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Rapid auxin signaling: Unknowns old and new

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There are known unknowns; that is to say, we know there are some things we do not know. But there are also unknown unknowns—the ones we don't know we don't know.

Donald Rumsfeld

Despite there likely being more people aware of Donald Rumsfeld and his controversial political views than those appreciating the importance of auxin as the major regulator of plant development, in the grand scheme of things, the latter is more important. Nevertheless, as it became obvious with a fleur of unexpected recent discoveries—also in the case of auxin and, in particular, auxin signaling—there are many unknown unknowns next to the mysteries that we are aware of.

Among prominent recently solved known unknowns is the structure of PIN auxin transporters [1–3], which has been eagerly awaited since their discovery 25 years ago [4]. It put to rest lingering doubts, if any, as to whether PINs are *bona fide* auxin exporters and provided a long-sought molecular insight into their transport mechanism. No less exciting are unknowns

pertaining to the decades-old enigma of perception and signaling mechanisms behind rapid, non-transcriptional auxin responses, which we review here.

The auxin signaling contract: 'time is of the essence'

Efforts to understand auxin signaling began in the pre-molecular era with investigations of near-instantaneous auxin responses (typically shorter than one minute) such as protoplast swelling or electrical events at the plasma membrane (PM) [5,6]. With the advent of classical genetics, however, there was a major focus shift towards developmental and growth responses which take hours or even days to manifest. The reason for this is simple yet profound: rapid electrophysiological and cellular phenotypes were incompatible with large-scale forward genetic screens which examined hundreds of thousands of *Arabidopsis* seedlings [7]. Researchers thus favored long-term growth phenotypes for their screens. Subsequent cloning and biochemical endeavors gave rise to 'the central dogma of auxin signaling' as we know it today. It comprises a double negative logic motif in which auxin activates transcription by degrading a repressor of gene expression [8].

In more detail: Auxin binds to F-box protein subunits of SCF E3 ubiquitin ligase complexes. This confers on SCF complexes the ability to bind and degrade Aux/IAA transcriptional co-repressors, relieving ARF transcription factors at auxin-responsive promoters from inhibition. Auxin binding occurs in F-box proteins from the TIR1/AFB family via their auxiliary leucine-rich repeat (LRR) domain while their F-box domain interacts with the rest of the SCF through the ASK1 adaptor protein [9]. Experiments with reporter genes demonstrate that this transcriptional pathway takes about 20–30 minutes to produce proteins and associated phenotypic responses [10]. Remarkably, the explanatory power of this mechanism nearly suffices to account for the rather impressive plethora of developmental roles of auxin [11].

If one considers the slow TIR1/AFB pathway for transcriptional regulation as an arising dogma of the early 21st century, then it is fair to label rapid auxin responses as the 'elephant in the room' because they were difficult to reconcile with this model. Among known rapid effects were PM depolarization, membrane proton fluxes, cytosolic Ca^{2+} transients, cytoplasmic streaming, and the alteration of endomembrane trafficking [6,12]. One possibility to accommodate rapid responses was the existence of alternative auxin-binding sites in the cell such as the AUXIN BINDING PROTEIN 1 (ABP1), a cupin-family protein with 50 years of controversial history, originally identified by its ability to bind radiolabeled

auxin in various species [13]. Nevertheless, the signaling mechanism behind rapid responses remained a known unknown.

Dirty little secret of TIR1/AFB auxin receptors: a fast non-transcriptional branch

While auxin-responsive transcription became the mainstream of the field as time passed, there were also continuing efforts to understand how cells respond to auxin within tens of seconds or faster. Pioneering experiments demonstrated that auxin triggers a rapid alkalinization of the root extracellular space (apoplast), mirrored by a cytosolic Ca^{2+} transient. Blockage of the Ca^{2+} response prevented the pH response, indicating that these are causally linked [14]. On the other hand, rapid auxin-mediated PM depolarization was known to occur already from studies conducted during the late 1970s [6].

A striking realization came with the finding that also auxin-triggered root growth inhibition is too rapid to involve transcription and that it actually depends, together with the even faster cytosolic [Ca^{2+}] increase and PM depolarization, on TIR1/AFB auxin receptors [15,16]. Accordingly, these effects were triggered by intracellular auxin because the loss of the AUX1/LAX auxin permease prevented them from happening [16]. Importantly, the CNGC14 membrane channel previously implicated in root gravitropism [17] was shown to be responsible for Ca^{2+} transients downstream of TIR1/AFBs [16]. All this was surprising given that TIR1/AFBs were identified in screens for long-term auxin phenotypes and always linked exclusively with transcriptional regulation. There was therefore no precedent to expect TIR1/AFB involvement in the rapid responses.

A comprehensive account of TIR1/AFB subcellular localization patterns suggested a division of labor among TIR1/AFBs and showed that it is the dominantly cytosolic AFB1 member which displays a major phenotype in rapid root growth inhibition [18]. Follow-up research placed AFB1 upstream of rapid membrane depolarization and apoplast alkalinization [19,20]. More recent work then elaborated that AFB1 orchestrates the formation of discrete alkaline and acidic pH zones along the root tip and that the *cngc14-1* mutant phenocopies an *afb1-3* zonation defect [21], thus establishing the AUX1-AFB1-CNGC14 pH-orchestrating module in rapid root growth.

These exciting discoveries posed an important question: What exactly distinguishes AFB1 from its TIR1/AFB counterparts and allows it to activate rapid signaling? It turns out that its

role in rapid root growth inhibition is an intrinsic property of the AFB1 protein and is not simply conferred by its cytoplasmic localization [22,23]. Cumulative data also suggest that AFB1 shows decreased ability to assemble into a full SCF complex [22–24]. Such a situation is highly reminiscent of non-canonical F-box proteins from the budding yeast which perform SCF-independent functions but still exist in a complex with the homolog of the ASK1 protein [25].

An unexpected and textbook-revising unknown unknown emerged with the discovery that TIR1, AFB1, and likely all other TIR1/AFB-type auxin receptors present in land plants harbor an adenylate cyclase (AC) center which, following auxin perception, synthesizes a second messenger familiar from animal cells: cAMP [26]. Despite being originally identified during a search for a non-transcriptional functionality of TIR1/AFBs, the TIR1 AC activity is unexpectedly required for classic, transcriptional responses, and dispensable for rapid ones.

The previously entirely unsuspected role of cAMP in transcriptional auxin signaling was therefore added to the list of now-known unknowns, next to the mystery of TIR1/AFB-mediated rapid auxin effects.

A phoenix reborn from its ashes: the ABP1 auxin receptor

The fascinating versatility of auxin has inspired biologists to search for auxin receptors for over half a century. Since the recovery of ABP1 using old-school biochemistry (more than twenty years before TIR1/AFB genetics), ABP1 has experienced a convoluted history including the incorrect identification of an embryo-lethal insertion mutant [13]. Importantly, the notorious re-evaluation of *abp1* mutant phenotypes with only minor defects [27–30] poured fuel on the fire of a community that was already, as human nature sometimes dictates, divided by its favoritism for either ABP1 or TIR1 as the more relevant auxin receptor. As a result, most ABP1 research was suspended, and all previous ABP1-related studies were called into question, including those that never used the erroneous mutant alleles.

