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REVIEW PAPER

Signal transduction networks during stress combination

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Abstract

Episodes of heat waves combined with drought can have a devastating impact on agricultural production worldwide. These conditions, as well as many other types of stress combinations, impose unique physiological and developmental demands on plants and require the activation of dedicated pathways. Here, we review recent RNA sequencing studies of stress combination in plants, and conduct a meta-analysis of the transcriptome response of plants to different types of stress combination. Our analysis reveals that each different stress combination is accompanied by its own set of stress combination-specific transcripts, and that the response of different transcription factor families is unique to each stress combination. The alarming rate of increase in global temperatures, coupled with the predicted increase in future episodes of extreme weather, highlight an urgent need to develop crop plants with enhanced tolerance to stress combination. The uniqueness and complexity of the physiological and molecular response of plants to each different stress combination, highlighted here, demonstrate the daunting challenge we face in accomplishing this goal. Dedicated efforts combining field experimentation, omics, and network analyses, coupled with advanced phenotyping and breeding methods, will be needed to address specific crops and particular stress combinations relevant to maintaining our future food chain secured.

Key words: Abiotic stress, drought, global warming, heat stress, network, stomata, stress combination, transcription factor, transcriptomics.

Global climatic changes and the physiology of stress combination

Heat waves, combined with acute periods of drought stress, have previously occurred in many regions of our planet, resulting in devastating outcomes to agriculture, plant and animal life, and economic stability (Mittler, 2006; https://www.ncdc.noaa.gov/billions/). Such catastrophic episodes have been a major driving force in the attempt by classical breeders to develop crops such as maize (*Zea mays*) and soybean (*Glycine max*) with enhanced tolerance to a combination of drought and heat stress (Mittler and Blumwald, 2010; Suzuki *et al.*, 2014). Although progress has been made in the past by different breeders on this front, the constant and steady increase in global average temperatures, driven by the increase in greenhouse gases (i.e. global warming), could make future events of stress combinations, such as drought and heat waves, much more intense and frequent (http://www.ipcc.ch/; Teuling, 2018). In addition to drought combined with high temperatures, the intensity and frequency of other abiotic and biotic stresses, as well as their combinations, could also increase in the future. These include combinations of heat stress with attack by different insects or pathogens, high soil salinity, nutrient stress, flooding, and other stressors that are predicted to become more pronounced in the future (Choudhury *et al.*, 2017; Noctor and

Mhamdi, 2017; Zhang and Sonnewald, 2017). The alarming rate at which global warming is currently progressing makes the development of many plants and crops with enhanced tolerance to stress combination a high priority in agriculture, especially considering the predicted future increase in global population and the decrease in resources such as good quality agricultural land and freshwater supply.

