Proteomics perspective on auxin biology

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2020

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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences.

Proteomics perspective on auxin biology

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof.Dr. A.P.J. Mol
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 30 March 2020
at 1:30 p.m. in the Aula.

Mark Roosjen
Proteomics perspective on auxin biology,
164 pages.

PhD thesis, Wageningen University, Wageningen, NL (2020)
With references and summary in English

ISBN: 978-94-6395-270-5
DOI: https://doi.org/10.18174/511017

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Chapter 1

Introduction

Auxin

Already in the 19th century, Charles and Francis Darwin hypothesized the existence of a mobile signal that promotes the elongation of grass coleoptiles (Darwin Charles, Darwin Francis, 1880). In 1935, Thimann and Koepfli discovered this plant hormone to be auxin (derived from the Greek word auxano, meaning "to grow or to expand") (Thimann and Koepfli 1935; reviewed in Abel and Theologis 2010). The most studied auxin is indole-3-acetic acid (IAA). It has a structure that is similar to the amino acid trypthophan and is a weak organic acid (reviewed in Paque and Weijers 2016). The discovery of auxin paved the way to many physiological, genetic and biochemical studies that show that auxin is involved in almost all aspects of plant development and growth (reviewed in Weijers and Wagner 2016). Combined efforts by many teams have revealed the mechanisms of hormone synthesis and inactivation (Korasick, Enders, and Strader 2013), directional auxin transport (Adamowski and Friml 2015), crosstalk with other plant hormones (Vert and Chory 2011) and the role of auxin in interactions of plants with microorganisms and viruses (Boivin, Fonouni-Farde, and Frugier 2016). One of the most striking conclusions of all these studies is that a complex panel of processes is regulated by a single, chemically simple hormone.

Many auxin activities are mediated by effects on gene regulation through the nuclear auxin pathway (NAP). This NAP consist of three dedicated core components: the TRANSPORT INHIBITOR RESISTANT1/ AUXIN SIGNALLING F-BOX (TIR1/AFB) F box proteins, the AUXIN/INDOLE-3-ACETIC-ACID (Aux/IAA) co-regulators and the AUXIN RESPONSE FACTORS (ARFs). The pathway (extensively reviewed in chapter 2) ultimately triggers alteration in gene expression and thus controls developmental and growth responses. The NAP relies on the inhibitory action of Aux/IAA proteins on ARF transcription factors. At low auxin levels, Aux/IAA's hetero-dimerize with the ARFs through their shared PB1 domains (Tiwari et al. 2001). Further recruitment by Aux/IAAs of TOPLESS (TPL) and histone deacetylases results in a non-permissive transcriptional environment and repression of ARF target genes (Szemenyei, Hannon, and Long 2008; Krogan, Hogan, and Long 2012; Wu et al. 2015). When auxin levels increase, it binds to the TIR1/AFB receptor complexes, serving as "molecular glue" with domain II of Aux/IAAs, and subsequently leads to ubiquitination and degradation of Aux/IAAs through the 26S proteasome complex (Gray et al. 2001; Zenser et al. 2001). This relieves the ARFs from transcriptional repression and allows activation of target genes.

Auxin-dependent transcriptional processes

The transcriptional auxin output has many roles in plant growth and development. One of the classical responses described for auxin is cell wall acidification, leading to increased growth rates of above-ground tissue (the acid growth theory) (Arsuffi and Braybrook 2017). The transcriptional mechanism underlying this response depends on the auxin-induced expression of SMALL AUXIN UP-RNA (SAUR) genes. SAUR proteins trigger apoplastic acidification through activation of the plasma membrane-localized H+-ATPase proton pump (Spartz et al. 2014). Specifically, SAUR proteins promote threonine phosphorylation of the pumps and inactivation of inhibitory phosphatases (Spartz et al. 2014; Takahashi, Hayashi, and Kinoshita 2012). Besides this classical growth response, auxin is responsible for many developmental processes such as embryo development, vascular tissue formation and differentiation, lateral root formation and reproductive development (reviewed in Weijers and Wagner 2016). Especially the organogenesis of the reproductive shoot apex exemplifies a well-studied auxin response. The central stem cell pool within the shoot apex give rise to new organs when auxin is perceived (Reinhardt, Mandel, and Kuhlemeier 2000). The perception of an auxin maximum dictates flower primordia initiation, and ARF5 is a prominent regulators of this process (Przemeck et al. 1996). The establishment of maxima is achieved through the polar auxin transport system (PAT) (Reinhardt et al. 2003). Both in shoot and in root tissues, this system is critical for correct growth and development (Muday and DeLong 2001). Directional transport of auxin, mediated by auxin influx and efflux PIN FORMED (PIN) proteins, causes maxima, minima and concentration gradients.

Recently, large-scale transcriptomic analysis of 1000 plant species allowed the study of the origin and evolution of the NAP (Mutte et al. 2018). This analysis showed that the NAP is not limited to angiosperms, but that the origin of all three NAP components can be traced back to charyophytic green algea, with the earliest land plants harbouring the first functional auxin response system (Mutte et al. 2018; Kato et al. 2015). Earlier reports already described phenotypic auxin effects in mosses (Johri and Desai 1973; Thelander, Landberg, and Sundberg 2017). In the moss Physcomitrella patens, auxin stimulates the transition of chloronema to caulonema tissues (reviewed in Thelander, Landberg, and Sundberg 2017). This effect has been shown to depend on PpPIN proteins and the components of the NAP (Viaene et al. 2014; Prigge and Bezanilla 2010; Lavy et al. 2012). Similarly, auxin controls many developmental processes in the liverwort Marchantia polymorpha (Flores-Sandoval, Eklund, and Bowman 2015). In contrast to other plant species, Marchantia only contains a single copy of each of the components

of the NAP, and it has been shown that some of these are crucial in morphogenetic processes (Flores-Sandoval, Eklund, and Bowman 2015; Kato et al. 2015; Mutte et al. 2018). Strikingly, it was recently shown that exogenous auxin application can alter growth in the green alga Klebsormidium nitens (Ohtaka et al. 2017). This finding is of prime interest since this species does not contain components of the NAP (Ohtaka et al. 2017; Mutte et al. 2018). This finding begs the question whether there are additional auxin receptors and signalling pathways.

Regulation beyond the NAP

The lag time of TIR1 dependent transcription is about 10-15 minutes, which can explain the majority of auxin dependent responses (Badescu and Napier 2006; Napier 1995). However several observations were made that cannot be explained by the TIR1-dependent NAP. Some of these must be independent of transcription because of their speed, while for others it was explicitly shown that auxin response does not require TIR1/AFB. For example, measurements in protoplasts membrane potential showed hyperpolarization following auxin addition after 2 minutes (Ephritikhine et al. 1987). At the time, such rapid effects were attributed to another auxin receptor, the AUXIN BINDING PROTEIN1 (ABP1;Dahlke, Luethen, and Steffens 2010; Leblanc et al. 1999). However, new insights question the involvement of the ABP1 protein (X. Dai et al. 2015; Michalko et al. 2015). Recent studies revealed rapid auxin effects that are TIR1 dependent and yet are too quick to depend on transcription. Using microfluidic devices, Fendrych et.al. (2018) showed that root growth rates rapidly halt after addition of IAA. Through analysis on the aux1 auxin uptake carrier mutant and tir1/afb mutants, it was shown that the auxin signal is perceived inside the cell and is TIR1/AFB-dependent (Fendrych et al. 2018). Interestingly Dindas et.al. (2018) reached similar conclusions while investigating membrane depolarization in response to auxin (Dindas et al. 2018). They showed that IAA induced membrane depolarization in root hair cells is AUX1- and TIR1/AFB-dependent, and involves transient Ca^{2+} ion influxes mediated through the cyclic nucleotide-gated channel 14 (CNGC14). The role of Ca^{2+} in auxin signalling has been reported before in maize (Felle 1988) and Arabidopsis (Monshausen et al. 2011; Vanneste and Friml 2013). These recent insights suggest dual mechanisms for TIR1 auxin perception, one rapid and one slow. In analogy to rapid and slow abscisic acid signalling (Cutler et al. 2010; Finkelstein, Gampala, and Rock 2002) rapid auxin action might be mediated through TIR1 dependent kinase activation (Dindas et al. 2018).

Kinase action in auxin signalling

It has been established that the localisation and activation of PIN-FORMED (PIN) auxin efflux facilitator proteins can be rapidly changed by their phosphorylation status (Armengot, Marquès-Bueno, and Jaillais 2016). Involvement of several kinases has been implicated within this process, including the D6 protein kinase, PM-associated kinases, PINOID and mitogen activated kinases (MAPK)(Barbosa, Hammes, and Schwechheimer 2018). Generic cellular MAPK activity increases in response to exogenous auxin application (Mockaitis and Howell 2008). Genetic studies implicated the MAP kinases MPK12, MPKK7, YODA, MEKK1 and MPK4 in auxin-dependent processes (Lee et al. 2009; Nakagami et al. 2006; Smékalová et al. 2014). A recent study reported that MPK1 phosphorylates ROP Binding protein Kinase 1 (RBK1), which in turn activates members of the Rho-like GTPases from plants (ROPs) (Enders, Frick, and Strader 2017). Modulation of ROP activity by auxin has also been reported in different context. The receptor –like kinases (RLKs) subfamily transmembrane kinases (TMK) are linked to auxin signalling (N. Dai et al. 2013). It was shown that ROP localisation and activity was abolished in tmk mutants (Xu et al. 2014). More recently, the involvement of TMK1 has been directly linked to ARF-dependent gene regulation (Cao et al. 2019). In the apical hook, auxin induces cleavage of the C-terminal region of TMK1 (including the kinase domain) leading to translocation to the nucleus. Within the nucleus, the cleaved TMK1 domain phosphorylates the non-canonical Aux/IAAs 32 and 34, leading to their stabilisation and subsequent inhibition of ARF activity (Cao et al. 2019). Although these examples highlight the involvement of auxin in kinase dependent signalling, a direct link between rapid auxin signalling and kinase action has not yet been made.

Plant (phytohormone) proteomics

The transcriptional responses to auxin have been well characterized (Bargmann et al. 2013; Lewis et al. 2013; Nemhauser, Hong, and Chory 2006), yet transcriptional output is a very indirect proxy for fast processes in cells, and may not always reflect ultimate protein changes. This is exemplified by a recent study where auxin-induced proteomic changes were compared to transcriptomic changes (Clark et al. 2019). Of the dynamically regulated proteins only very little overlap was observed with changes in mRNA levels (Clark et al. 2019). Post-transcriptional regulation, protein stability and post-translational regulation can all affect protein abundance. Especially with the identification of rapid physiological auxin responses, it is important to include

measurements of protein abundances and their modifications to gain better understanding of phytohormone regulation.

Classically, relative changes in abundance and modification of single proteins can be resolved by western blotting techniques. However, quantitative mass spectrometry-based proteomics is required in order to gain detailed insight. The goal of proteomics is to study the "how, when, where, what for and with whom" or proteins (Jorrín-Novo et al. 2015). Indeed, proteomics-based studies have filled substantial knowledge gaps in the field of plant research (Walton et al. 2015). Proteomics studies have for example contributed to the identification of phosphorylated residues of the BRI1 receptor (Wang et al. 2008) and its protein-protein interactions with BAK1 in brassinosteroid signalling (Li et al. 2002).

In general, a proteomics workflow consists of protein extraction, protein purification, peptide generation, mass spectrometry-based identification and quantification of peptides, and data analysis. Within this pipeline, variations can be added to answer specific questions. For example, besides comparative protein abundances changes, post translational modification (PTM) enrichment techniques can be employed to dissect the specific PTM status of proteins in response to phytohormones or other triggers. Especially within phytohormone research, the phosphorylation status is extensively researched due to its involvement in rapid signal amplification and transduction. Indeed, transient regulation of phosphorylation are the key mechanisms in cytokinin, brassinosteroid and abscisic acid signalling (Černý et al. 2016).

Another important and dedicated field within plant proteomics is the study of protein-protein interactions (PPIs) (Bontinck et al. 2018). Most proteins interact with other proteins, and it is therefore important to study PPIs to be able to understand protein function. Although techniques such as yeast-2-hybrid (Y2H; Causier and Davies 2002) or bimolecular fluorescence complementation (BiFC; Ohad, Shichrur, and Yalovsky 2007) are able to detect PPIs, they rely on a priori knowledge of potential interactions. Affinity purification followed by mass spectrometry (AP-MS/MS) enables detecting interactions in an unbiased manner. A clear example of this in a plant developmental context has been the interactomic analysis of MADS-box transcription factors (Smaczniak et al. 2012). The unbiased approach identified other TF interactions showing that the MADS-box do not act alone to achieve regulation of flower development (Smaczniak et al. 2012). In other work it was shown through an unbiased AP-MS/MS approach that an auxin regulated bHLH complex is important in regulating vascular development (De

Rybel et al. 2013).

Scope of the thesis

From our current understanding of auxin signalling, it is clear that many questions remain unanswered. Conventional genetic and molecular biology approaches have given many answers, yet protein-centred "omics" approaches have only been sparsely applied in the understanding of auxin signalling. Within this thesis, a proteomics approach was undertaken to shed more light on both the NAP and on non-canonical auxin signalling.

In *Chapter 2* we review the current understanding on the effectors of the NAP, the auxin response factors (ARFs). From this review, it is clear that critical principles of ARF protein functioning must be investigated in order to understand how the simple NAP determines specificity. We further highlight that the middle regions of ARFs are predicted to be intrinsically disordered, which offers an explanation for specificity determination in the NAP. Such domains can function as signalling hubs by regulation through PTMs or attracting specific co-factors. The latter option was investigated in *Chapter 3*.

Within *Chapter 3* we aimed to identify ARF co-factors through unbiased quantitative mass spectrometry to provide possible clues on ARF specificity determination. Conventional AP-MS/MS principles proved to be too cumbersome for this. We therefore tried to integrate crosslinking techniques and finally optimized BioID based proximity labelling to identify ARF co-factors.

The field of proteomics, in general, has gained momentum due to the development of state of the art sensitive mass spectrometers and reproducible sample preparation protocols. Yet, the field of plant proteomics is lagging behind in its application. We therefore, in *Chapter 4*, tested and integrated shotgun and phosphoproteomic protocols. Our investigation shows that simple and cost-effective protocols can be used to obtain good quality plant shotgun and phosphoproteomic data.

The optimized proteomic protocols were then integrated to investigate rapid non-canonical auxin signalling in *Chapter 5*. Our analysis reveals that many phosphorylation events occur, which involves several proteins that are evolutionary conserved. Through this analysis, we identified PB1-containing proteins other than ARFs and Aux/IAAs, to be rapidly regulated through phosphorylation by auxin.

Finally, in *Chapter 6*, the results obtained within this thesis are put in a broader context and critical reflection is given on how the plant proteomics field should move forward.

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Chapter 2

Auxin Response Factors - output control in auxin biology

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This chapter is published as:

Mark Roosjen, Sébastien Paque, Dolf Weijers, Auxin Response Factors: output control in auxin biology, Journal of Experimental Botany, Volume 69, Issue 2, 5 January 2018, Pages 179–188, https://doi.org/10.1093/jxb/erx237

The phytohormone auxin is involved in almost all developmental processes in land plants. Most, if not all, of these processes are mediated by changes in gene expression. Auxin acts on gene expression through a short nuclear pathway that converges upon the activation of a family of DNA-binding transcription factors. These AUXIN RESPONSE FACTORS (ARFs) are thus the effector of auxin response and translate the chemical signal to the regulation of a defined set of genes. Given the limited number of dedicated components in auxin signaling, distinct properties among the ARF family likely contributes to the establishment of multiple unique auxin responses in plant development. In the two decades following the identification of the first ARF in Arabidopsis much has been learnt about how these transcription factors act, and how they generate unique auxin responses. Progress in genetics, biochemistry, genomics and structural biology have helped to develop mechanistic models for ARF action. However, despite intensive efforts, many central questions are yet to be addressed. In this review we highlight what has been learnt about ARF transcription factors, and identify outstanding questions and challenges for the near future.

In the past decade, the auxin signaling pathway that leads to gene expression responses has been characterized in detail (Weijers and Wagner, 2016). The core of the auxin pathway, which takes place in the nucleus, is centered around three different factors (Figure 1). The pathway relies on the inhibiting role of Aux/IAAs, inhibitors of the Auxin Response transcription Factors (ARFs) that allow auxin-dependent gene expression. To unlock the system, auxin binds directly to the SCF (TIR1/AFB) ubiquitin ligase and hence increases the affinity for Aux/IAAs proteins, leading to their subsequent degradation by the 26S proteasome. Released from Aux/IAA inhibition, ARFs can then modulate auxin-dependent gene transcription. Based on this model, ARFs are considered as the output of the nuclear auxin pathway.

To date, these three signaling components appear to be sufficient to trigger nuclear auxin signaling in a heterologous system (Pierre-Jerome et al., 2014). The fact that these three components belong to multigene families offers some explanations for how such a simple pathway can control such a wide array of different developmental processes. Importantly, there may be significant functional specialization among ARFs. However, the precise mechanisms that generate dynamics and specificity to auxin output are largely unknown, but the community is currently addressing this challenge. This review will focus on the effectors of the nuclear auxin pathway in Arabidopsis. Given their position in the auxin pathway, we focus our discussion on the mode of action of the ARFs. Recent insights in the past years have allowed the community to see these transcription factors in a new light. This review will give a comprehensive overview of the work that has been done and will raise questions that need to be tackled in the future.

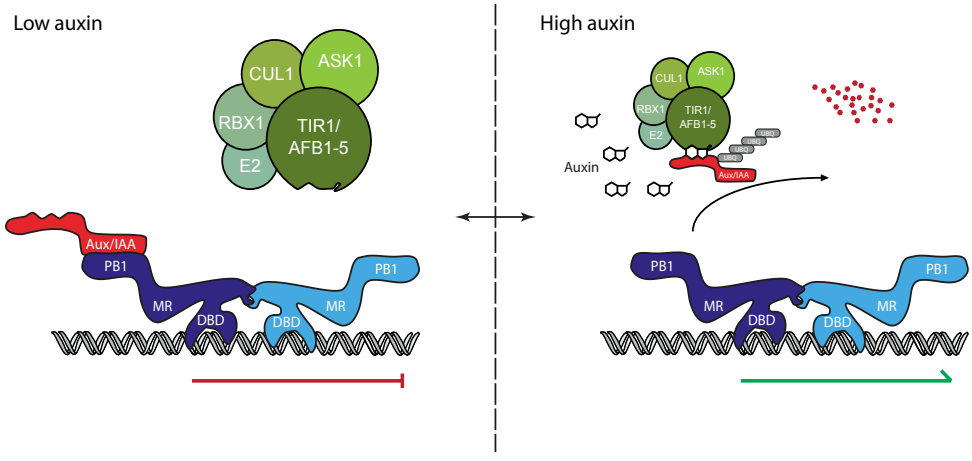

Figure 1: The nuclear auxin pathway. Regulation of auxin output is executed by ARFs. Under low auxin levels, the Aux/IAA transcriptional co-repressors prevent ARFs from controlling auxin-regulated genes. When auxin levels increase, auxin serves as "molecular glue" between the TIR1/AFB receptor and the Aux/IAA protein. This leads to subsequent ubiquitination and degradation of the Aux/IAAs, releasing ARFs from inhibition. Protein abbreviations: ARF, AUXIN RESPONSE FACTOR; ASK1, ARABIDOPSIS SKP1 HOMOLOGUE; Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID; CUL1, CULLIN 1; RBX1, RING-BOX 1; TIR1/AFB, TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX.

Domain organization of ARF transcription factors

The Arabidopsis genome encodes 23 ARFs that fall into three subclasses called A, B and C. Importantly, only few loss of function mutants show an obvious growth phenotype, and double mutants have revealed gene redundancy between close relatives (Okushima et al., 2005). However, a combination of promotor-swap, misexpression and loss-of-function approaches suggested that ARFs are not interchangeable and lead to specific phenotypes (Rademacher et al., 2011, 2012). Most ARFs share a similar topology with three conserved protein domains and the properties of these need to be understood in detail. Here, the three representative domains will be introduced separately.

All ARFs possess at their N terminus a conserved DNA binding domain (DBD) (Okushima et al., 2005; Boer et al., 2014). Surprisingly, a phylogenetic tree using only DBD protein sequences appears similar to that using full-length protein sequences (Boer et al., 2014). This suggests that some functional specificities could be provided by this domain. Crystal structures of the DBDs of ARF1 and ARF5 revealed an unique 3D conformation of the DBD and highlight the presence of three different subdomains: a B3 subdomain showing similarity with the DNA-contacting domain of bacterial endonucleases, a dimerization domain (DD) allowing ARF dimerization and a Tudor like ancillary domain (AD) of unknown function which might be involved in an interaction with the DD. The DBD of ARFs fulfils a critical role for a transcription factor: Recognition of a DNA motif, called the auxin responsive element (AuxRE). In addition, the DBD allows dimerization of ARFs that mediates biological activity.

Specific DNA binding through the DNA-binding domain

One of the functions of a transcription factor is to bind DNA with sequence specificity. The B3 subdomain is involved in the recognition of the ARF-specific AuxRE DNA motif. The crystal structures of the DBD of ARF1 and ARF5 homodimers, as well as the complex of ARF1 DBD with DNA allowed to visualize the mode of protein-DNA interaction. This ARF-DNA crystal confirmed results obtained two decades ago when domains involved in ARF

DNA binding had been discovered (Ulmasov et al., 1997a) and shows how amino acids in the DBD interact with the DNA binding motif TGTCTC (Boer et al., 2014). Mutations in these DNA-interacting amino acids indeed affect their DNA binding properties and their biological activity.

