Final report of the project NKFI KH-129510.

Title: Characterization of transcription factors, which control stress responses of Arabidopsis

Principal Investigator: Dr. László Szabados

Institute of Plant Biology, Biological Research Centre, Szeged

Introduction

In a natural environment plants are exposed to adverse conditions which affect their growth, fertility and survival. Adverse conditions are usually developing in combinations. Drought is often accompanied by high summer temperatures and deleterious effect of soil salinity can also be enhanced by heat. Until recently, effects of abiotic stresses on plants have been studied separately, as methods were not available which are suitable to study more complex regulatory interactions. In our research program we studied the role of HEAT SHOCK FACTOR A4A (HSFA4A) and ERF-type transcription factors RAP2.2, RAP2.3 and RAP2.12. These factors have previously been isolated in our laboratory and were shown to influence responses to several types of abiotic stresses. In this project function of these transcription factors have been investigated to decipher their role in coordination of responses to salt, osmotic, heat and combination of these stresses.

ERFVII class transcription factors RAP2.2, RAP2.3 and RAP2.12 control osmotic and combined, osmotic and heat responses of Arabidopsis plants.

Transcription factors belonging to the ERFVII subfamily are known to control responses to hypoxia and play essential role in surviving submergence. RAP2.12 was suggested to function as sensor of hypoxic conditions through its N-end rule regulation. Previously we found, that RAP2.12 and its homologues RAP2.2 and RAP2.3 is implicated in ABA signaling and can modulate tolerance not only to hypoxia but also to osmotic stress conditions. We found that RAP2.12, RAP2.2 and RAP2.3 can act redundantly in multiple stress responses. Alternative protein degradation pathways may provide inputs to the RAP2 transcription factors for the distinct stresses (Papdi et al., 2015). This research was trying to decipher the role of RAP2-type transcription factors in drought tolerance and responses to combination of osmotic and heat stresses.

Stress tolerance of transgenic plants overexpressing the RAP2.2, RAP2.3 and RAP2.12 factors.

To test responses to single and combined stresses, transgenic plants overexpressing the RAP2.2, RAP2.3 and RAP2.12 transcription factors and their wild type control were subjected to heat (37^{o}C), osmotic (300mM mannitol) and combination of these stresses for 4 days *in vitro*, than cultured in standard conditions for 7 days. Survival rates were determined by calculating percentages of recovered, growing plants and dead or damaged plants. Heat treatment alone had only slight influence on survival rates. Osmotic stress reduced survival of wild type plants by 23%, while plants overexpressing RAP2.2 and RAP2.12 factors had significantly higher survival rates. Combination of osmotic stress with high temperature reduced further survival of all plants tested. 57% of wild type plants could recover in such conditions, while survival of RAP2.2 and RAP2.12 overexpressing plants was 82% and 74%, respectively. Overexpression of RAP2.3 have not changed survival significantly after these treatments (Figure 1).

Mutations in RAP2.3 and RAP2.12 genes compromise stress tolerance.

Responses of knockout mutants of the RAP2.3 and RAP2.12 genes to osmotic, heat and combined stresses were compared to wild type plants in experimental system described above. Heat treatment have only minimal effect on plant survival, which was similar in all genotypes. Viability of *rap2.3rap2.12* double mutant was more reduced by osmotic stress than wild type plants and single rap mutants. When plants were subjected to combined stress, survival of wild type and single mutants was reduced by 25 to 30 %, while nearly 40% of double mutants died in such conditions (Figure 2). These results demonstrate that double *rap2.3rap2.12* is more sensitive to osmotic and combined osmotic and heat stresses than either the wild type or the single mutants and effect of these mutations is additive.

Evaluation of drought tolerance of soil-grown plants with plant phenotyping.

An automatic plant phenotyping platform has recently been installed in the BRC (PSI, Czech Republic), which allows the evaluation of growth and physiological parameters of large number of tested plants using non-destructive imaging technologies. Difference in osmotic stress tolerance of RAP2 overexpressing plants and rap mutants prompted us to test drought tolerance of soil-grown mutants. In this experiment Col-0 wild type, r*ap2.3* and *rap2.12* single mutants and *rap2.3rap2.12* double mutants were tested for growth and changes in selected physiological parameters.

Plants were cultured in small pots for three weeks in controlled conditions. Watering of half of the plants was stopped, allowing gradual drying of the soil for 18 more days. The control plants were watered without interruption. Water content of the drying plants dropped to 50% after 10 days and was reduced to 25% after 15 days of drying (Figure 4A). Growth of plants was monitored by regular imaging with RGB, thermal (infrared) and fluorescence cameras.

To monitor plant growth, rosette areas of 20 plants were determined by RGB imaging. RGB images were converted to color-segmented images, which were used to measure green areas. Until 10th day of drying only minor difference in rosette size could be observed in stressed and control plants, and genotype-dependent differences could not be established. In later timepoints control plants continued growing, while green area of the drought-treated plants became reduced, due to scattered pattern of the color-segmented images. Loss of chlorophyll content and enhanced senescence lead to reduction of green color and loss of green area. Genotype-dependent differences became obvious after 12th day of drying, as more green was lost in the rap2 mutants. After 15th days of drying residual green could still be detected in Col-0 and single *rap2.3* and *rap2.12* mutants, which was completely missing in the double *rap2.3rap2.12* mutant. Visual inspection of the images could therefore reveal genotype-dependent differences, showing enhanced sensitivity of the *rap2.3rap2.12* mutant to drought (Figure 3).

Quantitative evaluation of rosette areas was made by detecting green areas of color-segmented images. Growth of well-watered plants was similar in the genotypes tested although some differences could be observed after 10th days of observation (Figure 4B). Rosette growth or drought-treated plants was similar to watered plants in the first few days, but started to decline after 6-7th days of water withdrawal. After 8th days of drying anthocyanin accumulation became apparent in drought-treated plants, which increasingly masked the green areas of the rosettes. Due to anthocyanin accumulation and loss of chlorophyll of drought-treated plants, calculated rosette areas declined after 10th days of water withdrawal. Calculated rosette size of rap mutants however declined faster than wild type plants, and difference was most striking when wild type and double rap mutants were compared (Figure 4C). These results suggest, that the *rap2.3* and *rap2.12* mutants, in particular the *rap2.3rap2.12* double mutant are hypersensitive to drought.

Thermal camera measures surface temperatures and allows determination of small differences in leaf temperatures. Cooler temperatures

indicates faster water evaporation, which usually coincides with open stomata. Monitoring of leaf temperatures therefore can indicate differences in stomata closure and water loss. Drought stress have not changed leaf temperatures until day 10-11, but has increased afterward, suggesting reduction of water evaporation due to loss of viability. Temperature of the mutants increased faster than the wild type plants, suggesting that their viability was more affected by the drought conditions applied (Figure 5A).

Chlorophyll fluorescence is a sensitive indicator of photosynthetic processes and can be monitored by fluorescent camera system. Chlorophyll fluorescence was monitored after 5th days of drying. PSII quantum yield is a frequently used physiological parameter, used to measure the stability of PSII and associated light reactions. Fv/Fm values of drying plants were around 0.8 until 12th days, which was similar to watered plants. Afterward Fv/Fm values started to decline, indicating gradual damage of PSII due to increasing water deficiency. Differences in wild type and mutant plants were however not significant. These data indicate, that PSII quantum yield is a quite stable parameter, and is not very sensitive to water scarcity. Based on the collected data, we could not determine differences between the genotypes tested in this experiment (Figure 5B).

HEAT SHOCK FACTOR A4A (HSFA4A) regulates combination of salt and heat responses in Arabidopsis

Heat shock factors in plants are components of complex regulatory networks with various levels of control including transcription regulation, posttranslational modifications, intracellular transport, intra- and intermolecular interactions, homo and heteromeric trimer formation (Akerfelt *et al.*, 2010; Scharf *et al.*, 2012). Their function in stress combinations is however not known. The Arabidopsis HEAT SHOCK FACTOR A4A (HSFA4A) was previously reported to regulate responses to salt and oxidative stresses. Moreover, HSFA4A was found to be a substrate of MAP kinases MPK3 and MPK6, and phosphorylation influence its activity (Perez-Salamo *et al.*, 2014). Our research aimed at the better understanding how HSFA4A regulates

stress responses, whether it can modulate tolerance to stress combination and how phosphorylation can influence its activity.

Regulation of HSFA4A

To study stress-dependent regulation of HSFA4A, wild type Arabidopsis plants were treated by high salinity, high temperature and combination of these conditions. *HSFA4A* expression changed dramatically in these conditions. Salt enhanced *HSFA4A* transcription which remained elevated for up to 24 hours. Heat and combination of heat and salt stress had no effect on transcript levels in the first hours but enhanced *HSFA4A* transcription after 24 hours (Figure 6A). These results suggest that heat and salinity regulates *HSFA4A* through different signaling pathways. To study the HSFA4A protein *in vivo*, a transgenic Arabidopsis line was generated which expresses the YFP-tagged HSFA4A under the control of its own promoter (pHSFA4A::HSFA4A-YFP, Figure 6B). Western hybridization confirmed the presence of the HSFA4A-YFP chimeric protein in transgenic plants which was more abundant in salt-stressed plants correlating with stress-dependent induction of the endogenous *HSFA4A* gene (Figure 6C).

Confocal microscopic observations revealed detectable YFP signal in roots. Several hours of salt treatment lead to stronger fluorescence in root cells which became particularly strong in nuclei (Figure 7A). Heat shock factors are shuttling proteins with predominant cytoplasmic localization in non-stressed conditions and nuclear accumulation upon heat and other stresses. Salt stress led to a fast and temporal accumulation of HSFA4A-YFP in nuclei while fluorescence pattern did not change significantly in non-treated control cells (Figure 7B,C). These results suggest rapid transfer of HSFA4A to nuclei which starts within minutes upon onset of salt stress and takes place before gene activation and *de novo* protein biosynthesis.

Binding of HSFA4A to promoter elements of target genes

Whole-genome transcript profiling has identified genes which were upregulated by HSFA4A overexpression (Perez-Salamo *et al.*, 2014). Three HSFA4A-induced genes were selected to test promoter binding: the *HSP17.6A, ZAT12* and *WRKY30* genes encoding a small heat shock protein, a zinc finger and a WRKY-type transcription factors, respectively. Promoter binding was tested *in vivo* by chromatin immunoprecipitation (ChIP) assays, using transgenic plants expressing the pHSFA4A::HSFA4A-YFP gene construct. ChIP assay revealed specific enrichment of HSE-containing promoter regions of all three tested genes, but not on TUA gene promoter, which lacks HSE elements (Figure 8). These results demonstrate that the HSFA4A factor can directly bind to the promoters of the three target genes, which is enhanced depending on the type of stress.

HSFA4A is phosphorylated by MAP kinase 4

Earlier we showed that MAP kinases MPK3 and MPK6 can interact with and phosphorylate HSFA4A (Perez-Salamo *et al.*, 2014). We found that HSFA4A can be phosphorylated not only by MPK3 or MPK6 but also by MPK4 (Figure 9A). Analysis by mass spectrometry identified six amino acid residues of HSFA4A which were phosphorylated by MPK4: Thr124, Ser198, Ser239, Ser309, Thr396 and Ser397 (Figure 9B). Four of the identified sites coincided with amino acid residues phosphorylated also by MPK3 (Ser198, Ser239, Ser309, Thr396). Our results suggested that Ser309 is the primary phosphorylation site for MPK4, as it was found for MPK3 and MPK6 (Perez-Salamo *et al.*, 2014). Phosphorylation was confirmed by *in vivo* phosphorylation detection assays using mass spectrometry, confirming that these amino acids are indeed i*n vivo* targets of MAP kinases. Phosphorylation of several other amino acid residues was also revealed *in vivo* which were not phosphorylated by MAPKs (Figure 9C). These results suggest that HSFA4A is under complex post-translational control as it can be phosphorylated not only by MAPKs but also by other classes of protein kinases (Figure 9D).

