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Review

Tansley insight

Stress-adaptive gene discovery by exploiting collective decision-making of decentralized plant response systems

Author for correspondence:
Stephen Chivasa
Tel: +44 (0)191 334 1275
Email: stephen.chivasa@durham.ac.uk

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Stephen Chivasa and Heather Louise Goodman

Department of Biosciences, Durham University, South Road, Durham, DH1 3LE, UK

Contents

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Summary

Despite having a network of cytoplasmic interconnections (plasmodesmata) facilitating rapid exchange of metabolites and signal molecules, plant cells use the extracellular matrix as an alternative route for cell–cell communication. The need for extracellular signalling in plasmodesmata-networked tissues is baffling. A hypothesis is proposed that this phenomenon defines the plant extracellular matrix as a 'democratic space' for collective decision-making in a decentralized system, similar to quorum-sensing in bacteria. Extracellular communication enables signal integration and coordination across several cell layers through ligand-activated plasma membrane receptors. Recent results from drought stress-adaptive responses and light-mediated signalling in cell death activation show operational utility of this decision-making process. Opportunities are discussed for new innovations in drought gene discovery using platforms targeting the extracellular matrix.

I. Introduction

Although capable of solitary existence, prokaryotic cells in a colony often work cooperatively to trigger synergistic responses benefitting the entire community (Whiteley *et al.*, 2017). Likewise, eukaryotic cells in a tissue work cooperatively to ensure that the correct developmental processes are activated and appropriate stress response programmes are timeously deployed. These collaborations are underpinned by complex cell–cell communications. An understanding of how cells interact with each other in the context of their immediate physicochemical environment can provide fundamental insights into how biological systems work and spark new ideas for new biotech innovations.

Plant cells have cytoplasmic interconnections (plasmodesmata) enabling rapid exchange of metabolites and propagation of signal molecules to neighbouring cells. The cellular networks in different tissues and organs are interlinked by phloem across the entire plant body. This enables proximal and distal signal transmission for coordinating growth processes and stress-adaptive responses. Restriction of plasmodesmata-dependent transfer of molecules ≥ 524 Da, by mutation of the plasmodesmata size exclusion limit-regulatory *DECREASED SIZE EXCLUSION LIMIT 1 (DSE)*

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gene, triggers growth retardation and formation of defective floral organs in *dse1* Arabidopsis mutants (Xu *et al.*, 2012). Impedance of diverse plasmodesmata-routed molecules (reviewed by Sager & Lee, 2018), such as transcription factors (Sessions *et al.*, 2000; Wu *et al.*, 2003) gene regulatory RNAs (Xoconostle-Cázares *et al.*, 1999; Klahre *et al.*, 2002) and other proteins (Ishiwatari *et al.*, 1998) may underpin the extreme phenotype of plasmodesmata constriction. Despite the availability of such a well-developed symplastic transport network, plants additionally use extracellular signalling via the apoplast.

Signalling initiated by extracellular microbial ligands binding to plant cell surface receptors characterizes many interactions with symbiotic partners or pathogenic microbes. Nevertheless, extracellular signalling between plant cells via secreted signals acting on plasma membrane receptors has emerged in diverse processes, such as maintenance of apical meristem cell populations (Li *et al.*, 2017), immune responses (Zhang *et al.*, 2018) and responses to wounding (Toyota *et al.*, 2018; Hander *et al.*, 2019; Hou *et al.*, 2019). Furthermore, apoplast transport and propagation of signal molecules with intracellular receptors and/or targets also has been reported. For example salicylic acid (Lim *et al.*, 2016) and glutamate (Wudick *et al.*, 2018) are transported via the apoplast even though the target/receptor proteins may be intracellular. Although the need for cell surface signalling in plant interactions with microbes is clear, the advantages of apoplast signal transmission are not obvious. This article highlights occurrence of this phenomenon in plant responses to abiotic factors (drought and light) and discusses how similarities with prokaryotic signalling systems support a new hypothesis to rationalize extracellular signalling. Framing research questions using this hypothesis could redirect focus to the emerging importance of the plant extracellular matrix, yielding tremendous insights to understand complex plant physiological processes.

