## Tansley review

# Abiotic and biotic stress combinations

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## Summary

Environmental stress conditions such as drought, heat, salinity, cold, or pathogen infection can have a devastating impact on plant growth and yield under field conditions. Nevertheless, the effects of these stresses on plants are typically being studied under controlled growth conditions in the laboratory. The field environment is very different from the controlled conditions used in laboratory studies, and often involves the simultaneous exposure of plants to more than one abiotic and/or biotic stress condition, such as a combination of drought and heat, drought and cold, salinity and heat, or any of the major abiotic stresses combined with pathogen infection. Recent studies have revealed that the response of plants to combinations of two or more stress conditions is unique and cannot be directly extrapolated from the response of plants to each of the different stresses applied individually. Moreover, the simultaneous occurrence of different stresses results in a high degree of complexity in plant responses, as the responses to the combined stresses are largely controlled by different, and sometimes opposing, signaling pathways that may interact and inhibit each other. In this review, we will provide an update on recent studies focusing on the response of plants to a combination of different stresses. In particular, we will address how different stress responses are integrated and how they impact plant growth and physiological traits.

## I. Introduction

Owing to their sessile lifestyle, plants are continuously exposed to a broad range of environmental stresses. The main abiotic stresses that affect plants and crops in the field are being extensively studied (Cavanagh *et al.*, 2008; Munns & Tester, 2008; Chinnusamy & Zhu, 2009; Mittler & Blumwald, 2010). They include drought, salinity, heat, cold, chilling, freezing, nutrient, high light intensity, ozone ($O_3$) and anaerobic stresses (Wang *et al.*, 2003; Chaves & Oliveira, 2004; Agarwal & Grover, 2006; Nakashima & Yamaguchi-Shinozaki, 2006; Hirel *et al.*, 2007; Bailey-Serres & Voesenek, 2008). Nevertheless, field conditions are unlike the controlled conditions used in the laboratory. Under natural conditions, combinations of two or more stresses, such as drought and salinity, salinity and heat, and combinations of drought with extreme temperature or high light intensity are common to many

agricultural areas around the world and could impact crop productivity. A comparison of all major US weather disasters that exceeded a billion dollars each, between 1980 and 2012, indicates that a combination of drought and heat stress caused extensive agricultural losses of *c.* $200 billion. By contrast, over the same period, drought alone caused $50 billion worth of damage to agricultural production (Fig. 1a; http://www.ncdc.noaa.gov/billions/events). In addition, current climate prediction models indicate a gradual increase in ambient temperature, and an enhancement in the frequency and amplitude of heat stress in the near future (Ahuja *et al.*, 2010; Mittler & Blumwald, 2010; Mittler *et al.*, 2012; Li *et al.*, 2013). Moreover, high temperatures will be accompanied by other weather disasters, such as extended droughts, that could drastically impact crop production worldwide (IPCC, 2008). An urgent need to generate crops with enhanced tolerance to stress combinations therefore exists.

In addition to abiotic stresses, under natural conditions, plants face the threat of infection by pathogens (including bacteria, fungi, viruses and nematodes) and attack by herbivore pests (Atkinson & Urwin, 2012). The habitat range of pests and pathogens can be influenced by climate changes. For example, increasing temperatures are known to facilitate pathogen spread (Bale *et al.*, 2002;

Luck *et al.*, 2011; Madgwick *et al.*, 2011; Nicol *et al.*, 2011). Moreover, many abiotic stress conditions were shown to weaken the defense mechanisms of plants and enhanced their susceptibility to pathogen infection (Amtmann *et al.*, 2008; Goel *et al.*, 2008; Mittler & Blumwald, 2010; Atkinson & Urwin, 2012). Major crops growing in our future fields are therefore likely to be exposed to a greater range and number of abiotic and biotic conditions, as well as their combination.

Because different stresses are most likely to occur simultaneously under field conditions, a greater attempt must be made to mimic these conditions in laboratory studies (Mittler, 2006; Mittler & Blumwald, 2010; Atkinson & Urwin, 2012). In 2002 and 2004, it was revealed that the molecular response of plants to a combination of drought and heat stress is unique and cannot be directly extrapolated from the response of plants to drought or heat stress applied individually (Rizhsky *et al.*, 2002, 2004). Since then, many studies have uncovered the responses of plants to different combinations of stresses involving drought, salt, extreme temperature, heavy metals, UV-B, high light, $O_3$, $CO_2$, soil compaction and biotic stresses (Mittler, 2006; Mittler & Blumwald, 2010; Alameda *et al.*, 2012; Atkinson & Urwin, 2012; Kasurinen *et al.*, 2012; Srivastava *et al.*, 2012; Perez-Lopez *et al.*, 2013; Rivero *et al.*,

