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Review

*Tansley insight*

# Masters of perception: phosphorylation-dependent signaling in plants

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## Summary

Plants are masters of perception, reacting to a myriad of biochemical and physical cues in a constantly changing environment. Plants rely on local cell-based signal processing to perceive and react sufficiently fast to a multitude of stimuli. The ability to respond quickly is crucial for sustaining growth, defense, and metabolism and thereby the ability to survive challenges associated with pathogens, resource limitations, environmental fluctuations, and mechanical perturbations. Protein phosphorylation networks are prominent in mediating these fast responses, shaping the cellular response. In the past decade, plant sciences have moved our understanding of these networks further; however, current understanding is largely limited to minutes- and hour-long timescales, and hardly illuminates the (sub-)second timescales at which the initial signaling processes take place. In this Tansley insight, we discuss how quantitative mass spectrometry-based phosphoproteomics can be utilized to study the rapid and dynamic initial steps of protein phosphorylation networks in plants. We highlight rapid responses and show how bioinformatic approaches and the integration of other proteomics approaches can be utilized to deconvolve phosphorylation-based signaling in a data-driven approach. We offer an outlook on how to experimentally address rapid signaling and hope to inspire new approaches in the study of phosphorylation-dependent plant signaling networks.

## I. Introduction

To be able to grow in a constantly changing environment, plants must effectively perceive, relay, and integrate signals quickly to adjust cellular growth and developmental responses. With a little over 1000 kinases encoded in the Arabidopsis genome, a multitude of combinatorial signaling networks can be generated to perceive and transform developmental, biotic, and abiotic signals (Zulawski *et al.*, 2014). Many such signals are perceived at the cell membrane by cell-surface receptor-like kinases that transmit information across the membrane and trigger complex phosphorylation-dependent kinase signaling cascades that intersect with other pathways and other post-translational modifications, eventually giving rise to altered cellular responses (Vu *et al.*, 2018; Zhang *et al.*, 2023). Much of what we know about phosphorylation-dependent signaling has started from genetic screens identifying mutants that fail to respond to treatments (e.g. flagellin) (Gómez-Gómez & Boller, 2000). Biochemical assays have further connected receptors with downstream components, spurring textbook views on plant signaling modules. The culmination of these studies has shown that perception is diverse, using a multitude of plasma membrane-localized receptor kinases (RKs) and receptor proteins (RPs), triggering and regulating downstream physiological, immune, and developmental responses. Well-known examples are the FLS2 and BRI1 RKs, which bind to and mediate response to bacterial flagellin and the phytohormone brassinosteroid, respectively (Hohmann *et al.*, 2017; Bender & Zipfel, 2023). However, from the hundreds of RKs/RPs present in the plant genome, only a limited number have been connected to a ligand and/or downstream components. Quantitative mass spectrometry-based phosphoproteomics is a proven technique to fill this knowledge gap by exploring phosphorylation-dependent signaling in an unbiased manner (we refer the reader to excellent reviews covering this topic; Chen *et al.*, 2021; Zhang *et al.*, 2023; Sang *et al.*, 2025). Using biochemical enrichment techniques and the latest instrumentation and bioinformatic tools allows the mapping and quantification of thousands of phosphorylation sites in an unbiased manner. Such approaches have already uncovered downstream signaling components in osmotic stresses, biotic elicitors, nutrient signaling, and phytohormone responses (Benschop *et al.*, 2007; Van Leene *et al.*, 2019; Clark *et al.*, 2021; Nikonorova *et al.*, 2021; Sang *et al.*, 2024; Watkins *et al.*, 2024). However, these studies have primarily been conducted on time scales of tens of minutes to hours following signal perception, leaving a large knowledge gap regarding the dynamics of the first steps in plant signaling (Fig. 1a). In this insight, we aim to provide a framework for how these fast signaling events can be uncovered using phosphoproteomics and multi-proteomics data integration.