Phosphorylation affects intramolecular interactions of HSFA4A

Recognition of heat shock elements and transcriptional activation of target genes requires trimerization of heat shock factors. To study multimer formation of HSFA4A *in vivo*, protein extracts from HSFA4A-YFP-expressing plants were separated on non-denaturing gels. High molecular weight complex was detected by Western hybridization which was eliminated leading to low molecular weight signal in reducing environment (Figure 10A). In plant cells most HSFA4A protein seem to exist in high molecular weight complexes, which can be monomerized in reducing environment. BiFC studies revealed that MAPK-mediated phosphorylation on HSFA4A enhanced multimerization *in vivo* (Figure 10B). Nevertheless our data suggest that MAPK-mediated phosphorylation is not essential for HSFA4A dimerization but has positive effect on it.

HSFA4A can enhance tolerance to combined salt and heat stresses

Overexpression of HSFA4A in Arabidopsis could enhance tolerance to salt, heavy metal and oxidative agents while knockout mutant showed hypersensitivity to salt (Perez-Salamo *et al.*, 2014). To evaluate responses to combined salt and heat stresses, tolerance of wild type and HSFA4A overexpressing Arabidopsis lines were tested. Wild type seeds (Col-0), and HSFA4A overexpressing lines were germinated on standard culture medium and 10 days-old plantlets were exposed to different doses of salt, heat and combined stresses followed by transfer to standard culture medium to allow recovery. Heat stress (37^{o}C) had only minor effect on plant viability in these conditions, while salinity affected plants in a concentration-dependent manner. Damage was clearly alleviated by HSFA4A overexpression. When salt was combined with high temperature, HSFA4A overexpression clearly reduced damage and enhanced survival of stressed plants (Figure 11). These results indicate that overexpression of HSFA4A not only improved tolerance to salt but could increase viability under simultaneous heat and salt stresses.

Reactive oxygen species (ROS) are generated by adverse environmental conditions imposing oxidative damage to stressed plants. To assess the effect of HSFA4A on ROS-triggered damage, lipid peroxidation rates were compared in wild type and HSFA4A overexpressing plants. HSFA4A overexpression reduced lipid peroxidation in salt-stressed plants as well as in plants exposed to simultaneous salt and heat stress (Figure 12). These results indicate, that HSFA4A can reduce oxidative damage imposed not only by individual salt or heat stresses, but also by stress combinations.

Testing drought tolerance of the *hsfa4a* mutant

Our results convincingly showed, that HSFA4A regulates salt and combination of salt and heat stresses. Whether drought tolerance is influenced by this heat shock factor was however was not known. We have therefore performed a phenotyping experiment to compare drought responses of wild type and *hsfa4a* mutant. In order to impose drought stress, watering of 3 weeks-old plants was stopped and plant growth was monitored by RGB, infrared and fluorescence imaging using a plant phenotyping platform (PSI, Czech Republic). 20 plants were simultaneously imaged and numerical data were processed to compare plant growth and several physiological parameters.

Growth of wild type (WS ecotype) and *hsfa4a* mutant plants was comparable in both control and drought conditions, suggesting that HSFA4A does not influence drought responses (Figure 13). When leaf surface temperature and PSII quantum yields were compared in watered and drought-stressed plants, no significant changes could be detected between the wild type and mutant plants (Figure 14). These results suggest, that there is no difference in drought tolerance of the wild type and mutant plants. HSFA4A therefore has no influence on drought tolerance of Arabidopsis plants.

Our results shows, that HSFA4A overexpression can enhance tolerance to individually and simultaneously applied heat and salt stresses

through reduction of oxidative damage. HSFA4A is most probably not involved in regulation of drought tolerance.

As conclusion, HSFA4A is a component of a complex stress regulatory pathway, connecting upstream signals mediated by MAP kinases MPK3/6 and MPK4 with transcription regulation of a set of stress-induced target genes (Figure 15) **(Andrási et al., 2019).**

Functional diversity of plant heat shock factors.

While yeast and Drosophila have a single HSF and mammalian cells live with four, in plants large gene families with 18 to 52 members encode HSFs, which are divided into A, B and C classes (Scharf et al., 2012). HSFs are components of complex signaling systems which control responses not only to high temperatures but also to a number of abiotic stresses such as extreme temperatures, drought, hypoxic conditions, soil salinity, toxic minerals, strong irradiation and to some pathogen threats. Transcriptional regulation of the Arabidopsis HSF family shows great diversity. Transcript profiles have been downloaded from public databases and compared to establish gene-specific differences in stress-induced expression patterns (Figure 16).

As transcription regulators, HSFs are involved in complex web of protein-protein interactions which add considerable complexity to their biological function (Figure 17). Model in Figure 18 depicts the regulatory network of class A HSFs illustrating their regulation, interactions and function. Stress-responsive HSFs are induced by HSFA1 factors, and a number of less-known TFs. While the role of HSFA1 and several DREB2-type factors in transcriptional activation of stress-induced HSFs is well established, our knowledge about the effect of other TFs, chromatin structure and epigenetic regulation is scarce (Scharf et al., 2012). Activities of HSFs are regulated primarily on transcript levels, but alternative splicing and posttranslational modifications provides further variability. Heat stress memory can rely on epigenetic regulation, controlled by HSFA2-associated signals. Involvement of other HSFs in epigenetic control of stress responses and acquired stress tolerance is however still unexplored.

HSFAs function as positive regulators of stress responses which amplify signals and activate sets of target genes which encode defense proteins, or other TFs which further propagate stress signals. In such a network HSFs are subject of modifications such as phosphorylation, sumoylation or oxidation, interactions with other proteins which modulate their activity, stability or localization. Transcriptional activation of their targets can be modulated by chromatin remodeling, epigenetic regulation, heterotrimerisation with other HSFs, or inhibited by HSFB factors (Figure 18) **(Andrási et al., 2020).**

Conclusions

Our results revealed, that RAP2.2, RAP2.3 and RAP2.12 can influence drought, osmotic and combination of heat and osmotic stress tolerance in slightly different way. While results depended on the use of overexpressing constructs or knockout mutants, it is clear that these factors are implicated in responses to stresses which are different from previously published hypoxia. Overexpression of RAP2.2 and RAP2.12 enhanced osmotic and heat tolerance, while the double *rap2.3rap2.12* mutant was clearly hypersensitive to these stresses, their combinations as well as to drought, imposed by water withdrawal. These results suggest that this subgroup of ERFVII factors have broader function in stress regulation than thought before. We showed, that HSFA4A is part of a complex regulatory pathway, which modulate ROS-dependent stress signaling. HSFA4A influence salt and combination of salt and heat responses, but does not seem to have influence on drought tolerance. Our results open the way to count with the transcription factors, characterized in the present project as promising tools in biotechnology programs to engineer tolerance to adverse environmental conditions.

Publications supported by the project

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Perez-Salamo I, Papdi C, Rigo G, Zsigmond L, Vilela B, Lumbreras V, Nagy I, Horvath B, Domoki M, Darula Z, Medzihradszky K, Bogre L, Koncz C, 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

Scharf KD, Berberich T, Ebersberger I, Nover L (2012) The plant heat stress transcription factor (Hsf) family: structure, function and evolution. Biochim Biophys Acta 1819: 104-119

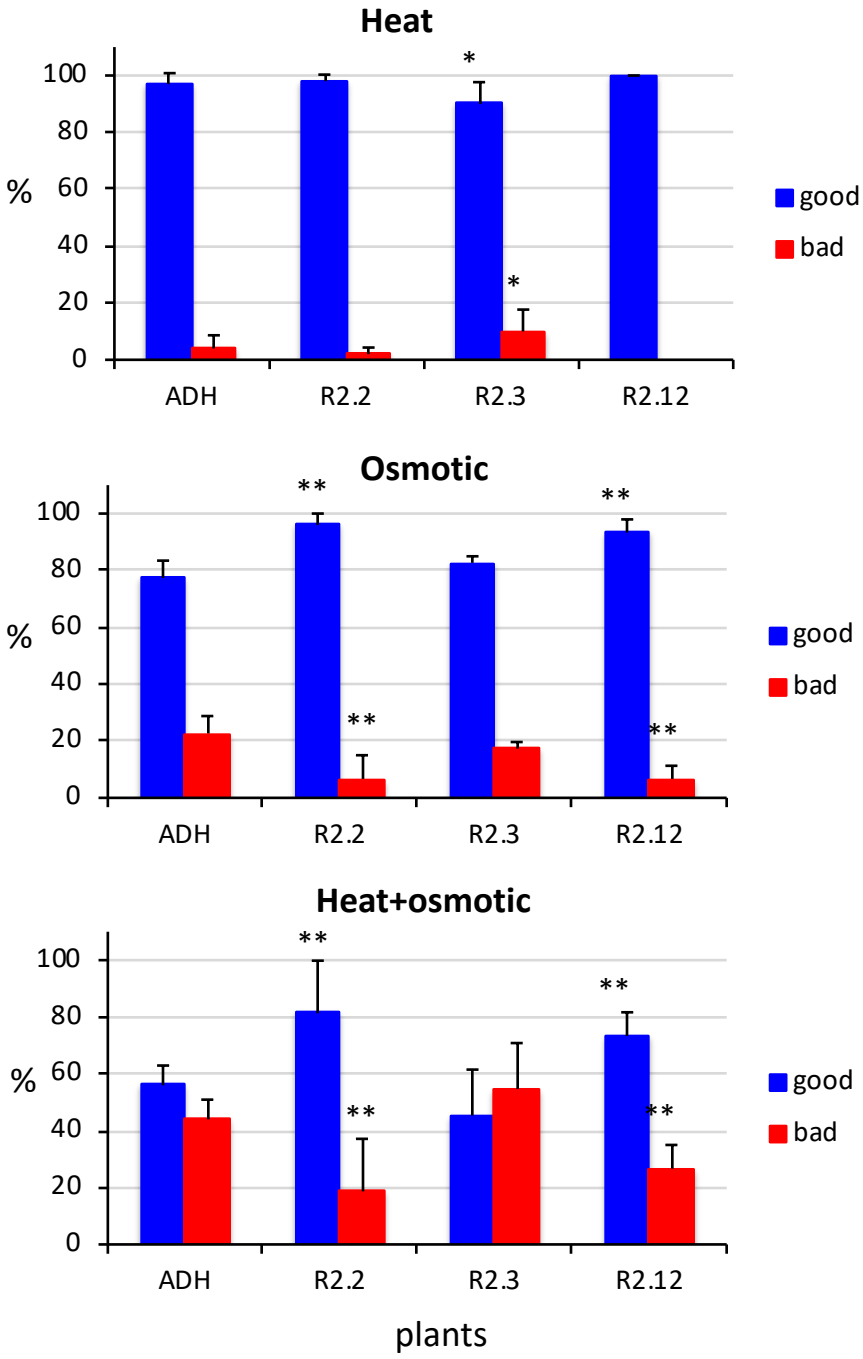


Figure 1. Effect of RAP2.2, RAP2.3 and RAP2.12 overexpression on plant survival under heat, osmotic and combined osmotic and heat stress. 2 weeks-old, in vitro-grown plants were treated by osmotic stress (300 mM mannitol), heat (37^{o}C) for 4 days. Plant survival was scored after 7 days of recovery. ADH: control plants, considered wild type, R2.2: RAP2.2, R2.3: RAP2.3, R2.12: RAP2.12. . Categories: good: recovered, growing, green plants, bad: dead or heavily damaged plants.

