New Phytologist

Forum

Viewpoint

Nonbinary fungal signals and calcium-mediated transduction in plant immunity and symbiosis

Summary

Chitin oligomers (COs) are among the most common and active fungal elicitors of plant responses. Short-chain COs from symbiotic arbuscular mycorrhizal fungi activate accommodation responses in the host root, while long-chain COs from pathogenic fungi are acknowledged to trigger defence responses. The modulation of intracellular calcium concentration – a common second messenger in a wide variety of plant signal transduction processes – plays a central role in both signalling pathways with distinct signature features. Nevertheless, mounting evidence suggests that plant immunity and symbiosis signalling partially overlap at multiple levels. We here elaborate on recent findings on this topic, highlighting the nonbinary nature of chitin-based fungal signals, their perception and their interpretation through Ca^{2+}-mediated intracellular signals. Based on this, we propose that plant perception of symbiotic and pathogenic fungi is less clear-cut than previously described and involves a more complex scenario in which partially overlapping and blurred signalling mechanisms act upstream of the unambiguous regulation of gene expression driving accommodation or defence responses.

Calcium as a universal signalling hub in plants

The modulation of intracellular calcium ion concentration ($[Ca^{2+}]$) has gradually emerged and developed as a ubiquitous and versatile signalling mode across the evolutionary tree of life, from prokaryotes to eukaryotes (Plattner & Verkhratsky, 2015; Luan & Wang, 2021). In plants, Ca^{2+}-mediated signalling plays a major role in the transduction of a broad range of very diverse environmental stimuli, including abiotic stresses and biotic interactions, such as herbivore bite or the perception of microbe-associated molecular patterns (MAMPs; Edel *et al.*, 2017). One possible explanation for the convergence of so many signalling pathways into one single ion is that Ca^{2+} is a rather reactive cation that can precipitate essential anions such as PO_4^{3-} and severely interfere with metabolic processes (Edel *et al.*, 2017). As such, cellular homeostasis maintains cytosolic and nuclear $[Ca^{2+}]$ in the range of 100 nM (roughly 10 000-fold lower than that in the extracellular space) through the constant action of ATP-powered Ca^{2+} pumps and/or Ca^{2+} exchangers (Luan & Wang, 2021). Any local increase above that baseline triggers immediate effects on structural and functional proteins, activating downstream responses. In fact, a long list of sensor proteins is known to be affected by Ca^{2+}, including Ca^{2+}-dependent protein kinases, calcineurin B-like proteins, calmodulin and calmodulin-like proteins (Ravi *et al.*, 2023). One major challenge in plant cell biology is the decoding of Ca^{2+}-mediated signals. In fact, converging evidence indicates that the correct coupling of upstream stimulation by environmental cues with downstream responses is achieved by encoding information in the transient changes in intracellular $[Ca^{2+}]$. The subcellular localisation, duration, amplitude and steepness of Ca^{2+} transients, as well as their frequency and regularity in the case of repeated oscillations (spiking), are therefore believed to contribute to the so-called 'calcium signature' that encodes information and transduces it downstream (Jiang & Ding, 2023).

Through the decades, the study of Ca^{2+} signalling in plants occurred through a number of different approaches, as new probes and technologies were developed. Pioneering studies based on cell loading with fluorescent Ca^{2+} dyes (Calcium Green-1, Fura-2 etc.) or Ca^{2+}-sensitive bioluminescent proteins (aequorin) were progressively complemented using genetically encoded Ca^{2+} indicators (GECIs), starting from the recombinant expression of aequorin and, more recently, fluorescence-based sensors such as Cameleon, GCaMP and GECO proteins (reviewed by Grenzi *et al.*, 2021). A consequence of this constant technological development is that, over the last few decades, different biological questions have often been addressed using different approaches, depending on which Ca^{2+} probes were available at the time. Some of the earliest investigations were based on the injection of fluorescent Ca^{2+} indicators such as Calcium Green-1 in individual cells before its exposure to pathogenic (Xu & Heath, 1998) or symbiotic elicitors (Ehrhardt *et al.*, 1996). Knight *et al.* (1991) were the first to demonstrate that aequorin-expressing *Nicotiana plumbaginifolia* generated measurable photon emissions upon cytosolic Ca^{2+} increases in response to touch, cold shock and biotic elicitors.

In the field of plant–microbe interactions, these pioneering studies revealed the onset of Ca^{2+}-mediated responses to several pathogen-associated elicitors, such as Ca^{2+} transients in suspended tobacco cells treated with oligogalacturonic acid (Chandra *et al.*, 1997), sustained elevations in $[Ca^{2+}]$ upon the perception of β-glucan (Mithöfer *et al.*, 1999), or biphasic Ca^{2+} variations in response to different *Phytophthora* elicitors (Lecourieux *et al.*, 2002). In the following years, a new class of ratiometric fluorescent GECIs, the most used being Yellow Cameleon (YC), was developed and successfully expressed in plants (Miyawaki *et al.*, 1999), ushering in a new era of high quantum yield and high-resolution Ca^{2+} imaging that provided subcellular scale details at the expense of the loss of precise quantitative information offered