Several reports noticed that the legitimate ABP1 gain-of-function lines present, conditional to an intact auxin-binding pocket, a wide range of developmental malfunctions including altered trafficking of PIN auxin exporters [28,30,31]. A recent biochemical revision of our knowledge of ABP1 focused on the *Arabidopsis* ABP1 protein and confirmed previous notions from other species: that ABP1 binds auxin at the acidic, apoplast-like pH and that it

partially localizes to the apoplast [32]. The use of verified *Arabidopsis abp1* mutants revealed strong and specific defects in processes related to the auxin-induced formation of new vasculature, such as its regeneration after wounding, or its establishment from an external auxin source. Importantly, an ABP1 version with an engineered lack of auxin binding proved inefficient in complementing the phenotypic defects, highlighting the crucial importance of auxin binding to ABP1 for its function [32]. These experiments reinstated ABP1 as a valid auxin receptor for processes underlying the formation of vascular strands via auxin canalization. What remains unsolved, however, is a notoriously known ABP1 unknown: the function of ABP1 in its main home organelle, the endoplasmic reticulum, whose near-neutral pH disfavors auxin binding to ABP1. Furthermore, according to available reports, the known interaction partners of ABP1 (see next paragraph) do not seem to reside to a significant extent in the endoplasmic reticulum.

Vasculature formation-related phenotypes were uncovered also in the PM interactors of ABP1 known as TRANSMEMBRANE KINASES (TMKs) [32]. Although the canalization phenotypes of single *tmk* mutants are comparable to those of *abp1*, higher-order *tmk* mutants show strong additional developmental defects. This suggests either ABP1-independent functions of TMKs or a functionally redundant action of potential auxin binders other than ABP1 from the same cupin family [33, preprint: 34]. Excitingly, the ABP1-TMK1 module responds to auxin by mediating an ultrafast phosphorylation cascade [32] which represents another unexpected unknown in the universe of rapid auxin signaling.

Essential TRANSMEMBRANE KINASES do what it says on the tin

The *Arabidopsis* TMK family comprises four LRR receptor-like kinases which were initially shown to exhibit genetic redundancy [35], but more detailed investigations revealed also their specific and separable functions in plant development. For instance, only TMK1 is responsible for the maintenance of the apical hook [36] and for auxin-dependent enhancement of abscisic acid signaling [37]. Conversely, TMK4 but not TMK1 regulates auxin biosynthesis by interacting with and phosphorylating the auxin biosynthetic enzyme TAA1 [38]. We finally note that TMK1-mediated phosphorylation has a prominent role in the transcriptional response to auxin within the context of the apical hook [36], as reviewed elsewhere [39].

The action of TIR1/AFB receptors can now be placed directly upstream of a subset of rapid auxin effects [40]. How can we, however, account for the remaining rapid auxin responses such as the activation of PM H^+-ATPases and apoplast acidification, the auxin effect on endomembrane trafficking, or the activation of cytoplasmic streaming? A major indication of TMK involvement in rapid auxin signaling emerged with the discovery that auxin induces the formation of an ABP1-TMK1 complex at the cell surface, rapidly activating small G-proteins called ROPs [41]. Recent investigations indicate that this rapid ABP1-TMK module does exactly what it says on the tin: phosphorylates downstream targets.

ABP1-TMK-mediated ultrafast phosphorylation

Novel evolutionarily conserved rapid auxin response: Renaissance of MAPK signaling

In a quest to understand rapid auxin responses, new studies used cutting-edge phospho-proteomics to probe the involvement of protein phosphorylation, an intrinsically rapid mechanism known to act within 2 minutes of various stimuli in both plants and animals [42,43]. The results were unprecedented: auxin specifically activated the phosphorylation of over 1700 proteins with hundreds of differential phospho-sites already 30 seconds after treatment. This required intact ABP1 and TMK1 but not TIR1/AFBs [32, preprint: 44,45]. However, AFB1 was found to buffer against ABP1-TMK1-mediated phosphorylation [preprint: 45], paralleled by the recent evidence that AFB1 inhibits the transcriptional long-term auxin response [22]. It is possible that AFB1 evolved as a molecular brake for various branches of auxin signaling.

The auxin phospho-response is conserved from land plants to at least basal Streptophyte algae and predates the nuclear TIR1/AFB pathway. In most species examined, it converges on mitogen-activated protein kinase kinase kinases (MAPKKKs) [preprint: 46]. Indeed, the auxin-induced phospho-proteome is enriched in the consensus MAP kinase (MAPK) phosphorylation motif and contains both MPK8 and MPK16 as prominent targets [preprint: 45,46]. Therefore, the ABP1-TMK1 complex activates the MAPK cascade, constituting a potentially ancestral auxin signaling pathway. Multi-level redundancy is a recurring theme during the phospho-response—likely reflecting the fact that its robustness is essential for life—but complicating genetic analysis. Reminiscent of TMKs, a septuple MAPKKK mutant had to be analyzed to see appreciable auxin phenotypes [preprint: 46,47]. A similar problem is also behind the notoriety of ABP1 but was recently addressed by the discovery of two

apoplastic structural ABP1 homologs whose mutants produce conditional synergistic sick phenotypes in combination with the *abp1* mutant [preprint: 34]. We anticipate that all these exciting discoveries will soon allow researchers to probe the mystery of why massive deregulation of protein phosphorylation in *abp1* and *tmk1* mutants produces only mild phenotypes under standard growth conditions [32].

Twenty to thirty years ago, plant biologists were inspired by the animal field to study MAPK signaling in plants, and discovered rapid MAPK activation by auxin within 2-5 minutes of treatment [48,49]. After some initial excitement [50,51], however, MAPKs fell out of fashion being swamped by the successful advent of TIR1/AFB signaling. A recent noteworthy report showed that auxin-activated MAPK signaling participates in lateral root organogenesis downstream of TMK1/4 [52], but the connection between MAPKs and rapid auxin signaling remained in the Middle Ages. Identification of the rapid ABP1-TMK-MAPK phosphorylation, therefore, represents the Renaissance of MAPK signaling in rapid auxin biology.

Auxin effect on pH and growth

Auxin derives its name from its trademark effect: growth regulation [53] which also illustrates a textbook auxin paradox—that it activates growth in shoots but inhibits growth in roots. A recent addition to the 'acid growth theory' (which postulates that auxin mediates growth by acidifying the apoplast) includes a rapid ABP1-TMK1-mediated activation of PM H^+ ATPase pumps (AHAs) through their phosphorylation [20,32,54]. In shoot growth regulation, the functional importance of this rapid effect is unclear as it is overlaid on a more dominant slow TIR1/AFB-dependent mechanism, explaining why hypocotyl growth responds to auxin only after 20-30 minutes [10]. In roots, conversely, the growth-promoting TMK-AHA pathway antagonizes the growth-inhibiting TIR1/AFB-dependent apoplast alkalinization, providing a gas-brake pedal machinery for rapid root navigation [20]. What distinguishes the root from the shoot in terms of auxin growth effects and what fine-tunes the relative contribution of TMK versus TIR1/AFB root signaling remains a notorious known unknown.

