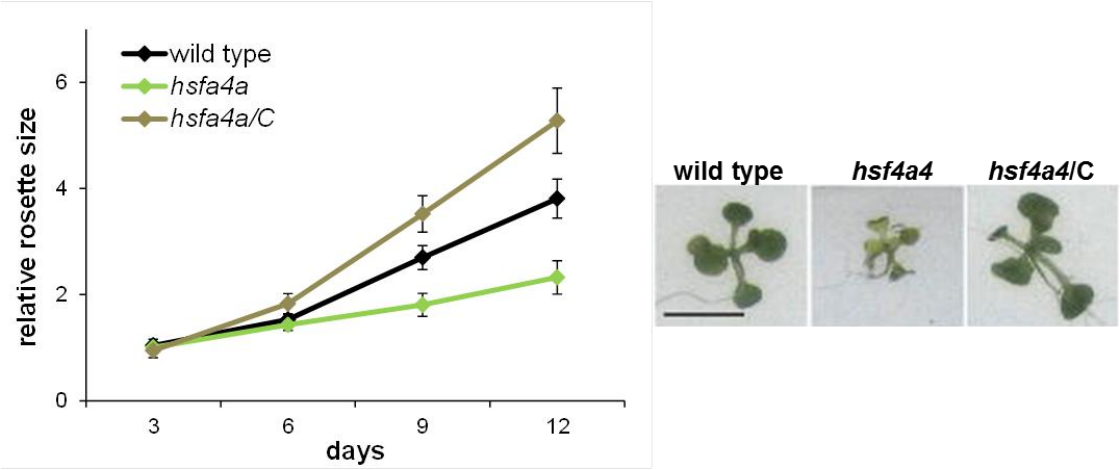

Figure 3.5.3 Restoration of salt-sensitivity by genetic complementation
Rosettes of wild type, *hsfa4a* mutant and complemented mutant (hsfa4a/C). 5 day-old seedlings were transferred to and grown on medium supplemented with 100 mM NaCl for 9 days. Graph shows relative rosette sizes on 100 mM NaCl. Bars indicate standard errors, size marker shows 1 cm

Survival of the *hsfa4a* mutant and the complemented line was subsequently tested upon exposure to high concentrations of salt, mannitol and anoxia. Whereas survival of *hsfa4a* mutant was markedly reduced on high salt medium (200 mM NaCl) compared to wild type and complemented mutant (**Figure 3.5.4**), the *hsfa4a* mutation did not confer hypersensitivity to osmotic stress (300 mM mannitol) and to the anoxic conditions applied (**Figure 3.5.4**). The complemented line showed a higher survival rate comparing to the wild type and the *hsfa4a* mutant in the presence of high salt, but did not confer significant tolerance to high osmotic or anoxia comparing to the the wild type (**Figure 3.5.4**).

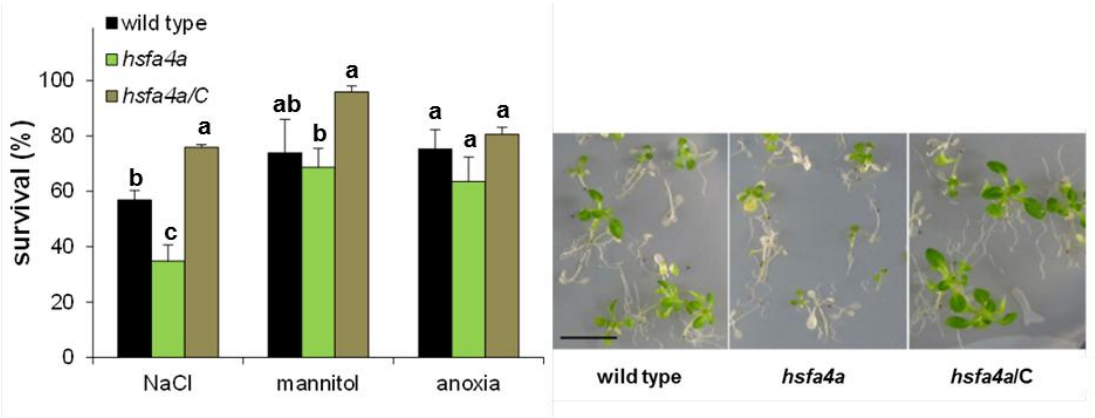

Figure 3.5.4 Genetic complementation of *hsfa4a* mutant results in tolerance to salt but not to osmotic stress and anoxiaDiagram shows survival rates of wild type, *hsfa4a* mutant and *hsfa4a*/C plants after high salt (200 mM NaCl, 5 days), osmotic (600 mM mannitol, 5 days) and anoxia treatments (8 hours). Image illustrates survival of wild type, *hsfa4a* mutant and *hsfa4a*/C plants on high salt medium. Viability of plants was scored 7 days after stress recovery. Bars show standard errors, different letters show significant differences at $p<0.05$ (Duncan-test), size marker shows 1 cm.

Finally, since HSFA4A overexpression reduced oxidative damage in HSF4ox plants, the accumulation of H_2O_2 and the rate of lipid peroxidation were measured in wild type, in *hsfa4a* mutant plants and in the complemented line. Following the same experimental settings used with HSF4ox lines, plants were harvested before transferring to 150 mM NaCl (0 hours, control) and after 6 and 24 hours salt treatment. As shown in **Figure 3.5.5**, H_2O_2 levels were already higher in the *hsfa4a* mutant before stress treatment in comparison to the wild type, while the complemented line exhibited the lowest H_2O_2 content. The same tendency was observed after 6 and 24 hours salt treatment. Lipid peroxidation rates before salt treatment were similar in the wild type and in the *hsfa4a*/C line, but in the *hsfa4a* mutant was significantly enhanced (**Figure 3.5.5**). However, after 6 and 24 hours salt treatment, the lowest lipid peroxidation levels were scored in *hsfa4a*/C, followed by wild type and being *hsfa4a* the line which accumulated the highest MDA levels.

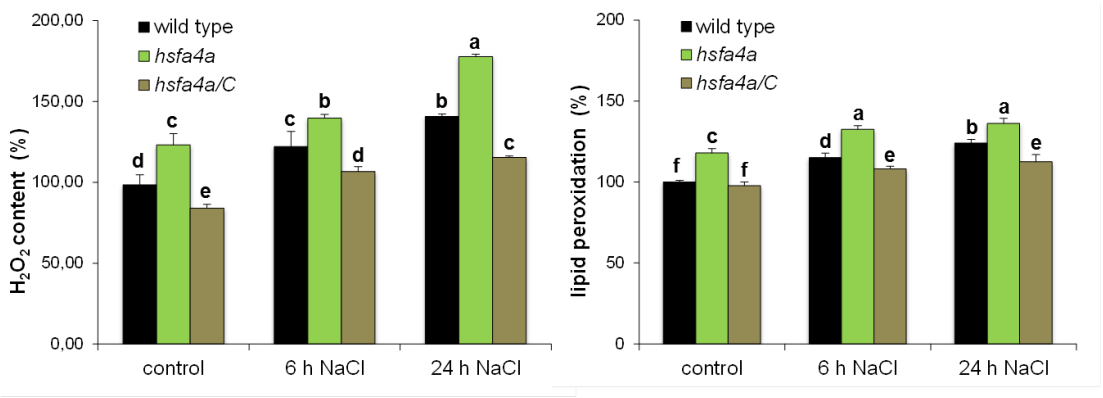

Figure 3.5.5 Genetic complementation of *hsfa4a* mutant reduces H_2O_2 accumulation and lipid peroxidation during salt stress
Accumulation of H_2O_2 (left) and lipid peroxidation rates (right) in wild type, *hsfa4a* mutant and *hsfa4a*/C plants subjected to salt stress (150 mM NaCl) during 6 and 24 hours. Control samples were taken before starting the treatments (0 hours). Bars show standard errors, different letters show significant differences at $p<0.05$ (Duncan-test).

3.5.3 Gene expression studies in *hsfa4a* background

The next experiment was to determine how expression of selected HSFA4A target genes was affected by *HSFA4A* knockout. With this purpose, two weeks-old wild type and *hsfa4a* plants were transferred to 0.5 MS media (control) and to 100 mM NaCl, and samples were harvested after 6 hours treatment. RNA levels were detected by quantitative RT-PCR, for 6 genes that we previously identified as regulated by HSFA4A and as salt-responsive: *WRKY30*, *CTP1*, *ZAT6*, *CRK13*, *ZAT12* and *HSF17.6A*. Transcripts levels of the selected genes in the *hsfa4a* mutant indicated that salt-induced expression of *WRKY30*, *CTP1*and *ZAT6* genes was reduced, whereas *CRK13*, *ZAT12* and *HSF17.6A* was similar in the *hsfa4a* mutant and wild-type (**Figure 3.5.6**). These data suggested that HSFA4A is required for proper regulation of some genes that play important roles in the control of oxidative stress responses (Miller, 2010). The fact that only three from the five examined salt and HSFA4A-induced target genes showed down-regulation in the *hsfa4a* mutant (**Figure 3.5.6**) indicated that in wild-type plants probably other HSF factors contribute also to the regulation of a large fraction of target genes that show differential expression in response to HSFA4A overexpression.

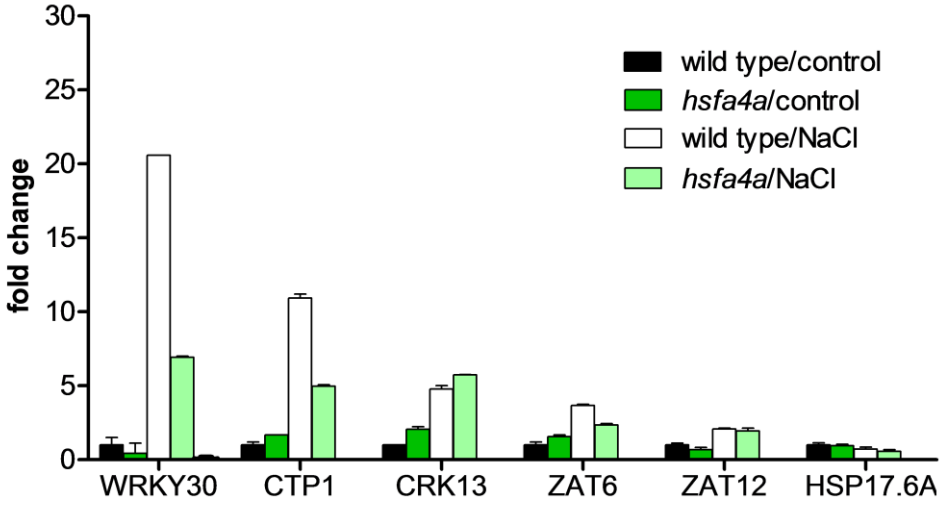

Figure 3.5.6. Gene expression analysis in *hsfa4a* mutant plants.
Expression of the selected genes in *hsfa4a* mutant and wild type plants (Ws, Wassilewskija ecotype), in control conditions or treated with 100 mM NaCl for 6 hours. Bars indicate standard deviation.

3.6 Protein localization and homodimerization studies

3.6.1 Subcellular protein localization

Conserved structural domains of HSFA4A included signal peptides, which determine the cellular distribution of HSFA4A. The presence of a nuclear localization sequence (NLS) and a nuclear export signal (NES) indicated that HSFA4A is transported bidirectionally between the nucleus and the cytosol.

To verify the cellular distribution, an in-frame C-terminal fusion of HSFA4A and the yellow fluorescent reporter protein (YFP) was generated. HSFA4A-YFP protein fusions were transiently expressed under the control of the CaMV35S promoter in Arabidopsis protoplasts and the subcellular localization of HSFA4A protein was monitored with a confocal laser scanning microscope (CLSM). As expected, transformed cells exhibited

YFP-derived fluorescence in both nucleus and cytosol (**Figure 3.6.1**), as previously reported for other plant HSFs (Scharf, 2012).

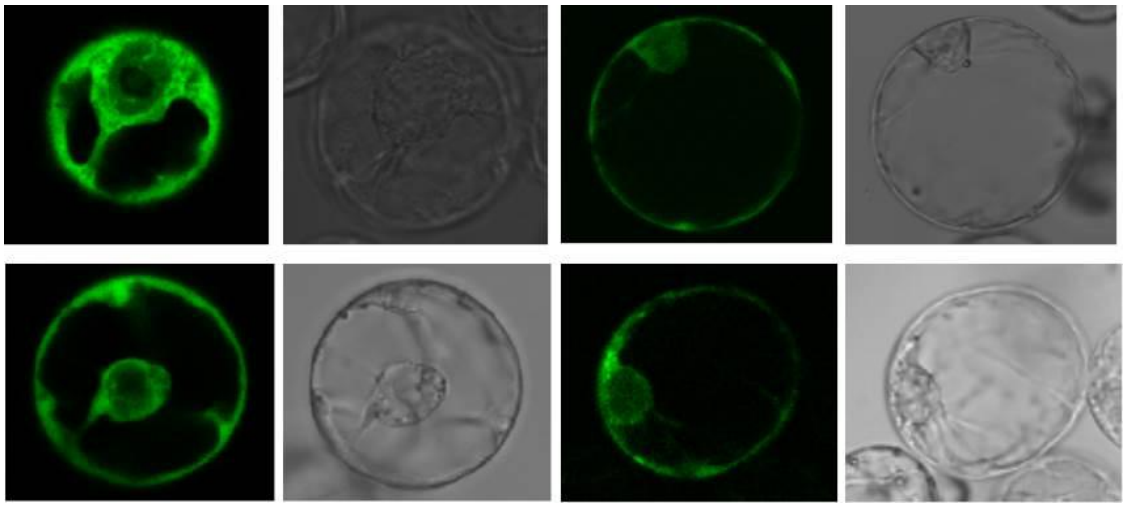

Figure 3.6.1. Intracellular localization of HSFA4A-YFP fusion protein.
Transient HSFA4A-YFP expression in Arabidopsis protoplasts, images show YFP fluorescence and bright field.

3.6.2 Redox-dependent HSFA4A homodimerization

During their activation preceding nuclear import, animal HSFs undergo trimerization, which is stimulated through oxidation of their cysteine residues by H_2O_2, accumulating during heat stress (Ahn, 2003),(Larson, 1988). In *Drosophila* and in yeast, similar mechanisms of HSFs regulation have been reported (Zhong, 1998), (Lee, 2000). It was previously suggested that plant HSFs might share the conserved feature of redox-dependent oligomerization feature, and function as ROS sensors in plants (Miller, 2006). Following the described models of post-translational regulation of eukaryotic HSFs, we used several approaches in order to investigate whether HSFA4A undergoes indeed the anticipated oligomerization.

3.6.2.1 HSFA4A oligomerization studies using yeast two-hybrid

First, I studied the protein-protein interaction of HSFA4A by using Yeast Two-Hybrid system (Y2H). For that purpose, the cDNA of *HSFA4A* gene was cloned into both yeast

expression vectors pGAD424 and pGBT9 to generate protein fusions of the Activation Domain (AD) and the DNA Binding Domain (BD) of the yeast transcription factor GAL4 and HSFA4A, respectively. As a control, cells were transformed with plasmid combinations of pGAD-HSFA4A and empty pGBT9 and pGBT9-HSFA4A and empty pGAD. Successfully co-transformed *S .cerevisiae* cells were able to grow in medium lacking Leucine and Tryptophan (-Leu, -Trp, **Figure 3.6.2A and 3.6.2C,** lower panels) confirming the presence of both plasmids in yeast cells. To test specific interaction of the protein fusions, co-transformed cells were transferred to medium lacking either Histidine (-Leu, -Trp, -His) or both Histidine and Adenine and containing 3-amino trazole (-Leu, -Trp, -His, -Ade, 3-AT). On these selective conditions auxotroph cells expressing HSFA4A-AD and HSFA4A-BD fusions were able to grow but, contrarily, yeast cells co-transformed with pGAD-HSFA4A and pGBT9 or pGBT9-HSFA4A and pGAD failed to propagate (**Figure 3.6.2A, 3.6.2C and 3.6.2E** upper panels). These results demonstrated that reconstitution of GAL4 was due to the specific interaction between chimeric HSFA4A-AD and HSFA4A-BD proteins, proving that HSFA4A forms homodimers in yeast.

