STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, 70, 2, 2025 (p. 101-114)
doi:10.24193/subbbiol.2025.2.07

Overexpression of *HSFA4A* and *RAP2.12* transcription factors in *Arabidopsis thaliana* confers tolerance to various abiotic stresses

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Article history: Received 18 February 2025; Revised 11 November 2025;
Accepted 15 November 2025; Available online 20 December 2025

Abstract. Transcription factors are part of stress-signaling pathways, controlling activation of stress-responsive target genes. Heat shock factors and ethylene response factors can regulate responses to extreme temperature, salinity, drought, heavy metals, oxidative damage and anoxia. *Arabidopsis* HEAT SHOCK FACTOR A4A (HSFA4A) is part of the mitogen-activated protein kinase signaling pathway and was previously shown to regulate responses to salt, oxidative and heat stresses as well as their combinations. The RELATED TO APETALA2.12 (RAP2.12) factor was shown to be involved in anoxia, oxidative and osmotic stresses, and to modulate sensitivity to abscisic acid (ABA). Here we show that overexpression of *HSFA4A* and *RAP2.12* can increase the survival rate of *Arabidopsis* plants exposed to heat, salt or osmotic stresses and combinations of high temperature with salt or osmotic stresses. Moreover, overexpression of these factors improved photosynthetic activity in such adverse conditions. Photosynthetic performance of the *hsfa4a* and *rap2.12-2* mutants was variable in plantlets stressed in sterile conditions and less affected in soil-grown mutants when exposed to drought stress. Our data clearly indicate that these factors are implicated in stress response control, although their precise function remains to be elucidated.

Keywords: abiotic stress tolerance, *Arabidopsis*, combined stress, heat shock factor, RAP

Introduction

Extreme environmental conditions can considerably hinder plant growth and development. Plants evolved various mechanism to cope with such challenges, including a number of physiological, transcriptional, biochemical and molecular responses (Nawaz *et al.*, 2023). Effect of and responses to individual abiotic stresses, such as high temperature, drought, osmotic and salinity stress, are well documented (Mareri *et al.*, 2022; Zhang *et al.*, 2022). In natural environments such stress conditions often act simultaneously, resulting in a more dramatic and distinctive impact on plants. Combined stresses lead to novel and unique transcriptome and metabolome profiles, suggesting that simultaneous effects generate more complex responses (Rasmussen *et al.*, 2013; Rivero *et al.*, 2014; Sewelam *et al.*, 2014; Suzuki *et al.*, 2014; Barah *et al.*, 2016; Georgii *et al.*, 2017).

The success to adapt to extreme environmental conditions depends on cascades of molecular networks, stress-signaling pathways. Transcription factors (TFs) play a pivotal role in these pathways, regulating the activation of specific stress-related target genes by recognizing and binding to their *cis*-regulatory elements located in their promoter regions (Gujjar *et al.*, 2014; Khan *et al.*, 2018). TFs are essential components of the signal transduction networks, often regulated by various types of protein kinases, such as mitogen-activated protein kinase (MAPK) cascades, CBL-interacting protein kinases (CIPK) or calcium-dependent protein kinases (CDPKs). Based on genome wide analyses we can distinguish a large number of TFs belonging to different TF families, like HSF, AP2/ERF, MYB, bHLH, WRKY, bZIP, NAC and others (Lindemose *et al.*, 2013; Khan *et al.*, 2018).