The canonical TGTCTC was originally identified in promotors of auxin-responsive genes in pea and soybean, and was shown to mediate ARF-activated gene expression (Ulmasov et al., 1995, 1997a, 1999a). In the past few years, different techniques have broadened the spectrum of known AuxREs. For example, protein-binding microarrays (PBMs) showed that the original AuxRE was not the sequence with the highest ARF-binding affinity, and instead identified the TGTCGG element as a high-affinity binding site (Boer et al., 2014; Franco-Zorrilla et al., 2014). Likewise, TGTCGG also appeared as a representative DNA binding motif of ARF2 and ARF5 in a "cistrome" analysis that measured in vitro binding to genomic fragments (O'Malley et al., 2016).

This higher affinity for TGTCGG has been translated into an optimized artificial auxin response reporter where the 9 TGTCTC repeats in the widespread "DR5" tool have been replaced by TGTCGG repeats (DR5v2) (Liao et al., 2015). This subtle change leads to improvement of the sensitivity of the marker. The coexistence of these two AuxRE's does not conflict with the numerous results showing the involvement of TGTCTC, but rather enlarge the scope of cis elements in auxin response. In fact, the TGTCGG motif appeared to be only present in a third of the strong cistrome peaks of ARF2 and ARF5 and its presence was distinct from the AuxRE sequence TGTCTC (Boer et al., 2014). The significance of AuxRE diversification is still unknown but gene ontology enrichment analysis of genes from auxin transcriptomes suggest that there is a correlation between particular AuxRE's and specific processes (Zemlyanskaya et al., 2016).

PBMs on ARF1 and ARF5 DBD's tested all the variants possible from TGTCNN and show that ARFs are in fact able to bind various variants. At the same time, an indirect proof that other TGTCNN variants could be involved in auxin response came from a meta-analysis of auxin transcriptomes published previously (Zemlyanskaya et al., 2016), as well as from cell type specific root transcriptomes (Bargmann et al., 2013). Correlation with auxin up/down regulation and overrepresentation of AuxRE's highlights putative new AuxRE that will need to be biologically tested. Most of the examples of biological relevance used, as a proof of concept, the canonical AuxRE TGTCTC. e.g. (Weiste and Dröge-Laser, 2014; Ripoll et al., 2015). Understanding the code hidden behind the disposition of AuxREs along the genome is of great importance to understand ARFs

mode of action and how auxin responsiveness is specified.

As the crystals structures of ARF1 and ARF5 DBDs show a high degree of similarity, Boer et al. tested the ability of the ARF1 and ARF5 dimers to bind differently spaced AuxREs. Surprisingly, ARF1 and ARF5 did not behave the same regarding the difference in space between two palindromic AuxRE's. ARF5 seemed to be more lenient than ARF1. This result gave birth to the caliper model where different ARFs can bind different AuxRE motifs with affinity depending on spacer length. This model is supported by the analysis of the cistrome of ARF5 and ARF2 where analysis of the enrichment of AuxRE in promotor of genes bound by the two ARFs show distinct patterns (O'Malley et al., 2016). This caliper theory emphasizes the cooperative binding of two AuxREs where this interaction enhances the binding of the homodimers to DNA compared to binding on the DNA independently (Boer et al., 2014).

In addition to sequences of the AuxRE and the spacing between two AuxRE's, the orientation of the elements is also an important parameter for binding specificity. Since the discovery of the AuxRE, it is known that differently oriented AuxREs are auxin inducible (Guilfoyle et al., 1998). Cistromes for ARF2 and ARF5 clearly show that both proteins do not bind the same motif (O'Malley et al., 2016). The difference in orientation between direct repeats and inverted repeats should impact the interactions between two AuxREs. The fact that ARF2 and ARF5 do not have the same motifs preferences could reflect specific conformation for homo/hetero dimerization of the ARF on composite AuxREs. However, structural information is at present only available for binding of the ARF1 DBD to an inverted repeat (Boer et al., 2014), and it remains an open question whether alternative dimerization modes underly binding to alternative repeats.

Some correlation seems to exist between the number of AuxRE in a promotor region and its auxin inducibility (Berendzen et al., 2012; O'Malley et al., 2016) . If several variants of AuxRE's confer auxin responsiveness, and the spacing or orientation of AuxRE modules lead to different affinities for the ARFs, it can explain the functional diversity of ARFs and how every ARF could be involved in different developmental processes and why they have specific transcriptomes.

Crystallography of the DBD of ARF1 and ARF5 show that they homodimerize through their DD mediated by hydrophobic interactions. A critical question is whether this homodimerization is biologically relevant. One of the arguments could be that point mutations on amino acids involved in the homodimerization of ARF5 failed to rescue

the strong phenotype of the loss of function mutant of ARF5 and without causing any change in the protein folding (Boer et al., 2014). Another piece of evidence to support the biological role of the ARF dimerization is provided by a study in the crop Brassica napus where a variant lacking 55 amino acids in the N-terminal domain of ARF18 was unable to dimerize. This dimerization seems to be a requirement for activity, as truncated ARF18 was not able to either bind the DR5 element or inhibit the expression of an auxin response reporter like the wild-type protein (Liu et al., 2015). Moreover, this deletion leads to decreased fruit size and seed weight. While some studies show some heterodimerization between different ARFs, currently it is not known whether the DBD is involved in this interaction.

Modulating gene activity through the middle region

While the ARF DBD is highly similar in structure and sequence, the middle Region (MR) shows a strongly contrasting property in that it displays the highest divergence in amino acid composition of the ARFs. Thus far, research has primarily focused on the functional properties of the DBD and the PB1 domain, and the properties of the MR have largely remained elusive. However, the MR has offered a framework to categorize the ARF family into either activators or repressors. This classification has been based on the enrichment of specific amino acids in the MR, as well as on the ability of some tested ARFs to either activate or repress transcription from promotors containing the canonical AuxRE TGTCTC (Ulmasov et al., 1999b; Tiwari et al., 2003). The activator/repressor categorization correlates with the division in subgroups A/B/C. Those ARFs tested as activators belong to class A, while class B ARFs encompass the ones tested as repressors (Tiwari et al., 2003).

The class A ARFs, regarded as activators, carries MR's that are enriched in glutamines, while MR's in class B and C ARFs have a strong enrichment in serines, prolines and threonines. This observation has not yet gone beyond a correlation, and it is unclear what mechanisms underlie activation and repression. Transient expression experiments of class B ARFs on a few known auxin-dependent promotors did not show a strong gene induction after auxin treatment. However, no genome-wide analysis of transcriptomes has been conducted on class B/C ARFs. It is worth to point out that the promotors used in transient expression assays mainly contained TGTCTC motifs and that, based on the recent knowledge on ARF binding sites preferences, other motifs would perhaps be better suited for analyzing class B/C ARF activity. This should be thoroughly studied to gain better insight into the mode of action of the different classes of ARFs. The important

fundamental question of how ARFs function cannot be answered only with a study in heterologous systems on a small set of specific genes. Particularly because genetic studies show that class B and C ARFs can be linked to auxin regulated processes, and that class A ARFs are able to repress certain genes (Sessions and Zambryski, 1995; Sessions et al., 1997; Nemhauser et al., 2000; Zhao et al., 2010; Zhang et al., 2014), the categorization of ARFs into activator and repressor categories should be exercised with caution.

An emerging concept in eukaryotic transcription factor biology is the usage of intrinsic disorder (ID) to elicit specific and rapid conformational changes to allow for adaptive interaction surfaces, conditional DNA binding or modulation of protein function through posttranslational modifications (Liu et al., 2008). In light of ARF biology such mechanisms might provide an additional layer of specificity determination in auxin output control. An example of ID in contribution to signaling diversity is the p53 tumor suppressor, which is involved in a wide set of cell fate decisions. Both the N- and C-terminal domains (comprising a third of the total protein sequence) are intrinsically disordered and contribute to most of the know protein-protein interactions (Dunker et al., 2008). Furthermore, most of the post-translational modifications cluster on the intrinsic disordered regions (Dunker et al., 2008). Besides a role in signaling diversity, intrinsically disordered domains can affect DNA binding. For example, the Drosophila transcription factor Ultrabithorax (Ubx) contains two intrinsically disordered domains that modulate the binding affinity of the structured DNA binding homeodomain (Liu et al., 2008; Hsiao et al., 2014).

The steroid hormone receptor (SHR) family is another class of proteins exemplifying the importance of ID in signaling. Similar to the MR of ARFs, the N-terminal transactivation domain (NTD), which can either activate or repress transcription, shows the least sequence homology among the SHR family and no structure of this region is available (Gallastegui et al., 2015). The SHR have a modular structure and among 400 analyzed vertebrate and invertebrate SHR family members the NTD showed the highest level of disorder (69%) (Krasowski et al., 2008). Induced folding of the NTD upon co-factor binding has been shown for the androgen-receptor (Reid et al., 2002; McEwan et al., 2007; Tantos et al., 2012). Similar to p53, most post translational modifications fall within the NTD of SHR proteins (Lavery and Mcewan, 2005; McEwan et al., 2007). The nature and convergence of different types of regulation on the ID domains implicate a focal point of extensive signal enhancement/diversity. To elaborate on the presence of intrinsic disorder, ARF protein sequences were analyzed using the disordered prediction

algorithm PONDR-FIT (Xue et al., 2010). The prediction, quite strikingly, shows a high degree of disorder in the MR of class A ARFs, which also seems to be conserved in the liverwort Marchantia polymorpha (Figure 2). There is a strong contrast to class B/C ARFs, which do not show this strong predicted disorder. Although there is no functional data supporting the existence of intrinsic disorder in the MR of activator ARFs, it provides a new concept in the explanation to the wide set of responses an ARF can elicit in specific cell types in response to auxin. Functional analysis of these ID regions should also help to define if ID is connected to the ability to activate gene expression.

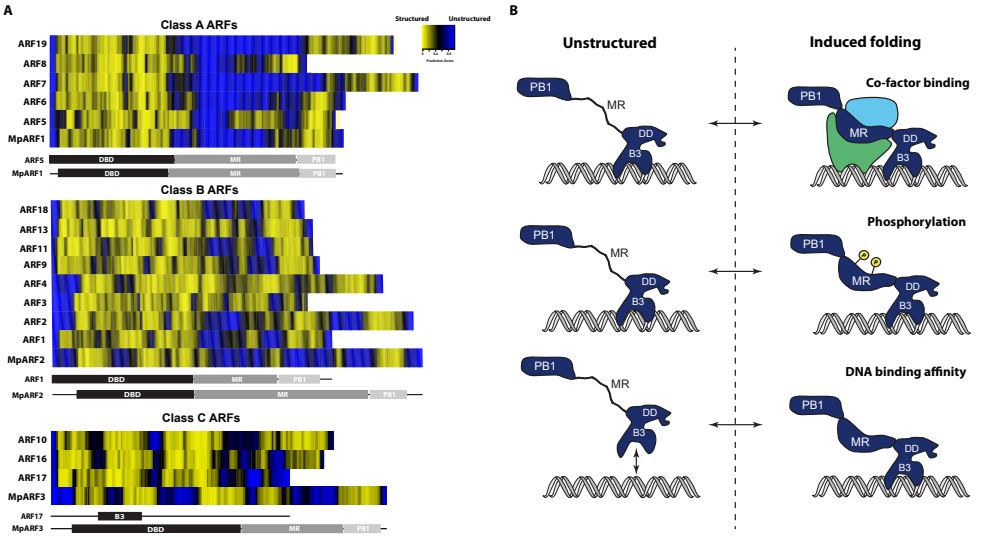

Figure 2: Intrinsic disorder in the ARF middle region. (A) Predicted disorder in the middle region appears to be a prominent and conserved feature in the class A "activator" ARFs. Full-length protein Arabidopsis ARF sequences, as well those from Marchantia polymorpha (MpARF) were used as input in the disorder prediction tool DisProt using the PONDR-FIT algorithm (Xue et al., 2010). Disordered values were used in R to generate a heatmap. Domain locations were retrieved from UniProt. (B) Disordered regions can serve as a focal signaling hub by obtaining induced structure with cofactors, modulation by posttranslational modifications or aid in DNA binding affinity/specificity. Protein abbreviations: ARF, AUXIN RESPONSE FACTOR; III/IV;C-TERMINAL PHOX AND BEM 1 DOMAIN; MR, MIDDLE REGION, DD, DIMERIZATION DOMAIN.

Regulation of ARF activity through the C-terminal domain

It has long been known that the C-terminal ARF domain mediates interactions with Aux/IAA proteins (Ulmasov et al., 1997b). Structural analysis on the C-terminal domain recently revealed the structural basis of such heterotypic interaction of ARF5 (Nanao et al., 2014), ARF7 (Korasick et al., 2014), IAA17 (Han et al., 2014) and PsIAA4 (Dinesh

et al., 2015). The structural analysis of ARF5 and ARF7 revealed type I/II PB1 domains and the chemical basis of dimerization (Korasick et al., 2014; Nanao et al., 2014). The domain has both acidic and basic motifs, which form a tertiary β-grasp-fold structure. The sidedness of the structure, with an acidic and a basic face that can interact with other PB1 domains via electrostatic interactions, creates a front to back arrangement. This arrangement underlies homo- and hetero-dimerization between ARFs and with Aux/IAAs that also carry a PB1 domain and use it to interact with ARFs.

Several studies explored interaction specificity between Aux/IAA and ARF proteins, in an effort to map pathway complexity that might explain diverse auxin outputs. Two comprehensive studies utilizing large scale yeast 2-hybrid (Y2H) assays showed the variety at which these interactions can occur (Vernoux et al., 2011; Piya et al., 2014). Interestingly, in this assay, class B and C ARFs have limited to no interactions with Aux/IAAs (Vernoux et al., 2011; Piya et al., 2014). This suggests that auxin regulation within the nuclear pathway exclusively converges upon class A ARFs. Taken at face value, this finding would suggest that class B and C ARFs are disconnected from auxin regulation, and act by counteracting class A ARFs, for example by competing for DNA binding or blocking through heterodimerization (Richter et al., 2013). It should be noted that in these large-scale interaction studies, proteins are expressed at much higher levels than naturally occurring and might also have increased stability. From studies in the moss Physcomitrella patens, a model was suggested wherein class A and B ARFs either compete or cooperate to repress or induce transcription respectively (Lavy et al., 2016). It appears that more in vivo studies are dearly needed to determine if and how class B and C ARFs are wired into the auxin response network, and what purpose their PB1 domains have.

An interesting finding in the structural analysis of ARFs and Aux/IAA proteins was that PB1 domains have the capacity to oligomerize in vitro, in crystal and in solution (Korasick et al., 2014; Nanao et al., 2014). The biological significance of such oligomerization is still an open question. ARF5 that lacks the PB1 domain has reduced capacity to bind DNA in vitro, and this could be overcome by antibody-induced dimerization (Ulmasov et al., 1999a). Thus, PB1-interactions, in addition to being the site for auxin regulation through Aux/IAA binding, could potentiate DNA binding. Mathematical modeling of TIR1/AFB, auxin, ARF and Aux/IAA interactions provide a conceptual basis for significance of ARF oligomerization on auxin output (Farcot et al., 2015). Aux/IAA-ARF interactions may determine the amplitude, Aux/IAA-Aux/IAA interactions the speed and ARF-ARF interactions the sensitivity of the response. Since the parameters depend

on the PB1 domain interaction, oligomerization may significantly affect the auxin output (Weijers and Wagner, 2016). On the other hand, questions can be raised about the relevance of mediated ARF DNA binding by the homo/hetereodimerization through the PB1 domain. For example the truncated ARF5 (ΔPB1) is hyperactive and still able to activate transcription (Krogan et al., 2012). Also, ARF4 and ARF3 act redundantly in establishing leaf polarity (Pekker et al., 2005). Since ARF3 naturally lacks a PB1 domain it appears that this domain is not required for ARF function in this context. A kinetic analysis of ARF-ARF, ARF-Aux/IAA and Aux/IAA-Aux/IAA interactions in vitro showed that the affinity of ARF:ARF homo-dimers is ~10 to ~100 fold lower than ARF:AuxIAAs hetero-dimers (Han et al., 2014). This suggests that equilibria will tend to favor heterotypic interactions, thus endowing auxin regulation upon ARFs.

Dynamic control of auxin-dependent genes in a chromatin context

An important question is how auxin – and ARFs – can regulate genes in the context of chromatin. It had previously been shown that Aux/IAA proteins recruit the co-repressor TOPLESS (TPL), and likely repress expression through histone de-acetylation (Long et al., 2006; Szemenyei et al., 2008). Recently, a chromatin switch mechanism has also been proposed to direct ARF-dependent gene activation. Chromatin can be configured in a bipartite manner; either closed marking an inactive state or an open configuration marking an active state. Recently a switch in this state was found in which ARF5 is able to unlock closed chromatin in concert with the SWI/SNF chromatin remodelers BRHAMA (BRM) and SPLAYED (SYD) (Wu et al., 2015). Aux/IAA proteins compete with SWI-SNF recruitment to ARF5, and thus Aux/IAA degradation allows chromatin remodeling (Wu et al., 2015). Furthermore, the GRE motif-binding bZIP transcription factors can recruit the histone acetyltransferase (HAT) SAGA complex to a GH3 gene and induce auxin responsive transcription (Weiste and Dröge-Laser, 2014). Interestingly, a conserved bZIP motif was shown to be occluded prior to ARF5-dependent chromatin unlocking (Wu et al., 2015). From these two studies it follows that there may be a concerted action of ARF5-induced nucleosome remodeling followed by HAT-dependent histone modification during developmental reprogramming. Since this mechanism has so far only been demonstrated for ARF5, it will be interesting to see if all class A ARFs, and possibly class B/C ARFs, operate in a similar manner.

Conversely, it was recently shown that histone deacetylation plays a role in the regulation of genes by other class A ARFs (Fukaki et al., 2006). The ARF7/19 and IAA14 proteins play a critical role in lateral root initiation (Okushima et al., 2005). Through phenotypic

analysis and exogenous histone deacetylase inhibitor application it was shown that the chromatin remodeler PICKLE (PKL) and histone deacetylation are required for IAA14-mediated ARF7/19 inhibition. Since PKL strongly resembles the mammalian CHD3/Mi-2 protein of the Nucleosome Remodeling Deacetylase complex (NuRD), consisting of several histone deacetylases, it is conceivable that such concerted action of remodeling and histone deacetylation takes place on ARF target loci.

Interactions between ARFs and chromatin regulators appear to be multi-layered and complex. For example, under low auxin levels, the TPL co-repressor bridges the CDK8 kinase module (CKM) of the MEDIATOR complex with the ARF7/19 - IAA14 module (Ito et al., 2016). The CKM Mediator module prevents the association of the core Mediator subcomplex with RNA polymerase II (Ito et al., 2016). The TPL-mediated interaction is probably distinct from the proposed recruitment of histone de-acetylases by TPL (Long et al., 2006), and importantly it might not involve covalent histone modifications. Under high auxin levels, IAA14 becomes degraded thus leading to loss of the TPL-CKM bridge followed by active transcription (Ito et al., 2016). Such a sequences of events resembles a primed transcriptional state that can accommodate quick transcriptional responses. It is clear from the few examples given here that we are only beginning to scratch the surface of chromatin-level control in ARF action, and further exploration in this area is likely to give much more insight into the fast and dynamic regulation of auxin-responsive genes.

No protein is an island – ARF cofactors shape auxin response

Other than interaction with chromatin regulators, transcription factors (TF) usually cooperate with co-factors that can modulate DNA binding specificity or transcriptional activity. Such interactions can assemble into higher-order protein complexes that can regulate the local chromatin environment and activate or repress gene transcription. In some instances, as reported for the Drosophila Hox TFs, co-factors can modulate the TF to gain novel DNA binding specificities (Slattery et al., 2011). In comparison with other TFs, the number of reported co-factors for ARFs is limited and, if reported, the precise functionality of the interaction not completely elucidated (Figure 3). Since co-factors are important in modulating TF activity, it is conceivable that ARF co-factors play a significant role in modulating activity.

Interactions between TFs can occur within and between families (Bemer et al., 2016). For ARFs, such (ARF-ARF) interactions have only been shown in vitro and appear to be

a requirement for high-affinity DNA binding (Boer et al., 2014). Interactions between transcription factors of different families are also frequently reported, extending the repertoire of TF activity and integrating several developmental, environmental and hormonal pathways. For ARFs this has been shown in several instances. An example is the interaction between MYB77 and ARF7. It was shown that this interaction is important for the regulation of auxin-dependent genes and might integrate abscisic acid signaling with auxin response (Shin et al., 2007; Zhao et al., 2014). A more complex integration was shown for ARF6, which interacts with the bHLH factor phytochrome interacting factor 4 (PIF4) and brassinazole resistant 1 (BRZ1) to regulate a common set of target genes (Oh et al., 2014). It was further shown by genetic studies and Y2H that gibberellic acid signaling integrates in the ARF6-PIF4-BZR1 complex by disrupting ARF6-PIF4 interaction through the DELLA protein repressor of GA (RGA). Of note is that the PIF4 and RGA interactions predominantly occur through the middle region and that RGA also interacts with ARF7 and ARF8 (Oh et al., 2014). Another bHLH (big petal (BPE)) - has also been shown to support ARF function. ARF8 and BPE synergistically act during petal organ growth (Varaud et al., 2011). It was further shown that ARF8, but also ARF6, interacts with the MADS-box transcription factor FRUITFULL (FUL) to promote fruit valve growth (Ripoll et al., 2015). Although the primary focus of the described ARF-TF interactions all relate to class A ARFs interactions with class B ARFs have also been described to a lesser extent. For example, ARF3 has been studied in the context of polarity determination where it interacts with the GARP family member ABERRANT TESTA SHAPE. In two studies, ARF2 has been shown to interact with MADS-box TF FUL and AP1 (Smaczniak et al., 2012; Ripoll et al., 2015).