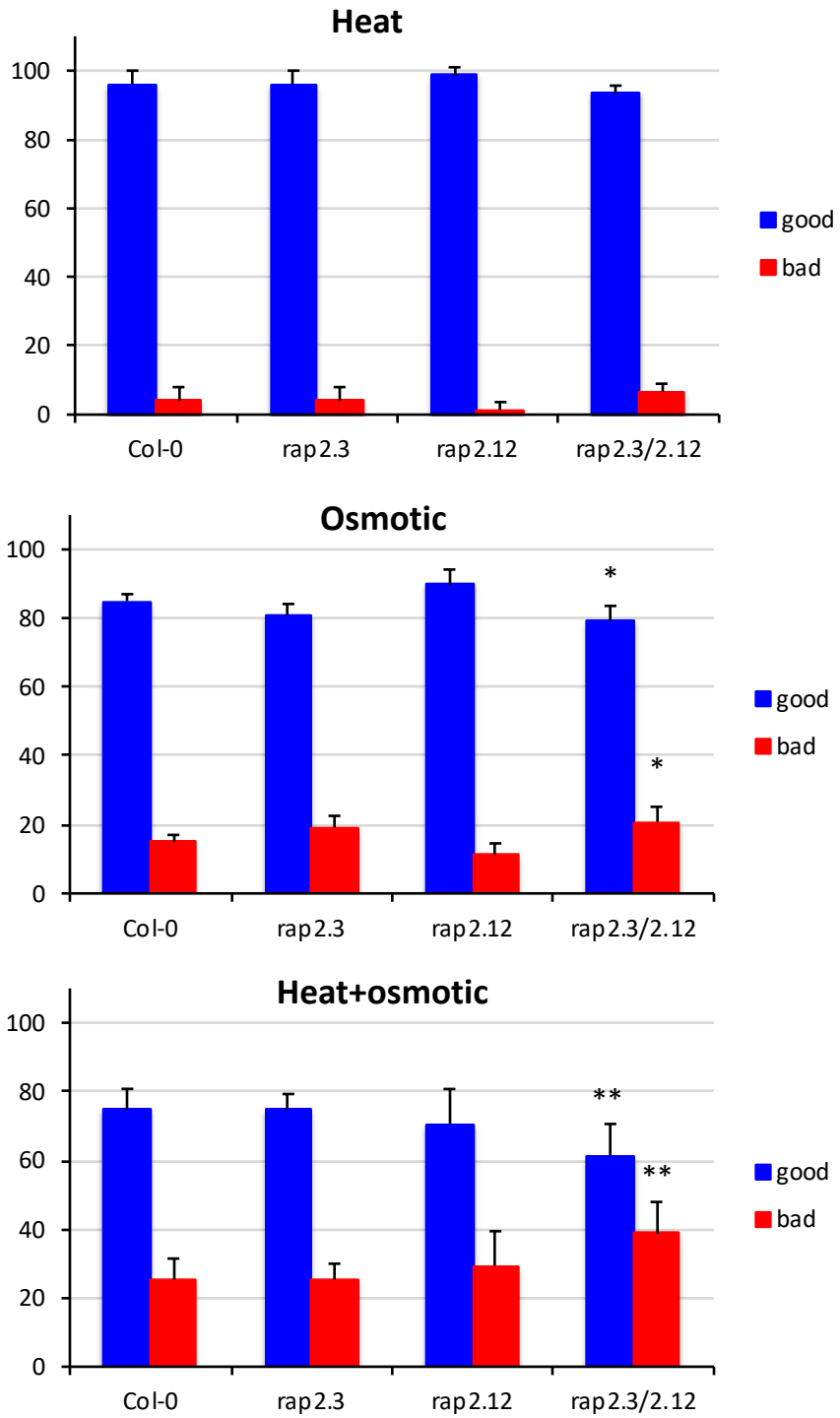


Figure 2. Analysis of osmotic stress and heat tolerance of rap2 mutants. 2 weeks-old in vitro-grown plants were treated by 300 mM mannitol (osmotic stress), 37oC (heat stress) and a combination of these treatments (heat+osmotic stress) for 4 days. Survival was scored 7 days after recovery by counting the green, growing plants. Categories: good: recovered, growing, green plants, bad: dead or heavily damaged plants.

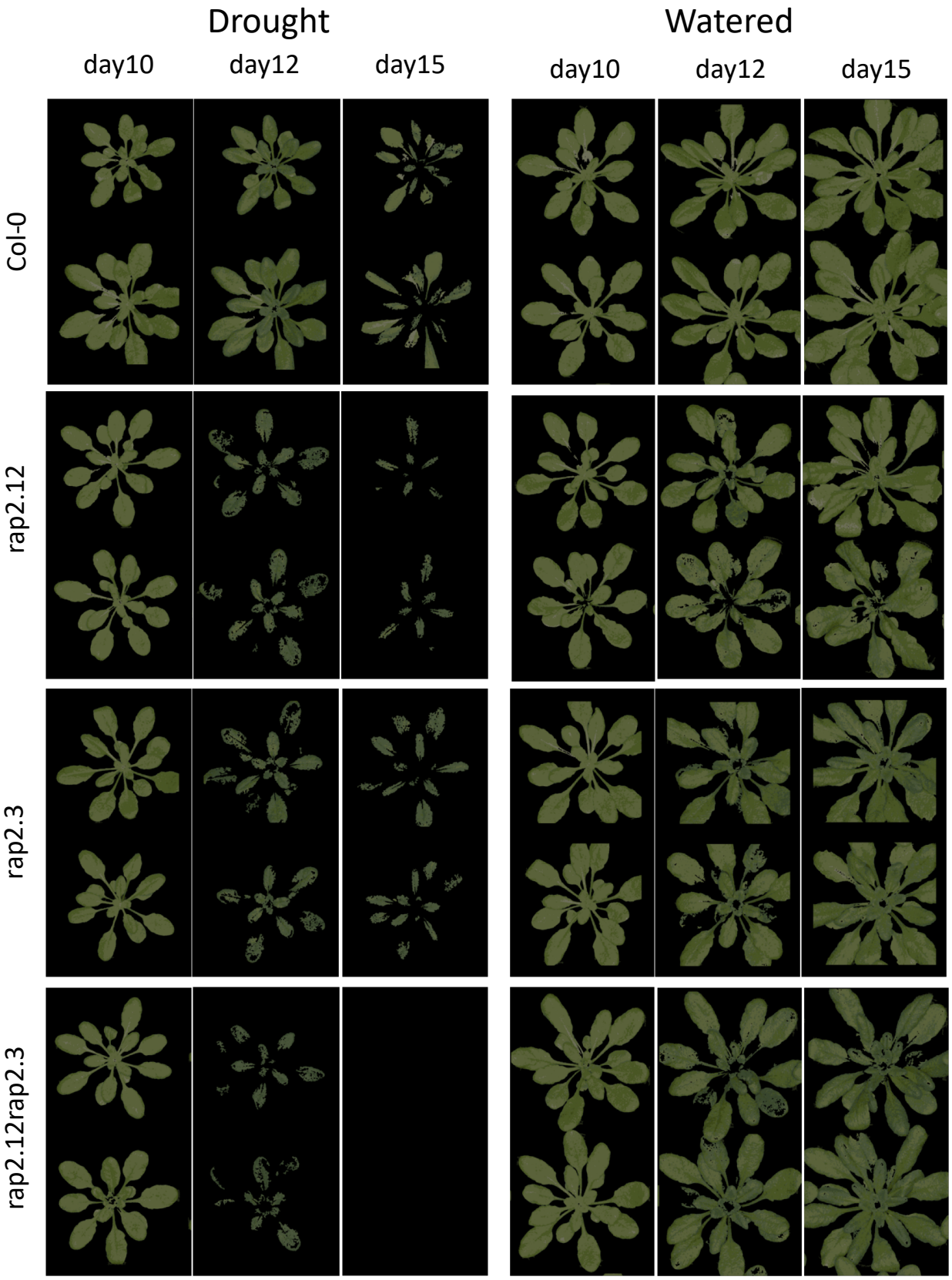


Figure 3. Color-segmented RGB images of Col-0, rap2.12, rap2.3 and rap2.3rap2.12 mutants. Color images were color segmented to determine green areas to measure rosette sizes. Plants were cultured in pots, and were either watered (control) or dried to generate drought stress. Images are shown 10, 12 and 15 days after watering was stopped. Note, that green areas are shrinking in drought-treated plants indicating chlorophyll loss. Well watered plants are growing. The double mutant rap2.3rap2.12 lost green area faster than wild type or single mutant plants, indicating hypersensitivity to water withdrawal.

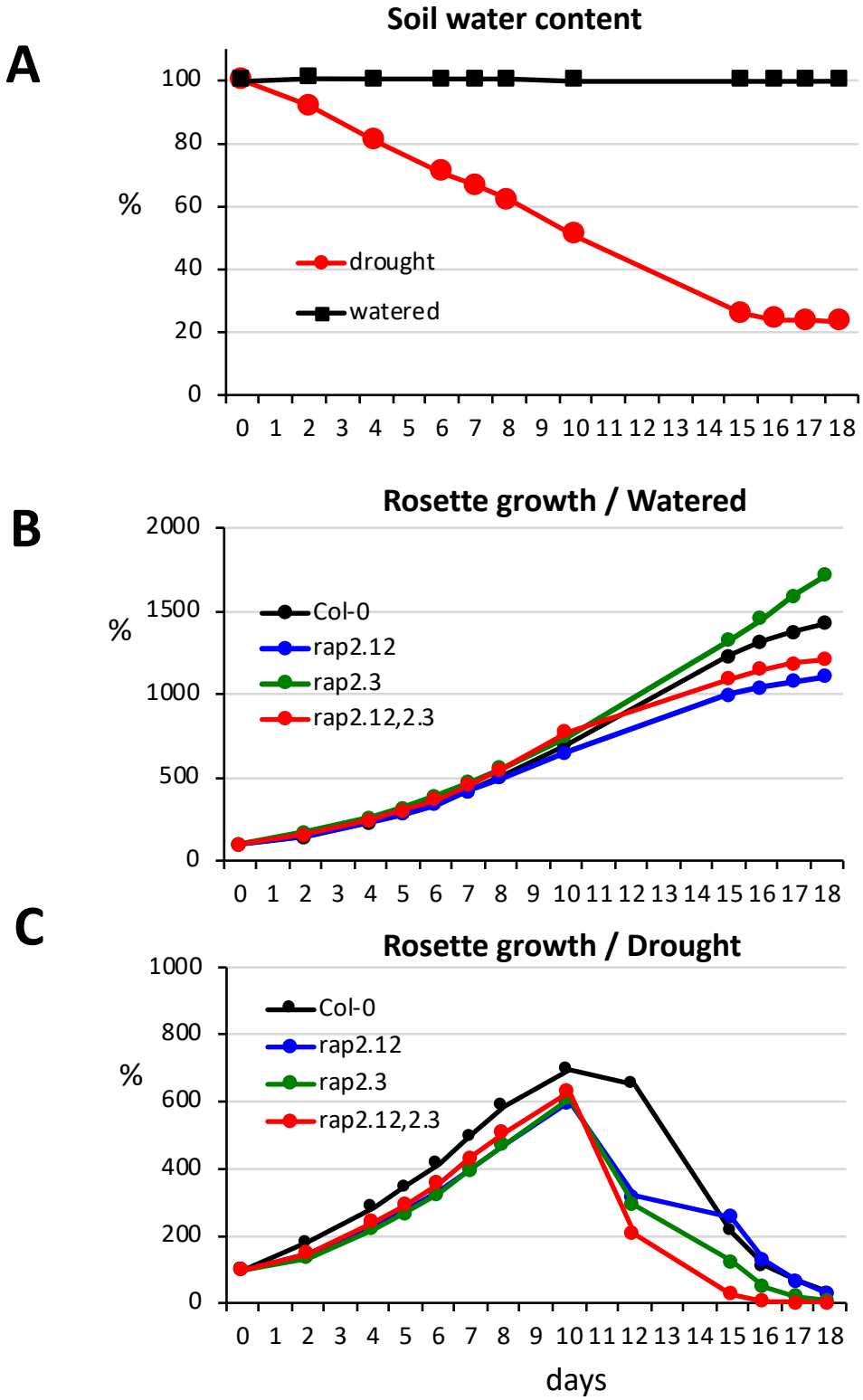


Figure 4. Phenotyping of rap2 mutants. A) Water content of well watered and drying soils. B,C) Growth of wild type (Col-0) and rap mutants (rap2.12, rap2.3 and rap2.12,rap2.3 double mutant) in well watered (B.) and drought (C.) conditions. Plants were grown in the automatic phenotyping equipment and RGB photos were taken regularly. Relative growth of average rosette sizes are shown, expressed as % increase comparing to the initial rosette sizes. Note, that rosete size of drought-treated plants drops after 10-12 days of drought, indicating loss of green chlorophyll in leaves. Soli water content is shown as % of the original value.