As highlighted by many recent studies, reviews, and special issues on the subject (e.g. Huber and Bauerle, 2016; Suzuki, 2016; Chaturvedi *et al.*, 2017; Choudhury *et al.*, 2017; Loudet and Hasegawa, 2017; Mao *et al.*, 2017; Zhang and Sonnewald, 2017; Chojak-Koźniewska *et al.*, 2018; Elferjani and Soolanayakanahally, 2018; Lawas *et al.*, 2018, 2019; Sehgal *et al.*, 2018, 2019; Zandalinas *et al.*, 2018; Zhang *et al.*, 2018; Yuan *et al.*, 2018; Balfagón *et al.*, 2019;Fábián *et al.*, 2019; Ibrahim *et al.*, 2019; Jumrani and Bhatia, 2019; Mahalingam and Bregitzer, 2019; Nieves-Cordones *et al.*, 2019; Rivero *et al.*, 2019;Trachsel *et al.*, 2019; Zhou *et al.*, 2019), the co-occurrence of two or more different stresses (be it abiotic and/or biotic) can pose a special challenge to plants. This challenge may be resolved by additive, subtractive, and/or combinatorial effects of different pathways, networks, and mechanisms that are activated by each of the different stresses, or by the activation of new and dedicated pathways and genes that are explicitly activated during the stress combination. Recent advances in using omics tools such as metabolomics, proteomics, and transcriptomics, coupled with machine learning and improved phenotyping and breeding methods, could make it possible to dissect, identify, and use different pathways and genes for the improvement of tolerance to stress combination in different crops. However, to understand the different challenges facing such endeavors we must first understand the physiological, developmental, and anatomical challenges plants may face during different types of stress combinations. For example, different stresses may require different stomatal responses to mitigate their impact and these could be conflicting (Fig. 1A). While previous studies have shown that a combination of drought (that requires stomatal closure to prevent water loss) and heat (that requires stomatal opening to cool leaves by transpiration) resulted in an overall response of stomatal closure (Rizhsky *et al.*, 2004), a new study has shown that during a combination of heat and high light stress (that requires stomata to close; Devireddy *et al.*, 2018), stomata remained open (Balfagón *et al.*, 2019). Changes in stomatal aperture are also a major player in responses to bacterial pathogens (require a closure response to prevent infection; Kollist *et al.*, 2019), and these could also be conflicting with responses to heat or high CO_2 levels (Fig. 1A). Because stomata play such as key role in regulating transpiration, leaf temperature, and photosynthesis (Sussmilch *et al.*, 2019), stomata represent an excellent example of converging pathways during stress combination. Future studies of stress combinations should therefore include a reference to, or a focus on, stomatal responses and their underlying mechanisms. Photosynthesis and respiration during stress combination are two other physiological processes that require attention, especially since both could be drastically affected by heat, or stress combinations that involve extreme temperatures as one of their components (Mittler, 2006). The effect of high light and heat stress was recently shown, for example, to result in enhanced photoinhibition that was coupled with the inability of plants to recover photosynthesis following recovery from the stress combination episode (Balfagón *et al.*, 2019). This phenomenon could be the result of high light damaging PSII, with heat stress simultaneously making it harder to repair the D1 protein of PSII (Balfagón *et al.*, 2019).

Among the many experimental tools available for us to dissect the response of plants to stress combination, transcriptomics is becoming more affordable and accessible. Pending the proper application of stresses and their combination, the right choice of tissues and cell types, and the coupling of RNA sampling with physiological, biochemical, and yield measurements, transcriptomics could become very useful in the analysis of plant responses to stress combination. Below we will discuss some of the recent advances in the study of stress combination by transcriptomics.

Transcriptomics of stress combination

The field of stress combination in plants has seen a recent increase in the number of transcriptomics studies attempting to dissect the response of plants to different types of abiotic and/or biotic stress combinations. These studies include stress combinations such as light and heat (Balfagón *et al.*, 2019), salinity, drought, and heat (Shaar-Moshe *et al.*, 2017), salinity and heat (Suzuki *et al.*, 2016), water stress combined with S deficiency (Henriet *et al.*, 2019), drought or flooding combined with herbivore attack (Nguyen *et al.*, 2016), drought and salinity (Osthoff *et al.*, 2019), drought and heat (Ashoub *et al.*, 2018; Liu *et al.*, 2018; Wang *et al.*, 2018), cold and drought (Zheng *et al.*, 2016), salinity and ozone (Natali *et al.*, 2018), drought and pathogen (Sinha *et al.*, 2017), high temperature and nitrogen (Yang *et al.*, 2015), and heat, drought, and virus infection (Prasch and Sonnewald, 2013). A variety of plants were used in these studies, including rice (*Oryza sativa*), soybean, pea (*Pisum sativum*), and Arabidopsis. In addition, different plant tissues such as roots, leaves, and reproductive tissues were used. In almost all of these studies, additive effects between the two different stresses were observed during stress combinations. In addition, in almost all of the studies listed above, as well as in the initial transcriptomics studies on drought and heat combination (Rizhsky *et al.*, 2004), the simultaneous application of two different stresses resulted in the altered expression of many transcripts that were unique to the stress combination and were not expressed when each of the different stresses was applied individually. These findings highlight the possible existence of dedicated pathways and genes that may have evolved to mitigate the effects of stress combination. Within this growing reservoir of transcriptomics data sets, several answers to key questions related to stress combination in plants may lie hidden. For example, how do different transcription factor (TF) families respond to stress combination? How do they regulate transcript expression during stress combination? To begin addressing some of these questions, we performed a meta-analysis of three transcriptomics studies conducted in our laboratory on stress combination, all including heat stress

