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***Title*:** ARF degradation fine-tunes auxin response in land plants

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***Standfirst**:*

Two recent studies in phylogenetically distant plants integrate a new layer of control into the canonical auxin pathway. A deeply conserved degron affecting the stability of repressors regulates transcriptional responses and plant development.

Main (1141w):

Plants display an incredible capacity for post-embryonic developmental plasticity, allowing them to adapt to a diverse array of environments. This spatiotemporal modulation of growth, which is orchestrated by phytohormone signalling, is the result of integration of numerous environmental inputs into systemic signals[1]. Amongst plant phytohormones, auxin refers to a class of small molecules that regulate multiple aspects of plant development, ranging from cell fate decisions and organ initiation, from embryogenesis to modulation of plant architecture. First named in the 1920s as a diffusible chemical messenger[2], the details of how auxin is able to regulate such a diverse array of growth responses still holds many challenging, unanswered questions[3]. Writing in *Nature Plants*, de Roij *et al.* [4] and Prigge *et al.* [5] provide new insights into this process.

Auxin signalling occurs through the activity of a number of deeply conserved transcriptional regulator protein families found in all land plants. These multigene families include the AUXIN/INDOLE-3-ACETIC-ACID auxin co-receptor/transcriptional co-repressor proteins (AUX/IAAs), AUXIN RESPONSE FACTORS (ARFs) transcription factors, and the TRANSPORT INHIBITOR RESPONSE 1 /AUXIN-SIGNALING F-BOX (TIR1/AFB) auxin co-receptors.

In flowering plants, ARFs fall into three classes: class A, B and C. Class A ARFs are thought to be activators, while class B ARFs are potential repressors and antagonists to class A ARFs. It has been suggested that class C ARFs are not involved in mediating auxin response[6]. Because class A and B ARFs compete for binding to the same auxin response elements (AuxREs) in the promoters of auxin responsive genes, the mechanisms underpinning the regulation of the stoichiometry of these ARFs are key parameters that

govern the specificity of the auxin response (Figure 1). A systems-level analysis of protein interactions in Arabidopsis has revealed that auxin signalling more strongly influences A-class ARFs compared to B/C-class ARFs. In the moss Physcomitrella patens, individual class A- and B- ARFs can also act antagonistically by competitively binding to the same promoter regions. Yet, these trends are not absolute – some class A-ARFs have been shown to directly repress gene targets, Aux/IAAs can interact with some class B/C-ARFs, and A- and B- ARFs can bind distinct DNA sequences[3,7]. These diverse and conflicting reports highlight a key uncertainty – how the core biochemical structure of the auxin response has evolved across multigene families that have acquired novel and specialised functions over time[7].

The auxin-dependent regulation of the Aux/IAA family of auxin co-receptors/co-repressors has already been demonstrated to be critical to modulate cell and tissue specific auxin responses in multiple land plants[8]. However, to date, the regulation of protein stability of ARFs has received relatively little attention. Here, de Roij *et al.* and Prigge *et al.* shed first light on this process in diverse land plants, including Marchantia, moss and maize[4,5]. Crucially, they demonstrate that the mechanisms governing class B ARF instability are conserved across all the species under study, suggesting ancient evolutionary origin. Both groups independently found that conserved mutations within the DNA binding domain (DBD) of class B ARFs were sufficient to cause protein accumulation by preventing proteasome-dependent degradation. Further, these mutations were found to be outside the previously identified core DBD motif, in a previously uncharacterised outward facing loop towards the C terminus of an alpha helical loop within the DBD. Interestingly, Prigge *et al.* further showed that complete functionality of all other known domains in moss class B ARFs was required for protein accumulation[5].

Both de Roij *et al.* and Prigge *et al.* used gene-editing and protein engineering approaches in moss, maize[5] and Marchantia[4], to demonstrate that selectively mutating specific residues within this region had profound effects on the stability of class B ARFs. The stabilisation of these ARFs strongly impacted growth and development in maize and moss, including reduced plant height as well as increased number of leaves and ear-traits in tassels[5]. Given that engineering an equivalent mutation in Arabidopsis conferred stability to ARF2, another class B ARF, the authors concluded that instability in B ARFs has a single evolutionary origin[5].

Interestingly, a previous study had already showed that MpARF1 and MpARF2 were both degraded via the proteasome in Marchantia[9]. Therefore, as both class A and B ARFs are unstable in Marchantia, de Roij *et al.* asked if they shared this origin of instability. They addressed this question by performing domain swap experiments between the class A, B and C ARFs in Marchantia, and demonstrated convincingly that homologous regions between class A and B, but not C ARFs confer protein instability, indicating that this feature might have a single origin predating the divergence of A and B ARFs. Finally, to test this hypothesis, the authors swapped a homologous domain from an algal ARF (SmARF2) into a Marchantia class B nuclear fusion construct[4]. This fusion was highly stable, indicating two distinct possibilities. First, the ancestral A/B-ARF protein may have been unstable via the DBD region, a feature that was later lost in the lineage leading to SmARF2. Alternatively, the ancestral A/B-ARF may have been stable, with instability emerging in the lineage that led to land plants before the A/B divergence. However, de Roij *et al.* rightly conclude that the extensive evolutionary divergence in this algal group (>600 million years ago) and limited species sampling make such inferences challenging.

Collectively, it is clear that these evolutionarily conserved mechanisms that regulate the stability of auxin signalling proteins are key processes to fine-tune auxin dependent growth and development across land plants. Over two decades of research has already established that the regulation of Aux/IAA protein stability and turnover via multiple signalling inputs has dramatic effects on numerous aspects of auxin-mediated plant development. These works show that the regulation of ARF (in)stability is also critical in this regard. Similar to Aux/IAA degradation, further work will doubtless focus on trying to establish how the minimal degron region within class A/B ARFs confers protein instability. Both groups propose that this is likely to be dependent on co-evolution and binding with a partner, proteolytic adaptor or ubiquitin ligase protein.

In the case of Aux/IAAs, it is well established that their degradation is auxin-dependent, responding to fluctuations in auxin levels driven by both internal developmental cues and external environmental signals[10]. However, how these intrinsic and extrinsic signals are integrated into the auxin signalling pathway - particularly in regulating ARF protein levels in a cell- and tissue-specific manner - remains an open question. For instance, a previous study has shown that stresses such as temperature, salinity, and drought can directly influence some class A ARF protein levels[11]. Yet, it is unclear whether this fluctuation in protein levels occurs through changes in transcription, post-translational modifications, and/or proteasome-mediated degradation. Further work is required to establish which, if any, of these is most likely. In any case, identifying additional components involved in ARF degradation in the future will enhance our understanding of the mechanisms, diversity, and biological significance of how the different pieces of the auxin puzzle fit together.

Figure legend:

Figure 1. **Class A and B Auxin Response Factors (ARFs) can compete for binding to Auxin Response Elements (AuxREs) in the promoters of auxin-responsive genes.** AuxRE-bound class B ARFs repress transcription while class A ARFs typically activate transcription, unless they are bound by Aux/IAA transcriptional co-repressor proteins. Aux/IAAs are degraded in response to auxin in a dose-dependent manner, allowing class A ARFs to promote transcription. New studies show that class B ARFs contain a degron domain that allow them to be targeted by an unknown mechanism that leads to their degradation.

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