## II. Phosphorylation on biochemical time scales

A central tenet in biology is that the timing of cellular responses should match the nature of the trigger. The dynamic range of biological time is large: developmental processes are usually slow but are preceded by fast perception of different hormonal cues or elicitors. Critical decisions in plant responses can take place very

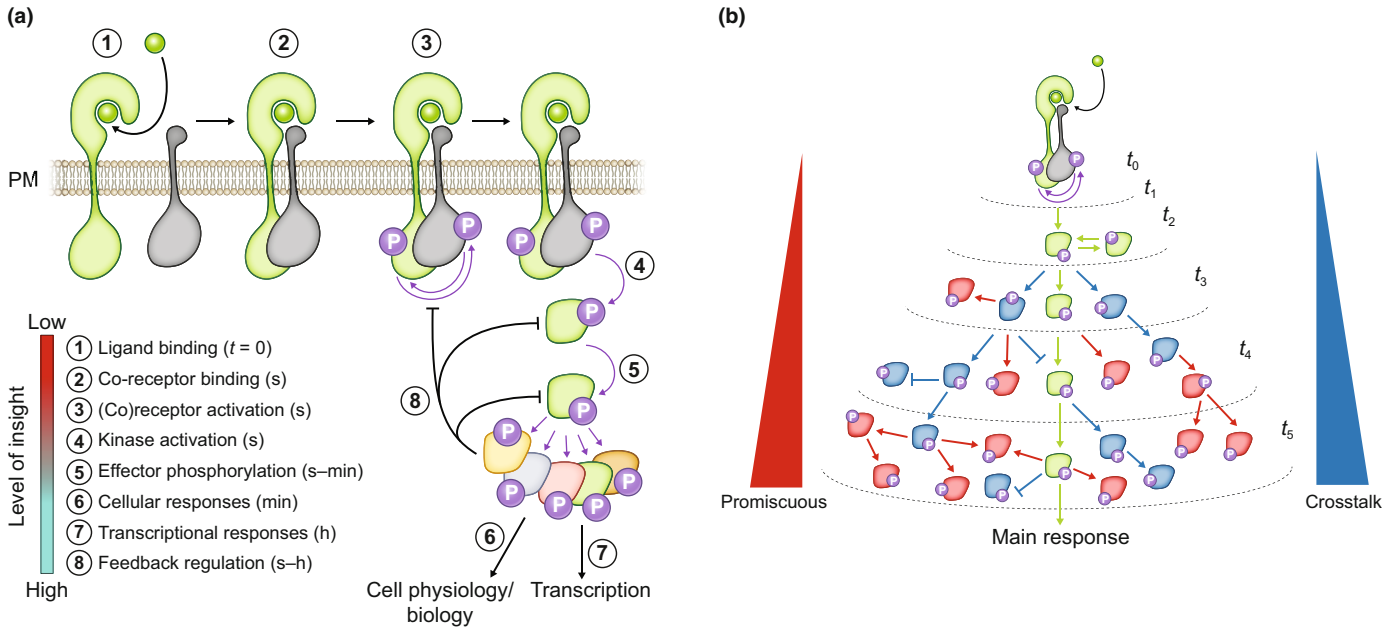


**Fig. 1** Time-dependent nature of phosphorylation-based signaling. (a) In generic receptor kinase-mediated signaling, a receptor kinase (green) associates with a ligand (green) and subsequently a co-receptor (grey). Through conformational changes and *trans*-phosphorylation, downstream kinases trigger specific cellular responses. Relevant steps are numbered and timing indicated. Our current knowledge on phosphorylation-mediated signaling largely encompasses later stages in the signaling cascade. (b) Stimulus-specific signaling occurs at earlier stages of the phosphorylation-dependent signaling cascade. Kinase signaling promiscuity and crosstalk increase over time, clouding the stimulus-specific events at later stages. Figure adapted from Kanshin *et al*. (2015, under the terms of CC BY-NC-ND 3.0).