**Fig. 1** The effects of stress combination on agricultural production and plant growth. (a) Total cost of all US weather disasters exceeding US $1 billion each between 1980 and 2012 (excluding hurricanes, tornadoes, winter storms, and wildfires). A combination of drought and heat wave is shown to have caused more damage to agricultural production than drought, freezing, or flooding. Total damages were normalized to the 2013 US dollar value (http://www.ncdc.noaa.gov/billions/events). (b) The effects of drought, heat stress, and their combination on growth and development of maize and wheat. Drought primarily causes a decrease in plant height, spike number and grain weight. By contrast, heat stress causes an increase in aborted spikes (indicated by dark gray) and a decrease in grain numbers. In reproductive tissues, drought primarily impacts pistil development, whereas heat stress primarily impacts pollen development (indicated by dark gray). The different individual effects of drought and heat stress impact upon plants simultaneously when these stresses are combined. These general effects, described for maize and wheat, could be different depending on the intensity and duration of stresses and the plant species involved. Data summarized in (b) were obtained from Westgate (1994) and Prasad *et al.* (2011).
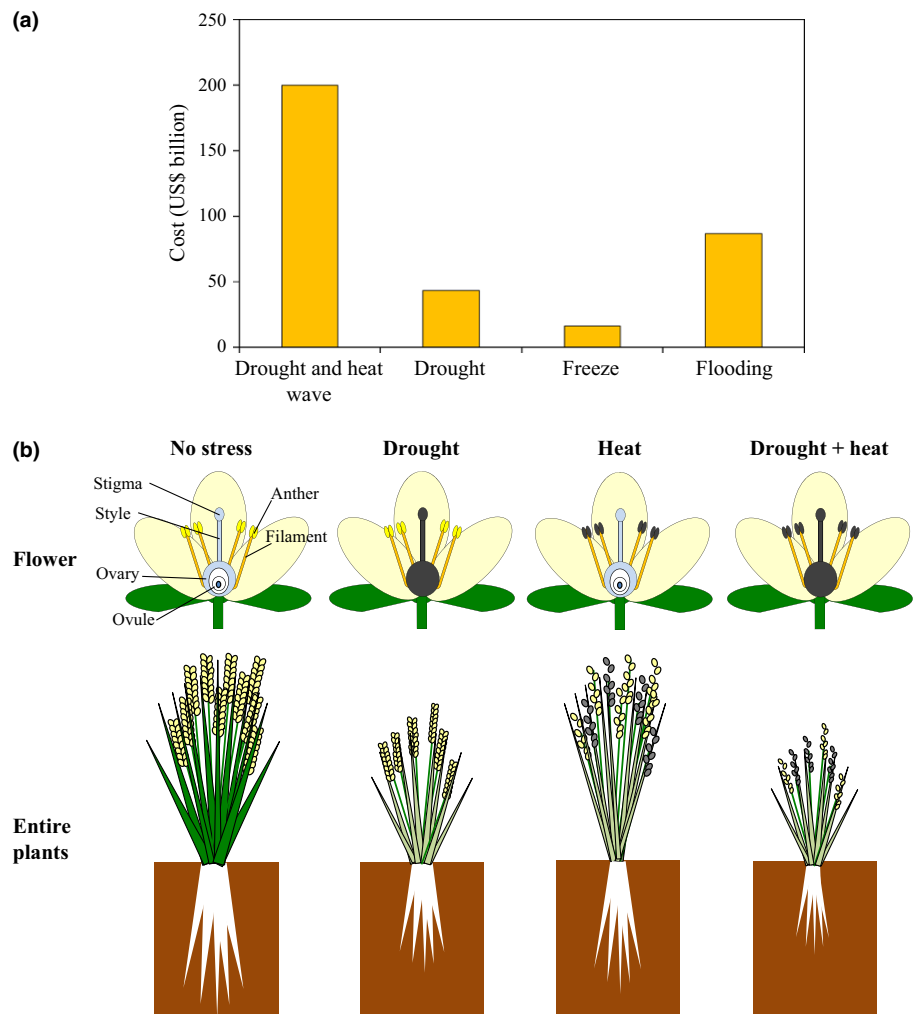
2013). These studies demonstrated that, despite a certain degree of overlap, each stress condition required a unique mechanism of response, tailored to the specific needs of the plant, and that each combination of two or more different stresses may also require a specific response. In addition, the simultaneous occurrence of different biotic and abiotic stresses was shown to result in a high degree of complexity in plant responses, as the responses to these combined stresses are largely controlled by different signaling pathways that may interact and inhibit one another (Mittler, 2006; Atkinson & Urwin, 2012; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013). Metabolic and signaling pathways involved in the response of plants to stress combination were found to include transcription factors, photosynthesis, antioxidant mechanisms, pathogen responses, hormone signaling ,and osmolyte synthesis (Rizhsky *et al.*, 2004; Koussevitzky *et al.*, 2008; Atkinson *et al.*, 2013; Iyer *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013). However, the majority of the mechanisms underlying the tolerance of plants to stress combinations are still unknown and further studies are required to address them.

This review provides an update on recent findings related to the growth and physiological responses of plants to abiotic and biotic stress combinations. In particular, we will attempt to address how different stress responses are combined and affect the growth and physiological traits of plants and crops. In addition, we will discuss recent studies employing systems biology analyses to uncover the complex mode of signaling pathways that underlie the response of plants to stress combinations.

## II. Effects of stress combination on growth, yield and physiological traits in plants and crops

The various effects of different stress combinations are summarized in Figs 1, 2 and Table 1. In this section, we will describe some of the negative and positive impacts of stress combination on plant growth, physiology, productivity, and yield (see also Mittler, 2006; Mittler & Blumwald, 2010).

### 1. Negative interactions of multiple stresses

The ability of plants to recognize and respond to specific stress combinations is particularly important when those individual stresses could elicit a negative effect on plant growth and reproduction. Climate change models predict that the occurrence and intensity of drought and heat waves will increase in the future and lead to a reduction in agricultural production (IPCC, 2007, 2008). High temperature and drought are perhaps the two most major environmental factors limiting crop growth and yield worldwide, and the combination of these stresses causes many physiological changes that affect crop yield and quality (Rizhsky *et al.*, 2002, 2004; Mittler, 2006; Prasad *et al.*, 2011; Vile *et al.*, 2012). Thus, to meet the global food demand for increasing future populations, it would be necessary to develop crops with enhanced tolerance to drought, heat stress, and their combination. Recent studies have characterized the effects of drought, heat, and their combination on the growth and physiological traits of different plants and crops (Fig. 1b). Vile *et al.* (2012) dissected the effects of
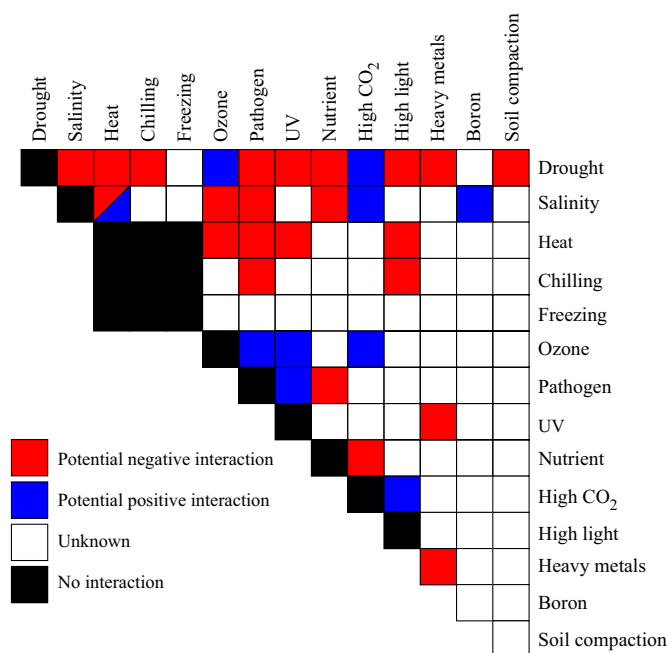


**Fig. 2** The stress matrix. Different combinations of potential environmental stresses that can affect crops in the field are shown in the form of a matrix. The matrix is color-coded to indicate stress combinations that were studied with a range of crops and their overall effect on plant growth and yield. References for the individual studies are given in the text and in Table 1.