Afterwards, we focused on determining the degree of relevance of cysteine oxidation in HSFA4A homodimerization. Alignments of HSFA4A orthologous protein sequences among several plant species are shown in **Figure 3.6.8** and structural domains of plant HSFs are marked with a colour code. Remarkably, the DNA-binding domain (DBD) at the amino-terminal end exhibits almost a perfect match between the orthologous protein sequences, suggesting that this highly conserved gene region is essential for the protein function. The oligomerization domain (OD) and the activation motifs (AHA) exhibit higher sequence diversification among the species, but still maintain a certain degree of conservation, since most of the residues are similar (equal chemical properties) or are fully conserved. The highly conserved nuclear localization sequence (NLS) contains a cluster of identical basic amino acids, on the contrary the nuclear export signal (NES) exhibits similarity but less sequence identity.

The protein sequence of Arabidopsis HSFA4A contains six cysteine residues and the multiple sequence alignment revealed that not all of them are conserved. However, three of the cysteine residues (C229, C267 and C295) remain constant in the *Brassicaceae* species *Arabidopsis thaliana*, *Thellungiella halophila* and *Brassica napus* (**Figure 3.6.8,** marked in red colour). Multiple sequence alignment of HSFA4A among

Brassicaceae resulted in high sequence identities, with scores of 88,8% between Arabidopsis and Thellungiella and 80,46% between Arabidopsis and rapeseed, implying that HSFA4A is very conserved in this plant family (data not shown).

To study the importance of these conserved cysteine residues in HSFA4A oligomerization, we performed site-directed mutagenesis of HSFA4A cDNA. Cysteines at positions 229, 267 and 295 were replaced with the non-reactive amino acid alanine. Point mutations were introduced in the cDNA sequence of HSFA4A, and the modified sequence was cloned into the yeast expression vectors pGAD424 and pGBT9. The modified protein (mHSFA4A) was expressed in yeast cells, along with wild type HSFA4A, in fusion with the GAL4 transcription factor AD and BD domains. As **Figure 3.6.2** depicts, after 72 hours culture, co-transformed cells grew on control media lacking Leucine and Tryptophan (lower panel, -Leu, -Trp) and only cells expressing HSFA4A-AD and HSFA4A-BD were able to grow at the same level on 3-AT-selective medium (-Leu, -Trp, -His, -Ade, 3-AT) (**Figure 3.6.2A)**. However, yeast cells expressing the mutant version of HSFA4A exhibited a largely reduced growth on the same circumstances, meaning that the three mutated cysteine residues have a functional importance in homomeric interaction of HSFA4A (**Figure 3.6.2B)**. On the other hand, increasing the culturing time on 3-AT medium to more than 3 days allowed yeast cells carrying the mHSFA4A protein to reach the similar growth rate of HSFA4A-expressing cells. These results suggest that even if these mutations can affect the homodimerization, do not fully impair the protein-protein interaction. As can be seen in **Figure 3.6.2D** and **3.6.2F**, control aliquots co-transformed with combined empty vectors were included, and failed to grow on 3-AT-selective media.

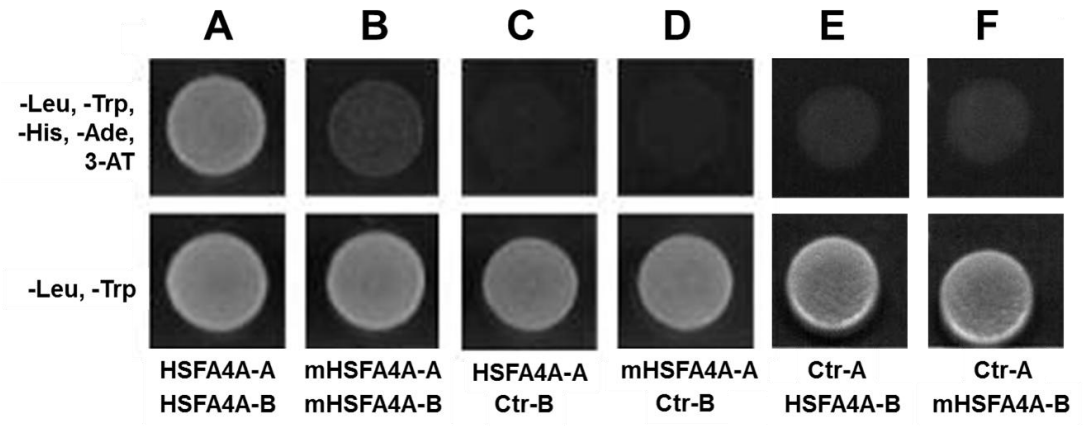

Figure 3.6.2. Dimerization of HSFA4A in yeast cells
Homodimeric interaction of HSFA4A in yeast two-hybrid system. Yeast growth on selective medium (-Leu, -Trp, -His, -Ade, 3-AT) indicates interaction of the assayed proteins (upper panels). (A)HSFA4A-A, HSFA4A-B: HSFA4A fused to Activation and DNA Binding domain of GAL4, respectively. (B)mHSFA4A-A, mHSFA4A-B: triple Cys mutant HSFA4A (C229A, C267A, C295A). (C) and (D)Ctr-B: empty vector with DNA Binding domain. (E) and (F)Ctr-A: empty vector with Activation domain.

3.6.2.2 HSFA4A dimerization studies in plant cells

3.6.2.2.1 BIFC in a transient expression system

The second approach was to test whether HSFA4A form homodimers in plant cells. Bimolecular Fluorescence Complementation (BIFC) technology was chosen for that purpose, as it represents a highly valuable tool to visualise protein-protein interactions *in vivo*. In BIFC assays, proteins are fused to the N-terminal or the C-terminal domains of the split YFP, positive protein-protein interactions lead to reconstitution of YFP signal and can be monitored by fluorescence microscopy . The cDNAs of *HSFA4A* and *mHSFA4A* were cloned into the BIFC vectors pSAT1A-cEYFP-N1 and pSAT1A-nEYFP-N1 (Citovsky, 2006) and HSFA4A/mHSFA4A-nYFP and HSFA4A/mHSFA4A-cYFP protein fusions were constitutively expressed in Arabidopsis protoplasts. As a control, protoplasts were also transformed with a combination of either pSAT1A-cEYFP-N1-HSFA4A and empty pSAT1A-nEYFP-N1 or pSAT1A-nEYFP-N1-HSFA4A and empty pSAT1A-cEYFP-N1. Images created with CLSM are depicted in **Figure 3.6.4**. Cells co-transformed with HSFA4A-cYFP and the

empty vector carrying the N-terminal domain of the YFP did not produce fluorescence (**Figure 3.6.4A**). Similar results were obtained with the other control plasmid combination (not shown). However, when protoplasts co-transformed with HSFA4A-nYFP and HSFA4A-cYFP protein fusions under the same technical conditions, strong fluorescent signals were detected, pointing to the reconstitution of split YFP by specific dimerization of HSFA4A-nYFP and HSFA4A-cYFP (**Figure 3.6.4B and 3.6.4C**). HSFA4A homodimers were localized in the nucleus and in the cytosol, coinciding with the previously determined subcellular localization of HSFA4A. Accordingly with the results obtained by yeast two-hybrid and BIFC assays, we positively concluded that HSFA4A creates homodimers in vivo.

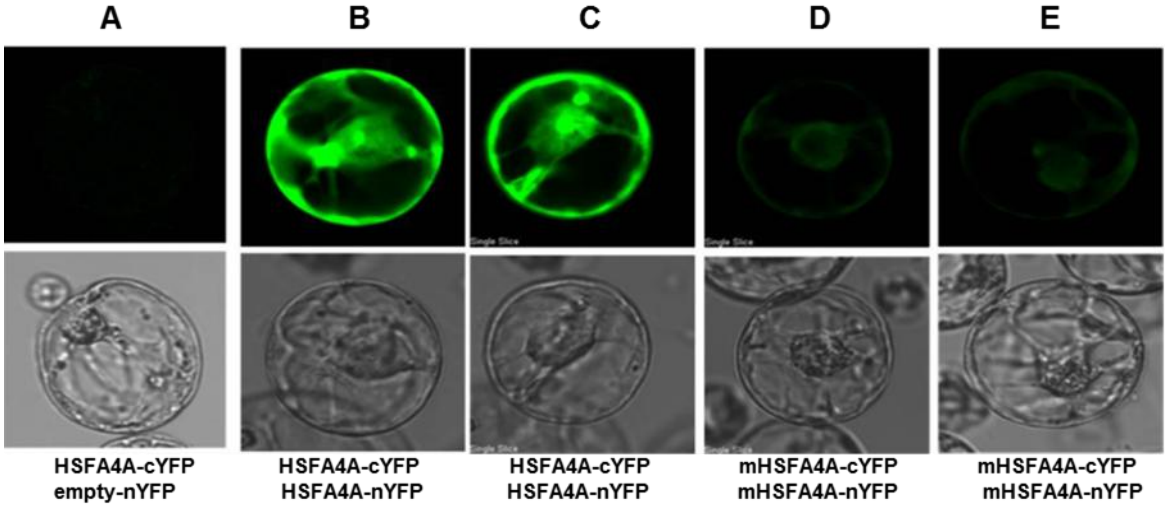

Figure 3.6.4. Oligomerization of HSFA4A in plant cells.
BIFC in Arabidopsis protoplasts: transient expression of HSFA4A-nYFP and empty cYFP vectors (A), HSFA4A-nYFP and HSFA4A-cYFP (B, C), triple Cys mutant mHSFA4A-nYFP and mHSFA4A-cYFP (D, E). Images show YFP fluorescence and bright field.

In comparison with the fluorescence intensity emitted by cells transformed with HSFA4A fusions, the BIFC signal intensity was considerably lower in cells expressing the mutant version mHSFA4A (**Figure 3.6.4D and 3.6.4E)**. To quantify the differences in fluorescence intensity, green fluorescent signals of transformed cells were measured and their average values compared. As indicated in **Figure 3.6.5A**, BIFC signal intensity was 75% lower in mHSFA4A-expressing cells compared to wild type HSFA4A. This result implied that *in vivo* homomeric interaction was indeed affected by the replacement of the conserved Cys residues to Ala. To make sure that the differences in fluorescence intensity were reliable we performed a control immunoblotting with

anti-GFP antibody. Western blot signals showed no difference between wild type and mutant HSFA4A-YFP samples (**Figure 3.6.5B**), revealing that low fluorescence intensity emitted by mHSFA4A-expressing protoplasts was not due to a lower level of protein expression or stability of mutant mHSFA4A.

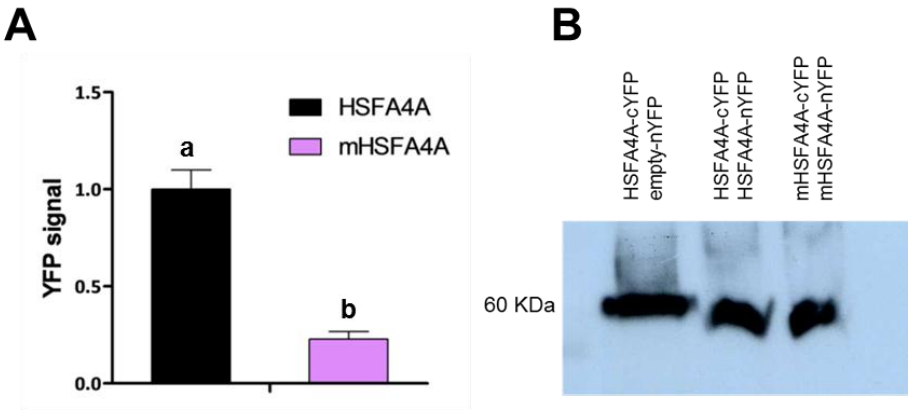

Figure 3.6.5. Dimerization of HSFA4A in plant cells.
(**A**) Quantitative evaluation of YFP signals in protoplasts transfected by wild type (HSFA4A) and triple Cys mutant HSFA4A-YFP gene constructs (mHSFA4A). (**B**) Western detection of HSFA4A-cYFP in protoplasts of the transient BiFC experiment. The GFP specific antibody (Roche) detects C terminal fragment of YFP.

These data demonstrate that conserved cysteine residues are functionally important for the homomeric interaction of HSFA4A. However, in agreement with results obtained by yeast two-hybrid, substitutions of cysteine to alanine did not abolish completely the protein-protein interaction, which means that they are not essential to basic protein oligomerization.

To investigate the direct effect of ROS on HSFA4A homodimerization, I used BIFC technology expressing the fused HSFA4A-cYFP and HSFA4A-nYFP in Agrobacterium-infiltrated *Nicotiana benthamiana* leaves. By using this technology, it was possible to monitor in real time the influence of stress treatments on a given group of transformed cells, observing the same microscopic field all along the experiment.

These series of experiments were focused in salt stress because HSFA4A was identified on a salt screen. To verify that salt treatment on tobacco leaves induces ROS accumulation, tobacco leaves were infiltrated with water (as control) or with a 100 mM NaCl solution and the salt-treated leaves were harvested after 20 and 45 minutes.

Afterwards, H_2O_2 accumulation was detected by *in situ* histochemical staining with 3,3-diaminobenzidine (DAB) (Ren, 2002). As shown in **Figure 3.6.6**, DAB-staining revealed that salt-infiltrated leaves accumulated H_2O_2 after the very first 20 minutes, contrarily to water-treated tobacco leaves. Therefore, it was confirmed that in these experimental conditions salt treatments resulted in ROS accumulation.

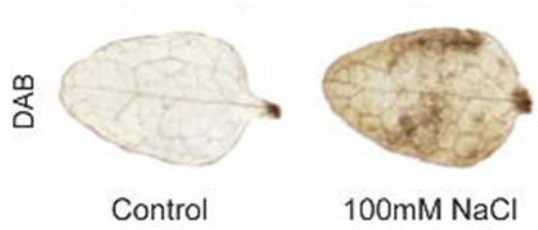

Figure 3.6.6. DAB staining of control and salt-treated tobacco leaves.
H_2O_2 peroxide is accumulated after 20 minutes of salt infiltration. The experiment was performed to monitor the ROS production in salt-treated tobacco leaves.

Nicotiana benthamiana leaves were *Agrobacterium*-infiltrated with the described BIFC constructs to co-express HSFA4A-nYFP and HSFA4A-cYFP proteins. Three days after transformation, leaf cuts excised from the infected area were fixed onto modified glass slides by the underside of the leaf; the upper side epidermis was removed with a blade exposing the mesophyll cells. Leaf-cuts were observed under CLSM and transformed cells emitted fluorescent signals, characteristic of a reconstituted YFP activity. As observed in protoplasts, HSFA4A oligomers were localized in the nucleus and in the cytosol of transformed tobacco cells. Once selected the microscopic field, which would contain several cells, an image was created representing the start time (**Figure 3.6.7,** left panel) and, subsequently, salt treatments started. After 20 minutes salt treatment, the resulting ROS accumulation enhanced the nuclear localization of HSFA4A-nYFP and HSFA4A-cYFP oligomers (**Figure 3.6.7** (right panel)). Nuclear BIFC signal intensity was quantified among several cells and, as it is represented in **Figure 3.6.7**, it increased to 2-fold after the stress treatment. In this context, we concluded that nuclear accumulation of HSFA4A oligomers occurred simultaneously with the production of ROS. This finding could suggest that ROS accumulation positively influence the

concentration of the active homomeric form in the nucleus, where HSFA4A acts as a transcription regulator.