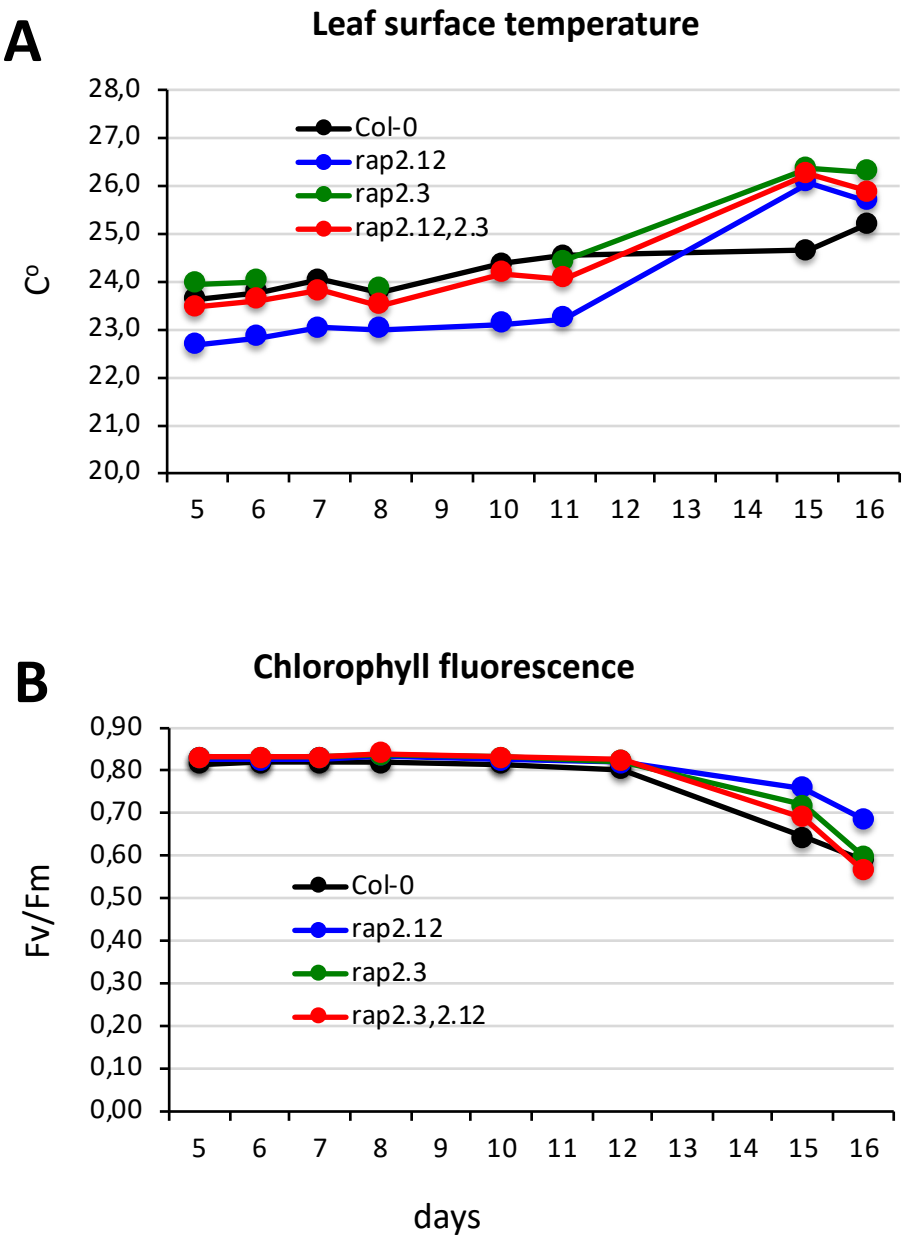


Figure 5. Phenotyping of rap2 mutants. Analysis of surface temperature and PSII quantum efficiency of drought-treated rap2 mutants. A) Temperature was monitored by thermo camera. B) Chlorophyll fluorescence was used to monitor PSII quantum efficiency. Note, that after 12 days of water withdrawal sudden increase in leaf temperature and drop in Fv/Fm values suggests drop in viability of the plants.

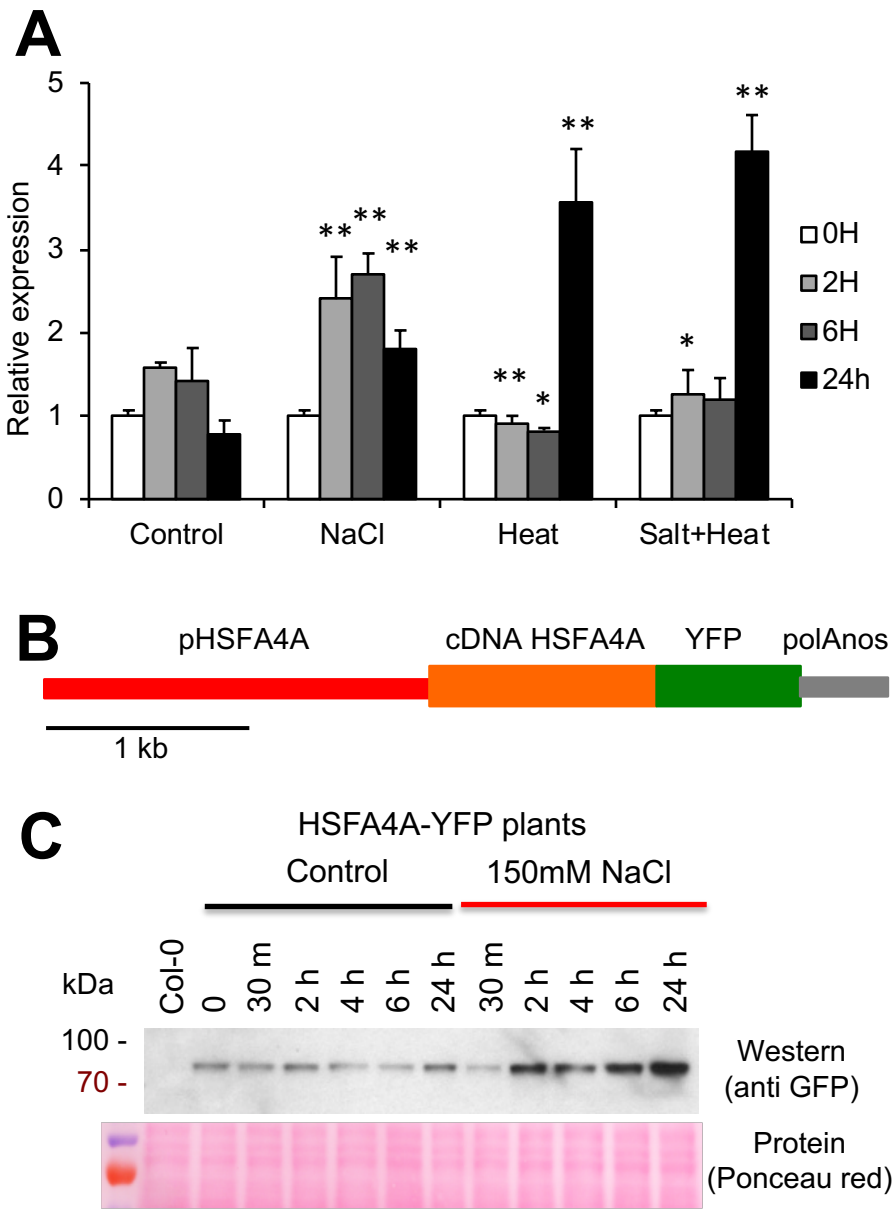


Figure 6. Regulation of HSFA4A. A) Transcriptional regulation of *HSFA4A* gene in wild type Arabidopsis plants treated by salt (150 mM NaCl), heat stress (37°C in light and 30°C in dark), and their combination for 2, 6 and 24 hours. Relative expression is shown where 1 corresponds to transcript level at 0 hour. Bars on diagrams indicate standard error, * and ** show significant differences to control at $p<0.05$ and $p<0.01$, respectively (Student t-test). B) Schematic map of the pHSFA4A::HSFA4A-YFP gene construct. C) Detection of HSFA4A-YFP fusion protein in ten days-old control and salt-stressed plants (150 mM NaCl, 0 to 24 hours) transformed with the pHSFA4A::HSFA4A-YFP gene construct. Salt treatment lead to enhanced HSFA4A-YFP specific Western signal.