drought, heat stress and a their combination on growth traits in Arabidopsis. Plant growth was significantly reduced under both stresses, and their combination resulted in even more detrimental effects. The combined effects of these two stresses were generally additive, suggesting a certain degree of independence between the mechanisms regulating the responses of plants to drought or heat stress. Indeed, some growth traits were altered specifically in response to one but not the other stress. For example, the proportion of root biomass increased in response to drought, whereas the proportion of reproductive tissues, hyponasty, and specific leaf area increased in response to heat stress. Different effects of drought or heat stress on growth traits were also observed in barley (Rollins *et al.*, 2013). Drought caused significant reductions in biomass, plant height, and spike numbers, but heat stress alone did not significantly affect these traits. By contrast, heat stress significantly increased the number of aborted spikes and decreased kernel weight, while drought did not have significant effects on these traits. Prasad *et al.* (2011) demonstrated detrimental effects of drought, heat stress, and their combination on crop yield in spring wheat. Drought or heat stress caused a significant decrease in grain number, spikelet fertility, grain yield and harvest index as well as Chl contents. The combined effects of these stresses were greater than the effects of drought or heat stress alone. Interestingly, reproductive tissues seem to be more sensitive than vegetative tissues to drought, heat stress, and their combination. Although both heat stress and drought can influence reproductive processes, these stresses affected reproductive traits differently (Westgate, 1994; Barnabas *et al.*, 2008). For example, in maize and spring wheat, heat stress decreases pollen fertility and grain

**Table 1** References showing the negative or positive interactions of stress combinations

|  | Stress combinations | References |
|---|---|---|
| Negative interaction | Drought + salinity | Ahmed *et al.* (2013) |
|  | Drought + heat | Rizhsky *et al.* (2002, 2004), Prasad *et al.* (2011), Vile *et al.* (2012) |
|  | Drought + chilling | Sales *et al.* (2013) |
|  | Drought + pathogen | Anderson *et al.* (2004), Prasch & Sonnewald (2013) |
|  | Drought + UV | Bandurska *et al.* (2013) |
|  | Drought + nutrient | Mittler & Blumwald (2010) |
|  | Drought + high light | Giraud *et al.* (2008) |
|  | Drought + heavy metals | de Silva *et al.* (2012) |
|  | Drought + soil compaction | Alameda *et al.* (2012) |
|  | Salinity + heat | Keleş & Öncel (2002), Wen *et al.* (2005) |
|  | Salinity + ozone | Mittler & Blumwald (2010) |
|  | Salinity + pathogen | Xiong & Yang (2003), Mittler & Blumwald (2010) |
|  | Salinity + nutrient | Mittler & Blumwald (2010) |
|  | Heat + ozone | Kasurinen *et al.* (2012) |
|  | Heat + pathogen | Zhu *et al.* (2010), Prasch & Sonnewald (2013) |
|  | Heat + UV | Mittler & Blumwald (2010) |
|  | Heat + high light | Hewezi *et al.* (2008), Mittler & Blumwald (2010) |
|  | Chilling + pathogen | Szittya *et al.* (2003) |
|  | Chilling + high light | Haghjou *et al.* (2009) |
|  | Pathogen + nutrient | Amtmann *et al.* (2008), Mittler & Blumwald (2010) |
|  | UV + Heavy metals | Srivastava *et al.* (2012) |
|  | Nutrient + high $CO_2$ | Mittler & Blumwald (2010) |
|  | Heavy metals + heavy metals | Cherif *et al.* (2011) |
| Positive interaction | Drought + ozone | Pääkkönen *et al.* (1998), Löw *et al.* (2006), Iyer *et al.* (2013) |
|  | Drought + high $CO_2$ | Brouder & Volenec (2008) |
|  | salinity + heat | Rivero *et al.* (2013) |
|  | Salinity + high $CO_2$ | Perez-Lopez *et al.* (2013) |
|  | Salinity + boron | del Carmen Martinez-Ballesta *et al.* (2008) |
|  | Ozone + pathogen | Bowler & Fluhr (2000), Mittler & Blumwald (2010) |
|  | Ozone + UV | Mittler & Blumwald (2010) |
|  | Ozone + high $CO_2$ | Ainsworth *et al.* (2008) |
|  | Pathogen + UV | Bowler & Fluhr (2000) |
|  | High $CO_2$ + high light | Perez-Lopez *et al.* (2013) |

number, whereas drought inhibits pistillate flower development, ovule functions, and grain weight (Westgate, 1994; Prasad *et al.*, 2011; Fig. 1b). Taken together, these findings suggest that different patterns of growth inhibition caused by drought and heat stress might occur simultaneously under the combination of these stresses, resulting in more severe damage to plant growth, and especially to reproductive tissues.

Drought, heat stress, and their combination cause alterations in physiological traits in plants. Photosynthesis was shown to be sensitive to drought or heat stress. Under drought conditions, stomatal closure imposed a limitation on photosynthesis by decreasing the availability of $CO_2$ (Chaves *et al.*, 2003). By contrast, heat stress inhibits photosynthesis mainly through alterations in nonstomatal traits, such as electron transport capacity and activity of Rubisco (Salvucci & Crafts-Brandner, 2004a,b; Way & Oren, 2010). In a recent study, photosynthetic performance under stress conditions was compared between different cotton cultivars that are tolerant or sensitive to drought (Carmo-Silva *et al.*, 2012). Photosynthetic rate and stomatal conductance decreased and leaf temperatures increased under heat stress and a combination of drought and heat stress, with a more severe impact under the combined stress condition. Drought-sensitive cultivars showed greater changes in these traits than drought-tolerant

cultivars. In addition, drought-sensitive cultivars showed lower activity of Rubisco compared with drought-tolerant cultivars under the stress combination. These results suggest that maintenance of photosynthetic activity is significant for the acclimation of plants to a combination of drought and heat stress, and both drought- and heat-induced limitations act simultaneously to inhibit photosynthesis under the combined stress condition in the field. The effects of drought, heat stress, and their combination on photosynthesis could, however, be different depending on plant species. In a recent study, photosynthetic activity was analyzed in European oaks subjected to drought, elevated daytime temperature, and their combination (Arend *et al.*, 2013). Elevated daytime temperature enhanced photosynthetic rate, whereas drought caused a decline in photosynthesis as well as stomatal conductance, maximum quantum efficiency of photosystem II (PSII) ($F_v/F_m$), and leaf water potential. The negative effects of drought on these physiological traits were exacerbated when drought was combined with elevated daytime temperature. These results suggest that European oaks may benefit from elevated temperature, but that negative effects can occur when elevated temperatures are combined with drought. Tobacco plants also showed relatively similar physiological responses to drought, heat stress, and their combination (Demirevska *et al.*, 2010). Drought induced a decrease in leaf

relative water content, photosynthesis rate and Chl content, and an increase in the accumulation of malondialdehyde and proline. Heat stress alone did not affect the plants significantly, but intensified the effect of drought when combined. In barley, drought or heat stresses reduced plant growth, with a more severe effect as a result of drought. The combination of drought and heat stress reduced plant growth to a much greater extent than drought or heat applied individually. By contrast, the effects of heat or drought and heat combination on photosynthetic rate were more severe than the effect of drought on this parameter (Rollins *et al.*, 2013).