II. Intercellular signalling – where a single cell can call the shots

Cytoplasmic signalling enables a single cell to initiate signalling and control physiological processes, as demonstrated by some multicellular photosynthetic prokaryotic systems, which can fix nitrogen (N). Nitrogen-fixing cyanobacteria in the genus *Anabaena* grow as filaments consisting of chains of pluripotent photosynthetic cells when supplied with sufficient nitrate or ammonium in the growth medium (Abedi *et al.*, 2019a). Nitrogen limitation activates differentiation of one in every 10–20 photosynthetic cells into thick-walled heterocysts, which are capable of fixing atmospheric N_2 (Abedi *et al.*, 2019b). Because N-fixing nitrogenase enzymes are sensitive to O_2, photosynthesis in the heterocysts is diminished and thick cell walls form a diffusion barrier to exclude dissolved oxygen uptake. The photosynthetic cells exchange sugars and nutrients for nitrates with heterocysts through septal junctions, which are plasmodesmata equivalent structures (Nieves-Morión *et al.*, 2017).

Although all vegetative cells are capable of differentiating into heterocysts, tightly regulated cell–cell signalling along the filament controls the process to ensure only a few cells undergo transformation (Fig. 1). Sensing N deficiency requires the transcriptional regulator NtcA, which activates the master regulator (*hetR*) controlling vegetative cell differentiation into heterocysts. A positive feedback loop between HetR and NtcA amplifies the signal, with HetR ultimately activating many genes required for the morphological and metabolic transformation to heterocysts. Among the downstream genes activated by the transcriptional factor HetR are genes encoding two regulatory proteins PatS and HetN (Muñoz-García & Ares, 2016). Both PatS and HetN possess the bioactive pentapeptide RGSGR, which is transported from the emerging heterocyst to neighbouring vegetative cells to suppress their differentiation into heterocysts. The pentapeptide directly binds to HetR (Hu *et al.*, 2015), destabilizing its interactions with DNA sequences to inhibit establishment of the transcriptome necessary for vegetative cell morphogenesis into heterocysts. PatS inhibits proximal vegetative cell differentiation in the immediate aftermath of N step-down, whereas HetN is deployed at later time points to maintain the status quo (Muñoz-García & Ares, 2016).

The bioactive peptide has a long-range inhibitory activity, suppressing differentiation of *c.* 10–20 vegetative cells on both flanks of the producing heterocyst. This example illustrates cell communication in a simple multicellular prokaryotic system, where a single cell 'makes decisions' that direct cell fate of its neighbours and influence what happens to the entire filament. The signal (RGSGR pentapeptide) is generated by the single preheterocyst/heterocyst cell and transported presumably via septal junctions to vegetative cells in the neighbourhood (Nieves-Morión *et al.*, 2017). Although this type of signalling is suited for rapid metabolic adjustments in cyanobacterial filaments to exploit the changing nutrient profile of their fairly homogenous aquatic environment, such a strategy might not always be appropriate for land plants that have to contend with the heterogeneous soil substratum and canopy growth habit creating variable microenvironments across different parts of the same plant. Thus, for plants certain 'decisions' have to be made on the basis of signals from several cells to ensure that the response reflects the needs of the entire tissue/plant. Although the symplastic pathway could efficiently route signals from multiple cells, the danger is that a minimum of a single cell is enough to stimulate the response. This would not be problematic in the case of pathogen defence, as plants with early detection and swift responses are likely to be successful in repelling pathogen attack. However, in response to aerial or subterranean abiotic stresses, a different approach is required due to heterogeneity characterizing these environments.

III. Extracellular signalling – a more democratic decision-making process

Quorum sensing bacteria demonstrate a prokaryotic cell communication system that entails signal transmission by a majority of individual cells in a community to synchronize collective response when a signal threshold has been attained (reviewed by Mukherjee & Bassler, 2019). The cells secrete a signal molecule known as an autoinducer, which binds to specific plasma membrane receptors to activate intracellular signalling when a sufficiently high cell density has been reached. It is not coincidence that the signal is secreted into the extracellular space, and is subsequently perceived by receptors