rapidly – as initial events in, for example, receptor activation and immediate substrate phosphorylation are only limited by the diffusion of one or two proteins. However, the earliest events in signaling are often overlooked, not only in plant sciences but also in other fields studying signaling cascades. Multiple cellular and physiological responses have been recorded which happen too fast to be explained by the transcriptional or even the translational machinery. A clear example is the calcium-dependent leaf closure of *Mimosa pudica*, touch-me-not plant, which occurs within seconds after mechanical triggering of trichomes (Hagihara *et al.*, 2022). In a similar manner and like an animal nervous system, upon herbivore attack, plants use glutamate receptor-like ion channels to trigger a cascade of calcium ion changes which propagates through the plant (Toyota *et al.*, 2018). The perception and propagation of the glutamate and calcium signal occurs within minutes (Toyota *et al.*, 2018). Since plants lack a nervous system, the rapid transduction of calcium waves can only be explained by rapid changes in enzyme and channel activity. More recently, it was shown using phosphoproteomic and cellular assays that auxin can cause rapid changes in cytoplasmic streaming, mediated by a RAF-like protein kinase (Kuhn *et al.*, 2024). These examples demonstrate that fast enzymatic changes must be at play to exert specific cellular responses.

The first molecular changes, including receptor conformational shifts and kinase activation, are thought to take place in the order of seconds (Hohmann *et al.*, 2017; Bender & Zipfel, 2023; Fig. 1a). Using immune-based assays, it was shown that the Insulin-like Growth Factor 1 Receptor (IGFR) is activated within 15 s upon insulin addition, while activation of the downstream AKT kinase occurs within 30 s (Blazek *et al.*, 2015). Using phosphoproteomics, it was further shown that activation of the insulin receptor and the insulin receptor substrate is detectable as early as 5 s after insulin stimulation, shortly followed by the downstream signaling components (Humphrey *et al.*, 2015). Another study probed the phosphorylation dynamics of the high-osmolarity glycerol (HOG) pathway in budding yeast using 5-s intervals during 1 min (Kanshin *et al.*, 2015). In plants, the earliest time point probed using phosphoproteomics is 30 s after auxin addition (Kuhn *et al.*, 2024) and phosphorylation changes in response to touch happen in as little as 40 s (Wang *et al.*, 2018). Despite the many publications utilizing phosphoproteomics to understand kinase signaling, the aforementioned limited set of publications on fast signaling equals the amount of understanding we have about early phosphorylation dynamics. For plants, such fast responses are likely crucial for orchestrating an acute response to acute stresses. Since many phosphorylation events already take place within seconds, these initial events are likely the most decisive for response specificity, signal transmission, and integration for a coordinated outcome. This notion was corroborated by Kanshin *et al.* (2015), who showed that putatively functional kinase- and phosphatase-substrate interactions take place more rapidly, within 60 s, while later time points include both specific, on-pathway phosphorylation changes; these time points may also include spurious or promiscuous signaling events (Kanshin *et al.*, 2015; Fig. 1b). Therefore, investigating the molecular basis of second-scale signaling events will fill a key knowledge gap.