From this non-exhaustive list of examples, it is apparent that ARFs are not the sole entities in regulating auxin dependent transcription. One prominent question that can be raised from the studies reported thus far is whether there is a common mode of regulation on auxin target genes. It appears that hetereotypic TF interactions are common, especially for class A ARFs. Cooperative DNA binding of two TFs can result in a net increase in affinity for their motifs while the specificity for the motifs remains unchanged (Spitz and Furlong, 2012). On the other hand cooperative binding can also create new specificities. It appears that cooperative binding plays a role in ARF dependent transcriptional activity as is the case for many other plant related TFs (Bemer et al., 2016). MYB77 has interaction with ARF7 and bZIP-dependent SAGA complex recruitment induces auxin transcription (Shin et al., 2007; Weiste and Dröge-Laser, 2014). The binding motifs of MYB and bZIP have been shown to be enriched and

evolutionary conserved near AuxRE (Berendzen et al., 2012).

Currently a comprehensive analysis on ARF/cofactor interactions is lacking. An unbiased in planta approach on all ARFs, as was for example performed on several MADS-box TFs (Smaczniak et al., 2012), could promote our understanding on how ARFs regulate transcription. In perspective, the BioGrid interaction database lists over 1000 interactions for the human p53 protein while ARFs only have a small portion of that number listed (Figure 3). This exemplifies that the field is currently far from understanding ARF biology.

Is it really that simple?

Historically, ARF1 was first found in a yeast 1-hybrid screen to identify transcription factors which bind on a synthetic DNA (P3[4x]) known to be highly auxin-responsive (Ulmasov et al., 1997a). All others ARFs have been found by sequence homology to ARF1 (Guilfoyle et al., 1998). This history urges an existential question: are all ARFs really ARFs? Do all ARFs mediate auxin response? Is an ARF that is not able to interact with Aux/IAA proteins still connected to the auxin response network? The PB1 domain is lacking in ARF3, ARF13, ARF17, and ARF23. ARF23 is different from all others as it is heavily truncated from its DBD.

It has been show that deletion between DBD and MR can affect dimerization of ARF18 (Liu et al., 2015), so there is good chance that ARF23 is not able to dimerize. Moreover its biological function or its ability to bind DNA is not known, and given that this gene is part of a recently duplicated cluster near the centromere of chromosome I (Okushima et al., 2005), there is a chance that ARF23 is becoming a pseudogene.

For ARF3 and ARF17, it appears that despite lack of the PB1 domain, these proteins do control auxin-dependent development (Mallory et al., 2005; Simonini et al., 2016). Y2H showed that ARF17 was able to interact with Aux/IAAs, despite it is lacking the conserved PB1 (Piya et al., 2014). Moreover, truncated ARF5 or ARF7 (lacking the PB1 domain) could still be activated by auxin, though less efficiently than the full-length protein (Wang et al., 2013). Even if in planta proof is lacking, these findings raise the possibility that Aux/IAAs can even interact with truncated ARFs. Thus, it appears that the lack of PB1 can not be used as a criteria to discriminate ARF from non-ARF.

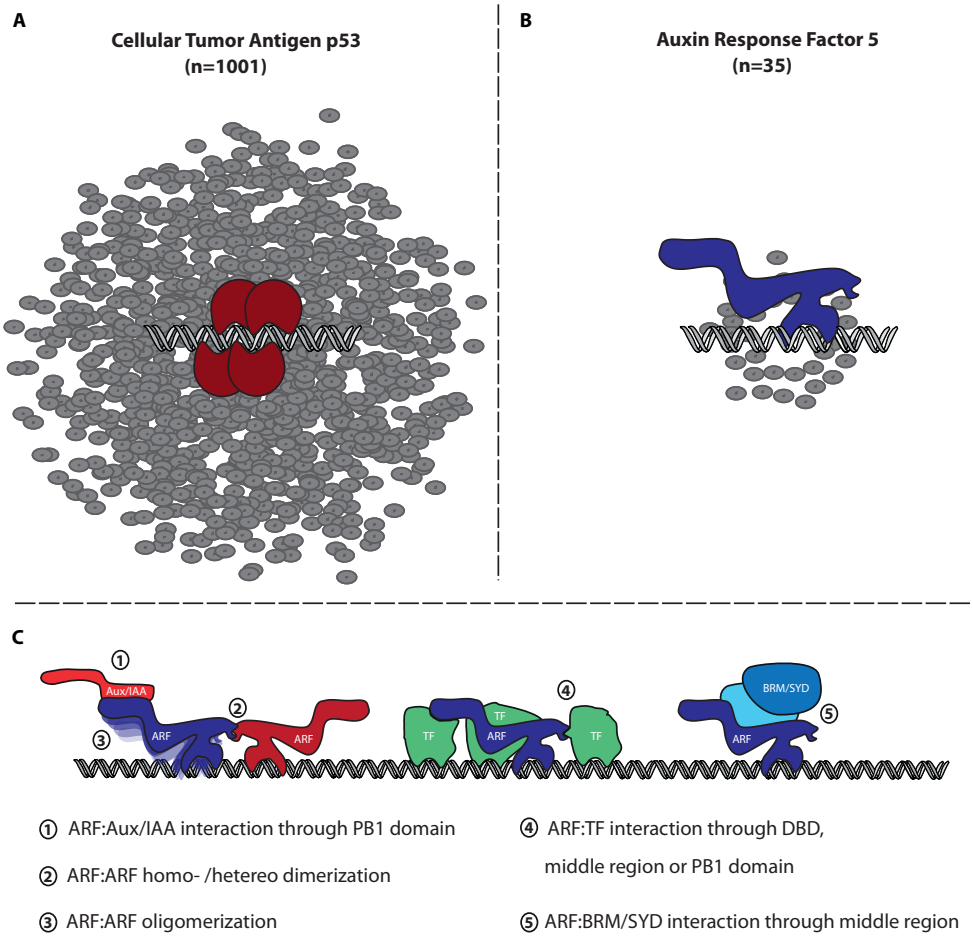

Figure 3: ARF cofactors. (A,B) Complete interactome of the human tumour suppressor p53 (A) and ARF5 (B) depicts the limited state of our knowledge on ARF functioning in comparison with p53. Figure was made utilizing Cytoscape by selecting direct neighbours and using the BioGrid database (last accessed march 2017). (C) Current known modes of interactions and interactions surfaces of ARFs. Protein abbreviations: ARF, AUXIN RESPONSE FACTOR; Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID, BRM, BRAHMA; SYD, SPLAYED; TF, TRANSCRIPTION FACTOR

In the past decades, research efforts characterized the canonical auxin signaling pathway wherein, under high auxin levels, repressive Aux/IAAs become degraded, relieving ARFs from repression. Although this auxin perception mechanism is well known, the regulatory mechanism by which ARFs control auxin output is still vaguely understood. Another aspect that is not currently investigated is the biological relevance

of ARF heterodimerization. Few studies have demonstrated the ability of distinct ARFs to interact in vitro. Heterodimerization has been observed in gel shift assays between ARF1 and ARF4 (Ulmasov et al. 1999) or between different ARFs in Y2H experiments (Ouellet et al., 2001; Hardtke et al., 2004; Vernoux et al., 2011). While it is thus clear that ARFs can heterodimerize, it needs to be established whether they do so in vivo, and the biological relevance of heterodimerization must be understood.

Besides the mechanism that concern the homeostasis of the nuclear auxin pathway, recent research revealed non-canonical pathways that effect ARF regulated gene expression. In the canonical pathway, control by posttranslational modifications have been identified, such as cis-trans proline isomerization of Aux/IAAs (Dharmasiri et al., 2003), S-nitrosylation of TIR1 (Terrile et al., 2012) and phosphorylation of Aux/IAAs (Colón-Carmona et al., 2000). For ARFs, phosphorylation events have been shown to be important for their function. During low potassium availability the K+ transporter HAK5 is upregulated to compensate for K+ deficiency (Gierth et al., 2005). The control of the HAK5 gene is modulated by ARF2. In the presence of sufficient K+ levels, ARF2 represses HAK5 transcription (Zhao et al., 2016). In K+ deficiency environments ARF2 becomes phosphorylated blocking ARF2 DNA binding activity (Zhao et al., 2016). This mechanism of modulation of DNA binding activity by phosphorylation has been shown on ARF2 by the brassinosteroid (BR) -regulated BIN2 kinase (Vert et al., 2008). The integration of BR signaling components and activity modulation on activator ARFs has also been reported (Cho et al., 2014). During lateral root organogenesis ARF7 and ARF19 play pivotal roles and it was shown that the auxin module does not solely control the activity of these ARFs during this process. The BIN2 kinase phosphorylates these ARFs and inhibits Aux/IAA interaction potentiating ARF activity (Cho et al., 2014). Quite surprisingly is that BIN2 in this process is not activated by BR but by the tracheary element differentiation inhibitory factor (TDIF) peptide (Cho et al., 2014).

Other than phosphorylation, a recent finding revealed an alternative auxin sensing mechanism resembling the animal thyroid hormone receptor pathway. The atypical (class B) ARF3/ETT is involved in auxin regulated gynoecium patterning (Sessions et al., 1997; Simonini et al., 2016). Since ETT lacks a PB1 domain, canonical auxin signaling is not likely to regulate ETT activity. ETT interacts with the basic helix-loop-helix (bHLH) transcription factor INDEHISCENT (IND) and this interaction is auxin-sensitive (Simonini et al., 2016). In a bimolecular fluorescence complementation experiment, upon addition of auxin, the ETT:IND dimer appeared to dissociate. Further Y2H experiments showed similar results for the ETT:IND dimer but also for other ETT:TF dimer complexes

(Simonini et al., 2016) .

These results show how elaborate ARF activity can be modulated beside the core nuclear auxin module. An interesting question is whether these non-canonical pathways represent a general mode of action in ARF activity modulation.

Concluding remarks

The past few years, many studies gave new details about ARFs mode of action and functions of their conserved domains. They confirmed the key role of the ARF as an output of the nuclear auxin pathway but particularly emphasizes new characteristics of ARF that were not suspected before. The mode of action of the ARFs was seen more like an on/off mechanism on TGTCTC motif while now, it is believed that ARF are more flexible than that and could be part of larger protein complex (chromatin switch or TF-TF). However, these recent breakthroughs raise new questions and need to be challenged first. Even if these findings brought new insights into ARF mode of action, it is still difficult to give a precise definition to describe this family. One of the reasons is that only little is known about the universality of these mechanisms. Testing these hypothesis on different ARFs classes (A,B,C) or “activators”/”repressors” ARFs will probably help to draw a mugshot of an ARF. It is also worth to highlight that some ARFs still have not been biologically characterized. It will be necessary to extend this knowledge to other species phylogenetically distant from Arabidopsis in order to understand how the auxin signaling pathway has evolved into a complex and apparently fine tuned system.

Acknowledgements

We apologize to colleagues whose important contributions we could not discuss in this review. Research on auxin response in D.W.’s lab is funded by the Netherlands Organization for Scientific Research (NWO; VICI 865.14.001). S.P. is supported by a long-term fellowship from the European Molecular Biology Organization (EMBO; ALTF-535-2014) and the Bettencourt Schueller Foundation.

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Chapter 3

Finally meeting the neighbours - proximity labelling identifies ARF interactors

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The nuclear auxin pathway (NAP) controls the genomic responses to auxin. Consisting of only three major components a pertinent question in the field is how specificity in this pathway is determined. As the effectors in NAP, the auxin response transcription factors (ARFs) are probably mediating this specificity. Being transcription factors, ARFs are likely taking part in multi-protein complexes controlling the specificity output in NAP. To identify these co-factors we employed unbiased mass spectrometry based affinity pulldowns. Conventional and crosslinking techniques proved to be cumbersome leading to minimal interactor identifications. Reasoning that ARF interactions might be transient in nature we implemented and optimized BioID to tag ARF interactors within the cell. This approach allowed to identify ARF hetereodimerization and in vivo ARF-TPL interactions. Further optimization and integration of newly developed and more efficient proximity labeling techniques would probably allow to capture a more exhaustive ARF interactome.

INTRODUCTION

The nuclear auxin signalling pathway (NAP) has been characterized in detail over the past few decades. Consisting of only three major components, it controls almost every aspect of auxin signalling through gene expression. The canonical signalling relies on the degradation of the Aux/IAA proteins which subsequently relieves the auxin responses factors (ARFs) from gene regulatory inhibition. The pathway is activated by the third component, the auxin SCF (TIR1/AFB) ubiquitin ligase receptor complex. In chapter 2, a detailed insight is provided on the functioning and regulation of ARF proteins from current research (Roosjen, Paque, and Weijers 2018). It can be distilled from this that a major question in NAP research is how specificity within this simple pathway is determined (Roosjen, Paque, and Weijers 2018). Although, specific cellular expression of NAP components (Rademacher et al. 2011), promotor architecture (Boer et al. 2014) and NAP interactions (Piya et al. 2014) might explain the specificity to some point, the contribution of co-factors have not been fully elucidated.

That co-factors can have important effects on the DNA binding properties of transcription factors (TFs) was shown for the Drosophila Hox TFs, wherein dimeric cofactor formation with Extradenticle-Homothorax (Exd) obtained novel recognition properties (Slattery et al. 2011). It is not unimaginable that for ARFs such mechanisms are also in play. Indeed, numerous reports have identified ARF interactors from different classes of TF families (Shin et al. 2007; Zhao et al. 2014; Oh et al. 2014; Varaud et al. 2011; José Ripoll et al. 2015; Smaczniak et al. 2012) as well as chromatin regulators (Wu et al. 2015; Weiste and Dröge-Laser 2014; Fukaki, Taniguchi, and Tasaka 2006; Ito et al. 2016). Although these reports shed some light on ARF functioning and the ARF-protein interaction landscape, the number of identified interactions thus far are in stark contrast with the numbers identified for a similar important TF protein p53 (Roosjen, Paque, and Weijers 2018).

Thus far, identifications of ARF co-factors have primarily been made by classical yeast 2 hybrid (Y2H) or genetic screens. An unbiased approach to identify novel ARF interactors such as performed by Smaczniak et. Al. 2012 has not been conducted yet (Smaczniak et al. 2012). This methodology, affinity purification followed by mass spectrometry (AP-MS), relies on a tagged protein which is used as an affinity handle to identify interactors. The rationale is that interactors stay bound to the tagged “bait” protein during sample processing wherein the interactor “prey” proteins are subsequently identified by mass spectrometry. Although this approach has been successfully applied in numerous studies, cumbersome to this approach is that weak or transient interactors are easily lost.

To overcome this limitation crosslinking strategies have been applied to stabilize interactors within the cell (Smith et al. 2011) or after lysis (Makowski et al. 2016). These crosslinking strategies combined with quantitative MS approaches even allowed to determine structural interfaces within protein complexes (Makowski et al. 2016; Kloet et al. 2016). The usage of cross linkers is technically challenging since large 'cross-linked' protein complexes might aggregate and reduces successful co-factor identifications. On the other hand cross-linked peptides are difficult to resolve in mass spectrometers. Another approach is to tag interactors within a living cell circumventing the transient or weak nature of the interaction. These proximity-dependent labelling approaches utilize enzymes that produce reactive biomolecules covalently interacting with neighbouring proteins. To date, several enzymes have been utilized; engineered ascorbate perxodiase (APEX), horseradisch peroxidases (HRP) and promiscuous biotin ligase (BirA). While the former two utilizes reactive radicals, BirA utilizes reactive biotin which can covalently interact with primary amines within a ~10nm range (D. I. Kim et al. 2014). Biotin is especially usable for proteomic screens since the biotin group can easily be purified utilizing streptavidin coated matrices.

To date, the BirA proximity-dependent interactions screens have shown to be complementary to conventional AP strategies. Recently, this approach has also been adopted for rice (Lin et al. 2017) and Arabidopsis thaliana (Khan et al. 2018).

In this study, we aim to identify ARF co-factors to shed more light on the ARF protein interaction landscape. Initially, we used in planta GFP tagged ARF1 to identify novel interactions. However, this strategy proved to be cumbersome for ARF1. We therefore integrated and optimised the BioID methodology in Arabidopsis cell suspension cultures. Our in vivo BioID approach confirms previous TPL-ARF interactions and ARF heterodimerization interactions

RESULTS

In planta AP-MS on ARF1

Our aim was to identify novel ARF interactions that could contribute or explain in part the specificity determination in the NAP. Since an unbiased in planta AP-MS approach, as done by Smazcniak et.al. 2012, for ARFs has not been published to date we first performed GFP pulldowns on ARF-GFP tagged lines (Smaczniak et al. 2012). We focused on the class B ARF1 since it is quite prominently expressed in the root. To this end, instead of commercially available GFP antibodies, we utilised the alpaca based

GFP-Trap® beads. These beads have high affinity allowing for shorter incubation times and give no antigen-based peptides from standard GFP antibodies. All pulldowns were performed in technical triplicate to allow for label free quantification. Due to the shorter generation time, GFP-pulldowns were first performed on pARF1-ARF1-GFP expressing roots. As expected, the pulldown showed a good enrichment, ~4 fold, of ARF1. In addition, along with ARF1 we further identified ARF2 (Figure 1A). Since ARFs have a high degree of sequence similarity, we looked whether peptides belonging to ARF2 were shared or unique. ARF1 was identified with 31 peptides from which 1 is unique for ARF1. ARF2 was identified with 34 peptides from which 31 were unique. Although there is a high degree of overlap, the unique identifications show that ARF1 interacts with ARF2 in planta. Beside the identification of ARF2 no other significant interactors were identified. We reasoned that, although prominently expressed in the root, the low abundance of ARF1 might explain these results. We therefore chose to use the floral buds since these have higher nuclei content than other tissue types. The enrichment of ARF1 (~10 fold, 13 unique peptides) indeed increased in comparison to roots (Figure 1A). The results were further comparable to roots wherein we identified ARF2 (with 3 unique peptides) but no other significant interactors. Although increasing input by using floral buds, the low abundance of ARFs might still lead to these results. Next, since driving TFs from a constitutive promotor can lead to aberrant phenotypes in planta, we decided to use the Arabidopsis cell suspension PSB-D culture. The latter allows the use of constitutive promotors and is rapidly scaled-up. We used a p35S-ARF1-YFP translational fusion to transform the cell cultures. Pulldown on the ARF1-expressing cell culture reveals a good enrichment of ARF1 but no identifications of co-factors.

Since over different tissue types we could not identify any known or novel interactors, we reasoned that these interactions might be too transient or too weak to maintain association during AP procedures (Figure1B). To circumvent these issues, crosslinking strategies (Figure1C) or proximity labelling techniques (Figure1D), such as BioID, can be applied wherein the co-factors are fixed or tagged within the cell, allowing for direct enrichment of the co-factors. We therefore first focused on integrating and optimizing crosslinking strategies.

Crosslinking-MS

Since conventional AP-MS failed to maintain co-factor associations during the affinity procedure, we opted to integrate crosslinking strategies to maintain labile protein interactions. A commonly used crosslinker is formaldehyde. Although effective,

we searched for a more selective crosslinker with greater spacer length to increase the chances of success. The N-hydroxysuccinimide (NHS) ester reactive crosslinker dithiobis[succinimidyl propionate] (DSP) and the non-cleavable variant disuccinimidyl suberate (DSS) have been used effectively in stabilizing proteins in vivo (Jafferali et al. 2014; Salazar et al. 2009; Percipalle et al. 2002). DSP in combination with a maleimide crosslinker dithio-bismaleimidoethane (DTME) have been used in a technique called Reversible Cross-Link Immuno-Precipitation (ReCLIP)(Smith et al. 2011). Due to the cell permeability and possible thiol cleavage this approach provides a good alternative to stabilize ARF interacting proteins before cell lysis.

At first, we reasoned that the cell wall provides an obstacle for crosslinker penetrance. Therefore, to circumvent the cell wall, we opted to crosslink on intact nuclei since ARFs are nuclear localized. To that end, we first optimized the concentration and timing of DSP and DTME on Columbia-0 (Col-0) wildtype isolated nuclei and probed crosslinking efficiency by using histone 3 (H3) antibody and western blotting. Nuclei were isolated and incubated for 30 minutes with increasing concentrations of DSP or DTME. A dose dependent reduction in monomeric H3 can be observed in conjunction with the appearance of large crosslinked complexes at the top of the membrane (Figure 2A). At a concentration of 0.5mM, DSP is already effective in crosslinking most H3 and monomeric H3 is successfully recovered by the addition of the reducing reagen

DTT. For DTME, it is expected to be less effective since cysteine residues are less prominent in proteins than lysines. However, at concentrations of 1.0-1.5 mM extra bands can be observed, which disappear after reducing conditions (Figure 2B).