The combination of drought and heat stress could require antagonistic responses of plants (Mittler & Blumwald, 2010). For example, during heat stress, plants increase their stomatal conductance in order to cool their leaves by transpiration. However, if the heat stress occurred simultaneously with drought, plants would not be able to open their stomata and their leaf temperature would be 2–5°C higher (Rizhsky *et al.*, 2002, 2004). In addition, proline that accumulated in plants under drought did not accumulate during a combination of drought and heat stress (Rizhsky *et al.*, 2004). Heat stress might ameliorate the toxicity of proline to cells, and, instead of proline, sucrose accumulated in plants subjected to the stress combination, perhaps to protect hyperactive and susceptible mitochondria from the build-up of potentially toxic pyrroline-5-carboxylate (Rizhsky *et al.*, 2004; Mittler, 2006). These findings suggest that during a combination of drought and heat stress, sucrose replaces proline in plants and functions as a major osmoprotectant. However, a recent study demonstrated the involvement of proline in the protection of plants against a combination of drought and heat stress (Cvikrova *et al.*, 2013). Growth and physiological traits were compared between wildtype (WT) and transgenic tobacco plants with high proline accumulation during drought, a combination of drought and heat stress, and recovery from these stresses. Transgenic plants showed higher relative water content and biomass during these stress treatments and the recovery period compared with WT plants. Drought induced an increase in proline accumulation, and heat stress at the end of the drought period caused a further increase in proline concentration in both genotypes. The rate at which the elevated proline concentration returned to normal during the recovery period was slower in the transgenic than in the WT plants. In addition, polyamine synthesis was inhibited under these stresses, but increased quickly during this recovery period. This increase in polyamine synthesis was more pronounced in transgenic plants than in WT plants. These results might suggest that proline plays an important role in the protection of tobacco plants against a combination of drought and heat stress by modulating polyamine biosynthesis. Unfortunately, the authors did not include a proper control for heat stress in the absence of drought, making it impossible to draw more definitive conclusions from this study.

Antioxidant mechanisms play an important role in the response of plants to a combination of drought and heat stress. Cytosolic ascorbate peroxidase 1 (APX1) protein was shown to accumulate during drought, heat stress, and their combination, and a null mutant of APX1 was more sensitive to this stress combination than WT plants (Koussevitzky *et al.*, 2008). In addition, transgenic tobacco plants expressing the cysteine protease inhibitor oryzacystatin I (OC-I) showed reduced accumulation of $H_2O_2$ and increased activity of glutathione peroxidase (GPX) under conditions of drought, heat stress and high light combinations, suggesting that expression of OC-I in tobacco results in the protection of the antioxidant enzyme GPX under this stress combination (Demirevska *et al.*, 2010).

In addition to a combination of drought and heat stress, other stress combinations can have a significantly higher detrimental effect than that caused by each of the stresses applied individually. For example, the effects of salinity stress could be exacerbated when combined with heat stress, because enhanced transpiration could result in enhanced uptake of salt (Keleş & Öncel, 2002; Wen *et al.*, 2005). High light intensity could prove problematic to plants subjected to drought or cold stress (Giraud *et al.*, 2008; Haghjou *et al.*, 2009). Under these conditions, the dark reactions are inhibited as a result of the low temperature or insufficient availability of $CO_2$, and the high photosynthetic energy absorbed by the plant (as a result of the high light intensities) enhances oxygen reduction and thus reactive oxygen species (ROS) production (Mittler, 2002; Mittler *et al.*, 2004). In addition, nutrient deficiency could pose a serious problem to plants when combined with other stresses, because energy and resources are required for the acclimation response in plants, and micronutrients are essential for the activation of many ROS scavenging enzymes (Mittler & Blumwald, 2010).

In a recent study, the effects of a combination of drought and salt stress were compared between wild and cultivated barley (Ahmed *et al.*, 2013). Either drought or salinity alone, and their combination significantly decreased plant growth, Chl content, photosynthetic rate, maximal photochemical efficiency of PSII ($F_v/F_m$), water potential and osmotic potential, with the largest suppression occurring under the stress combination. Wild barleys showed higher tolerance than cultivated barley to this stress combination. Higher stress tolerance of the wild barley might be attributable, at least partially, to a lower $Na^+ : K^+$ ratio, and enhanced osmoprotectant concentration and water-use efficiency. In addition, wild barley showed higher activities of ROS scavenging mechanisms such as superoxide dismutase (SOD), catalase (CAT), GPX, guaiacol peroxidase (POD), and glutathione reductase (GR) under a combination of drought and salt stress than did cultivated barley, with less accumulation of $H_2O_2$ and lipid peroxidation. Negative interaction of drought and salt stress was observed in wheat (Yousfi *et al.*, 2010). Biomass was positively related to nitrogen concentration as well as $K^+ : Na^+$ ratio under drought or salt stress; nevertheless, these correlations between growth and physiological parameters were weaker when drought was combined with salt stress, suggesting a complex mode of correlation between growth and physiological traits under this stress combination. In a recent study, growth and physiological traits were compared between drought-tolerant and drought-sensitive sugarcane cultivars under drought, cold stress, and their combination (Sales *et al.*, 2013). Reduction in biomass under drought conditions was observed in drought-sensitive cultivars, but not in drought-tolerant cultivars. The negative effect on drought-sensitive cultivars was exacerbated when drought was combined with cold stress. Under the stress combination, lipid peroxidation in the drought-sensitive cultivar

was significantly higher than that in the drought-tolerant cultivar. Activity of APX in the drought-tolerant cultivar increased under drought and the combined stress, and the highest activities were observed when the two stresses were combined. By contrast, activity of APX did not increase in the drought-sensitive cultivar under the same stress conditions. These findings support the emerging role of antioxidant mechanisms in the protection of plants during stress combinations.