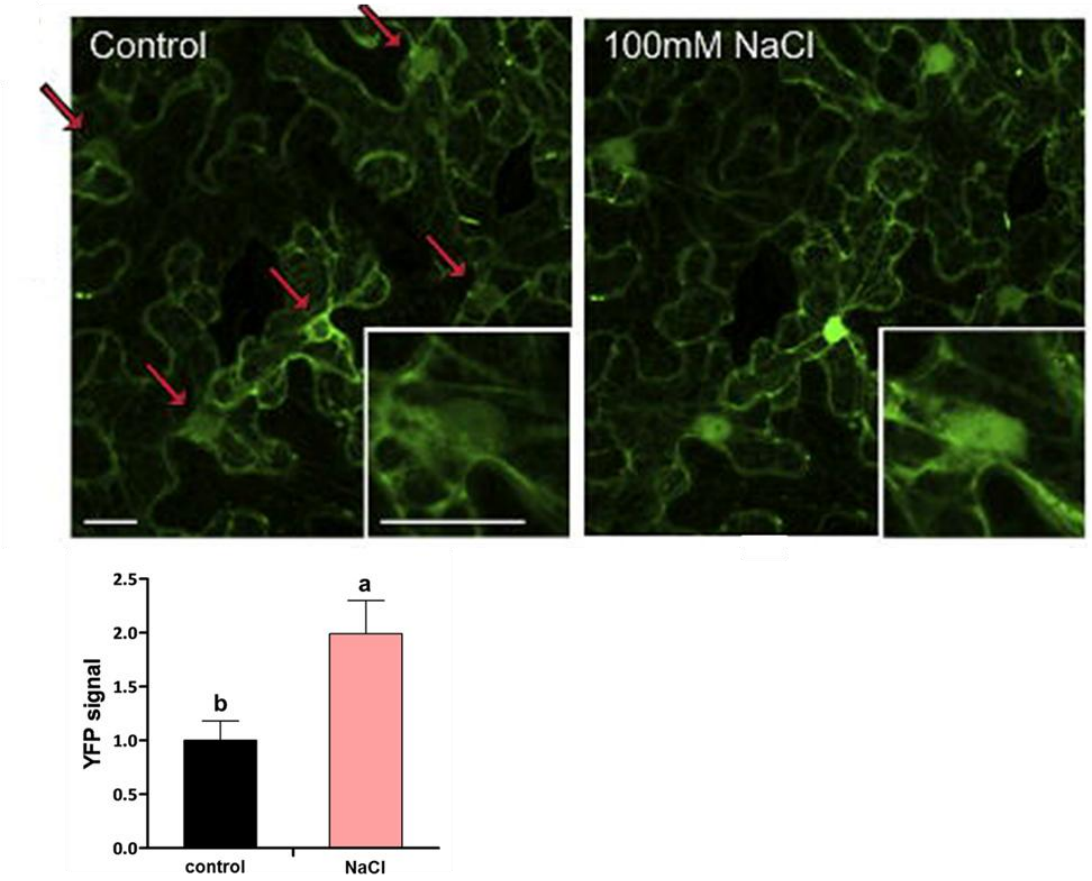

Figure 3.6.7. ROS-dependent HSFA4A homodimerization in tobacco cells
BIFC in tobacco cells. Transient expression of HSFA4A-nYFP and HSFA4A-cYFP in tobacco mesophyll cells. Leaves were treated with 100 mM NaCl for 20 min, images were taken at time zero (control, left panel) and after 20 minutes (100 mM NaCl, right panel). Arrows indicate positions of nuclei, insert shows enlarged image of a cell nucleus. Microphotographs in all figures show representative images, bars indicate 20μm. (Diagram) Quantitative evaluation of YFP signal in nuclei of 20 transfected tobacco cells.

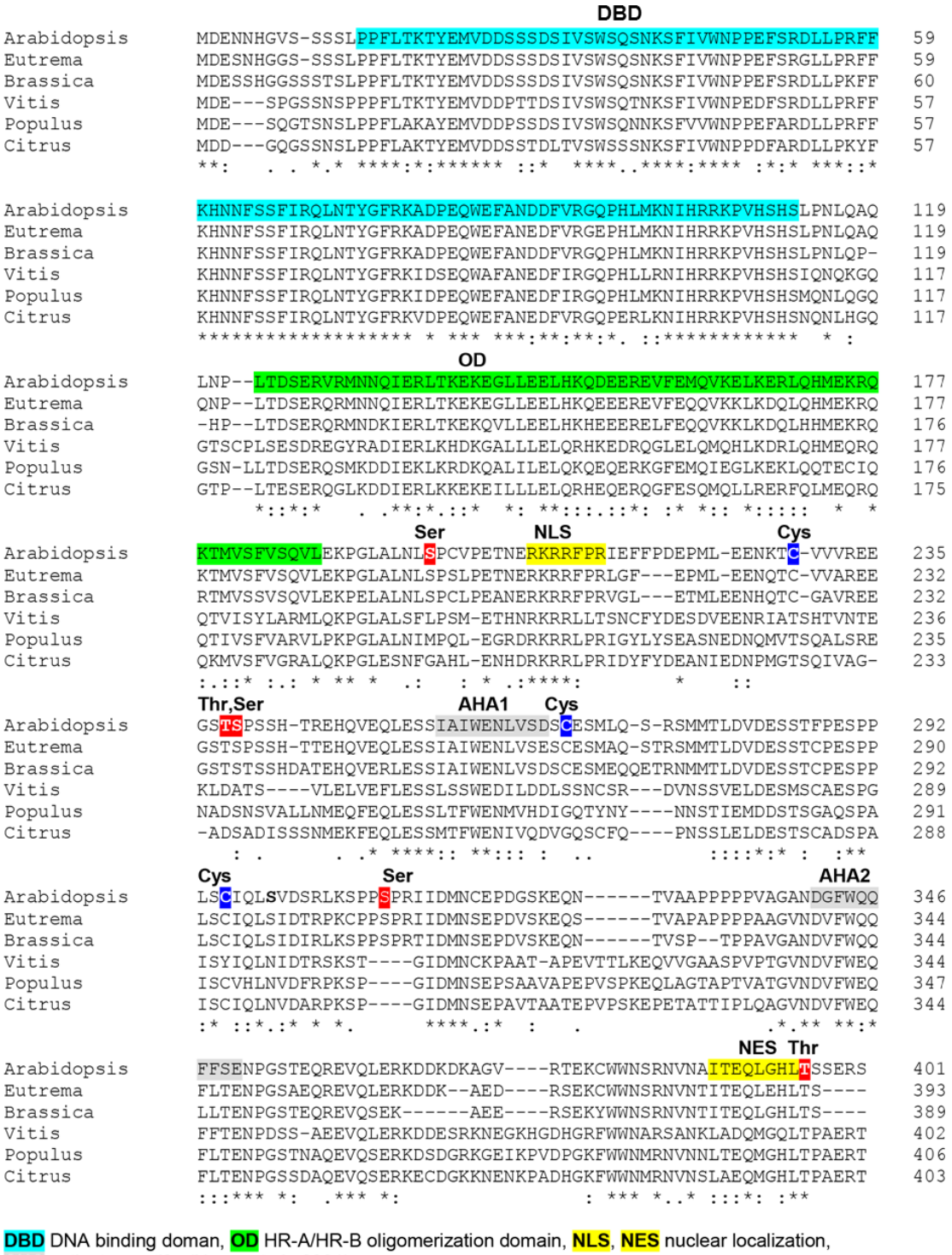

Figure 3.6.8.Multiple protein sequence alignment of the Arabidopsis HSFA4A and related transcription factors from five plant species

CLUSTAL O(1.2.0)multiple sequence alignment was made with the following sequences: *Arabidopsis thaliana*(AEE84101), *Eutrema salsugineum* (*Thellungiella salsuginea*, ESQ55497), *Brassica napus*(ADX69244), *Vitis vinifera* (XP_002267171), *Populus trichocarpa* (EEE97421), *Citrus sinensis*(XP_006467594). Domain structure of the Arabidopsis HSFA4A is indicated according to Nover et al.,(2001) Cell Stress Chaperones 6: 177-189.

3.7 HSFA4A interacts with and is phosphorylated by MPK3 and MPK6

In yeast and mammals, phosphorylation by either mitogen-activated protein kinases MAPK) or the glycogen synthase kinase 3 (GSK3) is required for activation or repression of HSFs (Kim, 1997; Hashikawa, 2004). In Arabidopsis, the mitogen-activated protein kinases MPK3 and MPK6 were implicated in ROS signalling (Liu, 2010).

The results presented in chapters 3.2-3.6 placed HSFA4A in ROS signalling pathways, and suggested a role for HSFA4A as a modulator of plant responses to oxidative stress. In this scenario, it was examined whether HSFA4A can be a substrate for the ROS-stimulated MPK3 and MPK6.

3.7.1 HSFA4A is phosphorylated by MPK3 and MPK6 *in vitro*

In the first place, I tested whether HSFA4A was phosphorylated by MPK3 and MPK6 using in-gel kinase assay. Essentially, this technique is based on imbedding a substrate peptide in a polyacrylamide gel, and the protein samples (which contain the kinases) are separated by SDS-PAGE in the substrate gel. After incubating the gel with radiolabeled ATP, the autoradiography will indicate bands corresponding to the molecular weight of the kinases (Fujii, 2007). To generate the substrate for this experiment, the cDNA of *HSFA4A* was cloned into the pET28A expression vector and fused to a polyhistidine tag (6xHis tag), generating a chimeric protein of 47 KDa. 6xHis-HSFA4A was expressed in *Escherichia coli* and purified by Ni^{2+}-affinity chromatography. Optimization of the protein expression protocol was required since expressed 6xHis-HSFA4A precipitated and formed inclusion bodies. Protein solubility was highly improved when a heat-shock step was included during bacterial culturing, prior to IPTG addition (Chen, 2002). After adjusting cell culture and protein expression conditions, relatively pure fractions of 6xHis-HSFA4A were recovered in soluble fractions, as can be seen in **Figure 3.7.1**, although most of it was insoluble.

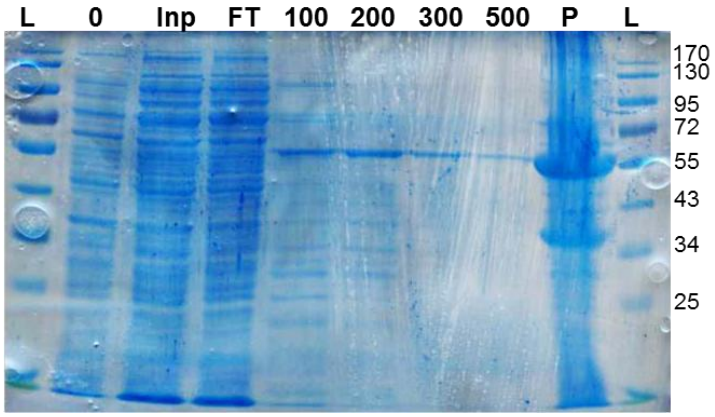

Figure 3.7.1 6xHis-HSFA4A purification using Ni-NTA agarose (Qiagen).
PAGE-BLUE-stained SDS gel. Purification of recombinant 6xHis-HSFA4A (47 KDa but migrates at about 55 KDa), from left to right: (L) prestained protein ladder (SM0671), (0) before IPTG induction, (Inp) input, (FT) flow through, 100, 200, 300, 500 mM imidazole gradient used to elute 6xHis-HSFA4A, (P) pellet, (L) protein ladder.

Most of the contaminating proteins were eluted during the washing step (not shown) while 6xHis-HSFA4A started to elute at 100 mM and up to 500 mM imidazole, increasing its purity with the linear imidazole gradient. The purest fractions recovered using higher concentrations of imidazole (usually with 300 to 500 mM) were used for the assays. Arabidopsis MPK3 and MPK6 were immunoprecipitated from wild type plant protein extracts and total protein samples were added. In parallel, control gels without substrate were included, to discard kinase autophosphorylation. As shown by the autoradiography on **Figure 3.7.2**, bands corresponding to MPK6 and MPK3 (at 45 and 43 kDa respectively) were present, indicating these kinase activities in HSFA4A-containing gels. Due to cross-reactivity of commercial antibodies, both MPK3 and MPK6 were immunoprecipitated by the anti-MPK3 and anti-MPK6 IgGs, but could nevertheless be clearly identified by their different molecular masses. The in-gel assays indicated that HSFA4A was used as a phosphorylation substrate by both MPK3 and MPK6, though the two kinases showed slightly different activities.

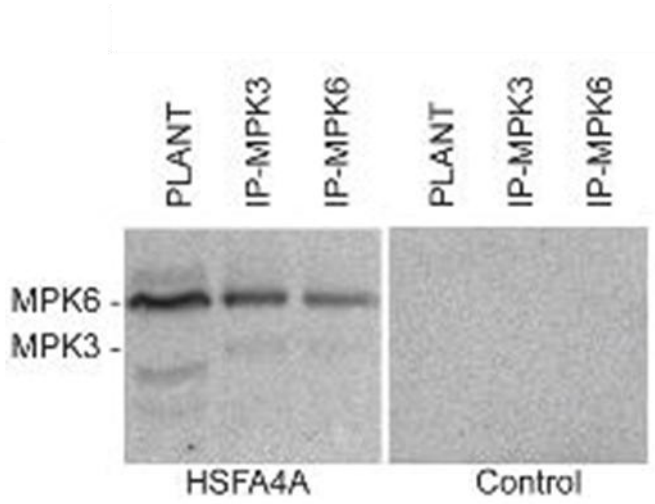

Figure 3.7.2 In-gel kinase assay using 6xHis-HSFA4A as substrate and plant MPK3 and MPK6. Phosphorylation of 6xHis-HSFA4A substrate with immunoprecipitated MPK3 and MPK6 kinases, analysed by in-gel kinase assay. Anti-MPK3 and anti-MPK6 antibodies (Sigma), partially recognize both kinases. PLANT indicates extracts obtained from wild type Arabidopsis (LER). Control: gel without 6xHis-HSFA4A substrate.

Next, we corroborated by *in vitro* kinase assays the finding that HSFA4A was a MPK3 and MPK6 substrate. 6xHis-tagged forms of MPK3 and MPK6 were produced and purified using Ni-NTA agarose. To exclude size overlapping with MPK3 and MPK6, HSFA4A was fused to the maltose-binding protein tag (MBP), generating a protein fusion of about 95 KDa that was purified using amylose resin. MBP-tagging of HSFA4A greatly improved protein solubility (**Figure 3.7.3)**.

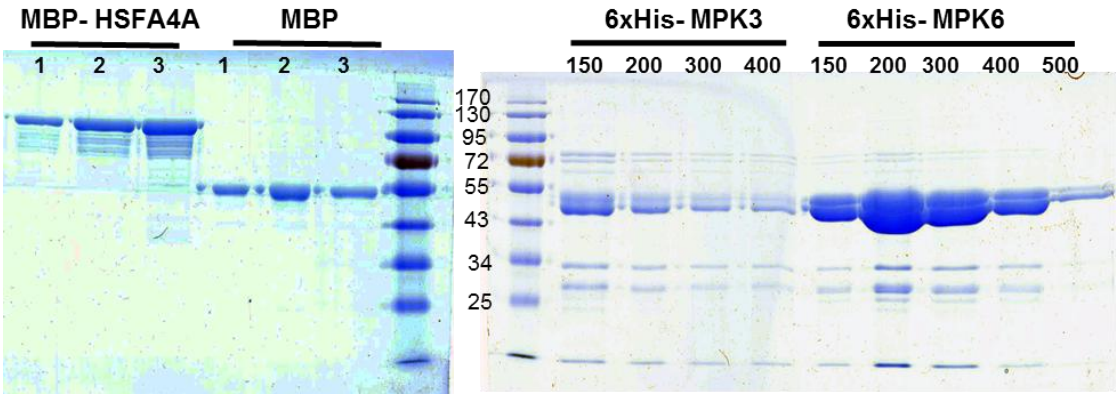

Figure 3.7.3. Protein elutions MBP-HSFA4A, 6xHis-MPK3 and 6xHis-MPK6 purification. (Left gel) Recovery of MBP-HSFA4A (95 KDa) and MBP (empty tag, around 50 KDa) was performed with three elutions of 10 mM maltose (1,2,3). (Right gel) Elutions of 6xHis-MPK3/6 (43 KDa and 46 KDa) were performed with an imidazole gradient of 150, 200, 300, 400 and 500 mM.

In vitro kinase assays confirmed that HSFA4A was specifically phosphorylated by both MPK3 and MPK6. In **Figure 3.7.4** are shown the autoradiograms of phosphorylation assays, performed with MPK3 on the left and with MPK6 on the right. The assays contained several control reactions, that are described as follows: lane 1 corresponds to a kinase reaction without substrate, which shows autophosphorylation activity by MPK6 but not by MPK3. Lane 2 contains a kinase reaction with the general kinase substrate myelin basic protein (MyelinBP), used as a positive control and indicating that MPK3 and MPK6 were active. The third lane shows no bands, which demonstrates that the kinases did not phosphorylate the MBP tag, since this reaction contained the MBP tag alone as substrate. To perform this kinase reaction, MBP was bound onto amylose resin and loaded, excluding the kinases in this sample to avoid size overlapping, which explains why there is no autophosphorylation signal by MPK6 on this lane. Finally, after an empty 4 lane, the kinase reaction containing HSFA4A shows a phosphorylation signal at 96 kDa, corresponding to MBP-HSFA4A and, on the right autoradiography, MPK6 autophosphorylation (lane 5). Another control reaction consisting in a kinase reaction without kinases was included (right autoradiography, lane 6), to demonstrate that purified MBP-HSFA4A sample did not contain any additional kinases which could phosphorylate HSFA4A.