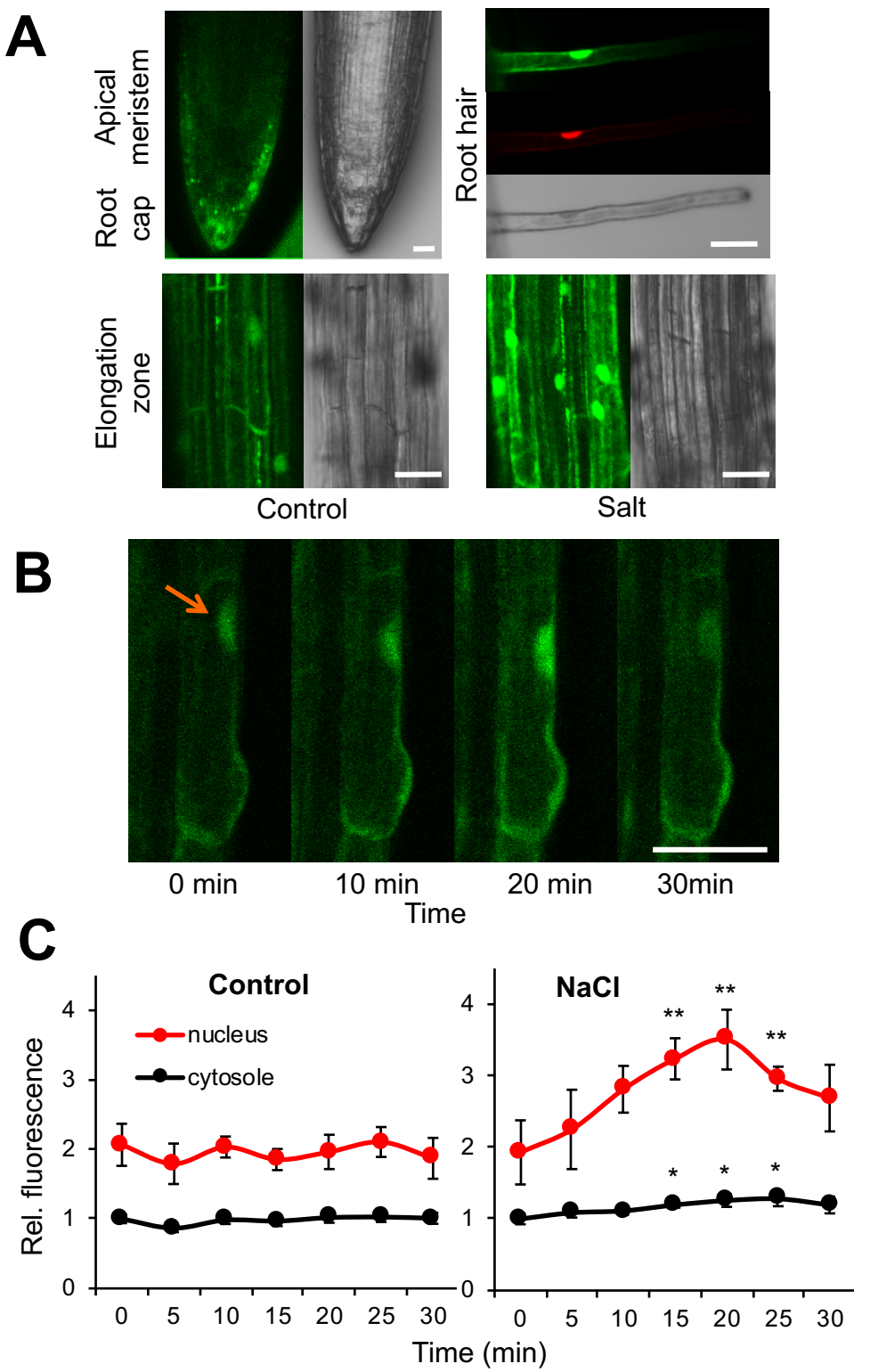


Figure 7. Intracellular localization and transfer of HSFA4A. A) Confocal microscopic detection of the HSFA4A-YFP fusion protein in roots. Root hair is stained with propidium iodide. Segments of elongation zone are shown with and without salt treatment (100 mM NaCl, 2 hours). B) HSFA4A is transported into nuclei during salt stress. Roots were treated with 100 mM NaCl and HSFA4A-YFP-derived fluorescence was monitored at regular intervals. C) Quantitative evaluation of YFP fluorescence in cytosol and nuclei. Relative fluorescence is shown where 1 corresponds to intensity measured in cytosol at time 0. Bar on photos indicates 20 µm. Bars on diagrams indicate standard error, * and ** show significant differences T0 at $p<0.05$ and $p<0.01$, respectively (Student t-test).

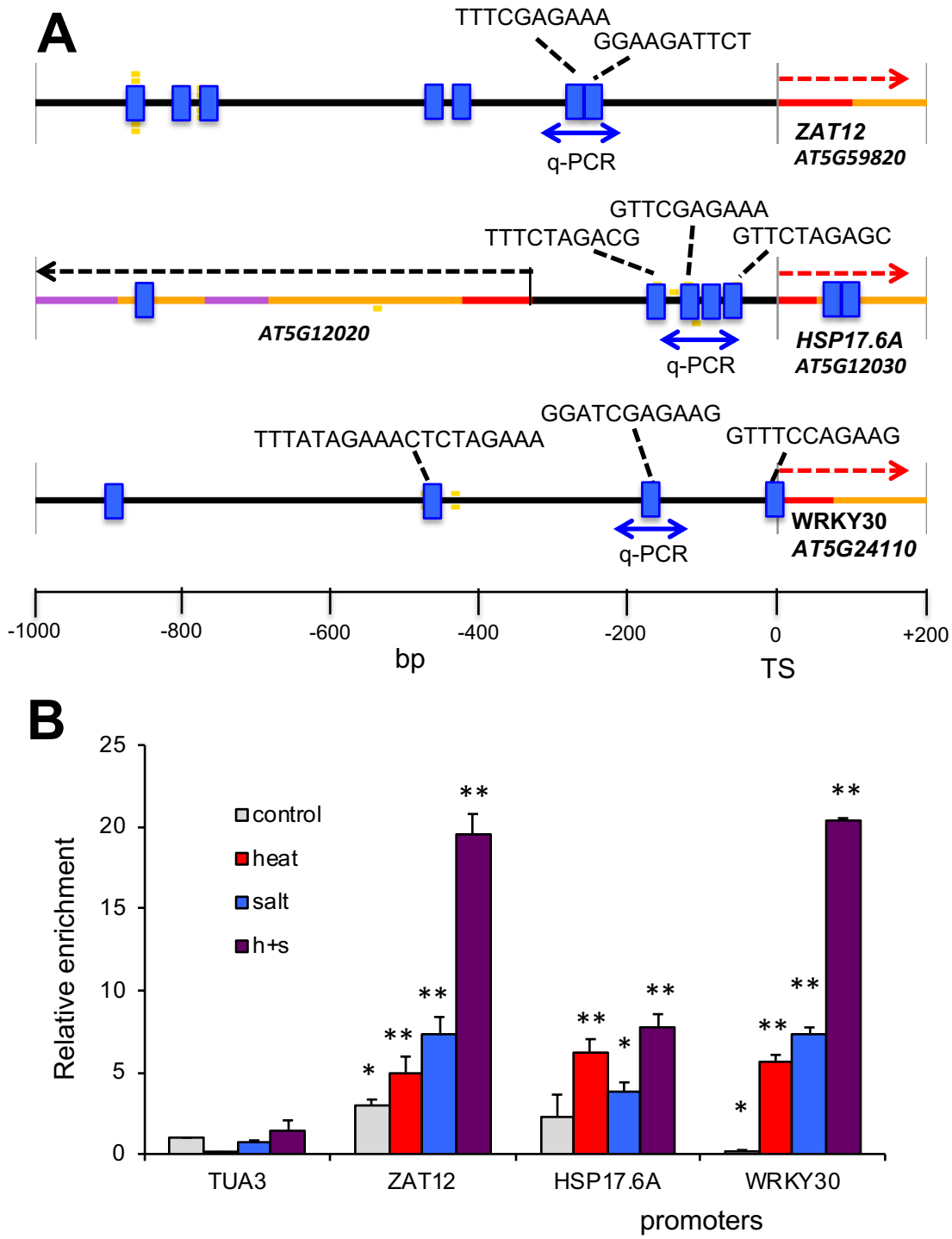
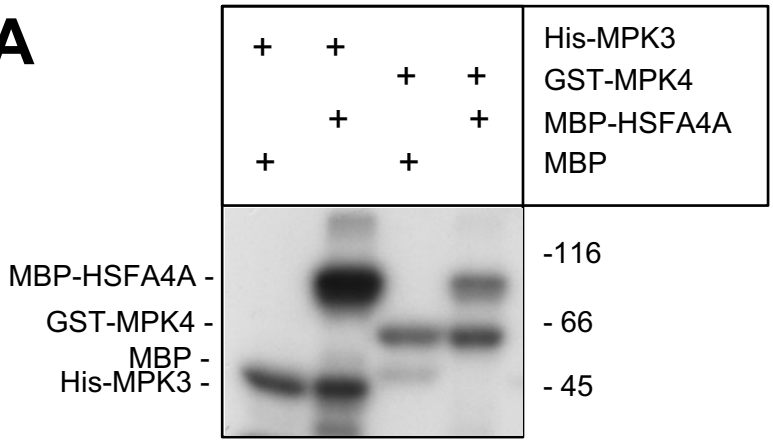


Figure 8. Binding of HSFA4A on target gene promoters. A) Schematic map of ZAT12, HSP17.6A and WRKY30 promoters. HSE motifs are indicated by blue boxes and sequences connected to the amplified regions are shown above the target region. Dashed arrows indicates transcription initiation. Amplified target sequences by q-PCR are indicated by blue double arrows. B) ChIP assay with YFP-tagged HSFA4A using transgenic plant expressing the pHSFA4A::HSFA4A-YFP gene construct (see: Figure 1B,C). Plants were treated by salt (150 mM NaCl, 6 h), heat stress (37°C, 6 h), and their combination before ChIP assay. ChIP results are shown as relative enrichment by q-PCR, where reference (value 1) is the q-PCR value of the TUA3 promoter, which lacks any HSE motif, at control conditions. Note enrichments on different promoter regions, which can be enhanced by salt or heat treatments. * and ** show significant differences to ChIP values of TUA3 promoter at $p<0.05$ and $p<0.01$, respectively (Student t-test).

A



B

Site	Phosphopeptide detected	signal%
T124	KPVHSHSLPNLQAQLNPL**pT**DSER	0.1
S198*	TMVSFVSQVLEKPGLALNL**pS**PCVPETNER	0.4
S239*	TCVVVREEGST**pS**PSSHTR	8.0
S239*	EEGST**pS**PSSHTR	4.5
S309*	LKSPP**pS**PR	79.9
T396*	NVNAITEQLGHL**pT**SSER	0.3
T396*	NVNAITEQLGHL**pT**SSERS	1.0
S397	NVNAITEQLGHLT**pS**SERS	0.2

C

Site	Phosphopeptide detected
S112	RKPVHSH**pS**LPNLQAQLNPLTDSER
S112	KPVHSH**pS**LPNLQAQLNPLTDSER
S239	TCVVVREEGST**pS**PSSHTR
S239	EEGST**pS**PSSHTR
S306,S309	LK**pS**PP**pS**PR
S306	LK**pS**PPSPR
S309	LKSPP**pS**PR

D

MDENNHGVSSSSLPPFLTKTYEMVDDSSSDSIVSWSQSNKSFIVWNPPEF
SRDLLPRFFKHNNFSSFIRQLNTYGFRKADPEQWEFANDDFVRGQPHLMK
NIHRRKPVHSH**S**LPNLQAQLNPL**T**DSERVRMNNQIERLTKEKEGLLEELH
KQDEEREVFEMQVKELKERLQHMEKRQKTMVSFVSQVLEKPGLALNL**S**PC
VPETNE**RKRRFPR**IEFFPDEPMLEENKTCVVVREEGST**S**PSSHTREHQVE
QLESSIAIWENLVSDSCESMLQSRSMMTLDVDESSTFPESPPLSCIQLSV
DSRLK**S**PP**S**PRIIDMNCEPDGSKEQNTVAAPPPPPVAGANDGFWQQFFSE
NPGSTEQREVQLERKDDKDKAGVRTEKCWWNSRNVNAITEQLGHL**TS**SER
S

Figure 9. Phosphorylation of HSFA4A. A) *In vitro* phosphorylation of HSFA4A by MAP kinases MPK3 and MPK4. MBP-tagged HSFA4A was phosphorylated *in vitro* by His-MPK3 or GST-MPK4. B) List of phosphopeptides identified by mass spectrometry. Phosphorylated amino acids are indicated with red letters. MBP-tagged HSFA4A was phosphorylated *in vitro* by MPK4, in-gel digested by trypsin and analyzed by mass spectrometry. Phosphopeptide signal % was calculated from MS signal areas of the unmodified and phosphorylated peptides. C) Detection of phosphopeptides *in vivo* by mass spectrometry. Blue letters indicate phosphorylated amino acids. D) Amino acid sequence of HSFA4A. Amino acids, phosphorylated by MPK3 (Perez-Salamo *et al.*, 2014) and MPK4 (this study) are shown by red letters, while phosphorylated amino acid detected only in immunoprecipitated samples are shown by blue S or T letters. Boxed letters indicate amino acids which were detected in both *in vitro* and *in vivo* phosphorylation assays.