New Phytologist

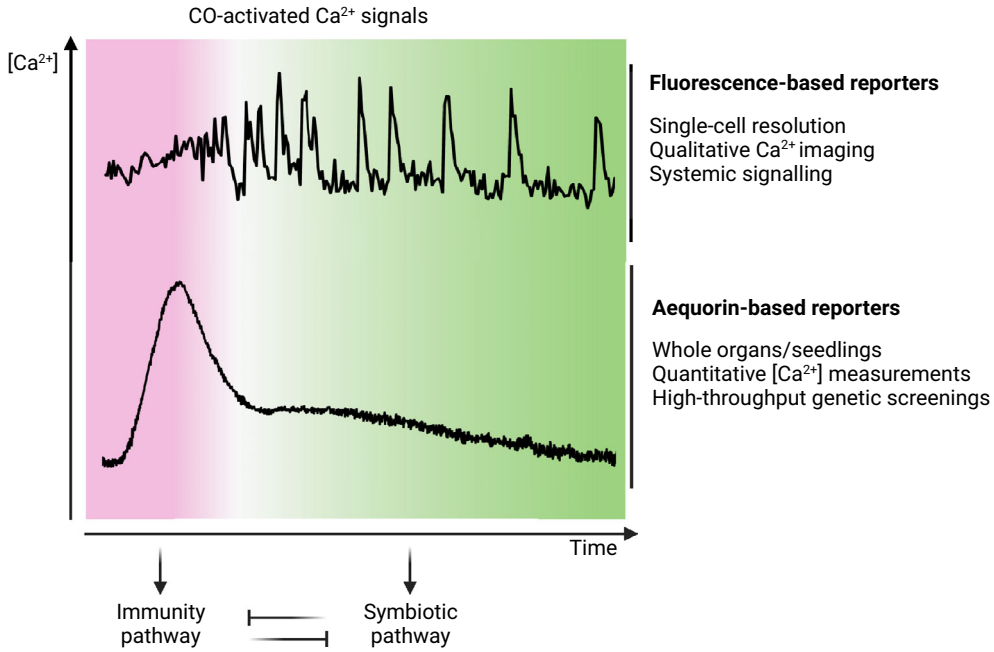

Fig. 1 Intracellular Ca^{2+} signatures underlying the plant defence-symbiosis continuum. Ca^{2+} signatures evoked in response to chitin oligomers (COs) leading towards defence or symbiotic responses reveal different patterns and dynamics depending on the Ca^{2+} reporter used. Fluorescence-based reporters such as cameleon or GECO (upper panel) allow the dissection of Ca^{2+} traces within single cells, revealing local and individual oscillations in $[Ca^{2+}]$. By contrast, bioluminescence-based reporters, such as aequorin, quantitatively monitor $[Ca^{2+}]$ variations as the sum of asynchronous traces from large cell populations within whole organs or seedlings. The comparison between the two approaches uncovers the biphasic nature of Ca^{2+}-mediated signals, associating an early increase in $[Ca^{2+}]$ (magenta background) with immunity-related responses and later spiking (green background) with symbiotic responses (Binci *et al*., 2023).

by aequorin. Studies based on such Ca^{2+} probes focussed for several years on the triggering of nucleus-centred Ca^{2+} spiking signals in diverse beneficial plant–microbe interactions (Barker *et al*., 2017). The most recently introduced intensiometric GECIs (such as GECO and GCaMP families) further improved quantum yield, allowing even more detailed studies of subcellular Ca^{2+} oscillations in defence- (Keinath *et al*., 2015) and symbiosis-related signalling (Kelner *et al*., 2018).

This diversity in methodological approaches to the investigation of Ca^{2+} signalling in plant–microbe interactions generated a corpus of literature data that is difficult to compare (Fig. 1) and the first attempts in this direction are only starting to appear (Binci *et al*., 2023).

The role of chitin-derived molecules in plant–fungus interactions

Microbe-associated molecular patterns (MAMPs) are well known to trigger Ca^{2+}-mediated signalling in plant cells. One of the most widespread and active fungal MAMP is chitin, the *N*-acetyl-glucosamine (GlcNAc) polymer representing the most abundant structural component of fungal cell walls. In particular, while the low solubility of fibrillar chitin is believed to limit its elicitation range to direct cell-to-cell contacts, diffusible chitin oligomers (COs) have been demonstrated to stimulate a number of plant responses when applied as water solutions. A quick cytosolic $[Ca^{2+}]$ elevation links the perception of pathogen-associated long-chain COs ($\geq$8 GlcNAc residues) with defence responses (Cao *et al*., 2014), while short-chain COs (4–5 GlcNAc residues) from symbiotic arbuscular mycorrhizal (AM) fungi trigger repeated oscillations in nuclear $[Ca^{2+}]$, known as Ca^{2+} spiking (Barker *et al*., 2017). Evidence in support of this clear-cut binary distinction led to a model where the alternative recognition of short vs long COs triggers a sophisticated competition mechanism in the assembly of alternative receptor complexes (Feng *et al*., 2019; Zhang *et al*., 2021; Fig. 2). However, the ability of both symbiotic and pathogenic fungi to release COs of variable length – and the technical challenge of determining the relative abundance of each molecule in raw fungal exudates – has prevented the correlation of an enrichment in short or long COs with the early recognition of symbionts and pathogens, respectively. One possible explanation comes from the identification of lipochito-oligomers (LCOs) in arbuscular mycorrhizal (AM) fungal exudates. These short-chain CO-derived molecules – very similar to rhizobial Nod factors – also activate nuclear Ca^{2+} spiking and have been proposed as a more specific signal, reinforcing – if not outcompeting – the symbiotic message of short COs (Oldroyd, 2013). Nevertheless, LCOs have also been shown to be present in the exudates of both symbiotic and nonsymbiotic fungi (Rush *et al*., 2020). Furthermore, the occurrence of additional signalling molecules specifically characterising AM vs pathogenic fungi has long been postulated on the basis of experimental evidence (Bonfante & Requena, 2011). Lastly, it is important to underline that most of the studies on plant responses to pathogenic fungi have been focussed on foliar interactions, whereas direct comparisons between root responses to symbiotic and pathogenic fungi remain very limited (Genre *et al*., 2009). This implies that any comparison between such responses has to take into account the distinct cellular, anatomical