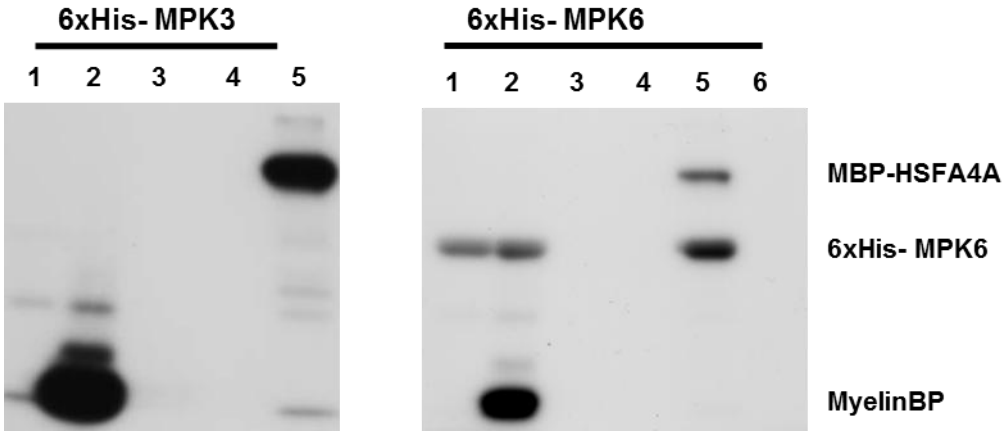

Figure 3.7.4. *In vitro* phosphorylation of MBP-HSFA4A by 6xHis-MPK3 and 6xHis-MPK6.
Left panel: kinase assays using 6xHis-MPK3, right panel, using 6xHis-MPK6. (1)kinase reaction without substrate, (2) kinase reaction using myelin basic protein (MyelinBP) as substrate, (3) kinase reaction using the empty MBP tag as substrate, (4) empty lane, (5) kinase reaction using MBP-HSFA4A as substrate, (6, right) kinase reaction without kinases.

3.7.2 HSFA4A interacts with MPK3 and MPK6 *in vivo*

Next, it was investigated whether the MAP-kinases form a stable complex with their HSFA4A substrate. I started with yeast two-hybrid assays, which revealed that MPK3 and MPK6 interact with HSFA4A. This protein-protein interaction successfully reconstituted Gal4 only when the HSFA4Awas fused with the Gal4 DNA-binding domain (DBD) and the MAP kinases to the Gal4 activation domain (AD) (**Figure 3.7.5.**). On the contrary, cells transformed with another combination of constructs, expressing HSFA4A-AD and MPK3-DBD or MPK6-DBD, failed to grow on –LTHA restrictive media similarly to those aliquots of cells co-transformed with the control plasmid combination pGAD-MPK3/MPK6 and empty pGBT9.

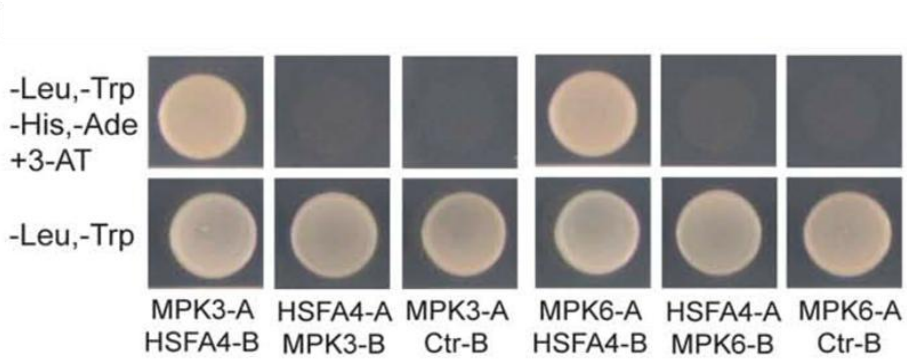

Figure 3.7.5. HSFA4A interaction with MPK3 and MPK6 in yeast cells.
A: proteins fused to activation domain (AD), B: proteins fused to DNA binding domain (DBD). Ctr: empty vector with DBD

Formation of HSFA4A-MPK3 and HSFA4A-MPK6 complexes was subsequently confirmed in planta by BIFC. After *Agrobacterium*-infiltration of *Nicotiana benthamiana* leaves with HSFA4A-nYFP and MPK3-cYFP or MPK6-cYFP (harbouring the C-terminus of split YFP), strong fluorescent signals in the nuclei and occasionally in the cytoplasm of leaf epidermal cells were observed. That pointed to the reconstitution of split YFP due to the specific interaction between HSFA4A and MPK3 and MPK6, since no fluorescence was detected on leaves co-transformed with HSFA4A-nYFP and c-YFP alone (**Figure 3.7.6.**) or with the other control combinations

HSFA4A-cYFP and empty n-YFP, MPK3/6-cYFP and empty n-YFP and MPK3/6-nYFP and empty c-YFP (not shown).

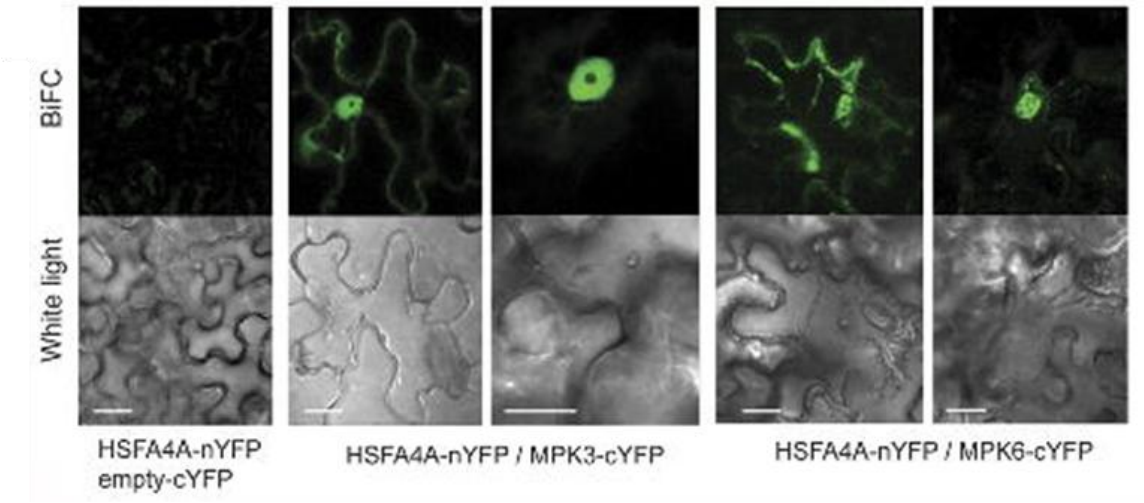

Figure 3.7.6.Interaction of HSFA4A with MPK3 and MPK6 in plant cells.
BIFC of HSFA4A-nYFP, MPK6-cYFP and MPK3-cYFP in *Agrobacterium*-transformed tobacco leaf cells. Bars on microphotographs indicate 20μm.

3.7.3 Identification of MPK3 and MPK6 phosphorylation sites

Next, the MPK3 and MPK6 phosphorylation sites in HSFA4A were identified. Purified MBP-HSFA4A was phosphorylated with non-radioactive ATP, separated on an SDS-PAGE and subjected to mass spectrometry. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of HSFA4A tryptic digests with and without previous enrichment of phosphopeptides was optimized to reach a high sequence coverage (>90%). MS analysis of the TiO2-enriched phosphopeptides identified four candidate MPK3 phosphorylation sites. Ser198, Thr238 and/or Ser239 were found to be phosphorylated exclusively in the MPK3 treated samples (incomplete fragmentation of the corresponding phosphopeptide prevented exact assignment for the Thr238 and Ser239 sites). Two further sites, Ser309 and Thr396 proved to be phosphorylated at a very low level before the MAPK kinase reactions, pointing that bacterial kinases might

have transferred phosphate groups to those sites during the protein expression procedure. The representation of peptides carrying phosphorylated Ser309 and Thr396 residues was however increased by an order of magnitude followed MPK3 and MPK6 phosphorylation indicating that MPK3 might phosphorylate HSFA4A at four or five motives and MPK6 at two motives. Identified putative phosphorylation sites are shown on **Figure 3.7.7**. These target serine and threonine residues are conserved only among the *Brassicaceae* family and are not located in the structural domains of HSFA4A (see **Figure 3.6.8**). The fact that mitogen-activated protein kinases usually phosphorylate a serine or threonine residue that is followed by a proline (Caspersen, 2007), suggested that Thr396 was not targeted by MAPKs.

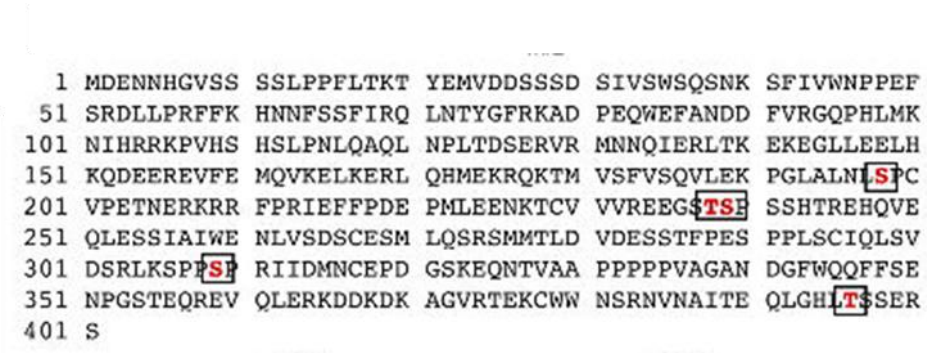

Figure 3.7.7. Identification of MPK3 and MPK6 phosphorylation sites.
Amino acid sequence of HSFA4A with MPK3 phosphorylation sites boxed. Mass Spectrometry identified 5 putative phosphorylation sites in the HSFA4A sequence.

In order to validate the results obtained by mass spectrometry, site-directed mutagenesis was performed. First, since five candidate phosphorylation sites were identified, five variants of HSFA4A were generated. In each mutant construct, four of the target residues were replaced to alanine, leaving one of the sites intact and available for phosphorylation. Finally, to create a non-phosphorylable version of HSFA4A, a construct which contained all five sites substituted to alanine was prepared. All constructs were cloned into pMAL-C2 generating six different versions of MBP-tagged HSFA4A. Recombinant proteins were purified and subjected to the *in vitro* kinase

assays that are shown in **Figure 3.7.8.** Alanine exchange of all five candidate phosphorylation sites completely abolished HSFA4A phosphorylation by MPK3 and MPK6 (**Figure 3.7.8**.). HSFA4A mutated at four predicted phosphosites except Ser309 was phosphorylated almost as well as wild type HSFA4A by MPK3 and MPK6. By contrast, when unaltered Ser198, Ser238 or Thr239 residues were combined with Ala-replacements of other four predicted phosphosites, phosphorylation of HSFA4A by MPK3 was strongly reduced but remained still detectable (**Figure 3.7.8**). Consequently, this combinatorial analysis of phosphosite Ala-replacements indicated that Ser309 is the most preferred site in HSFA4A for phosphorylation by both MPK3 and MPK6.

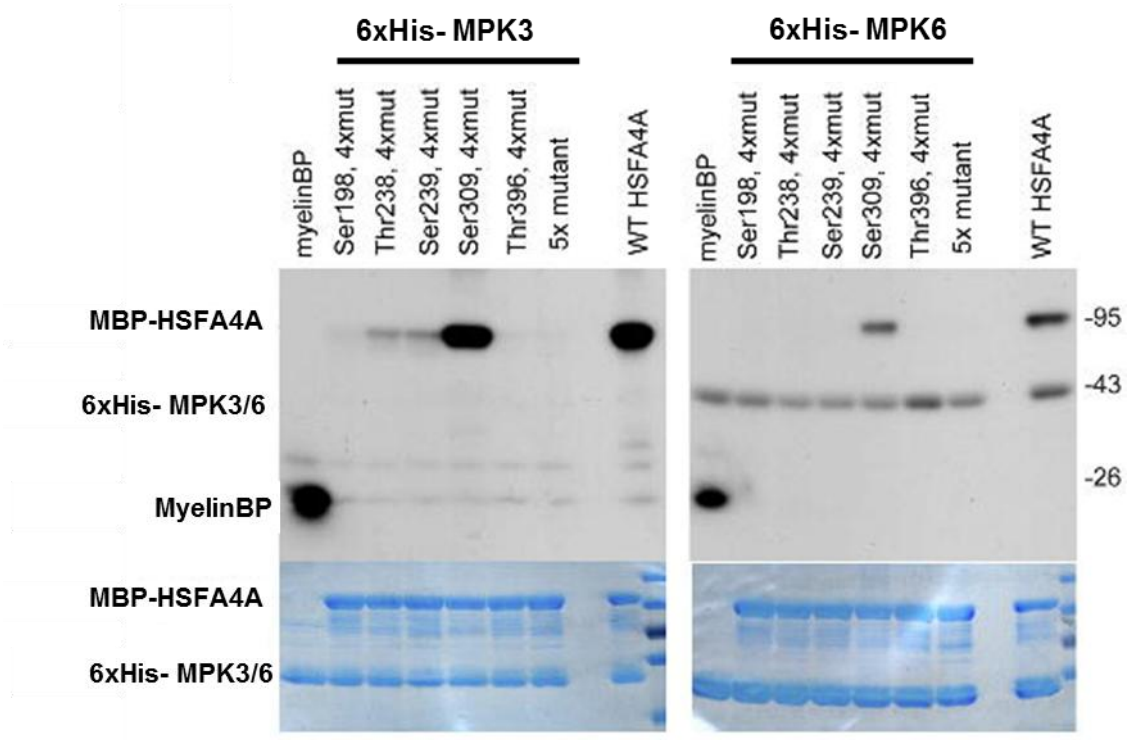

Figure 3.7.8 . Identification of MPK3 and MPK6 phosphorylation sites
Phosphorylation of wild type and mutant HSFA4A by MPK3 and MPK6. 5xmutant: all identified Ser and Thr residues changed to Ala. 4xmutant: all Ser and Thr changed to Ala, except the indicated one. MyelinBP was used in positive control reactions. Upper panel shows phosphorylation reaction, lower panel shows coomassie-stained gels indicating equal loading.

3.7.4 Phosphorylation of HSFA4A enhances transient trans-activation of *HSP17.6A* transcription

In order to characterize the *in vivo* relevance of HSFA4A phosphorylation by MPK3 and MPK6, I performed a transient promoter trans-activation assay. Based on the set of HSFA4A-regulated genes identified by RNA-Seq, *HSP17.6A* was chosen to perform a promoter trans-activation experiment using Arabidopsis protoplasts. The *HSP17.6A* promoter fragment 2 Kb upstream of translation start was fused to a firefly luciferase (LUC+) reporter gene. In parallel, a site directed mutagenesis was performed, to substitute Ser309 to alanine, and the mutagenized cDNA was cloned under the control of CaMV35S promoter. Arabidopsis protoplasts were co-transformed with the pHSP17.6A-LUC reporter construct along with CaMV35S promoter-cDNA constructs expressing either wild type or Ser309Ala mutant HSFA4A (HSFA4m). By monitoring luciferase activity at different time points up to 40 hours after transformation (**Figure 3.7.9**), I observed that trans-activation by wild-type HSFA4A increased the activity of pHSP17.6A-LUC reporter construct 15-fold compared to basic LUC levels. In comparison, HSFA4m carrying the Ser309Ala replacement showed 30% to 40% lower trans-activation of the reporter gene (**Figure 3.7.9**). As MAPK-kinase MKK4 is known to activate the MPK3 and MPK6 (Kim, 2011), I transformed cells with constitutive active $MKK4^{EE}$ to activate downstream MPK3 and MPK6 (Bartels, 2009) together with either wild type or Ser309Ala HSFA4A mutant. Co-expression of MKK4 and HSFA4A led to a gradual increase of pHSP17.6A-LUC trans-activation reaching a 18-fold increase after 40 hours; however, the level of pHSP17.6A-LUC trans-activation was 50% lower after co-expression of MKK4 and HSFA4m (**Figure 3.7.9**). These results suggested that MPK3 and MPK6 activation increases the HSFA4A trans-activation function *in vivo*, while mutating the Ser309 to non-phosphorylable alanine abrogates it.