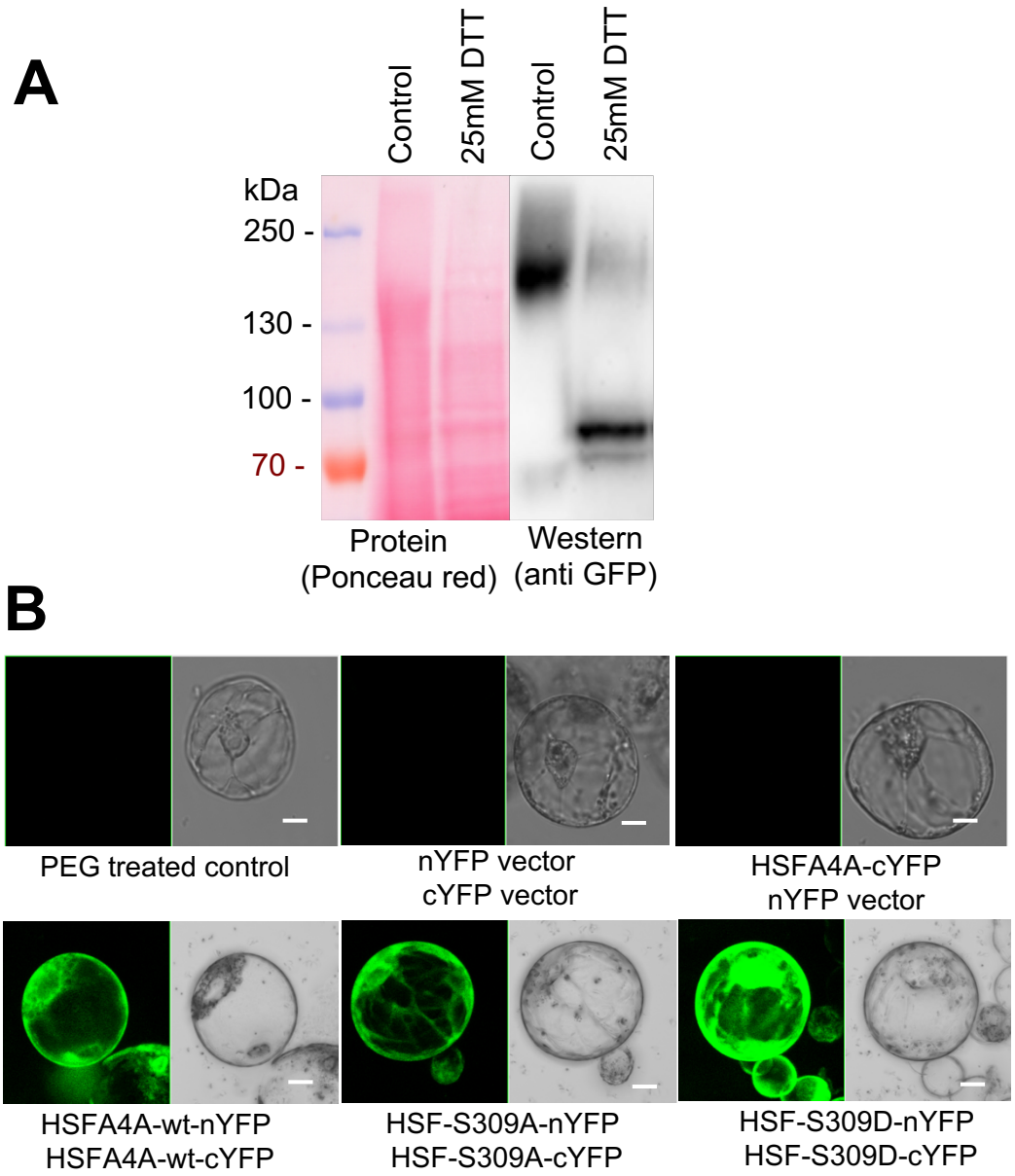


Figure 10. Multimerisation of HSFA4A. A) Detection of HSFA4A-YFP multimers in Arabidopsis plants transformed with the pHSFA4A::HSFA4A-YFP gene construct. Protein extracts were treated with or without DTT and separated on non-denaturing polyacrylamide gels. HSFA4A-YFP was detected by western hybridization with anti-GFP antibody. Separated and membrane-blotted proteins were stained with Ponceau Red. B) BiFC assay of wild type HSFA4A (HSFA4A-wt), and mutants in which Ser309 was changed to Ala (HSF-S309A) or Asp (HSF-S309D). nYFP and cYFP indicates N and C terminal half of YFP protein. Controls include PEG-treated protoplasts without plasmids, protoplasts transformed with plasmids having nYFP and cYFP fragments or protoplasts expressing HSFA4A-cYFP in combination with the empty nYFP plasmid (upper row). Typical BiFC images are shown.

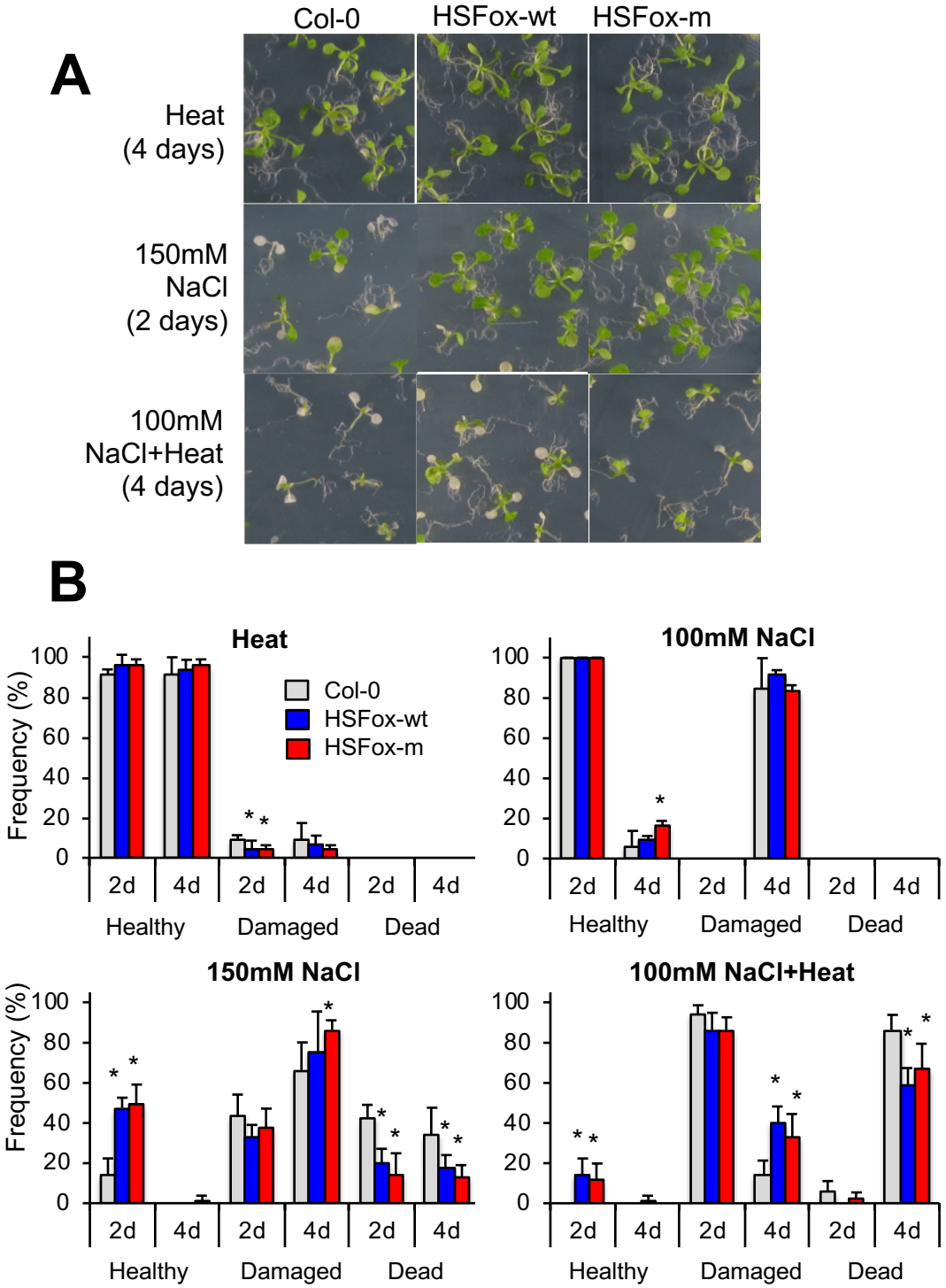


Figure 11. HSFA4A overexpression enhances tolerance to heat and salt stresses. 10 days-old *in vitro*-grown plantlets were treated by salt (100 mM, 150 mM NaCl), heat (37°C in light, 30°C in dark) or their combinations for two or four days. Rates of surviving healthy (vigorous growth with several new green leaves), damaged (small plants with retarded growth and/or chlorotic leaves) and dead plants (completely chlorotic with no green leaves) were scored 10 days after recovery. Similar results were obtained with independent transgenic lines of both constructs and one representative transgenic line was used for each construct in this experiments. A) Growth of wild type (Col-0) and transgenic plants overexpressing the wild type (HSFox-wt) and S309D mutant (HSFox-m) forms of HSFA4A after heat, 150 mM NaCl and combined 100 mM NaCl and heat treatments. B) Frequencies of healthy, damaged and dead plants after heat, salt and combined heat and salt stresses applied for 2 or 4 days. Survival frequencies of control, non-stressed plants (all survived and healthy) and plants treated by 150 mM NaCl and heat (all dead) are not shown.

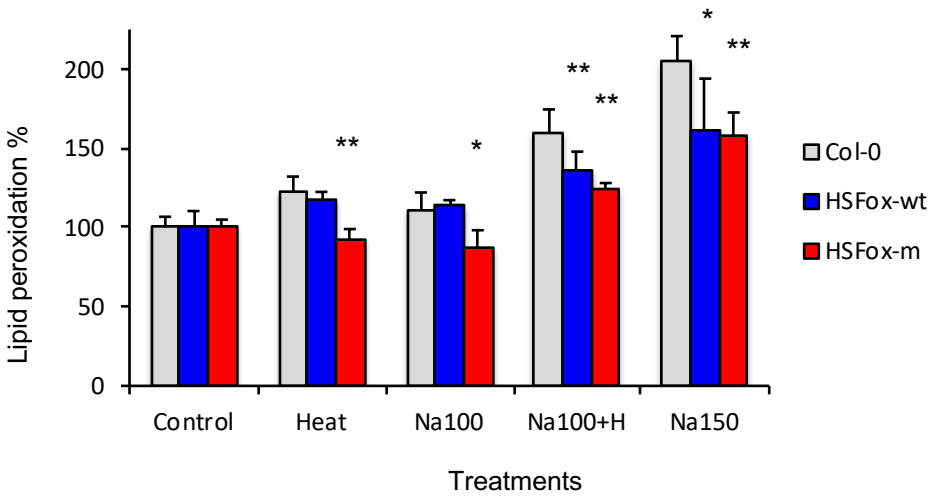


Figure 12. Lipid peroxidation rates of wild type and HSFA4A overexpressing lines (HSFox-wt, HSFox-m). Values are normalized to control, non-treated plants. Error bars indicate standard deviation, * and ** show significant differences to Col-0 wild type plants, at $p<0.05$ and $p<0.01$, respectively (Student t-test).

