Invited Review Article

Synergism and antagonism in plant acclimation to abiotic stress combinations

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Abstract: Acclimation of plants to their environment involves mechanisms at all molecular levels and only the complete temporal and spatial pattern adequately describes the acclimation state. The diversity of physiological states realized in plants increases by the essentially infinite combinations of abiotic and biotic stresses, considering duration, impact order, and strength. The interference between stressors involves phenomena of synergism, antagonism, and indifference. This review exemplarily sketches principles of interference considering posttranslational modifications, the formation of molecular assemblies in regulosomes, the regulation of gene expression, and the vast number of other posttranscriptional controls e.g., in RNA processing and recruitment to ribosomes. Small molecules such as hormones, reactive molecular species as ROS, ions, and metabolites are decisive players in this network. Available methodology for global analysis combined with machine learning approaches offers a perspective to identify the central regulatory modules, particularly if the multiple layers of molecular regulation are included in parallel, and to better understand the underlying mechanisms of stress interference in an unbiased manner.

Key words: Abiotic stress, gene expression, posttranslational modification, RNA fate, signaling, small molecules, stress combinations

1. The variable environment of plants

Combinations of stressful parameters are the common rather than the exceptional growth scenario in nature (Suzuki et al. 2014). Abiotic and biotic stresses to plants range from nutrient element deficiency to salinity, exposure to a wide variety of toxic compounds, including gaseous noxae or particulate matter, temperature extremes, water deficit, and hypoxia to biotic stresses such as herbivory or viral, bacterial, and fungal diseases. Consequently, the number of realistically possible abiotic stress interactions may reach 5000 dual combinations and exorbitant scenarios assuming about 100 relevant stressors if most permutations of stresses are allowed. The assumed "infinite" number of possible combinations is realistic, if duration, stressor intensity and order of stress occurrence, cell specificity and developmental state of the plant are considered as well. This complex environmental potpourri poses a physiological challenge to the plant, namely, to coordinate its response in an optimal manner and to distinguish the main challenges from less significant impact and, in the end, requires elasticity, plasticity, and robustness. Our understanding of such phenomena of interference is scarce and often limited to specific settings of plant growth history and stress treatments.

Apart from monitoring plant performance in the natural environment, researchers mostly rely on two types of experiments. Field studies are designed within the complex natural environment and only single or few parameters are manipulated, e.g., by UV shielding, rain shelter, N-supply, or CO_2 enrichment (Leakey et al. 2008, Dehariya et al. 2012, Teixeira et al. 2014). Alternatively, experiments in climate chambers occur in a fully controlled environment, and mostly single parameters are altered to explore their effect on plant performance and physiological and biochemical features.

The most important output parameters are growth and yield. Plants may encounter synergistic including additive effects, antagonistic interactions, or indifference of combined stresses (Figure. 1). In the depicted scenario, a stressor 1 inhibits plant growth by more than 50%, while a stressor 2 has only mild effects. The effect of combined stressors 1 and 2, however, strongly enhances growth retardation in (D), depicts antagonism in (E) or lacks interaction in (F). To give an example for each of these scenarios: combination of drought and heat has a synergistic effect on growth inhibition in maize (Hussain et al. 2019) and drought stress antagonized ozone toxicity in spruce (Otu-Larbi et al. 2020). Suzuki et al. (2014) compiled a matrix of such positive and negative interactions between some stress types. The available interaction matrix is rudimentary and needs extension, e.g., arsenic toxicity and combined hypoxia application

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Interference in stress response
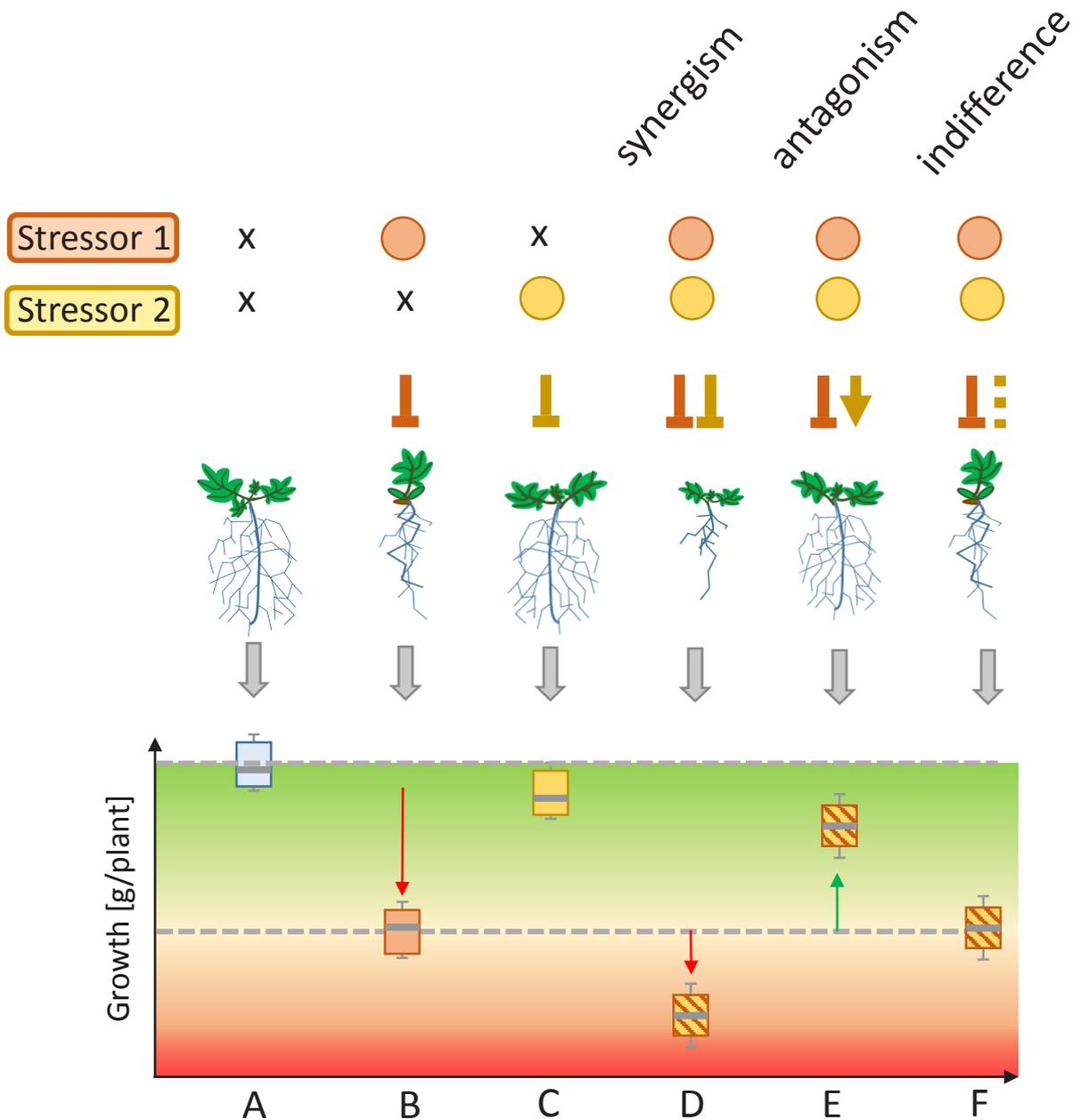
Figure 1. Schematics of interactions between two stressors. The two stresses 1 and 2 may be absent (A), occur individually (B, C), or in combination (D-F). The combination may lead to synergistic (D), antagonistic (E), or indifferent responses (F) of the growth phenotype (middle) and quantified biomass (lower graph).