Heavy metals were also reported to cause a higher detrimental effect on plant growth when combined with other abiotic stresses. Growth inhibition was observed in both the shoot and root of pea seedlings by nickel (Ni) or UV-B alone, and the combined stresses resulted in more severe damage (Srivastava *et al.*, 2012). Growth inhibition under a combination of Ni and UV-B was accompanied by a decrease in Chl content and photosynthetic activity. Both Ni and UV-B can cause damage to Chl and the photosynthetic apparatus, however, via a different mechanism. Magnesium (Mg) in the Chl molecule can be replaced with Ni (Küpper *et al.*, 1996), and Ni increases the degradation of Chl and the damage to the thylakoid membranes (Molas, 2002; Gajewska *et al.*, 2006). On the other hand, UV-B causes damage to the thylakoid lumen as well as alterations in mRNA turnover of the Chl*a/b*-binding proteins (Renger *et al.*, 1989; Jordan *et al.*, 1991). A combination of Ni and UV-B resulted in a reduction in carotenoid content (Srivastava *et al.*, 2012), and might also have detrimental effects on Chl concentration and photosynthetic apparatus. These findings suggest that the additive effects of Ni and UV-B on plant growth under the combined stress might be at least partially attributable to the different negative effects on Chl and thylakoid membranes caused by these stresses. Heavy metals were also found to aggravate the effects of drought on plant growth (de Silva *et al.*, 2012). Drought and different heavy metals (Ni, copper (Cu), cobalt (Co) and chromium (Cr)) reduced growth of red maple in an additive manner by altering xylem structure and hydraulic conductivity. Chl content decreased under heavy metal stress and a combination of drought and heavy metal stress, but not under drought. These findings indicated that maintenance of Chl concentration and photosynthetic activity might be important in the response of plants to combinations of heavy metals and other abiotic stresses. Moreover, high concentration of zinc (Zn) in combination with cadmium (Cd) resulted in enhanced oxidative stress, which was higher than that caused by high concentrations of Cd or Zn alone (Cherif *et al.*, 2011), suggesting that high Zn and Cd are synergistic in their effect on plant growth and oxidative stress. On the other hand, low concentration of Zn resulted in a decrease in oxidative stress and the restoration of Chl content. The effects of Zn on Cd toxicity in plants might be altered in a concentration-dependent manner.

Negative interactions of $O_3$ and heat stress were addressed in previous studies (Mittler, 2006; Mittler & Blumwald, 2010; Kasurinen *et al.*, 2012). In silver birch trees, high temperature increased biomass and delayed leaf senescence, whereas $O_3$ negatively affected these traits (Kasurinen *et al.*, 2012). In combination, $O_3$ partly cancelled temperature effects on leaf biomass. Changes in biomass might be the result, at least in part, of alterations in carbon allocation, because high temperature increased carbon fixation in the foliage and roots, whereas $O_3$ seemed to change tree carbon allocation, as it decreased foliar carbon amounts. These results suggest that high temperature has a potential to increase carbon accumulation and enhance silver birch growth, but allocation of the carbon is partly altered by a simultaneous $O_3$ stress.

Similar to other abiotic stresses, such as drought, salt and extreme temperature, soil compaction is commonly considered to have negative effects on plant growth and crop yield. A recent study demonstrated that the effects of soil compaction on tobacco plants were different depending on the occurrence of other stresses, such as shading, drought and mechanical stress (Alameda *et al.*, 2012). Drought and mechanical stress, but not shading stress, accelerated growth inhibition caused by soil compaction. Soil compaction reduced fine root proportion when combined with drought or mechanical stress, and the xylem area was reduced only under a combination of soil compaction and mechanical stress.

Negative interactions between biotic and abiotic stresses have also been addressed previously (Atkinson & Urwin, 2012). For example, both cold and heat stresses were found to lower the resistance of plants to biotic stresses. Cold stress was found to impair gene silencing, a potent plant defense against viral pathogens (Szittya *et al.*, 2003). In addition, it was recently demonstrated that the heat sensitivity of the N gene-mediated tobacco mosaic virus (TMV) resistance of tobacco is caused by heat-induced conformational changes in the R protein (Zhu *et al.*, 2010). Prasch & Sonnewald (2013) investigated the response of Arabidopsis to heat stress, drought, virus and their different combinations. A significant reduction in biomass was found in all single stress conditions, which was further exaggerated when the different stresses were combined. Extensive reduction in biomass was particularly observed under heat stress and a combination of virus and heat stress, as well as a combination of all three stresses that showed the lowest biomass. Stomata were closed during a combination of virus and drought, and virus and heat, as well as during the triple stress, while heat stress alone or virus infection resulted in stomata opening.

## 2. Positive interactions of multiple stresses

Some stress combinations might have beneficial effects on plants, when compared with each of the individual stresses applied separately. Drought stress, for example, would cause a reduction in stomatal conductance, thereby enhancing the tolerance of plants to $O_3$ stress (Pääkkönen *et al.*, 1998; Löw *et al.*, 2006). In a recent study, the response of *Medicago* to $O_3$, drought, and their combination was analyzed (Iyer *et al.*, 2013). Short-term $O_3$ stress resulted in chlorosis and small necrotic lesions, whereas drought led to wilting and collapse of entire leaves as well as chlorosis. Interestingly, the effects caused by $O_3$ or drought alone were dramatically cancelled when these stresses were combined. Lowered stomatal conductance might be an important mechanism to overcome the combined effects of $O_3$ and drought. In addition, ROS concentrations enhanced by drought or $O_3$ alone were decreased under the stress combination to a value comparable to the controlled condition. Reduction in ROS concentration under the

stress combination was accompanied by an increase in reduced ascorbate and glutathione.

In contrast to the combined effects of drought and heat stress on plant growth, a combination of salt and heat stress provides a significant degree of protection to tomato plants against the effects of salt stress alone (Rivero *et al.*, 2013). Accumulation of glycine betaine and trehalose might be important for the specific response of plants to this stress combination. The accumulation of these compounds under the stress combination was linked to the maintenance of a lower $Na^+ : K^+$ ratio, with a better performance of the cell water status and photosynthesis compared with salt stress alone. Previous studies demonstrated the role of glycine betaine in the protection of PSII against photo- and heat-induced inactivation (Allakhverdiev *et al.*, 2003), and inhibition of salt-induced $K^+$ efflux (Cuin & Shabala, 2005). In addition, trehalose was also shown to be involved in the maintenance of photosynthesis capacity and PSII efficiency (Lunn, 2007). Moreover, these compounds play important roles in the protection of cells against oxidative stress (Garg *et al.*, 2002; Chen & Murata, 2008). Indeed, $H_2O_2$ accumulation and protein oxidation were inhibited under a combination of salt and heat stress, suggesting that protection of tomato plants against this stress combination might be at least partially attributable to the inhibition of oxidative stress.

