Non-canonical auxin signalling: fast and curious

Martin Kubeš[1, 2, 3] and Richard Napier[1]

[1] School of Live Sciences, University of Warwick, Coventry, United Kingdom

[2] Laboratory of Growth Regulators, The Czech Academy of Sciences, Institute of Experimental Botany & Palacky University, Slechtitelu 27, CZ-78371 Olomouc, Czech Republic

[3] University Hradec Kralove, Fac Sci, Dept Biol, Rokitanskeho 62, CZ-50003 Hradec Kralove, Czech Republic

Correspondence: Martin.Kubes@warwick.ac.uk or Richard.Napier@warwick.ac.uk

Many plant biologists might think that the auxin signalling pathway has been resolved. Activation of gene expression as a result of indole-3-acetic acid (IAA)-mediated assembly of Transport Inhibitor1 (TIR1)/Auxin F-Box (AFB) proteins with AUX/IAA transcriptional regulators has become accepted as the canonical auxin signalling pathway. However, the evidence strongly suggests that non-canonical pathways will still prove to be important, and this theme ran through the 2018 Auxins and Cytokinins in Plant Development conference held in Prague (ACPD 2018).

There is no doubt that the canonical pathway deserves its title because most auxin-mediated phenotypes can be accounted for by TIR1/AFB-mediated ubiquitination of AUX/IAAs (Leyser, 2018). The diversity of responses to auxin is then attributed to differential gene expression brought about as a consequence of the multiplicity of AUX/IAAs, a similar multiplicity of Auxin Response Factors (ARFs) with which AUX/IAAs heterodimerize, cellular context and the varying dose dependencies of TIR1/AFB co-receptor formation (Calderon *et al.*, 2012) (see Box 1).

Historically, Auxin-Binding Protein1 (ABP1) offered another possible auxin signalliong pathway, but the ABP1 story was foreshortened when new *ABP1* knockout lines were found to have no gross phenotype (Gao *et al.*, 2015), and subsequent publications explained why some earlier tools in ABP1 research had been misleading (Dai *et al.*, 2015; Michalko *et al.*, 2016). Yet there have always been observations of responses to auxin which are difficult to rationalize with the canonical pathway, such as those too rapid to be the consequence of transcription and translation (Badescu and Napier, 2006). Details of such non-canonical pathways are now appearing in abundance. Somewhat surprisingly, TIR1 remains at the heart of some of these mechanisms.

Root responses too quick for the canonical pathway

Fendrych and Friml have developed a microscopy platform that allows roots to be imaged *in vivo* growing vertically (von Wangenheim *et al.*, 2017*a,b*). A microfluidic perfusion system allows the root medium to be switched on demand (Grossmann *et al.*, 2012) and these technical advances allowed them to record real-time images of primary roots responding to auxin by reducing elongation growth within 30 seconds (Fendrych *et al.*, 2018). This is far too fast for the canonical pathway of auxin signalling. Just as remarkable is that the growth rate recovered within two minutes after removing IAA from the bathing medium, illustrating strong and rapid homeostatic control of cellular auxin concentrations. Auxin responses were induced by nanomolar IAA concentrations, with an IC50 of 1.4 nM.

Given that the response was so rapid and so sensitive it was pertinent to ask whether the site of perception was intracellular or extracellular, and so a knockout line for the auxin uptake carrier, AUX1, was used. Rapid changes in root elongation growth were shown to depend on AUX1, indicating that the auxin signal was detected inside the cells. Exactly the same conclusion was reached in work from the Hedrich lab which used root hair cells impaled with microelectrodes to demonstrate similarly rapid and AUX1-dependent changes in plasma membrane (PM) potential in response to auxin challenge (Dindas *et al.*, 2018). Both groups went on to use single and multiple

TIR1 and *AFB* mutant lines show that the responses were also dependent on TIR1/AFBs. This is remarkable because it suggests that these receptors must have a second mechanism of action, a mechanism that is very rapid and exercised at the PM (see Box 2). The canonical TIR1 pathway is certainly based within the nucleus (Wang *et al.*, 2016) and is expected to take many minutes to complete transcription and translation, even of early auxin response genes.