To prevent too much background both concentration and timing are critical. We next looked how long DSP and DTME incubation is necessary to achieve sufficient crosslinking. Initially, 30 minutes incubation time was used, but since nuclei have good permeability we investigated whether short incubation, up to 10 minutes, is sufficient. Crosslinking can already be observed after 1 minute incubation, while after 9-10 minutes crosslinking is more effective as observed by the increasing reduction of

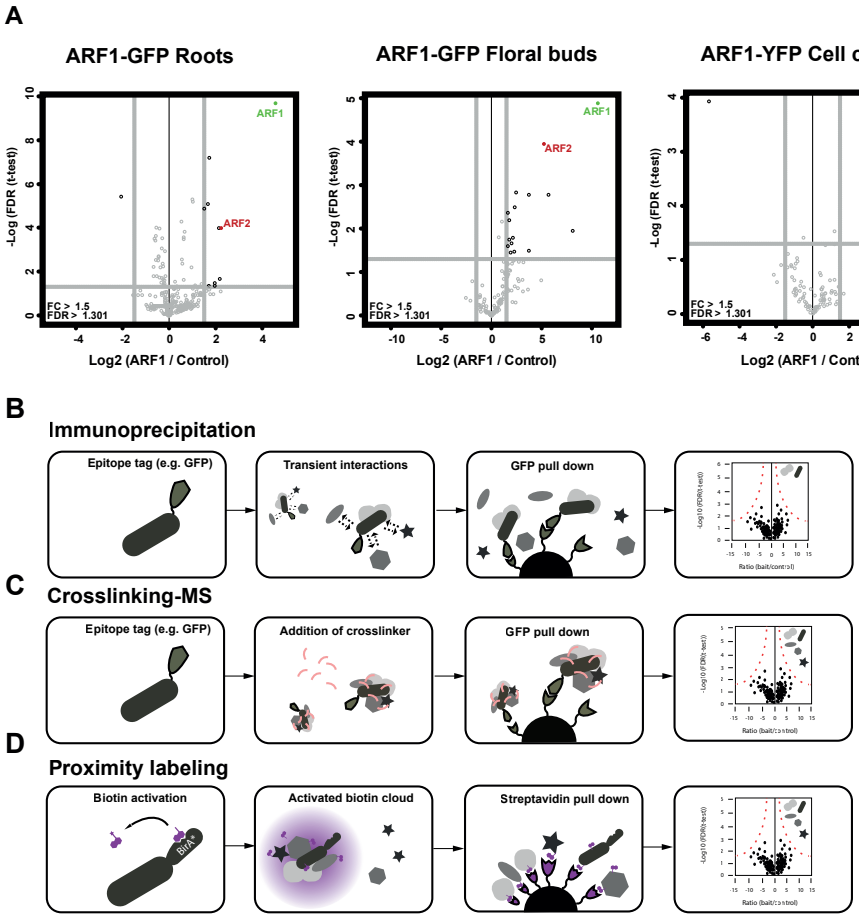

Figure 1: In planta AP-MS on ARFs fails to detect novel co-factors. Conventional AP-MS procedures on ARF1 from different tissue types fail to detect novel interactions (A,B). Volcano plots show fold changes (FC, x-axis) and significance (FDR, y-axis). Proteins passing the threshold of FDR 0.05 and fold change >1.5 are marked and have their name displayed. Alternative strategies such as the usage of crosslinkers (C) or the use of proximity labelling (D) might be useful in identifying labile ARF protein interactionS.

monomeric H3 (Figure 2C). Based on these results, we chose 10 minutes incubation with 0.5mm DSP and 1.0mM DTME as optimal crosslinking parameters.

Before applying the ReCLIP strategy to ARFs, we were interested to test how well the strategy performed on a nuclear protein from which the interactors are known. For this,

we utilized plants expressing the OBERON1 (OBE1) protein with a C-terminal fused GFP under its native promotor (pOBE1-OBE1-GFP). OBE1 is a plant homeodomain (PHD)-finger class of protein. In planta pulldowns showed that OBE1 interacts with OBERON2 (OBE2) and its close homologs TITANIA1 (TTA1) and TITANIA2 (TTA2)(Saiga et al. 2012, 2008). From 5-day-old root tissue, nuclei were extracted and treated with or without DSP/DTME. Lysate was subsequently submitted to GFP enrichment and mass spectrometry analysis. In conventional AP samples OBE1 was identified with ~21 peptides (Figure 2D). We further identified OBE4 with ~ 3 peptides but no other known interactors (not shown). In the crosslinked samples OBE1 peptides drastically decreased (~4 peptides) and no peptides belonging to OBE4 or other interactors were identified. Although, it is expected that crosslinking reduces peptide identifications, this drastic reduction was not expected. In a similar way, we tested the ATP dependent chromatin remodeler BRAHMA (BRM). Similar to OBE1, ~7 peptides for BRM were identified in conventional samples, while none were identified in crosslinking conditions.

From this, we reason that the optimized conditions are good for crosslinking H3 but that successful crosslinking requires a protein by protein optimization. Such strategy is too cumbersome for a holistic ARF in planta interaction screen.

Integration and optimisation of BioID in Arabidopsis cell suspension cultures

Other than fixing protein interactions in the cell by crosslinkers, vicinal proteins can be tagged in vivo by a technique called BioID allowing a history of interactions. The basis of BioID relies on a promiscuous biotin ligase (BirA*) which can readily release bioAMP and covalently interacts with primary amines on neighbouring proteins. Due to the quick generation of stable expressing cell suspension cultures we chose the PSB-D cells to integrate and optimize BioID for ARFs. To that end, we used an Arabidopsis codon optimized promiscuous BirA* (R118G) C-terminally tagged with 3xMyc (mBirA*-3xMyc) (Palovaara et al. 2017) (Figure S1). The mBirA*-3xMyc cassette was integrated in the pPLV26 vector (de Rybel et al., 2011; Wendrich et al., 2015) allowing constitutively active expression from a 35S promotor.

At first, we established the timing and concentration of exogenous biotin addition since long incubation and high exogenous biotin concentrations might increase false positives in interaction screens. Being an E. coli enzyme, the optimal activity temperature is 37 °C with an almost ~10 fold reduction of activity at 25°C (D. I. Kim et al. 2016). It was further reported that BioID was nearly undetectable in yeast growing at 30°C (Opitz et al. 2017; Branon et al. 2018).

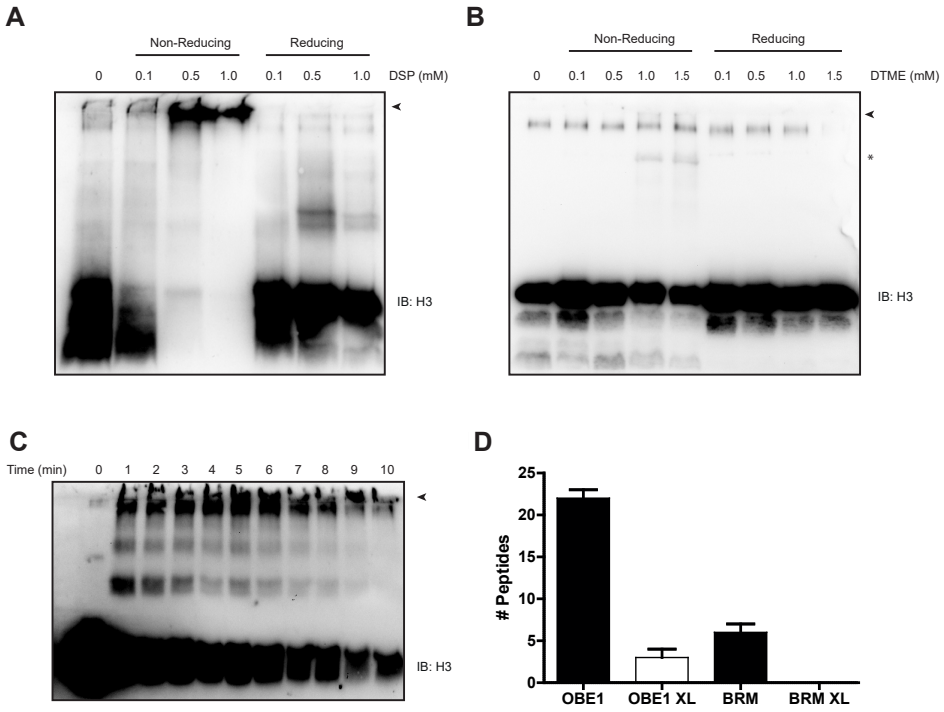

Figure 2: Optimizing crosslinking strategies. Isolated nuclei were subjected to different timing and concentrations of DSP or DTME. Nuclei protein extracts were subjected to SDS-PAGE separation, treated with or without DTT and subsequently probed with Histone 3 (H3) antibody. Concentrations of >0.5mM DSP are sufficient to create H3 crosslinked complexes (A). At concentrations >1.0mM of DTME crosslinked H3 can be observed as marked by asterisk (B). DSP and DTME crosslinking is sufficient after 9 minutes as observed by the decrease in monomeric H3 signal (C). Optimised crosslinking conditions were tested on OBE1 and BRM (D). Crosslinking resulted in a significant reduction of peptide identifications. Affinity pulldowns were performed in technical triplicate. Note: black arrows mark stacking part of SDS-PAGE gel.

We therefore first tested whether biotinylation within the PSB-D cell cultures occurred

with different timing (24, 48 and 72 hrs) and concentrations (0, 1, 10 and 100 µM) of exogenously applied biotin. Already without exogenous biotin a signal was observed corresponding to mBirA*-3xMyc (Figure 3A). With the exogenous addition of biotin, signal intensities increased with increasing biotin concentrations. No major increase in signal intensities was observed between the different timing of biotin incubation and we thus chose to use 24hrs incubation for further optimisation (Figure 3A).

In mammalian cell cultures a concentration of 50µM biotin is often used (Roux et al. 2012; Khan et al. 2018). Recently, BioID was established in Arabidopis thaliana where concentrations of 2mM Biotin was infused in the leaves (Khan et al. 2018). We reasoned that biotin is more accessible in suspension cultures and therefore employed a gradient ranging from 10-3µM to 100µM biotin for 24 hrs (Figure 3B). We observed that from a concentration of 1µM biotin, streptavidin-HRP signal intensities increased which already levelled at a concentration of 10µM biotin (Figure 3B). To ensure sufficient biotinylation, we employed 50 µM biotin for 24 hrs in further experimentation.

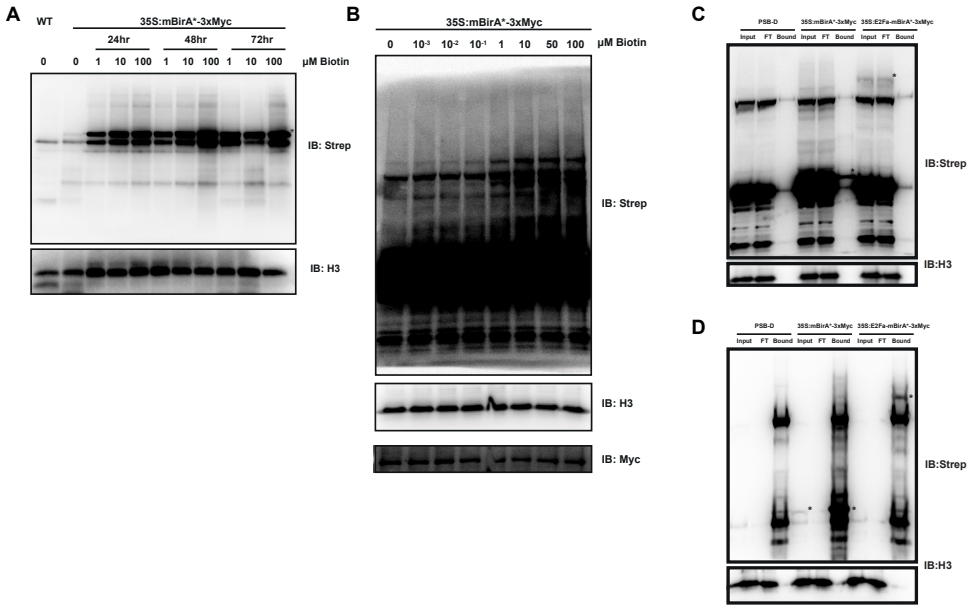

Figure 3: Optimisation of biotinylation conditions. PSB-D cells expressing mBirA*-3xMyc were used to optimize biotinylation conditions. Whole cell extracts were used to monitor biotinylation by streptavidin-HRP immunoblotting. H3 immunoblotting was used as loading control. Maximum biotinylation occurred after

24 hours as seen by the signal intensity of mBirA* marked by asterisk (A). After concentrations of 50 µM Biotin no discernible streptavidin signal was observed (B). Removing free biotin from whole cell extracts is paramount for successful streptavidin enrichment (C without clearance, D with biotin clearance).

Proof of principle

Following the successful expression and biotinylation in PSB-D cells, we were next interested whether this platform is amenable to streptavidin purification. Since the interaction landscape of ARFs is still unclear, we first opted to use a more defined plant TF where the interactions have been well described before. The TF E2Fa has been utilized before in PSB-D cells for tandem affinity purification (TAP-MS) and tandem chromatin affinity purification (tChAP) (Van Leene et al. 2010; Verkest et al. 2014). The E2Fa TF is part of the conserved E2F/DP/RBR pathway and a previous AP-MS strategy confirmed the interaction of E2Fa with DPa , DPb and RBR in PSB-D cells (Van Leene et al. 2010; Verkest et al. 2014; Inzé and De Veylder 2006; van den Heuvel and Dyson 2008). We therefore expect that BioID on E2Fa should identify these factors.

Initially, we tested whether streptavidin pulldowns can sufficiently enrich biotinylated proteins form PSB-D protein extracts. Protein extracts were made in RIPA lysis buffer to allow sufficient solubilisation. Lysates of wildtype PSB-D, mBirA*-3xMyc expressing and E2Fa-mBirA*-3xMyc expressing cells were subjected to overnight streptavidin pulldowns and probed with streptavidin-HRP on a western blot. Surprisingly, very pore enrichment was observed for all tested extracts (Figure 3C). Since specific bands for mBirA*-3xMyc and E2Fa-mBirA*-3xMyc can be observed in the flow-through fractions, expression of the construct or insufficient biotinylation cannot be causative. We therefore considered that the protein extracts might still contain high amounts of free biotin competing with biotinylated proteins for streptavidin beads. To test this, we utilized 10kDa Amicon® filters to allow buffer exchanges of the protein extract. Indeed, when the extracts were depleted from biotin, high signal intensities can be observed in the bound fractions (Figure 3D).

With the successful enrichment of biotinylated proteins, we next tested the efficacy of

BioID for MS based protein interaction identification. First we tested whether, through conventional AP-MS, we could identify the known E2Fa interactors DPa, DPb and RBR. LFQ pulldowns on p35S-E2Fa-YFP expressing cells indeed highly enriched E2Fa, DPb, DPa and RBR (Figure 4). This further shows that our single affinity approach is effective for co-factor identification and that failure to identify ARF interactors is not due to experimental set-up. Next mBirA*-3xMyc and E2Fa-mBirA*-3xMyc protein extracts were submitted for streptavidin pulldowns and LFQ based protein identification. In both pulldowns mBirA* was highly enriched showing that mBirA* can self-biotinylate and that enrichment procedures are effective (Figure 4). In E2Fa-mBirA*-3xMyc enrichments both E2Fa and one co-factor DPb could be identified (Figure 4). Unfortunately, no other known interactors could be identified using BioID on E2Fa. Since biotinylation occurs within a ~10nm range (D. I. Kim et al. 2014), it could very well be that the 3D conformation of the complex is out of range for the other factors. On the other hand, primary amines within the known co-factors could be buried within the protein structure shielding it from biotinylation. Although only one co-factor is identified, this shows that BioID works although not efficient as expected, in Arabidopsis cell suspension cultures.

ARF BioID

Since structural reasons could explain the sub-optimal co-factor identification on E2Fa, we continued on preforming BioID on ARFs. From the class A/B ARFs we chose two representatives per class to increase chances of positive co-factor identification and to be able to cross-compare general and specific ARF interactions. Full length ARF1, ARF2, ARF5 and ARF6 were cloned in the mBirA*-3xMyc vector and transformed in PSB-D cell cultures. Over all pulldowns, mBirA* was identified indicating a good enrichment. Strikingly only in ARF1- and ARF2-mBirA*-3xMyc the respective ARF was identified, while in BioID of ARF5 and ARF6 the respective ARFs were not identified. Since mBirA* was enriched this could indicate non-accessible primary amines for ARF5 and ARF6. With the in planta AP-MS of ARF1 we identified ARF2 as a heterodimerization partner, this finding was also observed in PSB-D cells (Figure 5). Interestingly, ARF2 was also identified in the BioID of ARF5 showing class A/B

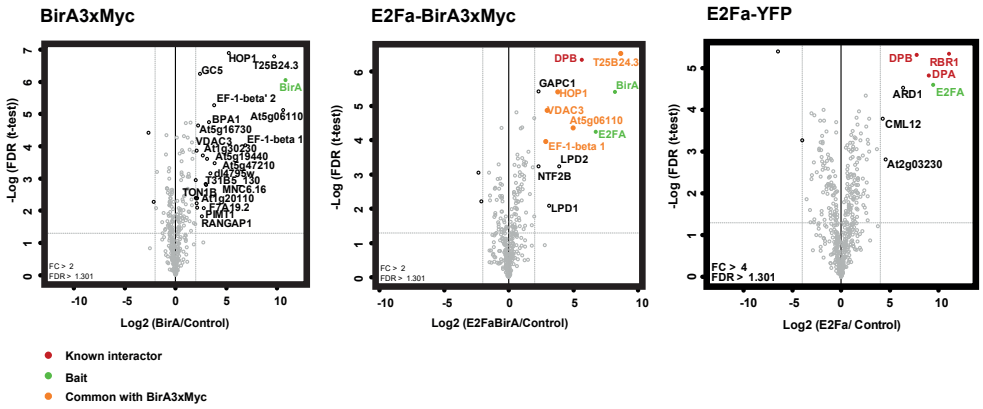

Figure 4: Proof of principle shows successful integration of BioID in suspension cultures. Optimized biotinylation conditions were tested on cultures expressing mBirA*-3xMyc and E2Fa-mBirA*-3xMyc, as comparison a conventional pulldown on E2Fa was performed. Streptavidin and GFP pulldowns were conducted in technical triplicate on biotin cleared whole cell lysates and subjected to mass spectrometry analysis. Proximity labelling on E2Fa identified the known interactor DPb while GFP pulldown identified the known complex consisting of DPa, DPb and RBR. Volcano plots show fold changes (FC, x-axis) and significance (FDR, y-axis).

heterodimerization. ARF-ARF interactions have been previously reported (Vernoux et al. 2011) and it has been suggested, based on a study in Physcomitrella patens, that class A/B interaction could co-operate or compete to induce or repress transcription respectively (Lavy et al. 2016).

BioID usually results in a large list of potential candidate proteins (Roux, Kim, and Burke 2013). Since probably not all are relevant proteins, we compared ARF- mBirA*-3xMyc to mBirA*-3xMyc to reduce non-specific mBirA* biotinylated proteins. This still results in a large list of candidate proteins and we further curated manually on what is known about the respective candidates (e.g. localisation, function, etc.). In ARF1 BioID we identified TOPLESS (TPL) and TPL-related (TPR) to be interactors (Figure 5). It is generally believed that TPL is recruited by Aux/IAA proteins yet class B ARFs have shown to have limited or not at all interaction with Aux/IAAs (Piya et al. 2014). Recently, it was shown that ARF2 can directly interact with TPL/TPR through TPL binding motifs in the middle region, and this motif is also present in ARF1 (Choi, Seo, and Cho 2018). This result shows for the

first time the in planta interaction between an ARF and the co-repressor TPL. We further identified histone deacetylases (HDA3 HDT2,3 and 4) in all ARF BioIDs (Figure 5). In the canonical NAP signalling scheme HDA19 interacts through TPL binding. Although we identified TPL in ARF1 BioID, it is absent in the other ARF BioID. This could either mean that the primary amines of TPL are not accessible or out of range or HDAs can interact through other means with the ARFs. In addition, subunit components of the Mediator complex were also retrieved (Figure 5). Since mediators are part of the general transcription factor machinery that connects specific TFs, these results imply that ARFs can both engage in active and repressive transcription complexes. In summary, we here show that the BioID methodology is effective in identifying ARF co-factors.

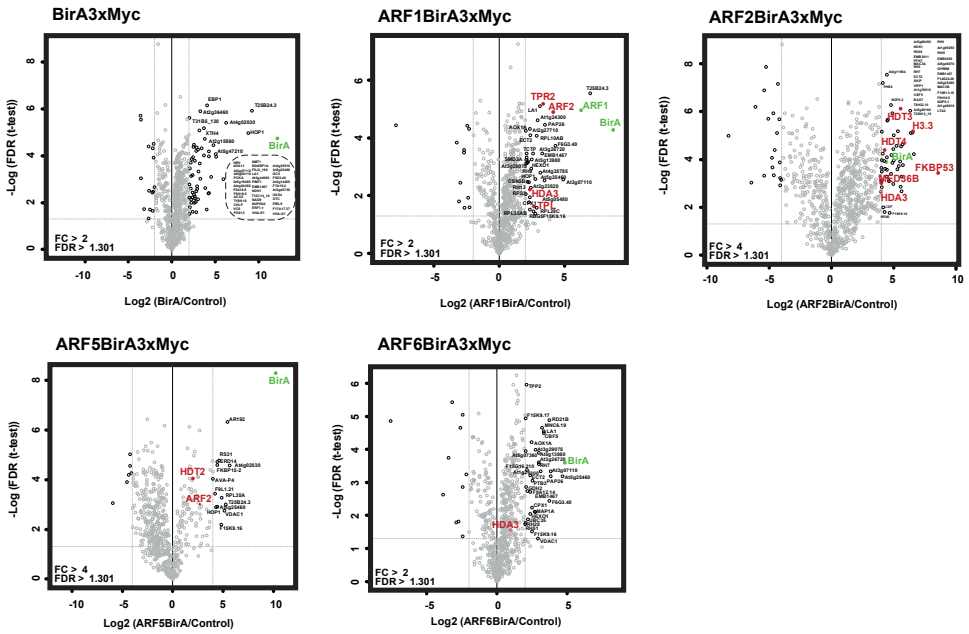

Figure 5: Proximity labelling identifies ARF interactors. Proximity labelling on representatives of class B (ARF1 and ARF2) and class A (ARF5 and ARF6) identifies ARF interactors. Volcano plots show fold changes (FC, x-axis) and significance (FDR, y-axis). Green dots mark the baits, while red dots mark known or potential interactors. mBirA* expressing cells were compared to PSB-D wild type control. Streptavidin enrichments were performed in technical triplicate.