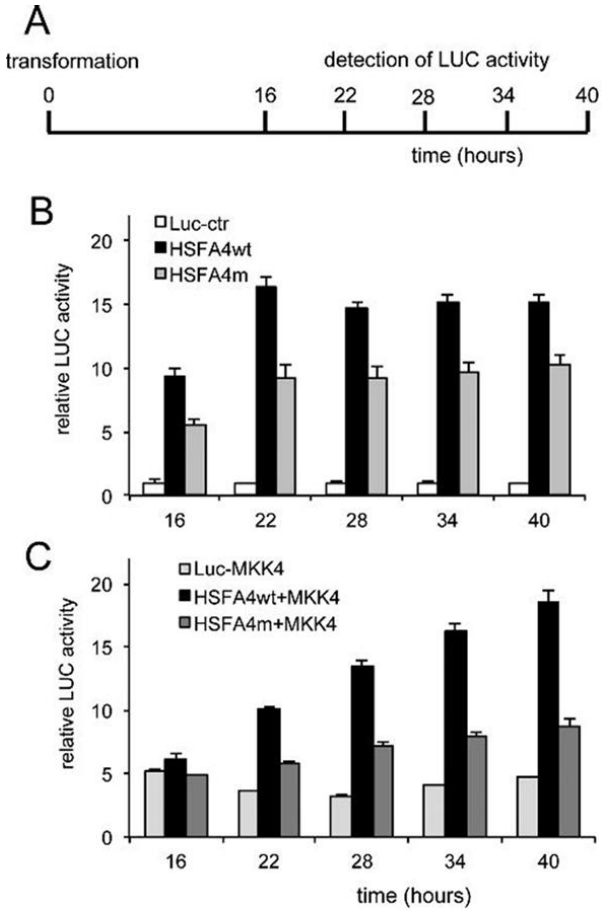

Figure 3.7.9 . HSP17.6A-LUC reporter construct trans-activation assay.
(A) Scheme of the experiment. Arabidopsis protoplasts were transformed with the LUC reporter construct and the wild type HSFA4A (HSFA4wt) or Ser309 mutant HSFA4A (HSFA4m) under the control of CaMV35S promoter on 0 hours, luciferine (3 mM) was added at 16 hours, and LUC activity was detected 16, 22, 28, 34 and 40 hours after transformation. **(B)** Trans-activation of HSP17.6A-LUC with wild type and mutant HSFA4A constructs. **(C)** Trans activation of HSP17.6A by HSFA4A constructs and constitutively active MAP kinase kinase 4 ($MKK4^{EE}$). In both diagrams normalized LUC activities are shown, where activities Luc-ctr is 1.

4 DISCUSSION

4.1 Identification of Stress Response Regulatory Genes using the COS System

4.1.1 Genetic screens using the COS technology in Arabidopsis cell cultures

In order to identify novel regulatory factors involved in stress responses, we used the COS technology (Controlled cDNA Overexpression System, (Papdi, 2008)) to conditionally express a cDNA library in Arabidopsis cell cultures. Our strategy was based on the selection of clones with enhanced salt tolerance, phenotype strictly dependent on cDNA overexpression. About three weeks following *Agrobacterium*-mediated transformation we obtained 1.2 million transformed colonies, which were screened for salt tolerance, finally resulting in 4 independent colonies capable of proliferating only in the presence of estradiol on high salt medium. Isolation of cDNAs from the T-DNA inserts of these salt-tolerant colonies identified potential regulatory factors implicated in salt tolerance. As our main aim was to identify transcription factors involved in stress response, we decided to further investigate the function of the an uncharacterized heat shock transcription factor, HSFA4A. We re-cloned the *HSFA4A* cDNA into the estradiol inducible vector pER8GW (Papdi, 2008), re-transformed Arabidopsis cell suspension, and confirmed the salt-tolerance phenotype associated with *HSFA4A* overexpression. Therefore, the COS technology proved to be a simple and efficient approach for identifying regulatory factors mediating plant stress responses. Expression of cDNA libraries in plants has been rarely used to perform genetic screens. By screening a population of transgenic lines for a given gain-of-function phenotype, several genes involved in stress response regulation have been identified (Kuhn, 2006; Fujita, 2007). However, the establishment of a transformed plant population, followed by screening for altered phenotypes and further re-transformation and plant propagation steps make this technology highly time consuming. An alternative to plant transformation is the expression of plant cDNA libraries in yeast cells. This approach has been proven to be successful for identifying stress related genes, especially transporters (Quintero, 1996), (Obata, 2007). However, there are a number of disadvantages associated with using heterologous expression systems for genetic

screens (Bassham, 2000). For example, if target proteins require to interact with other plant proteins for their function, they will not be identified in such screen (Lambers, 2003). These reasons have made expression of cDNA libraries in plant cell cultures an attractive approach for gene identification. First, cell cultures are rapidly transformed and propagated, and secondly, the natural environment provides all cellular components required for protein modification, activity regulation and function (Lee, 2012). Our cDNA library screening method using an Arabidopsis cell culture was a rapid and effective approach, which allowed us, in relatively short time, to screen a large number of transformed cells. The fact that the salt-tolerance phenotype, conferred by *HSFA4A* overexpression, was further confirmed in Arabidopsis plants, supports the efficiency of this method.

4.1.2 Conditional overexpression of HSFA4A confers tolerance to Arabidopsis plants

Expression of *HSFA4A* in Arabidopsis plants recapitulated the enhanced salt tolerance phenotype observed in cell cultures. Study of the effects of estradiol-inducible *HSFA4A* overexpression, revealed that induction of *HSFA4A* confers enhanced tolerance not only to salt but also to osmotic stress, paraquat, H_2O_2 and to anoxia, which generate oxidative stress. Thus, a tolerance trait selected in cell culture could be simply validated in transgenic plants. It is therefore not surprising that potential HSFA4A orthologs from wheat and rice were reported to confer cadmium tolerance when expressed in yeast and transgenic rice; and the putative rice *hsfa4a* mutant was found to be hypersensitive to cadmium (Shim, 2009). In comparison, overexpression of *HSFA2* enhances salt and osmotic stress tolerance (Ogawa, 2007), while HSFA3 confers drought tolerance (Yoshida, 2008), suggesting that particular members of HSF family are implicated in responses to various environmental stress conditions. These findings point to the existence of a high degree of specialization in the response of a HSF to a specific stress. Similarly to overexpression of *CBF* transcription factors, which confer tolerance to cold stress (Achard, 2008), estradiol-induction of *HSFA4A* conferred thus some growth inhibitory effect in the absence of stress while decreased growth inhibition under salt stress. The overexpression of *HSFA4A* could prevent oxidative damage in Arabidopsis plants, as indicated by the lower content of H_2O_2 and reduced lipid peroxidation after

salinity stress. These data indicates that *HSFA4A* overexpression upgrades the plant antioxidant capacity, making plants more tolerant to environmental stress. Similarly, it was previously reported that overexpression of transcription factors such *Zat12* and *Zat10* enhanced the expression of ROS-scavenging genes, which improved plant tolerance to salt, drought and osmotic stress (Miller, 2010). Besides, HSF-dependent regulation of *APX* genes have been previously documented (Panchuk, 2002), underlining the importance of HSFs as mediators of oxidative stress responses.

4.2 Transcriptional regulation of *HSFA4A*

Induction of gene expression in response to a external stimuli is a key step for the activation of the downstream molecular events that integrate plant responses. Therefore, gene transcription analysis is a powerful tool to estimate gene function, as gene activation in response to a given stimuli implies a function in mediating a specific response. In our gene expression studies, *HSFA4A* was found to be induced by all stress treatments performed, including salt, mannitol, H_2O_2, paraquat, and heat, all of them inducing ROS accumulation. The expression pattern during the different stress treatments indicated that *HSFA4A* expression is highly flexible, and HSFA4A may control the response to diverse stress conditions. *HSFA4A* expression was shown to be tightly regulated by stress, as indicated by the fast response upon H_2O_2 treatment, which triggered *HSFA4A* transcript accumulation within 30 minutes (**Figure 3.4.1**.). In parallel, public transcript profiling data showed that *HSFA4A* is activated by other stressors, such cold, UV-B and pathogens (**Figure 3.3.2).** The data obtained on the spatial expression of *HSFA4A*, visualized using the pHSFA4A-GUS construct, revealed that *HSFA4A* was ubiquitously expressed in seedlings, especially in adult leaves and in meristems. According to the Genevestigator database, *HSFA4A* expression in Arabidopsis is mainly localized in green tissues, especially in adult leaves, senescent leaves and some root tissues. The spatial distribution of *HSFA4A* strongly supports a role of a regulator of oxidative stress responses. Expression in green tissues, with high photosynthetic activity and therefore main sites of ROS production, and in meristems indicates that HSFA4A is required to regulate ROS homeostasis in these tissues. Similarly, ascorbate content, a powerful antioxidant, is highest in photosynthetic cells

and meristems (Shao HB, 2008). The expression of *HSFA4A* in senescent leaves is not surprising, as senescence is tightly associated with oxidative stress, and a higher ROS accumulation during this process (Zimmermann, 2005). Also, ROS production is involved in the regulation of root tip growth (Swanson, 2010). Therefore, *HSFA4A* expression pattern narrowly correlates with the function of HSFA4A as a modulator of plant oxidative stress response.

4.3 HSFA4A-regulated genes

I identified genes that are controlled by HSFA4A and H_2O_2 signals and are annotated to respond to diverse range of stresses, such as heat, salt, heavy metals and/or pathogens. qRT-PCR analysis confirmed that HSFA4A is involved in transcriptional activation of *HSP17.6A*, *ZAT6*, *ZAT12/RHL41*,*CTP1*,*WRKY30*, and *CRK13*, which play pivotal roles in the regulation heat, salt, oxidative, osmotic, heavy metal, pathogenic and starvation stress responses (Sun et al., 2001; Libault et al., 2007; Peng et al., 2012). Most of these genes are upregulated by salinity indicating that they are involved in defences to salt stress. Similarly to H_2O_2, *ZAT12* stimulates the induction of the *APX1* gene in response to light stress providing a feedback regulatory circuit for H_2O_2 removal (Davletova et al., 2005). *WRKY30* is induced by superoxide anions generated in damaged chloroplasts (Scarpeci et al., 2008), whereas *ZAT6* is activated by osmotic and salt stress (Liu et al., 2012). These results suggest that HSFA4A can act in common pathways with these transcription factors. The expression of several APX and HSP genes are known to be controlled by HSF complexes in response to H_2O_2-derived signals during heat stress (Volkov et al., 2006). Similarly, HSFA4A seems to modulate transcription of a set of target genes, involved in mounting defense to abiotic and biotic stress stimuli. Data obtained from RNA-Seq showed that promoters of many HSFA4A-regulated genes contained one or more HSE motifs, suggesting that HSFA4A and/or other HSFs might directly control their transcription.

4.4 The *hsfa4a* T-DNA mutant is hypersensitive to salt

A knockout mutant of *HSFA4A* from the INRA Versailles T-DNA collection was identified. HSFs exhibit a high functional redundancy (Scharf, 2012) and, due to

overlapping regulatory functions, the classical genetic approach based on characterization of individual gene mutations has a relatively low resolution power (Liu, 2011). Subsequent characterization of the *hsfa4a* T-DNA insertion mutant confirmed that HSFA4A plays an important role in the regulation of salt tolerance in Arabidopsis, as the mutation conferred markedly enhanced sensitivity to salt treatment. The *hsfa4a* mutant does not show enhanced sensitivity to H_2O_2, $CdCl_2$, paraquat, osmotic stress and anoxia, indicating that other HSFs could compensate for the lack of HSFA4A to mount sufficient tolerance to these stress stimuli. Nonetheless, the *hsfa4a* mutant displays enhanced H_2O_2, accumulation and higher lipid peroxidation rate compared to wild-type and genetically complemented mutant, suggesting that the threshold of oxidative stress tolerance is clearly lowered by inactivation of HSFA4A. Therefore, these data also supports the finding that HSFA4A is involved in the modulation of ROS metabolism and responses to oxidative stress.

I tested a set of selected genes and we found that activation some of these genes was compromised in the *hsfa4a* mutant, while others were unaffected, which can be explained by observations that subsets of plant heat shock factors can have at least partially overlapping functions (Scharf, 2012). By testing a set of salt-induced genes, I found that salt-induced transcript levels of WRKY30 and ZAT6 transcription factor genes are notably reduced in the *hsfa4a* mutant. As WRKY30 is induced rapidly by superoxide anions generated in damaged chloroplasts (Scarpeci, 2008), whereas ZAT6 is activated by osmotic stress in addition to salt (Liu, 2013), these results suggest that HSFA4A, as upstream regulator, acts in common pathways with WRKY30 and ZAT6.

4.5 Homomeric interaction of HSFA4A

Activation of HSFs involves the formation of homotrimers, which is required for their nuclear import and high affinity binding to conserved heat shock elements (HSE) in the promoters of target genes (Anckar, 2011). Yeast and BiFC protein interaction studies illustrated that HSFA4A forms oligomers in both yeast and plant cells, which is stimulated by salt-treatment. In addition to the described salt treatments, I also performed experiments using 10 mM H_2O_2. Usually 10 minutes treatment increased BIFC signal intensity, but led to the accumulation of cytosolic protein aggregates,

which were most likely cytosolic stress granules or processing bodies (P-bodies) caused by the harsh effect of using directly H_2O_2. Although H_2O_2 treatments would have been a most direct approach to show ROS-mediated HSFA4A oligomerization, technically resulted challenging and I could not obtain clear results. It would be necessary to standardize both concentration of H_2O_2 and length of the treatment if future experiments are to be performed. On the other hand, salt treatments resulted in reproducible results, most likely because they induced a milder and gradual ROS accumulation which work better in my experimental set up.

Arabidopsis HSFA1A and HSFA1B were reported to form homo- and heterodimers, which is thought to be essential for transcriptional activation of their target genes (Li, 2010). Repression of the activity of HSFA4 through interaction of HSFA5 was reported in tomato and Arabidopsis (Baniwal, 2007). Such interaction can be responsible for the regulation of HSFA4 in other species as well. Our RNASeq data showed thattranscript levels of HSFA5 were not affected significantly by enhanced HSFA4A expression (data not shown), therefore balance between the two factors was probably changed leading to HSFA4A excess in the overexpressing plants. Importance of proper control of HSFA4A activity can be illustrated by the fact that HSF4ox plants displayed growth deficit in non-stress conditions when compared to wild type.

The oxidation of two cysteine residues promotes the formation of disulphide bonds, which leads to conformational changes that alters enzyme activity. The formation of intramolecular disulphide bonds modify enzyme activity by conformational changes, but intermolecular disulphide bond formation promotes oligomerization (Bienert, 2006). Several well characterized redox-regulated proteins are transcription factors. The *Escherichia coli* transcription factor OxyR is only active upon oxidation, when a disulphide bond is generated between its Cys199 and Cys208 residues. Following oxidation, OxyR regulates a set of H_2O_2-inducible genes; OxyR deactivation is achieved by disulphide bond reduction (Aslund, 1999). In *Saccharomyces cerevisiae*, the Yap1 transcription factor regulates the expression of antioxidant genes in response to oxidative stress, and is activated by oxidation and intramolecular disulphide bond formation when H_2O_2 levels increase (Delaunay, 2000). However, Yap1 is not directly oxidized by H_2O_2; the thiol peroxidase Gpx3 was identified as a H_2O_2 sensor, which upon oxidation bridges its Cys36 and the Yap1 Cys598 by a transient intermolecular disulphide bond. Eventually, the disulphide is converted to an intramolecular Cys303-Cys598 bond within Yap1, which leads to its activation (Delaunay, 2002). In plants, two

transcription regulators involved in biotic stress responses are regulated by redox modifications induced by salicylic acid. The protein NPR1 (Non Expressor of Pathogenesis Related Genes 1) is inactive in an oligomeric state maintained by intermolecular disulphide bonds. Reduction of cysteine residues releases the monomeric NPR1, which accumulates inside the nucleus. Also following cysteine reduction, TGA transcription factors interact with NPR1, which stimulates their DNA-binding activity (Fobert, 2005).