Auxin regulation of cytoplasmic streaming and post-endocytic trafficking

Increased cytoplasmic streaming is one of the oldest known but mostly neglected rapid auxin effects first documented in 1937 [12]. It requires specific myosin XI family members and it has been linked to the size of individual cells and whole plants [55]. An important insight originating from the ultrafast auxin phospho-proteomes is that the ABP1-TMK1 module rapidly phosphorylates myosin XIK [preprint: 44] as mirrored by defects in auxin-regulated cytoplasmic streaming in *abp1* and *tmk1* mutants [32, preprint: 46]. This implies that ABP1-TMK1 cell surface signaling mediates the auxin effect on cytoplasmic streaming and its hitherto elusive cellular and physiological consequences.

Modulation of endomembrane trafficking by auxin is a more recent and incompletely understood chapter of auxin biology. It was originally found in studies on constitutive PIN recycling indirectly visualized by the trafficking inhibitor Brefeldin A, backed by the use of direct endocytic tracers [56], and later verified with photoswitchable tagged proteins [31]. These approaches revealed that higher auxin levels (predominantly of synthetic auxins) interfere with the internalization and trafficking of multiple cargoes, including PINs. It also became clear that this effect does not require components of the canonical TIR1/AFB-Aux/IAA pathway but rather binding to a distinct site, implicating ABP1 [31].

The development of novel state-of-the-art techniques to study trafficking and individual endocytic events showed that auxin does not directly target the process of endocytosis at the PM [31,56] but rather downstream endocytic trafficking processes [57]. In addition, these studies revealed a highly specific positive auxin role in rapid PIN2 internalization [57]. Auxin thus appears to have two distinct effects on trafficking: (i) high-affinity/high-specificity promotion of PIN2 internalization and (ii) lower-affinity and rather general modulation of bulk post-endocytic traffic. The latter is likely linked to ABP1-TMK1-mediated phosphorylation of myosin XI [preprint: 44], but whether it indeed occurs downstream of ABP1-TMK1 auxin perception and whether it is mechanistically linked to the myosin XI-dependent cytoplasmic streaming is a current unknown.

Auxin feedback on auxin transport

Auxin is famous for its ability to self-organize the formation of polarized auxin-transporting channels, providing positional information for the subsequent development of complex vasculature during organogenesis, leaf venation, shoot branching, and vascular regeneration [58]. The mechanism of such ‘auxin canalization’ necessitates a molecular feedback of auxin

on its own transport, likely through the polarization of PIN auxin transporters [59]. To provide a possible framework for how individual cells might polarize co-ordinately with their neighbors during canalization, mathematical models proposed a hypothetical role of extracellular/apoplastic auxin perception for auxin canalization [60], but mechanistic details remained elusive. A tandem of two manuscripts now provides an elegant explanation for this feedback: extracellular ABP1-based auxin perception, downstream activation of TMK1, and its direct interaction with and phosphorylation of PINs. What is striking is the developmental context dependence of this mechanism. First, ABP1-TMK1 effect on PIN1 modulates PIN1 polarity and presumably explains the canalization phenotypes of *abp1* and *tmk1* mutants [32, preprint: 61]. Second, ABP1-TMK1 feedback on PIN2 seems to rather act as a molecular rheostat that adjusts PIN2 levels to mediate robust root gravitropism [preprint: 62]. These insights will allow for the first time the replacement of speculative parameters in auxin canalization models with solid biological ones to truly 'deconstruct' canalization.

Conclusion

Throughout the text, we highlighted recent discoveries of unknown unknowns in rapid auxin signaling and beyond. These included: (i) adenylate cyclase activity of TIR1/AFB receptors and (ii) ultrafast global phosphorylation downstream of ABP1-TMK cell surface auxin perception. We also provided an outlook on current known unknowns, the most prominent among them: (i) the mechanism behind non-transcriptional effects of the TIR1/AFB pathway, and (ii) the exciting unknown roles of the rapid auxin phospho-response. What we could not do, by definition, is to outline future unknown unknowns of the field. Who knows what exciting breakthroughs tomorrows will bring?

Declaration of competing interests

Nothing declared.

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The opening quote is not intended to reflect any political views of the authors. The authors by no means endorse the rhetoric of Donald Rumsfeld or the 2003 invasion of Iraq by the United

States. Nevertheless, Rumsfeld's quote led to both public and academic debates on the concept of known and unknown unknowns, which can be applied to the recent unexpected developments in the auxin signaling field. We thank Linlin Qi and Huihuang Chen for suggestions on figure presentation and inspiring discussions of TIR1/AFB signaling. Finally, we thank Aroosa Hussain for discussion of Greek mythology.

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•• This study provides a molecular mechanism for auxin feedback on its own transport. This relies on auxin binding to ABP1, TMK1 activation, and auxin-induced interaction of TMK1 with and phosphorylation of PIN2. While auxin feedback on its transport is usually thought to be required for canalization, it appears that in this case the feedback also fine-tunes gravitropic root bending.