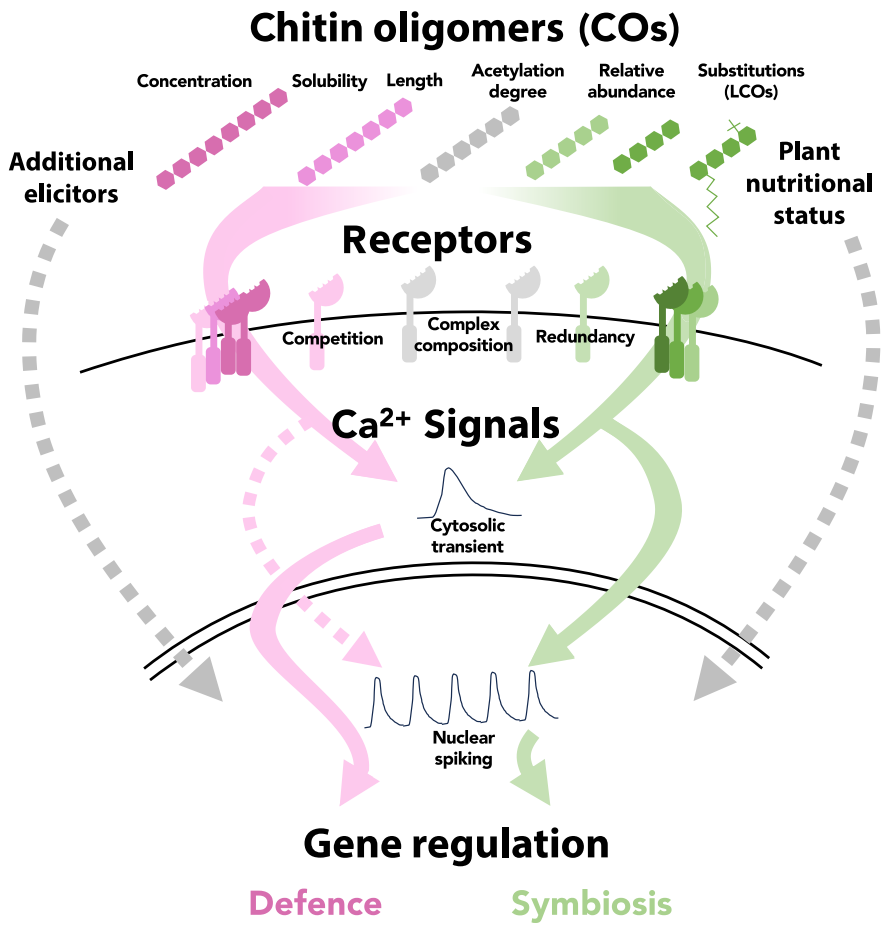

Fig. 2 Nonbinary signalling in plant immunity and symbiosis. Symbiotic and pathogenic fungi both release a mix of short- and long-chain chitin oligomers (COs). While a major distinction has been proposed between symbiosis-related (green) short-chain molecules (with optional substitutions comprising a lipid tail and a sulphate group, as in lipochito-oligosaccharides, LCOs) and defence-associated (magenta) long-chain COs, recent evidence suggests that this clear-cut distinction is not evident and additional factors such as CO concentration, the relative abundance and solubility of each molecule in fungal exudates, as well as their acetylation degree, may play crucial roles in the elicitation of downstream responses. A second level of complexity is emerging from the study of plant receptors involved in CO perception. Besides the variability observed between plant species, several studies suggest that the same receptors take part in different complexes, depending on the availability of individual COs, competition dynamics between membrane-associated complexes and functional redundancy. Changes in intracellular [Ca^{2+}] have been described as a central hub in signal transduction of both symbiosis and immunity signalling. In more detail, cytosolic Ca^{2+} transients are elicited by both types of fungi. A hallmark of symbiotic signalling is the induction of nuclear-centred Ca^{2+} spiking, even if long-chain COs have also been shown to induce this mechanism when applied as purified molecules (dashed magenta arrow). A major challenge in the biology of plant–microbe interactions is the disentanglement of this complex signalling scenario, which underpins the efficient and unambiguous regulation of gene expression towards defence or symbiotic responses. A possible contribution can come from the simultaneous perception of additional (nonchitinous) signals that are more specific to symbionts or pathogens and the physiological conditions of the plant, such as its nutritional status (dashed grey arrows).

and physiological background of the experiments (Zhang & Kong, 2021; Tehrani & Mitra, 2023).

Volpe *et al.* (2020), however, showed that the application of short-chain COs alone is sufficient to promote AM development, whereas exogenous long-chain COs can reduce AM colonisation (Zhang *et al.*, 2021), demonstrating that – in spite of our limited understanding of fungus-plant signalling – symbiotic MAMPs are interpreted univocally by the plants. Furthermore, a recent investigation by Yu *et al.* (2023) demonstrated the role of host plant-released extracellular lysin motif (LysM) proteins in specifically intercepting long-chain COs and quenching defence responses during arbuscule development. Whether an analogous mechanism also occurs during earlier steps of AM colonisation remains to be demonstrated, but the hypothesis is very intriguing.

Two additional aspects have anyway to be taken into consideration, which can explain the importance of short-chain COs as symbiotic signals. The first one is the ability of chitin-derived molecules to diffuse in soil solutions. Indeed, CO solubility in water is high for short-chain COs (as for most analogous oligosaccharides), but drops quickly as the chitin backbone grows beyond 6 GlcNAc residues (Lodhi *et al.*, 2014). This implies that selective pressure would favour the use of short-chain COs as diffusible signals for their ability to reach the host root earlier than longer molecules. Second, the strategies for host infection by fungal pathogens seemingly converge into a stealth approach, with multiple mechanisms aimed at preventing plant recognition of defence elicitors. In some of the best-studied models, these include the fungal release of LysM proteins that bind long-chain COs

released from the fungal wall by the action of plant chitinases, thus competing with plant receptors and suppressing the activation of host defence signalling (Volk *et al.*, 2019). In other words, fungal symbionts take a critical advantage in revealing their identity as early as possible, whereas pathogens are favoured by the opposite strategy. In line with this, AM fungi possess a number of chitin synthases and chitinases (Tisserant *et al.*, 2013; Sun *et al.*, 2019) granting autonomous and active production of short-chain COs – besides the long chitinous chains that make up their hyphal walls – and indeed AM fungi are known to release short-chain COs even in the absence of a plant host (Genre *et al.*, 2013).

Nevertheless, recent evidence suggests that the boundaries between the signalling cascades activated in the plant host in the two above-mentioned cases are less defined than previously thought. Both short- and long-chain COs can trigger a transient elevation of cytosolic [Ca^{2+}] (Binci *et al.*, 2023). Moreover, not only short- but also long-chain COs activate nuclear Ca^{2+} spiking in *Medicago truncatula*, rice and barley (Feng *et al.*, 2019; Zhang *et al.*, 2021; Li *et al.*, 2022). Nevertheless, the genetic repertoire needed in either case seems to differ (Feng *et al.*, 2019; Binci *et al.*, 2023). However, the few reports about CO application to plants in the presence of AM fungi show that short-chain COs promote, whereas long-chain COs inhibit symbiotic responses (Volpe *et al.*, 2020; Zhang *et al.*, 2021). This testifies that the plant perception of these fungal signals is likely more complex than previously thought, possibly involving factors such as their concentration, relative composition of bioactive mixtures, acetylation degree, plant nutritional status, competition between redundant receptors and the activation of additional signalling pathways acting in parallel (Fig. 2). Remarkably, while most studies on plant responses to chitin-based fungal signals have been performed by exposing whole organs to water-based solutions, under natural conditions the most intense signal exchange is likely to occur very locally, in the presence of direct contact or very close interaction between fungal hyphae and plant cells, that is a situation where the combined perception of multiple, soluble and surface-associated MAMPs has to be taken into consideration. Altogether this may contribute to the generation and shaping of the observed Ca^{2+}-mediated signals, as much as their downstream decoding. In short, the long-standing paradigm based on the classical subdivision of fungal signals between pathogenic and symbiotic molecules needs a careful and timely revision, with a reinterpretation of the elusive barriers between symbiosis and immunity, that are possibly better represented by a continuum or, rather, nonbinary identities. Furthermore, an analogous consideration may apply to the evoked intracellular Ca^{2+} signals, thus questioning the traditional specificity of stimulus–response coupling via unique Ca^{2+} signatures. Indeed, the emerging scenario suggests a unifying role of Ca^{2+} at the nexus of signalling circuits in plant–fungus interactions, thereby acting as a common interpreter in the multiple communication pathways of pathogenic and beneficial fungi with their host plants.