Boron (B) was also shown to inhibit the negative effects of salt stress (del Carmen Martinez-Ballesta *et al.*, 2008; del Carmen Rodríguez-Hernández *et al.*, 2013). Antagonism between boron and salt might lead to inhibition in their relative toxicity. $Na^+$ accumulation in leaves decreased with the addition of B to the soil, probably as a result of the inhibition in root growth caused by the B treatment (del Carmen Martinez-Ballesta *et al.*, 2008). On the other hand, reduction in B accumulation in leaves in the presence of salt has been reported in broccoli, tomato, jack pine, and grapevines (del Carmen Martinez-Ballesta *et al.*, 2008). It could result from the reduction in transpiration as a consequence of the osmotic effects that inhibit transport of boron via xylem. In addition, a recent study suggested that B at the plasma membrane enhanced the tolerance of plants to salt stress by altering root hydraulic conductivity through the function of aquaporin (del Carmen Rodríguez-Hernández *et al.*, 2013).

Elevated $CO_2$ concentrations might also be considered as beneficial when combined with different stresses. High $CO_2$ decreases stomatal conductance and inhibits diffusion of $O_3$ into leaves (Ainsworth *et al.*, 2008), as well as decreasing transpiration rate and improving water-use efficiency (Brouder & Volenec, 2008). In addition, high $CO_2$ was shown to increase the biomass of lettuce when combined with salt or high light (Perez-Lopez *et al.*, 2013). Under the combined stress conditions, increase in biomass was accompanied by enhanced water-use efficiency and antioxidant capacity.

## III. The complexity of stress response signaling during stress combination

When plants are exposed to a combination of stresses, their response to each of the individual stresses comprising the stress combination must be modulated to take into consideration all other factors imposed on the plant by the other individual stresses in the combination. In addition, novel mechanisms activated only during the stress combination were reported to be found during stress combination (Schenke *et al.*, 2011; Atkinson *et al.*, 2013; Prasch & Sonnewald, 2013). Systems biology analyses revealed a complex mode of integration of the different signaling pathways triggered in plants during stress combination (Rizhsky *et al.*, 2004; Atkinson *et al.*, 2013; Iyer *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013). The transcriptome analysis of plants subjected to drought and heat combination agrees with the physiological and metabolic analyses of this stress combination and suggests that it requires a unique acclimation response involving over 770 transcripts that are not altered by drought or heat stress (Rizhsky *et al.*, 2004). Similar changes in metabolite and protein accumulation were also found, with several unique metabolites and at least 53 different proteins accumulating specifically during the stress combination (Rizhsky *et al.*, 2004; Koussevitzky *et al.*, 2008). In addition, at least one protein, cytosolic APX1, was found to be specifically required for the tolerance of Arabidopsis plants to the combination of drought and heat stress (Koussevitzky *et al.*, 2008). A transcriptome analysis of sunflower plants subjected to a combination of heat and high light stress supported the results obtained from the analysis of Arabidopsis plants exposed to drought and heat combination, and identified a large number of transcripts that specifically responded to this combination (Hewezi *et al.*, 2008).

More recently, responses of plants to multiple stress exposures (cold, heat, high light, salt, and pathogen infection) were studied in combination or individually using a large-scale microarray analysis (Rasmussen *et al.*, 2013). This analysis identified different patterns of transcript responses. Approximately 25% of transcripts showed similar responses to the different stresses applied individually, but had a different response to their combination. These transcripts are associated with defense mechanisms such as systemic acquired resistance, programmed cell death, and salicylate biosynthesis. The highest proportion of transcripts (29%) responded differently to the different stresses applied individually, with their expressions returning to control levels in response to the combined stress. These transcripts are primarily associated with the regulation of secondary metabolism, such as IAA and phenylpropanoids (including anthocyanin), and growth regulation involving ethylene and auxin signaling. This may indicate that different stresses activate different secondary metabolic pathways and differentially affect growth via auxin and ethylene signaling. In addition, IAA is known to influence phenylpropanoid biosynthesis, and the link between auxin signaling and anthocyanin synthesis was addressed in a previous study (Liu *et al.*, 2013), suggesting that auxin might play a key role in the regulation of secondary metabolism. On the other hand, only 7% of transcripts responded differently to individual stresses and remained at one of these levels in response to their combination (Rasmussen *et al.*, 2013). Importantly, > 60% of the transcripts belonged to one of the response patterns already described, suggesting that a large proportion of the plant transcriptional changes in response to stress combination cannot be predicted from the plants' responses to the different stresses applied individually. Approximately 28% of transcripts were

regulated by either of the individual stresses, and their regulation was unaffected by the stress combination. These transcripts are associated with the thylakoid membrane organization and the response of plants to high light stress, suggesting that pathways regulated by these transcripts involve the maintenance of photosynthetic machinery.

Transcriptome analysis of Arabidopsis plants subjected to drought, heat stress, virus infection and double or triple combinations of these stresses revealed that the expression pattern of transcripts under stress combination cannot be predicted from that under each of the single stress conditions applied individually (Prasch & Sonnewald, 2013). Expression of 11 transcripts was found to be altered under all the different single, double or triple stress conditions. Among these transcripts, G-BOX BINDING FACTOR3 (GBF3) was strongly up-regulated, whereas Rap2.9 was strongly down-regulated under all stress conditions. GBF3 was shown to be involved in ABA signaling and is responsive to multiple stress conditions (Fujita *et al.*, 2005). Rap2.9 is known to be a homolog of DEAR1 that functions as a transcriptional repressor (Magnani *et al.*, 2004; Tsutsui *et al.*, 2009). DEAR1 was shown to inhibit transcription of cold response genes, and overexpression of DEAR 1 resulted in higher sensitivity of transgenic plants to cold stress, accompanied by decreased abundance of cold response transcripts (Tsutsui *et al.*, 2009). Rap2.9 might function as a transcriptional repressor that is expressed under normal conditions to keep stress responses under tight control, but which is down-regulated to avoid further inhibition of stress response genes under stress conditions. Twenty-three transcripts were specifically up-regulated under the combination of all three stresses (Prasch & Sonnewald, 2013). These transcripts include DREB2A and two zinc finger proteins, as well as other transcripts associated with stress responses. In addition, a combination of all three stresses inhibited the expression of transcripts involved in the R-mediated disease response, but enhanced the expression of transcripts associated with the heat stress response. These responses in transcript expression were not observed under each of the individual stresses or the different combinations of two different stresses. These results suggest that abiotic stress factors significantly altered pathogen-related signaling networks, which could lead to the deactivation of defense responses and the higher susceptibility of plants.

