Responses of plants to climate change: Metabolic changes during abiotic stress combination in plants

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Highlight

The specific and complex metabolic responses of plants to a combination of different abiotic stresses, that is predicted to worsen with climate change, is reviewed.

Abstract

Climate change is predicted to increase the frequency and intensity of abiotic stress combinations that negatively impact plants and pose a serious threat to crop yield and food supply. Plants respond to episodes of stress combination by activating specific physiological and molecular responses, as well as by adjusting different metabolic pathways, to mitigate the negative effects of stress combination on plant growth, development, and reproduction. Plants synthesize a wide range of metabolites that regulate many aspects of plant growth and development, as well as plant responses to stress. Although metabolic responses to individual abiotic stresses have been studied extensively in different plant species, recent efforts have been directed at understanding metabolic responses that occur when different abiotic factors are combined. In this review we examine recent studies of metabolomic changes under stress combination in different plants and suggest new avenues for the development of stress combination-resilient crops based on metabolites as breeding targets.

Abbreviations

ABA, abscisic acid; ABR1, ABA Repressor 1; APX1, Ascorbate Peroxidase 1; ET, ethylene; GABA, γ-aminobutyric acid; JA, jasmonic acid; MBF1c, Multiprotein Bridging Factor 1c; RD26, Responsive to Desiccation 26; ROS, reactive oxygen species; SA, salicylic acid; TCA, tricarboxylic acid.

Introduction

Plant growth, yield and survival are highly affected by global warming and climate change that cause a constant increase in global atmospheric CO_2 levels, as well as the intensity and frequency of different abiotic stresses including drought, extreme temperatures, and flooding; resulting in many cases in a multifactorial stress combination phenomenon (Steg, 2018; Masson-Delmotte *et al.*, 2021; Zandalinas *et al.*, 2021*b,a*; von der Gathen *et al.*, 2021; Houtan *et al.*, 2021; Rivero *et al.*, 2022). The predicted increase in soil-associated stresses such as salinity, inorganic nutrient imbalances or heavy metals, alongside biotic stresses, further adds to the complexity of the future plant environment and make it difficult to forecast how plants will respond to the challenges posed by the interactions of multiple stresses. In addition to the activation of specific physiological and molecular responses to stress, plants adjust several metabolic pathways to mitigate the effects of stress on growth and development and to adapt to new energetic demands imposed by different climatic and environmental scenarios (Dusenge *et al.*, 2019; Fernie *et al.*, 2020). The efficiency of reconfiguring metabolic networks, resuming active growth and seed production, and restoring a new state of homeostasis after stress release will further determine how successful a plant is in acclimating to stress (Shulaev *et al.*, 2008; Dusenge *et al.*, 2019).

Plants produce a wide range of different metabolites in a spatiotemporal- and/or environment-dependent manner (Fang *et al.*, 2019), including carbohydrates, amino acids, phenolics, polyols, polyamines, lipids, and others, with many different biological functions. This large diversity is traditionally divided into primary metabolites, which are directly required for plant growth [*e.g.*, carbohydrates, tricarboxylic acid (TCA) cycle intermediates or amino acids]; secondary metabolites, which are involved in regulating plant–environment interactions (*e.g.*, phenolics, terpenes or nitrogen-containing compounds); and hormones, that control many aspects of plant growth and development, as well as plant responses to stress [*e.g.*, abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) or ethylene (ET)] (Fang *et al.*, 2019; Erb and Kliebenstein, 2020). It was recently proposed that some secondary metabolites are multifunctional and could function as potent regulators of plant growth and development processes, as well as defense against pathogens, thus acting as plant regulatory compounds or hormones. For example, secondary metabolites such as flavonoids were implicated in regulating plant growth and development, as well as environmental responses (Erb and Kliebenstein, 2020). In

addition to being considered as downstream targets of many stress-response signaling pathways, it was suggested that different metabolites also function as stress signals. In this sense, metabolic responses generated by specific abiotic stresses that persist in the plant could prime metabolic responses for subsequent environmental stresses (Schwachtje *et al.*, 2019). Examples of stress-induced metabolic signals include the amino acid proline, that is triggered by drought in *Periploca sepium* (An *et al.*, 2013) or tobacco plants (Vanková *et al.*, 2012). Other metabolic signals consisting of primary metabolites (*e.g*., sugars, sugar phosphates and different amino acids) and/or secondary metabolism, were previously described in response to drought (*e.g.,* Moyankova *et al.*, 2014; Wedeking *et al.*, 2018), cold (*e.g.,* Kaplan *et al.*, 2004; Mazzucotelli *et al.*, 2006) or heat stress (*e.g.,* Hemme *et al.*, 2014). Plants can therefore produce a wide array of variable and flexible regulators to adjust their growth and development, and to survive challenging environmental habitats.

Metabolic responses to single abiotic stresses are well described for many plant systems and in both perennial and annual plants (*e.g*., Wang *et al.*, 2016*b*, 2020; Kang *et al.*, 2019; Muchate *et al.*, 2019; Itam *et al.*, 2020; Cai *et al.*, 2020). However, plants growing in the field are typically subjected to more than one abiotic stress condition at a time (*i.e*., stress combination), imposing new and unique metabolic demands on plants (Mittler, 2006; Zandalinas *et al.*, 2018; Rivero *et al.*, 2022). Although considerable research efforts have been invested in the last decade in dissecting plant responses to a combination of two or three different stresses (*e.g*., Vile *et al.*, 2012; Iyer *et al.*, 2013; Zandalinas *et al.*, 2020*a*,*b*, 2021*b*; Cohen *et al.*, 2021), a deeper understanding of the metabolic response of plants to multiple stress factors under field conditions is crucial for improving stress-tolerance of different crops. Because the plant metabolome plays such a crucial role in defining the phenotype of plants during stress (Fang *et al.*, 2019; Sharma *et al.*, 2021; Carrera *et al.*, 2021), studies of the metabolome of plants grown under multiple stress combinations could be used to identify stress resistance-associated metabolites, which can then be used in future metabolic engineering strategies, or breeding programs, as selective markers (Abdelrahman *et al.*, 2018). In addition, since annual and perennial plant species generally adopt different strategies to cope with abiotic stresses, future research should consider these differences in dissecting and identifying stress-specific markers under multiple stress combinations.