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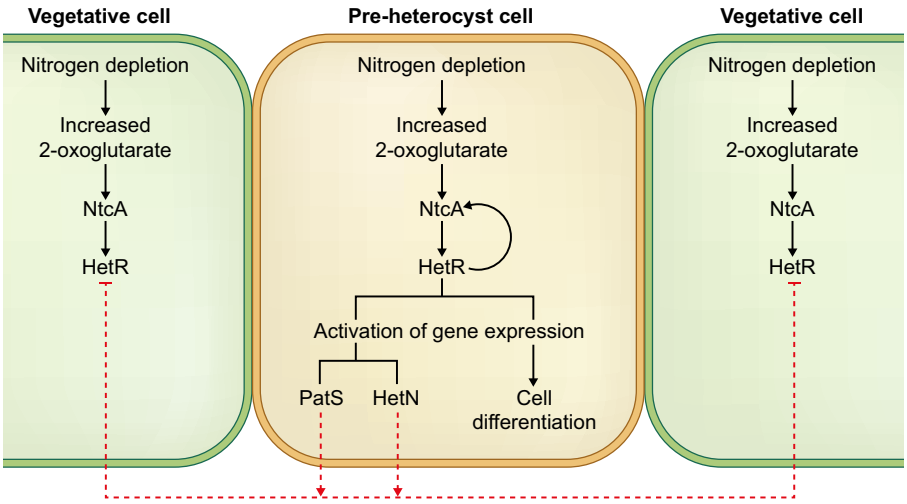

Fig. 1 Schematic representation of heterocyst differentiation in filamentous cyanobacteria. Depletion of combined nitrogen in the growth medium triggers metabolic accumulation of 2-oxoglutarate, which leads to activation of two key transcription factors, NtcA and HetR. NtcA activates transcription of *HetR*, which in turn activates *NtcA* transcription to amplify the signal. HetR directly binds to promoter sequences of downstream genes responsible for differentiation of the vegetative cell to a heterocyst. However, two genes downstream of HetR encode regulatory proteins (PatS and HetN), which diffuse into neighbouring vegetative cells where the bioactive pentapeptide (RGSGR) directly binds HetR to inhibit activation of gene expression. The mechanism by which HetR in the producing cell is not inhibited by PatS and HetN proteins derived from the same cell is not currently known. PatS is produced early during heterocyst differentiation, whereas HetN is produced after differentiation is complete, to perpetuate inhibition of neighbouring vegetative cells from developing into heterocysts. Black lines connect interlinked components/processes, with the arrow pointing at the component/process activated by the preceding factor; dashed red lines with arrows indicate that PatS and HetN move out from the producing pre-heterocyst; dashed red lines with a 'T-junction' at the end indicate that PatS and HetN move into neighbouring vegetative cells to bind and inhibit HetR. The red lines do not chart the path of movement of PatS and HetN. Both proteins and/or the derived bioactive peptides are suspected to move from cell-to-cell via the septal junctions.

localized at the cell surface. This caters for integration of signal molecules from multiple cells in the same space, thereby 'democratizing' decision-making. Sensing the signal at the cell surface allows all cells in the community sharing the same volumetric space to be synchronously activated by ligand molecules with no diffusion barrier, as is the case for intracellular receptors. This community-wide coordination is invoked in physiological processes, such as biofilm formation, bioluminescence and production of virulence factors during host tissue invasion. Solitary bacterial cells would not be able to perform such processes. It is proposed that the extracellular matrix provides plants with a functional mechanism to modulate cooperative signalling and benefit from quorum processes in a similar fashion to quorum sensing seen in some prokaryotes.

Plants operate a decentralized system in which 'decisions' for certain physiological processes are undertaken locally and the response restricted to tissues in the vicinity of the primary stimulus. However, certain 'decisions' require propagation to the entire plant body, exacting an enormous energy burden that could be catastrophic if repeatedly deployed on false alarm. The present authors contend that by integrating signals arising from several cells in the apoplast, a more representative response averaged across cells located in different micro-environments is triggered. For example, the drought response is initiated in the roots by sensing the soil water deficit followed by activation of biosynthesis of a mobile signal to trigger adaptive responses in aerial organs (Takahashi *et al.*, 2018). Secreted signal integration in the apoplast would dampen-down unrepresentative spurious signals emanating from roots in contact with drier soil clumps or air pocket in an otherwise reasonably watered environment.