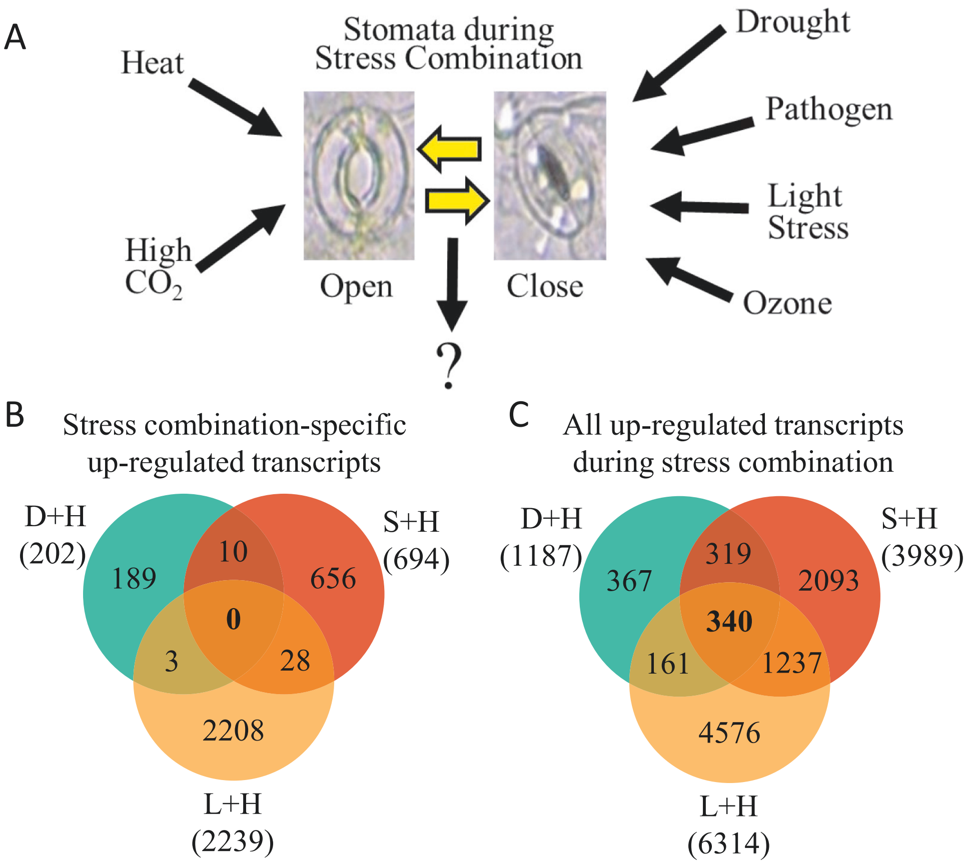

Fig. 1. Regulation of stomata during stress combination and overlap between abiotic stress-specific transcripts. (A) The opposing demands on stomatal aperture regulation during different types of stress combination. (B) Venn diagram showing the overlap between stress combination-specific transcripts up-regulated during different types of stress combination. (C). Same as (B) but for all transcripts up-regulated during different types of stress combination. Drought and heat (D+H); salinity and heat (S+H); and high light and heat stress (L+H). Data were obtained from Rizhsky *et al.* (2004), Suzuki *et al.* (2016), and Balfagón *et al.* (2019), and subjected to meta-analysis as described in Zandalinas *et al.* (2019).

as one of their components (Rizhsky *et al.*, 2004; Suzuki *et al.*, 2016; Balfagón *et al.*, 2019).

Unique transcriptomic responses to stress combination

To compare the transcriptomic response of plants to different stress combinations, we used three transcriptomic data sets generated in our laboratory over the years. These were drought and heat stress (Rizhsky *et al.*, 2004), salinity and heat stress (Suzuki *et al.*, 2016), and high light and heat stress (Balfagón *et al.*, 2019). Because all stress combinations included heat stress as one of their components, we expected some degree of overlap among the transcripts unique to each of the different stress combinations. Nonetheless, as shown in Fig. 1B, not even one transcript was found to be common among all three groups of stress combination-specific transcripts. This finding highlights the uniqueness of the plant response to stress combination and its dependency on the particular stress combination the plant experiences. Interestingly, several TF families, including heat shock factors (HSFs), MYBs, and ethylene response factors (ERFs), were represented in each of the different groups of stress combination-specific transcripts (Supplementary Fig. S1; Supplementary Table S1 at *JXB* online). Because transcriptional regulators such as HSFs, MYBs, and ERFs belong to large gene families in Arabidopsis, we examined whether the pattern of expression of these gene families during stress combination could shed new light on how the transcriptome of plants is modulated during stress combination.