120 In this review, we describe recent research efforts focused on studying the metabolomic
121 responses of plants to different abiotic stress conditions and their combination and
122 propose stress combination-resistance metabolites as potential targets for plant breeding.
123 Unraveling the complexities of the plant metabolomic responses to a combination of
124 multiple abiotic stresses could facilitate the development of climate-resilient crops,
125 improving global food supply.

126

Primary metabolic pathways impacted by abiotic stress combination

128 The general primary response of plants to abiotic stress includes a reduction in energy
129 consumption and overall protein biosynthesis in order to shift from plant growth to
130 protective mechanism, adjusting various primary metabolic pathways, including
131 carbohydrate, amino acid and polyamine metabolism (Zhang *et al.*, 2022). Below we
132 describe specific and common changes in primary metabolic pathways in response to
133 different abiotic stresses and their combination (Fig. 1).

134 *Sugars*

135 Different abiotic conditions and their combinations induce stomatal closure to minimize
136 water loss. Examples for these include drought (*e.g.,* Flexas *et al.*, 2002, 2004; Carmo-
137 Silva *et al.*, 2012; Medina and Gilbert, 2016; Zhang *et al.*, 2021), high light (*e.g.,*
138 Devireddy *et al.*, 2018, 2020*a*; Zandalinas *et al.*, 2020*a*), and salinity (*e.g.*, Flexas *et al.*,
139 2004; Orsini *et al.*, 2012; Zhu *et al.*, 2014; Hedrich and Shabala, 2018), as well as
140 combined drought and heat (Rizhsky *et al.*, 2002, 2004; Carmo-Silva *et al.*, 2012;
141 Zandalinas *et al.*, 2016*a*; Zhou *et al.*, 2017; Shaar-Moshe *et al.*, 2017), salinity and heat
142 (Shaar-Moshe *et al.*, 2017; Balfagón *et al.*, 2019*b*), or salinity and drought (Shaar-Moshe
143 *et al.*, 2017; Dugasa *et al.*, 2019). This response is accompanied by a decrease in the
144 intercellular CO_2 concentration of plants, limiting photosynthesis (Chaves *et al.*, 2003),
145 that in turn alters carbohydrate levels. Carbohydrates are direct products of photosynthetic
146 CO_2 fixation and are substrates for biomass accumulation and biosynthesis of other
147 organic molecules such as sugar alcohols. In addition, they play a central role in stress
148 perception and signaling, function as a regulatory hub for stress-mediated gene
149 expression, and act as stabilizers of membranes and proteins as well as osmoprotectants
150 and reactive oxygen species (ROS) quenchers (Sami *et al.*, 2016; Pommerrenig *et al.*,
151 2018). Therefore, a fine-tuned reconfiguration of sugar accumulation under stress

combination is crucial for plant acclimation and survival. Different abiotic stresses result in distinct, and sometimes opposing, patterns of sugar accumulation (Fig. 1), and this pattern depends on the type of stress as well as the plant species. For example, in response to cold stress, the pathway leading to raffinose was shown to be crucial for freezing tolerance in Arabidopsis (Korn *et al.*, 2010). Heat stress caused a decrease in glucose in soybean plants (Das *et al.*, 2017), but an increase in soluble sugars in Arabidopsis plants (Wang *et al.*, 2020*a*). In addition, in both plant species (Arabidopsis and soybean), heat stress inhibited the accumulation of metabolites involved in the TCA cycle (Das *et al.*, 2017; Wang *et al.*, 2020*a*). In response to drought stress, many of the major carbohydrates such as glucose, fructose and sucrose accumulated in soybean (Mutava *et al.*, 2015; Das *et al.*, 2017) as well as in Arabidopsis (Rizhsky *et al.*, 2004). In addition to drought, changes in the amounts of carbohydrates (including trehalose, glucose and fructose) were determined in leaves and roots of soybean plants subjected to flooding (Coutinho *et al.*, 2018). Salinity stress caused an increased abundance of sugars and sugar derivatives such as arabinose, inositol, mannose, sucrose, trehalose, xylose, and galactose in sugar beet (Hossain *et al.*, 2017). Moreover, high amounts of hexoses, sugar alcohols and organic acids were found in the salt-tolerant *Thellungiella halophila* plants (Gong *et al.*, 2005), demonstrating the protective functions of sugars under salt stress.

In contrast to individual stresses, different combinations of adverse conditions displayed unique patterns of sugar accumulation (Rizhsky *et al.*, 2004; Wulff-Zottele *et al.*, 2010; Rivero *et al.*, 2014; Obata *et al.*, 2015; Zandalinas *et al.*, 2016*c*; Zinta *et al.*, 2018; Weiszmann *et al.*, 2018; Balfagón *et al.*, 2022*a*) (Fig. 1). A metabolite profiling analysis of Arabidopsis subjected to a combination of drought and heat stress revealed that plants accumulated sucrose and other sugars such as maltose and gulose exclusively under this stress combination. In addition, it was suggested that a combination of drought and heat stress required sucrose rather than proline (accumulated only under drought applied individually) as an osmoprotectant, and that sucrose biosynthesis could occur from starch in Arabidopsis plants (Rizhsky *et al.*, 2004). In maize subjected to a combination of drought and heat, metabolite profiles were analyzed and contrasted with grain yield in the field (Obata *et al.*, 2015). Metabolic responses under this stress combination could be mostly predicted from the sum of those in single stresses, but eight metabolites, including succinate, raffinose and xylose, were specifically regulated under the combined stress conditions (Obata *et al.*, 2015). Interestingly, elevated CO_2 levels reduced the impact of

185 a combination of drought and heat on sugar metabolism, showing less accumulation of

186 soluble sugars in Arabidopsis plants, pointing towards the role of high CO_2 in stress

187 combination mitigation (Zinta *et al.*, 2018). In addition to the possible beneficial role of

188 CO_2 in alleviating damages from a combination of drought and heat stress, it was reported

189 that elevated CO_2 decreased iron and zinc concentration in soybean seeds when plants