DISCUSSION

Although the NAP has been studied in detail, an important question in the field of NAP research is how specificity is determined. Insights in binding site topology shed some light on this question, yet it does not give a conclusive answer (Boer et al. 2014). As transcription factors, ARFs are likely taking part in large protein complexes which mediate chromatin alteration and transcriptional activation or repression. Cross TF family interactions indeed occur with ARFs (Shin et al. 2007; Zhao et al. 2014; Varaud et al. 2011; José Ripoll et al. 2015; Oh et al. 2014; Smaczniak et al. 2012). Such cooperativity can increase or reduce binding, blocks binding or results in latent specificity as has been shown for the Hox-Exd interaction (Lelli, Slattery, and Mann 2012; Bemer et al. 2016). Oh et.al. 2014 showed that ARF6 cooperatively binds to DNA with the bHLHs Phytochrome-interacting factor 4 (PIF4) and Brassinazole-resistant (BZR) (Oh et al. 2014). Although such studies show interactions between specific ARF co-factors and their phenotypic outcomes, an unbiased interaction screen for ARFs has not been conducted to date.

In this study, we set out to perform an unbiased in planta affinity purification quantitative MS based approach to identify the ARF protein interaction landscape. Cumbersome to this approach are weak or transient interactions which are easily lost during affinity procedures. We showed that indeed the ARFs are not suitable for this approach. Although in planta ARF1-ARF2 heterodimerization could be showed, no other interactors were found. This interaction was additionally confirmed in ARF1 BioID screen. Further, ARF heterodimerization was identified in ARF5 BioID screen. Previously, it has been reported in a large Y2H screen that there are interactions between class A/B factors (Vernoux et al. 2011). It was further suggested in Physcomitrella patens that this type of heterodimerization compete or cooperate for transcriptional repression/induction (Lavy et al. 2016). It is however interesting to ask whether such interaction attracts different co-factors than those of individual ARFs alone. Although conventional AP-MS and BioID strategies are not applicable, a BioID based protein fragment complementation assay (PCA) called Split-BioID could answer such question (Schopp et al. 2017; De Munter et

al. 2017). This methodology relies on the successful reassembly of the BirA* allowing proximity labelling only when the interactions occurs.

Although BioID allows the tagging of co-factors within the cell, the technique, from site of synthesis to its functional location, provides a complete history of the BioID tagged protein (Chojnowski et al. 2018). Therefore cumbersome to this approach are false positives. In our ARF-BioID experiments we indeed have a high amount of false positives (Figure 5). Although we optimized and reduced incubation times to 24 hours, this is still a relatively long incubation period and does not only provide a true interactomic snapshot within this time frame. To address this, improvements in specificity of proximity labelling have been made. BioID2 for example, is a BirA* smaller in size and can achieve better results as BioID with a reduced addition of exogenous biotin and a larger biotinylation range (D. I. Kim et al. 2016). BioID2 is primarily beneficial due to its smaller size but still requires relatively long incubation periods. Other developments made in yeast resulted in TurboID (Branon et al. 2018). The development of a biotin ligase in yeast is beneficial for organisms that do not grow at 37°C since activity kinetics are higher at lower temperatures. Successful TurboID was performed in flies and worms growing at 25°C and 20°C respectively (Branon et al. 2018). For in planta proximity labelling such temperatures are ideal. They further showed that labelling times can be reduced in mammalian cell cultures to 10 minutes. Such short incubation times allow true snapshots of interactomes and provide a means to analyse temporal protein complexes.

In our BioID on E2Fa and ARFs, we identified a limited set of interactors. We showed with a conventional AP-MS that the core co-factors of E2Fa (RBR, DPa and DPb) could be identified while with the BioID approach only DPb could be identified. Structural inaccessibility might be causative for this observation. On the other hand, the labelling range for BirA* has been predicted to be 10nm (D. I. Kim et al. 2014). This range can be too limiting for the identification of complete protein complexes. Although BirA* can be fused to either the N-terminal or C-terminal sight of the bait protein increasing spatial coverage of the bait surrounding protein complex, a flexible linker was shown

to increase the identification of a subcomplex of the nuclear pore complex (D. I. Kim et al. 2016). A 13 repeat GGGGS linker increased the distance by ~25nm allowing substantially more identifications of the subcomplex (Amet, Lee, and Shen 2009; D. I. Kim et al. 2016).

We identified in vivo ARF heterodimerization and for the first time show in vivo ARF-TPL interactions. We believe that integration and further optimization of improved proximity labelling techniques such as TurboID and flexible linkers will provide an indispensable tool for unbiased MS based in planta protein complex identification. Indeed, when this manuscript was in preparation 3 additional works appeared in BioRxiv making use of TurboID in plants and in Arabidopsis cell suspension cultures (Arora et al. 2019; T.-W. Kim et al. 2019; Mair et al. 2019). The appearance of these works underline the usefulness of proximity labelling to identify protein-protein interactions in plants.

MATERIAL AND METHDOS

Plant material and growth

Arabidopsis seeds were surfaced sterilized and dry seeds were subsequently grown on half strength Murashige and Skoog (MS) plates. Plates were vertically grown in a grow chamber at 22◦C in standard long day lighting (16h:8h light:dark). Five days after germination roots were harvested. For floral bud harvesting, 7-day-old seedlings were transferred to soil and grown in standard long day lighting at 22◦C. Floral buds were harvested after ~1 month of growth.

PSB-D cell suspension culture were weekly maintained in MS minimal organics (MSMO) growing in the dark in an incubator at 25◦C, while shaking at 130 RPM. Transformation of PSB-D cells was conducted as previously described (Van Leene et al. 2010).

Molecular cloning

For generating translational fusions, CDSs of genes were amplified from cDNA using Phusion flash PCR master mix (Thermo Scientific). Amplified gene fragments were sliced into pPLV26 or the mBirA* modified pPLV26 vector using ligation independent cloning (LIC). Sequence of modified mBirA* are listed is supplementary table 1

Nuclei isolation

For nuclei isolation, plant material was grinded to fine powder in liquid nitrogen using mortar and pestle. To the powder, 3ml per gram of tissue weight nuclei isolation buffer (NIB) was added (20 mM HEPES (pH 7.5), 25% glycerol, 20 mM KCL, 2 mM EDTA, 2.5mM MgCl2 250 mM Sucrose and 1mM PMSF) and rotated head over tail for 20 minutes at 4◦C. The homogenate was subsequently filtered through 100µM and 50µM nylon mesh to remove large cell debris. Nuclei were spin down for 15 minutes, 3000RPM at 4◦C. Nuclei were subsequently washed 3 times in NIB supplemented with 0.3% Triton-X (Sigma) for 5 minutes at 3000 RPM at 4◦C. Crude nuclei were next suspended in PBS or directly suspended in RIPA lysis buffer (50 mM Tris pH 7.5, 150mM NaCl, 2mM MgCl2,

0.2mM EDTA, 1xCPI, 0.5mM DTT, 1%NP40, 0.5% SodiumDeoxycholate, 0.1% SDS, 1mg/ml DNAse).

Crosslinking

For crosslinking on nuclei dithiobis(succinimidyl propionate) (DSP) (Thermo Scientific) or dithiobismaleimidoethane (DTME) (Thermo Scientific) was dissolved in DMSO and added to the nuclei and rotated head over tail for desired time at 25◦C. Crosslinking reaction was quenched by adding 50mM Tris pH7.5 and rotating for 15min at 25◦C. Nuclei were spun down for 10 minutes at 3000RPM at 25◦C. Next nuclei proteins were extracted in RIPA lysis buffer and sonicated using a Biorupter (Diagenode) Decrosslinking of DSP and DTME was achieved by the addition of 50mM DTT for 15 minutes rotating at 25◦C.

Western blotting

For western blotting, protein concentrations were determined using Bradford reagent (Bio-Rad). 10 µg of nuclei or whole cell lysate was resolved on a precast 4-12% Bolt™ gradient gel (Invitrogen) in MES running buffer (Invitrogen). Transfer of proteins was achieved using the Transblot Turbo transfer system (Bio-Rad) on pre-cut nylon membranes (Bio-Rad). Membranes were subsequently blocked for 1 hour in 5% milk or 5% bovine serum albumin in Tris buffered saline Tween-20 (TBST) (50mM Tris pH 7.5, 150mM NaCl and 0.05% Tween-20). Primary antibodies were either incubated for 5 hours at 25◦C or overnight at 4◦C while gently rotating. Antibodies used were c-Myc (clone 9E10) (Thermo Scientific), Histone 3 (Agrisera), Streptavidin-HRP (GE Healthcare). Secondary antibodies y anti-rabbit HRP (A0545 Sigma) or anti-mouse HRP (A9917 Sigma) were incubated for 1 hour at 25◦C. Membranes were visualized using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) on a Chemidoc™ XRS+ (Bio-Rad).

GFP pulldowns

For pulldowns on YFP and GFP translational fusions, grounded material was lysed in

mild lysis buffer (50 mM Tris pH 7.5, 150mM NaCl, 2mM MgCl2, 0.2mM EDTA, 1xCPI, 0.5mM DTT, 0.2%NP40 and 1mg/ml DNAse) and mildly sonicated using a Biorupter (Diagenode). After lysate clearance supernatant was submitted to enrichment using GFP-Trap agarose beads (Chromotek) for 45 minutes at 4◦C while gently rotating. Beads were subsequently washed twice in lysis buffer, twice in lysis buffer without detergent and trice in 50mM Ammoniumbicarbonate (ABC)(Sigma) for 2 min at 2000xg at 4◦C. For crosslinked reactions reducing reagents were omitted during lysis and enrichment procedures. After ABC wash, 50mM DTT was added for crosslinked samples for 15 min at 25◦C. After final wash, bead precipitated proteins were alkylated using 50mM Acrylamide (Sigma). Precipitated proteins were next submitted to on-bead trypsin digestion using 0.35µg trypsin (Roche) per reaction. After overnight incubation at 25◦C, peptides were desalted and concentrated using C18 Stagetips.

Streptavidin pulldowns

Wildtype or mBirA* transgene expressing PSB-D cells were treated for 24 hours with 50µM Biotin (Duchefa). Cells were harvested and ground to a fine powder using liquid nitrogen and a mortar and pestle. Cells were further lysed in RIPA lysis buffer and sonicated using a Biorupter (Diagenode). After sonication, lysate was spun down for 1 hour, maximum speed at 4◦C. Supernatant was next submitted to biotin clearance using 10kDa Amicon ® centrifugation filtration devices. Lysate was washed 3 times for 30 minutes, 3000xg at 25◦C in RIPA lysis buffer. The concentrate was next submitted to overnight streptavidin enrichment using magnetic streptavidin sepharose beads (GE Healthcare) at 4◦C. Following day, bead precipitated proteins were washed 2 times in RIPA lysis, 2 times in RIPA lysis without detergent and 3 times in 50mM ABC for 2 min at 2000xg at 4◦C. Next precipitated proteins were subjected to alkylation, on-bead trypsin digestion and C18 Stagetips as described in GFP pulldown section.

Mass spectrometry and data analysis

After stagetip processing, peptides were applied to online nanoLC-MS/MS using a 60 min acetonitrile gradient from 8-50%. Spectra were recorded on a LTQ-XL mass

spectrometer (Thermo Scientific) according to Wendrich et.al. 2017 (Wendrich et al. 2017). Data analysis of obtained spectra was done in MaxQuant software package according to Wendrich et.al. 2017 (Wendrich et al. 2017). Data visualization was performed in Adobe Illustrator and R.

Acknowledgments

We thank Dominique Bergmann and Shunsuke Saiga for providing pBRM:BRM-GFP and pOBE1-OBE1-GFP seeds respectively.

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SUPPLEMENTARY INFORMATION

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Supplementary figure 1. Nucleotide sequence of Arabidopsis codon optimized mBirA*. Sequence bold, italic and underlined shows the R118G position which makes BirA* promiscuous. Bold and underlined shows sequence of 3xMyc tag.

Chapter 4

A “hitchhikers” guide to plant proteomics

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Advances in mass spectrometry equipment and sample preparation have given the proteomics field wide momentum. Although state of the art sample preparation procedures and mass spectrometers are widely used in the field of animal research, plant sciences have lagged behind, and few optimised workflows are available. Here we integrated and optimized frequently used sample preparation techniques for plant proteomic studies. Our aim was to provide a guide with easy and robust sample preparation procedures for shotgun proteomic and phosphoproteomic studies. We show that single vessel approaches perform excellent for plant proteomics, even with sub-microgram sample amounts. To gain in-depth proteome coverage, we devised simple and cost-effective offline stagetip-based fractionation strategies. Eventually we tested enrichment strategies to study phosphorylation. Our results showcase a set of methods that enable deep proteomic studies in plant sciences. We envision that our technical guide provides a practical reference for plant proteomic studies.

INTRODUCTION

In the past decade, “omics” approaches have emerged as indispensable tools in plant and crop biological research (Rey et al. 2019). Particularly proteomics has gained wide momentum due to advances in quantitative mass spectrometry (MS) platforms and the completion of model plant and crop species genomes. The field of proteomics studies the dynamics in protein abundance, protein turnover or specific post-translational modifications (PTM’s) such as phosphorylation. Whilst not being as comprehensive as genome or transcriptome methodologies, current MS platforms allow the identification of ~10.000 proteins from small sample amounts (Deshmukh et al. 2015; Meier et al. 2018). Further advances have been made to measure absolute abundances of proteins, measure subcellular trafficking of proteins and measure single cell type proteomes (Jadot et al. 2017; Wiśniewski et al. 2014; Budnik et al. 2018).

Although such advances are technically challenging in plant proteomics, a more commonly applied strategy is comparative bottom-up peptide-based proteomics. The usual workflow consists of protein solubilisation, peptide generation, optional peptide fractionation, LC-MS/MS measurement and statistical analysis. Numerous strategies can be employed within this workflow, yet pivotal to the success of proteomics studies is the quality and purity of the samples to be measured. Contaminant substances such as detergents, lipids, cell wall polyphenols, secondary metabolites and salts need to be removed before MS/MS measurement (S. Chen and Harmon 2006). Therefore, the outcomes of proteomic measurements largely depend on the experimental sample preparation procedures being employed.

Advances in proteomics sample preparation have primarily been made for model micro-organisms and eukaryotic cell cultures. Examples are the matrix-aided sample preparation techniques such as Filter Aided Sample Preparation (FASP) (Manza et al. 2005; Wiśniewski et al. 2009) and Single-Pot Solid-Phase-enhanced Sample Preparation (SP3)(Hughes et al. 2014). These methods reduce sample losses and therefore yield high peptide recoveries as compared to e.g. protein precipitation techniques. Although such methodologies are already published and widely adopted, the general notion is that the field of plant proteomics is lagging behind in adopting these advances. This is exemplified by the still prominent use of gel-based protocols in phytohormone proteomics (Černý et al. 2016; Jorrín-Novo et al. 2015; Jorrin-Novo et al. 2019). Probable reasons are that the plant proteomics field is much smaller, and that knowledge of proteomics is limited or not readily accessible for non-specialized

research groups. Although some studies report the integration or optimisation of plant proteome methodology, these usually focus on a single method or a sub-set of existing methodologies (Balliau et al. 2018; Vu et al. 2016).

Therefore, in this chapter, we set out to investigate and integrate commonly applied proteomic sample preparation strategies in the field of mammalian proteomics to provide a "hitchhikers" guide for the field of plant proteome research. Our prime focus is to describe qualitative and quantitative differences in sample preparation, peptide fractionation and phosphopeptide enrichment workflows.

RESULTS

Proteomics sample processing with single vessel approaches.

The first step in a proteomic workflow consist of the solubilisation of proteins from whole plants or specific organ or tissue types. Although there are numerous strategies, the use of the strong detergent sodium dodecyl sulfate (SDS) is frequently used due to its excellent protein solubilisation properties. SDS or other ionic detergents must be removed before protein digestion and MS/MS measurement since both chromatography and MS measurement are negatively influenced by charged hydrophobic contaminants (Wiśniewski et al. 2009). Protein precipitation techniques such as methanol/chloroform, acetone or TCA are frequently used, yet the in-solution removal of SDS is not trivial and impurities can impede complete digestion of the proteome (Wiśniewski et al. 2009). Therefore, single-vessel approaches have been developed to allow for SDS lysis conditions whilst generating highly pure peptide samples.

Here we focused on three methodologies: FASP (Wiśniewski et al. 2009), SP3 (Hughes et al. 2014) and integrated StageTip (iST) (Kulak et al. 2014) sample processing (Figure 1A). The SP3 and iST workflows have been primarily developed to allow sub-microgram protein sample processing. Such methodologies would allow to conduct proteomics from tissues that are available only in limited quantties, such as individual tissues or mutant plants. For an equal comparison, we used 10µg, 1µg and 0.2µg Arabidopsis thaliana root protein extract, which roughly corresponds to the amount of HeLa cells used within the original SP3 publication (50.000, 5000 and 1000 cells, respectively)(Hughes et al. 2014). Our results show that FASP and SP3 have comparable reproducibility and protein identifications in the 10 µg and 1µg samples, yet SP3 outperforms FASP in the 0.2µg samples (Figure 1B, Figure S1A). These results, that SP3 outperforms in low-microgram range, are in agreement with the original SP3 publication (Hughes et

al. 2014) and a recent comparison of FASP, SP3 and iST (Sielaff et al. 2017). On the other hand, iST performed poorly over all measurements. This is in contrast with Sielaff et.al. (2017), where iST preformed equally with SP3 (Sielaff et al. 2017). Since the iST workflow cannot remove interfering compounds such as SDS, mild lysis buffers must be used. We reason that this is insufficient for plant protein extraction since cell wall disruption is a prerequisite for efficient protein solubilisation. On the other hand, the protease accessible area might be limited in iST.

We further focused on the qualitative differences between FASP and SP3. The nature of protein enrichment between these methods is different. While FASP uses a molecular weight cut-off filter to retain proteins, SP3 uses a hydrophilic carboxylated surface to trap proteins on the beads by a mechanism similar to hydrophilic interaction chromatography (HILIC) (Alpert 1990; Hughes et al. 2014). For equal comparison between the two methods, we used the 10 µg datasets since these showed comparable performance in protein identification. The identified proteins show a good overlap (83%) (Figure S1). Investigation into the biochemical parameters of the unique peptides belonging to the identified proteins shows an overall overlap, yet a small loss in hydrophilic peptides is observed with SP3 (Figure S1). This loss of hydrophilic peptides was also observed in the chromatogram (Figure 1C). This finding is at odds with the original publication, which found no biases between FASP and SP3 (Hughes et al. 2014) yet Sielaff et.al. (2017) found a substantial loss in the initial protein binding step (Sielaff et al. 2017). We reason that the optimized procedure as described by Sielaff et.al. (2017), using neutral pH and higher acetonitrile concentration (≥70%) for protein binding, will remedy the loss of proteins (Sielaff et al. 2017).

Besides the optimized protocols for SP3 (Sielaff et al. 2017; Moggridge et al. 2018), optimisation procedures have also been reported for FASP (Nel et al. 2015; Erde, Loo, and Loo 2014; Wiśniewski 2016; Wiśniewski and Mann 2012). Initially, enhanced FASP (eFASP) reported an improvement over FASP (Erde, Loo, and Loo 2014), however re-evaluation showed no discernible differences between the two methods (Nel et al. 2015). The improvement of proteome depth by utilizing multienzyme digestion FASP (MED-FASP) (Wiśniewski and Mann 2012; Wiśniewski 2016) is uncontested. In this approach, the first digestion is performed with endoproteinase Lys-C followed by cleavage with trypsin (Wiśniewski and Mann 2012). Our results show no discernible differences in increased proteome depth by MED-FASP (Figure 1D). This result might be attributed to the sample type used, as Wiśniewski (2016) observed differences in peptide yields from different sample types. On the other hand, differences in

manufacturer's proteases might result in different peptide yields. Overall, we show that FASP and SP3 are excellent single-vessel approaches for the protemic sample processing of plant protein extracts.

Increasing depth of proteome coverage

Compared to transcriptomic approaches like RNAseq, proteomics is not as comprehensive since there are no reliable multiplication strategies for proteins. Although current MS platforms allow great depth of proteome coverage, the numer of identified proteins relies heavily on the complexity of the sample. To reduce proteomic sample complexity, fractionation strategies can be performed on organelle, protein or peptide level. For the latter, this can be performed either online or offline while making use of different biochemical properties of the peptides. Since online methods require specialized equipment, we focused here on offline stagetip-based peptide fractionation strategies. Such a strategy does not require expert knowledge, is quick and cost-effective (Yasushi Ishihama, Juri Rappsilber, and Matthias Mann 2006).