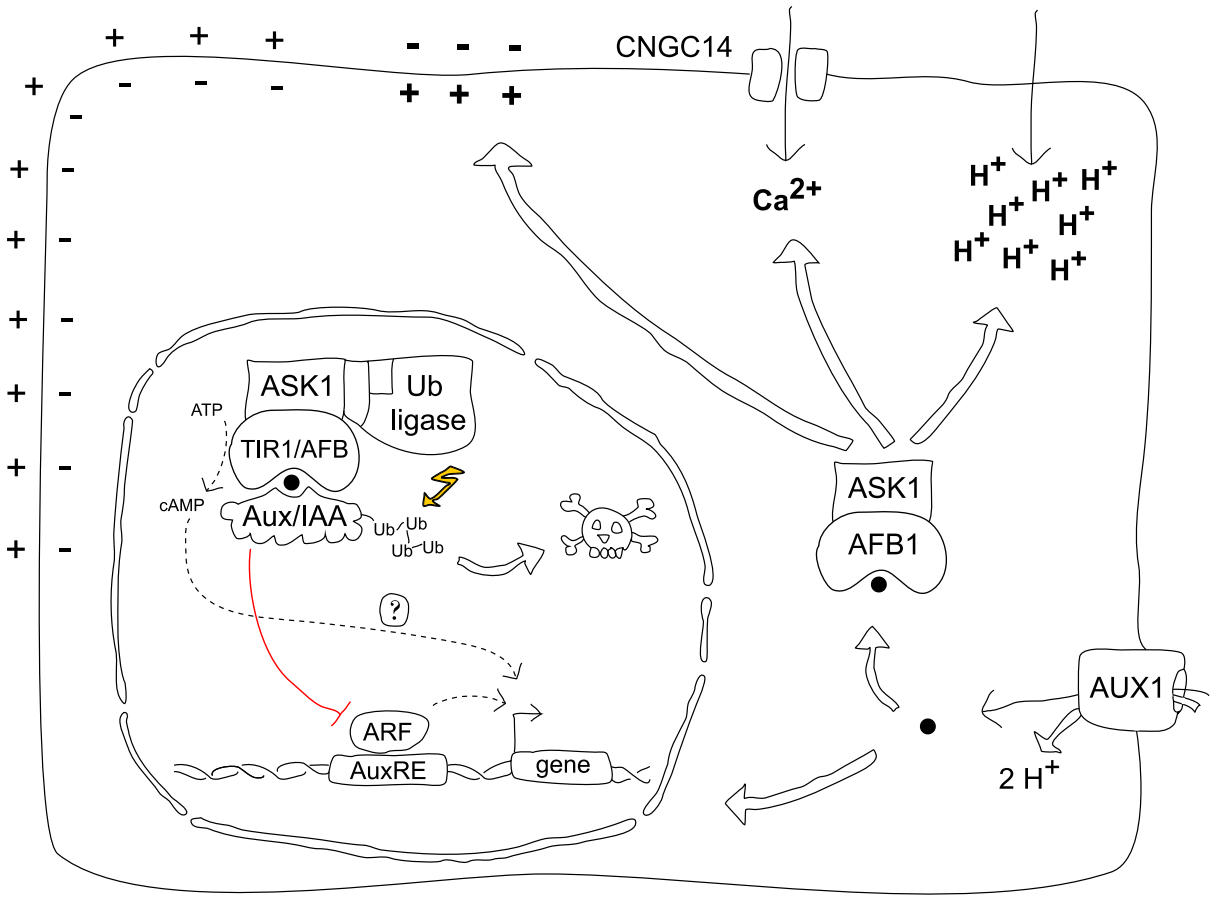

Figure 1. Comparison between rapid and slow intracellular TIR1/AFB-dependent auxin signaling branches.

Auxin (black circles) enters the plant cell through AUX1. In the nucleus, auxin binds to the TIR1/AFB specificity subunits of SCF E3 ubiquitin ligase complexes, allowing them to recognize, ubiquitinate, and degrade Aux/IAA transcriptional co-repressors. This allows ARF transcription factors at auxin response elements (AuxRE) to activate transcription. This slow, transcriptional cascade requires cAMP production by the TIR1/AFB-Aux/IAA complex but details of the cAMP involvement are unclear.

In the cytoplasm, auxin is recognized predominantly by the AFB1 F-box protein, which probably exists in a complex with ASK1 but does not assemble into a full SCF complex. Within seconds, AFB1 then activates a trio of rapid responses by an unknown mechanism: (i) plasma membrane depolarization, (ii) cytosolic CNGC14-dependent Ca^{2+} influx, and (iii) apoplast alkalinization. The main phenotypic readout of this non-transcriptional pathway is rapid root growth inhibition.

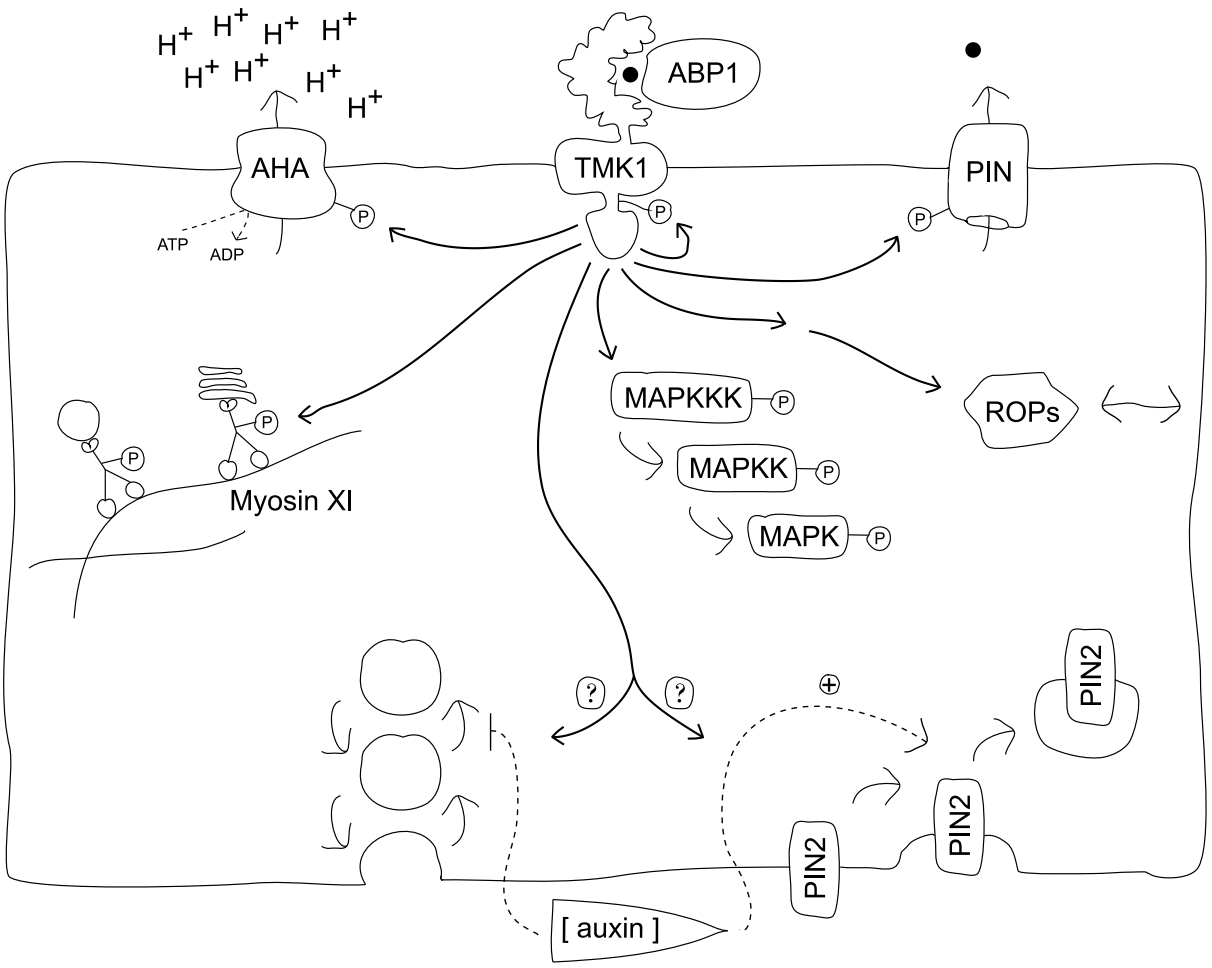

Figure 2. Overview of processes targeted by the ultrafast ABP1-TMK1 phosphorylation in response to extracellular auxin.

The low pH of the apoplast favors auxin (black circle) binding to ABP1, promoting the rapid association of ABP1 with its docking partner, the TMK1 kinase. Activated TMK1 undergoes auto-phosphorylation, and subsequently executes a complex phosphorylation program that targets around 1000 proteins within 2 minutes. In roots, TMK1-induced phosphorylation of AHA H^+ pumps causes rapid apoplast acidification, counteracting the AFB1-mediated rapid apoplast alkalinization (Figure 1), to achieve sensitive soil navigation. Among other direct targets of TMK1 are PIN proteins whose phosphorylation is important during gravitropism and auxin canalization. TMK1 also rapidly activates the MAPK cascade and, through an elusive mechanism, small G-proteins from the ROP family. Another rapid effect of ABP1-TMK1 is the promotion of cytoplasmic streaming; this occurs possibly through myosin XI phosphorylation. Finally, auxin has very rapid and concentration-dependent effects on protein trafficking. While high auxin concentrations inhibit bulk post-endocytic trafficking,

nanomolar auxin concentrations actually specifically promote PIN2 internalization in root cells. At present, it is not clear whether these trafficking effects of auxin depend on the ABP1-TMK1 pathway or not.