In addition to use of the *TIR1/AFB* mutant lines, Fendrych and Friml used an exciting new synthetic auxin switch based on TIR1 (Uchida *et al.*, 2018) to confirm the requirement for this receptor in rapid root responses. This version of TIR1 has been engineered to extend the auxin-binding pocket (concave TIR1). The partner to concave TIR1 is a novel auxin (convex auxin) which has been designed to match the extended pocket, but is unable to bind to wild type TIR1/AFBs. Thus, the synthetic convex auxin activates auxin signalling only in plants transformed to express concave TIR1, and in these lines convex auxin induces rapid inhibition of primary root extension confirming the involvement of TIR1 in this response (Fendrych *et al.*, 2018).

The events at the PM are initiated with a membrane depolarization, which was associated with the co-transport of protons with AUX1-mediated IAA uptake (Dindas *et al.*, 2018). Interestingly, the depolarization pattern was found to include transient influx of Ca^{2+} ions and this rise in intracellular calcium concentration may be associated with further mechanisms to drive longer and larger depolarization events. Root gravitropism in *Arabidopsis* requires the cyclic nucleotide-gated channel 14 (CNGC14; Shih *et al.*, 2015) and the Hedrich group confirmed that this channel protein was responsible for IAA-induced Ca^{2+} influx by showing that calcium influx was absent in *cngc14* root hairs.

It is intriguing that so many key features of rapid, TIR1-dependent non-canonical auxin signalling have been identified and yet there remain so many unanswered questions about the system. Principal amongst these are how TIR1/AFBs converse so rapidly with the PM to trigger ion fluxes, and how many elements comprise this signalling pathway. Suggestions have been made (Dindas *et al.*, 2018; Fendrych *et al.*, 2018; Retzer *et al.*, 2018) and a key realization is that TIR1 and AFBs are not found exclusively in the nucleus. With our eye on canonical nuclear signalling we have merely overlooked these proteins in the cytoplasm (Wang *et al.*, 2016) since this population is small (Yu *et al.*, 2015). Mutants of TIR1 have also been shown to promote dissociation of TIR1 from its Ubiquitin E3 ligase complex, and the untethered TIR1 is stabilized and accumulates (Yu *et al.*, 2015). Whilst this was found to lead to auxin-resistant phenotypes, no rapid responses were investigated. Given that mammalian HSP90 family members are mechanistically connected to, for example, estrogen receptor activity and migration between the cytoplasm and nucleus (Echeverria and Picard, 2010), it

is also interesting that *Arabidopsis* TIR1 has been shown to be chaperoned by Heat-Shock Protein 90s (HSP90s) and Suppressor of G2 allele SKP1 (SGT1; Wang *et al.*, 2016; Watanabe *et al.*, 2017).

Non-canonical auxin signalling beyond TIR1 and the AFBs: ETTIN

Not all reports of non-canonical auxin signalling have been linked to TIR1 and the AFBs. A prominent non-canonical pathway is associated with one of the auxin-response factors (ARFs). The ARFs are a family of transcription factors which bind to Auxin Response Elements (AREs) through a conserved N-terminal DNA-binding domain (Chandler, 2016; Weijers and Wagner, 2016; Leyser, 2018; Roosjen et al., 2018). These N-terminal domains also function as dimerization domains. Most ARFs also contain a conserved C-terminal Phox/Bem1p box (PB1) domain which is responsible for binding AUX/IAA proteins in the canonical auxin pathway. One variant is ARF3, also known as ETTIN (ETT), which has a long and intrinsically-disordered C-terminal domain (ETT-specific, ES domain; Simonini *et al*., 2018a) and this domain interacts with a set of alternative transcriptional regulators which include INDEHISCENT (IND), REPLUMLESS (RPL) and BREVIPEDICELLUS (BP; Simonini *et al*., 2016). All these transcription factors contribute to determining plant shape and pattern and the role of ETT and its interactions with these other tissue identity factors has been especially well described during gynoecium development (Simonini *et al.*, 2016, 2017, 2018b). Of particular interest is the observation that ETT controls growth and tissue patterning in an IAA-dependent mechanism that requires neither ubiquitination nor TIR1 (Simonini *et al*., 2016).