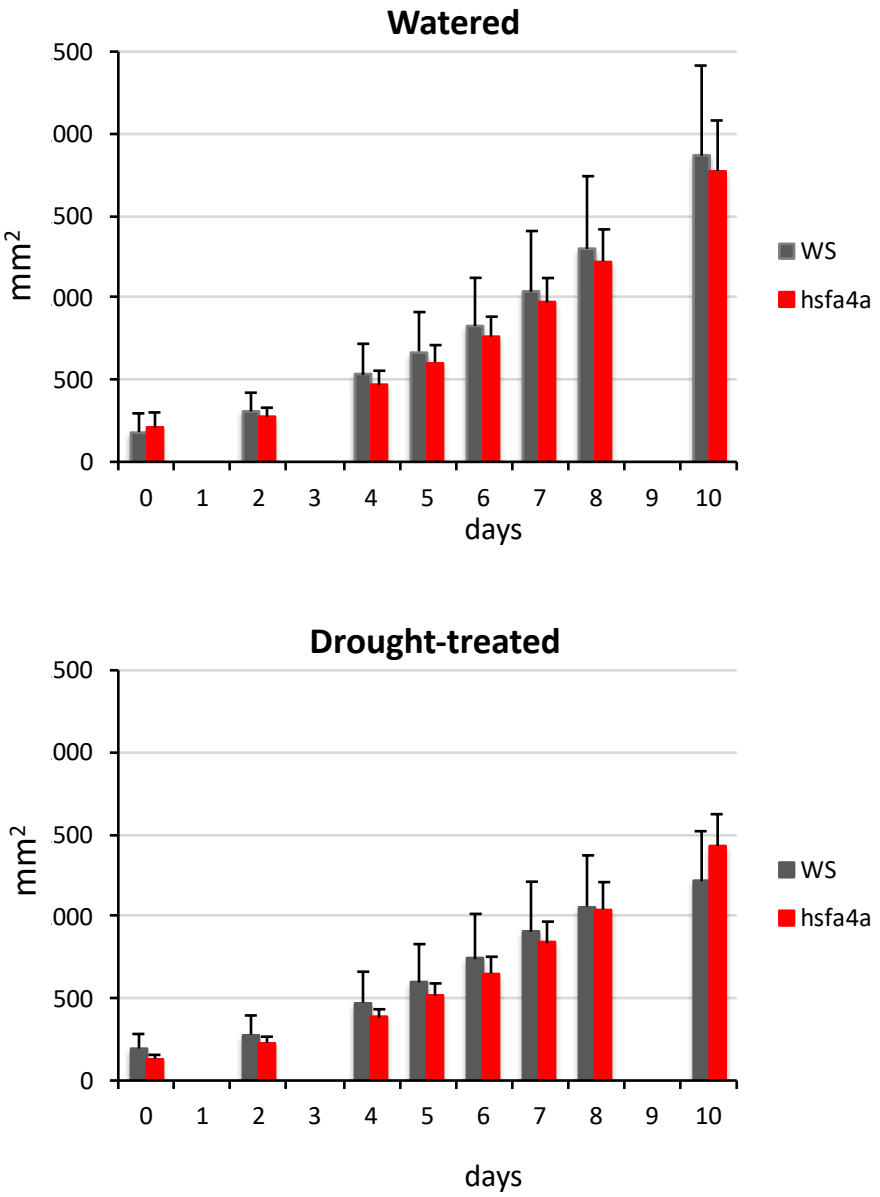


Figure 13. Growth of WS wild type and hsfa4a mutant plants in well-watered or drying conditions (drought). Watering of plants was stopped at day 0. RGB imaging was made regularly to record rosette growth.

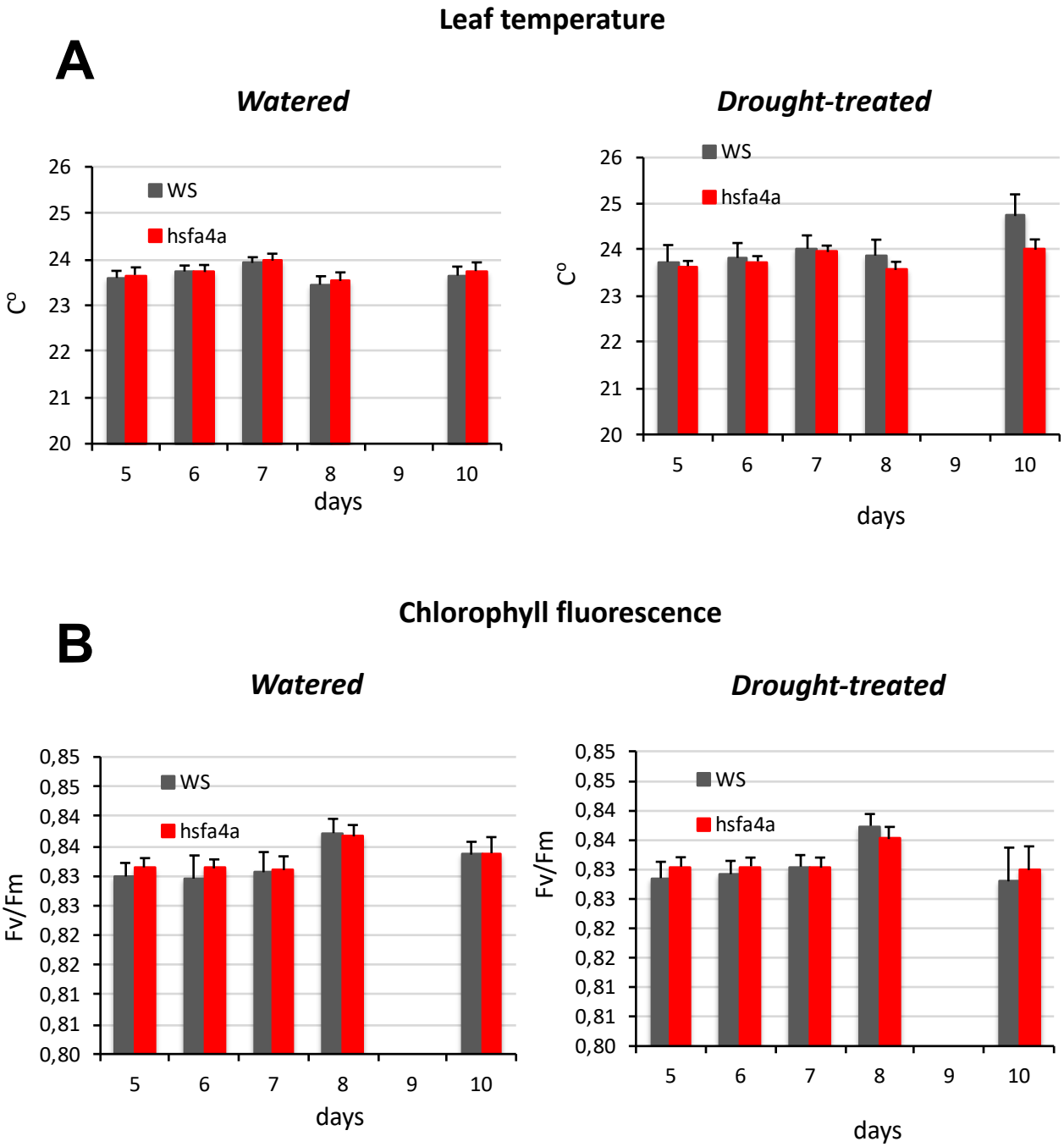


Figure 14. Physiological parameters of wild type (WS) and *hsfa4a* mutant plants in drought stress. Leaf temperatures were monitored by thermal imaging using infrared camera. Changes in PSII quantum yield was monitored by periodic recording of chlorophyll fluorescence.

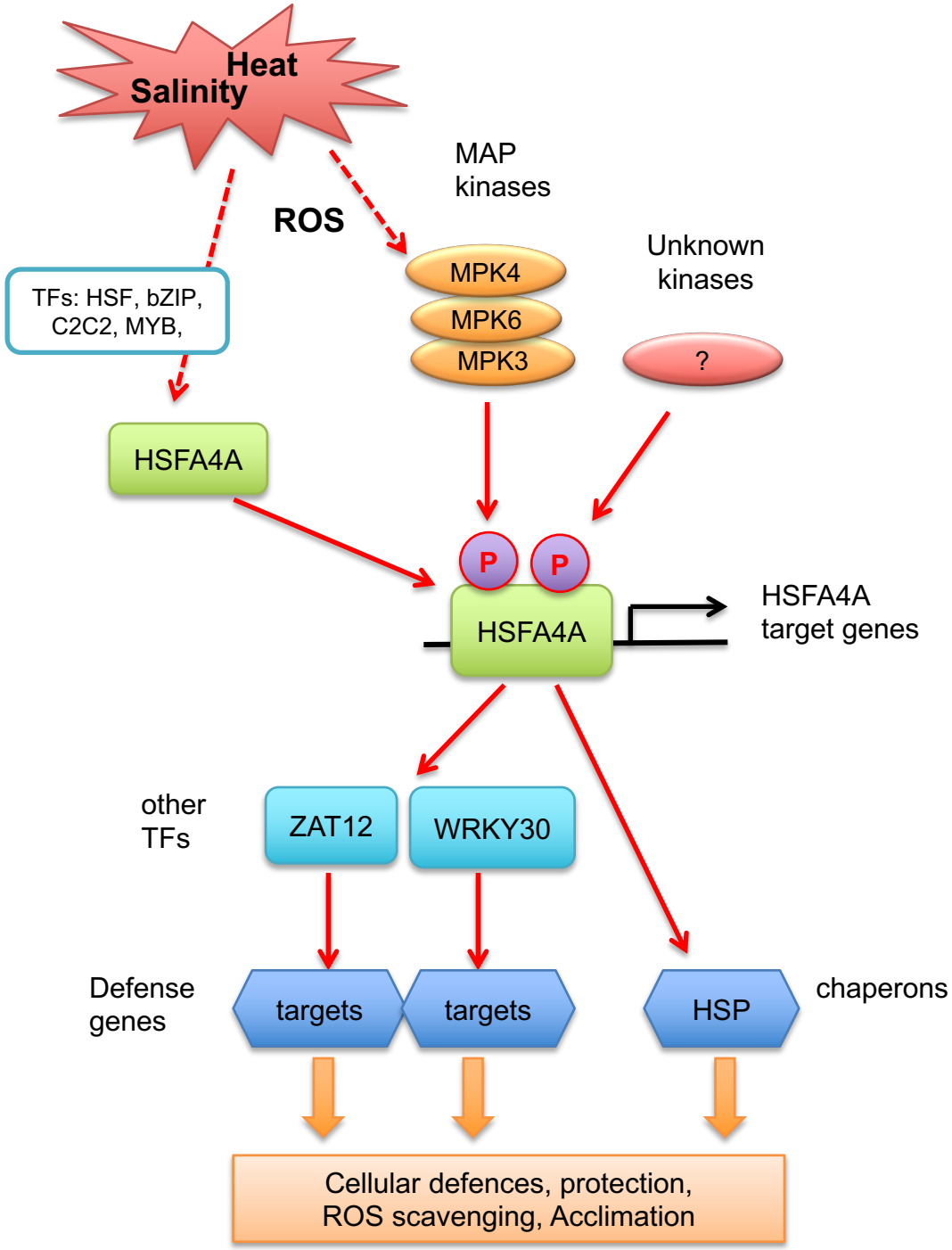


Figure 15. Model of stress signal transduction and transcription regulation mediated by HSFA4A. Environmental stresses such as salt and heat generate reactive oxygen species, which in turn can activate MAP kinases MPK3/6 and MPK4. Expression of HSFA4A is activated by stress conditions, in which different classes of TFs (eg. HSF, bZIP, C2H2, MYB) are implicated. Phosphorylation of HSFA4A by MPK3/6, MPK4 and other unknown kinases, modulates its activity and induction of target genes. HSFA4A binds to promoters of effector genes such as chaperons (eg. HSP17.6A) or other TFs (eg. WRKY30, ZAT12) in a stress-dependent manner and activate their transcription. Induction of these target genes contribute to stress tolerance either directly producing protective proteins or indirectly through activation of other defense-related genes.