Rapid auxin signaling: Unknowns old and new

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There are known unknowns; that is to say, we know there are some things we do not know. But there are also unknown unknowns—the ones we don't know we don't know.

Donald Rumsfeld

Despite there likely being more people aware of Donald Rumsfeld and his controversial political views than those appreciating the importance of auxin as the major regulator of plant development, in the grand scheme of things, the latter is more important. Nevertheless, as it became obvious with a fleur of unexpected recent discoveries—also in the case of auxin and, in particular, auxin signaling—there are many unknown unknowns next to the mysteries that we are aware of.

Among prominent recently solved known unknowns is the structure of PIN auxin transporters [1–3], which has been eagerly awaited since their discovery 25 years ago [4]. It put to rest lingering doubts, if any, as to whether PINs are *bona fide* auxin exporters and provided a long-sought molecular insight into their transport mechanism. No less exciting are unknowns

pertaining to the decades-old enigma of perception and signaling mechanisms behind rapid, non-transcriptional auxin responses, which we review here.

The auxin signaling contract: 'time is of the essence'

Efforts to understand auxin signaling began in the pre-molecular era with investigations of near-instantaneous auxin responses (typically shorter than one minute) such as protoplast swelling or electrical events at the plasma membrane (PM) [5,6]. With the advent of classical genetics, however, there was a major focus shift towards developmental and growth responses which take hours or even days to manifest. The reason for this is simple yet profound: rapid electrophysiological and cellular phenotypes were incompatible with large-scale forward genetic screens which examined hundreds of thousands of *Arabidopsis* seedlings [7]. Researchers thus favored long-term growth phenotypes for their screens. Subsequent cloning and biochemical endeavors gave rise to 'the central dogma of auxin signaling' as we know it today. It comprises a double negative logic motif in which auxin activates transcription by degrading a repressor of gene expression [8].

In more detail: Auxin binds to F-box protein subunits of SCF E3 ubiquitin ligase complexes. This confers on SCF complexes the ability to bind and degrade Aux/IAA transcriptional co-repressors, relieving ARF transcription factors at auxin-responsive promoters from inhibition. Auxin binding occurs in F-box proteins from the TIR1/AFB family via their auxiliary leucine-rich repeat (LRR) domain while their F-box domain interacts with the rest of the SCF through the ASK1 adaptor protein [9]. Experiments with reporter genes demonstrate that this transcriptional pathway takes about 20–30 minutes to produce proteins and associated phenotypic responses [10]. Remarkably, the explanatory power of this mechanism nearly suffices to account for the rather impressive plethora of developmental roles of auxin [11].

If one considers the slow TIR1/AFB pathway for transcriptional regulation as an arising dogma of the early 21st century, then it is fair to label rapid auxin responses as the 'elephant in the room' because they were difficult to reconcile with this model. Among known rapid effects were PM depolarization, membrane proton fluxes, cytosolic Ca^{2+} transients, cytoplasmic streaming, and the alteration of endomembrane trafficking [6,12]. One possibility to accommodate rapid responses was the existence of alternative auxin-binding sites in the cell such as the AUXIN BINDING PROTEIN 1 (ABP1), a cupin-family protein with 50 years of controversial history, originally identified by its ability to bind radiolabeled

auxin in various species [13]. Nevertheless, the signaling mechanism behind rapid responses remained a known unknown.

Dirty little secret of TIR1/AFB auxin receptors: a fast non-transcriptional branch

While auxin-responsive transcription became the mainstream of the field as time passed, there were also continuing efforts to understand how cells respond to auxin within tens of seconds or faster. Pioneering experiments demonstrated that auxin triggers a rapid alkalinization of the root extracellular space (apoplast), mirrored by a cytosolic Ca^{2+} transient. Blockage of the Ca^{2+} response prevented the pH response, indicating that these are causally linked [14]. On the other hand, rapid auxin-mediated PM depolarization was known to occur already from studies conducted during the late 1970s [6].

A striking realization came with the finding that also auxin-triggered root growth inhibition is too rapid to involve transcription and that it actually depends, together with the even faster cytosolic [Ca^{2+}] increase and PM depolarization, on TIR1/AFB auxin receptors [15,16]. Accordingly, these effects were triggered by intracellular auxin because the loss of the AUX1/LAX auxin permease prevented them from happening [16]. Importantly, the CNGC14 membrane channel previously implicated in root gravitropism [17] was shown to be responsible for Ca^{2+} transients downstream of TIR1/AFBs [16]. All this was surprising given that TIR1/AFBs were identified in screens for long-term auxin phenotypes and always linked exclusively with transcriptional regulation. There was therefore no precedent to expect TIR1/AFB involvement in the rapid responses.

A comprehensive account of TIR1/AFB subcellular localization patterns suggested a division of labor among TIR1/AFBs and showed that it is the dominantly cytosolic AFB1 member which displays a major phenotype in rapid root growth inhibition [18]. Follow-up research placed AFB1 upstream of rapid membrane depolarization and apoplast alkalinization [19,20]. More recent work then elaborated that AFB1 orchestrates the formation of discrete alkaline and acidic pH zones along the root tip and that the *cngc14-1* mutant phenocopies an *afb1-3* zonation defect [21], thus establishing the AUX1-AFB1-CNGC14 pH-orchestrating module in rapid root growth.

These exciting discoveries posed an important question: What exactly distinguishes AFB1 from its TIR1/AFB counterparts and allows it to activate rapid signaling? It turns out that its

role in rapid root growth inhibition is an intrinsic property of the AFB1 protein and is not simply conferred by its cytoplasmic localization [22,23]. Cumulative data also suggest that AFB1 shows decreased ability to assemble into a full SCF complex [22–24]. Such a situation is highly reminiscent of non-canonical F-box proteins from the budding yeast which perform SCF-independent functions but still exist in a complex with the homolog of the ASK1 protein [25].

An unexpected and textbook-revising unknown unknown emerged with the discovery that TIR1, AFB1, and likely all other TIR1/AFB-type auxin receptors present in land plants harbor an adenylate cyclase (AC) center which, following auxin perception, synthesizes a second messenger familiar from animal cells: cAMP [26]. Despite being originally identified during a search for a non-transcriptional functionality of TIR1/AFBs, the TIR1 AC activity is unexpectedly required for classic, transcriptional responses, and dispensable for rapid ones.

The previously entirely unsuspected role of cAMP in transcriptional auxin signaling was therefore added to the list of now-known unknowns, next to the mystery of TIR1/AFB-mediated rapid auxin effects.

A phoenix reborn from its ashes: the ABP1 auxin receptor

The fascinating versatility of auxin has inspired biologists to search for auxin receptors for over half a century. Since the recovery of ABP1 using old-school biochemistry (more than twenty years before TIR1/AFB genetics), ABP1 has experienced a convoluted history including the incorrect identification of an embryo-lethal insertion mutant [13]. Importantly, the notorious re-evaluation of *abp1* mutant phenotypes with only minor defects [27–30] poured fuel on the fire of a community that was already, as human nature sometimes dictates, divided by its favoritism for either ABP1 or TIR1 as the more relevant auxin receptor. As a result, most ABP1 research was suspended, and all previous ABP1-related studies were called into question, including those that never used the erroneous mutant alleles.