190 where growing in the field, heat stress had the opposite effect, and the combination of

191 elevated CO_2 and heat generally restored seed iron and zinc concentrations to levels

192 obtained under ambient CO_2 and temperature conditions (Köhler *et al.*, 2019). These

193 results suggest that increased levels of CO_2 due to climate change could be beneficial for

194 the nutritional quality of crops. However, it should be noted that C3 grains and legumes

195 had lower concentrations of zinc and iron when grown under field conditions at elevated

196 atmospheric CO_2 concentration (Myers *et al.*, 2014), highlighting the complexity of

197 predicting climate change effects on food and nutritional security. In addition to the

198 combination of drought and heat, it was recently reported in Arabidopsis that a

199 combination of high light and heat stress resulted in a unique metabolic response that

200 included increased accumulation of sugars (including maltose, glucose and fructose),

201 coupled with decreased levels of metabolites participating in the TCA cycle (including

202 citrate and fumarate), compared to the individual stresses (Balfagón *et al.*, 2022*a*).

203 Similarly, the combination of high light and sulfur depletion had a profound effect on

204 central metabolic pathways including the TCA cycle and glycolysis. Specifically, sugars

205 such as glucose, fructose and raffinose were elevated compared to control (Wulff-Zottele

206 *et al.*, 2010). Another stress combination that affected the accumulation of sugars in

207 Arabidopsis was cold and high light stress. The freezing-tolerant Arabidopsis Rsch

208 accession significantly increased sucrose, fructose and glucose concentrations in response

209 to this stress combination, and a role for vacuolar invertase activity in preventing a

210 limitation in cytosolic hexose metabolism under stress was suggested (Weiszmann *et al.*,

211 2018). An increased content of fructose, glucose and UDP-glucose was also found in the

212 combined stress-tolerant tomato line RIL-76 under a combination of heat and salinity,

213 indicating that the level of this specific sugar could be used for the selection of varieties

214 more resistant to conditions of climate change (Lopez-Delacalle *et al.*, 2021).

215 Furthermore, tomato plants subjected to a combination of heat and salinity specifically

216 accumulated trehalose, that could have an important role in protecting photosynthetic

217 proteins from this stress combination (Rivero *et al.*, 2014).

Taken together, a general increase in soluble sugar accumulation seems to be a common response to different stress combinations, that could be a result of reduced plant growth (decreasing the demand for primary metabolites) as well as impaired photosynthetic rate. In turn, the decreased photosynthetic rate under stress combination would indicate that the increase in sugar levels could come from starch breakdown instead of carbon fixation. Therefore, remobilizing starch reserves to produce energy, and sugar and derived metabolites when photosynthesis is suppressed could provide metabolites that act as osmoprotectants and compatible solutes to alleviate the negative effect of different stress combinations (Thalmann and Santelia, 2017). It should be noted that in the future the combination of different abiotic stresses will be accompanied by an elevation in CO_2 levels under climate change conditions, and high CO_2 concentrations are expected to increase leaf photosynthetic rates. However, the degree to which this will occur is unknown, given that the stimulation of photosynthesis by CO_2 depends on leaf temperature, and water and nutrient availability (Dusenge *et al.*, 2019). Plant respiration could be also altered by elevated CO_2 levels due to its effects on stomatal aperture. Dusenge *et al.* (2019) reviewed recent literature focused on increased CO_2 levels and plant respiration, concluding that responses of respiration to changes in CO_2 are highly variable and that a consistent theoretical basis for making strong predictions for how respiration will change when CO_2 levels are high are still unclear (Dusenge *et al.*, 2019).

Amino acids

Like sugars, the accumulation of amino acids during different abiotic stresses can also contribute to osmotic adjustments and ROS scavenging (Hildebrandt, 2018). Moreover, amino acids can function as alternative substrates for mitochondrial respiration when carbohydrate supply is insufficient due to a decrease in photosynthesis rate, as well as serve as precursors of secondary metabolites (Hildebrandt, 2018; Batista-Silva *et al.*, 2019; Heinemann and Hildebrandt, 2021). The accumulation of amino acids resulting from protein breakdown or *de novo* biosynthesis can therefore minimize the adverse effects of different abiotic stresses (Lugan *et al.*, 2010; Krasensky and Jonak, 2012; Obata and Fernie, 2012; Hildebrandt, 2018). For example, during cold stress, amino acids play an important role in N fixation into glutamine, and the synthesis of different proteins as well as polyamines, phenylpropanoids, glucosinolates, auxins and indole alkaloids (Alcázar *et al.*, 2006; Bernard and Habash, 2009; Amir, 2010; Tzin and Galili, 2010; Hildebrandt, 2018). In Arabidopsis, the catabolic intermediates of lysine and the

branched-chain amino acids valine, leucine and isoleucine represent key factors in drought tolerance (Pires *et al.*, 2016), and the activation of biosynthetic pathways of serine, arginine, glutamine and alanine during osmotic and salt stress are associated with storage of precursors for protein synthesis to prepare for rapid recovery of plant metabolism following stress (Batista-Silva et al., 2019). Proline is thought to be crucial for acclimation to drought, cold stress and salinity but not to heat stress (Rizhsky *et al.*, 2004; Kaplan *et al.*, 2004; Gagneul *et al.*, 2007; Usadel *et al.*, 2008; Kempa *et al.*, 2008; Urano *et al.*, 2009; Szabados and Savouré, 2010; Lugan *et al.*, 2010; Lv *et al.*, 2011; Per *et al.*, 2017). In addition, the non-proteinogenic amino acid γ-aminobutyric acid (GABA) accumulates in response to drought, hypoxia, anoxia, heat stress, salinity, zinc, osmotic stress, wounding and oxidative stress (Bouché and Fromm, 2004; Xing *et al.*, 2007; Miyashita and Good, 2008; Fait *et al.*, 2008; Allan *et al.*, 2008; Bor *et al.*, 2009; Renault *et al.*, 2010; Akçay *et al.*, 2012; Nayyar *et al.*, 2014; Vijayakumari and Puthur, 2016; Daş *et al.*, 2016; Mei *et al.*, 2016; Mekonnen *et al.*, 2016; Salvatierra *et al.*, 2016; Bown and Shelp, 2016; Wang *et al.*, 2017; Scholz *et al.*, 2017; Priya *et al.*, 2019; Xu *et al.*, 2021; Wu *et al.*, 2021) (Table 1).