Heat shock factors (HSFs) have been identified first as essential regulators of responses to high temperatures, but were found later to be implicated in salt, heavy metal, high light and other abiotic and biotic stresses (Andrási *et al.*, 2021). HSFs share a well conserved domain structure, consisting of DNA binding domain, oligomerization domain and nuclear localization signal. Heat shock factors activate target genes by recognizing and binding special regulatory elements in their promoters, called heat shock elements (HSEs). HSEs consist of palindromic cis-regulatory binding domains, 5′-AGAAnnTTCT-3′ (Nover *et al.*, 2001; Akerfelt *et al.*, 2010; Anckar and Sistonen, 2011). *Arabidopsis* HEAT SHOCK FACTOR A4A (HSFA4A) was identified in a salt screen using a special genetic tool, allowing controlled cDNA overexpression (COS system). HSFA4A confers tolerance to salt, oxidative, high irradiance and combined stress and it is phosphorylated by MPK3, MPK4 and MPK6 protein kinases. In response to individual or combined stresses HSFA4A can activate other TFs such as *WRKY30* and *ZAT12* or molecular chaperons such as heat shock protein 17.6A (*HSP17.6A)* by directly binding their HSE-containing promoter regions (Papdi *et al.,* 2008; Pérez-Salamó *et al.*, 2014; Huang *et al.*, 2018; Andrási *et al.*, 2019).

The ethylene response factor (ERF) family is a plant-specific group of TFs, which regulates hormone response, development, and tolerance to biotic and abiotic stresses (Licausi *et al.*, 2013; Dey and Vlot, 2015). The members of RAP-type ERF-VII TFs are involved in oxygen sensing, and they are distinguished by their conserved APETALA2 (AP2) domain that plays an essential role in protein–DNA interactions (Gibbs *et al.*, 2011; Licausi *et al.*, 2013). RELATED TO APETALA2.12 (RAP2.12) is a member of the ethylene response factor VII (ERF-VII) protein family. RAP2.12 was identified in *Arabidopsis* plants in a screen using the COS system and the *ADH1 promoter::luciferase* (*ADH1-LUC*) reporter (Papdi *et al.*, 2008, Papdi *et al.*, 2015). RAP2.12 confers tolerance to anoxia, oxidative and osmotic stress and enhances abscisic acid (ABA) sensitivity (Papdi *et al.*, 2015).

Although HSFA4A and RAP2.12 belong to distinct transcription factor families, both have been reported to participate in various stress responses (Pérez-Salamó *et al.*, 2014; Papdi *et al.*, 2015; Andrási *et al.*, 2019). Therefore, we selected these two genes to compare and characterize their role in response to heat, salt, osmotic and combined stresses.

Materials and methods

Plant material and growth conditions

Arabidopsis Col-0 ecotype was used in all experiment. The overexpressing *Arabidopsis* lines, HSFA4Aox and RAP2.12ox, were generated in our lab (Papdi *et al.,* 2008; Pérez-Salamó *et al.*, 2014). The *hsfa4a* (GK-181H12) mutant line was a kind gift of prof. Wu (Huang *et al.,* 2018) and the *rap2.12-2* (SAIL-1215-H10) mutant line was identified and deposited in our lab collection by Papdi *et al.,* (2015).

Arabidopsis plants were grown in sterile conditions in growth chambers with the following settings: 8 h light–16 h dark light cycle at 22 °C and 100 µE m^{-2} s^{-1} light intensity (control condition). Seedlings were grown on half-strength Murashige and Skoog (½MS) medium. To control the transcription of *HSFA4A* and *RAP2*.12 in overexpressing *Arabidopsis* lines, the culture medium was supplemented with 5 µM estradiol at the beginning and during stress treatments (Pérez-Salamó *et al.*, 2014; Andrási *et al.,* 2019).

Stress conditions

Stress treatments were carried out *in vitro* in growth chamber, with the same light/dark cycle and light intensity as indicated above. Conditions for stress treatments were optimized on wild-type *Arabidopsis* plants (Col-0). A nylon mesh (SEFAR 07-20/13) was placed on the surface of agar-solidified standard ½MS medium. Col-0 seeds were germinated, and seedlings were grown on this nylon mesh for 12 days and then placed to ½MS medium, containing 0.5% agar and the following supplements: 150mM or 200mM NaCl (salt stress), 300mM or 400mM Sorbitol (osmotic stress). Heat stress was implemented as incubation of seedlings on high temperature: 37 °C in light and 30 °C in dark (Andrási *et al.,* 2019).