For quorum sensing to work, the effective concentration (EC) giving half-maximal response (EC_{50}) of the autoinducer is set high to ensure that only in high density bacterial populations, which accumulate a high secreted ligand concentration, is the physiological response activated. The EC_{50} of an agonist is the molar concentration producing 50% of the maximal possible stimulatory/inhibitory effect in a biological system (Neubig *et al.*, 2003). In this context, it provides a relative measure of the concentration at which receptors become responsive to cognate ligands. The EC_{50} of autoinducer-1 (3-hydroxybutanoyl homoserine lactone) of *Vibrio harveyi* is 23 nM, which likewise is in the nanomolar–micromolar range as secreted plant signalling ligands, such as extracellular ATP (EC_{50} of 2.6 μM), extracellular NAD^+ (> 436.5 nM), and brassinosteroids (*c.* 50–100 nM) (Table 1). Even peptide signals secreted into the extracellular matrix have EC_{50} values in the nanomolar range, for example INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), the peptide controlling Arabidopsis floral abscission through plasma membrane receptor signalling has an EC_{50} of 33 nM (Table 1). It stands to reason that extracellular plant cell signalling requires a high EC_{50} for secreted signals to ensure that signal molecules from several cells are integrated before physiological stimulation. By contrast, pathogen- or plant-derived ligands associated with defence tend to have relatively low EC_{50} values (in the picomolar range), presumably because single cell detection and signal activation are desirable to mount a swift robust defence. For example, systemin, the wound-induced defence signal, and the pattern-triggered immunity-inducing 22-amino acid peptide of flagellin (flg22) have EC_{50} values of *c.* 30 pM, whereas the bioactive 18-amino acid

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Table 1 Characteristics of plant and microbial ligand-receptor pairs in cell–cell signalling

Ligand[1]	Origin	Receptor(s)	EC_{50}[2]	Reference
flg22	Bacterial	FLS2	30 pM	Felix *et al*. (1999)
elf18	Bacterial	EFR1	300 pM	Schwessinger *et al*. (2015)
AI-1	Bacterial	LuxN	23 nM	Swem *et al*. (2008)
IDA	Plant	HAE/HSL2	33 nM	Butenko *et al*. (2014)
Systemin	Plant	SYR1/SYR2	30 pM	Wang *et al*. (2018)
Extracellular NAD^+	Plant	LecRK-1.8	> 436.5 nM	Wang *et al*. (2017)
Extracellular ATP	Plant	DORN1	2.6 μM	Demidchik *et al*. (2003)
Brassinosteroids	Plant	BRI1	50–100 nM	Clouse *et al*. (1996); Nam & Li (2002)

Ligand-receptor pairs were selected from the literature on the basis of having a published EC_{50} value and the receptor being on the plasma membrane.
[1]flg22, the bioactive 22 amino acid peptide of bacterial flagellin; elf18, the bioactive 18-amino acid peptide of bacterial elongation factor-Tu; AI-1, autoinducer-1 (3-hydroxybutanoyl homoserine lactone) from *Vibrio harveyi*; IDA, inflorescence deficient in abscission peptide.
[2]EC, effective concentration; EC_{50}, effective concentration activating half maximal (50%) response.

residue peptide (elf18) of bacterial ELONGATION FACTOR-Tu activates plant defences at an EC_{50} of 300 pM (Table 1).

IV. Exploiting extracellular signalling for gene discovery in drought

The recent publication by Takahashi *et al.* (2018) shows that water deficit perception in roots leads to root production of a secreted mobile peptide signal, CLAVATA3/EMBRYO-SURROUNDING REGION-RELATED 25 (CLE25), and its export to leaves. On arrival in leaves, the CLE25 peptide signals via plasma membrane BARLEY ANY MERISTEM (BAM) receptors to modulate abscisic acid production and stomatal control. This study confirms that extracellular signalling is quite important and is likely invoked to ensure signal integration across root tissue in the highly heterogeneous rhizosphere to avoid unnecessary activation of drought stress responses. Sorghum cell cultures exposed to osmotic stress activated increased protein secretion, with the level of expression of the encoding genes showing a strong positive correlation with the level of drought-tolerance achievable across different sorghum lines (Ngara *et al.*, 2018). These results suggest that osmotic stress-induced gene expression and a surge in protein secretion could play a wider role in drought stress-adaptive responses. Most of these proteins have not been characterized biochemically, but could turn out to be pivotal signal-regulatory proteins.