Regulation of different TF families during stress combination

Transcriptional regulators and the transcriptional networks they control play a major role in plant acclimation to stress. To study the regulation of different TF families in Arabidopsis during different stress combinations, we expanded our meta-analysis to all transcripts up-regulated in plants during stress combination. As shown in Fig. 1C, an overlap of 340 transcripts was found among all transcripts up-regulated in response to each of the different stress combinations. This finding suggests that although none of the stress combination-specific responses had an overlap with the other (Fig. 1B), some responses to stress combination, that were not stress combination specific, did occur in response to all stress combinations (Fig. 1C; Supplementary Fig. S2). These included transcripts involved in responses to heat, salinity, and abscisic acid (ABA) (Supplementary Fig. S2). Similar to the stress combination-specific transcripts (Supplementary Fig. S1), TFs belonging to the HSF, MYB, and ERF families were represented among the 340 transcripts up-regulated in all stress combinations (Supplementary Table S2). We therefore examined the expression pattern of each of these families during stress combination, as well as comparing them with the WRKY TF family that is also proposed to be involved in stress combination (Bai *et al.*, 2018).

The HSF family of transcriptional regulators plays a canonical role in the response of plants to heat and other stresses by regulating both immediate responses and epigenetic control of heat stress memory (Jacob *et al.*, 2017; Ohama *et al.*, 2017; Zhu *et al.*, 2017; Duan *et al.*, 2019). It is composed of 21 genes in Arabidopsis and can function as a regulatory network under different conditions and in different tissues. As shown in Fig. 2A, out of the 16 HSFs included in our meta-analysis, two (HSFA2 and HSFB1) were up-regulated by all stress combinations. These two HSFs play a major role in orchestrating the HSF network and are known to have distinct roles in the regulation of heat stress responses in Arabidopsis (Ohama *et al.*, 2017). Interestingly, although many HSFs are up-regulated during heat stress, when heat is combined with other stresses the expression of some of these HSFs is suppressed. In addition, although the expression of some HSFs during each of the individual stresses is repeated when the two stresses are combined (e.g. HSFA1D during light, heat, and light combined with heat; Fig. 2B), in many cases the effect of the stress combination is not additive or similar between the two different individual stresses and their combination (e.g. HSFA4C during salinity, heat, and salinity combined with heat, or HSFA4A during drought, heat, and drought combined with heat). The HSF network could therefore be using an additive, subtractive, and/or combinatorial manner to regulate gene expression during stress combination.