As shown for sugar metabolism, a combination of different abiotic stresses results in different patterns of amino acid accumulation compared to those caused by individual stresses. These unique patterns can be correlated with the specific demands the combined stress imposes on plants. For example, valine, asparagine and glutamine significantly accumulated in the tomato tolerant line RIL-76 under a combination of salinity and heat, and this accumulation was correlated with better nitrogen use efficiency compared to that of the sensitive tomato line RIL-66 (Lopez-Delacalle *et al.*, 2020). In response to a combination of drought and heat stress, the specific accumulation of certain amino acids including glutamate, tyrosine, valine, and tryptophan in Purslane plants was thought to play a role in the cellular osmotic adjustment aimed at maintaining leaf turgor during stress combination (Jin *et al.*, 2016). In addition, a stress combination induced by two metals, Co and Cu, led to a higher amino acid accumulation in the tolerant barley genotype Yan66 (Lwalaba *et al.*, 2020), suggesting that amino acids could confer resistance to toxic levels of metals due to their potential metal-binding capacity (Anjum *et al.*, 2015; Yuan *et al.*, 2020; Lwalaba *et al.*, 2020). Special attention has been recently given to GABA in response to different abiotic stress combinations (Table 1). The tomato tolerant line RIL-76 subjected to a combination of salinity and heat (Lopez-Delacalle *et*

al., 2020), as well as Arabidopsis plants subjected to a combination of high light and heat stress (Balfagón et al., 2022a), specifically accumulated this amino acid in response to stress combination. GABA also accumulates under combinations of salinity and drought, and heat and drought, as well as salinity, drought and heat, in *Brachypodium distachyon* plants (Shaar-Moshe et al., 2019). In addition to its involvement in plant growth regulation and stomatal responses, and its proposed role in enhancing plant tolerance to different environmental stresses (Bouché and Fromm, 2004; Shi et al., 2010; Shang et al., 2011; Yu et al., 2014; Mekonnen et al., 2016; Salvatierra et al., 2016; Li et al., 2016b; Seifikalhor et al., 2019, 2020; Priya et al., 2019; Fromm, 2020; Xu et al., 2021), GABA was also proposed to regulate autophagy during stress and stress combination (Signorelli et al., 2019; Li et al., 2020; Wang et al., 2021; Balfagón et al., 2022a). Therefore, GABA could play a key role regulating plant responses to individual as well as combined abiotic stresses (Table 1), emerging as a potential metabolic marker to assist breeding programs in the development of climate-resilient crops. Further research is needed to decipher the role of GABA in plant tolerance of crops growing in the field subjected to a multifactorial stress combination.

Polyamines

Amino acids such as arginine and ornithine are precursors of polyamines, including spermine, spermidine and putrescine. These metabolites are aliphatic compounds induced by several abiotic stresses such as salt, heat, drought and oxidative stress (Alcázar et al., 2010; Fu et al., 2014; Glaubitz et al., 2015; Sang et al., 2017; Ebeed et al., 2017; Podlešáková et al., 2019; Jing et al., 2020; Upadhyay et al., 2020). Elevated levels of the main polyamines putrescine, spermidine and spermine have been shown to confer tolerance to different abiotic stresses. For example, a T-DNA mutational analysis of *Oryza sativa* Lysine Decarboxylase-like Protein 1 (OsLDC-like 1) revealed that an increased polyamine biosynthesis enhanced oxidative stress tolerance by preventing the accumulation of ROS (Jang et al., 2012). In addition, spermidine could be involved in the expressions of proteins related to cell rescue and defense and the activation of antioxidant enzymes in tomato seedlings exposed to high temperatures (Sang et al., 2017) and has also been suggested to protect Arabidopsis from heat stress by increasing the expression of genes encoding heat shock proteins (Sagor et al., 2012). In addition to high temperatures, drought responses can be modulated by polyamines in wheat plants growing in the field through osmolytes accumulation (Ebeed et al., 2017). Spermidine

has been proposed to alleviate salinity damages in tomato plants (Zhang *et al.*, 2015) and in sorghum seedlings (Yin *et al.*, 2016), as well as to reduce chilling injury during low temperature storage of grape berries from vineyards (Harindra Champa *et al.*, 2015). In addition, an Arabidopsis mutant plant (*acl5/spms*), which cannot produce spermine, was hypersensitive to high salt and drought (Jang *et al.*, 2012). Therefore, the role of polyamines in protecting plant cells from a wide range of different stress-associated damages in several plant species has been extensively demonstrated. However, the potential role of polyamines in regulating plant responses to abiotic stress combination remains unclear. It was previously suggested that proline could be involved in the protection of tobacco plants against a combination of drought and heat stress by modulating polyamine biosynthesis (Cvikrová *et al.*, 2013). In addition, spermine could confer tolerance to combined high temperature and drought stress in trifoliate orange seedlings by modulation of antioxidant capacity (Fu *et al.*, 2014). However, the combination of high light and heat stress repressed the accumulation of putrescine in Arabidopsis plants, suggesting that the role of polyamines under this stress combination might be marginal (Balfagón *et al.*, 2022*a*).

Although different transgenic approaches indicated that polyamines could be essential for abiotic stress tolerance, underlying the possibility of exploiting them as markers to improve plant tolerance (Alcázar *et al.*, 2006), further research is needed to identify the role of polyamines in plants subjected to stress combination.