reveal synergistic inhibition in *Arabidopsis thaliana* (Kumar et al. 2019).

The occurrence of no effect (indifference) is seen in nutrient interactions obeying the "law of the minimum" originally phrased by Justus von Liebig stating that plant growth is limited by the scarcest nutrient element. In this

case, another limitation will not exert control on biomass production if combined with the strong deficiency of the first one. However, there are exceptions from this law of the minimum (Gorban et al. 2011). At the level of whole plant performance, the interaction may be quantified in a straight-forward manner and includes cross-hardening

in various combinations, e.g., if cold treatment increases resistance of triticale to the fungal pathogen *Microdochium nivale* (Szechynska-Hebda et al. 2014). But in this scenario, plants are often considered as black boxes and molecular details are not revealed. The following sections will deal with molecular mechanisms in the "box" contributing to the stress interference and the readouts.

2. Combinatorial stress and the molecular principles of response optimization

The number of plant studies aiming to explore synergistic, antagonistic, or indifferent effects of combinatorial stress treatments increased exponentially in the last two decades. It should be noted that experiments quantifying the whole plant performance and assessing simple physiological or biochemical features under such scenarios are of high importance despite their design, often disrespectfully,

classified as "descriptive". The identification of such novel interferences is the ground on which reasonable mechanistic studies can be built. Experimental strategies commonly employ dual combinations but occasionally address triple combinations (Vogel et al. 2012; Sewelam et al. 2020). In parallel, researchers aim to identify molecular mechanisms underlying the whole plant response. The following section addresses four principles mediating interaction, namely posttranslational modifications (PTM), regulosome assembly, the role of small molecules and promoter control in gene expression.

3. Posttranslational modifications of proteins

Central signaling or regulatory elements that participate in stress acclimation usually undergo switching by PTMs (Figure 2). The high number of PTMs and the diversity of stress-triggered signal transduction pathways on the
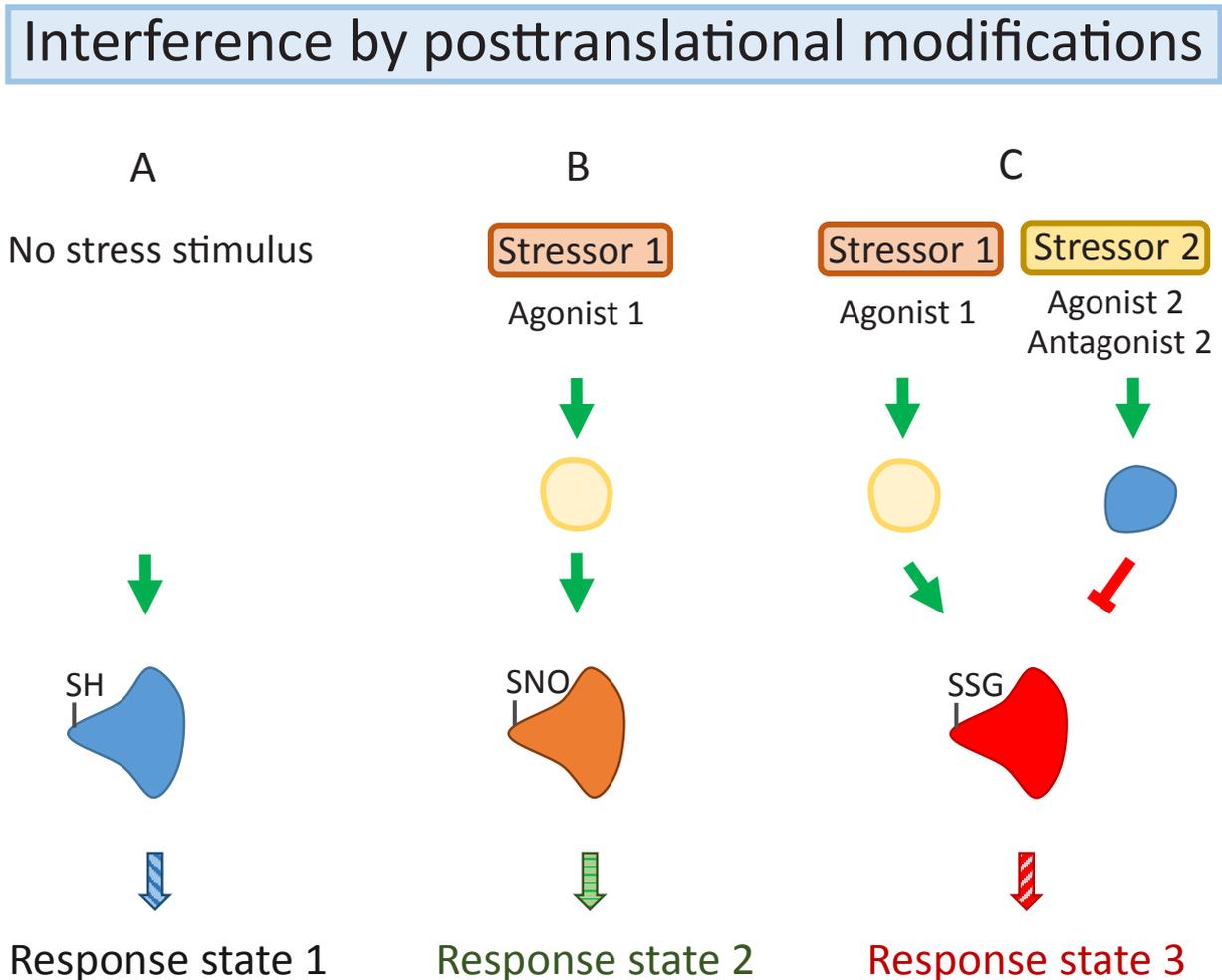
Figure 2. The effect of posttranslational modifications of a protein on the stress response. The schematics compares the state of a cellular protein in the absence of the stressor, the presence of stressor 1 and the presence of the combination of stressors 1 and 2. The reduced thiol form (A) has a different activity or fate than the S-nitrosylated form (B) or the S-glutathionylated form (C) form, where either the activation state observed under B may be enhanced (left hand side of C) or antagonized (right hand side of C).