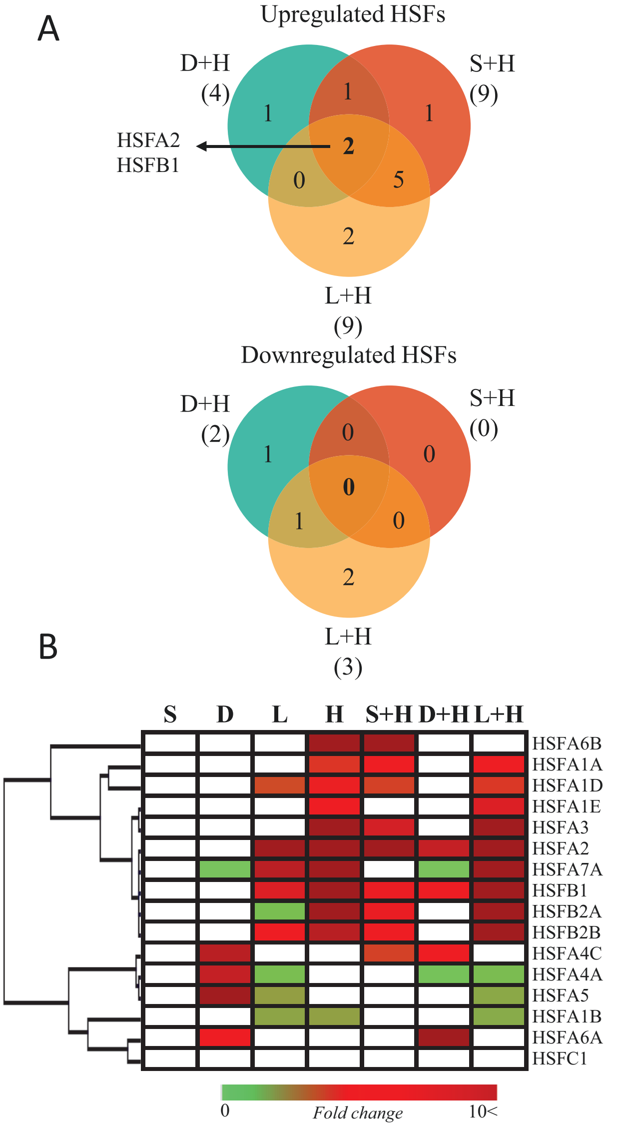

Fig. 2. Regulation of heat shock transcription factors (HSFs) during stress combination. (A) Venn diagrams showing overlap between HSFs expression (top, up-regulated; bottom, down-regulated) during the different abiotic stress combinations in Arabidopsis. Drought and heat (D+H); salinity and heat (S+H); and high light and heat stress (L+H). (B) Heat maps showing the response of the different HSFs in each stress condition and their combinations (relative to control). Data were obtained from Rizhsky *et al.* (2004), Suzuki *et al.* (2016), and Balfagón *et al.* (2019), and subjected to meta-analysis as described in Zandalinas *et al.* (2019).

In contrast to the HSF TF family, the MYB (Baldoni *et al.*, 2015; Li *et al.*, 2019; Millard *et al.*, 2019) and AP2-EREBP (that includes classical ERF TFs; Mizoi *et al.*, 2012; Agarwal *et al.*, 2017; Srivastava and Kumar, 2018; Takahashi *et al.*, 2018) TF families are much larger and thought to be involved in many more complex and different functions that include responses to stress and regulation of development (under stressful and non-stressful conditions). Analysis of the MYB family (Fig. 3) revealed that two MYBs (7 and 32) were up-regulated by all stress combinations, whereas two other MYBs (30 and 51) were suppressed by all stress combinations. MYB7 was previously found to negatively regulate ABA-induced inhibition of seed germination by blocking the expression of ABI5 (Kim *et al.*, 2015), and MYB32 is required for normal pollen development in Arabidopsis (Preston *et al.*, 2004). In contrast, MYB30 plays a key role in regulating reactive oxygen signaling, root cell elongation, and plant immune responses (Mabuchi *et al.*, 2018), and MYB51 regulates glucosinolate biosynthesis in Arabidopsis (Frerigmann and Gigolashvili, 2014). Although it is possible that regulating these pathways in a negative or a positive manner is involved in the response of plants to different stress combinations (similar to HSFs, MYBs can be activators or suppressors of gene expression), a much more inclusive view of the MYB network as a whole should be adopted when studying the response of plants to stress combination. When viewing the heat map for MYB expression (Fig. 3B), it is apparent that similar to HSFs, an additive, negative, and/or combinatorial manner of regulation is most probably used by MYBs to control transcript expression, acclimation, and development during stress combination.

Analysis of the AP2-EREBP family of TFs, that includes the DREB TFs important for heat and drought responses in plants (Takahashi *et al.*, 2018; Mizoi *et al.*, 2019), revealed that four different AP2-EREBP TFs (RAP2.10 and DREB 2A, 2B, and 2C) were up-regulated by all stress combinations (Fig. 4).