The transcriptional activation of the mammalian heat shock transcription factor 1 (HSF1) depends on redox regulation. Upon heat or H_2O_2 treatment, the inactive monomeric form of HSF1 is oxidized and undergoes a conformational change. The intramolecular disulphide bond between Cys35 and Cys105 facilitates multimerization, required for its nuclear accumulation and transcriptional activity (Ahn, 2003). Although oligomerization domains (OD) were shown to be essential for interactions between HSFA1a and HSFA1b in Arabidopsis (Li, 2010), and HSFA1 and HSFA2 in tomato (Chan-Schaminet, 2009), the importance of conserved Cys residues in homomeric interactions of plant HSFs has not been clearly demonstrated. Our data support a likely role of Cys residues in dimerization of Arabidopsis HSFA4A because exchanging conserved Cys residues to Ala greatly reduces the formation of HSFA4A oligomers without affecting protein stability. However, the fact that these residues are conserved in HSFA4A homologs in Arabidopsis-related species but not in HSFA4A proteins of citrus, grapevine and poplar suggest that formation of redox-sensitive disulfide bonds of cysteine residues is probably not a sole requirement for HSFA4A di/trimerization. Therefore, the prediction that plant HSFs function as molecular sensors of ROS signals (Miller, 2006) remains to be revisited by further studies.

4.6 Phosphorylation of HSFA4A by interacting MPK3 and MPK6

In Arabidopsis, MAPK cascades are involved in signalling pathways activated by multiple environmental stimuli and specifically, the implication of MAPK cascades in biotic stress signalling has been extensively documented. For example, a complete MAPK signalling pathway consisting of MEKK1, MKK4/MKK5 and MPK3/MPK6 was identified downstream of the flagellin receptor FLS2, and was shown to promote the expression of *WRKY29* and *flg22-induced receptor-like kinase 1* (*FRK1*) genes,

enhancing the resistance to bacterial and fungal pathogens (Asai, 2002). Another study demonstrated that flagellin activates MKK1, which in turn phosphorylates MPK4, therefore suggesting an alternative signalling module with MEKK1-MKK1-MPK4, and placing this pathway as a negative regulator of pathogen responses (Meszaros, 2006).

MAPK cascades are also involved in signalling pathways activated by abiotic stresses such as salt, cold, water stress, oxidative stress and high temperature (Colcombet, 2008). Under salt and cold stresses, MPK4 and MPK6 are phosphorylated by MKK2, which was shown to be activated by salt, cold, and the stress induced MEKK1 (Teige, 2004). The MAPKs MPK3 and MPK6 have been connected to ABA-mediated signalling processes: MPK3 was shown to participate in ABA and ROS perception during stomatal closure in Arabidopsis guard cells (Gudesblat, 2007) and a MAPK cascade consisting of MKK1 and MPK6 mediated the ABA-induced expression of catalase 1 (CAT1) and H_2O_2 production, in an ABA-dependent signalling cascade (Xing, 2008). Oxidative stress activates MPK3, MPK4 and MPK6 although by different MAPKs pathways , (Kovtun, 2000). MPK3 and MPK6 are activated by ozone (O_3) and translocated to the nucleus after O_3 treatment, which suggests that MPK3 and MPK6 are involved in the phosphorylation of transcription factors that regulate O_3-responsive genes (Ahlfors, 2004). Recently, it was shown that followed by heat activation, the Arabidopsis MPK6 phosphorylated the heat shock transcription factor 2 (HSFA2), therefore regulating its nuclear accumulation (Evrard, 2013).

The presented data demonstrate that HSFA4A interacts with MPK3 and MPK6 in both yeast and plant cells, and that HSFA4A is a substrate of MPK3 and MPK6 *in vitro*. Phosphorylation of heat shock factors by MAP kinases was reported in several organisms including mammalian cells (Kim, 1997), yeast (Hashikawa, 2004) and plants (Evrard, 2013). While phosphorylation repressed HSF1-dependent transcriptional activation in human cells (Chu, 1996) multimerisation of yeast HSF in connection with hyperphosphorylation enhanced induction of target genes (Hashikawa, 2004). Heat-activated MAP kinases were shown to transduce heat stress signals by phosphorylating HSFs in tomato or promoting HSP gene expression in tobacco (Link, 2002), (Suri, 2008). MPK3- and MPK6-dependent phosphorylation of HSFA4A and their physical interaction, therefore correlates with earlier observations indicating that some HSFs can be substrates for MAP kinases. Ser309 was found as the preferential MPK3 and MPK6 phosphorylation site in HSFA4A *in vitro*, which is located between two activator domains. This residue is conserved in closely related homologs of *Eutrema salsugineum*

and *Brassica napus*, but not in the more distantly related plants. Whether HSFA4A indeed is phosphorylated on Ser309 site after MPK3 and MPK6 activation *in vivo*, remains to be confirmed, but these experiments are challenging (Dephoure, 2013). To investigate the *in vivo* relevance of Ser309 phosphorylation, *in vivo* functional studies by site directed mutagenesis were performed. These functional studies were based on trans-activation assays in cell culture-derived protoplasts. To establish this system, three different promoters were fused to the luciferase gene and tested for trans-activation by HSFA4A. The selected genes were *HSP17.6A* (AT5G12030), *ATL31* (AT5G27420) and a flagellin-induced FAD-binding and BBE domain-containing protein (AT1G26420). Among the three promoter-LUC fusions, pHSP17.6A exhibited the highest trans-activation by HSFA4A and therefore was chosen to perform the experiments. Using the pHSP17.6A-LUC reporter construct for a transient transformation assay in Arabidopsis protoplasts, I found that exchange of serine 309 to alanine reduced the trans-activation of pHSP17.6A-LUC by HSFA4A. In addition, the constitutively active form of MKK4 activated HSP17.6A promoter driven LUC expression, which was further enhanced when MKK4 was co-expressed with HSFA4A. These results suggested that HSFA4A could act in concert with the MKK4-MPK3/6 phosphorylation cascade regulating *HSP17.6A* expression, but further studies are necessary to characterize the function of HSFA4A phosphorylation by MPK3/6 in transcription regulation of other genes.

MPK3 and MPK6 were identified as central regulators of plant innate immunity, hypoxia, salt and osmotic stress responses, which control cross-talk between different stresses, hormonal signals and second messengers, such as ROS (Chang, 2012), (Rasmussen, 2012). MAP kinases were shown to phosphorylate transcription factors such as WRKY33, involved in ethylene biosynthesis (Li, 2012), or MYB44 which regulates ABA sensitivity (Nguyen, 2012). MPK3 and MPK6 can form *in vivo* complexes with ZAT10/STZ (Nguyen, 2012), while under salt and osmotic stress, MPK6 interacts with and phosphorylates ZAT6 (Liu, 2013).Thus, MPK3 and MPK6 appear to regulate a wide-range of biotic and abiotic defence responses mediated by ROS signals, and coordinate the activity of various transcription factors such as WRKY, MYB, ZAT and HSF, that in turn control the transcription of a large set of target genes.

4.7 Proposed model of HSFA4A-mediated stress signalling

Figure 4.1 shows a suggested model of regulation of HSFA4A, based both on our data and the literature. First, different stress stimuli cause ROS accumulation, which triggers the activation of downstream ROS-dependent signalling pathways. Second, ROS activate both HSFA4A and MPK3 and MPK6. Based on results obtained from HSFA4A homomerisation studies, we conclude that ROS induce HSFA4A di/trimerisation, and the accumulation of these oligomers in the nucleus. Following ROS-mediated activation MPK3 and MPK6 interact with and phosphorylate HSFA4A. I did not investigate whether MPK3 and MPK6 phosphorylate HSFA4A in its monomeric or oligomeric state, but based on the proposed mechanism of regulation of HSFs by (Miller, 2006), (**Figure 1.2.4),** and similarly to yeast HSF1, HSFA4A may be phosphorylated in the oligomeric state (Batista-Nascimento, 2011). Finally, HSFA4A regulates the transcription of a set of stress response genes (**Figure 3.4.4)** either by directly bind to their promoter HSEs or through the activation of other regulatory proteins.

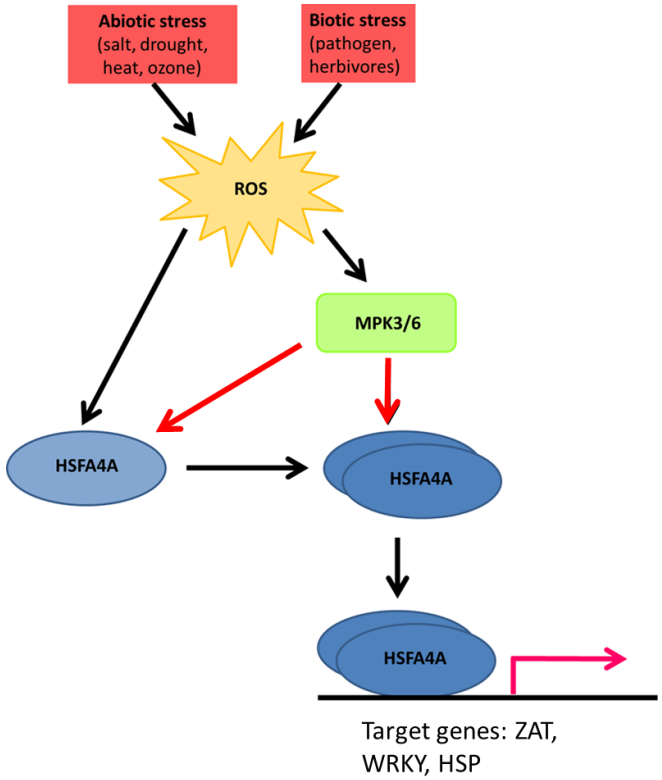

Figure 4.1 Model of HSFA4A-mediated Stress Signalling.
Reactive Oxygen Species such as H_2O_2 are produced in plant cells as consequence of abiotic stress or pathogen infection. Upon stimulation by ROS signals, MPK3/6 are activated and phosphorylate HSFA4A, which forms multimers when exposed to stress-derived ROS. Activated HSFA4A triggers the expression of target genes, encoding other transcriptional regulators or defense-related proteins

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7 SUMMARY

Plants, as sessile organisms are constantly subjected to external stimuli, which determine their growth, development and survival. The environmental conditions frequently fluctuate beyond optimal parameters, becoming a source of stress for the plants. To cope with the unfavourable environment, plants have developed several strategies, which involve different biochemical and physiological adjustments. Mechanisms of stress acclimation and tolerance are controlled by complex signalling pathways that regulate gene expression. Our knowledge of stress perception is limited, downstream components of the signalling cascade are activated by specific receptors that are mostly unknown. The signal is transmitted by secondary messenger molecules such as phospholipids, cyclic nucleotides, hormones, Ca^{2+} and reactive oxygen species (ROS) and activates enzymes involved in post-translational modifications, i. g. protein phosphorylation by CIPKs, CDPKs and MAP kinases. Phosphorylation regulates the activity of several stress response molecules, including transcription factors. In general, mechanisms of stress tolerance are based on the maintenance of homeostasis, ROS detoxification and direct protection of cellular components (Wang, 2003).

A substantial amount of information on abiotic stress signalling pathways has been obtained using *Arabidopsis thaliana* as a model plant. Publication of the genome sequence of Arabidopsis, together with development of genetic tools has been essential to dissect molecular mechanisms behind stress responses (Papdi, 2009). Particularly, genetic screens have proven to be very useful tools to identify stress-related genes (Sreenivasulu, 2007; Papdi, 2008).

The aim of this project was to identify new components of plant stress tolerance using a novel genetic approach. For this purpose, an Arabidopsis random cDNA library under the control of an estradiol induced promoter (Papdi, 2008) was expressed in Arabidopsis cell suspension, and 1.2 million transformed cells were screened for enhanced salt tolerance. Four cell suspension derived microcolonies were identified with salt tolerant growth that was dependent of estradiol. One of the colonies carried the full-length cDNA of the heat shock transcription factor HSFA4A, this gene was chosen for further functional characterization.

The conditional salt-tolerance phenotype was confirmed by re-transforming the estradiol-induced ER8-HSFA4A into Arabidopsis cell suspension. Thereafter, ectopic expression of HSFA4A in Arabidopsis plants provided tolerance to salt, paraquat, H_2O_2, anoxia, $CdCl_2$ and mannitol, all these stressors converge in oxidative stress. During salt stress, HSFA4A overexpression resulted in lower lipid peroxidation rate and reduced H_2O_2 content indicating a decreased effect of the salt-induced oxidative damage. These results suggested that HSFA4A contributes to Arabidopsis oxidative stress tolerance.

Corresponding with public available transcript profiling, gene expression analysis showed that *HSFA4A* expression can be induced by several stress conditions. After H_2O_2 treatment, rapid induction of *HSFA4A* was detected and high levels of HSFA4A mRNA was sustained for several days.

To find target genes of HSFA4A, a transcript profiling was performed, comparing HSFA4A overexpressing plants with wild type. A set of stress response genes was identified as transcriptional targets of HSFA4A: *ZAT6, ZAT12, HSP17.6A, ATL31, CRK13, WRKY30* and *CTP1.*

Characterization of *hsfa4a* T-DNA insertion mutant revealed that the inactivation of *HSFA4A* causes salt hypersensitive growth, higher lipid peroxidation and increased H_2O_2 accumulation during salt stress. However, genetic complementation of *hsfa4a* could restore the wild type phenotype. Salt-treated *hsfa4a* plants showed reduced levels of *ZAT6, ZAT12, WRKY30* and *CTP1* comparing to wild type.

A model previously proposed that in plants, ROS accumulation results in multimer formation of heat shock factors, which can be connected to their function (Miller, 2006). Performing yeast two-hybrid assay and Bimolecular Fluorescence Complementation (BIFC), it was demonstrated that HSFA4A is able to create homodimers, furthermore, during salt stress, nuclear accumulation of HSFA4A homodimers was observed. Multimer formation occurs between cysteine residues of two or more HSFA4A proteins. Substitution of 3 highly conserved cysteine residues to alanine greatly reduced HSFA4A homodimerization in yeast cells and in Arabidopsis protoplasts.

Using yeast two-hybrid assay and BIFC in tobacco leaves, was showed that HSFA4A interacts with two mitogen-activated kinases MPK3 and MPK6. With *in-gel* and *in vitro* kinase assays, was demonstrated that MPK3 and MPK6 phosphorylate HSFA4A,

and phosphorylation sites were identified by mass spectrometry. Site-directed mutagenesis followed by an *in vitro* kinase assay revealed Ser309 as the major phosphorylation site. *Trans*-activation of pHSP17.6A-LUC reporter construct by the Ser309Ala mutant HSFA4A was significantly reduced compared to the wild type HSFA4A.

8 ÖSSZEFOGLALÁS

A helyhez kötött életmódjuk miatt, a növények növekedése, fejlődése és túlélése is a környezetüktől függ. A környezeti viszonyok gyakran kedvezőtlenné válnak és ez stresszt okoz a növények számára. A megváltozott körülményekkel szemben a növények számos stratégiát fejlesztettek, amelyek biokémiai és fiziológai változasokat idéznek elő. Azokat a folyamatokat, amelyek a stresszhez való alkalmazkodást és toleranciát biztosítják egy komplex jelátviteli hálózat szabályozza ami végsősoron génkifejeződések megváltozásához vezet. Jelenleg keveset tudunk arról, hogy a növények hogyan érzekelik a stresszt, valószínűleg stressz-specifikus receptorok aktiválják a jelátviteli hálózatokat. A szignálokat másodlagos hírvivő molekulák közvetítik, ilyen molekulák a foszfolipidek, a ciklikus nukleotidok, a hormonok, a Ca^{2+} és a reaktív oxigén fajták (ROS). Ezek sokszor poszt-transzlációs módosításokért felelős enzimeket aktiválnak, mint pl. a fehérje foszforilációt végző CIPK, CDPK and MAP kinázokat. A fehérje foszforilációk számos stressz-válasz molekula aktivitását tudják szabályozni, többek között transzkripciós faktorokét is. Általánosságban elmondható, hogy a stressz tolerancia mechanizmusok a homeosztázis fennartására, a ROS detoxifikálásra és a sejtkomponensek közvetlen védelmére irányulnak (Wang, 2003).

Nagy mennyiségű információ áll rendelkezésre az abiotikus stressz toleranciáról, ami főleg a növényi modell, az *Arabidopsis thaliana* kutatásokból származik. Az Arabidopsis genom megszekvenálása és hatékony genetikai módszerek kifejlesztése fontos szerepet játszik abban, hogy a stressz válaszok molekuláris mechanizmusait vizsgáljuk (Papdi, 2009). A genetikai szűrések nagyon hasznosnak bizonyultak a stressz válasz gének azonosításában (Papdi, 2008)(Sreenivasulu, 2007).