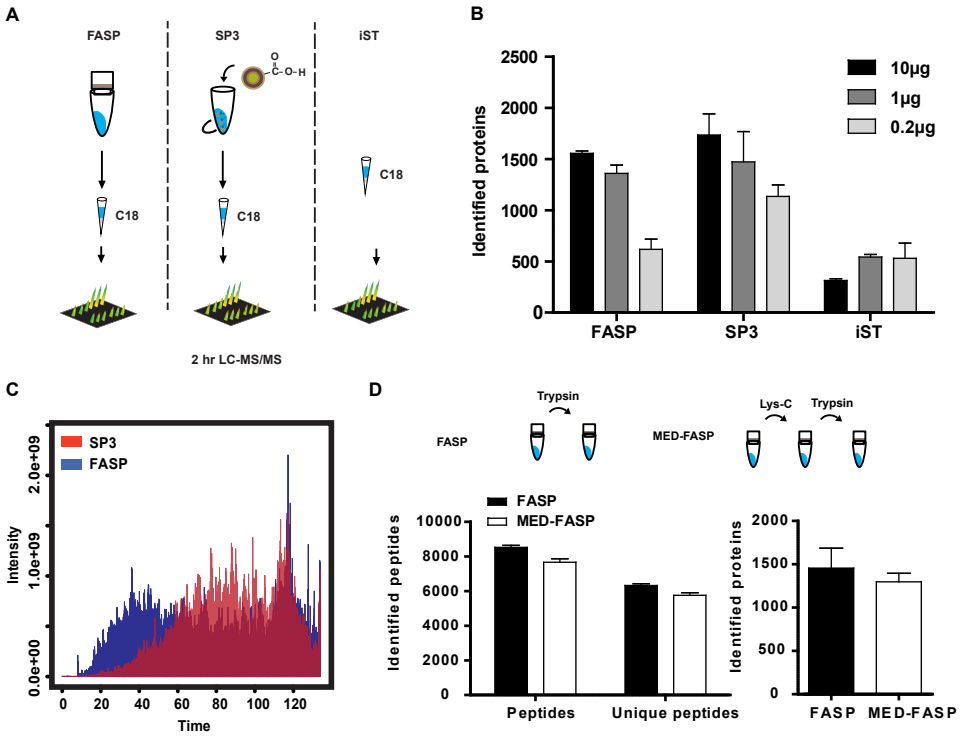

Figure 1: Comparison of single-vessel proteomic approaches. Three commonly used single-vessel approaches were compared for their performance with plant material (A). FASP and SP3 have similar performances in terms of protein identifications while iST performs poorly (B). Chromatographic retention time shows losses of hydrophilic peptides for SP3 enriched samples (C). No discernable differences between FASP and MED-FASP were observed on peptide or protein identification. All analysis were performed in technical triplicates.

Commonly used methods include ion exchange strategies, such as strong cation exchange (SCX) and strong anion exchange (SAX), or high pH reverse phase (HpRP) strategies employing C18 materials. In combination with FASP, Wiśniewski et.al. (2009) showed a ~2-fold increase in protein identifications using a stagetip-based SAX peptide fractionation (Wiśniewski, Zougman, and Mann 2009). They further showed that SAX had increased protein identifications over a SCX approach, which has also been described in another publication (Wiśniewski, Zougman, and Mann 2009; Dimayacyac-Esleta et al. 2015). We therefore chose to compare the performance of FASP-SAX and FASP-HpRP (Figure 2A).

As input we used 10 µg FASP digest and used 5 buffers with increasing pH (pH11,8,5 and 2) for SAX and 5 buffers with increasing Acetonitrile concentration (5,8,11,18 and 40%) for HpRP (Figure 2A). Based on protein identification in techincal triplicates, SAX increased the identifications ~2-fold while HpRP increased the identifications ~2.6-fold (Figure 2B). Analysis of the peptide properties from the single fractions show that seperation occurred based on isoelectric point for SAX and based on the hydrophobic nature of the peptides for HpRP (Figure 2C). The efficiency of fractionation relies on how well the peptides are separated between the fractions, since this reduces sample complexity. To investigate the efficiency of peptide seperation we determined the peptide overlap between adjacent fractions. Indeed, the percentage overlap between subsequent fractions in SAX is ~50%, while for HpRP this is ~20%. This explains the higher increase in protein identifications (Figure 2E). This finding is in good agreement with Dimayacyac-Esleta et al. (2015), who also showed a lower peptide overlap with HpRP. We further investigated how much each fraction contributed to the total amount of identified peptides (Figure 2F). Low contributions of single fractions might be omitted in fractionation or combined with other fractions, which should reduces total measurement time. Overall, the fractions show equal contributions except for the first fractions in both SAX and HpRP (Figure 2F). For SAX, the flow-through fraction probably consists of peptides that do not bind well to the anion material while for HpRP the 5% acetonitrile might be too low to give a significant elution from C18 material. Indeed Dimayacyac-Esleta et al. (2015) started fractionation from 11%-80% acetonitrile

concentrations.

In summary, we showed here that the combination of FASP with HpRP in stagetip-based peptide fractionation allows for a rapid and cost-effective increase of proteome depth. Eventually, peptide fractionation increases measurement time 5-fold, and therefore choices should be made whether increased coverage is required at the expense of measurement time.

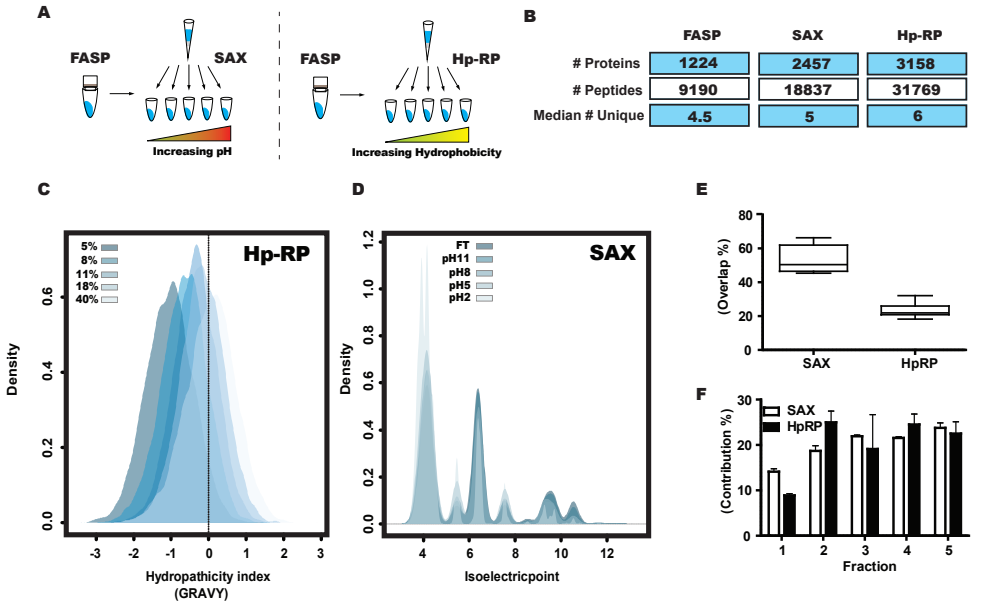

Figure 2: Comparison of peptide fractionation strategies. For peptide fractionation strategies, FASP digested peptides were submitted to offline in stagetip SAX using decreasing pH or offline in stagetip reversephase using an increasing ACN gradient (A). ¬Hp-RP fractionation outperfroms SAX by increasing protein identification ~2.6 fold (B). Based on peptide properties fractionation is achieved by increasing hydrophobicty for Hp-RP (C) or acidity for SAX (D). Adjacent fractions 1-2,2-3,3-4 and 4-5 were compared for overlap to determine fractionation efficiency (E). Overall each fraction contributes equally to the number of identified peptides (F). All samples were performed in technical triplicates.

Phosphopeptide enrichment strategies

Other than comparitive shotgun proteomics approaches to investigate protein abundance differences, post-translational modification (PTM) research is widely applied using MS-based platforms. Since these PTMs are usually in relatively low abundance, it is technically challenging to identify and investigate dynamics of PTMs. One of the

most-studied PTMs is phosphorylation, since it can have a wide range of biochemical effects on proteins (Nilsson 2012).

Numerous protocols have been published, describing phosphopeptide enrichment strategies. Yet, as with shotgun proteomics, protein solubilisation and enrichement of phosphopeptides can have a great impact on the quality of the proteomic measurement. Here we first investigated how well protein enrichment strategies performed. For phosphopeptide enrichment, we used the protocol as described by Vu et.al. (2016), utilizing immobilized metal ion affinity chromatography (IMAC)-based $Ti4^+$ beads (Vu et al. 2016). Especially for IMAC based protocols, interfering compounds such as nucleic acids can hinder efficient phosphopeptide enrichment (Potel et al. 2018). We tested the commonly applied protein precipitation methods, TCA, acetone and methanol/chloroform pericipitation. We further included FASP since it retains DNA and RNA on the filter after peptide elution (Wiśniewski et al. 2014). After protein digestion, aliqots were submitted for DNA agarose gel analysis. Our results clearly show that common precipitation techniques fail to completely remove DNA from samples (Figure 3A). On the other hand, almost no signal was detected in TCA precipitation while no signal at all was visible in FASP samples. We further reasoned that the presence of DNA must interfere with phosphopeptide enrichment and therefore submitted the samples to $Ti4^+$ -IMAC enrichment and subsequent LC-MS/MS measurement (Potel et al. 2018). Indeed, interference is also reflected in the identification of phosphopeptides with a ~1.5-fold reduction for acetone and methanol/chloroform precipitated samples (Figure 3B). Although benzonase can be used with precipitation methods (Potel et al. 2018) we decided based on these results to further employ FASP to compare different phosphopeptide enrichment strategies.

Due to the low abundance of phosphopeptides in whole cell lysate mixtures, phosphopeptides need to be enriched. Most commonly applied are targeted metal oxide affinty (MOAC) or IMAC chromatography techniques. While IMAC approaches relies on the attraction between metal cations and negatively charged phosphate groups, MOAC relies on metal attraction with oxygen in the phosphate groups. Some studies report differences between the two methods (Liang et al. 2007; Negroni et al. 2012) while others do not (Matheron et al. 2014). Recently Vu et.al. (2016) described the usage of $Ti4^+$-IMAC for phosphopeptide enrichment in plant species (Vu et al. 2016) however they did not compare the performance of these beads with other enrichment strategies. We therefore set out to compare commercially available IMAC and MOAC resins (Figure 4A). For the IMAC approach we used the magnetic $Ti4^+$-IMAC

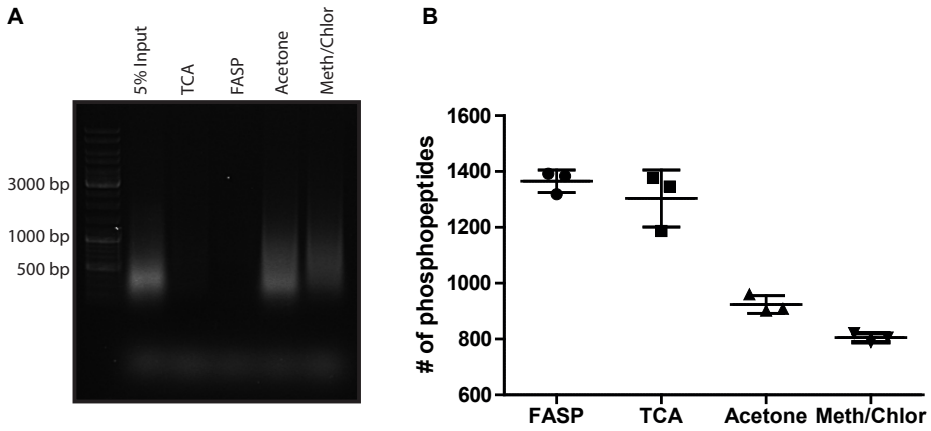

Figure 3: Comparison of nucleic acid interferences with different protein enrichment strategies. Peptide digested samples were compared to 5% protein lysate input material for nucleic acid interference on DNA agarose gel (A). Phosphopeptide identifications supports DNA gel analysis by poor perfromance of acetone and methonol/chlorform precipiation techniques (B). All samples were performed in technical triplicate and phosphopeptides were enriched by the $Ti4^{+}$ -IMAC method.

beads from MagReSyn® as described by Vu et.al. (2016). For the MOAC approach, we chose the commonly applied TiO_2 Titansphere™ (TS) and TiO_2 magnetic beads from MagReSyn® (Mg) (Figure 4A). For TiO_2 (TS), it was reported that peptide to bead ratios are critical for phosphopeptide enrichment (Li et al. 2009; Yue, Schunter, and Hummon 2015). We therefore chose the optimized ratio of 1:2 peptide to TiO_2 (µg/µg) (Yue, Schunter, and Hummon 2015). For MagReSyn® beads, we followed manufacturer's instructions.

Based on the number of identified phospopeptides with a localisation probability score of ≥75% and identified in at least 2 out of the 3 technical replicates, it is evident that $Ti4^{+}$-IMAC enriches ~2-3 fold and ~5 fold more phosphopetides than TiO_2 (Mg) and TiO_2 (TS) respectively (Figure 4C). Although a marginally higher identification of phosphopeptides has been reported before with $Ti4^{+}$-IMAC (Yue, Schunter, and Hummon 2015), this is in contrast with the difference we found. A possible explanation could be that peptide interaction with MOAC resin is limited in stagetip format for TiO_2 (TS). On the other hand, TiO_2 (Mg) also showed reduced identifications. Another explanation could be that the TiO_2 methodology retains more nonspecifically bound acidic peptides. This has been remedied by the usage of 2,5-dihydroxybenzoic acid (DHB), which is capable of displacing nonspecifically bound acidic peptides (Larsen et al. 2005). However with the technique EasyPhos, employing TiO_2, DHB proved to be unneccassary (Humphrey,

Azimifar, and Mann 2015). Another comparison showed that elution efficiencies differ drastically between Ti4^{+}-IMAC and TiO_2-MOAC (Ti4^{+} > TiO_2) strategies, and further showed that the order of identified phosphopeptides follows Ti4^{+}-IMAC > TiO_2(Mg) > TiO_2(TS) (Tape et al. 2014). This is in good agreement with our results.

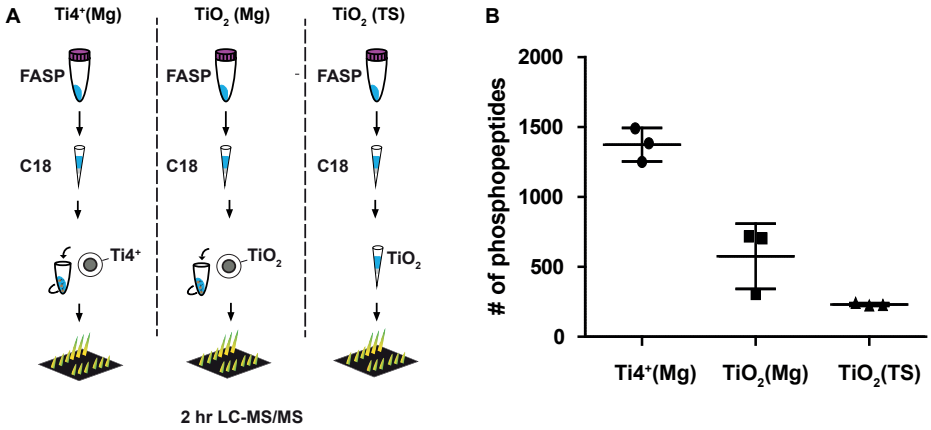

Figure 4: Comparison of phosphopeptide enrichment strategies. Magnetic IMAC, MOAC (Mg) and agrose MOAC (TS) enrichment strategies were compared for phosphopeptide enrichment performance (A). IMAC outperfromes the MOAC method by identifying ~3 fold more phosphopetides. All enrichment were conducted in technical triplicate.

We next examined whether there are any preferential biases of the different methods (Figure 5). The overall nature of phosphorylated amino acids followed previously published percentages (Serine ~90%, Threonine ~7 and Tyrosine ~1%) (Figure 5.) (Matheron et al. 2014; Vu et al. 2016; Wu et al. 2016; Nakagami et al. 2010). Further evaluation of the identified phosphopeptides shows a high degree of overlap and similiarity in chemical properties (Figure 5) A deeper look at the subsets showed that the motif preferences has a proline at +1 for Ti4^{+}(Mg) and TiO_2(Mg) (Figure 5.) . For TiO_2(Mg) the +2 position onwards shows acidic amino acids, this acidic preference is also reflected in the isoelectricpoint of the subset (Figure 5.). This implies that there are indeed biases between the used affinity matrixes.

DISCUSSION

It is genereally noted that the potential of proteomics in the field of plant and crop research is not fully exploited (Jorrin-Novo et al. 2019). Although the tendency of using older methodology (e.g. gel based proteomics) is decreasing, it is still the most

prominent methodology used (Jorrin-Novo et al. 2019; Černý et al. 2016). Here we set out to integrate, investigate and compare state of the art sample preperation methods for use in the field of plant proteomics.

Our results show that single-vessel approaches perform very well in a plant proteomics setting. We further highlight that these approaches also allow for minimal, sub-microgram, sample input. This is adventageous especially when limited amount of samples are available as would be the case for e.g. fluorescent activated cell sorted (FACS) sorted plant cells or plant mutants. Recently, FASP was compared to a tube-gel based method for plant proteome purposes wherein FASP gave higher identifications (Balliau et al. 2018). Indeed, here we show that FASP in terms

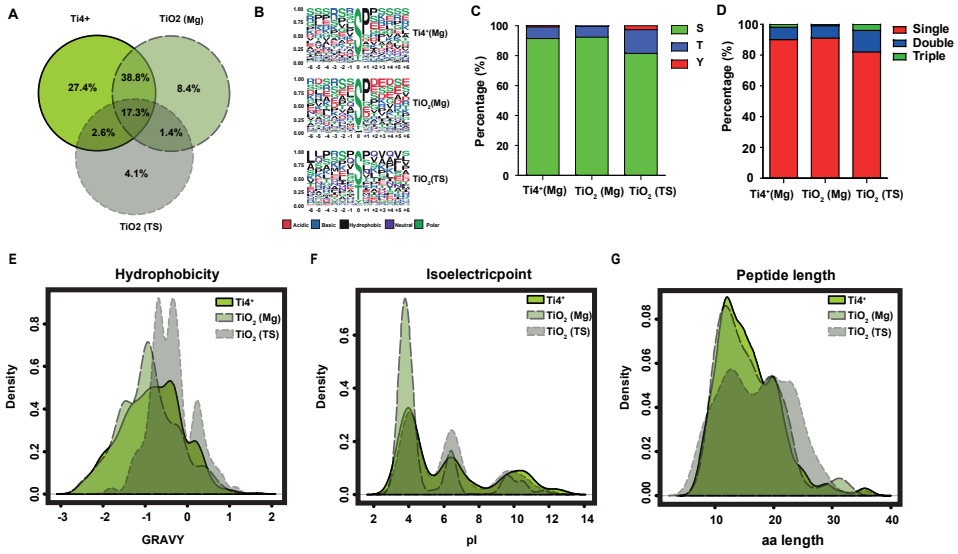

Figure 5: Phosphopeptide enrichment strategies reveals biases. (A) Venn diagram of enriched phosphopeptides show litlle overlap between the different enrichment methods. Motif analysis from the specific enriched peptides shows a bias towards acidic peptides for TiO2(Mg) (B). No discernbale differences were observed in phosphorylated amino acid (C) or charge state (D). Biochemical properties of specific peptides show a bias in acidic peptides for TiO2(Mg) (E,F and G).

of number of identifications and reproducibility performs excellent within the microgram range. The FASP method has a widespread utility in the field of mammalian/animal proteomics. An example of this is the use of lectins on the FASP filter to enrich for glycosylated proteins which allowed the mapping of N-glycosylation sites of seven species including Arabidopsis thaliana (Zielinska et al. 2010, 2012). Although questions have

been raised about the losses of proteins on the FASP filter (Krahmer et al. 2015), it has been well documented that the peptide yields are around 70%. In contrast, in-solution protocols have peptide yields ranging between 40-50% (Wiśniewski 2016). We further show that the usability of FASP can be further intergrated into phosphoproteomics methodology. That the FASP methodology works well has been shown before in a DNA damage response phosphoproteome screen in Arabidopsis thaliana (Roitinger et al. 2015) while another report, investigating tomato phosphosignalling, showed that methanol/chloroform precipitation outperformed FASP (Hsu et al. 2018). We reason that this discrepancy with our results relies in the MW cut-off filter used, since it has been shown that 30kDa cut-off filters perform better than 10kDa cut-off filters (Wiśniewski 2018; Wiśniewski, Zielinska, and Mann 2011; Hsu et al. 2018).

FASP-generated peptides provide an excellent sample for single shot proteomics, yet when greater depth is necessary, sample complexity needs to be reduced. Although it can be stated that with the increased sensitivity and measurement speed of modern mass spectrometers, fractionation is becoming obsolete, still a comprehensive proteome requires reduction in complexity. A simple off-line stagetip based fractionation strategy was compared here. Our results show that high pH reversed phase peptide separation outperforms the SAX. This is in good agreement with a previous report comparing stagetip-based SCX, SAX and HpRP strategies (Dimayacyac-Esleta et al. 2015). Although HpRP on FASP-mediated protein digests resulted in a ~2.6 fold increase of identifications, we reason that optimisation of LC gradients and MS parameters might improve this, as has been shown before (Smits et al. 2014; Batth, Francavilla, and Olsen 2014).