In the following paragraphs, we try to outline this intertwined scenario, starting from the best established aspects of Ca^{2+}-mediated signalling in plant transduction of symbiotic and pathogenic fungal signals.

Nuclear Ca^{2+} spiking: a hallmark of symbiotic signalling

Following the progress of Ca^{2+} monitoring and imaging tools, Ca^{2+} spiking has been first described in root hairs for rhizobium-legume symbiosis (Ehrhardt *et al.*, 1996; Miwa *et al.*, 2006). The more refined and reliable approach allowed by GECIs clarified the synchronicity between nuclear and perinuclear Ca^{2+} oscillations (Sieberer *et al.*, 2009; Kelner *et al.*, 2018); moreover, it revealed that analogous Ca^{2+} signalling occurs in non-root-hair epidermal cells (atrichoblasts) during early establishment of the AM symbiosis (Chabaud *et al.*, 2011), in inner root cells during root colonisation by both AM fungi and rhizobia (Sieberer *et al.*, 2012), in actinorhizal symbioses (Chabaud *et al.*, 2016) and during the interaction with an endophytic *Fusarium* (Skiada *et al.*, 2020). Importantly, for the two symbioses where microbial elicitors have been characterised – Nod factor and Myc factors for symbiotic nitrogen fixation and AM, respectively – Ca^{2+} spiking activation has been consistently demonstrated to be part of the earliest signalling mechanism within the so-called Common Symbiotic Signalling pathway (CSSP; Oldroyd, 2013). Overall, the triggering of nucleus-centred oscillations in [Ca^{2+}] remains a hallmark of signalling pathways governing beneficial plant–microbe interactions (Oldroyd, 2013).

Cytosolic Ca^{2+} responses: plant immunity and beyond

Transient elevations in cytosolic [Ca^{2+}] are well known to be part of the plant immunity response to most fungal pathogens (Zipfel & Oldroyd, 2017). Plant immunity is made of two components: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI; Yuan *et al.*, 2021). PTI is activated upon MAMP recognition by pattern recognition receptors (PRR) in the plasma membrane of plant cells. By contrast, ETI is mediated by intracellular plant immunity receptors containing a nucleotide-binding domain and a leucine-rich repeat domain, called NLRs, which are able to recognise pathogen-released effectors. This model, splitting PTI and ETI mechanisms, is now being revised, based on recent evidence suggesting a mutual potentiation of PTI and ETI to properly mount the plant immunity response to pathogens (Ngou *et al.*, 2022). Whatever the eventual update of this scheme, both PTI- and ETI-associated changes in cytosolic [Ca^{2+}] are crucial mediators of downstream responses that are activated through a Ca^{2+}-regulated phosphocode, involving calcium-dependent protein kinases and possibly epigenetics mechanisms (Erickson *et al.*, 2022; Hannan Parker *et al.*, 2022).

PTI-activated cytosolic Ca^{2+} signals are characterised by a steep and rapid elevation in [Ca^{2+}], which is tightly interconnected with the activation of other typical PTI responses such as reactive oxygen species (ROS) burst, MAPK cascade and immunity marker gene expression (Bjornson *et al.*, 2021; Köster *et al.*, 2022). Microbes as well as purified elicitors of different origins, including chitin-derived molecules, have been shown to activate PTI-induced Ca^{2+} responses (Ranf *et al.*, 2011; Cao *et al.*, 2014; Keinath *et al.*, 2015). From a historical perspective, such studies have mainly used aequorin as a Ca^{2+} probe, which contributed to defining a fast and

steep transient change in [Ca^{2+}] as the acknowledged PTI-associated Ca^{2+} signature. More recently, the use of fluorescent GECIs has revealed the oscillatory nature of such Ca^{2+} signals at the single-cell level (Thor & Peiter, 2014; Keinath *et al.*, 2015), under the control of a partially characterised repertoire of Ca^{2+}-permeable channels and Ca^{2+} pumps (reviewed by Köster *et al.*, 2022).

By contrast, the dynamics of ETI-associated cytosolic Ca^{2+} signals are much slower, with a later onset and longer duration (Köster *et al.*, 2022). The effector-triggered cytosolic Ca^{2+} influx has been shown to depend on the activity of a set of plasma membrane Ca^{2+} channels (Kim *et al.*, 2022) as well as on the formation of NLR oligomers called resistosomes, which are crucial for hypersensitive response and cell death (Ngou *et al.*, 2022).

Inside the symbiosis-immunity continuum

Whereas the plant molecular pathway underpinning the symbiont accommodation and the one triggered by the interaction with pathogens are highly divergent and mainly controlled in different intracellular compartments by a specific Ca^{2+} signature, the existence of several levels of interactions between the two processes is widely accepted. The first hints pointing at an involvement of the plant immunity machinery during the establishment of AM symbiosis came from cytological observations (Gianinazzi-Pearson, 1996) and, later on, from gene expression analyses: Indeed, the presence of AM fungal structures in root cells induces the transcription of dozens of immunity-related genes in different plant species (reviewed by Rey & Jacquet, 2018) with contrasting level of expressions depending on the developmental stage (Giovannetti *et al.*, 2015).