one hand and the infinite environment-versus-plant states on the other hand set the scene for versatile responses of involved individual players and activity adjustments of groups of involved players. To give some examples, S-nitrosylation synergistically or antagonistically affects stress-responses and "regulates regulators" (Gupta et al. 2019). S-nitrosylation can enhance or suppress pathogen susceptibility and abiotic stress tolerance and this type of regulation and manipulation is exploited by both the plant as host and the pathogen (Lubega et al. 2021). Nitrosylation, nitration and redox switches affect the central cellular signal amplification pathway of mitogen activated protein kinase (MAPK) cascades.

Another more specific example is the cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC), which functions in glycolysis and photosynthetic carbohydrate metabolism but, in addition, displays moonlighting activities in the control of gene expression and in posttranscriptional regulation as RNA binding protein. Antagonistically effective PTMs appear on the catalytic cysteine of GAPC: S-nitrosylation of Cys149 occurs in the presence of elevated levels of NO and nitrosoglutathione (GSNO), inhibits its catalytic activity and likely eases taking over non-glycolytic functions by GAPC (Zaffagnini et al. 2013). S-glutathionylation of Cys149 in the presence of H_2O_2 and subsequent disulfide formation triggers protein aggregation, while hyperoxidation to sulfinic acid at Cys149 initiates degradation (Zaffagnini et al. 2019). In contrast, synergistic effects of certain PTMs could play a role upon heat and salt stress where S-nitrosylation of GAPC might induce translocation to the nucleus and foster its moonlighting activity in the control of gene expression (Wawer et al., 2010; Kim et al., 2020). Other stress-induced nuclear localization events were also linked to S-nitrosylation, albeit the significance of this PTM for nuclear translocation was questioned based on maintained translocation in Cys→Ser-mutants (Aroca et al. 2017, Testard et al. 2016, Vescovi et al. 2013).

Another group of PTMs needs attention as well, namely the attachment of ubiquitin or SUMO to proteins under stresses like salinity, drought, or heat (Li et al. 2017). SUMOylation and ubiquitination alter protein functions, including assembly with other proteins, subcellular localization, and turnover (Yu et al. 2020).

Other prominent examples for entities under control of established interfering signaling pathways and PTMs are transcription factors and modulator proteins such as TGACG-BINDING FACTORs (TGA-TFs) in combination with NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) (Shearer et al. 2012; Budimir et al. 2021), RADICAL INDUCED CELL DEATH 1 (RCD1) (Shapiguzov et al. 2019) or G-group of BASIC REGION LEUCINE ZIPPER 16 (bZIP16) (Shaikhali et al. 2012).

The number of such proven or suggested integrating elements is high. The dependency of the whole plant performance under combinatorial stress conditions on these specific PTMs often awaits validation or is established under certain growth conditions only. The significance of the PTMs for the whole plant performance depends on the centrality of the considered protein in the signaling and regulatory network mediating the acclimation.

4. Interference engages modules and networks

Cellular signal integration at the molecular scale often occurs in functional modules of interacting elements, mostly consisting of proteins but also containing other components such as nucleic acid and small molecules (Dietz et al. 2010, Vandereyken et al. 2018). Alterations in molecular interactions elicit, enhance, or suppress specific responses within a signalling network. The dynamic interactome to a significant extent is governed by PTMs as discussed before. The molecular site of interference or crosstalk is represented by signal integration modules which are essential components of signalling networks and may also be called regulosomes if assembled for longer than the short-lived interactions in catalysis. Graph theoretical approaches describe the interactions between the involved elements (= nodes) as edges (Ribeiro and Ortiz 2014). A module is a functional signalling unit consisting of several nodes, which cooperate for the processing (Figure 3). Information processing in its straightest implementation employs very few nodes with sparse edges and shortest paths.

In bacteria, cellular responses are predominantly linked to sensing of chemical and physical stimuli. The participating sensory systems often involve simple modular structures consisting of a sensory domain as input node, a transmitter, and a receiver domain-associated response regulator (Lengeler 2000). The input nodes are highly specific, and each bacterial cell expresses many sensors (Kurjan and Taylor 1993), e.g., the *Corynebacterium glutamicum* genome codes for 150 two component systems. The signalling pathways contain few nodes and, at first glance, often seem to have a linear structure from an input to response regulation. However also in prokaryotes signalling may be more complex and involve integration steps from various input and process parameters, e.g., in the regulation of the lac or tryptophan operons, where several regulatory circuitries interfere. A good example is bacterial chemotaxis, which can be described through a small network of basically six signal transduction proteins. This network is characterized by sensing, adaptation, feedback regulation, memory, and robustness (Vladimirov et al. 2009).