Following the succesfull integration of single-shot and peptide fractionation strategies we next compared different phosphopeptide enrichment strategies. It has been debated that different enrichment strategies might give specific biases (Liang et al. 2007; Negroni et al. 2012; Matheron et al. 2014). Our results indeed show that in terms of identifications, the $Ti4^{+}$-IMAC approach as described by Vu et.al. (2016) outperformed the TiO_2 approaches. These findings are in good agreement with previously published comparisons (Tape et al. 2014). In our set-up, we aimed for simplicity in sample handling and MS measurement. Although in our platform we could identify ~1500 phosphopeptides, such numbers can be increased by using fractionation prior or after phosphopeptide enrichment (Batth, Francavilla, and Olsen 2014), or by using tandem enrichment approaches. It was for example reported that tandem MOAC enrichment using Al(OH)3-based MOAC and TiO_2-based MOAC increased the amount of quantified

phosphopeptides (Y. Chen and Hoehenwarter 2018). Such tandem or combinatoral approaches have also been reported before for IMAC to provide enhanced coverage of the phosphoproteome (Ye et al. 2010; Thingholm et al. 2008). Altough this enhances coverage, it is at the expence of increased measurement time.

In summary, we here interogated different proteomic sample processing procedures for the integration into plant proteomics workflow. We have shown that FASP provides an excellent approach for both shotgun and phosphoproteomics sample preparations. When sub-microgram samples are to be handled, SP3 is beneficial due to its minimal losses. In terms of increasing coverage stagetip based HpRP fractionation provides an easy, rapid and efficient tool. Eventually Ti4+-IMAC outperformed other commonly applied phosphoenrichment strategies. We envision that our investigation will provide a useful reference for non-specialized plant proteomics groups to make choices on proteomic sample processing procedures.

MATERIAL AND METHODS

Plant growth and material

Arabidopsis seeds were surfaced sterilized and dry seeds were grown on half strength Murashige and Skoog (MS) plates. Plates were vertically grown in a growth chamber at 22◦C in standard long day lighting (16h:8h light:dark). Five days after germination roots were harvested.

Protein extraction

Harvested roots were grounded to fine powder in liquid nitrogen with a mortar and pestle. Powder was suspended in SDS lysis buffer (100mM Tris pH8.0, 4%SDS and 10mM DTT). Protein extract was next sonicated using a cooled Biorupter (Diagenode) for 10 minutes using high power with 30 seconds on 30 seconds off cycle. Lysate was cleared by centrifugation at maxiumum speed for 30 minutes. Protein concentrations were determined using the Bradford reagent (Bio-Rad).

Protein precipitation

Acetone precipitation was done according to Humphrey et.al. (2015). Methanol chloroform precipitation was done according to Vu et.al. (2016). For trichloroacetic acid (TCA) precipitation 1 volume of ≥99% TCA was added to 4 volumes of protein lysate. Mixture was incubated on ice for 10 minutes and spin down at maximum speed for 5 minutes at 4◦C. Pellet was washed twice with acetone at maximum speed for 5 minutes at 4◦C. Pellet was air dried and suspended in 50mM ammoniumbicarbonate (ABC) (Sigma).

Filter aided sample preparation (FASP)

For FASP 30kDa cut-off amicon filter units (Merck Millipore) were used. Filters were first tested by appling 50µl urea buffer UT buffer (8M Urea and 100mM Tris pH8.5) and centrifuging for 10 minutes on 11000 RPM at 20◦C. Desired amount of protein sample was next mixed with UT buffer until a volume of 200 µl, applied to filter and

centrifuged for 15 minutes on 11000 RPM at 20◦C. Filter was washed with UT buffer for 15 minutes on 11000RPM at 20◦C. Retained proteins were alkylated with 50mM acrylamide (Sigma) in UT buffer for 30 minutes at 20◦C while gently shaking. Filter was centrifuged and after that washed trice with UT buffer for 15 minutes on 11000RPM at 20◦C. Next filter was washed trice in 50mM ABC buffer. After last wash proteins were cleaved by adding trypsin (Roche) in a 1:100 trypsin:protein ratio. Digestion was completed overnight. The following day filter was changed to a new tube and peptides were eluted by centrifuging for 15 minutes on 11000RPM at 20◦C. Further elution was completed by adding two times 50mM ABC buffer and centrifuging for 10 minutes on 11000RPM at 20◦C.

Single pot Solid phase enhande Sample preparation (SP3)

Sera-Mag Carboxylate hydrophilic and hydrophobic beads (50µg/µl, Thermo Scientific) were mixed to a concentration of 10µg/µl and washed twice in milli-Q water on a magnetic rack. For protein enrichment, desired amount of alkylated protein was added to 20 µg bead mixture and 1% formic acid was added to acidify the mixture. Acetonitrile (ACN) was added to a final percentage of 50%. Mixture was incubated at 25◦C for 10 minutes. Next 70% ethanol was added twice and incubated for 30 seconds. After that acetonitrile was added and incubated for 15 seconds. Beads were subsequently air dried for 30 seconds and reconstituted in 50mM ABC. Digestion was completed overnight as described under FASP section.

In Stagetip (iST)

For iST harvested and grinded roots were lysed in 50mM ABC using a cooled Biorupter (Diagenode) for 10 minutes using high power with 30 seconds on 30 seconds off cycle. Lystae was subsequently cleared by centrifugation. Protein content was determined by Bradford reagent (Bio-rad) and desired µg loaded onto a 200µl tip containing 2 plugs of C18 octadecyl 47mm Disks 2215 (Empore™). iST tip was washed with 50mM ABC by centrifugation for 4 minutes at 1500xg. Next Proteins were reduced with 50mM DTT in 50mM ABC and incubated at 60◦C for 30 minutes. Alkylation was achieved

using 50mM Acrylamide in 50mM ABC in the dark for 30 minutes at 25◦C. Trypsin was added (1:100, trypsin:protein) and digested overnight at 37◦C. Following day iST tip was washed twice with 0.1% formic acid and peptides eluted with 80% ACN in 0.1% formic acid.

Peptide fractionation

FASP digested peptides (10 µg) were submitted to offline in stage-tip based strong anion exchange (SAX) or high pH reversed phase (Hp-RP) fractionation. For SAX, Britton–Robinson buffers (B&R) of pH11, 8,5 and 2 were used. SAX tips were made with 20 plugs of Anion-SR 47mm Extraction DISKs 2252 (Empore™) material in a 200 µl tip. StageTips were washed with B&R pH11 for 24 minutes at 1000xg. To the peptides 140 µl B&R pH11 and 10 µl 1M NaOH was added until pH 11-12. Sample was loaded on SAX StageTip and centrifuged for 45 minutes at 500xg. Next peptides were sequentially eluted by adding 200 µl B&R pH11,8,5 and 2 and centrifuged for 24 minutes at 1000xg. SAX fractionated peptides were next submitted to C18 Stagetips for peptide deslating and concentrating.

For Hp-RP tips, 2 plugs of C18 octadecyl 47mm Disks 2215 (Empore™) material and 1mg:10 µg of LiChroprep® RP-18 (Merck) : peptide were added to a 200 µl tip. Tips were equilibrated with methanol for 4 minutes at 1000xg. Next buffer containing 0.1% formic acid and 80% acetonitrile was added and centrifuged for 4 minutes at 1000xg. Final equilibration was achieved with two washes of 0.1% formic acid and two washes of 20mM Ammonium formate (Optima®) pH10 for 4 minutes at 1000xg. Peptides were acidified with 10% trifluoracetic acid (TFA)(Alfa Aesa) to pH 2-3 before loading onto Hp-RP tip. Sample was loaded by centrifugation for 20 minutes at 400xg. Peptides were subsequently eluted with ammonium formate buffers containing 5%,8%,11%,18% and 40% ACN.

Phosphopeptide enrichment

For magnetic $Ti4^{+}$-IMAC (MagResyn) and TiO_2-MOAC (MagResyn) approaches manufactures protocol was used without modifications (Resyn biosciences). For stage

tip based TiO_2 Titansphere™ (GL Sciences) a 1:2 peptide to TiO_2 (µg/µg) was used. FASP eluted peptides were mixed with ACN and TFA until a concentration of 50% ACN and 6%TFA. TiO_2 columns were made with double C8 membrane and desired amount of beads in 100% methanol. The columns were washed and equilibrated with 100% ACN and 80% ACN in 6% TFA using centrifugation for 4 min at 1500xg. Sample was loaded at 400xg for 30 minutes. Non-specifically bound peptides were washed with 80% ACN in 6% TFA by centrigugation for 4 min at 1500xg and 2 times with 60% ACN in 1% TFA for 4 min at 1500xg. Next bound phosphopeptides were eluted three times in 40% ACN and 15% NH4OH. After the last elution samples were concentrated using a vacuum concentrator for 30 minutes at 45◦C. Samples were subsequently acidfied using 10% TFA and processed with C18 Stagetip clean up.

C18 Stagetip clean up

For peptide desalting and concentrating 200 µl tips were fitted with 2 plugs of C18 octadecyl 47mm Disks 2215 (Empore™) material and 1mg:10 µg of LiChroprep® RP-18 (Merck) : peptides. Tips were sequentially equilibrated with 100% methanol, 80% ACN in 0.1% formic acid and twice with 0.1% formic acid for 4 min at 1500xg. After equilibration peptides were loaded for 20 minutes at 400xg. Bound peptides were washed with 0.1% formic acid and eluted with 80% ACN in 0.1% formic acid for 4 min at 1500xg. Eluted peptides were subsequently concentrated using a vacuum concentrator for 30 minutes at 45◦C and resuspended in 50µl of 0.1% formic acid.

Mass spectrometry and data analysis

After stagetip processing, peptides were applied to online nanoLC-MS/MS using a 120 min acetonitrile gradient from 8-50%. Spectra were recorded on a LTQ-XL mass spectrometer (Thermo Scientific) according to Wendrich et.al. (2017). Data analysis of obtained spectra was done in MaxQuant software package according to Wendrich et.al. (2017) with the addition of phosphorylation as a variable modification. Data visualization was performed in Adobe Illustrator and R.

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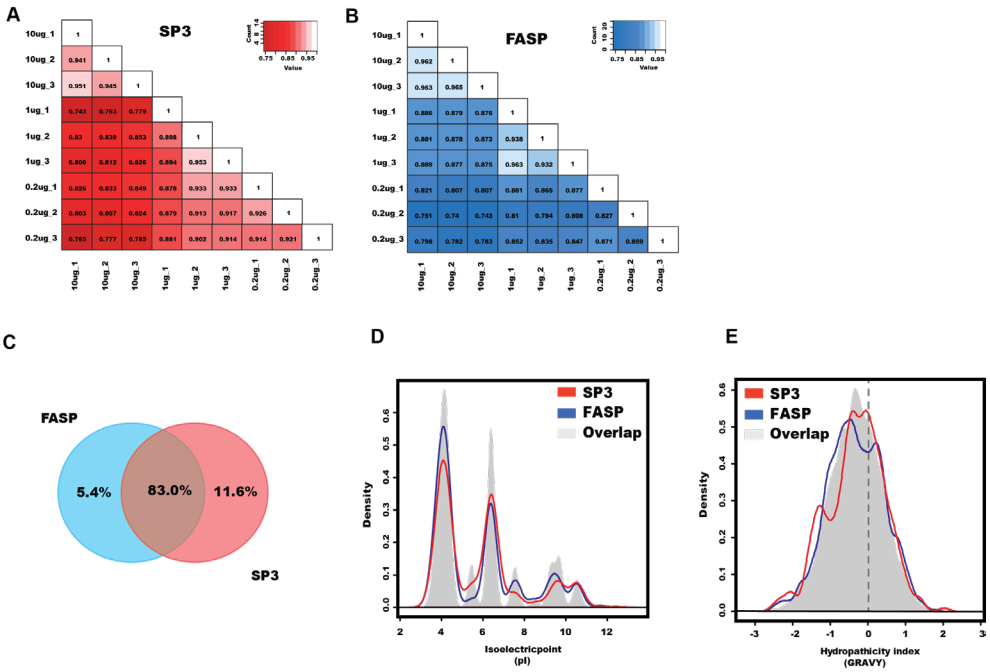
Supplementary information



Supplementary figure 1: Comparison of FASP and SP3 single vessel approaches. Correlations show good reproducibilty of the SP3 (A) and FASP (B) approaches. From the 10µg set biochemical properties were determined. FASP and SP3 enrich similar proteins (C). No differences were oberved in acidity of peptides (D) yet, a loss of hydrophilic peptides can be obeserved in SP3 (E). All sampels were analyzed in technical triplicate.

Chapter 5

A deeply conserved, rapid phosphorylation-based auxin response mediates plant growth

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The plant hormone auxin profoundly regulates plant growth and development. Many auxin responses require the control of auxin-dependent gene expression, for which a pathway has been well elucidated. However, there are several auxin-dependent cellular and physiological responses that occur within seconds or minutes, and are too quick to be mediated by auxin-dependent gene expression. Furthermore, there are growth responses to auxin in algae, that do not have the components for auxin-dependent gene regulation. Here, we used phosphoproteomics to identify early auxin responses. We found that numerous auxin-dependent phosphorylation changes occur within 2 minutes of treatment. Through the use of an inhibitor and an orthologous ligand/receptor system, we show that these responses do not depend on the TIR1 receptor that mediates gene expression responses. By comparing rapid phosphorylation responses to auxin responses across algal and land plant species, we show that this response is ancient and has been conserved in both green algae and all land plants tested. We identified a PB1 domain-containing MAP kinase (MARK) to have conserved auxin-dependent regulation. Phenotypic and (phospho) proteomic mutant analysis demonstrates a prominent role in mediating this novel auxin response and identifies phosphorylation targets that are involved in multiple cellular processes. This work identifies a novel auxin response that, unlike gene expression responses, is deeply conserved. This response likely predates other auxin responses and may mediate fast physiological responses to the auxin signal.

INTRODUCTION

Over a century ago, a mobile substance was proposed to regulate growth in plants (Darwin Charles, Darwin Francis, 1880). This substance was later identified as indole-3-acetic acid (IAA), and named auxin (Cholodny 1926; Went 1926). Since then, numerous aspects of auxin-regulated growth and development have been unravelled (Teale, Paponov, and Palme 2006; Weijers and Wagner 2016). From these studies it is clear that auxin is a prominent signalling molecule in most plants studied. Much of auxin-dependent growth and development is mediated by the nuclear auxin pathway (NAP), and leads to altered expression of thousands of genes (Weijers and Wagner 2016). The NAP consists of only a few dedicated components and has been discussed in detail in chapter 2 and chapter 3. Although signalling through the NAP can explain much of the long-term developmental and growth activities of the hormone, there are other, fast auxin responses that may not use this signalling pathway.

For example, changes in root growth can be observed within two minutes after exogenous auxin application in Arabidopsis (Fendrych et al. 2018). Given that the first auxin dependent transcript can be detected after ten to fifteen minutes (Abel and Theologis 1996), such fast response is unlikely to be based upon NAP-dependent gene regulation. The use of the auxin uptake carrier aux1 mutant, tir1/afb mutants and an orthogonal ccvIAA-cvxTIR1 system revealed that signal perception for this response occurs inside the cell and does depend on TIR1 (Fendrych et al. 2018).

There are a number of other fast auxin responses that may operate through pathways independent of the NAP. For example, the involvement of Calcium in auxin signalling was reported three decades ago (Felle 1988). Recently it was shown that AUX1, TIR1/AFB and the cyclic nucleotide-gated channel (CNGC14) all mediate fast auxin response involving membrane depolarization and Calcium fluxes in the Arabidopsis root (Dindas et al. 2018). On the other hand, Calcium involvement in auxin signalling and root surface pH changes were also previously reported to be TIR1/AFB-independent (Monshausen et al. 2011). This begs the question of whether there may even be separate TIR1-dependent and independent pathways for fast auxin response.

No receptor for fast auxin responses has yet been identified. Until recently, the auxin binding protein 1 (ABP1), together with the transmembrane kinase 1 (TMK1) were thought to constitute a receptor/effector module to perceive extracellular auxin and relay to non-transcriptional responses (Xu et al. 2014). However, genetic analysis of

abp1 mutants have called earlier results into question (Gao et al. 2015; Dai et al. 2015; Michalko et al. 2015). Nonetheless, TMK1 was recently shown to be involved in an alternative, TIR1/AFB-independent transcriptional pathway. Auxin promotes C-terminal cleavage of TMK1, which in turn regulates the stability of non-canonical Aux/IAA32 and 34 through phosphorylation, ultimately leading to altered gene expression (Cao et al. 2019).

Beyond TMK1, other protein kinases have been described to be involved in auxin action. For example, the AGC kinases PINOID and D6 protein kinase contribute to polar auxin transport by modulating the phosphorylation status of the PIN auxin efflux proteins (Barbosa, Hammes, and Schwechheimer 2018). Furthermore, the involvement of mitogen activated protein kinases (MPKs) has been reported. For example, MPK1 phosphorylation of ROP BINDING PROTEIN KINASE1 (RBK1) which interacts with Rho-like GTPases, mediates auxin-responsive cell expansion (Enders, Frick, and Strader 2017). Although several protein kinases are involved in different aspects of auxin action, to date no kinase has been directly linked to rapid signalling.

Since phosphorylation offers a mechanism for fast responses, we asked if there is a clear and fast phosphorylation response to auxin. Through an unbiased quantitative phosphoproteomics strategy, we find that within 2 minutes of exogenous auxin application, numerous phosphorylation events occur. Most of these however, are not mediated by TIR1, suggesting an independent pathway. Indeed, we find this response to be conserved among algae and land plants, and therefore to likely predate the NAP. We identified a Phox-Bem1 (PB1) domain containing mitogen activated protein kinase kinase kinase (MAPKKK) as a key component in this conserved, fast auxin response.

RESULTS

Identification of a fast, phosphorylation-based auxin response

Several proteomic analyses have been performed on auxin-treated cells. These studies however focused on timeframes where changes in abundance can be explained by altered gene expression (Xing and Xue 2012; Clark et al. 2019; Pu et al. 2019) . To investigate whether there are rapid proteomic effects on auxin we employed phosphoproteomic analysis on roots that were treated with 100 nM IAA for only two minutes, well before transcriptional changes can be detected (Abel and Theologis 1996)(Figure 1A). Following treatment, phosphopeptides were enriched by Ti4+-IMAC and identified and quantified using label-free quantitive mass spectrometry (see

Chapter 4). This analysis identified ~3100 phosphopeptides which were next subjected to a hybrid data analysis approach (Nikonorova et al. 2018). This approach takes into account identified phosphosites which are only found in one condition, and that are otherwise missed due to stringent filtering of missing values. After filtering, ~2157 phosphopeptides were subjected to FDR-controlled statistical comparison resulting in ~10% statistically significant (FDR ≤0.05) differentially abundant phosphopeptides (Figure 1B, Supplemental figure 1).

Global analysis showed that auxin primarily causes hyperphosphorylation (Figure 1B), suggesting the rapid activation of phosphorylation events. Among the phosphopeptides that were specific to IAA-treated conditions, we identified phosphorylation of SORTING NEXIN1 (SNX1) on serine position 16 (Ser-16). This position in SNX1 was previously reported to be differentially phosphorylated after 2 hours auxin treatment (Zhang et al. 2013), and thus validates our dataset. Within this study 20 proteins were found differentially phosphorylated from which 8 proteins overlap with our data (not shown). We further identified an auxin-dependent phosphopeptide corresponding to an activation site, Ser-35, of the MOB kinase activator-like 1A (MOB1A). MOB1A in both plant and animal species is an important regulator of growth and has been implied in auxin signalling (Cui et al. 2016; Praskova, Xia, and Avruch 2008). Hyperphosphorylation on aquaporin (PIP2-1 and PIP2-7) was detected on serine residues know to be involved in PIP membrane targeting (Ser280-Ser283)(Supplemental table 1). This suggest auxin regulated hydraulic control through PIP targeting as reported previously in lateral root emergence (Prak et al. 2008; Péret et al. 2012). We also identified hyperphosphorylation on Guanine nucleotide exchange factor SPIKE 1 (SPK1) which has been implicated in a ROP GTPase auxin dependent PIN internalisation pathway (Lin et al. 2012).

To gain more insight into this fast auxin response we employed the auxin antagonist α-phenylethyl-2-one-IAA (PEO-IAA). This compound acts as a competitive auxin inhibitor for gene expression responses. It can bind to the auxin binding pocket in TIR1/AFB (Hayashi et al. 2012), but may of course also bind and inhibit other, yet unknown auxin receptors. Additionally, to determine what part of the fast phosphorylation response is mediated by TIR1, we used the orthogonal concave-TIR (ccvTIR)/convex-IAA (cvxIAA) pair. This pair allows selective activation of TIR1-dependent signalling (Uchida et al. 2018). While the IAA-treated phosphoset shows preferential hyperphosphorolation, we did not observe the same trend in the PEO-IAA and cvxIAA sets (Figure 1B). PEO-IAA and cvxIAA sets show a more equal distributed regulation (Figure1B).