One of the possible reasons behind the regulation of immunity-related genes is that AM fungal components are also able to induce cellular responses that are typically associated with plant defence, such as ROS burst and MAPK phosphorylation (Bozsoki *et al.*, 2017). Interestingly, the same molecules that have been described as purely 'symbiotic' such as tetrameric COs (CO4; Genre *et al.*, 2013) – able to trigger nuclear and perinuclear Ca^{2+} spiking also at nanomolar concentration – can also induce plant cellular ROS burst and MAPK cascades, and this activation fully depends on the chitin receptor presence, similarly to what happens with octameric COs (CO8) and chitin (Bozsoki *et al.*, 2017). Consistently, this immunity cascade triggered by fungal COs clearly overlaps with a strong and quick cytosolic Ca^{2+} influx that can be equally induced by CO4 or CO8 and that critically depends on the elicitor concentration (Binci *et al.*, 2023). Indeed, signals from AM fungi activate a cytosolic Ca^{2+} influx similar to the PTI-triggered one: in aequorin-expressing soybean suspension cultures, a steep cytosolic [Ca^{2+}] elevation is activated after stimulation with germinating spore exudates from *Gigaspora margarita* (Navazio *et al.*, 2007).

It remains unclear whether and how cellular processes that are usually associated with plant immunity could also play an active signalling role during the recognition of symbiotic fungal molecules and, subsequently, during the regulation of arbuscular development and degeneration. This is the case with a class of LysM receptors, such as *M. truncatula* LYK9 (Bozsoki *et al.*, 2017; Feng *et al.*, 2019; Gibelin-Viala *et al.*, 2019) and *L. japonicus* CERK6 (Bozsoki *et al.*, 2017; Binci *et al.*, 2023), with strong affinity for chitin and mediating CO-induced ROS burst, MAPK phosphorylation, cytosolic Ca^{2+} influx and showing different degrees of involvement in the regulation of mycorrhizal colonisation. A similar role is played by LYRIIIA proteins that regulate plant defence in response to the perception of LCOs, thereby facilitating colonisation by AM fungi in mycotrophic plant species. Interestingly, their involvement in plant defence remains conserved also in nonmycotrophic plants (Wang *et al.*, 2023). Altogether, it is tempting to speculate that an initial cytosolic [Ca^{2+}] increase could mediate a cytosolic signalling cascade partially facilitating the establishment or the tuning of AM symbiosis, being linked with cytosolic intermediate messenger(s), such as mevalonate (Venkateshwaran *et al.*, 2015). The picture gets even more complicated when considering that CO4 (and Nod factor) also possess a strong capacity of suppressing plant immunity in nonmycorrhizal plants (Liang *et al.*, 2013).

In addition, it has also to be considered that a notable contrast is arising in the regulation of nuclear Ca^{2+} spiking patterns between Fabaceae plants, commonly employed as primary models for endosymbioses, and monocots. For example, in barley, nutrient homeostasis, and particularly phosphorus and nitrogen depletion, strongly impacts plant cell responsiveness to short-chain COs by increasing the percentage of nuclei that actively respond to LCO and, partially, to CO4 treatment. This has been correlated with an increment of AM fungal colonisation that is controlled by NSP1 and NSP2 (Li *et al.*, 2022). It will now be interesting to understand whether the competitive interaction between OsCERK1 and OsCeBiP/OsMYR1, mediating plant immune or symbiotic response (Zhang *et al.*, 2021), is also controlled by the plant nutritional status.

Research outlook

Our current understanding of plant–microbe interactions mainly derives from studies conducted in controlled laboratory conditions, where plants are exposed to purified signals – often tested at arguable doses – or subjected to single microorganism challenges. A stimulating, albeit arduous, direction for future studies lies in investigating the intricate mechanisms employed by plants to integrate multiple signals from diverse microorganisms. The use of combinations of molecules and mixtures of microbes to unravel these complex processes will likely represent one of the forthcoming challenges in gaining further insights into how plants adapt to their dynamic and multifaceted biotic surroundings.

It appears reasonable to speculate that part of the specificity in plant recognition of fungal signals does not solely rely on chitin-based molecules and that a multifactorial signalling scenario may indeed drive the plant response in the appropriate direction (Fig. 2). Besides the possible existence of additional, symbiosis-specific fungal signals that have so far eluded our efforts, the plant nutritional status has been proposed to play a critical role: nutrient starvation appears to lower plant defences, favouring symbiosis development at the risk of suffering a pathogenic attack; by

contrast, high phosphate and nitrogen availability are known to favour plant immunity and knockdown AM symbiosis (Oldroyd & Leyser, 2020; Dejana *et al.*, 2022). Furthermore, one consistent feature of AM symbiosis is the formation of large fungal adhesion structures, called hyphopodia, that develop on the root epidermis a few hours before the appearance of a penetrating hyphal tip. This broad and relatively long-lasting surface-to-surface contact between fungal and plant cells is associated with local cell wall remodelling (Bonfante & Genre, 2010) and – most likely – intensive bi-directional secretion of signals, including extracellular vesicles (Holland & Roth, 2023). Unveiling what messages are being exchanged in such microscopical interfaces remains a biological (and technical) challenge that may indeed shed light on key processes in AM fungal recognition by their host plants.

An additional frontier for further investigations concerns a thorough analysis of the contribution of different plant intracellular compartments, such as plastids and the endoplasmic reticulum (ER), in the regulation and shaping of the complex intracellular Ca^{2+} signatures triggered by fungal molecules. Indeed, plastids and ER are known to be involved in the modulation and dissipation of cytosolic Ca^{2+} signals in plant cells (Sello *et al.*, 2016; Cortese *et al.*, 2022).

Furthermore, the urgency to combine complementary approaches (e.g. aequorin-based Ca^{2+} quantification and cameleon-based imaging) to measure and visualise $[Ca^{2+}]$ at subcellular resolution is emerging. This appears as a key step towards the disentangling of Ca^{2+} signalling events in plant–fungus interactions. The use of next-generation GECIs endowed with brighter fluorescence, such as GECO and GCaMP (Grenzi *et al.*, 2021), has just started to allow the investigation of long-range Ca^{2+} signals on a systemic scale in different experimental systems (Toyota *et al.*, 2018; Bellandi *et al.*, 2022) and has the potential to reveal the occurrence of analogous signalling mechanisms between root epidermis and cortex (Carotenuto *et al.*, 2019) or between root and shoot (Gutjahr *et al.*, 2009) in response to AM fungal signals. The development of bioluminescence resonance energy transfer (BRET)-based aequorin-GFP reporters, together with the introduction of high-sensitivity photon-counting cameras, revived the use of aequorin as the only available GECI that can so far generate quantitative subcellular resolution mapping of $[Ca^{2+}]$ over time (Grenzi *et al.*, 2021). Moreover, the constant development of novel Ca^{2+} biosensors with maximised performance (Chai *et al.*, 2023) opens up new perspectives in plant symbiotic and immunity studies, by allowing the detection of systemic signalling events at the level of the entire plant during different types of plant–microbe interactions.