Signalling in eukaryotes mostly engages pathways linked to processing networks and seems to have little
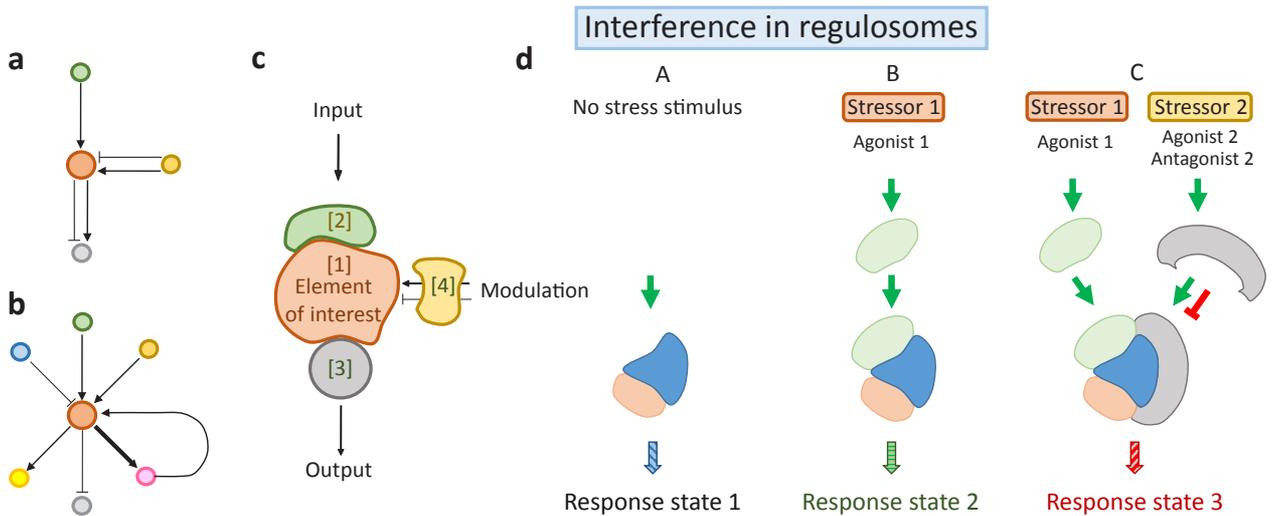
Figure 3. Minimal local structure of an integrating module and scenarios of hub activity. (a) The minimal module consists of a computational core element (red ball) with three interacting nodes (input: green; output: grey; modulator: orange). (b) This module is comprised of several interactors and is under control of a positive feedback loop. (c) Module like to (a) consisting of the core element [1] and partners linked to each connected pathway, i.e. the input [2], output [3] and modulator site [4]. Lines mark interactions or flow of information, arrowheads indicate activation, blunt ends indicate inhibition. (d) Schematics depicting how stresses affect the build-up of the signalling module. A different module is built in the absence of stress (A), the presence of a single stress (B) or the impact of combined stresses 1 and 2 (C). The different assemblies trigger the different response states 1-3.

in common with that in prokaryotes. Nevertheless, comparative studies indicate that many of the elements and principles typical of eukaryotic signalling are present in prokaryotes (Shpakov and Pertseva 2008, Hwang et al. 2002). Thus, the complexity of signal processing in multicellular eukaryotes is not explained by the number of genome-encoded genes but by the use of multiple and diverse combinatorial interactions of nodes also in signalling. Such interactions are often visualized as simplified two dimensional networks, e.g., gene activation networks, metabolic networks, and protein interaction networks (Kitano et al. 2005).

Despite their apparent dissimilarity, all cellular networks feature major communalities. (i) Each network is built from a few basic mechanistic motifs (Milo et al. 2002) (Figure 3). (ii) The functionality of each network implicates participation of specific interacting nodes such as proteins. (iii) Some proteins execute few, while others perform many interactions. Those with many interactions (≥5) are called hubs. (iv) Networks operate in the dimension of time. (v) Network performance in agreement with cell requirement demands regulation of its activity, e.g., by positive feedback mechanisms to enable reliable cell fate decisions (Brandman et al. 2005) and negative feedback to improve information transmission, e.g., in yeast signalling (Yu et al. 2008). Thus, network regulation depends on additional interactions. (vi) Linear pathways combine to 2D-networks, 2D-networks to

multidimensional networks. However, the number of connections between functional pathways is kept low in order to develop sufficient specificity and robustness in the cell information system (Maslov and Sneppen 2002).

5. The mechanistic concept of signal integration modules

While signal transduction has been studied in many systems in detail, the mechanisms of integrating interactions and modulation of protein activity often remain to be explored in detail with biochemical and genetic experiments focusing on the specific system. As the first and most employed experimental approach, the implicated signalling pathways are reconstructed and analyzed via reverse engineering. In a biological context, reverse engineering means the use of gain-of-function and loss-of-function mutants. Also, biochemical methods are used to establish linear cascades of biochemical reactions within cells. However, on the level of the organ, tissue, or organism, the specific significance of any particular input signal depends on the context of other endogenous and exogenous parameters and varies enormously in time and space.

Parameters such as nutrient status, chemical milieu, environmental stimuli, cell cycle numbers, or developmental stimuli gradually modulate or digitally enable or disable signalling within a specified pathway and in specific cell types and may have quite different

effects. Such mechanisms are important for antagonistic or synergistic phenomena in a complex environment. Decision making is regulated by few principal mechanisms: (i) The signal integration may result in gradual modulation of the output, e.g., during starvation, in dependence on the intracellular redox milieu or state of a co-receptor (Um et al. 2004, Romero-Puertas et al. 2007, Lamanna et al. 2007). (ii) Signal interpretation may lead to digital decisions in cell fate. An example is the life or cell death decision (Martianez et al. 2009). Another example is the expression of *GlsA* and *RegA,* which, in a digital manner, controls the differentiation between somatic and reproductive cells in *Volvox* based on environmental cues (Babinger et al. 2006). (iii) In eukaryotic cells, signal gating may occur in a cell type and state specific manner. The circadian clock enables or disables developmental and acclimation programs of plants (Sharma and Bhatt 2015). (iv) Positive feedback regulation enables stable switching between two states, e.g., activation of cell differentiation programs (Brandman et al. 2005), while negative feedback loops readjust homeostasis and can contribute to oscillatory behaviour (Schöning et al. 2008). These principal mechanisms in signal processing theoretically are realized by few functional nodes in signalling networks (Milo et al. 2002) which must be reflected by local structures and interactions, and which define the global context including properties such as centrality, essentiality, specificity, and redundancy within the pathway (Perkins et al. 2009).