Changes in secondary metabolites in response to stress combination

A wide variety of secondary metabolites in higher plants are synthesized from primary metabolic pathways such as glycolysis, TCA cycle, aliphatic and aromatic amino acids, pentose phosphate pathway and shikimate pathway (Aharoni and Galili, 2011; Erb and Kliebenstein, 2020). The major secondary metabolites groups generated by plants are typically divided into three groups: phenolic compounds (including flavonoids/isoflavonoids, lignins and tannins), terpenes (including terpenoids or isoprenoids), and nitrogen- or sulfur-containing compounds including alkaloids and glucosinolates, respectively (Aharoni and Galili, 2011). In addition to their role in regulating plant defense against herbivores and pathogens, secondary compounds are essential for the acclimation of plants to different abiotic stresses (including temperature,

drought, nutrient deficiencies, elevated CO_2, salinity and UV light) by protecting key biomolecules such as proteins and nucleic acids from stress-induced oxidative damage (Ramakrishna and Ravishankar, 2011; Austen *et al.*, 2019; Ahanger *et al.*, 2020; Jan *et al.*, 2021). For example, flavonoids are accumulated in response to almost all abiotic stresses (Nakabayashi and Saito, 2015) and their antioxidant function has been experimentally identified under different stress conditions (Hernández *et al.*, 2004; Kusano *et al.*, 2011; Nakabayashi *et al.*, 2014; Nakabayashi and Saito, 2015; Wang *et al.*, 2016*a*). Terpenoids are also involved in plant responses to different abiotic stresses providing antioxidant protection to drought, temperature, light and salt stresses (Schuh *et al.*, 1997; Loreto and Delfine, 2000; Munné-Bosch *et al.*, 2001; Vallat *et al.*, 2005; Zhang *et al.*, 2019; Ahanger *et al.*, 2020). Similarly, nitrogen-containing compounds such as alkaloids increase in their levels in response to different stresses and are considered potent mitigators of oxidative damage resulting from stress (Kleinwächter and Selmar, 2015; Zhu *et al.*, 2015; Benjamin *et al.*, 2019; Patel *et al.*, 2020; Yadav *et al.*, 2021). In addition to alkaloids, glucosinolates accumulate in response to different stresses (Ramakrishna and Ravishankar, 2011) to mediate tolerance to, for example, low temperatures (Ljubej *et al.*, 2021), high temperatures (Rao *et al.*, 2021), or drought stress (Eom *et al.*, 2018; Salehin *et al.*, 2019). Metabolic changes of Lonicerae Japonicae Flos exposed to salt stress included altered patterns of the biosynthesis of phenolic acid, flavonoids, and iridoids (Cai *et al.*, 2020).

Besides their protective role during different individual abiotic stresses, several studies provided evidence for the function of secondary metabolites in the response of plants to stress combination. Some reports showed that, depending on the plant species and the type of the abiotic stresses involved, the secondary metabolism response to one of the single stresses prevails over the other under combined stress. For example, Austen *et al.*, (2019) reported that heat responses (inducing the accumulation of isoprene) overcame the effects of elevated CO_2 (suppressing the accumulation of isoprene) under controlled growth conditions of combined heat and elevated CO_2 in a woody plant (Salix spp.), indicating that the antioxidant function and capacity to protect cell membrane of isoprene compensates for its energetic cost under this stress combination (Austen *et al.*, 2019). In contrast, phenylpropanoid accumulation in tomato plants under a combination of heat and salinity was more similar to that raised by salinity than that caused by heat. While several compounds belonging to the caffeoylquinic acids family were repressed under heat, they

were induced under salinity or the combination of salinity and heat. By contrast, flavonol-related compounds were accumulated under heat, whereas under salinity and salinity combined with heat these metabolites were repressed, correlating with higher oxidative damage (Martinez et al. 2016). In addition, whereas phenylpropanoids were important for plant tolerance to a combination of salinity and heat (Martinez et al. 2016), a key role for tocopherols was found in the tolerance of tomato plants to the combination of light and heat stress (Spicher *et al.*, 2017). Different stress combinations that included heavy metals resulted in different patterns of accumulation of secondary metabolites depending on the plant species (Zhao *et al.*, 2016; Chrysargyris *et al.*, 2019). For example, in *Rhodiola rosea* seedlings growing under semi-controlled conditions, heat stress enhanced the negative effects of heavy metals, showing a higher accumulation of secondary metabolites such as phenolic compounds under this stress combination (Zhao *et al.*, 2016). The combination of salinity stress and Cu exposure altered the primary metabolic pathways in favor of production of different secondary metabolites including limonene in *Mentha spicata* plants (Chrysargyris *et al.*, 2019). Furthermore, the combination of drought and heat resulted in activation of secondary metabolites that led to tolerance or sensibility to this stress combination depending on the plant tested. For example, tolerance to a combination of drought and heat stress was correlated with elevated levels of flavonoids and phenols in the cotton genotype Zhongmian 23 (Ibrahim *et al.*, 2019), whereas the activation of secondary metabolism was associated to sensitivity to this stress combination in citrus plants (Zandalinas *et al.*, 2016*c*).

Overall, the ability of plants to synthesize a wide range of largely specialized secondary compounds is thought to facilitate their adaptation to a changing environment (Wink, 2010), at the expense of their growth (Panda *et al.*, 2021). The fact that plants invest large amounts of energy synthesizing secondary metabolites indicate the importance of these metabolites to plant survival, especially under combined stresses, in which damages tend to be higher compared to those caused by individual stresses. However, the complexity of metabolic networks, that include tens of thousands of plant secondary metabolites (Wink, 2010), and the findings that the production of secondary compounds is specific to the type of stress combination, as well as the plant species involved, render the use of secondary metabolites in breeding efforts to develop combined stress-resilient crops, a challenge that could be difficult to overcome.