Acknowledgements

We apologise to colleagues whose publications could not be cited due to space limitations. This work was supported by grants from the Ministry of the University and Research (MUR), Italy (Progetti di Ricerca di Rilevante Interesse Nazionale – PRIN) prot. 2022NW97JX to AG and LN; the University of Torino, Italy (Ricerca Locale 2022) to AG; the University of Padova, Italy (Progetti di Ricerca Dipartimentali – PRID) grant no. BIRD180317 to LN, grant no. BIRD214519 to MG; the European Union (NextGenerationEU 2021 STARS Grants@Unipd programme P-NICHE) to MG. Fig. 1 was created with Biorender.com.

Competing interests

None declared.

Author contributions

LN and AG designed the concept of the viewpoint. MG and FB contributed to the conceptual ideas. All authors wrote and revised the manuscript. AG and FB generated the figures.

ORCID

Filippo Binci https://orcid.org/0000-0002-9030-2496
Andrea Genre https://orcid.org/0000-0001-5029-6194
Marco Giovannetti https://orcid.org/0000-0001-5305-0341
Lorella Navazio https://orcid.org/0000-0001-5640-108X

Marco Giovannetti[1,2], Filippo Binci[2], Lorella Navazio[2]* and Andrea Genre[1]*

[1]Department of Life Sciences and Systems Biology, University of Torino, 10125, Torino, Italy;
[2]Department of Biology, University of Padova, 35131, Padova, Italy
(*Authors for correspondence: email lorella.navazio@unipd.it (LN); andrea.genre@unito.it (AG))

References

Barker DG, Chabaud M, Russo G, Genre A. 2017. Nuclear Ca^{2+} signalling in arbuscular mycorrhizal and actinorhizal endosymbioses: on the trail of novel underground signals. *New Phytologist* **214**: 533–538.

Bellandi A, Papp D, Breakspear A, Joyce J, Johnston MG, de Keijzer J, Raven EC, Ohtsu M, Vincent TR, Miller AJ *et al.* 2022. Diffusion and bulk flow of amino acids mediate calcium waves in plants. *Science Advances* **8**: eabo6693.

Binci F, Offer E, Crosino A, Sciascia I, Kleine-Vehn J, Genre A, Giovannetti M, Navazio L. 2023. Spatially and temporally distinct Ca^{2+} changes in *Lotus japonicus* roots orient fungal-triggered signalling pathways towards symbiosis or immunity. *Journal of Experimental Botany.* erad360. doi: 10.1093/jxb/erad360.

Bjornson M, Pimprikar P, Nürnberger T, Zipfel C. 2021. The transcriptional landscape of *Arabidopsis thaliana* pattern-triggered immunity. *Nature Plants* **7**: 579–586.

Bonfante P, Genre A. 2010. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. *Nature Communications* **1**: 48.

Bonfante P, Requena N. 2011. Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. *Current Opinion in Plant Biology* **14**: 1–7.

Bozsoki Z, Cheng J, Feng F, Gysel K, Vinther M, Andersen KR, Oldroyd G, Blaise M, Radutoiu S, Stougaard J. 2017. Receptor-mediated chitin perception in legume roots is functionally separable from Nod factor perception. *Proceedings of the National Academy of Sciences, USA* **114**: E8118–E8127.

Cao Y, Liang Y, Tanaka K, Nguyen CT, Jedrzejczak RP, Joachimiak A, Stacey G. 2014. The kinase LYK5 is a major chitin receptor in Arabidopsis and forms a chitin-induced complex with related kinase CERK1. *eLife* **3**: e03766.

Carotenuto G, Volpe V, Russo G, Politi M, Sciascia I, de Almeida-Engler J, Genre A. 2019. Local endoreduplication as a feature of intracellular

fungal accommodation in arbuscular mycorrhizas. *New Phytologist* **223**: 430–446.

Chabaud M, Genre A, Sieberer BJ, Faccio A, Fournier J, Novero M, Barker DG, Bonfante P. 2011. Arbuscular mycorrhizal hyphopodia and germinated spore exudates trigger Ca^{2+} spiking in the legume and nonlegume root epidermis. *New Phytologist* **189**: 347–355.

Chabaud M, Gherbi H, Pirolles E, Vaissayre V, Fournier J, Moukouanga D, Franche C, Bogusz D, Tisa LS, Barker DG *et al.* 2016. Chitinase-resistant hydrophilic symbiotic factors secreted by Frankia activate both Ca^{2+} spiking and NIN gene expression in the actinorhizal plant *Casuarina glauca*. *New Phytologist* **209**: 86–93.

Chai F, Cheng D, Nasu Y, Terai T, Campbell RE. 2023. Maximizing the performance of protein-based fluorescent biosensors. *Biochemical Society Transactions* **51**: 1585–1595.

Chandra S, Stennis M, Low PS. 1997. Measurement of Ca^{2+} fluxes during elicitation of the oxidative burst in aequorin-transformed tobacco cells. *Journal of Biological Chemistry* **272**: 28274–28280.

Cortese E, Moscatiello R, Pettiti F, Carraretto L, Baldan B, Frigerio L, Vothknecht UC, Szabo I, De Stefani D, Brini M *et al.* 2022. Monitoring calcium handling by the plant endoplasmic reticulum with a low-Ca^{2+}-affinity targeted aequorin reporter. *The Plant Journal* **109**: 1014–1027.

Dejana L, Ramírez-Serrano B, Rivero J, Gamir J, López-Ráez JA, Pozo MJ. 2022. Phosphorus availability drives mycorrhiza induced resistance in tomato. *Frontiers in Plant Science* **13**: 1060926.

Edel KH, Marchadier E, Brownlee C, Kudla J, Hetherington AM. 2017. The evolution of calcium-based signalling in plants. *Current Biology* **27**: R667–R679.

Ehrhardt DW, Wais R, Long SR. 1996. Calcium spiking in plant root hairs responding to Rhizobium nodulation signals. *Cell* **85**: 673–681.