Stress combinations were implemented by simultaneous application of salt or osmotic stress with high temperature. All treatments were carried out for 2-4 days. 40 wild-type plants were used for each stress treatment to optimize the treatments. In case of scoring plant survival, photosynthetic activity measurements and plant phenotyping, we used 70, 15 and 20 plants for each genotype (including the wild-type) in every treatment, respectively.

Scoring plant survival

Sensitivity to individual or combined stresses was evaluated by counting the percentage of recovered or dead plants after the treatments. Plants were subjected to different doses of stress and subsequently transferred to standard growth conditions for 7 days. Following the recovery period, we recorded the percentages of three groups: healthy plants, with several new green leaves, damaged plants, with decreased growth and possibly chlorotic leaves, and dead plants (Andrási *et al.,* 2019). 70 plants were assessed for each genotype in every treatment, divided into seven Petri dishes/treatment and 10 plants/genotypes in a Petri dish (13 cm diameter).

Photosynthetic activity

To assess the photosynthetic performance of control and stressed plants, we measured the PSII maximum quantum efficiencies (Imaging-PAM MAXI, M-Series, Heinz Walz GmbH, Germany; Baker 2008) using the same stress conditions as described above, except the treatments were carried out for three days and there was no recovery period. Fv/Fm values were measured on dark-adapted plants (incubation in dark for 15 minutes, 15 plants were used for each genotype in every treatment, divided into 3 Petri dishes as 5 plants/genotype in a Petri dish).

Plant phenotyping (drought stress)

Phenotypic traits of Col-0, *rap2.12-2* and *hsfa4a* mutants were recorded in controlled environmental conditions with the PlantScreen™ Compact Phenotyping System as described by Faragó *et al.,* (2022). Briefly, after germination plants were grown in soil filled pots for 21 days, in well-watered condition, then subjected to drought stress by suspending watering for 15 days. RGB and chlorophyll fluorescence (ChlF) images were obtained daily during the drought period. ChlF imaging was performed after dark-adaptation (15 minutes) with specific intermittent light pulses, as described by Kant *et al.,* (2024). 20 plants/genotype/treatment were used in the experiment, planted individually in soil filled pots.

Results

Stress treatment optimization using wild-type Arabidopsis plants

Treatments were optimized with *Arabidopsis* wild-type (Col-0) plants. When heat stress was applied alone, all wild type plants survived, although more damaged plants recorded (30%). Salt stress had time- and concentration-dependent effect on wild type plants. After 2 days of 150-200mM NaCl treatment, 70% and 50% of the plants recovered, respectively. 54% and 23% of the plants survived when 150mM or 200 mM NaCl was used for 4 days. Consequently, higher doses of salt stress increased plant lethality (Fig. 1).