Several reports noticed that the legitimate ABP1 gain-of-function lines present, conditional to an intact auxin-binding pocket, a wide range of developmental malfunctions including altered trafficking of PIN auxin exporters [28,30,31]. A recent biochemical revision of our knowledge of ABP1 focused on the *Arabidopsis* ABP1 protein and confirmed previous notions from other species: that ABP1 binds auxin at the acidic, apoplast-like pH and that it

partially localizes to the apoplast [32]. The use of verified *Arabidopsis abp1* mutants revealed strong and specific defects in processes related to the auxin-induced formation of new vasculature, such as its regeneration after wounding, or its establishment from an external auxin source. Importantly, an ABP1 version with an engineered lack of auxin binding proved inefficient in complementing the phenotypic defects, highlighting the crucial importance of auxin binding to ABP1 for its function [32]. These experiments reinstated ABP1 as a valid auxin receptor for processes underlying the formation of vascular strands via auxin canalization. What remains unsolved, however, is a notoriously known ABP1 unknown: the function of ABP1 in its main home organelle, the endoplasmic reticulum, whose near-neutral pH disfavors auxin binding to ABP1. Furthermore, according to available reports, the known interaction partners of ABP1 (see next paragraph) do not seem to reside to a significant extent in the endoplasmic reticulum.

Vasculature formation-related phenotypes were uncovered also in the PM interactors of ABP1 known as TRANSMEMBRANE KINASES (TMKs) [32]. Although the canalization phenotypes of single *tmk* mutants are comparable to those of *abp1*, higher-order *tmk* mutants show strong additional developmental defects. This suggests either ABP1-independent functions of TMKs or a functionally redundant action of potential auxin binders other than ABP1 from the same cupin family [33, preprint: 34]. Excitingly, the ABP1-TMK1 module responds to auxin by mediating an ultrafast phosphorylation cascade [32] which represents another unexpected unknown in the universe of rapid auxin signaling.

Essential TRANSMEMBRANE KINASES do what it says on the tin

The *Arabidopsis* TMK family comprises four LRR receptor-like kinases which were initially shown to exhibit genetic redundancy [35], but more detailed investigations revealed also their specific and separable functions in plant development. For instance, only TMK1 is responsible for the maintenance of the apical hook [36] and for auxin-dependent enhancement of abscisic acid signaling [37]. Conversely, TMK4 but not TMK1 regulates auxin biosynthesis by interacting with and phosphorylating the auxin biosynthetic enzyme TAA1 [38]. We finally note that TMK1-mediated phosphorylation has a prominent role in the transcriptional response to auxin within the context of the apical hook [36], as reviewed elsewhere [39].

The action of TIR1/AFB receptors can now be placed directly upstream of a subset of rapid auxin effects [40]. How can we, however, account for the remaining rapid auxin responses such as the activation of PM H^+-ATPases and apoplast acidification, the auxin effect on endomembrane trafficking, or the activation of cytoplasmic streaming? A major indication of TMK involvement in rapid auxin signaling emerged with the discovery that auxin induces the formation of an ABP1-TMK1 complex at the cell surface, rapidly activating small G-proteins called ROPs [41]. Recent investigations indicate that this rapid ABP1-TMK module does exactly what it says on the tin: phosphorylates downstream targets.

ABP1-TMK-mediated ultrafast phosphorylation

Novel evolutionarily conserved rapid auxin response: Renaissance of MAPK signaling

In a quest to understand rapid auxin responses, new studies used cutting-edge phospho-proteomics to probe the involvement of protein phosphorylation, an intrinsically rapid mechanism known to act within 2 minutes of various stimuli in both plants and animals [42,43]. The results were unprecedented: auxin specifically activated the phosphorylation of over 1700 proteins with hundreds of differential phospho-sites already 30 seconds after treatment. This required intact ABP1 and TMK1 but not TIR1/AFBs [32, preprint: 44,45]. However, AFB1 was found to buffer against ABP1-TMK1-mediated phosphorylation [preprint: 45], paralleled by the recent evidence that AFB1 inhibits the transcriptional long-term auxin response [22]. It is possible that AFB1 evolved as a molecular brake for various branches of auxin signaling.

The auxin phospho-response is conserved from land plants to at least basal Streptophyte algae and predates the nuclear TIR1/AFB pathway. In most species examined, it converges on mitogen-activated protein kinase kinase kinases (MAPKKKs) [preprint: 46]. Indeed, the auxin-induced phospho-proteome is enriched in the consensus MAP kinase (MAPK) phosphorylation motif and contains both MPK8 and MPK16 as prominent targets [preprint: 45,46]. Therefore, the ABP1-TMK1 complex activates the MAPK cascade, constituting a potentially ancestral auxin signaling pathway. Multi-level redundancy is a recurring theme during the phospho-response—likely reflecting the fact that its robustness is essential for life—but complicating genetic analysis. Reminiscent of TMKs, a septuple MAPKKK mutant had to be analyzed to see appreciable auxin phenotypes [preprint: 46,47]. A similar problem is also behind the notoriety of ABP1 but was recently addressed by the discovery of two

apoplastic structural ABP1 homologs whose mutants produce conditional synergistic sick phenotypes in combination with the *abp1* mutant [preprint: 34]. We anticipate that all these exciting discoveries will soon allow researchers to probe the mystery of why massive deregulation of protein phosphorylation in *abp1* and *tmk1* mutants produces only mild phenotypes under standard growth conditions [32].

Twenty to thirty years ago, plant biologists were inspired by the animal field to study MAPK signaling in plants, and discovered rapid MAPK activation by auxin within 2-5 minutes of treatment [48,49]. After some initial excitement [50,51], however, MAPKs fell out of fashion being swamped by the successful advent of TIR1/AFB signaling. A recent noteworthy report showed that auxin-activated MAPK signaling participates in lateral root organogenesis downstream of TMK1/4 [52], but the connection between MAPKs and rapid auxin signaling remained in the Middle Ages. Identification of the rapid ABP1-TMK-MAPK phosphorylation, therefore, represents the Renaissance of MAPK signaling in rapid auxin biology.

Auxin effect on pH and growth

Auxin derives its name from its trademark effect: growth regulation [53] which also illustrates a textbook auxin paradox—that it activates growth in shoots but inhibits growth in roots. A recent addition to the 'acid growth theory' (which postulates that auxin mediates growth by acidifying the apoplast) includes a rapid ABP1-TMK1-mediated activation of PM H^+ ATPase pumps (AHAs) through their phosphorylation [20,32,54]. In shoot growth regulation, the functional importance of this rapid effect is unclear as it is overlaid on a more dominant slow TIR1/AFB-dependent mechanism, explaining why hypocotyl growth responds to auxin only after 20-30 minutes [10]. In roots, conversely, the growth-promoting TMK-AHA pathway antagonizes the growth-inhibiting TIR1/AFB-dependent apoplast alkalinization, providing a gas-brake pedal machinery for rapid root navigation [20]. What distinguishes the root from the shoot in terms of auxin growth effects and what fine-tunes the relative contribution of TMK versus TIR1/AFB root signaling remains a notorious known unknown.