In order to exploit the propensity of plant cells for collective decision-making via the extracellular matrix in gene discovery, researchers are intercepting secreted metabolites and proteins to identify core cell death-regulatory proteins. Cell death induced by heavy metal ions (Wang *et al.*, 2013), a mycotoxin (Stone *et al.*, 2000) or herbicide (Chivasa & González-Torralva, 2018), is blocked by dark incubation, suggesting the existence of a shared light-dependent pathway or critical components. The mycotoxin fumonisin B1 (FB1) activates cell death in light-grown Arabidopsis cell cultures, with dark-grown cultures showing immunity (Fig. 2a). However, when the growth medium from light-grown cultures is applied to dark-grown cells, the latter behave like light-grown cells and become susceptible to FB1, even though they are exposed to the toxin under dark incubation (Fig. 2a). These results show that light-dependent secreted extracellular signals/factors control the response of cells to FB1 and ongoing research focuses on identifying putative candidate signals secreted in the extracellular matrix. It is expected that such signals are secreted in light-grown, but not dark-grown cell cultures, and would be possibly responsive to FB1 addition. A candidate gene with such an expression profile is *CYCLASE1*, which encodes a protein secreted into the extracellular matrix (Smith *et al.*, 2015). *CYCLASE1* is upregulated by FB1 treatment only in light-grown, but not dark-grown cells (Fig. 2b). Previous results from the present authors have demonstrated that loss-of-function gene knockout (*cyclase1*) mutants are immune to FB1-induced cell death (Smith *et al.*, 2015), suggesting that CYCLASE1 is part of the secreted machinery controlling Arabidopsis response to FB1.

A central focus of research by the present authors is understanding drought stress-adaptive responses in plants. Together with studying cell death in response to FB1, the focus also is on investigating programmed cell death invoked during drought-adaptive responses as a way to reconfigure root architecture by killing upper lateral roots to promote deeper root growth in pursuit of the receding water table. It remains to be seen if the secreted signals used in controlling FB1-induced cell death have any relevance in drought-induced cell death. Preliminary data using *CYCLASE1* show a strong response of this gene to drought and osmotic stress (data not shown). Thus, it is predicted that studying the extracellular matrix on the basis of the hypothesis proposed here could facilitate gene discovery in many plant stress-adaptive processes, including drought.

V. Conclusions

Cells in tissues constantly communicate with their neighbours, transmitting positional information, signals about their metabolic status and their perception of the external environment. A surge in cell–cell communication precedes adaptation to stress, with either the symplast or apoplast route providing signal passage. The symplast pathway can theoretically enable a single cell to initiate and propagate signalling (Fig. 3a). The contention herein is that a more democratic decision-making process is invoked by routing signals via the apoplast (Fig. 3b), particularly

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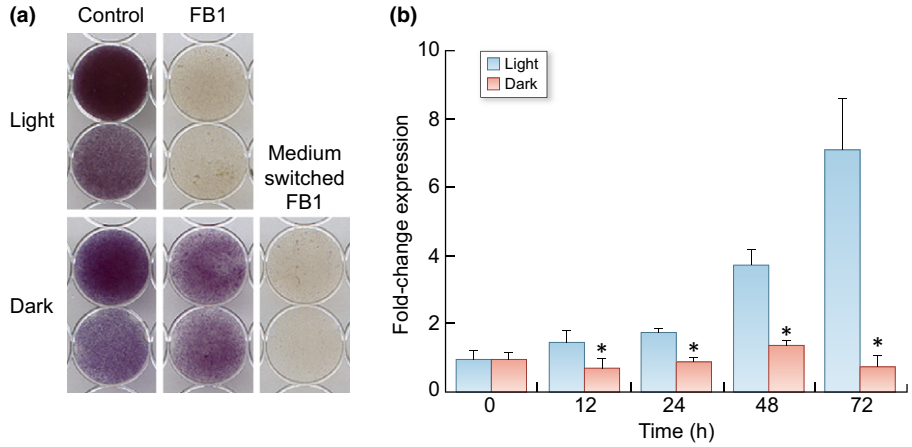

Fig. 2 Secreted light-dependent signals regulate FB1-induced cell death. (a) Arabidopsis cell cultures were grown as described previously (Smith *et al*., 2015) in light or darkness and then treated with 2 μM fumonisin B1 (FB1). After 72 h, a lawn of cells analyzed for cell death using the MTT assay (Chivasa *et al*., 2005) was photographed. Living cells convert the MTT solution to purple, whereas dead cells remain cream-coloured and unstained. FB1 killed cells incubated in the light, with dark-incubated cells showing immunity; however, when dark-grown cells had their growth medium removed and replaced with medium in which light-grown cells had been growing, they lost their immunity to the mycotoxin and died even though they were incubated in the dark. This shows that light-dependent signals secreted into the medium control Arabidopsis response to FB1. (b) Expression of *CYCLASE1* gene in FB1-treated light-grown or dark-grown cell cultures, showing significant suppression of the gene in darkness. Arabidopsis *cyclase1* knockout mutants are immune to FB1 (Smith *et al*., 2015). This confirms the importance of light-dependent secretions into the apoplast during stress responses. Quantitative PCR was conducted as described previously (Ngara *et al*., 2018) using At3g13920 and At3g18780 as constitutive reference control genes. Bars are mean ± SD ($n = 3$). Significant fold-change differences between light- and dark-grown cells: *, $P < 0.001$.