Thus, some elements in signalling pathways have additional functions in enabling crosstalk between separate pathways. Such modules "weigh" incoming information from two or several pathways. Therefore, the signal strength of their output into the pathway represents an integration event among all inputs.

On the mechanistic scale of molecules, physical interactions build the specific molecular machinery for signal integration. Each signal integration module consists of a central protein which interacts with an upstream input interactor. Interactors also exist on the output 'side' leading to the response (output interactor) while on the modulation 'side' interaction with signals from the crosstalking information source may occur. The chemical nature of the input interactor, the output interactor, and the modulator is variable, ranging from other proteins, metabolites, signalling molecules such as hormones and ligands to nucleic acids. The biochemical transformation of molecules within the integration module, e.g., via phosphorylation, allows the transformation of short-lived interactions (e.g., ligand and receptor) in the form of long-lasting biochemical cellular memory. The modules may also be constructed in a more complex manner with additional elements and transient or permanent interactions. The ABA signalling module may serve as example and consists of cytosolic ABA receptor proteins (PYR/PYL/RCAR) and PP2CA-phosphatases as negative regulators (Gosti et al. 1999; Merlot et al. 2001; Ma et al. 2009). In addition, other protein kinases, e.g., of the SnRK2 type, act as positive regulators (Yoshida et al. 2002). The module was also termed ABA signalosome (Romero et al. 2012).

On the physiological scale of the cell, the input signal feeds into the pathway that involves the signal integration module. After signal processing, downstream responses are evoked as outputs. One (or several) cross-talking pathway(s) modulate(s) the efficiency of information transfer within the pathway in a positive or negative manner (Figure 3). The state of the integrating module defines the combinatorial stress response. The experimental unit of research is frequently defined by specific players such as defined proteins or metabolites. The importance of the identified players is often proven in very narrow settings, e.g., for seedlings grown on sterile solidified medium. It may be questioned that conclusions from such experiments deserve generalization without deeper validation in a more natural growth environment.

6. Small molecules are central to the control of acclimation

The physicochemical state of the cell determines the pattern of PTMs that are triggered by suitable signals arising from external or internal input. In addition, reversible binding of ligands control many cellular processes and the function of regulatory modules. Diverse small molecules ranging from metabolites and ions to hormones play a particularly important role and coordinate the molecular response by targeting the regulatory elements in the networks (Fonseca et al. 2014). Small molecules serve as indicators or mediators of synergistic or antagonistic acclimation responses.

Prominent examples for small molecules are plant hormones such as ABA, auxin, salicylic acid, jasmonic acid and its precursor 12-oxophytodienoic acid (12-OPDA), strigolactones, ethylene, and brassinosteroids (Kohli et al. 2013). Additional messengers and regulators are reactive oxygen, nitrogen, sulphur, and carbonyl species (H_2O_2, H_2S, NO, $O_2^{\cdot-}$, R_2-C=CH-HC=OR) (Zhou et al. 2021, Meyer et al. 2021), glutathione (Foyer and Noctor 2011) and novel players such as melatonin (Arnao and Hernandez-Ruiz 2021, Sun et al. 2021) and γ-butyric acid (GABA) (Bor and Turkan 2019). Proline plays a major role in stress acclimation, and both its synthesis and its regulatory roles have been suggested to be subject to crosstalk with other signalling pathways (Roychoudhury et al. 2015). Many reviews have covered this topic, e.g., by summarizing the multiple interactions between auxin responses shaping development and other hormones such as brassinosteroids, jasmonic acid, ethylene, or abscisic

acid (Laxmi et al. 2013). These authors elaborate on molecular mechanisms of interference between different hormones. From these data and considerations, it is evident that hormonal crosstalk is central to stress acclimation including dual and multiple stress combinations.

A suitable example is the control of root architecture for optimized nutrient foraging under the impact of single or combinatorial stresses (Li 2021, Kumar et al. 2020). The effect of hormones on root patterning differs among species and genotypes (Ristova et al. 2018). Upon phosphate starvation, *Arabidopsis thaliana* inhibits primary and enhances lateral root development, while rice stimulates root elongation. Jasmonic acid accumulation in phosphate-starved Arabidopsis contributes to primary root growth inhibition, while, in rice, the JASMONATE ZIM-DOMAIN protein JAZ11 suppresses jasmonate signaling and allows for maintained root growth under phosphate starvation (Pandey et al. 2021).

Apart from hormones, root system development is affected by the rhizosphere and gaseous compounds such as H_2S, NO and CO (Mukherjee and Corpas 2020). NO reacts with $O_2^{\cdot-}$ to form $ONOO^-$ which triggers protein nitration and alters root patterning and is involved in aerenchyma formation under hypoxia (Wany et al. 2017). Exogenous addition of H_2O_2 stimulates lateral root formation (Su et al. 2016). Changes in glutathione redox potential occur within minutes after transfer of *Arabidopsis thaliana* to hypoxic conditions and together with ROS appear to shape the root system especially if combined with arsenic stress (Kumar et al. 2019, 2020). These examples prove the existence of an elaborate network of interacting signalling molecules. This network adopts distinct states in dependence on environmental conditions such as combinatorial stresses.

Powerful new technologies now allow for addressing cell type specificity. RNAseq analyses in laser dissected cell types (Berkowitz et al. 2021) provide information in specific cell types. Imaging with in vivo biosensors gives access to dynamic hormone levels, glutathione redox states, ROS transients and changes in other small molecules in real time with high spatial resolution (Levak et al. 2021). Dynamic imaging approaches provide the additional opportunity for high throughput analyses of many samples in microtiter plates (Wagner et al. 2019). Such technologies are needed if we wish to study the interaction of multiple combinatorial stresses and specific physiological states of the plants.