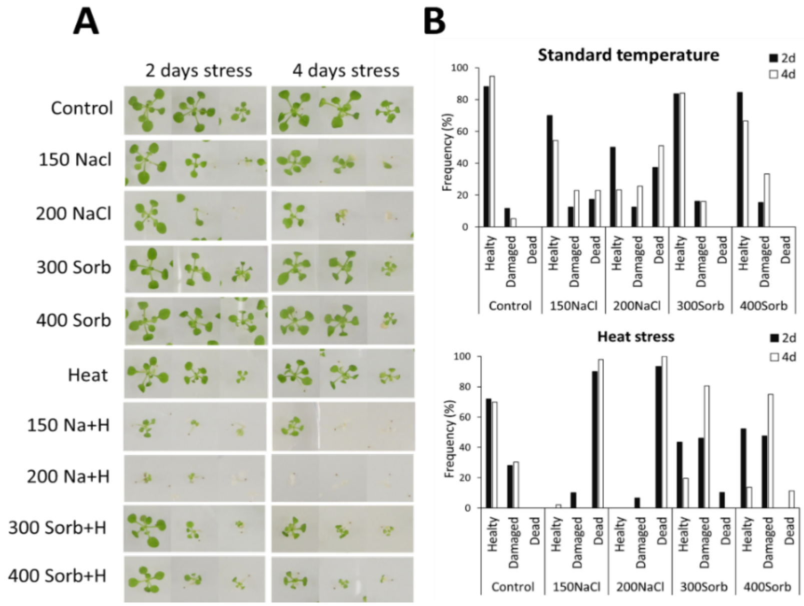

Figure 1. Stress response of wild-type *Arabidopsis* plants. Plants were grown *in-vitro* for twelve days, then exposed to different stress conditions. After 2 or 4 days of treatments plants were placed to standard culture conditions for recovery. Survival was scored by imaging the plants 7 days later. (A) Growth of wild-type (Col-0), after individual and combined stress treatments and recovery period. Treatments: 150 NaCl – 150mM NaCl; 200 NaCl – 200mM NaCl; 300 Sorb – 300mM Sorbitol; 400 Sorb – 400mM Sorbitol; 150 Na+H – 150mM NaCl+37°C Heat; 200 Na+H – 200mM NaCl+Heat; 300 Sorb+H – 300mM Sorbitol+Heat; 400 Sorb+H – 400mM Sorbitol+Heat. (B) Percentages of healthy, damaged, and dead plants after control, heat, salt, osmotic and combined stresses applied for 2 or 4 days.

Combination of salt stress with high temperature led to more severe damage: 90 to 93% of the plants died after 2 days, while nearly all of them was dead after 4 days of salt and heat stress. Surviving plants were heavily damaged after both 2 and 4 days of such treatments, regardless of salt concentration used (Fig. 1).

Effect of osmotic stress was slightly different. 300 and 400mM sorbitol concentration had similar effect, almost 85% of plants survived and were healthy after 2 days treatment, and around 15% of them were damaged. 4-days of 400mM sorbitol treatment had a bit more drastic effect, as it resulted in 34% damaged plants. Combination of osmotic and heat stress increased the ratio of damaged plants, although most of them still survived the treatments. When plants were simultaneously exposed to sorbitol and heat, 50% and 80% of the plants became damaged after 2 and 4 days of stress, respectively (Fig. 1B).

Based on the results of our trial experiment the following treatments were used to test the tolerance of the overexpressing and mutant *Arabidopsis* lines to various stresses: 125mM NaCl, 300mM Sorbitol, 37^{o}C (heat), 125mM NaCl+heat and 300mM Sorbitol+heat for 4 days.

HSFA4A and RAP2.12 can alleviate the adverse effect of certain individual and combined stresses

Transcription factors HSFA4A and RAP2.12 were reported to regulate responses to different adverse conditions including salt, osmotic, anoxic or oxidative stress (Pérez-Salamó *et al.*, 2014; Papdi *et al.,* 2015; Andrási *et al.,* 2019). Their role in different stress combination and drought condition is not well known. To study the effects of HSFA4A and RAP2.12 factors on stress tolerance, responses of overexpressing lines and knockout mutants to salt and heat or osmotic and heat stresses and their combinations were tested. Due to the high degree of lethality of 150mM NaCl and heat stress combination (Fig. 1), 125mM of NaCl was used in combination with 37°C in subsequent experiments. Number of damaged plants increased moderately when heat stress (10-20%), sorbitol (15-35%) or combination of heat and sorbitol treatment (17-27%) was applied. No differences in survival rates between the overexpressing lines and mutants were observed compared to wild type, when sorbitol and heat treatment was applied alone or in combination (Fig. 2). However, overexpression of *HSFA4A* and *RAP2.12* significantly increased the number of healthy plants (from 65% to 85%) after salt treatment. When salt and heat treatment was applied in combination, *HSFA4A* overexpression had significantly positive effect on survival: while 19% of Col-0 plants survived this stress combination, 46% of HSFA4Aox plants recovered completely. Survival frequencies of the *hsfa4a* and *rap2.12* mutants were similar to wild type plants under these conditions (Fig. 2A, B).