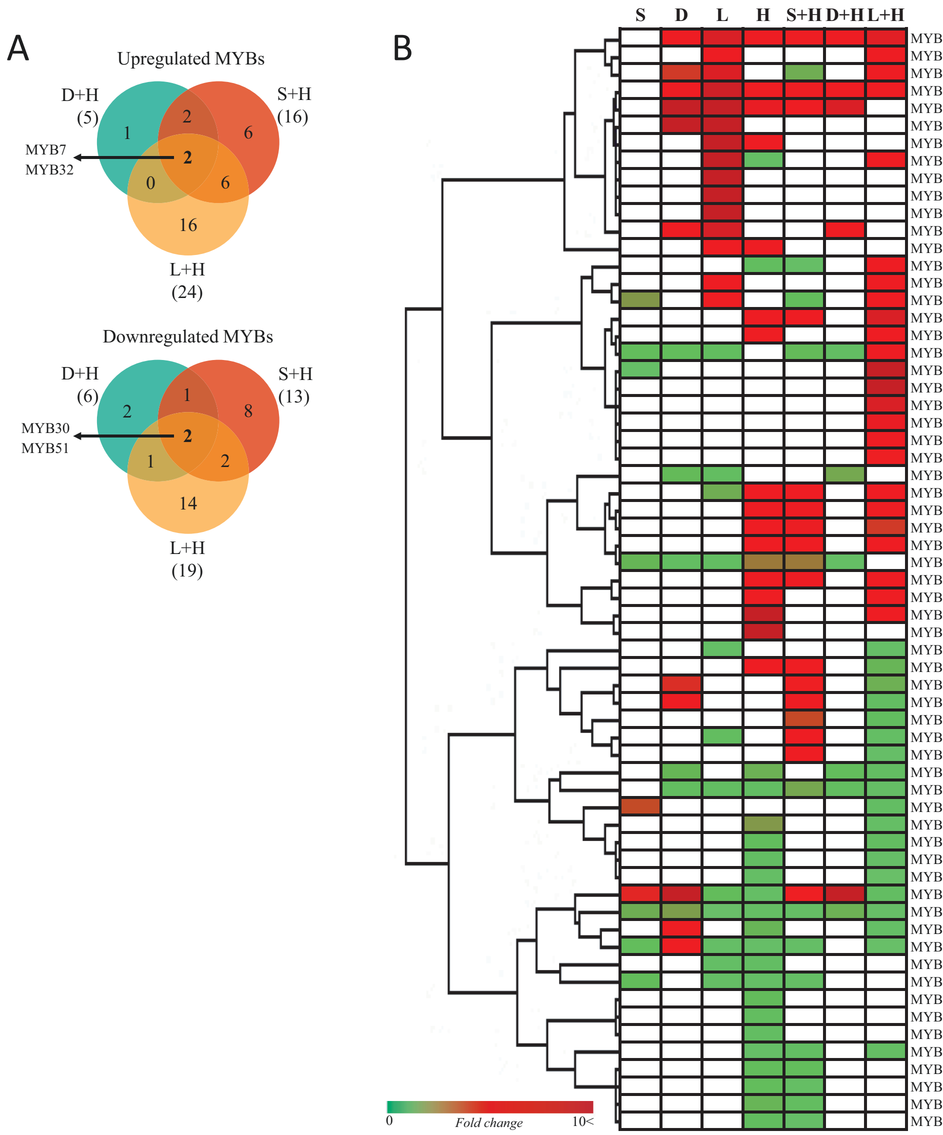

Fig. 3. Regulation of MYB transcription factors during stress combination. (A) Venn diagrams showing overlap between MYB expression (top, up-regulated; bottom, down-regulated) during the different abiotic stress combinations in Arabidopsis. Drought and heat (D+H); salinity and heat (S+H); and high light and heat stress (L+H). (B) Heat maps showing the response of the different MYBs in each stress condition and their combinations (relative to control). Data were obtained from Rizhsky *et al.* (2004), Suzuki *et al.* (2016), and Balfagón *et al.* (2019), and subjected to meta-analysis as described in Zandalinas *et al.* (2019).

In contrast, no AP2-EREBP TF was suppressed by all stress combinations. While not much is known about RAP2.10, DREB 2A, 2B, and 2C are known to regulate many responses to drought, salinity, and heat stresses (Takahashi *et al.*, 2018; Mizoi *et al.*, 2019). Similar to HSFs and MYBs, the pattern of AP2-EREBP expression during the different stresses (Fig. 4B) revealed an additive, negative, and/or combinatorial manner of regulation. In contrast to HSFs, MYBs, and AP2-EREBPs, no WRKY TF (Schluttenhofer and Yuan, 2015; Viana *et al.*, 2018) responded to all stress combinations, and the expression of the different WRKYs in response to the different stresses was not as extensive as that of the other TF families (Supplementary Fig. S3). It is therefore possible that WRKYs are mainly expressed using combinations of biotic and abiotic conditions (Bai *et al.*, 2018; as opposed to the abiotic stress combinations tested in Figs 1–4).