Another transcriptome analysis was recently performed using Arabidopsis plants subjected to drought, nematode infection, and their combination (Atkinson *et al.*, 2013). The majority of transcripts regulated by the stress combination were also regulated by drought alone, but not by nematode stress alone, suggesting that plant responses to this stress combination might prioritize the potentially more damaging abiotic stress when drought and nematode infection are combined. Nevertheless, a unique set of transcripts was altered in response to the combination of drought and nematode infection. These include RAPID ALKALINIZATION FACTOR-LIKE8 (AtRALFL8), METHIONINE GAMMA LYASE (AtMGL), and AZELAIC ACID INDUCED1 (AZI1). AtRALFL8, which was induced in roots by the combined stress conditions, might produce signal peptides to induce cell wall remodeling (Joshi *et al.*, 2010). The methionine homeostasis gene AtMGL was up-regulated by the combined stress conditions in

leaves. It may regulate methionine metabolism involved in osmolyte synthesis and signaling under multiple stress conditions (Pearce *et al.*, 2001). In addition, AZI1, involved in systemic acquired resistance (Jung *et al.*, 2009), was down-regulated in leaves as part of an ABA-induced repression of pathogen response genes (Yasuda *et al.*, 2008). Although expression of AtMGK enhanced the tolerance of transgenic plants to nematodes, AtRALFL8 and AZI1 conferred susceptibility to drought stress and nematode infection when overexpressed. These results highlight the complex nature of stress combination.

When plant cells were exposed to a combination of pathogen and UV-B, signals regulating pathogen defense were significantly activated (Schenke *et al.*, 2011). Application of the pathogen elicitor flg22 and its combination with UV-B induced the production of defense-related compounds, such as the phytoalexins, camalexin, and scopoletin, as well as of lignin, a structural barrier thought to restrict pathogen spread. By contrast, production of UV-protective flavonols, induced by UV-B, was attenuated by the simultaneous application of flg22. It appears that this crosstalk involved antagonistic regulation of two opposing MYB transcription factors, the positive regulator of the flavonol pathway MYB12 and the negative regulator MYB4.

Transcriptome analysis of drought, $O_3$, and their combination revealed specificity in signal transduction in the response of *Medicago* to the single or combined stresses (Iyer *et al.*, 2013). In response to drought, transcripts involved in ABA signaling, proline biosynthesis, and response to heat, high light and oxidative stress were specifically up-regulated. By contrast, $O_3$ specifically induced transcripts associated with phenylalanine ammonia-lyase biosynthesis, and glucose, sucrose and glucan metabolism. Transcripts involved in jasmonic acid (JA) signaling and innate immunity were specifically up-regulated under a combination of these stresses. Interestingly, transcription factors such as WRKY and MYC3 were uniquely up-regulated under the stress combination. WRKYs are known to be responsive to JA, biotic and abiotic stresses and stress combinations (Rizhsky *et al.*, 2002; Qiu & Yu, 2009; Gao *et al.*, 2011; Peng *et al.*, 2011). MYC3 was found to be a repressor of JAZ1 and JAZ2, which are involved in JA signaling (Figueroa & Browse, 2012; Kazan & Manners, 2012).

## IV. Conclusions

The major abiotic stresses that affect plants and crops in the field are being extensively studied in the laboratory in an individual manner. By contrast, the study of different stress combinations that mimic the field environment in a much more realistic manner has only recently been attempted by several different groups. Fig. 3 summarizes some of the key differences between the field environment and the laboratory and describes the flow of information and resources that are currently being used to develop plants and crops with enhanced tolerance to biotic or abiotic stresses. The extent of damage caused to agriculture by different stress combinations (Fig. 1a; Mittler, 2006; Mittler & Blumwald, 2010), conditions that are rarely studied in the laboratory, underscores the need to include the study of stress combination in the laboratory phase in order to develop crops with enhanced
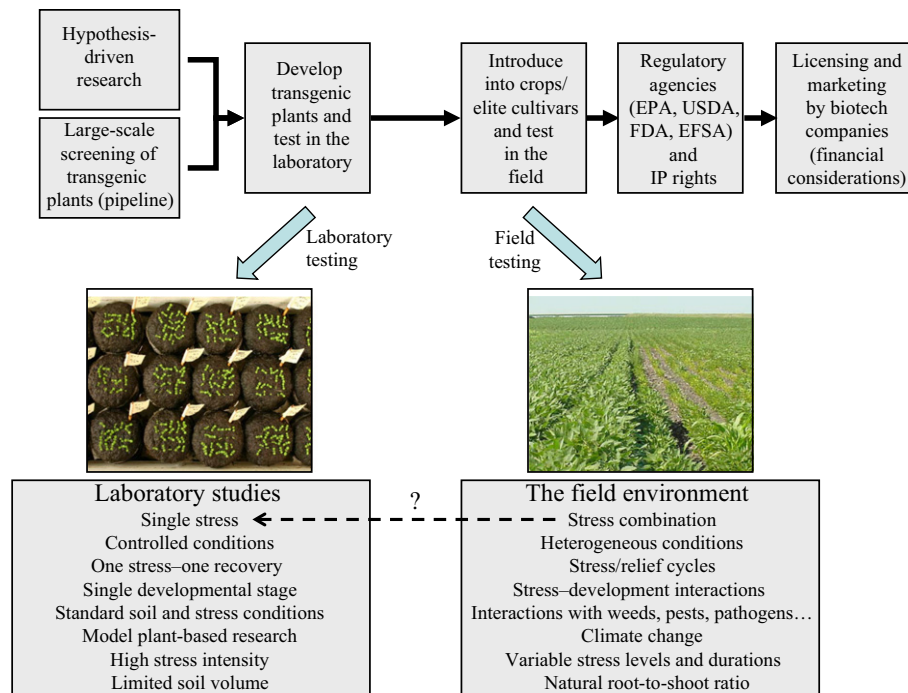
**Fig. 3** A general overview of translational research in the field of environmental stress tolerance in the form of a flow chart asks the question: should we move the stress combination concept from field studies to the laboratory? The chart contrasts the conditions used to study environmental stress in the laboratory using Arabidopsis or other model plants and those that prevail in the field environment. Parameters that influence plant growth in the field are shown to include stress combination; heterogeneous conditions within the same field plot; multiple stress and relief cycles; interactions between different developmental programs and abiotic stress programs; interactions with weeds, insects, and pathogens, such as viruses, bacteria, nematodes and fungi; extended periods of stress caused by climate change; variations in the type and intensity of stresses; and differences that result from growth parameters that are achieved in the field but not in the laboratory (e.g. a natural root-to-shoot ratio that may not be achieved in the laboratory). Data acquisition, model development, and selection of genes for large-scale testing in transgenic crops could therefore be suffering from a bias towards laboratory conditions, resulting in the production of transgenic crops that perform poorly in the field. In the opinion of the authors, a greater attempt should be made to use stress combination conditions in the laboratory phase so that specific molecular pathways that can enhance plant tolerance in the field would be identified (dashed arrow). EFSA, European Food Safety Authority; EPA, Environmental Protection Agency; FDA, Food and Drug Administration; IP, intellectual property; USDA, United States Department of Agriculture.