Erickson J, Weckwerth P, Romeis T, Lee J. 2022. What's new in protein kinase/phosphatase signalling in the control of plant immunity? *Essays in Biochemistry* **66**: 621–634.

Feng F, Sun J, Radhakrishnan GV, Lee T, Bozsóki Z, Fort S, Gavrin A, Gysel K, Thygesen MB, Andersen KR *et al.* 2019. A combination of chitooligosaccharide and lipochitooligosaccharide recognition promotes arbuscular mycorrhizal associations in *Medicago truncatula*. *Nature Communications* **10**: 5047.

Genre A, Chabaud M, Balzergue C, Puech-Pagès V, Novero M, Rey T, Fournier J, Rochange S, Bécard G, Bonfante P *et al.* 2013. Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca^{2+} spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. *New Phytologist* **198**: 190–202.

Genre A, Ortu G, Bertoldo C, Martino E, Bonfante P. 2009. Biotic and abiotic stimulation of root epidermal cells reveals common and specific responses to arbuscular mycorrhizal fungi. *Plant Physiology* **149**: 1424–1434.

Gianinazzi-Pearson V. 1996. Plant cell responses to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis. *Plant Cell* **8**: 1871–1883.

Gibelin-Viala C, Amblard E, Puech-Pages V, Bonhomme M, Garcia M, Bascaules-Bedin A, Fliegmann J, Wen J, Mysore KS, le Signor C *et al.* 2019. The *Medicago truncatula* LysM receptor-like kinase LYK9 plays a dual role in immunity and the arbuscular mycorrhizal symbiosis. *New Phytologist* **223**: 1516–1529.

Giovannetti M, Mari A, Novero M, Bonfante P. 2015. Early *Lotus japonicus* root transcriptomic responses to symbiotic and pathogenic fungal exudates. *Frontiers in Plant Science* **6**: 480.

Grenzi M, Resentini F, Vanneste S, Zottini M, Bassi A, Costa A. 2021. Illuminating the hidden world of calcium ions in plants with a universe of indicators. *Plant Physiology* **187**: 550–571.

Gutjahr C, Novero M, Guether M, Montanari O, Udvardi M, Bonfante P. 2009. Presymbiotic factors released by the arbuscular mycorrhizal fungus *Gigaspora margarita* induce starch accumulation in *Lotus japonicus* roots. *New Phytologist* **183**: 53–61.

Hannan Parker A, Wilkinson SW, Ton J. 2022. Epigenetics: a catalyst of plant immunity against pathogens. *New Phytologist* **233**: 66–83.

Holland S, Roth R. 2023. Extracellular vesicles in the arbuscular mycorrhizal symbiosis: current understanding and future perspectives. *Molecular Plant–Microbe Interactions* **36**: 235–244.

Jiang Y, Ding P. 2023. Calcium signaling in plant immunity: a spatiotemporally controlled symphony. *Trends in Plant Science* **28**: 74–89.

Keinath NF, Waadt R, Brugman R, Schroeder JI, Grossmann G, Schumacher K, Krebs M. 2015. Live cell imaging with R-GECO1 sheds light on flg22- and chitin-induced transient $[Ca^{2+}]_{cyt}$ patterns in Arabidopsis. *Molecular Plant* **8**: 1188–1200.

Kelner A, Leitão N, Chabaud M, Charpentier M, De Carvalho-Niebel F. 2018. Dual color sensors for simultaneous analysis of calcium signal dynamics in the nuclear and cytoplasmic compartments of plant cells. *Frontiers in Plant Science* **9**: 245.

Kim NH, Jacob P, Dangl JL. 2022. Con-Ca^{2+}-tenating plant immune responses via calcium-permeable cation channels. *New Phytologist* **234**: 813–818.

Knight MR, Campbell AK, Smith SM, Trewavas AJ. 1991. Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* **352**: 524–526.

Köster P, DeFalco TA, Zipfel C. 2022. Ca^{2+} signals in plant immunity. *EMBO Journal* **41**: e11074.

Lecourieux D, Mazars C, Pauly N, Ranjeva R, Pugin A. 2002. Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell* **14**: 2627–2641.

Li X-R, Sun J, Albinsky D, Zarrabian D, Hull R, Lee T, Jarratt-Barnham E, Chiu CH, Jacobsen A, Soumpourou E *et al.* 2022. Nutrient regulation of lipochitooligosaccharide recognition in plants via NSP1 and NSP2. *Nature Communications* **13**: 6421.

Liang Y, Cao Y, Tanaka K, Thibivilliers S, Wan J, Choi J, Kang C, Qiu J, Stacey G. 2013. Nonlegumes respond to rhizobial Nod factors by suppressing the innate immune response. *Science* **341**: 1384–1387.

Lodhi G, Kim Y-S, Hwang J-W, Kim S-K, Jeon Y-J, Je J-Y, Ahn C-B, Moon S-H, Jeon B-T, Park P-J. 2014. Chitooligosaccharide and its derivatives: preparation and biological applications. *BioMed Research International* **2014**: 1–13.

Luan S, Wang C. 2021. Calcium signaling mechanisms across kingdoms. *Annual Review of Cell and Developmental Biology* **37**: 311–340.

Mithöfer A, Ebel J, Bhagwat AA, Boller T, Neuhaus-Url G. 1999. Transgenic aequorin monitors cytosolic calcium transients in soybean cells challenged with β-glucan or chitin elicitors. *Planta* **207**: 566–574.

Miwa H, Sun J, Oldroyd GED, Downie JA. 2006. Analysis of Nod-factor-induced calcium signaling in root hairs of symbiotically defective mutants of *Lotus japonicus*. *Molecular Plant–Microbe Interactions* **19**: 914–923.

Miyawaki A, Griesbeck O, Heim R, Tsien RY. 1999. Dynamic and quantitative Ca^{2+} measurements using improved cameleons. *Proceedings of the National Academy of Sciences, USA* **96**: 2135–2140.

Navazio L, Moscatiello R, Genre A, Novero M, Baldan B, Bonfante P, Mariani P. 2007. A diffusible signal from arbuscular mycorrhizal fungi elicits a transient cytosolic calcium elevation in host plant cells. *Plant Physiology* **144**: 673–681.

Ngou BPM, Ding P, Jones JDG. 2022. Thirty years of resistance: Zig-zag through the plant immune system. *Plant Cell* **34**: 1447–1478.