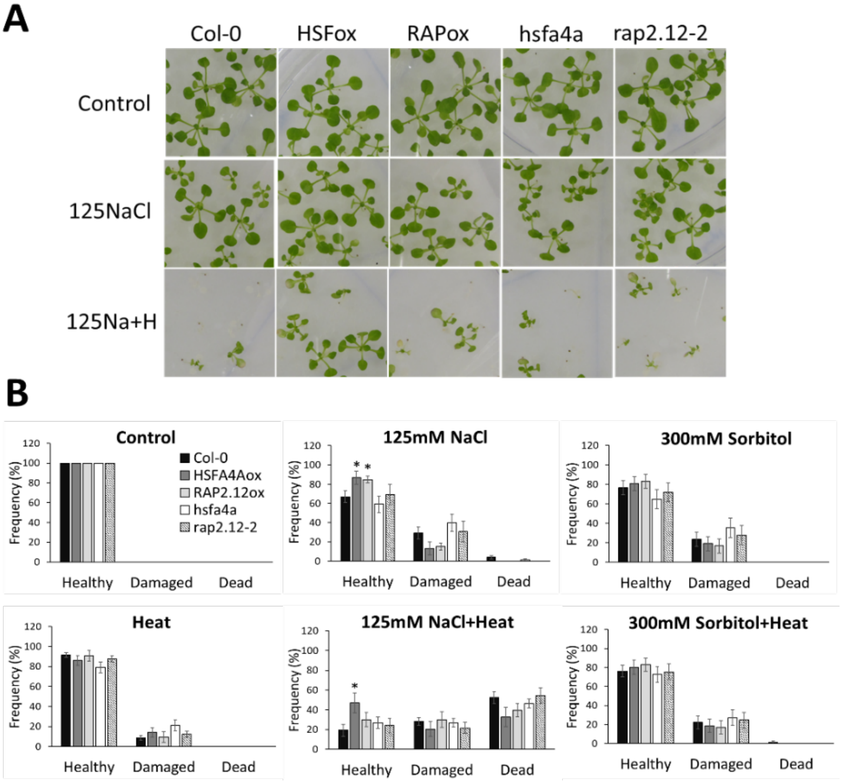

Figure 2. Survival of Col-0, transgenic overexpressing and mutant plants in salt, osmotic, heat stresses and their combinations. Survival rate was recorded after 7-day recovery. (A) Images of recovered Col-0, HSFA4Aox (HSFox), RAP2.12ox (RAPox), *hsfa4a* and *rap2.12-2* plants subjected to 125mM NaCl (125Na) and 125mM NaCl+Heat (125Na+H) treatments. (B) Percentages of established categories (healthy, damaged, and dead plants) after different stress conditions applied for 4 days. Standard errors are shown; overexpressing and mutant plants were compered to Col-0 plants (Student's *t*-test, **P*<0.05; n=7).