Auxin regulation of cytoplasmic streaming and post-endocytic trafficking

Increased cytoplasmic streaming is one of the oldest known but mostly neglected rapid auxin effects first documented in 1937 [12]. It requires specific myosin XI family members and it has been linked to the size of individual cells and whole plants [55]. An important insight originating from the ultrafast auxin phospho-proteomes is that the ABP1-TMK1 module rapidly phosphorylates myosin XIK [preprint: 44] as mirrored by defects in auxin-regulated cytoplasmic streaming in *abp1* and *tmk1* mutants [32, preprint: 46]. This implies that ABP1-TMK1 cell surface signaling mediates the auxin effect on cytoplasmic streaming and its hitherto elusive cellular and physiological consequences.

Modulation of endomembrane trafficking by auxin is a more recent and incompletely understood chapter of auxin biology. It was originally found in studies on constitutive PIN recycling indirectly visualized by the trafficking inhibitor Brefeldin A, backed by the use of direct endocytic tracers [56], and later verified with photoswitchable tagged proteins [31]. These approaches revealed that higher auxin levels (predominantly of synthetic auxins) interfere with the internalization and trafficking of multiple cargoes, including PINs. It also became clear that this effect does not require components of the canonical TIR1/AFB-Aux/IAA pathway but rather binding to a distinct site, implicating ABP1 [31].

The development of novel state-of-the-art techniques to study trafficking and individual endocytic events showed that auxin does not directly target the process of endocytosis at the PM [31,56] but rather downstream endocytic trafficking processes [57]. In addition, these studies revealed a highly specific positive auxin role in rapid PIN2 internalization [57]. Auxin thus appears to have two distinct effects on trafficking: (i) high-affinity/high-specificity promotion of PIN2 internalization and (ii) lower-affinity and rather general modulation of bulk post-endocytic traffic. The latter is likely linked to ABP1-TMK1-mediated phosphorylation of myosin XI [preprint: 44], but whether it indeed occurs downstream of ABP1-TMK1 auxin perception and whether it is mechanistically linked to the myosin XI-dependent cytoplasmic streaming is a current unknown.

Auxin feedback on auxin transport

Auxin is famous for its ability to self-organize the formation of polarized auxin-transporting channels, providing positional information for the subsequent development of complex vasculature during organogenesis, leaf venation, shoot branching, and vascular regeneration [58]. The mechanism of such ‘auxin canalization’ necessitates a molecular feedback of auxin

on its own transport, likely through the polarization of PIN auxin transporters [59]. To provide a possible framework for how individual cells might polarize co-ordinately with their neighbors during canalization, mathematical models proposed a hypothetical role of extracellular/apoplastic auxin perception for auxin canalization [60], but mechanistic details remained elusive. A tandem of two manuscripts now provides an elegant explanation for this feedback: extracellular ABP1-based auxin perception, downstream activation of TMK1, and its direct interaction with and phosphorylation of PINs. What is striking is the developmental context dependence of this mechanism. First, ABP1-TMK1 effect on PIN1 modulates PIN1 polarity and presumably explains the canalization phenotypes of *abp1* and *tmk1* mutants [32, preprint: 61]. Second, ABP1-TMK1 feedback on PIN2 seems to rather act as a molecular rheostat that adjusts PIN2 levels to mediate robust root gravitropism [preprint: 62]. These insights will allow for the first time the replacement of speculative parameters in auxin canalization models with solid biological ones to truly 'deconstruct' canalization.

Conclusion

Throughout the text, we highlighted recent discoveries of unknown unknowns in rapid auxin signaling and beyond. These included: (i) adenylate cyclase activity of TIR1/AFB receptors and (ii) ultrafast global phosphorylation downstream of ABP1-TMK cell surface auxin perception. We also provided an outlook on current known unknowns, the most prominent among them: (i) the mechanism behind non-transcriptional effects of the TIR1/AFB pathway, and (ii) the exciting unknown roles of the rapid auxin phospho-response. What we could not do, by definition, is to outline future unknown unknowns of the field. Who knows what exciting breakthroughs tomorrows will bring?

Declaration of competing interests

Nothing declared.

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The opening quote is not intended to reflect any political views of the authors. The authors by no means endorse the rhetoric of Donald Rumsfeld or the 2003 invasion of Iraq by the United

States. Nevertheless, Rumsfeld's quote led to both public and academic debates on the concept of known and unknown unknowns, which can be applied to the recent unexpected developments in the auxin signaling field. We thank Linlin Qi and Huihuang Chen for suggestions on figure presentation and inspiring discussions of TIR1/AFB signaling. Finally, we thank Aroosa Hussain for discussion of Greek mythology.

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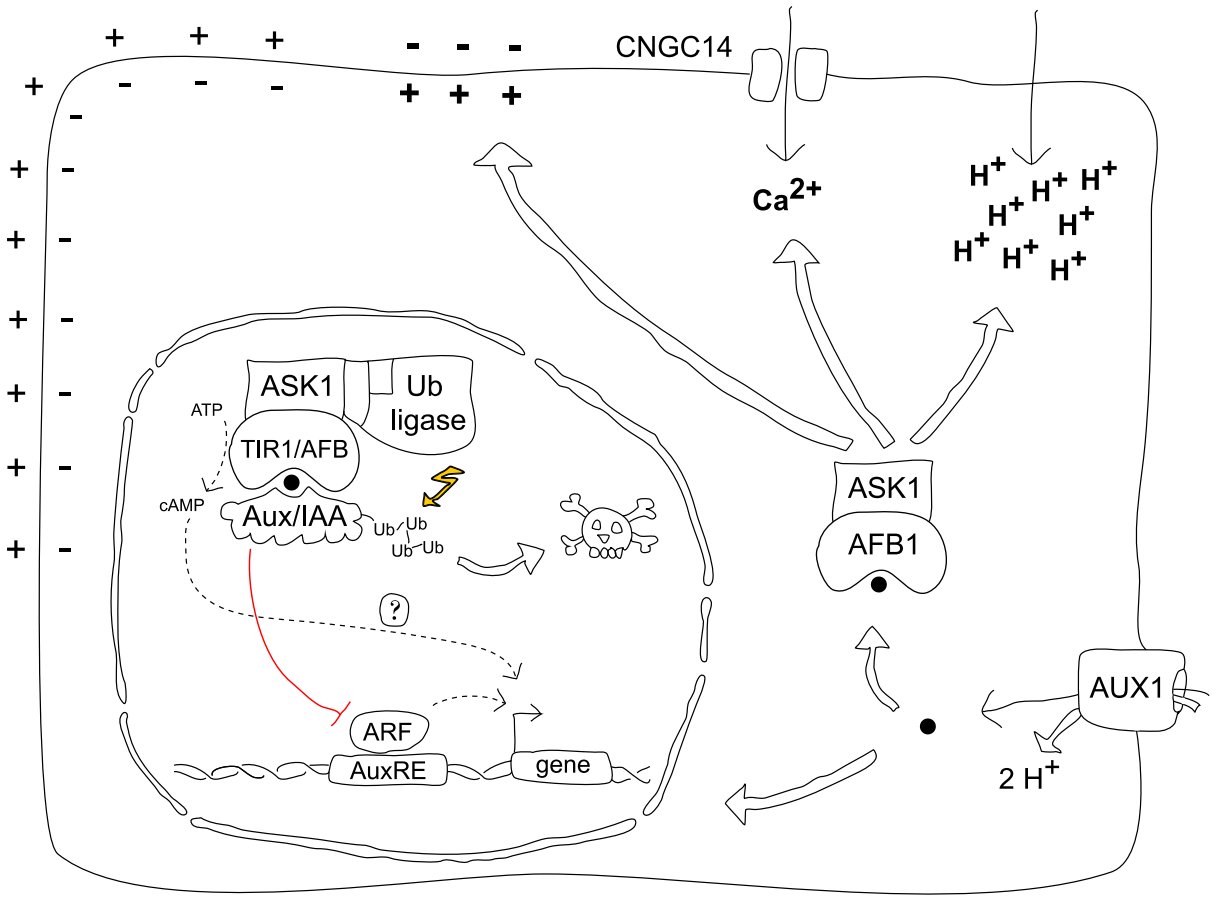