tolerance to these combinations. Drawing upon the limited physiological, molecular, and metabolic studies performed with plants simultaneously subjected to two or more distinct stresses, it is not sufficient to study each of the individual stresses separately (Keleş & Öncel, 2002; Rizhsky *et al.*, 2002, 2004; Hewezi *et al.*, 2008; Koussevitzky *et al.*, 2008). Different stress combinations should therefore be handled as a new state of stress in plants that requires novel types of defense and acclimation responses.

What stress combinations should we study? Fig. 2 summarizes many of the stress combinations that could have a significant impact on agricultural production (Fig. 2, the 'stress matrix'; taken from Mittler, 2006 and Mittler & Blumwald, 2010 and updated with new information; Table 1). Stress interactions that have a deleterious effect on crop productivity include drought and heat, $O_3$ and salinity, $O_3$ and heat, nutrient stress and drought, nutrient stress and salinity, nutrient stress and high $CO_2$, UV and heat, UV and drought, and high light intensity combined with heat, drought, or chilling. These deleterious stress combinations have been addressed in previous reviews (Mittler, 2006; Mittler & Blumwald, 2010; Bandurska *et al.*, 2013). Recent studies have revealed additional stress combinations that cause negative effects when applied to crops. These include drought and chilling, drought and salt, heavy metal combined with drought, UV-B and different types of heavy metals (Cd and Zn), and drought combined with soil

compaction (Cherif *et al.*, 2011; Alameda *et al.*, 2012; de Silva *et al.*, 2012; Srivastava *et al.*, 2012; Ahmed *et al.*, 2013; Sales *et al.*, 2013).

Perhaps the most studied interactions presented in Fig. 2 are those between different abiotic stresses and pests or pathogens (i.e. biotic stress; Atkinson & Urwin, 2012). In some instances, it was reported that a specific abiotic stress enhanced the resistance of plants to biotic stress (Bowler & Fluhr, 2000; Park *et al.*, 2001; Sandermann, 2004; Rouhier & Jacquot, 2008; Carter *et al.*, 2009). However, in most cases, prolonged exposure of plants to abiotic stresses, such as drought, extreme temperature, nutrient stress, or salinity, resulted in the weakening of plant defenses and enhanced susceptibility to biotic stresses (Szittya *et al.*, 2003; Xiong & Yang, 2003; Anderson *et al.*, 2004; Grodzki *et al.*, 2004; Sandermann, 2004; Amtmann *et al.*, 2008; Mittler & Blumwald, 2010; Zhu *et al.*, 2010). In addition, recent studies have revealed that, in some combinations of biotic and abiotic stresses, plants prioritize their responses to address one of the individual stresses involved in the stress combination (Schenke *et al.*, 2011; Atkinson *et al.*, 2013).

Environmental interactions that do not have a deleterious effect on yield, and that could actually have a beneficial impact on each other's effects, include drought and $O_3$, $O_3$ and UV, salt and heat, B and salt, and high $CO_2$ combined with drought, salt, $O_3$ or high

light (Pääkkönen *et al.*, 1998; Ainsworth *et al.*, 2008; Brouder & Volenec, 2008; del Carmen Martinez-Ballesta *et al.*, 2008; Wilkinson & Davies, 2009; Mittler & Blumwald, 2010; Perez-Lopez *et al.*, 2013; Rivero *et al.*, 2013). Although heat stress has been considered to ameliorate the effects of salt stress, a recent study demonstrated that the detrimental effects on tomato plants caused by salt stress were partially counteracted by heat stress when these stresses were combined (Rivero *et al.*, 2013). Our unpublished results have demonstrated that, in Arabidopsis, the combination of salt and heat stress is as lethal as that of drought and heat stress. These conflicting results suggest that the positive or negative effects of a particular stress combination could be dependent on the particular plant genotype, species, and/or timing and intensity of the different stresses involved.

The majority of stress combinations studied to date have a negative effect on crop yield under field conditions, suggesting that there is an urgent need to produce crops with enhanced tolerance to stress combinations. The higher sensitivity of reproductive tissues, as compared with vegetative tissues, to a combination of drought and heat stress (Prasad *et al.*, 2011) further underlines the detrimental effects of this stress combination on the yield of important crops such as grains and soybean in the field. Studying the response of reproductive tissues to stress combinations is therefore a high priority in the development of crops that will perform better in our current and future fields.

One of the key pathways that appears to underlie the tolerance of plants to stress combinations is the antioxidant defense machinery. Recent studies demonstrated the association of higher antioxidant capacity or lower ROS accumulation with tolerance of plants to stress combinations (Demirevska *et al.*, 2010; Ahmed *et al.*, 2013; Iyer *et al.*, 2013; Perez-Lopez *et al.*, 2013; Rivero *et al.*, 2013; Sales *et al.*, 2013). Accordingly, cytosolic APX1 was found to be required for the tolerance of plants to a combination of drought and heat stress (Koussevitzky *et al.*, 2008). The importance of carbon metabolism could also be deduced from the beneficial effects of high $CO_2$ on the response of plants to stress combination. Transcriptome, metabolome, and proteome analyses of plants subjected to different stress combinations revealed several different signaling pathways that are specifically activated by these combinations (Rizhsky *et al.*, 2004; Koussevitzky *et al.*, 2008; Atkinson *et al.*, 2013; Iyer *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013). These pathways include different transcription factors, defense responses, hormone signals and osmolyte synthesis. The response of plants to different stress combinations might therefore be regulated by the coordination of these different pathways and signals. Our knowledge of the molecular and biochemical mechanisms that regulate the response of plants to stress combinations is still very limited and further studies are required to address these mechanisms.

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