Photosynthetic parameters are known to be affected by environmental stresses. To find out how the studied TFs could influence photosynthetic efficiency in stress conditions, the maximum quantum yield of PSII photochemistry (Fv/Fm) was determined in mutant and overexpressing plants exposed to different stresses. *Arabidopsis* lines were exposed to the same stress conditions as described above, except that treatments were applied for 3 days, to get statistically more reliable results. In control condition the RAP2.12ox, *hsfa4a* and *rap2.12-2* had similar or slightly higher Fv/Fm values than Col-0 plants. 300mM sorbitol treatment slightly reduced Fv/Fm values of all plants, but the differences between the genotypes remained similar to control. Col-0 and HSFA4Aox had Fv/Fm 0.65 to 0.67, while the other overexpressing and mutant lines displayed Fv/Fm 0.70. Heat stress reduced the Fv/Fm values of Col-0, HSFA4Aox and *hsfa4a* mutant lines to similar degree, while *RAP2.12* overexpressing and mutant lines had slightly higher Fv/Fm values. When osmotic stress was combined with high temperature, PSII maximum yield of HSFA4Aox, RAP2.12ox and *rap2.12-2* mutants were significantly higher when compared to Col-0 plants. Salt stress imposed by 125mM NaCl lead to considerable reduction of Fv/Fm while combination of salt and heat stress reduced even more these values. There were no significant differences between photosynthetic activities of the studied lines when they were exposed to combined salt and heat stress (Fig. 3).

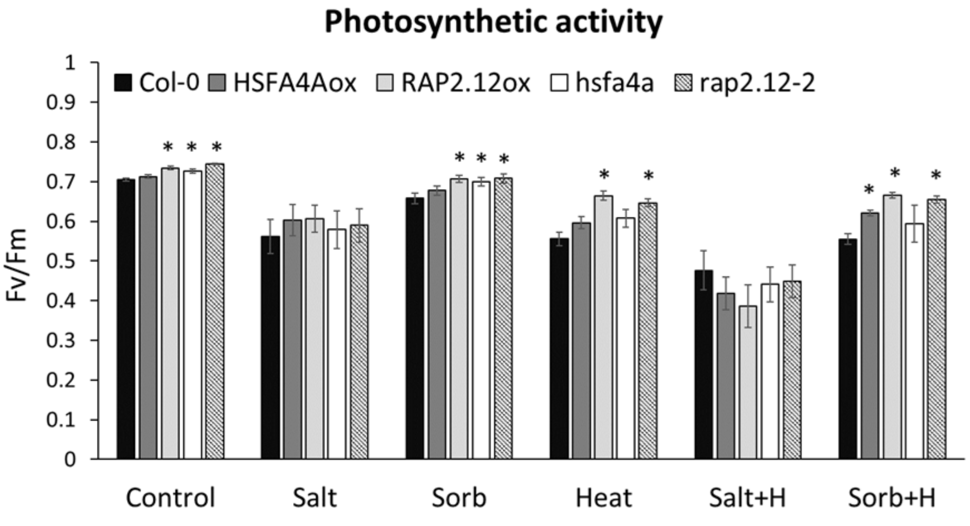

Figure 3. Photosynthetic activities of wild-type, HSFA4Aox, RAP2.12ox, *hsfa4a* and *rap2.12-2* plants after 72-hour of stress treatments (Salt: 125mM NaCl; Sorb: 300mM Sorbitol; Salt+H: 125mM NaCl+Heat; Sorb+H: 300mM Sorbitol+Heat). PSII maximum yields are shown. Standard errors are shown (n=3); significant differences to Col-0 wild-type plants were shown with statistical analyses, Student's *t*-test (**P*<0.05).

To further characterize the role of HSFA4A and RAP2.12 in stress tolerance, we conducted an image-based phenotyping experiment analyzing growth and photosynthetic performance of soil grown Col-0 wild type, *hsfa4a* and *rap2.12-2* mutant plants in water-limited conditions. The rosette growth of *hsfa4a* mutant in well-watered control condition was smaller than that of Col-0, throughout the whole experiment, while growth of *rap2.12-2* mutant was similar to Col-0 plants with the exception of last few days when its growth lagged behind the wild type. Water stress reduced growth of all lines to similar degree, with the exception of the *hsfa4a* mutant, which was less affected in the last few days of water limitation (Fig. 4A). The photosynthetic activity was monitored by chlorophyll fluorescence imaging. Fv/Fm values of all lines were similar in well-watered condition and were similar up to 9 days after watering was stopped. More severe water depletion has less deleterious effect on Fv/Fm of the studied mutants than Col-0 *Arabidopsis* plants (Fig. 4B).