A munkám célja az volt, hogy a növényi stressz tolerancia ezidáig nem ismert komponenseit azonosítsam, egy új genetikai módszer alkalmazásával. Ehhez, egy random Arabidopsis cDNS könyvtárat használtam, aminek a kifejeződését ösztradiol indukálható promóter szabályozta (Papdi, 2008). A cDNS könyvtárat Arabidopsis sejtszuszpenzióban túltermelve hozzávetőlegesen 1,2 millió transzformált sejt kolónia osztódási képességét vizsgáltam só stressz jelenlétében. Négy olyan sejtszuszpenzióból származó mikrokolóniát találtam, ami ösztradioltól függően, só toleráns módon növekedett. Az egyik kolónia sejtjeibe beépült T-DNS egy hő sokk transzkripciós faktor a *HSFA4A* teljes hosszúságú cDNS-ét tartalmazta, ennek a génnek a funkcionális jellemzését végeztem el.

Ösztradiol-függő só toleráns mikrokolóniákat kaptam, amikor az ER8-HSFA4A plazmidot újra Arabidopsis sejtszuszpenzióba transzformáltam. A HSFA4A Arabidopsis növényekben való túltermelése só, paraquat, H_2O_2, anoxia, $CdCl_2$ és mannitol toleranciát okozott, mindezek a körülmények összefüggésbe hozhatóak az oxidatív stresszel is. Só stressznek kitéve a HSFA4A túltermelése a lipid peroxidáció és a H_2O_2 tartalom csökkenését idézte elő Arabidopsisban. Ezek a megfigyelések arra utalnak, hogy az HSFA4A túltermelése csökkentette a só indukált oxidatív károsodás mértékét. Mindezekből arra következtettünk, hogy az HSFA4A hozzájárul az Arabidopsis oxidatív stressz toleranciájához.

Az *HSFA4A* génkifejeződés vizsgálata, a hozzáférhető microarray adatokkal összhangban arra engedett következtetni, hogy az *HSFA4A* számos stressz körülmény hatására indukálódik. Hidrogén-peroxid kezelés a *HSFA4A* kifejeződés gyors indukcióját okozta, és ezt követően a HSFA4A mRNS magas szintje több napon keresztül kimutatható volt.

RNS szekvenálással azonosítottuk az HSFA4A transzkripciós célpontjait. Az HSF4A4 többek között ismert stressz-válasz géneket szabályoz: *ZAT6, ZAT12, HSP17.6A, ATL31, CRK13, WRKY30* és *CTP1*.

Az HSFA4A inaktiválása a *hsfa4a* inszerciós mutánsban só-érzékeny növekedést, megnövekedett lipid peroxidációt és H_2O_2 felhalmozódást idézett elő. A *hsfa4a* mutáns genetikai komplementációja helyre tudta állítani a vadtípusra jellemző só-tartalmú táptalajon való növekedési tulajdonságokat. A só stresszel kezelt *hsfa4a* növényekben a

ZAT6, *ZAT12*, *WRKY30* és *CTP1* gének alacsonyabb szinten fejeződtek ki mint a vadtípusú növényekben.

Egy korábban felvázolt modell szerint a ROS felhalmozódás hatására a hő sokk faktorok multimerekké állnak össze, ami fontos a funkciójuk szabályozásában (Miller, 2006). Élesztő két-hibrid és kétmolekuláris fluoreszcencia komplementáció (BIFC) kísérlettel igazoltam, hogy az HSFA4A képes homodiméreket alkotni, és ezek a homodimérek só stress hatására a sejtmagban halmozódnak fel. A fehérje-fehérje kölcsönhatásokat cisztein oldalláncok közötti diszulfid-hídak stabilizálhatják. Három konzervált cisztein aminosav alaninra történő kicserélése a dimerizációs kölcsönhatások csökkenését idézte elő élesztő és Arabidopsis sejtekben is.

Élesztő kéthibrid és BIFC kísérletekkel azt is sikerült kimutatni, hogy az HSFA4A kölcsönhat két mitogen-aktivált kinázzal az MPK3-al és az MPK6-al. Két különböző módszerrel, *in-gel* és *in vitro* kináz assay-vel bizonyítottam, hogy mindkét MAP kináz képes az HSFA4A foszforilációjára. A HSFA4A foszforilációs helyeit tömeg spektrométerrel tudtuk meghatározni. Hely-specifikus mutagenezist követő *in vitro* kináz assay-vel kimutattam, hogy a 309 pozicióban levő szerin (Ser309) a legfontosabb foszforilációs hely. A túltermelt HSFA4A Ser309Ala aminosav cserélt változata a vadtípussal összehasonlítva kisebb mértékben tudta a HSP17.6A promóterét aktiválni.

Ezek az eredmények igazolták, hogy a HSFA4A fontos szerepet játszik a reaktív oxigének és a MPK3 és MPK6 MAP kinázok által közvetített jelátviteli folyamatok szabályozásában.

9 PUBLICATION LIST

This PhD thesis is based on the article:

Perez-Salamo I, Papdi Cs, Rigo G, Zsigmond L, Vilela B, Lumbreras V, Nagy I, Horvath B, Domoki M, Darula Z, Medzihradszky K, Bogre L, Koncz Cs, Szabados L (2014) The Heat Shock Factor A4A Confers Salt Tolerance and Is Regulated by Oxidative Stress and the Mitogen-Activated Protein Kinases MPK3 and MPK6. Plant Physiol 165: 319-334, MTMT: 2570455, IF: 6.555

Other publications related to this thesis :

Papdi Cs, Joseph MP, **Pérez-Salamó I**, Vidal S, Szabados, L (2009) Genetic technologies for the identification of plant genes controlling environmental stress responses. Funct Plant Biol 36:696-720., MTMT: 1920786, IF: 2.471

Papdi Cs, Leung, J, Joseph MP, **Pérez-Salamó I**, Szabados L (2010) Genetic screens to identify plant stress genes. In: *Methods in Molecular Biology,* vol. 639. New York: Humana Press. 639: 121-139, MTMT: 1921541

Other publications :

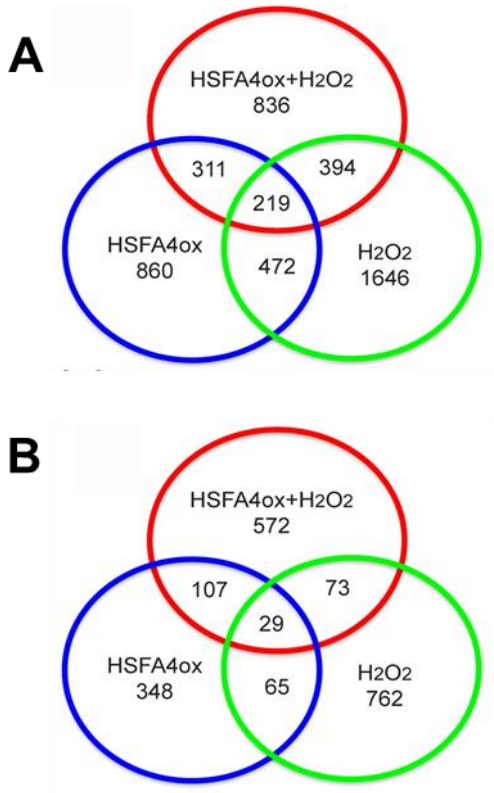
Ruibal C, **Pérez-Salamó I**, Carballo V, Castro A, Bentancor M, Borsani O, Szabados L, Vidal S (2012) Differential contribution of individual dehydrin genes from Physcomitrella patens to salt and osmotic stress tolerance. Plant Sci 190:89-102. MTMT: 2014259, IF: 2.922

Ábrahám E, **Pérez-Salamó I**, Koncz C, Szabados L (2011) Identification of Arabidopsis and Thellungiella genes involved in salt tolerance by novel genetic system. Acta Botanica Szegediensis 55:53-57, MTMT: 1922012

10 SUPPLEMENTAL DATA

10.1 Figure S1.

To dissect the role of HSFA4A in ROS signalling, RNA-Seq transcript profiling was performed using RNA samples from wild type and HSF4ox2 plants, which were treated with 5 µM estradiol in the absence or presence of 1 mM H_2O_2 for 6 hours. Venn diagrams depicting the distribution of Arabidopsis genes induced (A) or repressed (B) by both HSFA4A and H_2O_2 treatment



10.2 Table S1.

Genes upregulated by both HSFA4A overexpression and H_2O_2treatment**.**

AGI	Protein	HSFA4A	H_2O_2	HSFA4A +H_2O_2	HSE	Other TF
AT5G12030	small heat shock protein HSP17.6A	7,3	16,1	22,3	0	AP2/EREBP, MYB, C2C2(Zn) Dof
AT5G12020	small heat shock protein HSP17.6II	5,3	4,5	18,3	1	AP2/EREBP
AT1G64500	glutaredoxin-like protein	2,9	4,6	15,3	1	HD-HOX
AT3G46230	small heat shock protein HSP17.4	4,7	3,3	14,5	1	HSFB, C2C2(Zn) Dof

AT1G53540	HSP20-like chaperone protein	5,6	5,2	12,4	2	HSFB, HD-HOX
AT4G22960	Protein of unknown function (DUF544)	6,6	13,8	12,4	1	C2C2(Zn) Dof
AT1G17010	2-oxoglutarate and Fe(II)-dependent oxygenase	7,3	4,3	12,3	0	
AT1G69570	Dof-type zinc finger DNA-binding protein	3,2	4,3	11,1	1	bZIP, MYB, GATA
AT1G16030	heat shock protein HSP70B	2,9	7,5	8,6	0	HSFB
AT4G25200	small heat shock protein, HSP23.6-MITO	11,3	1,4	8,2	1	MYB
AT1G06160	ethylene response factor, ERF094, ORA59	2,7	2,1	6,9	1	AP2/EREBP, MYB, SBP,
AT5G27420	E3 ubiquitin-protein ligase, ATL31, CNI1,	5,1	4,2	6,8	1	bZIP, Trihelix, HD-HOX, C2C2(Zn) Dof
AT2G30770	Cytochrome P450 protein, CYP71A13	5,1	6,0	5,7	0	HD-HOX, TBP
AT3G15450	Aluminium induced, YGL and LRDR protein	2,3	1,8	5,7	0	Trihelix, MYB
AT3G28210	stress-associated protein 12, SAP12, PMZ	3,0	1,7	5,3	0	HSFB
AT1G71000	Chaperone DnaJ-domain protein;	3,5	2,6	4,8	1	AP2/EREBP, MYB, HD-HOX,
AT5G52640	heat shock protein, HSP90.1	5,1	1,5	4,3	2	AP2/EREBP, MYB, HD-HOX,
AT5G44310	Late embryogenesis abundant (LEA) protein	2,2	5,2	4,3	1	HSFB, Dof
AT2G29500	HSP20-like chaperone protein	2,0	2,1	4,1	1	HD-HOX
AT4G23210	cysteine-rich receptor kinase 13, CRK13	2,3	2,2	3,9	0	AP2/EREBP, MYB
AT2G04040	MATE family transporter related protein TX1	2,3	2,3	3,7	1	HD-HOX, C2C2(Zn) Dof
AT1G32460	unknown protein	3,3	3,0	3,5	1	
AT1G74310	heat shock protein HSP101	2,4	2,1	3,4	1	bZIP, AP2/EREBP, HD-HOX
AT1G27730	salt tolerance zinc finger, STZ/ZAT10	1,7	3,0	3,2	0	HSFB, AP2/EREBP, MYB, HD-HOX
AT5G41750	Disease resistance protein (TIR-NBS-LRR)	1,6	1,4	3,0	2	MYB, HD-HOX, C2C2(Zn) Dof, CBF
AT5G48570	peptidylprolyl isomerase ROF2	3,0	1,9	2,8	1	MYB, HD-HOX, C2C2(Zn) Dof
AT5G25930	LRR-type protein kinase	2,4	1,4	2,8	0	MYB
AT1G29060	Target SNARE coiled-coil domain protein;	2,0	2,3	2,8	0	bZIP, MYB, HD-HOX
AT4G21400	cysteine-rich receptor kinase 28, CRK28	1,9	2,2	2,7	0	HSFB, MYB, HD-HOX
AT1G22510	unknown RING/U-box protein (DUF 1232)	2,7	3,1	2,7	0	bZIP, MYB
AT3G17611	rhomboid-like protein 14, RBL14	2,1	2,2	2,6	0	AP2/EREBP, Trihelix, MYB, TCP
AT5G46350	WRKY transcription factor 8, WRKY8	2,6	2,8	2,6	2	HSFB, Trihelix,
AT2G27490	dephospho-CoA kinase, ATCOAE	1,9	1,8	2,6	1	HSFB, AP2/EREBP, SBP, HD-HOX, C2C2(Zn) Dof
AT1G72920	Toll-Interleukin-Resistance (TIR) protein	3,7	2,3	2,5	0	Trihelix, HD-HOX, C2C2(Zn) Dof
AT5G04340	C2H2 zinc finger transcription factor, ZAT6	2,5	2,1	2,5	1	AP2/EREBP, Trihelix, MYB
AT1G08315	ARM repeat superfamily protein;	2,8	2,2	2,5	1	
AT2G47180	galactinol synthase 1, GolS1	2,5	2,0	2,5	1	AP2/EREBP, MYB, HD-HOX
AT3G43230	RING/FYVE/PHD-type zinc finger protein;	2,2	2,0	2,4	0	HSFB, AP2/EREBP
AT1G33030	O-methyltransferase family protein;	2,6	4,4	2,4	0	HSFB, AP2/EREBP, HD-HOX
AT5G52760	Copper transport protein family;	2,8	6,9	2,4	1	
AT2G31990	Exostosin family protein;	2,8	2,2	2,3	1	MYB, HD-HOX
AT5G61270	phytochrome-interacting factor 7, PIF7	1,6	1,9	2,3	0	MYB
AT1G26420	FAD-binding Berberine family protein;	2,6	2,0	2,3	1	AP2/EREBP, MYB
AT1G02560	ATP-dependent Clp protease, CLPP5	3,0	3,4	2,1	0	bZIP, HD-KNOTTED, Trihelix
AT2G29990	alternative NAD(P)H dehydrogenase 2, NDA2	1,8	2,1	2,1	2	HSFB, C2H2(Zn), Trihelix, HD-HOX, CBF, HD-ZIP
AT1G68570	Major facilitator superfamily protein;	2,2	2,1	2,1	1	MADS, MYB,
AT2G43580	Chitinase family protein;	3,2	6,0	2,1	1	
AT1G60730	NAD(P)-linked oxidoreductase protein;	2,4	2,0	2,0	1	HD-HOX
AT4G18250	putative receptor serine/threonine kinase	2,8	1,9	1,9	0	Trihelix, HD-HOX
AT4G28020	unknown protein;	2,6	1,9	1,9	0	AP2/EREBP, MYB, HD-HOX
AT3G03440	ARM repeat protein	2,6	1,9	1,9	0	MYB
AT2G41640	Glycosyltransferase family 61 protein	2,6	1,9	1,9	1	MYB, HD-HOX

AT5G17370	Transducin/WD40 repeat-like protein	2,8	1,9	1,9	0	HD-HOX
AT4G25390	putative protein kinase protein	2,1	3,9	1,8	0	MYB
AT2G14290	Protein of unknown function DUF295	2,4	4,7	1,8	0	MYB, ABI3/VP1
AT5G59820	C2H2-type zinc finger protein, ZAT12, RHL41	1,3	1,4	1,5	2	MYB
AT5G24110	WRKY transcription factor 30, WRKY30	3,4	3,7	0,5	2	ABI3/VP1, HD-HOX, MADS

List of genes found commonly upregulated by HSFA4A overexpression and H_2O_2 treatment. RNA-Seq was performed with RNA from two weeks old Col-0 and HSF4ox2 plants after plus /minus 1mM H_2O_2 treatment. Plants for every sample were equally treated with 5 µM estradiol. Values indicate differential gene expression (fold change) relative to Col-0 minus H_2O_2 sample. Categories presented in columns: **AGI:** AGI number of genes. **Protein**: type of encoded protein, **HSFA4A:** fold change in HSF4ox2 minus H_2O_2 vs. Col-0 minus H_2O_2 samples. **H_2O_2:** fold change in Col-0 plus H_2O_2 vs. Col-0 minus H_2O_2 samples, **HSFA4A+H_2O_2**: fold change in HSF4ox2 plus H_2O_2 vs. Col-0 minus H_2O_2 samples, **HSE:** number of Heat Shock Elements identified in the 1000 bp promoter regions determined with Promomer (bar.utoronto.ca). **Other TF** category indicate other predicted TF binding sites (AthaMap promoter search tool, www.athamap.de, 75% restriction).