Changes in hormone levels in response to stress combination

Together with primary and secondary metabolites, hormones can display a stress-specific accumulation pattern in response to different stresses and their combination. Because the role of different hormones under individual abiotic and biotic stresses is too vast to cover here, readers are directed to excellent reviews on the subject (*e.g.*, Shi *et al.*, 2015; Dar *et al.*, 2015; Eremina *et al.*, 2016; Li *et al.*, 2016*a*, 2021; Pandey *et al.*, 2016; Verma *et al.*, 2016; Raja *et al.*, 2017; Shigenaga *et al.*, 2017; Shu *et al.*, 2018; Dermastia, 2019; Wang *et al.*, 2020*b*; Devireddy *et al.*, 2020*b*; Yu *et al.*, 2020). The ability of plants to regulate specific and unique hormonal responses when subjected to stress combinations has an important role in plant acclimation (Table 2). One of the main hormones involved in the response of plants to different abiotic stresses and their combination is ABA. It is thought to be involved in the acclimation of Arabidopsis to high temperatures combined with salinity (Suzuki *et al.*, 2016), or with drought (Zandalinas *et al.*, 2016*a*), by modulating gene expression and the accumulation of Ascorbate Peroxidase 1 (APX1) and Multiprotein Bridging Factor 1c (MBF1c), respectively (Suzuki *et al.*, 2016; Zandalinas *et al.*, 2016*a*). Furthermore, ABA accumulated in response to a combination of salinity and high temperatures in citrus plants (Balfagón *et al.*, 2019*b*), and in tomato plants subjected to salinity and drought (Xue *et al.*, 2021). The transcription of ABA-response genes such as Responsive to Desiccation 26 (RD26) and ABA Repressor 1 (ABR1) in poplar plants was also elevated under salinity and heat stress combination (Jia *et al.*, 2017), further suggesting that ABA could be a key component is signaling pathways involved in plant acclimation to different stress combinations. However, due to the complex (and sometimes opposing) interactions different stresses impose on hormonal signaling, other hormones can participate in plant responses to stress combination (Table 2). For example, in contrast to ABA, JA was shown to be involved in plant acclimation to a combination of high light and heat stress by modulating the expression of different JA-response genes (Balfagón *et al.*, 2019*a*). In citrus plants, SA accumulated under the combination of drought and heat stress (Zandalinas *et al.*, 2016*b*), and at low temperatures combined with wounding citrus plants accumulated JA (Balfagón *et al.*, 2019*b*). In contrast, wounded citrus plants subjected to high temperatures did not accumulate JA whereas SA levels increased, suggesting antagonistic interactions between SA and JA under a combination of heat and wounding (Balfagón *et al.*, 2019*b*). Interestingly, a recent study of signal integration during the combination of high light and heat stress revealed

that the interplay between JA and SA could be involved in how an Arabidopsis leaf simultaneously subjected to high light and heat stress initiates systemic signaling and plant acclimation (Zandalinas *et al.*, 2020*a*).

In summary, during stress combination, different hormone interactions specific for particular stresses may collide, making the study of plant responses to stress combination a complex challenge. In order to identify new targets for crop improvements, deciphering the unique crosstalk modes between the different hormones under different stress combinations should be addressed in future studies of plant acclimation to the combination of multiple stresses.

Interconnection among primary metabolism, secondary metabolism and hormones under stress

The biosynthesis of secondary metabolites and hormones is closely associated with pathways of primary metabolism (Aharoni and Galili, 2011) (Fig. 2). For example, increments in the sugar/amino acid ratio leads to elevation in phenylpropanoid levels and a decline in the concentration of the alkaloid nicotine. In situations where photosynthesis is inhibited, the decrease in carbohydrates is accompanied by a collapse in the absolute levels of carbon- and nitrogen-rich secondary metabolites (Matt *et al.*, 2002). In addition, amino acids represent major precursors for the synthesis of secondary metabolites and some hormones. Examples include the secondary metabolites nicotine, anthocyanins, glucosinolates, and terpenoid indole alkaloids, that are synthesized from proteinogenic amino acids (Wasternack and Strnad, 2019), or the hormones indole-3-acetic acid (IAA) and ET, that are synthesized from tryptophan and methionine, respectively (Yang *et al.*, 2020). Other primary metabolites involved in the biosynthesis of secondary metabolites are polyamines. The interaction of polyamines such as putrescine with secondary metabolism is well established in some plants. The enzyme Putrescine N-methyltransferase (PMT) catalyzes the methylation of putrescine, and the product of this reaction (N-methyl putrescine) is required for the synthesis of nicotine, tropane and nortropane alkaloids in Solanaceae and Convolvulaceae plants (Biastoff *et al.*, 2009). Hormones, in turn, can alter both primary and secondary metabolism (Fig. 2). The hormone ABA can trigger changes in the metabolism of sugars and organic acids in guard cells (Jin *et al.*, 2014) and crosstalk between hormones and secondary metabolism was

480   described in response to different stresses. For example, under drought stress, methyl
481   jasmonate (MeJA) and JA elicit the production of secondary metabolites such as
482   alkaloids, taxanes, terpenoids, coumarins, and phenolic phytoalexins in several plant
483   species (Jogawat *et al.*, 2021). Supporting the link between JA and secondary metabolism
484   reprograming, it was also reported that in tomato plants, constitutive activation of the
485   jasmonate signaling pathway enhanced the production of secondary metabolites (Chen *et*
486   *al.*, 2006) and that the jasmonate-responsive transcriptional regulator ORCA3 controlled
487   the production of terpenoid indole alkaloids (Van Der Fits and Memelink, 2000). In
488   addition to JA, SA together with putrescine can trigger the accumulation of primary and
489   secondary metabolites including sugars, succinate, leucine, and phenolic compounds to
490   protect plants against drought stress (Khan *et al.*, 2019). Other examples of the interaction
491   of hormones and secondary metabolites under abiotic stress conditions were described
492   previously in Arabidopsis plants (Hectors *et al.*, 2012; Salehin *et al.*, 2019). Auxins play
493   a key role in UV acclimation by regulating flavonoid concentration (Hectors *et al.*, 2012)
494   and the auxin-sensitive Aux/IAA repressors IAA5, IAA6, and IAA19 regulate the
495   biosynthesis of aliphatic glucosinolates under drought stress (Salehin *et al.*, 2019).