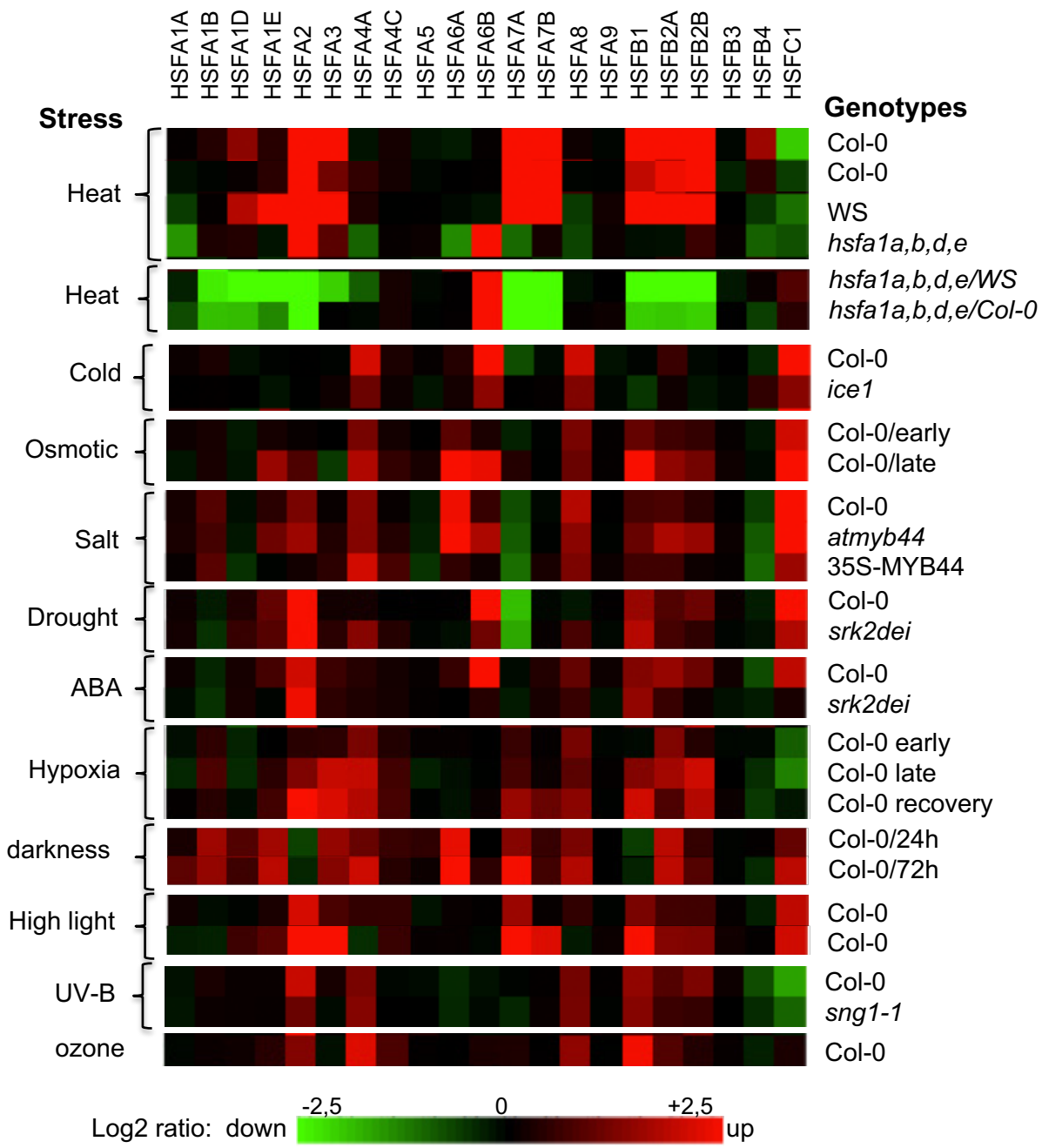


Figure 16. Effect of abiotic stress treatments on the expression of Arabidopsis HSF genes, compiled from Genevestigator. Treatments are indicated in the left side while genotypes such as ecotypes and mutants are listed in the right side of the color boxes. Mutants are discussed in the text. Color code: Red and green indicates up and downregulation, respectively.

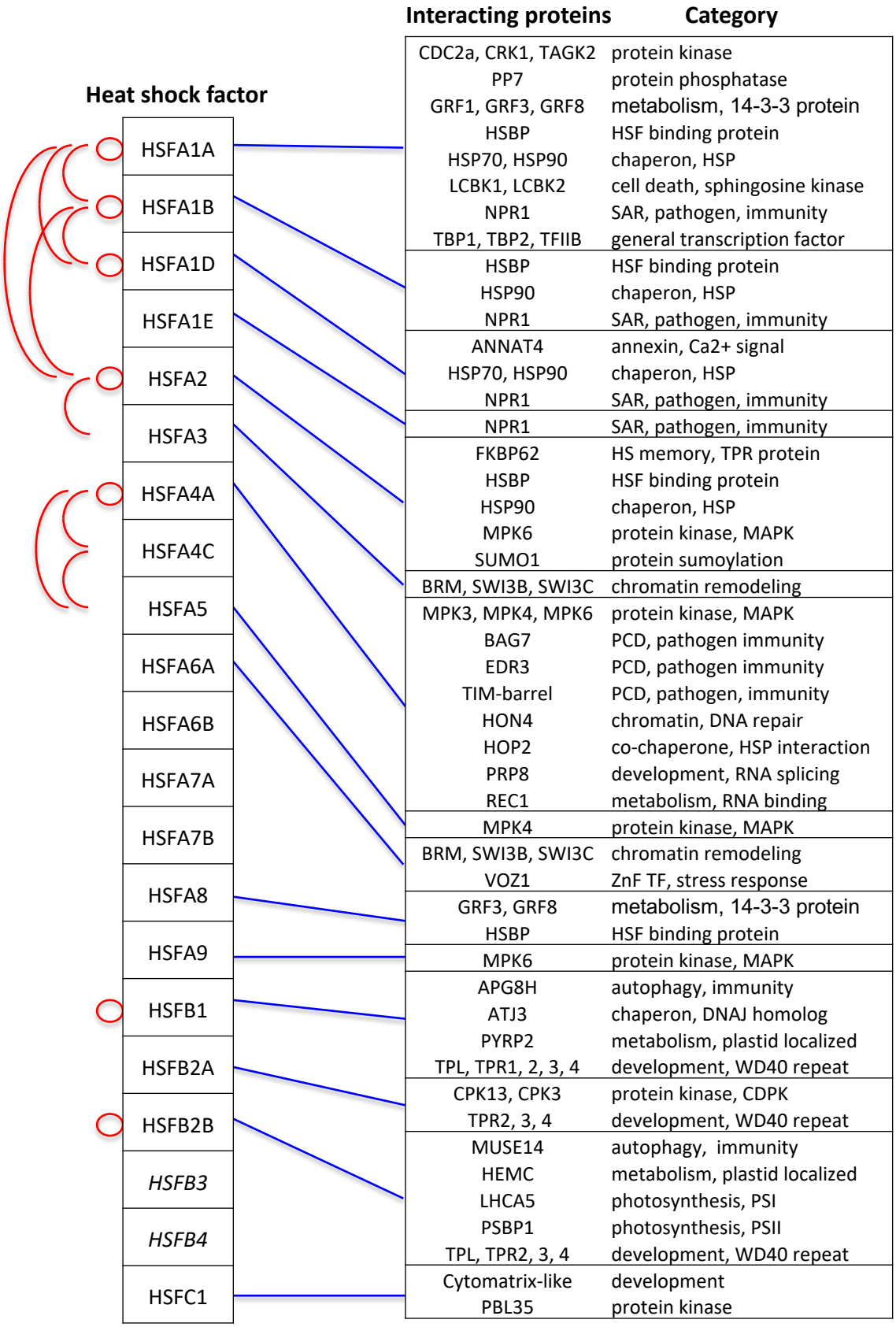


Figure 17. Protein-protein interactions of Arabidopsis HSFs. Homomeric and heteromeric HSF-HSF interactions are shown with red lines on left side (circles indicate homomerisation). Blue lines indicate interactions between HSFs and other proteins, listed in the column “Interacting proteins”.

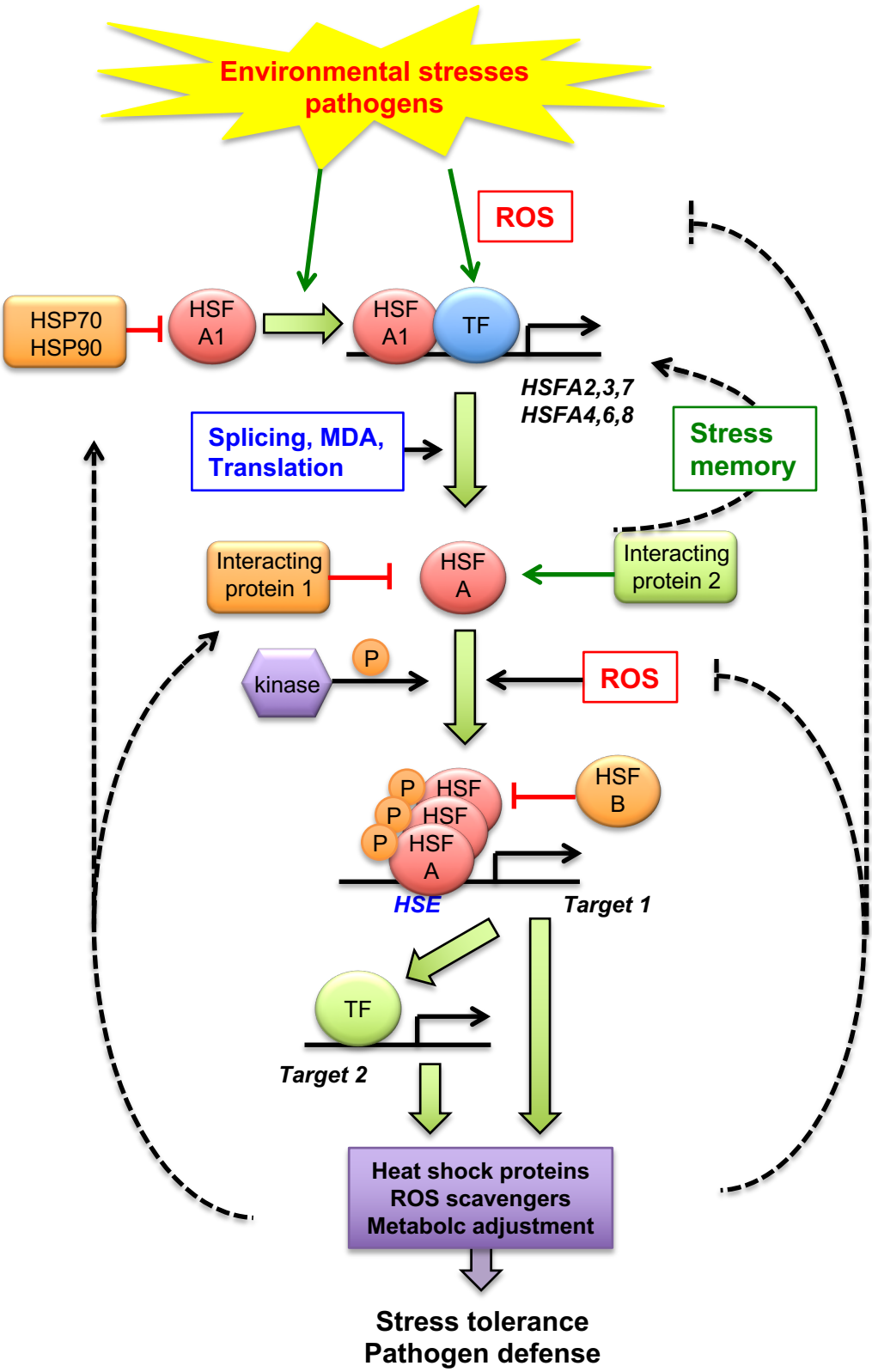


Figure 18. Regulatory interactions and the transcriptional network of class A HSFs in plants. Several stress-induced genes (eg. *HSFA2, A3, A7*) are regulated by HSFA1 factors, others (eg. *HSFA4A, A6, A8*) are controlled by different TFs such as DREB2. Stress-generated ROS can mediate induction. HSFA factors can positively or negatively be regulated by other interacting proteins, and modifications imposed by phosphorlation, ROS, etc. Some of them (eg. HSFA2) can enhance acquired stress tolerance by epigenetically controlled stress memory. Trimers bind to HSE motifs of target genes, which can be inhibited by class B HSFs. Target genes can encode defense-related proteins, enzymes or other TFs which themselves can induce expression of further targets. Feed-back regulation by encoded proteins such as HSPs or ROS scavengers may attenuate HSFA-mediated stress response.