According to Genevestigator (www.genevestigator.com) analysis of public transcript profiling data 80% of these genes are induced by several abiotic stresses (heat, ozone, UV light, hypoxia, osmotic and salt stress, H_2O_2), several pathogens (*P. syringe*, *B. graminis*), effectors (FLG22, HrpZ), in *cat2* and *flu* mutants, down-regulated during germination and by MeJA.

10.3 **Table S2.**

Genes downregulated by both HSFA4A overexpression and H_2O_2treatment.

AGI	Protein	HSFA4A	H_2O_2	HSFA4A +H_2O_2	HSE	Other TF
AT1G05250	peroxidase 1/2	-1,4	-4,3	-12,9	0	AP2/EREBP
AT4G30500	Protein of unknown function (DUF788);	-8,3	-5,3	-11,1	1	HD-HOX, MYB
AT4G02270	protein root hair specific 13, RHS13	-3,8	-4,0	-5,9	0	bZIP, MYB
AT2G13100	starvation-induced glycerol-3-phosphate permease	-5,1	-9,6	-5,2	0	MYB, HD-HOX
AT2G35810	unknown protein;	-2,7	-2,8	-5,1	0	HD-HOX, MYB
AT1G30870	peroxidase 7	-2,8	-2,4	-4,6	1	C2C2(Zn) Dof, HD-HOX, HD-ZIP
AT2G03420	unknown protein	-7,5	-2,2	-4,5	0	MYB, HD-HOX, C2C2(Zn) Dof
AT5G17820	peroxidase 57	-1,8	-2,0	-4,4	0	HD-HOX, MYB
AT1G48930	Glycosyl hydrolase 9C, endoglucanase 5, GH9C1	-1,6	-1,5	-4,3	0	AP2/EREBP, MYB, SBP
AT3G05950	RmlC-like cupins superfamily protein;	-2,2	-1,7	-3,4	0	TBP
AT4G01450	nodulin MtN21 /EamA-like transporter protein	-1,9	-1,8	-2,9	0	MYB
AT4G28040	nodulin MtN21 /EamA-like transporter protein	-1,8	-2,4	-2,7	0	AP2/EREBP, MYB, HD-ZIP
AT1G14345	NAD(P)-linked oxidoreductase superfamily protein	-8,2	-2,7	-2,6	0	bZIP, MYB
AT4G23270	cysteine-rich receptor-like protein kinase 19, CRK19	-2,4	-3,5	-2,5	0	HD-HOX
AT3G23800	selenium-binding protein 3, SBP3	-1,8	-1,8	-2,5	1	AP2/EREBP, MYB, HD-ZIP, MADS
AT5G44820	Nucleotide-diphospho-sugar transferase protein	-1,8	-4,1	-2,4	1	HSFB
AT5G59320	non-specific lipid-transfer protein 3, LTP3	-3,6	-2,2	-2,4	0	C2C2(Zn) Dof, TBP
AT4G30290	xyloglucan:xyloglucosyl transferase, XTH19	-2,7	-6,6	-2,4	2	HSFB
AT1G14960	major latex-related protein	-2,5	-2,1	-2,4	0	HD-ZIP
AT3G58100	Plasmodesmata callose-binding protein 5, PDCB5	-2,2	-2,0	-2,1	1	MYB, HD-HOX, bZIP
AT5G42180	peroxidase 64	-2,1	-1,9	-2,1	0	MYB
AT3G63110	IPT3, isopentenyltransferase 3	-2,2	-2,3	-2,0	0	ABI3/VP1, MYB, HD-HOX
AT4G30650	Low temperature and salt responsive protein	-4,3	-1,9	-2,0	0	AP2/EREBP, HD-HOX, MYB
AT5G22860	Serine carboxypeptidase S28 family protein;	-2,0	-2,6	-2,0	1	HD-HOX
AT5G64620	Cell wall/vacuolar inhibitor of fructosidase 2, C/VIF2	-3,2	-1,8	-1,9	0	HD-HOX, HD-ZIP
AT2G40080	Early flowering 4, ELF4	-2,5	-2,2	-1,9	0	HSFB
AT5G26260	TRAF-like family protein;	-2,0	-3,0	-1,9	0	
AT3G58990	isopropylmalate isomerase 1, IPMI1	-1,7	-2,7	-1,9	0	AP2/EREBP, MYB, HD-HOX
AT4G23690	Disease resistance-responsive (dirigent-like) protein	-2,8	-1,9	-1,8	1	HD-HOX, MYB, C2C2(Zn) Dof
AT1G53900	Eukaryotic translation initiation factor 2B (eIF-2B)	-1,8	-2,2	-1,8	0	HD-HOX
AT5G42250	Zinc-binding alcohol dehydrogenase family protein;	-2,2	-2,3	-1,8	3	MYB, HD-ZIP
AT3G21770	Peroxidase superfamily protein;	-1,8	-2,1	-1,8	0	
AT2G36485	ENTH/VHS family protein;	-1,8	-2,5	-1,8	0	AP2/EREBP, MYB, HD-HOX
AT4G30660	Low temperature and salt responsive protein	-2,1	-4,1	-1,8	1	HSFB, HD-HOX, HD-ZIP
AT3G03480	acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase, CHAT	-2,3	-2,9	-1,7	0	AP2/EREBP, MYB, HD-HOX
AT5G50160	ferric reduction oxidase 8, FRO8	-2,4	-1,8	-1,7	0	AP2/EREBP, HD-HOX, MYB
AT3G25805	unknown protein	-2,3	-2,9	-1,7	0	AP2/EREBP
AT1G49720	ABA responsive element-binding factor 1, ABF1	-2,3	-2,0	-1,7	0	AP2/EREBP, GATA
AT2G25210	Ribosomal protein L39 family protein	-2,2	-2,5	-1,6	0	MYB
AT3G53190	Pectin lyase-like superfamily protein	-2,4	-1,7	-1,6	0	MYB
AT3G05880	Rare-cold-inducible 2A, RCI2A	-2,2	-3,2	-1,6	1	HD-HOX, MYB
AT2G21210	SAUR-like auxin-responsive protein	-3,0	-1,9	-1,6	0	TBP, MYB

List of genes found commonly downregulated by HSFA4A overexpression and H_2O_2 treatments. **Categories:** AGI, Protein, HSFA4A, H_2O_2, HSFA4A+H_2O_2, HSE and Other TF are the same as in Table S3.According to Genevestigator (www.genevestigator.com) data, 75% of these genes are downregulated by abiotic stresses (UV light, drought, osmotic, hypoxia, ozone), repressed by SA, and induced during germination.

10.4 Table S3.

Occurrence of predicted transcription factor binding sites in promoter regions of HSFA4A and hydrogen peroxide induced or repressed genes (listed on tables S1 and S2). Bold letter indicate binding sites which were overrepresented in upregulated genes, while bold italics shows those which were more frequent in downregulated genes. Data were assembled using the Promomer (bar.utoronto.ca) and AthaMap promoter (www.athamap.de) search tools.

TF binding sites	% of sites in induced genes	% of sites in repressed genes
HSF	**70**	**33**
HSFB	**33**	**10**
Trihelix	**30**	**6**
MYC_MYB	**63**	**29**
ABI3/VP1	3	2
C2H2(Zn)	3	2
SBP	3	2
bZIP_DOF	103	69
LFY	33	27
C2C2(Zn) Dof	15	12
AP2/EREBP	55	49
HOX2a_HOX2a	115	110
HD-HOX	62	59
RAV	17	16
MYB	207	204
bZIP	27	27
TSS	100	102
GATA	15	16
GARP/ARR-B	10	12
CAMTA	3	4
WRKY(Zn)	23	29
MADS	***43***	***65***
C2C2(Zn) GATA	***8***	***24***
GRF	***3***	***10***
TBP	***2***	***6***
HD-ZIP	***2***	***16***
CBF	3	0
HD-KNOTTED	2	0
NAC	2	0
TCP	5	0

10.5 **Primer list**

Code	5' - 3' sequence	Use	Reference
HsfA4A-seq2F	GCTTGCTTTGAACCTATCGCCG	HsfA4A (AT4G18880) cDNA sequencing	This study
HsfA4A-seq4F	GACGGATTCAGAACGAGTGAGAATG	HsfA4A (AT4G18880) cDNA sequencing	This study
HsfA4A-seqR	CACTCGTTCTGAATCCGTCA	HsfA4A (AT4G18880) cDNA sequencing	This study
HsfA4A-seq7R	GCGATAGGTTCAAAGCAAGC	HsfA4A (AT4G18880) cDNA sequencing	This study
hsfa4a_gen_F	CATTCCACCAAACCCAATCTTTGT	*hsfa4a* mutant gene-specific (genotyping)	This study
hsfa4a_gen_R	TTGGTGAGGAAAGGTGGAAGTG	*hsfa4a* mutant gene-specific (genotyping)	This study
HsfA4A-RT-F	CGAGCAACGGAAGT TCAATTAGA	RT-PCR of HsfA4A gene	This study
HsfA4A-RT-R	CTGAACCATACCTAAGTACTGTAATCGAAAAC	RT-PCR of HsfA4A gene	This study
HsfA4A-qF	CTTTGAACCTATCGCCGTGT	HsfA4A (AT4G18880) Q-RTPCR	This study
HsfA4A-qR	TGTGTGTGAAGAAGGGCTTG	HsfA4A (AT4G18880) Q-RTPCR	This study
Hsf-prom-5(EcoRI):	TGACTGAATTCCCACATTCCACACTTGCCTTT	HsfA4A promoter cloning in pENTRY-BS (-)	This study
Hsf-prom-3(BamHI)_noATG	TGATTGGATCCCCTCTGAAAACACTTTTAACAACTCTCACACTC	HsfA4A promoter cloning in pENTRY-BS (-)	This study
Hsf-prom-seq1F	AATTCGTCCTACTCCGACCA	HsfA4A promoter sequencing	This study
Hsf-prom-seq2F	TGATCCGACTTTTTCTTGCAC	HsfA4A promoter sequencing	This study
Hsf-prom-seq3F	ATCGTCGTCGTCGTAAATCC	HsfA4A promoter sequencing	This study
HsfA4A-5'(BamHI)	TGAGCGGATCCATGGATGAGAATAATCATGGAGTTTC	Cloning in pPCV-Y/CFP-H, pET28a, pMALC2, pGAD424, pGBT9	This study
HsfA4A-5'(Hind III)	TGAGCAAGCTTATGGATGAGAATAATCATGGAGTTTC	Cloning in pSAT1A, pPILY	This study
HsfA4A-3' (SmaI)_ noSTOP	TGATTCCCGGGACTTCTCTCTGAAGAAGTCAGAT	Cloning in pSAT1A, pPILY, pPCV-Y/CFP-H	This study
HsfA4A-3'(HindIII)	AAGCTTATCAACTTCTCTCTGAAGAAGTCAGAT	Cloning in pET28a, pMALC2	This study
HsfA4A-3'(SalI)	TGAGCGTCGACTCAACTTCTCTCTGAAGAAGTCAGAT	Cloning in pGAD424, pGBT9	This study
MPK3-5'(BamHI)	TGAGCGG ATC CAA ATGAACACCGGCGGTGGCCAATAC	Cloning in pET28c, pGAD424, pGBT9	This study
MPK6-5'(BamHI)	TGAGCGG ATC CTC ATGGACGGTGGTTCAGGTCAACCGGC	Cloning in pET28c, pGAD424, pGBT9	This study

MPK3-3'(SalI)	TGAGCGTCGACCTAACCGTATGTTGGATTGAGTGC	Cloning in pET28c, pGAD424, pGBT9	This study
MPK6-3'(SalI)	TGAGCGTCGACCTATTGCTGATATTCTGGATTGAAAG	Cloning in pET28c, pGAD424, pGBT9	This study
3'C229_Hsf_R	CCTTCCTCTCTCACAACAACAGCAGTTTTGTTCTCTTCCAACA	HsfA4A Site-directed mutagenesis, C229A	This study
3'C267_Hsf_R	GATTGTAACATACTCTCAGCAGAATCCGATACAAG	HsfA4A Site-directed mutagenesis, C267A	This study
3'C295_Hsf_R	GAATCGACACTTAACTGTATAGCAGAAAGAGGAGGGCTCTCTG	HsfA4A Site-directed mutagenesis, C295A	This study
Hsp17.6A-qF	CCAAAGAAAAAGCCAAGAAGC	Hsp17.6A gene (AT5G12030) quantitative PCR	This study
Hsp17.6A-qR	TGGAAACCTTCCAAACTCCA	Hsp17.6A gene (AT5G12030) quantitative PCR	This study
Zat6-qF	GTGACCTTGACCTGCCTTCTTC	Zat6 gene (AT5G04340) quantitative PCR	This study
Zat6-qR	CTCCGGCAGATTGAGTAAGC	Zat6 gene (AT5G04340) quantitative PCR	This study
Zat12-qF	GACGCTTTGTCGTCTGGATT	Zat12 gene (AT5G59820) quantitative PCR	This study
Zat12-qR	GTGTCCTCCCAAAGCTTGTC	Zat12 gene (AT5G59820) quantitative PCR	This study
Hsp90-qF	GTGGTTCCTTCACTGTCACTAG	Hsp90 gene (AT5G52640) quantitative PCR	This study
Hsp90-qR	TTCACCAAGTCTTTGAGTCTCC	Hsp90 gene (AT5G52640) quantitative PCR	This study
ER8A	GCTTGGGCTGCAGGTCGAGGCTAA	amplification of inserts in pER8 vector	Papdi et al., 2008
ER8B	CTGGTGTGTGGGCAATGAAACTGATGC	amplification of inserts in pER8 vector	Papdi et al., 2008
GAPDH2_qF	AATGGAAAATTGACCGGAATGT	GAPDH2 (AT1G13440) Q-RTPCR reference	Papdi et al., 2008
GAPDH2_qR	CGGTGAGATCAACAACTGAGACA	GAPDH2 (AT1G13440) Q-RTPCR reference	Papdi et al., 2008
Tubulin2-RT-F	AGGAACTGGATCTGGTATGGGAACAT	tubulin 2 (AT5G62690) Q-RTPCR reference	Koncz C.,
Tubulin2-RT-R	GTCACACCAGACATAGTAGCAGAAATCAAG	tubulin 2 (AT5G62690) Q-RTPCR reference	Koncz C.,
DONR1	TCGCGTTAACGCTAGCATGGATCTC	pDONR201 sequencing	Invitrogen
DONR2	GTAACATCAGAGATTTTGAGACAC	pDONR201 sequencing	Invitrogen
LB-4	CGTGTGCCAGGTGCCCACGGAATAGT	Left border T-DNA-specific (genotyping)	INRA
WRKY30-qF	AGAGCGATGATTCCGATCAAG	WRKY30 gene (AT5G24110) quantitative PCR	Besseau et al., 2012
WRKY30-qR	CATCGTCCAGCGTTCTATCAA	WRKY30 gene (AT5G24110) quantitative PCR	Besseau et al., 2012
PDC-qF	CGATTATGGCACTAACCGGATT	PDC1 gene (At4g33070) quantitative PCR	Banti et al., 2010

PDC-qR	TGTTCACCACCGCCTGATAAC	PDC1 gene (At4g33070) quantitative PCR	Banti et al., 2010
ADH-qF	GAATCGCTGGTGCTTCTAGG	ADH1 gene (At1g77120) quantitative PCR	Papdi et al., 2008
ADH-qR	CTCAGCGATCACCTGTTGAA	ADH1 gene (At1g77120) quantitative PCR	Papdi et al., 2008
3' S198A _Hsf_R	GGGAACACACGGAGCTAGGTTCAAAGC	HsfA4A Site-directed mutagenesis	This study
3' T238A _Hsf_R	TGAAGAAGGGCTAGCAGAACCTTCCTC	HsfA4A Site-directed mutagenesis	This study
3' S239A _Hsf_R	GTGTGAAGAAGGAGCTGTAGAACCTTC	HsfA4A Site-directed mutagenesis	This study
5' S309A _Hsf_F	CAAATCTCCTCCTGCTCCAAGGATCATC	HsfA4A Site-directed mutagenesis	This study
3' T396A _Hsf_R-HindIII	AGTCAAAGCTTATCAACTTCTCTCTGAAGAAGCCAGATGTCCAAG	HsfA4A Site-directed mutagenesis	This study
5' Hsp-prom_EcoRI	TGACTGAATTCCGGACTGTTCCAAATTTCCT	Hsp17.6A promoter cloning in pPCV-LUC+	This study
3' Hsp-prom_SalI	TGATTGTCGACTCTTAGCTGTTGTGTTAACTTCC	Hsp17.6A promoter cloning in pPCV-LUC+	This study

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