Concluding remarks and future perspectives

The unique physiological and developmental demands imposed on plants during periods of stress combination could be reflected in their molecular response to these conditions. Opposing demands for stomatal responses (Fig. 1A) and their effects on leaf temperature, respiration, transpiration, and photosynthesis could, for example, be driving novel transcriptomic responses. Indeed, our meta-analysis of the response of plants to heat and drought, salinity and heat, and high light and heat stress combinations revealed that each different stress combination resulted in a unique transcriptomic response that included stress combination-specific transcripts; and that the three groups of stress combination-specific transcripts had no overlap between them (Fig. 1B). In addition, the three different stress combinations

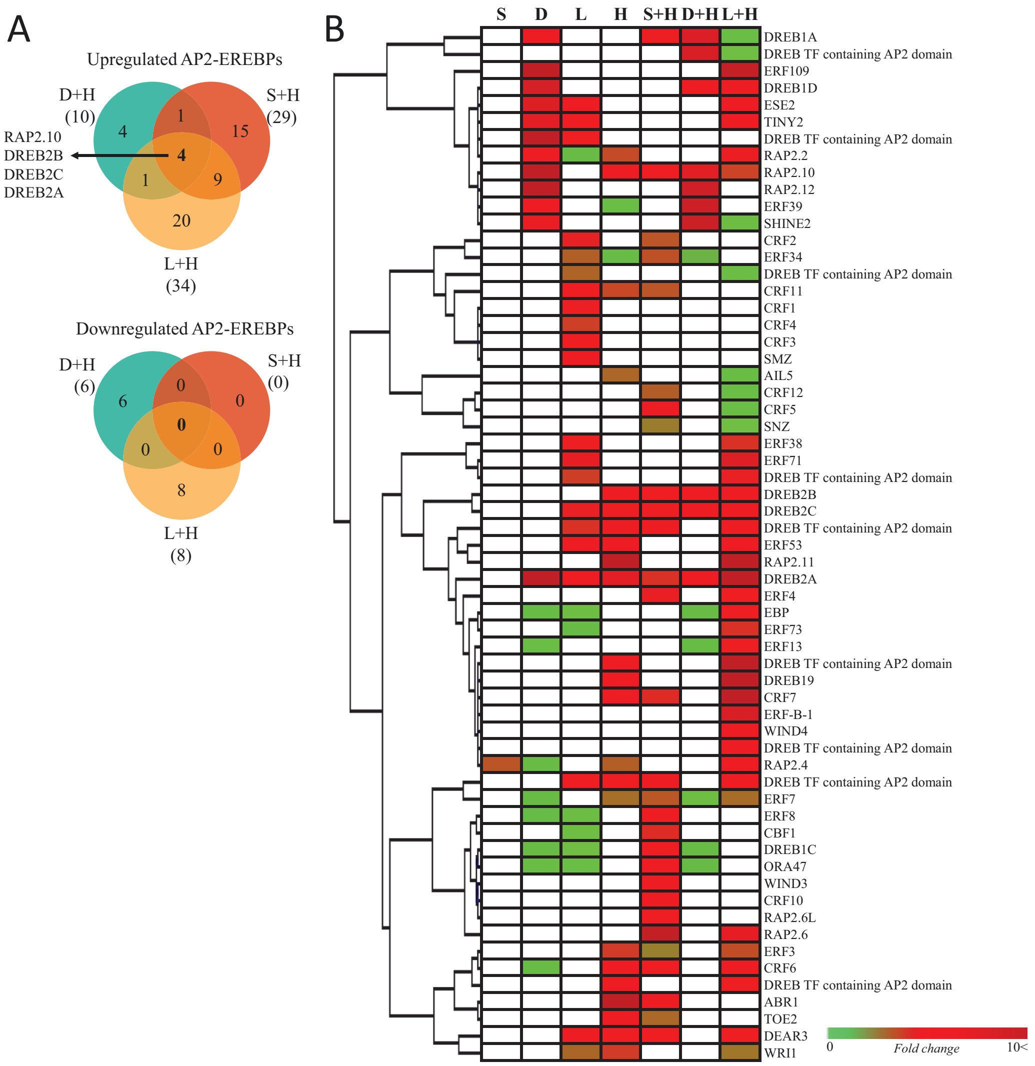

Fig. 4. Regulation of AP2-EREBP transcription factors during stress combination. (A) Venn diagrams showing overlap between AP2-EREBP expression (top, up-regulated; bottom, down-regulated) during the different abiotic stress combinations in Arabidopsis. Drought and heat (D+H); salinity and heat (S+H); and high light and heat stress (L+H). (B) Heat maps showing the response of the different AP2-EREBPs in each stress condition and their combinations (relative to control). Data were obtained from Rizhsky *et al.* (2004), Suzuki *et al.* (2016), and Balfagón *et al.* (2019), and subjected to meta-analysis as described in Zandalinas *et al.* (2019).

resulted in different combinatorial patterns of TF expression (i.e. a TF fingerprint unique to each stress combination; Figs 2–4; Supplementary Fig. S3), that could be mediating these unique transcriptomic responses. One possible conclusion that could be drawn based on these observations is that, because each stress combination is unique, attempting to generate crops with enhanced tolerance to stress combination would require a dedicated effort directed at each particular stress combination. No single 'magic bullet' might therefore be used to generate crops with enhanced tolerance to all different stress combinations.

The transcriptomic response of plants to the different stress combinations addressed in our meta-analysis could be regulated by unique TFs that are dedicated to each of the different stress combinations (Supplementary Table S1), by additive, subtractive, or combinatorial expression patterns of different groups of TFs (e.g. Figs 2, 3; Supplementary Fig. S3), generating a novel overall TF pattern of expression that is unique to the stress combination (a TF fingerprint unique to each stress combination), or by both of these mechanisms combined. Future studies are of course needed to decipher these TF regulatory networks and their regulated targets, and to learn how to use them in the development of crops with enhanced tolerance to stress combinations. Although, as discussed above, no one 'magic bullet' could be used for all different types of stress combination, at least when it comes to stress combinations that have one component in common (e.g. heat stress; Figs 2, 3), some candidates, for example MYB7 and MYB32 (Fig. 3), or HSFA2 (Fig. 1), could potentially be used in an attempt to enhance the tolerance of plants to different types of stress combinations that involve heat. Of course future studies are needed to address this hypothesis.