## III. Data-driven analysis of dynamic kinase-substrate interaction

A main reason why fast signaling events are understudied is the technical challenge associated with rapidly treating and harvesting biological materials within seconds. In Section V of this Tansley insight, we provide ideas on how to tackle this problem. Once this is overcome and dense temporal phosphoproteomes can be acquired, the next hurdle lies in extracting functional relationships from the data. Through years of research, the animal signaling field has developed numerous bioinformatic tools and known kinase-substrate relationships (Lachmann & Ma'ayan, 2009; Beekhof *et al.*, 2019); however, the plant field has sparse knowledge on kinase-substrate relationships and few dedicated tools for deducing this from phosphoproteomics data. Typically, a phosphoproteomics approach provides information on which proteins are phosphorylated and provides site-specific information on the amino acid location phosphorylated within the respective proteins. This is beneficial for targeted mutant validation and predicting the functional consequence of phosphorylation, but can also be used to predict the enzymatic activity of kinases. The activation loop (A-loop) is a well-conserved region in protein kinases where phosphorylation generally results in kinase activation, while dephosphorylation leads to inactivation (Adams, 2003). The A-loop typically starts with the amino acids DFG and ends with an APE motif (Montes *et al.*, 2022). This allows for easy prediction of the position of A-loops from kinases in available plant proteomes. Using the positional information from the A-loop and the site localization from phosphoproteomics data, one can then infer kinase (in)activation events from the data. This approach can be extended to infer kinase-substrate relationships using distance metrics (e.g. Pearson or Spearman correlation) within phosphopeptide intensity data, guiding research for further functional validation (Walley *et al.*, 2013). This approach has already been shown to be fruitful in deciphering kinase signaling networks in brassinosteroid (Clark *et al.*, 2021; Montes *et al.*, 2022) and auxin signaling (Kuhn *et al.*, 2024). An important assumption in this approach is that the phosphorylation profile of kinase substrates follows a similar intensity profile to the activity profile of the kinase. In a classical phosphorylation cascade, there is usually a time lag between successive kinase activation events. When having dense second-scale phosphoproteomics data at hand, such events can be deduced from the data using lag time cross-correlation analysis. In this approach, correlations are calculated by shifting time windows, potentially allowing for reconstructing cascading kinase events in a data-driven manner. Although promising and insightful, it was recently highlighted that similarity measures alone are poor predictors of kinase-substrate associations, but this can be significantly improved using *a priori* knowledge such as sequence similarity filters, validated kinase-interactions, and context-specific information (Sriraja *et al.*, 2023).

## IV. Multi-(prote)omics data integration

The predictive power of regulatory networks has already been shown to be improved by using multi-omics data integration

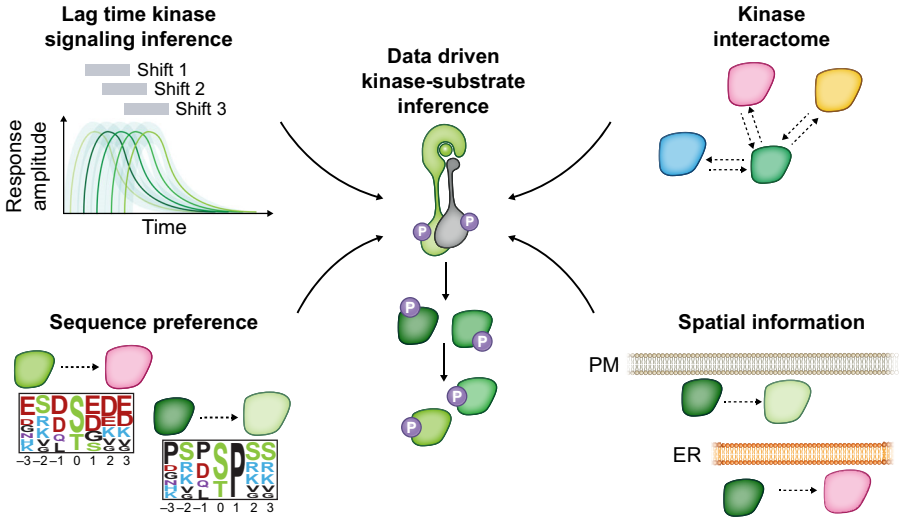


**Fig. 2** Integrative proteomics approaches for data-driven reconstruction of kinase signaling. Phosphoproteomics experiments generate large datasets from which it is not trivial to deduce functional relationships. Combining multiple proteomics and bioinformatic approaches covering different molecular contexts of kinases (location, interactions, sequence preferences, and kinase activity) allows for reconstructing receptor-kinase signaling in a data-driven manner.