Oldroyd GED. 2013. Speak, friend, and enter: Signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology* **11**: 252–263.

Oldroyd GED, Leyser O. 2020. A plant's diet, surviving in a variable nutrient environment. *Science* **368**: eaba0196.

Plattner H, Verkhratsky A. 2015. The ancient roots of calcium signalling evolutionary tree. *Cell Calcium* **57**: 123–132.

Ranf S, Eschen-Lippold L, Pecher P, Lee J, Scheel D. 2011. Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *The Plant Journal* **68**: 100–113.

Ravi B, Sanyal SK, Pandey GK. 2023. Calcium decoders and their targets: The holy alliance that regulate cellular responses in stress signaling. *Advances in Protein Chemistry and Structural Biology* **134**: 371–439.

Rey T, Jacquet C. 2018. Symbiosis genes for immunity and vice versa. *Current Opinion in Plant Biology* **44**: 64–71.

Rush TA, Puech-Pagès V, Bascaules A, Jargeat P, Maillet F, Haouy A, QuyManh Maës A, Carrera Carriel C, Khokhani D, Keller-Pearson M *et al.* 2020. Lipo-chitooligosaccharides as regulatory signals of fungal growth and development. *Nature Communications* **11**: 3897.

Sello S, Perotto J, Carraretto L, Szabò I, Vothknecht UC, Navazio L. 2016. Dissecting stimulus-specific Ca^{2+} signals in amyloplasts and chloroplasts of *Arabidopsis thaliana* cell suspension cultures. *Journal of Experimental Botany* **67**: 3965–3974.

Sieberer BJ, Chabaud M, Fournier J, Timmers ACJ, Barker DG. 2012. A switch in Ca^{2+} spiking signature is concomitant with endosymbiotic microbe

entry into cortical root cells of *Medicago truncatula*. *The Plant Journal* **69**: 822–830.

Sieberer BJ, Chabaud M, Timmers AC, Monin A, Fournier J, Barker DG. 2009. A nuclear-targeted cameleon demonstrates intranuclear Ca^{2+} spiking in *Medicago truncatula* root hairs in response to rhizobial nodulation factors. *Plant Physiology* **151**: 1197–1206.

Skiada V, Avramidou M, Bonfante P, Genre A, Papadopoulou KK. 2020. An endophytic Fusarium–legume association is partially dependent on the common symbiotic signalling pathway. *New Phytologist* **226**: 1429–1444.

Sun X, Chen W, Ivanov S, MacLean AM, Wight H, Ramaraj T, Mudge J, Harrison MJ, Fei Z. 2019. Genome and evolution of the arbuscular mycorrhizal fungus *Diversispora epigaea* (formerly *Glomus versiforme*) and its bacterial endosymbionts. *New Phytologist* **221**: 1556–1573.

Tehrani N, Mitra RM. 2023. Plant pathogens and symbionts target the plant nucleus. *Current Opinion in Microbiology* **72**: 102284.

Thor K, Peiter E. 2014. Cytosolic calcium signals elicited by the pathogen-associated molecular pattern flg22 in stomatal guard cells are of an oscillatory nature. *New Phytologist* **204**: 873–881.

Tisserant E, Malbreil M, Kuo A, Kohler A, Symeonidi A, Balestrini R, Charron P, Duensing N, Dit FNF, Gianinazzi-Pearson V *et al.* 2013. Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proceedings of the National Academy of Sciences, USA* **110**: 20117–20122.

Toyota M, Spencer D, Sawai-Toyota S, Jiaqi W, Zhang T, Koo AJ, Howe GA, Gilroy S. 2018. Glutamate triggers long-distance, calcium-based plant defense signaling. *Science* **361**: 1112–1115.

Venkateshwaran M, Jayaraman D, Chabaud M, Genre A, Balloon AJ, Maeda J, Forshey K, den Os D, Kwiecien NW, Coon JJ *et al.* 2015. A role for the mevalonate pathway in early plant symbiotic signaling. *Proceedings of the National Academy of Sciences, USA* **112**: 9781–9786.

Volk H, Marton K, Flajšman M, Radišek S, Tian H, Hein I, Podlipnik Č, Thomma BPHJ, Košmelj K, Javornik B *et al.* 2019. Chitin-binding protein of *Verticillium nonalfalfae* disguises fungus from plant chitinases and suppresses chitin-triggered host immunity. *Molecular Plant–Microbe Interactions* **32**: 1378–1390.

Volpe V, Carotenuto G, Berzero C, Cagnina L, Puech-Pagès V, Genre A. 2020. Short chain chito-oligosaccharides promote arbuscular mycorrhizal colonization in *Medicago truncatula*. *Carbohydrate Polymers* **229**: 115505.

Wang T, Gasciolli V, Gaston M, Medioni L, Cumener M, Buendia L, Yang B, Bono JJ, He G, Lefebvre B. 2023. LysM receptor-like kinases involved in immunity perceive lipo-chitooligosaccharides in mycotrophic plants. *Plant Physiology* **192**: 1435–1448.

Xu H, Heath MC. 1998. Role of calcium in signal transduction during the hypersensitive response caused by basidiospore-derived infection of the cowpea rust fungus. *Plant Cell* **10**: 585–598.

Yu H, Bai F, Ji C, Fan Z, Luo J, Ouyang B, Deng X, Xiao S, Bisseling T, Limpens E *et al.* 2023. Plant lysin motif extracellular proteins are required for arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences, USA* **120**: e2301884120.

Yuan M, Ngou BPM, Ding P, Xin XF. 2021. PTI-ETI crosstalk: an integrative view of plant immunity. *Current Opinion in Plant Biology* **62**: 102030.

Zhang C, He J, Dai H, Wang G, Zhang X, Wang C, Shi J, Chen X, Wang D, Wang E. 2021. Discriminating symbiosis and immunity signals by receptor competition in rice. *Proceedings of the National Academy of Sciences, USA* **118**: e2023738118.

Zhang M, Kong X. 2021. How plants discern friends from foes. *Trends in Plant Science* **27**: 107–109.

Zipfel C, Oldroyd GED. 2017. Plant signalling in symbiosis and immunity. *Nature* **543**: 328–336.

Key words: arbuscular mycorrhiza, calcium signalling, chitin oligomers, fungal signals, genetically encoded calcium indicators, plant immunity, plant symbiosis.

Received, 26 July 2023; accepted, 8 November 2023.