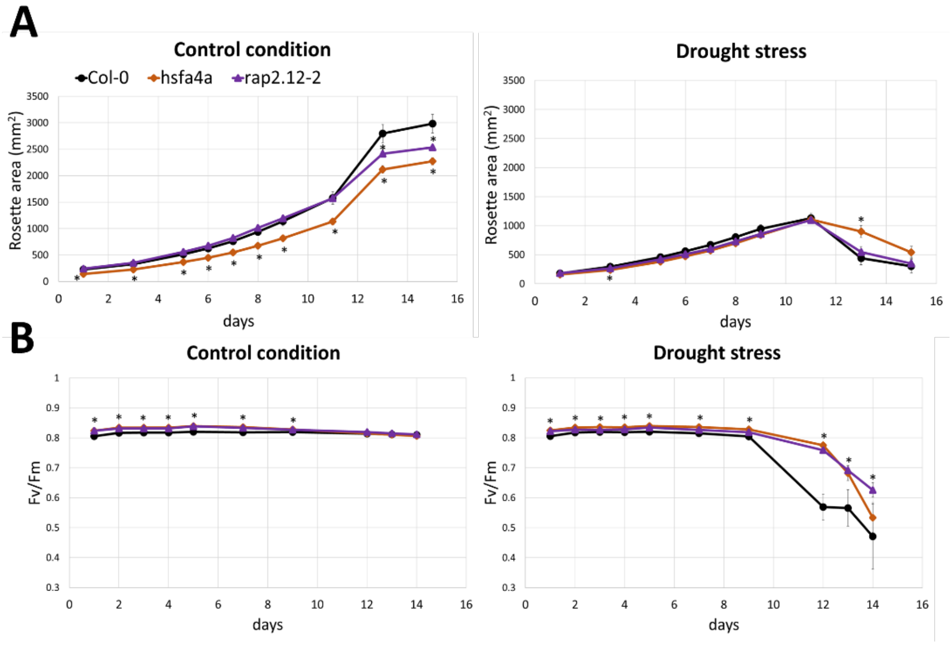

Figure 4. Image-based phenotyping of plants subjected to water deprivation. Plants were grown for 3 weeks in well-watered conditions, then watering was suspended to generate drought stress. RGB and chlorophyll fluorescence imaging was subsequently initiated and performed on daily intervals. (A) Rosette size and (B) photosynthetic activity of Col-0, *hsfa4a* and *rap2.12-2* mutant plants in control and drought conditions. Error bars represent standard error; **P*<0.05 represent significant differences compared to Col-0 (Student's *t*-test, n=20, individual plants).

Discussion

Transcription factors are essential components of stress-signaling pathways and stress responses (Khan *et al.*, 2018). Barah *et al.*, (2016) identified 294 TFs, including *HSFA1A* and *RAP2.7*, whose expression was differentially regulated by individual and combined stresses, including heat, salinity and cold. Many of these TFs controlled the activity of more than 1500 target genes, highlighting the importance of TFs in stress responses (Barah *et al.*, 2016). Heat shock factors are key regulators of stress-responsive signaling networks, with varying degrees of impact (Akerfelt *et al.*, 2010; Scharf *et al.*, 2012). HSFA4A is activated by various abiotic stress, including salt, oxidative, heavy metal, high irradiance, high temperature and combined salt and heat stress (Pérez-Salamó *et al.*, 2014; Huang *et al.*, 2018; Lin *et al.*, 2018 Andrási *et al.*, 2019). HSFA4A regulates a set of TFs and chaperon proteins, and its overexpression can reduce oxidative damages and enhance growth in salt, heat and combined stress (Pérez-Salamó *et al.*, 2014; Andrási *et al.*, 2019). Ethylene response factors (ERFs) are one of the largest transcription factor families in plants and plays indispensable role in plant growth, development and in responses to various stresses (Wu *et al.*, 2022). Several members of ERF-VII subfamily function in perception and transmission of low oxygen signals (Giuntoli & Perata, 2018). RAP2.12 modulates anoxic, oxidative and osmotic stress responses, acts as an oxygen sensor and was shown to be implicated in ABA signaling (Licausi et al., 2011; Kosmacz *et al.*, 2015; Papdi *et al.*, 2015).