496

Conclusions and future perspectives

498   Among the many processes affected by harsh environmental conditions, plant metabolism
499   is perhaps the most influenced, resulting in significant adjustments to the growth,
500   development, and reproduction of plants. The ability of plants to modulate their primary
501   and secondary metabolism in response to different stresses and/or stress combination is
502   key for the reallocation of resources from growth and reproduction to stress acclimation
503   that is crucial for plant survival (Fig. 2). Progress in our understanding of the changes that
504   occur in different metabolic processes in response to stress combination would identify
505   new targets for crop improvement. However, major challenges exist for metabolic studies
506   and engineering projects attempting to alter these processes. These include: (*i*) the
507   identification of the key metabolic processes occurring under multiple combined stresses;
508   (*ii*) designing the proper engineering strategy to increase flux of the metabolic processes
509   required to enhance plant acclimation to stress combination; and (*iii*) the identification of
510   necessary genetic manipulations to implement this strategy. In addition, due to the
511   conflicting nature of some metabolic changes triggered during different stress

combinations, together with the different stress intensities impacting plants in the field, it is difficult to predict the specific metabolites that could be good candidates for breeding programs. Perhaps special attention should be given to common metabolites that function during a wide range of single and combined stresses. In this sense, different studies of metabolic changes in plants subjected to different stress combinations have already provided clues regarding key metabolites that could play a key role in the tolerance of plants growing under multiple stress conditions. An example could be GABA, which accumulate in response to different abiotic stresses and their combination in several plant species (Table 1). Interestingly, GABA is not considered a simple metabolite (Bouché and Fromm, 2004; Bown and Shelp, 2016), and it has been suggested to provide a direct link with the metabolic status of plant cells under stress conditions as a bypass of different reactions of the TCA cycle (Fait *et al.*, 2008; Xu *et al.*, 2021). In addition, GABA has recently been proposed to be a stomatal aperture regulatory signal of economic significance, since genetic manipulation of cell-type specific GABA metabolism could reduce water loss by fine-tuning stomatal aperture (Xu *et al.*, 2021). GABA is therefore a good example for a primary metabolite that could be used for the improvement of plant stress resilience to multiple co-occurring environmental stress conditions. Other potential metabolites that may serve as candidates for crop improvement are secondary metabolites. These are known to have a role in protecting plants from oxidative stress caused by different abiotic stresses. Plant varieties containing high amounts of specific secondary metabolites could therefore provide new avenues for the development of crops with high tolerance to multiple abiotic stress combinations. However, due to the multiple functions secondary metabolites have in plant cells (*e.g.,* flavonoids are developmental regulators, but also function in the response of higher plants to a wide range of abiotic stresses as antioxidants; Agati and Tattini, 2010), future research should focus on how secondary metabolites affect plant survival under multiple stresses, and how these effects are linked to plant development and growth. In addition to primary and secondary metabolites, hormones have been reported to play a key role in plant acclimation to several stress combinations (Table 2), making them potential candidates to improve the climate-resilience of crops. Although ABA has been reported as key metabolite for plant acclimation to a wide range of abiotic stress combinations (Suzuki *et al.*, 2016; Zandalinas *et al.*, 2016*a*; Jia *et al.*, 2017; Balfagón *et al.*, 2019*b*), its integration with other hormones and/or other signaling pathways including ROS regulatory systems might be different depending on the different stress combinations (Suzuki, 2016; Devireddy *et al.*, 2020*b*).

Identifying essential master regulators that activate different hormone signaling pathways in response to different stress combinations could therefore provide an avenue to tailor responses of plants under multiple abiotic stress conditions. Further studies should be conducted to address this possibility.

Finally, it is important to keep in mind that all metabolic studies of stress combination reported to date focused on metabolite changes in plants subjected to two or at most three co-occurring stress conditions. By contrast, no study has revealed how the different metabolic pathways will be affected by a higher number of different abiotic factors (*i.e.,* multifactorial stress combination; Zandalinas *et al.*, 2021*b,a*). Future climate scenarios may include the simultaneous or sequential exposure of crops to high temperature in combination with many other stresses including salinity, drought, flooding, nutrient deficiency, high CO_2 and/or other biotic and abiotic stressors. It was recently reported in Arabidopsis that with the increasing number and complexity of multiple stress factors (up to 6 different stress factors) acting simultaneously, plant growth and survival declines, even though the individual level of each stress applied had a negligeable effect on plants (Zandalinas *et al.*, 2021*b*). In this study it was also found that the molecular response of Arabidopsis to the different multifactorial stress combinations was unique and may involve pathways or metabolites not identified yet (Zandalinas *et al.*, 2021*b*). In addition, biotic stressors can modify plant responses to abiotic stresses and vice versa (Rivero *et al.*, 2022 and references therein), making the study of multifactorial stress combination a difficult challenge to overcome. Further studies addressing the identification of potential metabolites acting under multifactorial abiotic/biotic stress combination could therefore be key to mitigating the impact of global warming and climate change on crop productivity.

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Author contributions

579   SIZ, DB, AG-C and RM wrote and approved the final version of the manuscript.

580

Conflict of interest

582   The authors declare no conflict of interest.

583

Data Availability

585   All data supporting the findings of this study are available within the paper.

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Tables

Table 1. GABA levels are enhanced in different plant species in response to different abiotic stresses and their combination. C letter in references means experiments conducted under controlled conditions; SM letters in references mean experiments conducted under semi-controlled conditions; F letter in references means experiments conducted in the field.

Species	Stress	References
Individual stresses		
Agrostis stolonifera	Heat	[C]Li *et al*., 2016*b*
Arabidopsis thaliana	Salinity	[C]Kempa *et al*., 2008; [C]Renault *et al*., 2010; [C]Allan *et al*., 2008
	Flooding	[C]Allan *et al*., 2008
	Cold	[C]Allan *et al*., 2008
	Heat	[C]Allan *et al*., 2008
	Drought	[C]Mekonnen *et al*., 2016; [C]Allan *et al*., 2008; [C]Xu *et al*., 2021
	Hypoxia	[C]Miyashita and Good, 2008; [C]Wu *et al*., 2021
	Wounding	[C]Scholz *et al*., 2017
Camellia sinensis	Anoxia	[C]Mei *et al*., 2016
Glycine max	Salinity	[C]Xing *et al*., 2007
Nicotiana sylvestris	Salinity	[C]Akçay *et al*., 2012
Nicotiana tabacum	Flooding	[C]Allan *et al*., 2008
	Zinc	[C]Daş *et al*., 2016
Oryza Sativa	Heat	[C]Nayyar *et al*., 2014
Piper nigrum	Osmotic stress	[C]Vijayakumar and Puthur, 2016
Prunus	Hypoxia	[SC]Salvatierra *et al*., 2016
Sesamum indicum	Osmotic stress	[C]Bor *et al*., 2008
	Salinity	[C]Bor *et al*., 2008
	Selenium	[C]Bor *et al*., 2008
	Heat	[C]Bor *et al*., 2008
Vigna radiata	Heat	[SC]Priya *et al*., 2019
Zea mays	Salinity	[C]Wang *et al*., 2017
	Drought	[F]Obata *et al*., 2015
	Heat	[F]Obata *et al*., 2015
Combined stresses		
Arabidopsis thaliana	High light + Heat	[C]Balfagón *et al*., 2022*a*
Brachypodium distachyon	Salinity + Heat	[C]Shaar-Moshe *et al*., 2019
	Salinity + Drought	[C]Shaar-Moshe *et al*., 2019
	Salinity + Drought + Heat	[C]Shaar-Moshe *et al*., 2019
Camellia sinensis	Anoxia + Wounding	[C]Mei *et al*., 2016
Citrus sinensis	Drought + Heat	[C]Balfagón *et al*., 2022*b*
Zea mays	Drought + Heat	[F]Obata *et al*., 2015