7. Combinatorial responses at the level of gene expression

The availability of annotated genome sequence information, transcriptome data and computational methods including machine learning approaches are paving the way for understanding gene expression regulation also under combinatorial stress conditions. Transcription per se is a combinatorial process (Pilpel et al. 2001). The accessibility of the DNA, the presence of motifs, and the matching activated transcription factors determine the activity of genes (Beer and Tavazoie 2004). Machine learning for improved pattern recognition based on large transcriptome data sets allowed for defining the local DNA landscape determining the binding sites of transcription factors (Sielemann et al. 2021). In addition, DNA-binding proteins often assemble in combinatorial manner as homo- or heteromers, activate or repress transcription and are focal points of supramolecular assemblies with properties of signal integration modules as discussed above.

Different signalling pathways innervate these modules (Figure 4). In addition, PTMs control interactions, subcellular partitioning and activity of the transcriptional control units. Thus, combinatorial relationships between cis-regulatory elements, location in the promoter, and frequency are better predictors for gene expression regulation than the mere presence or absence of cis-elements (Zou et al. 2011). In addition, stress acclimation is not controlled by few master combinatorial rules, rather several combinatorial patterns determine the transcriptional response to environmental stress (Zou et al. 2011). Our knowledge on these functional interactions has advanced to an extent that cis-trans engineering has been proposed for customized regulation of gene expression in transgenic plants (Shrestha et al. 2018).

The promoters of the subunit B of ADP glucose pyrophosphorylase (AGP) in maize and of ABSCISIC ACID INSENSITIVE 5 (ABI5) in *Arabidopsis thaliana* may serve as two examples of such combinatorial response regulation. The AGP is the committed step in starch synthesis in source and sink tissue, e.g., in the endosperm of maize kernels. AGP is composed of a large (maize mutant Shrunken-1) and small (maize mutant Brittle-2 [Bt-2]) subunit (Okita et al. 1990). Sucrose and ABA synergistically stimulate starch accumulation in maize endosperm. This synergism could be linked to two cis-regulatory regions in the Bt-2 promoter, the region between −370 and −186 mediates the sucrose response and the region between −186 and −43 the ABA response (Li et al. 2018).

ABI5 is a basic leucine zipper transcription factor that positively regulates ABA-dependent stress acclimation, especially under drought, along developmental processes such as seed development and germination (Finkelstein and Lynch 2000). ABI5 is expressed throughout the plant life cycle in many cell types and in response to multiple environmental stresses. Several independent and dependent signalling pathways antagonistically or synergistically modulate ABI5 gene expression. Important

Interference in gene expression
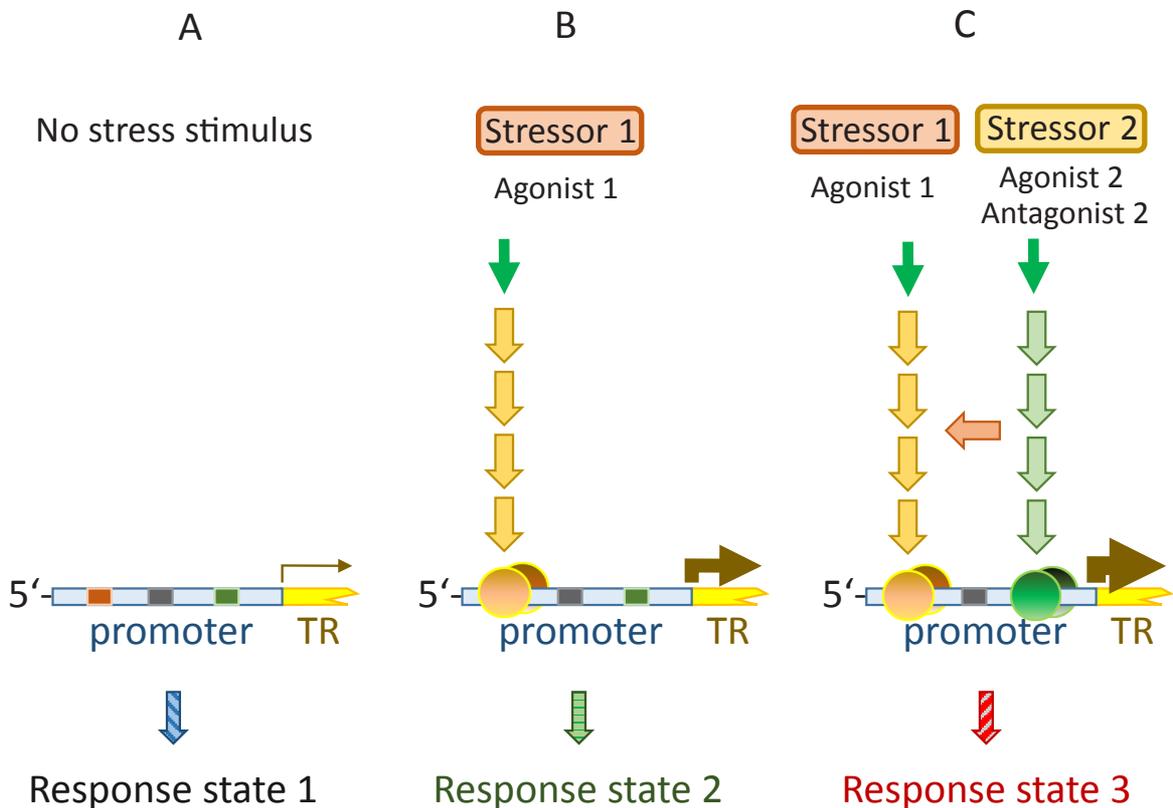
Figure 4. Combinatorial control of gene expression. The promotor driving the expression of the transcribed region (TR) contains three cis-regulatory elements (CRE, black, orange, green). Stressor 1 activates the signalling pathway causing the orange transcription factor (TF) to bind to the orange CRE and enhancing gene transcription. Stressor 2 activates another signalling pathway and the green TF binds at CRE and further stimulates gene expression in a synergistic manner. The binding could also have antagonistic effects; in this case, binding of the green TF would suppress gene expression even in the state of bound orange TF. Signalling crosstalk may occur upstream of the different TFs.

cis-regulatory elements are G-boxes and GT1-motifs (Bai et al. 2019). The B-box protein BBX19 suppresses seed germination via activating ABI5 gene expression. BBX19 binds to the light-responsive GT1-cis-regulatory element (Bai et al. 2019). In line with this finding, BBX19-overexpressors display ABA hypersensitivity. This pathway is independent of ELONGATED HYPOCOTYL 5 (HY5), which also directly activates expression of ABI5 and might antagonize the gibberellic acid signaling pathway promoting seed germination. In a converse manner, BBX21 suppresses ABI5 expression by interacting with HY5 (Xu et al. 2014). Another feedforward circuitry is an autoactivation of the ABI5 gene by ABI5 protein. Several other transcription factors bind to the promoter of ABI5, e.g., FHY3/FAR1 to the FHY3/FAR1-binding site (FBS; CACGCGC) and ABI4, an APETALA 2/ETHYLENE

RESPONSE ELEMENT BINDING PROTEIN to a CE1-like element (CACCG) (Xu et al. 2014). It is obvious, that a dedicated cross-talking regulatory network controls expression of ABI5 integrating several input signals such as ABA and light.