Simultaneously acting stress conditions impacts plants growth, development and survival more severely than individual stresses. The particular effect of stress combinations on transcript profiles demonstrated that plant responses are characterized by special signatures, not observed in conditions imposed by individual stresses (Rasmussen *et al.*, 2013; Shaar-Moshe et al., 2017; Zandalinas *et al.*, 2021; Jiang *et al.*, 2024). Withstand such conditions require special regulatory mechanisms with particular factors. Overexpression of *Arabidopsis HSFA2* can improve PSII activity and alleviate rosette wilting in short-lasting heat stress combined with high-light stress and methylviologen treatment (Nishizawa *et al.*, 2006). Transgenic *Arabidopsis* plants overexpressing *HSFA7b* have increased tolerance to salt and heat stresses, characterized by greater fresh weight and chlorophyll content, and longer roots, while *hsfa7a* mutant plants showed salt and heat hypersensitivity (Zang *et al.*, 2019). Zang *et al.*, (2019) identified 193 TFs, whose expression was positively regulated by HSFA7a, several of them with the capacity to improve salt tolerance, including *RAP2.6*, *WRKY38* and *ZFP3*. Overexpression of *Arabidopsis ERF1* and wheat *ERF3* enhanced tolerance to salt and drought stress, manifested in increased survival and growth rates, while stress tolerance of *erf1* and *erf3* mutants was considerably inferior to wild-type plants (Cheng *et al*., 2013; Rong *et al*., 2014). A recent study showed that *Arabidopsis* ERF95 and ERF97 factors are interacting with each other in heat-dependent manner, and overexpression of them can increase thermotolerance, by directly upregulating various heat responsive target genes including *HSFA2* (Huang *et al*., 2021). Our results show that overexpression of *HSFA4A* and *RAP2.12* improves survival and may play a role in rosette growth in salt and combined heat and salt stresses, suggesting an important role in plant growth and development during stress response (Fig. 2). We also showed that overexpression of these TFs protects photosynthetic performance by maintaining PSII activity during osmotic, high temperature and combined osmotic and heat stresses. Interestingly photosynthetic activity of *hsfa4a* and *rap2.12* mutants was similar to wild type during osmotic and drought stresses, while *rap2.12-2* mutant plants displayed slightly higher PSII activity when plants were exposed to heat and combined osmotic and heat stresses (Fig. 3). It is not uncommon, that individual gene mutations result in stress tolerance comparable to wild-type plants, and only multiple mutations leads to decreased stress tolerance, due to complementing function of TFs (Cheng *et al*., 2013; Rong *et al*., 2014, Papdi *et al*., 2015; Huang *et al*., 2021). Further research is required to decipher the precise molecular mechanisms which are controlled by these factors and the way they determine responses to stress combinations.

Conclusions

This study allowed us to understand better the role of two transcription factors in controlling tolerance to individual and combined stresses. We demonstrated that overexpression of *HSFA4A* and *RAP2.12* can enhance the tolerance of *Arabidopsis* plants to heat, salt, osmotic and combined stresses. Our knowledge could be extended to decipher the function of HSFA4A and RAP2.12 in stress signaling. However, to elucidate the role of these transcription factor in drought stress needs more research.

Acknowledgements. We would like to thank to Annamária Király for the excellent technical assistance. Research was supported by NKFI KH-129510 grant. Norbert Andrási was supported by Young Scientist Fellowship of Hungarian Academy of Sciences.

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