Table 2. Involvement of different hormones in plant responses to different abiotic stress combinations under controlled conditions.

Hormone	Species	Combined stress	Hormone involvement	References
ABA	*Arabidopsis*	Salinity + Heat	Regulation of gene expression	Suzuki *et al*., 2016
	Citrus	Salinity + Heat	Increased ABA accumulation	Balfagón *et al*., 2019*b*
	Poplar	Salinity + Heat	Increased ABA accumulation	Jia *et al*., 2017
	Tomato	Salinity + Drought	Stomatal closure	Xue *et al*., 2021
	Arabidopsis	Drought + Heat	Induction of acclimation proteins	Zandalinas *et al*., 2016*a*
JA	*Arabidopsis*	High light + Heat	Regulation of gene expression	Balfagón *et al*., 2019*a*; Zandalinas *et al*., 2020*a*
	Citrus	Cold + Wounding	Increased JA accumulation	Balfagón *et al*., 2019*b*
SA	*Citrus*	Drought + Heat	Increased SA accumulation	Zandalinas *et al*., 2016*b*
	Citrus	Heat + Wounding	Increased SA accumulation	Balfagón *et al*., 2019*b*

Figure legends

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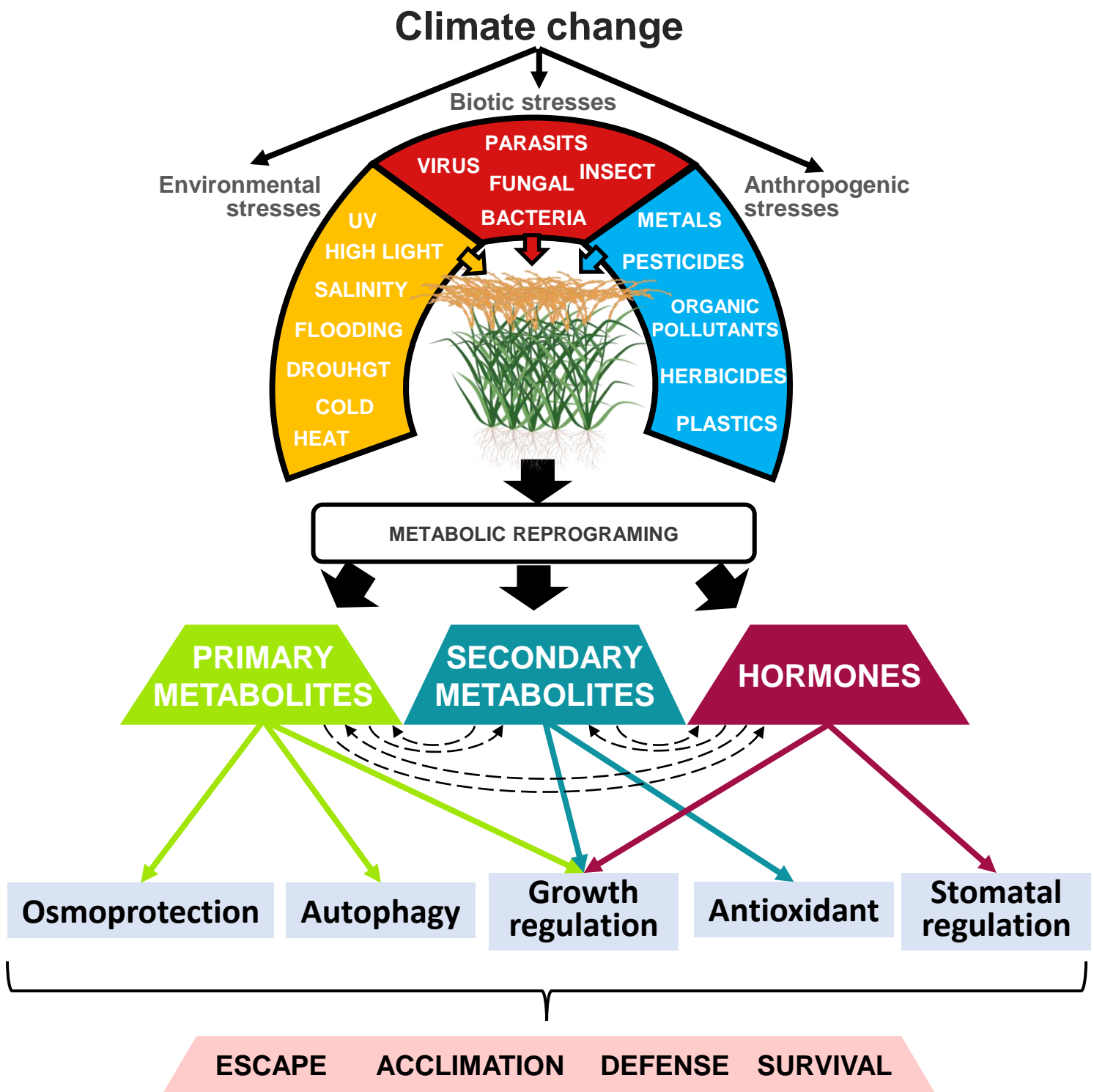
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