These two examples demonstrate the power of signal integration at the level of gene expression employing combinatorial input from diverse signaling pathways, mediating synergistic, or antagonistic responses in stress acclimation.

8. Interference in combinatorial stress acclimation must be considered as integrated phenomenon
The previous sections elaborated on principle mechanisms of signal integration with a focus on gene expression, protein assembly, PTMs and the role of small molecules.

Still, there usually remains a gap between individual molecular mechanisms and whole plant responses to single and combinatorial stresses. The reason is that each mechanism is embedded in a molecular hierarchy and consecutive order of events with regulation at each level (Figure 5). Each regulatory level may contribute to observed synergistic, antagonistic, or indifferent responses. It is noteworthy that many regulatory levels have not been scrutinized under the impact of combinatorial stressors.

Following gene expression, alternative splicing of transcripts in the nucleus produces several splice variants resulting in different properties such as distinct subcellular targeting, regulation, or activity (Filichkin et al. 2010). Combined heat and drought stress in wheat increased the number of alternative splicing events from 200 (drought) and 3576 (heat) to 4056 (Liu et al. 2018). The authors concluded that alternative splicing is a major mechanism

for tuning acclimation and a potential target for crop tolerance engineering (Liu et al. 2018). This conclusion was supported for grapevine exposed to drought and heat (Ju et al. 2021). Interestingly, a group of differentially expressed genes concerned the spliceosomes with 19 transcripts under heat stress relative to control, 4 under drought, but 36 under combined stress (Ju et al. 2021), underpinning the importance of alternative splicing for efficient combinatorial stress acclimation.

Messenger ribonucleo proteins (mRNP) associate with each mRNA and co-determine its fate for export from the nucleus and partitioning between ribosome association in functional polysomes, stress granules and processing bodies (P-bodies) and ultimately translation versus storage and decay. Stress granules are cytoplasmic assemblies of mRNAs and proteins formed under various stresses, e.g., heat (Hamada et al. 2018), while P-bodies target mRNA
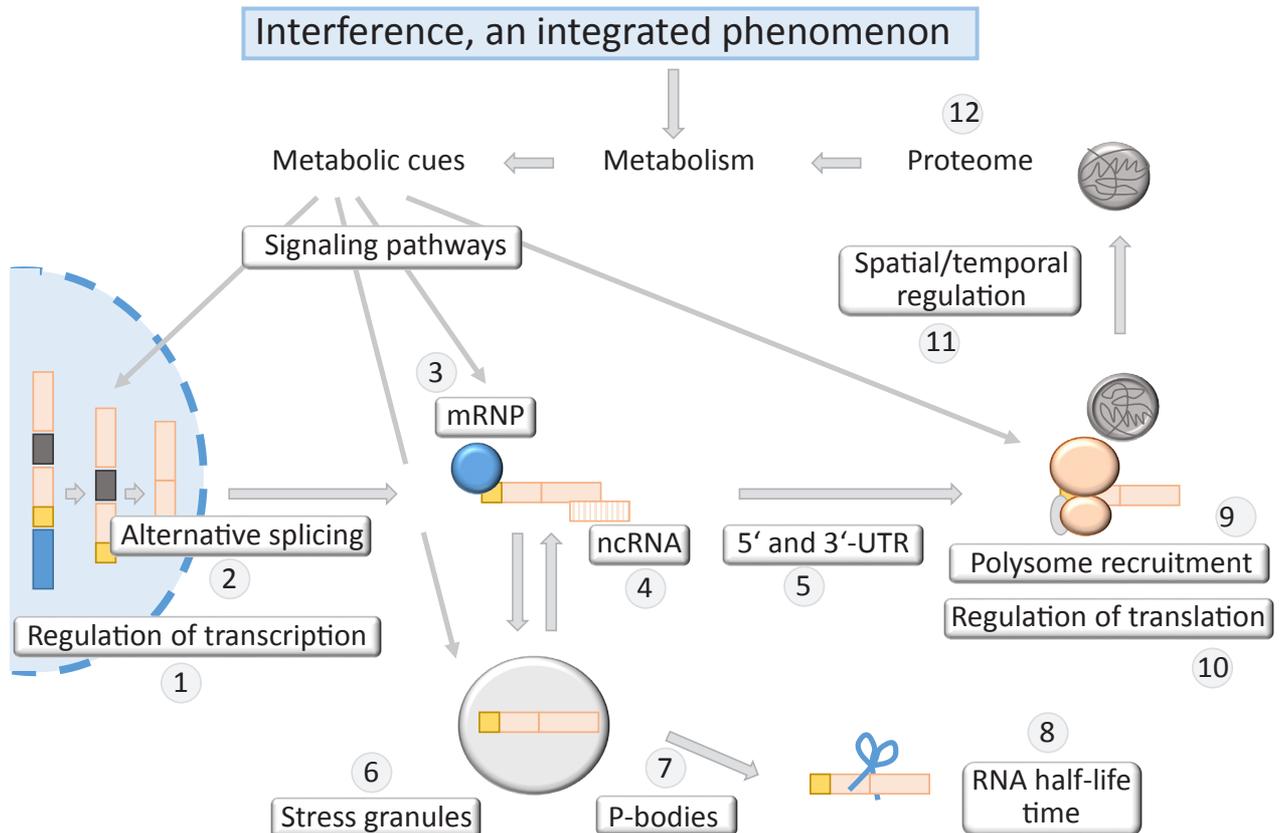
Figure 5. Necessity to consider the various molecular levels of regulation to understand stress interference. The majority of research focuses on transcript amounts which in fact reflect the balance between gene expression (1) and posttranscriptional activities, in particular degradation. Alternative splicing (2), binding of messenger ribonucleoproteins (mRNPs, 3), the action of noncoding RNAs (4) and the regulatory role of 5'- and 3' untranslated regions (5) are decisive features that determine the efficiency of recruitment to translationally active polysomes (9, 10), to stress granules for storage (6) or P-bodies for processing and degradation (7, 8). These different layers of regulation define the proteome (12) with an additional effect of the spatial and temporal partitioning of proteins, e.g., between cytosol and nucleus or by dual targeting (11). Indifference of response may be caused by antagonistic effects like upregulation of transcription but downregulation of recruitment to translationally active polysomes or decreased RNA half-life.