(Walley *et al.*, 2016; Clark *et al.*, 2021; Montes *et al.*, 2022). To obtain a proper understanding of fast kinase signaling and phosphorylation-dependent cascades, we need to experimentally probe the different molecular contexts in which this takes place. Phosphoproteomics data alone provide only a one-dimensional inference of molecular regulation, while protein interactions, cellular localization, and sequence context are of equal importance to the regulation and activity of kinases (Fig. 2). The plant research field has already widely adopted large-scale protein interaction (interactomics) approaches. The combinatorial integration of phosphoproteomics and interactomics data revealed complementary subspaces, but also an overlapping space, which allowed dissection of direct kinase-substrate interactions of the kinase BRASSINOSTEROID-INSENSITIVE2 (BIN2) and in the target of rapamycin signaling network (Van Leene *et al.*, 2019; Kim *et al.*, 2023). Among interactomics techniques, proximity labeling approaches such as TurboID are particularly promising because they can capture weak and transient interactions *in vivo*, which are often missed by traditional affinity capture methods (Mair *et al.*, 2019; Zhang *et al.*, 2019; Arora *et al.*, 2020; Kim *et al.*, 2023). Kinases can reside in different cellular locations, or be translocated upon perception of signals (Cao *et al.*, 2019). Understanding kinase residence under static conditions and perturbations provides additional context-specific information on their molecular regulation. Unbiased organellar mapping approaches can provide such information at a proteome-wide scale, but this has yet to be integrated in the plant field (Itzhak *et al.*, 2016; Geladaki *et al.*, 2019; Martinez-Val *et al.*, 2021; Crook *et al.*, 2022). Probably the most informative piece of information for inferring kinase-substrate networks is the sequence preference of kinases for their substrates. Traditionally, such information has been deduced from synthesized peptide arrays and incubation with an *in vitro* purified kinase (Johnson *et al.*, 2023), but new methodologies allow the use of peptides derived directly from plant proteome samples, which allows for a closer approximation of *in vivo* kinase-substrate relationships in an otherwise *in vitro* context (Wang *et al.*, 2020; Montes *et al.*, 2022). These approaches have been shown to be fruitful in reducing potential kinase-substrate interactions from phosphoproteomics data and in identifying sequence-specific motifs. To date, these approaches have only been applied in specific contexts in the plant field, while large-scale 'kinome' analysis has the potential to deconvolve the substrates of hundreds of kinases linked to phosphorylation networks (Sugiyama *et al.*, 2019; Jha *et al.*, 2025). The integration of the aforementioned approaches will ultimately help to deduce the important regulatory factors in a kinase signaling cascade from phosphoproteomics data (Fig. 2).

## V. Outlook

Although there is great potential for using multi-proteomics data to infer the core regulatory circuitry of phosphorylation-dependent signaling cascades, a major dependence and hurdle is to harvest plant materials on the second time scale. If we want to study molecular events on this time scale, many factors need to be controlled to allow proper comparative time studies (i.e. equal treatment, equal timing etc.). To fulfill these requirements, automated platforms should be employed. Recent years have seen the development and implementation of 'lab on a chip' approaches where organs, tissues, and single cells can be grown and manipulated in a controlled environment (Cardoso *et al.*, 2023; Lin *et al.*, 2025). For proteomics experiments, such microfluidics approaches should allow large-scale manipulation of protoplasts or suspension cell cultures. A good example of such a cell culture is the Arabidopsis PSB-D culture, as used in the study by Van Leene *et al.* (2019). We envision that combining cellular model systems with advanced microfluidics approaches would allow the capture of second-scale phosphorylation events. Combining time series phosphorylation data with other proteomics technologies (kinase sequence specificity, interactomics etc.) will allow deconvoluting kinase-substrate relationships and shed novel insights on phosphorylation-dependent signaling cascades.

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## Competing interests

None declared.

## Author contributions

MR, JWW and DW conceived the manuscript. MR wrote the initial draft, and JWW and DW edited the final manuscript.

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