Figure 1. Comparison between rapid and slow intracellular TIR1/AFB-dependent auxin signaling branches.

Auxin (black circles) enters the plant cell through AUX1. In the nucleus, auxin binds to the TIR1/AFB specificity subunits of SCF E3 ubiquitin ligase complexes, allowing them to recognize, ubiquitinate, and degrade Aux/IAA transcriptional co-repressors. This allows ARF transcription factors at auxin response elements (AuxRE) to activate transcription. This slow, transcriptional cascade requires cAMP production by the TIR1/AFB-Aux/IAA complex but details of the cAMP involvement are unclear.

In the cytoplasm, auxin is recognized predominantly by the AFB1 F-box protein, which probably exists in a complex with ASK1 but does not assemble into a full SCF complex. Within seconds, AFB1 then activates a trio of rapid responses by an unknown mechanism: (i) plasma membrane depolarization, (ii) cytosolic CNGC14-dependent Ca^{2+} influx, and (iii) apoplast alkalinization. The main phenotypic readout of this non-transcriptional pathway is rapid root growth inhibition.

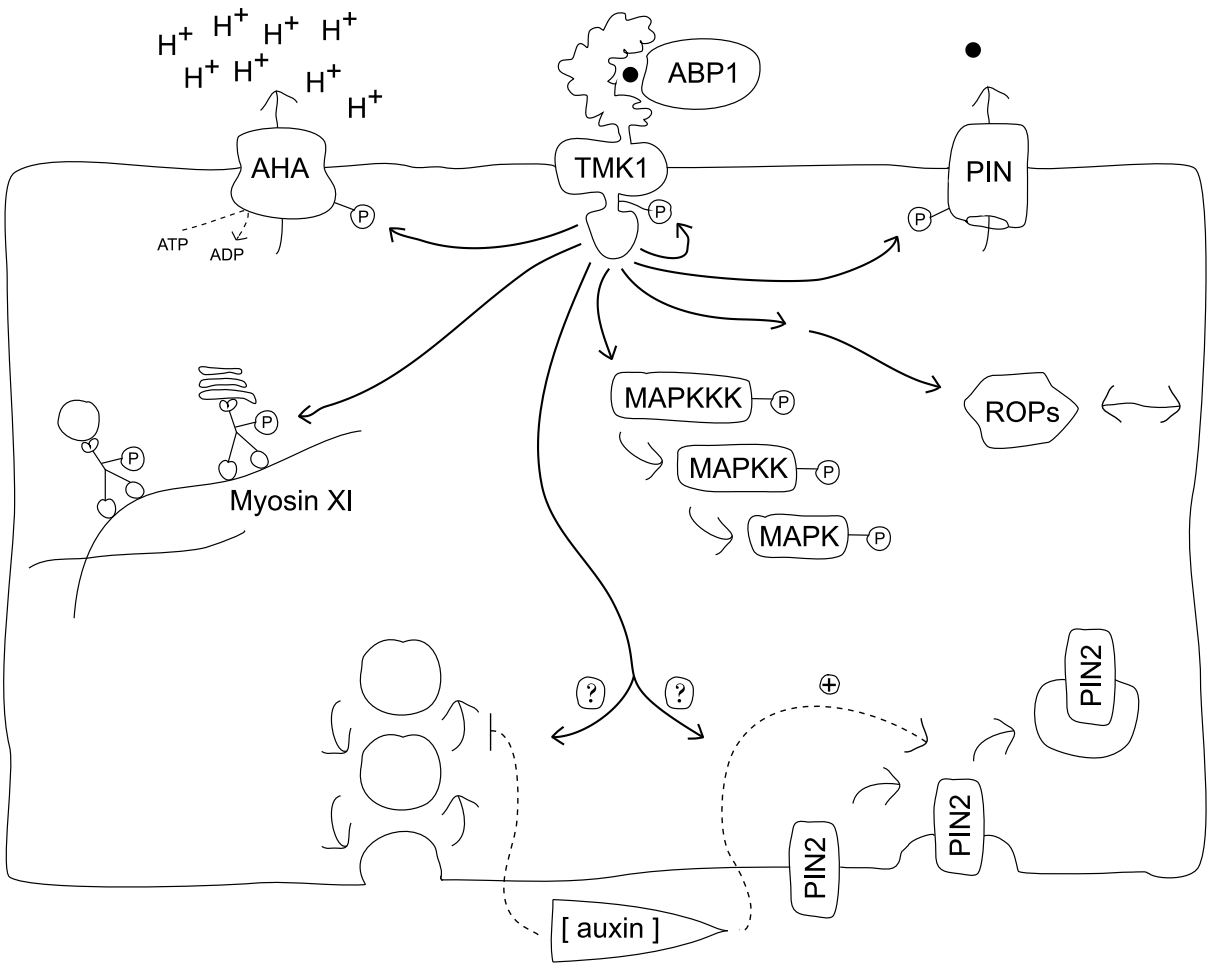

Figure 2. Overview of processes targeted by the ultrafast ABP1-TMK1 phosphorylation in response to extracellular auxin.

The low pH of the apoplast favors auxin (black circle) binding to ABP1, promoting the rapid association of ABP1 with its docking partner, the TMK1 kinase. Activated TMK1 undergoes auto-phosphorylation, and subsequently executes a complex phosphorylation program that targets around 1000 proteins within 2 minutes. In roots, TMK1-induced phosphorylation of AHA H^+ pumps causes rapid apoplast acidification, counteracting the AFB1-mediated rapid apoplast alkalinization (Figure 1), to achieve sensitive soil navigation. Among other direct targets of TMK1 are PIN proteins whose phosphorylation is important during gravitropism and auxin canalization. TMK1 also rapidly activates the MAPK cascade and, through an elusive mechanism, small G-proteins from the ROP family. Another rapid effect of ABP1-TMK1 is the promotion of cytoplasmic streaming; this occurs possibly through myosin XI phosphorylation. Finally, auxin has very rapid and concentration-dependent effects on protein trafficking. While high auxin concentrations inhibit bulk post-endocytic trafficking,

nanomolar auxin concentrations actually specifically promote PIN2 internalization in root cells. At present, it is not clear whether these trafficking effects of auxin depend on the ABP1-TMK1 pathway or not.