for de-capping and degradation (Weber et al. 2008). The assemblies in stress granules are dynamic and differ between stresses. For this reason, Weber et al. (2008) preferred the term heat stress granule for assemblies formed at elevated temperature. P-body dynamics plays a pronounced role in controlling plant immunity against bacterial infections by controlling transcriptome reprograming (Yu et al. 2019).

The cellular mRNA pool associated with actively translating ribosomes is termed translatome and undergoes rapid and profound reorganization upon environmental changes, e.g., upon wounding (Chen et al. 2021) or following the shift of low light-acclimated plants to high light (Moore et al. 2021). Only this pool is used to synthesize de novo proteins. The preferential recruitment of mRNAs depends on features of the mRNA, in particular sequence motifs and secondary structures in the 5'- und 3'-untranslated region. Comparison of total mRNA pools with translatomes reveals sets of transcripts that are preferentially associated with translating ribosomes under a given condition, while other mRNA species are underrepresented in the translatome (Kage et al. 2020, Moore et al. 2020). RNase-protected footprints are sequenced to differentiate stalled from actively translating ribosomes. If certain footprints are highly enriched, ribosomes are arrested at stalling sites and show no or low translation efficiency (Kage et al. 2020). Stress effects on translatomes are increasingly determined, e.g., under hypoxia or upon pathogen infection, but regulation under combinatorial stress conditions await future scrutiny (Mustroph et al. 2009, Meteignier et al. 2017).

Regulatory non-coding RNA molecules interfere with various posttranscriptional processes like RNA degradation and translation. microRNA guides the precise cleaving process on their target mRNAs (Sunkar et al. 2012). Global analyses of noncoding RNA have revealed highly dynamic responses of their transcription and stability to environmental stresses such as drought and heat (Hivrale et al. 2016, Seeve et al. 2019). Research on this type of regulation needs intensification.

An additional layer of regulation is given by adjusting the subcellular localization of the translated polypeptides in response to the prevailing environmental condition. Alternative splicing of mRNAs to generate proteins with different targeting addresses, piggybacking of proteins lacking transit information by proteins with transit or localisation sequences (Hackenberg et al. 2012), proteolytic cleavage and other PTMs can control subcellular distribution of proteins under stress (Klein et al. 2012, Giesguth et al. 2015). A highly dynamic process is the cytosol-nucleus partitioning of regulator proteins such as transcription factors, a process that responds to stresses (Yu et al. 2016), as, for instance, the degree of dual targeting of proteins between plastids and mitochondria (Van Aken et al. 2009).

Each of these processes and mechanisms has the potential to affect the stress acclimation response but their analysis under combinatorial stress treatments is often missing. The interaction between gene expression and posttranscriptional regulation, e.g., recruitment to actively translating ribosomes may synergize or antagonize each other. There is a need to integrate the regulatory levels to fully understand stress acclimation and to explain the frequently observed discrepancy between changes in transcript amounts, de novo protein synthesis and protein accumulation (Oelze et al. 2014).

9. Outlook

Considering the large number of possible interaction scenarios between different stressors, we are just at the beginning of understanding combinatorial stress responses for few selected examples. Obviously, it is needed to continue with taking the inventory of stress interactions at the phenomenological scale. Our mechanistic understanding of synergistic, antagonistic, and indifferent phenomena depends on qualitative, quantitative and theory-based description of those signal integration modules that display a high centrality in cellular communication. Knowledge of these modules as input signal integrators in intracellular communication has, then, to be projected into the global scale of physiological and postgenomic context and is considered as a decisive step towards understanding network function in order to finally approach a systems' biology type of understanding of signalling, regulation, and physiological acclimation in combined stress scenarios.

Several steps must be made toward this goal, namely (i) the qualitative and quantitative analysis of the molecular interactions and integration mechanisms, (ii) the establishment of the network environment of the signal integration module, and (iii) the elaboration of the global significance of the considered integration process. In the end, it is the question whether the experimental results and observations describe a robust, essential, and central mechanism or a phenomenon solely valid for a particular scenario of conditions such as seedling growth on sterile medium.

The accessibility of the mass of data from many studies is far from optimal. On the one hand, critical issues start with incomplete information contained in the searchable elements of the publications, continue with missing essential details in materials and methods and the lack of unbiased description of the type of independent replication and applied statistics (Rogers et al. 2021). Research reporting should adopt a unified policy of minimum information provision. This is particularly important for studies on stress combinations where the regulatory state of the plants may be particularly sensitive to perturbations and subtle differences.

On the other hand, the analytical methods, e.g., of metabolomics and proteomics reach unprecedented sensitivity. New tools for interactome prediction, visualization, and modelling ease the integrating view on big data from various data sources (Waese et al. 2017). Machine learning approaches identify patterns and regulators in gene regulatory networks based on large transcriptome data sets (Wulf and Bräutigam 2020). Based on such methodological progress and in light of the pressing issue of climate change, research on the impact of combinatorial stresses on plant performance must be propelled in the near future. This review provides an introduction to approaches needed to mechanistically understand plant elasticity, plasticity, and robustness in stress acclimation needed for sustained growth and to overcome the frequent conclusion in many studies that plant responses to combined stresses cannot be predicted from established responses to individual stressors.

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Conflict of Interest

The author encounters no conflict of interest.

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