The Ostergaard group has shown that non-canonical auxin signalling *via* ETT is mediated by IAA interfering with the interaction of ETT with other transcriptional regulators, such as IND (Simonini *et al*., 2016). In a mechanism which parallels that of AUX/IAA repression of transcription in the canonical TIR1 pathway, ETT forms a repressive complex that is released by IAA to change patterns of transcription. Intensive analysis of the ES domain using informatics, mutagenesis, yeast 2 hybrid (Y2H) and expression techniques revealed long stretches with little sequence conservation interspersed with a series of short conserved motifs (Simonini *et al*., 2018a). The conserved motifs include a nuclear localisation sequence and a serine-rich kinase site plus three others for which a function has yet to be identified. The low sequence conservation in the rest of the domain is consistent with a generally disordered structure which was confirmed using circular dichroism. Expressed and purified ES domain did not bind IAA, a finding that was attributed to the lack of structure. Yet, constructs of ES did confer full responsiveness to IAA when cloned in the Y2H system and assayed for interaction with IND. Most of the ES domain seems to be required for the interaction, although IAA sensitivity was lost when any of the serine residues was mutated from the kinase motif. Interestingly, single serine substututions at a site which appears to be specific to

Arabidopsis and the Brassica family also knocked out ETT-IND binding, but this was not IAA-dependent (Simonini *et al*., 2018b).

It is tempting to hypothesise that the residues necessary for IAA-dependent activity are directly involved in an auxin sensing mechanism, either by contributing directly to binding, or by contributing to a conformation conducive to interaction with IAA. However, the ETT effect has low sensitivity to auxin (treatments were generally between 50 and 100 μM IAA), is not triggered by the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) or 1-naphthylacetic acid (1-NAA) and evolution of the ARF3 clade arose far more recently than the canonical auxin pathway (Mutte *et al.*, 2018) making this an interesting, but somewhat idiosyncratic non-canonical pathway. The role of ETT at the centre of a vital transcriptional hub is not in doubt, nor that this could influence and be influenced by local auxin dynamics, but the mode of action of auxin on ETT *in planta* remains somewhat less certain. The lack of IAA binding by the ES domain does not rule out a conformationally-dependent binding pocket (Simonini et al., 2018a), but it is also possible, for example, that IAA is acting at these higher concentrations as a mimic of certain residues involved in intermolecular association, replacing them in the association and, hence, dissolving the interaction (Box 3).

Non-canonical auxin signalling: Kinases

The term 'non-canonical auxin signalling' has also been linked to different sets of protein kinases. It has been established for some years that the localization of the auxin efflux proteins known as PINs is, in some cases, determined by phosphorylation of target residues in their intracellular loops (Michniewicz *et al.*, 2007; Dhonukshe *et al.*, 2010). The D6 protein kinases, PINOID, mitogen-activated protein kinases (MAPKs) and PM-associated kinases are all implicated (Barbosa *et al.*, 2014; Armengot *et al.*, 2016; Dory *et al.*, 2018; Haga *et al.*, 2018; Marhavá *et al.*, 2018) and all contribute to auxin action by moderating PIN protein localization and activity, some of them rapidly. Clearly, regulation of auxin action via its transport affects auxin signalling indirectly. Yet, kinase cascades are rapid could be involved in rapid, non-canonical signalling. Indeed, not long ago transmembrane kinases (TMKs) were linked to auxin signalling (Dai *et al.*, 2013) and to rapid non-canonical auxin signalling via ABP1 (Xu *et al.*, 2014). The TMKs form a subfamily of the plant receptor-like kinases (RLKs; Dai et al., 2014). The RLKs are a large and diverse family sharing a few structural features which include an extracellular domain that frequently acts as an activation domain, a transmembrane domain and an intracellular kinase domain, sometimes with a phosphorelay receiver domain. The family does include receptors for other plant signals such as brassinosteroids, but since the foreshortening of the ABP1 story we await further reports on TMK involvement in auxin signalling with interest.

It is also clear that MAPK cascades are involved in many auxin regulated responses systems (Enders *et al*., 2017). The roles of MAPKs include phosphorylation of ROP Binding protein Kinase 1 (RBK1) leading to activation of members of the Rho-like GTPases from Plants (ROP) which are small GTPases often linked to regulation of the cytoskeleton and auxin transport (Dai *et al*., 2013; Huang et al., 2014). The rapidity of the TIR1/AFB-dependent non-canonical signal (Box 2) makes a kinase cascade an attractive candidate relay mechanism, although no kinase has yet been linked to these activities.

Perspective

The proliferation of results implicating non-canonical pathways shows acute and continuing interest in the immediate consequences of IAA perception and a readiness to believe that auxin signalling is not limited to TIRs (Badescu and Napier, 2006). Rapid responses to auxins have been discussed previously in terms of a two-receptor concept (Scherer, 2011), although it remains possible that plants rely on one receptor (family) with two mechanisms of action. Auxins remain important for food security as herbicides (Quareshy *et al.,* 2018) and so any new mechanistic understanding will contribute to how we manage these agrochemicals to retain utility and combat resistance (Busi et al., 2018). We now have some excellent new tools and assays to chase down unknown pathway contributors.