The three different studies used for our meta-analysis were all performed with leaf tissues of plants subjected to stress combination. Although all different stress combinations studied had a negative impact on plant growth and survival (Rizhsky *et al.*, 2004; Suzuki *et al.*, 2016; Balfagón *et al.*, 2019), the effect of these stress combinations on plant reproduction, and in particular the transcriptomic response of reproductive tissues to the different stress combinations, was not addressed in them. It is therefore possible that a different, as yet unknown, set of TF responses occurs in reproductive tissues, and that this response is more common or different than that of vegetative tissues. Because reproductive tissues are particularly sensitive to stress combination and are one of the major causes of crop loss (e.g. due to flower or seed abortion), studying the transcriptomic response of reproductive tissues to stress combination should be a major goal of future studies.

The alarming rate at which global average temperatures are increasing, coupled with predicted future weather events such as extensive periods of droughts, highlights the urgent need to develop many different crop plants with enhanced tolerance to stress combination. The uniqueness and complexity of the physiological and molecular response of plants to stress combination underline, however, the daunting challenge we face in accomplishing this goal. Dedicated efforts combining field experimentation, omics, and network analysis using machine learning, coupled with advanced phenotyping and breeding methods are therefore needed to address the many specific crops and particular stress combinations relevant to maintaining our food chain secured and viable worldwide. There is no lack of work waiting for us, so let's start working.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Overlap between transcripts encoding different stress combination-specific transcription factors (TFs), and representation of different transcriptional regulators within the different stress combination-specific transcript groups.

Fig. S2. Gene Ontology (GO) annotation of the 340 transcripts common to all stress combinations shown in Fig. 1C.

Fig. S3. Regulation of WRKY transcription factors during stress combination.

Fig. S4. Gene Ontology (GO) annotation of all transcripts up-regulated during the three different stress combinations shown in Fig. 1C.

Table S1. Transcription factors specifically up-regulated during different types of stress combinations.

Table S2. Transcripts up-regulated during all stress combinations analyzed.

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