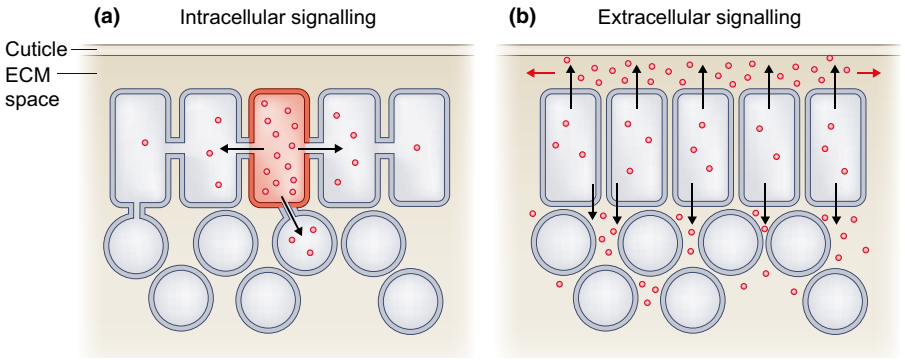

Fig. 3 Cell–cell signalling via the symplast and apoplast. (a) Intracellular signalling can be initiated in a single cell or a few cells and relies on signal diffusion between adjacent cells via plasmodesmata. In this schematic, signalling molecules are produced by the cell (red colour) and propagated to all its neighbours. In fairly homogenous systems, activation of physiological processes by a single cell does not run the risk of triggering false alarms and costly energy diversion because what the single cell has sensed is representative of the environment and the corresponding response is appropriate for the plant. For example, turbulence from wind action and waves can mix the water in aquatic systems, creating a fairly homogenous environment with respect to nutrient distribution. (b) Extracellular signalling initiated by multiple cells enables integration of signal molecules in the extracellular matrix, which then activate receptors on the cell surfaces to ensure a synchronous response. This type of signalling is more appropriate under heterogeneous environments within the soil or plant canopy. For example, a thick plant canopy is highly heterogeneous in terms of the amount of light penetrating through it, with leaves on the top or flanks of the canopy receiving more light than leaves inside the canopy. It is argued herein that the scenario in (a) is used when, as an example, an incompatible pathogen is detected. Theoretically, the stylet of a viruliferous aphid can deliver pathogenic viral particles in a single cell where activation of a hypersensitive response and systemic acquired resistance can be initiated. In this scenario, the energy cost associated with a swift response to immunize the entire plant against subsequent pathogen attack is a necessary investment. It is additionally argued that the scenario in (b) is used as a way to reduce or avoid triggering a costly response, such as a drought response on the basis of, for example, part of a root hair entering a dry air pocket while the rest of the plant is adequately watered. Please note that epidermal cells have been omitted from the schematic diagrams to draw attention to the extracellular matrix (ECM) space.

in response to heterogeneous abiotic factors, such as light and water availability. Although it is vital to set very low signal intensity thresholds for activating pathogen/pest counter-measures at the behest of a single or few cells, a much higher threshold is necessary for signal integration across a broad swathe of cells. This bodes well for the use of metabolomics and proteomics as tools to analyze extracellular signals in the run up to stress adaptation. It is predicted that targeting the extracellular

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compartment for discovery of new metabolites, peptides and signal-regulatory proteins important in growth and stress-adaptive responses could yield very exciting results.

Discovery of plant extracellular vesicles carrying cargo normally localized in the cytoplasm (Baldrich *et al.*, 2019) may define a previously unforeseen additional pathway for exchange of signal molecules and signal integration within the extracellular space. Furthermore, identification of plasma membrane receptors for extracellular ATP (Choi *et al.*, 2014) and extracellular NAD^+ (Wang *et al.*, 2017) is an indication that metabolite signals thought to be exclusively cytosolic may turn out to have authentic extracellular targets. Taken together, this shows that the plant extracellular matrix is emerging as a critical hub for signal integration to enable collective decision-making across diverse physiological processes, including stress adaptation.

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ORCID

Stephen Chivasa https://orcid.org/0000-0001-9284-4953

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