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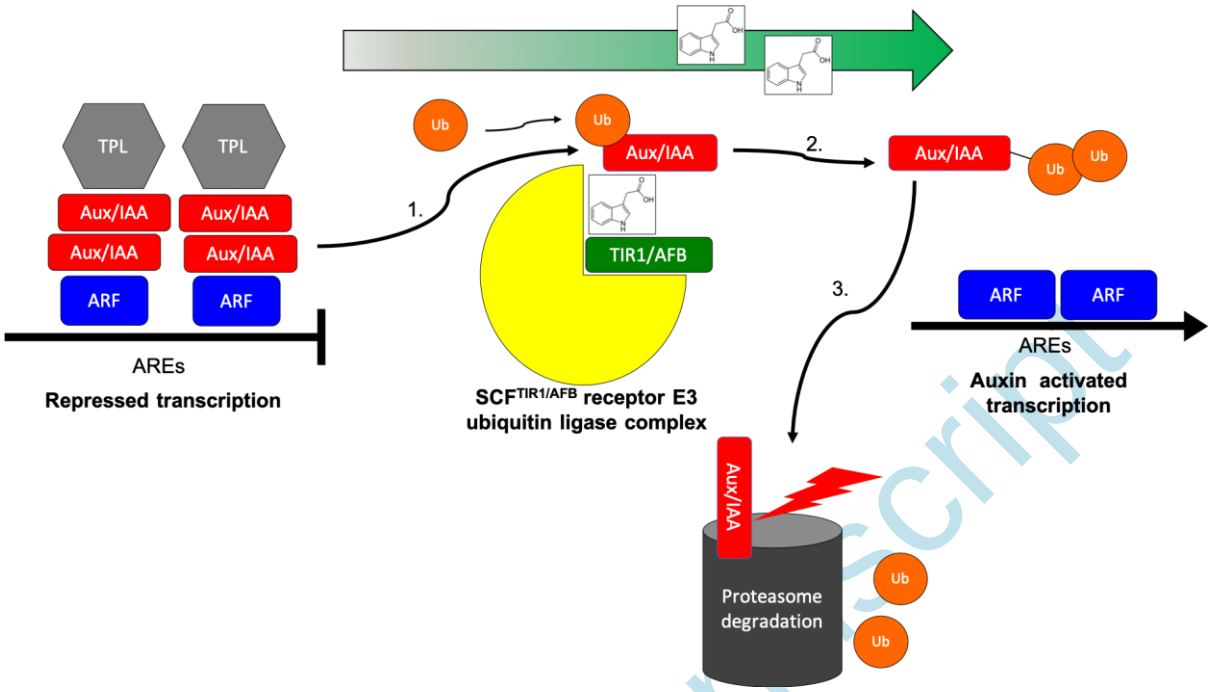
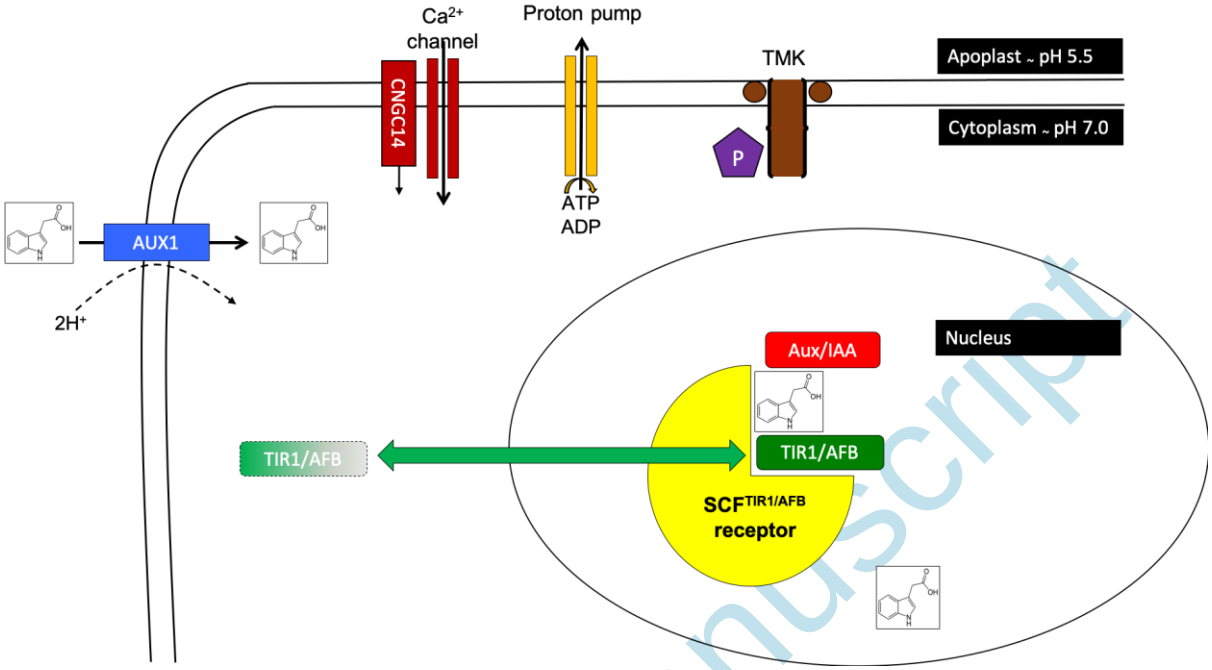
Box 1. Canonical TIR



1-dependent auxin signalling.

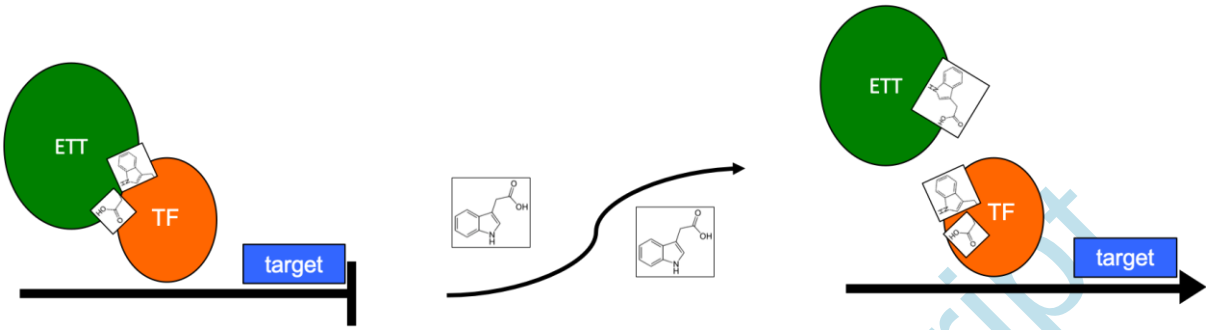
In the TIR1-dependent pathway auxin controls the transcription of auxin-inducible genes. Promoters of these genes contain auxin-response elements (AREs) and are bound by auxin-response factor (ARF) dimers. Expression is blocked by Aux/IAA transcriptional repressors via their interaction with ARFs and TOPLESS (TPL) corepressors. 1. Auxin brings together Aux/IAAs and F-box proteins of the TIR1/AFB family. 2. The $SCF^{TIR1/AFB}$ E3 ubiquitin ligase complex transfers activated ubiquitin (Ub) to the AUX/IAA. 3. Polyubiquitination of the Aux/IAAs results in their degradation in the proteasome. 4. The reduced Aux/IAA population releases repression of the ARFs and transcription is activated.

Box 2. Cytoplasmic and plasma membrane players in non-canonical auxin sensing



Auxin enters cells via AUX1 and can be perceived by the $SCF^{TIR1/AFB}$ receptor complex localized in nucleus and by TIR1 in the cytosol. One non-canonical pathway involves this cytosolic TIR1 activating the cyclic nucleotide-gated ion channel CNGC14 leading to Ca^{2+}-influx ands plasma membrane depolarisation. A second non-canonical pathway might involve trans-membrane kinases (TMK). In each case the events downstream from auxin binding remain unknown.

Box 3. The ETT non-canonical auxin sensing pathway



ETT interacts with transcription factors (TF) such as IND. The complex represses transcription. Tryptophan and acidic amino acids such as aspartic acid may contribute to these protein-protein interactions. These side groups are common with features of IAA, namely the indole ring and a free carboxylic acid group. At high IAA concentrations, IAA weakens the interaction by displacing these side groups and releasing ETT (based on the scheme in Simonini et al., 2016).