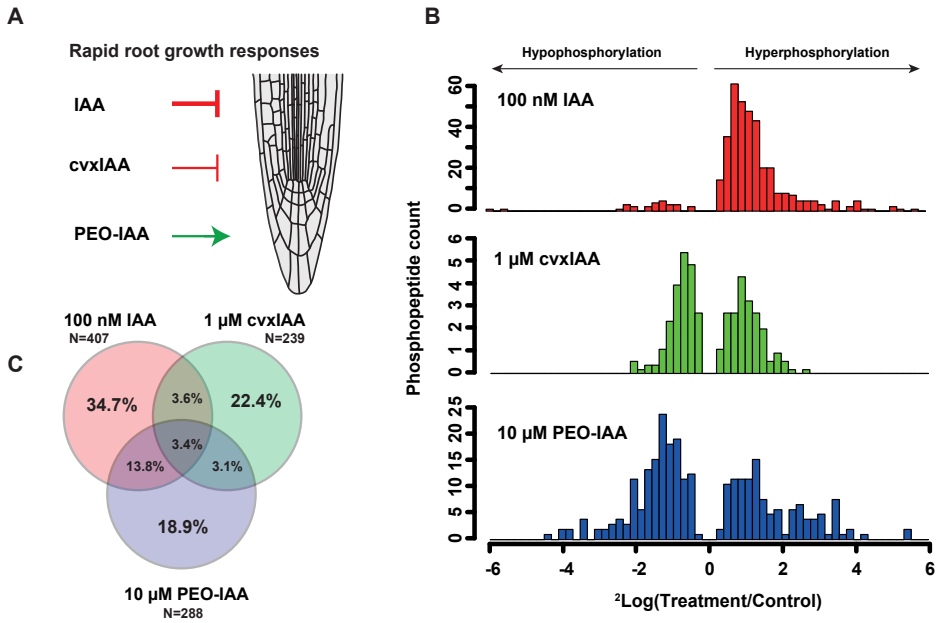


Figure 1: Phosphoproteomic analysis reveals rapid phosphodependent auxin signalling. (A) Schematic depiction of the effects of auxin (IAA), the auxin antagonist (PEO-IAA) and the chemical synthesized convexIAA (cvxIAA) on root growth. After 2 minutes exogenous treatment numerous proteins change their phosphorylation status (B). Histograms depict log2 fold changes of significantly regulated phosphopeptides (FDR ≤0.05). (C) Venn diagram of overlapping significantly regulated phosphopeptides (FDR ≤0.05).

5

We next compared differential phosphopeptides between IAA, PEO-IAA and cvxIAA treatments (Figure 1C). We found little overlap between the datasets with 17.2% overlap between IAA and PEOIAA and 7% between IAA and cvxIAA, which suggests that much of the fast phosphorylation response occurs independently of TIR1. It also suggests that an auxin binding site is used that cannot efficiently be inhibited by PEO-IAA. Analysis of heatmaps plotting differential phosphorylation (Figure 2) confirms this finding.

Some peptides, however, were oppositely affected by IAA and PEO-IAA. This included the penultimate threonine (T947) of H+-ATPase 2 (AHA2) that was hyperphosphorylated in response to IAA and hypophosphorylated after PEO-IAA treatment (Figure 2A). The T947 site has a regulatory function, with phosphorylation causing pump activation (Fuglsang et al. 1999). It was previously reported that pump activation is independent of TIR1/AFB in hypocotyls (Takahashi, Hayashi, and Kinoshita 2012), and this is confirmed by our analysis of the cvxIAA dataset (Figure 2A).

In a similar manner, we took all significantly regulated cvxIAA phosphopeptides and merged IAA and PEO-IAA phosphopeptides which should show TIR1 dependent regulation. Strikingly little overlapping regulation was observed between cvxIAA and IAA (Figure 2B). We next merged the datasets based on protein identifiers, rather than peptides, since the same protein might be regulated on different phosphosites. This however did not lead to larger overlap between datasets (data not shown).

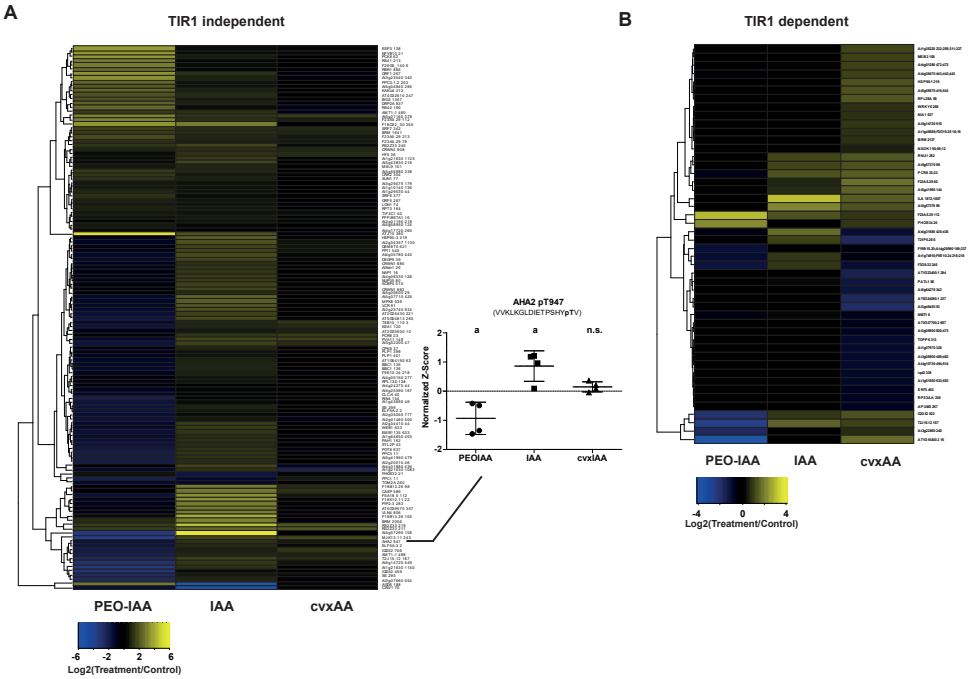


Figure 2: TIR1 dependent and independent rapid auxin signalling. (A) Overlapping significantly regulated phosphopeptides of IAA and PEO-IAA dataset with same phosphosite shows partial opposite regulation in a TIR1 independent manner. Corresponding sites show no regulation in the cvxIAA set as seen by predominantly black colour. (B) Significant TIR1 dependent phosphosites (cvxIAA) mapped to phosphopeptides of IAA and PEO-IAA dataset. Only non-significant IAA and PEO-IAA regulated phosphopeptides mapped to significantly regulated cvxIAA peptides.

Non-NAP roles for TIR1/AFB receptors?

While much of the fast phosphorylation response is independent of TIR1, a small part (7%) does seem to be mediated by TIR1. Such a role is difficult to explain by its residence in an SCF complex to target Aux/IAA substrates for degradation. We therefore asked if TIR1 has any other substrates or proteins interactions that may explain this rapid auxin effect. We therefore conducted AP-MSMS on roots from lines carrying functional pTIR1:TIR1-Venus and pAFB1:AFB1-Venus translational fusions in tir1 and afb1 background, respectively. To prevent degradation of substrates, plants were pre-treated for 1 hour with the proteasome inhibitor MG132 and subsequently for 2 minutes with 100 nM IAA. Pulldowns were conducted in a label free format utilizing non transgenic Col-0 as control.

In all conditions, we identified the baits TIR1 and AFB1 as well as known members of the SCF E3 ubiquitin ligase complex SKP1A, SKP1B, ASK4 and CUL1 (Figure 3). In addition, we identified the indole-3-butyric acid response (IBR3) protein, which is known to be involved in IBA to IAA conversion, to interact with AFB1 (Figure 3A). We further identified ribosomal proteins which are usually detected as “sticky” proteins in AP-MSMS procedures. Strikingly, we detected auxin-sensitive interaction both TIR1 and AFB1 with ribosomal protein L24 (RPL24B) (Figure 3A/B). This protein has previously been shown to mediate translational control of auxin-dependent transcripts (Nishimura et al. 2005).

To validate this interaction, we used biomolecular fluorescence complementation (BIFC). Unfortunately, no interaction was detected in our analysis (data not shown). BIFC can only test for direct interaction therefore it may be that RPL24B indirectly interacts with TIR1/AFB1. Since the rpl24b mutant shows defects in root growth rates, translational control by TIR1 might be an interesting avenue to investigate. However, this interaction analysis did not reveal any conspicuous TIR1/AFB substrates that may explain the fast phosphorylation responses observed, and it is therefore likely that this response is mediated by other receptors.

Fast auxin phosphorylation response involves MAPK activity

To dissect the potential mechanisms and regulation of the fast phosphorylation response, we performed motif analysis using Motif-X (Chou and Schwartz 2011; Schwartz and Gygi 2005).

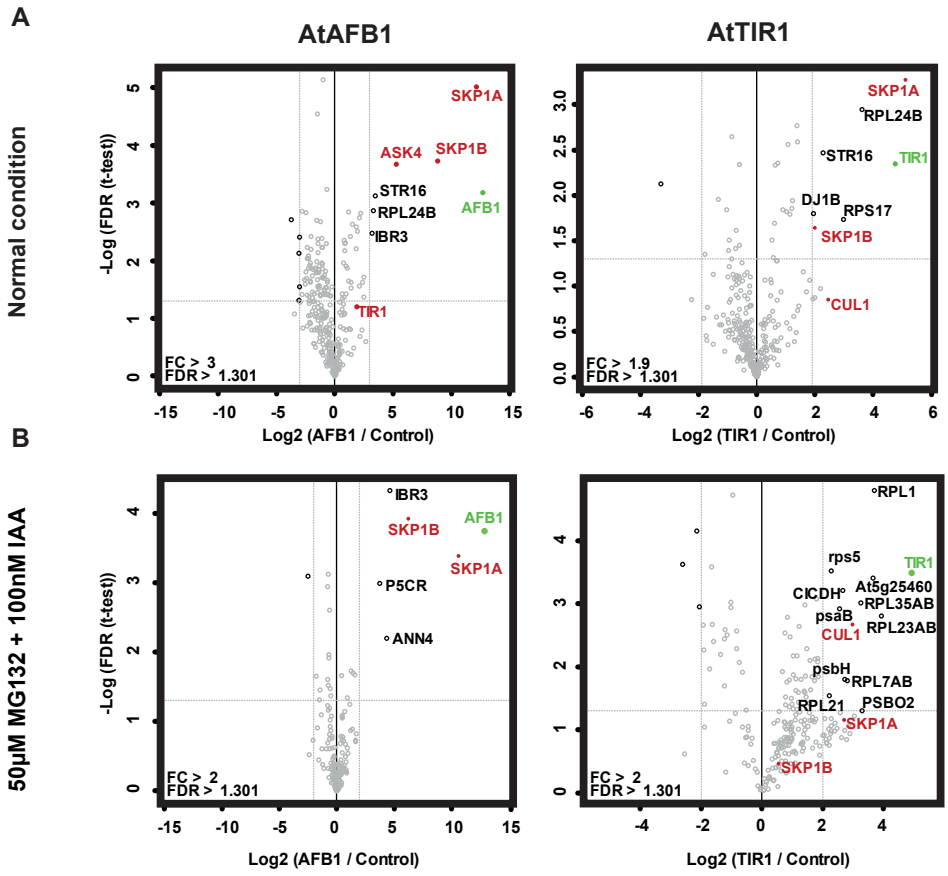


Figure 3: Interactomic analysis on auxin receptors TIR1 and AFB1. (A) AP-MS/MS on TIR1 and AFB1-Venus lines under normal (A) and MG132 pre-incubation and IAA treatment (B). Volcano plots show fold changes (FC, x-axis) and significance (FDR, y-axis). Proteins passing the threshold of FDR 0.05 and specific fold change are marked and have their name displayed. Green depicts the bait proteins and red depicts known members of the SCF E3 ubiquitin ligase complex. Pulldowns were performed in triplicate LFQ analysis.

This analysis revealed enrichment of a proline-directed serine motif (Figure 4A). This motif is one of the major regulatory phosphorylation motifs for kinases and is amongst others targeted by mitogen activated protein kinases (MAPKs) (K. P. Lu, Liou, and Zhou 2002). MAPK cascades were reported to be involved in auxin signalling (Enders, Frick, and Strader 2017; Smékalová et al. 2014; Mockaitis and Howell 2008). MAPKs are activated by dual phosphorylation on threonine and tyrosine residues in the activation loop. In our phosphoproteome, we indeed identified the MPK6 Thr-221

residue to be 5-fold hyperphosphorylated in response to IAA, yet the peptide did not pass the localisation probability threshold (0.73; threshold ≥ 0.75) for high-confidence phosphosite determination. Through activity assays, Mockaitis et.al (2008) showed that MPK activation in seedling roots peaks at 5 minutes after exogenous auxin treatment (Mockaitis and Howell 2008). It is possible that the 2 minutes treatment is relatively early during the activation of MPKs.

Since the proline-directed serine motif suggests the involvement of a MAPK cascade, we compared our data with a recent phosphoproteomic analysis on mpk mutants (Rayapuram et al. 2018). In their analysis a distinction was made between direct MPK targets having the (S/T)*P and indirect targets not containing (S/T)*P sites. The comparison revealed only a minor overlap between our and their data (Figure 4B). However, of interest is a common phosphoprotein MAPKKK (AT2G35050) within mpk3 and mpk6 datasets (Figure 4B). In our analysis, this MAPKKK has an opposite regulation being hyperphosphorylated when treated with IAA while hypophosphorylated when treated with PEO-IAA. This same phosphopeptide was found to be hyperphosphorylated after 2 hours with NAA (Zhang et al. 2013). Since this MAPKKK is probably involved in auxin signalling we renamed it to mitogen auxin-responsive kinase (MARK). These findings suggest that MAP kinase modules are involved in the fast auxin signalling.

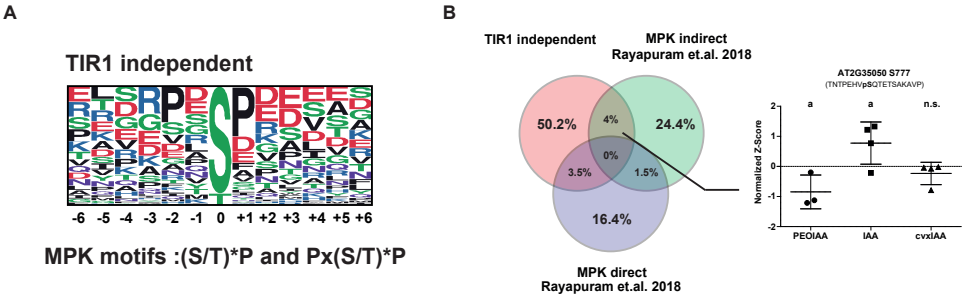


Figure 4: Motif analysis implicates MPK pathway in rapid auxin signalling. (A) TIR1 independent phosphopeptides motif analysis using Motif-X reveals proline serine directed kinase motif. (B) Cross comparison of TIR1 independent phosphopeptides and phosphopeptides regulated by mpk3 and mpk6 reveal a PB1-MAPKKK to be oppositely regulated in a TIR1 independent manner. Bar plot depicts Z-score normalized phospopeptide intensity values of Ser-777 of AT2G35050 under the three measured conditions. a. means significant regulation (FDR≤0.05), n.s. means not significant

Deep evolutionary conservation of fast phosphorylation response

Physiological responses to auxin are widespread in the plant kingdom, and can also be detected in algae that lack NAP components (Mutte et al. 2018; Ohtaka et al. 2017). We therefore asked if perhaps the fast phosphorylation response may be part of a more ancient auxin response system.

We performed phosphoproteomics on the moss Physcomitrella patens (Pp), the liverwort Marchantia polymorpha (Mp) and the charophycean alga Klebsormidium nitens (Kn). Tissues were exogenously treated with natural (IAA) and synthetic (2,4D) auxin, and phosphoproteomics was performed as in Arabidopsis roots. Analysis of the Pp, Mp and Kn datasets identified ~1600 phosphopeptides for Pp and Mp and ~800 phosphopeptides for Kn after hybrid filtering. The reduced identifications in comparison with Arabidopsis thaliana (At) is most likely due to incomplete annotation of the genomes causing lower peptide identifications. Further statistical analysis revealed that the phosphorylation status of numerous proteins was changed within 2 minutes after exogenous auxin application (Figure 5A). Motif analysis on the differentially regulated phosphopeptides reveals, as in Arabidopsis, enrichment of a proline-directed serine motif, thus implying involvement of MAPK regulation (Figure 5B).

We next analysed the phosphosets from Mp, Pp and Kn for best hits to Arabidopsis proteins and asked if there was conserved phosphoregulation. Strikingly this analysis showed that homologs of the identified Arabidopsis MARK were hyper-phosphorylated in response to auxin in all species analysed (Figure 6B). Although the exact phosphosites differ between species, all fall within the middle region of the protein (Figure 6A).

MARK belongs to the B4 group of MAPKKK consisting of six other uncharacterized members in Arabidopsis thaliana (Kazuya Ichimura et al. 2002)(Figure 6A). Recently, one member was identified to be involved in modulating plant flooding responses by regulating O2 supply in a K+-dependent manner (Shahzad et al. 2016). All B4 members contain an N-terminal Phox and Bem1 (PB1) domain and a C-terminal kinase domain that shares similarity with Raf kinases (Kazuya Ichimura et al. 2002).

To gain more insight into PB1-containing kinases we constructed a phylogenetic tree using the aforementioned and evolutionary distant plant species At, Pp, Mp and Kn (Figure 6A). Our analysis shows that MARK is conserved across all species from green algae to land plants. Both Marchantia polymorpha and Klebsormidum nitens contain a single copy of the gene.

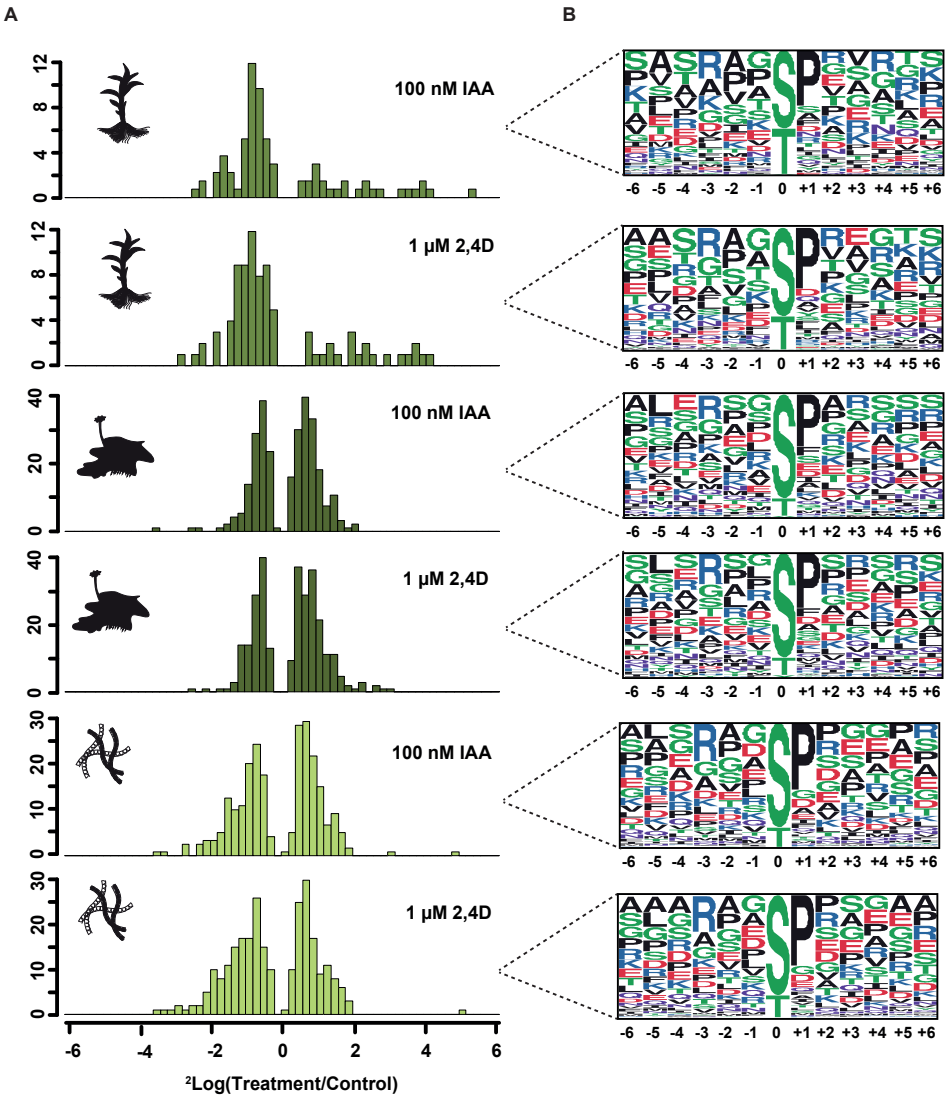


Figure 5: Evolutionary conservation of fast phosphorylation response. (A) Histograms depict log2 fold changes of significantly regulated phosphopeptides (FDR ≥0.05). (B) Motif analysis on significantly regulated phosphopetides reveal serine proline directed kinase motif.

The presence of a PB1 domain in MARK suggests possible hetero-dimerisation with other PB1-containing proteins important for signalling, as has been shown in animal and yeast systems (Moscat et al. 2006), as well as in Aux/IAA and ARF proteins (Korasick et al. 2015; Han et al. 2014). We therefore mined the measured phosphosets and

indeed identified multiple PB1-containing proteins (Figure 6C). Phylogenetic analysis of all PB1 domain-containing proteins in plant species tested, reveals that there is a clear distinction between the "NAP" PB1 domain protein and other proteins (Figure S1). From this phylogenetic analysis 6 subgroups can be made, NLP, CBS, NBR1, PHOX, Kinases and Kinase derived, based on other domains present or functionality (Figure 6C and Figure S1). From these subgroups, we identified members of the PHOX and kinases to be evolutionary conserved in their auxin-dependent phospho-regulation (Figure 6C). We therefore further focused on the characterisation of PHOX and MARK in rapid auxin dependent signalling.

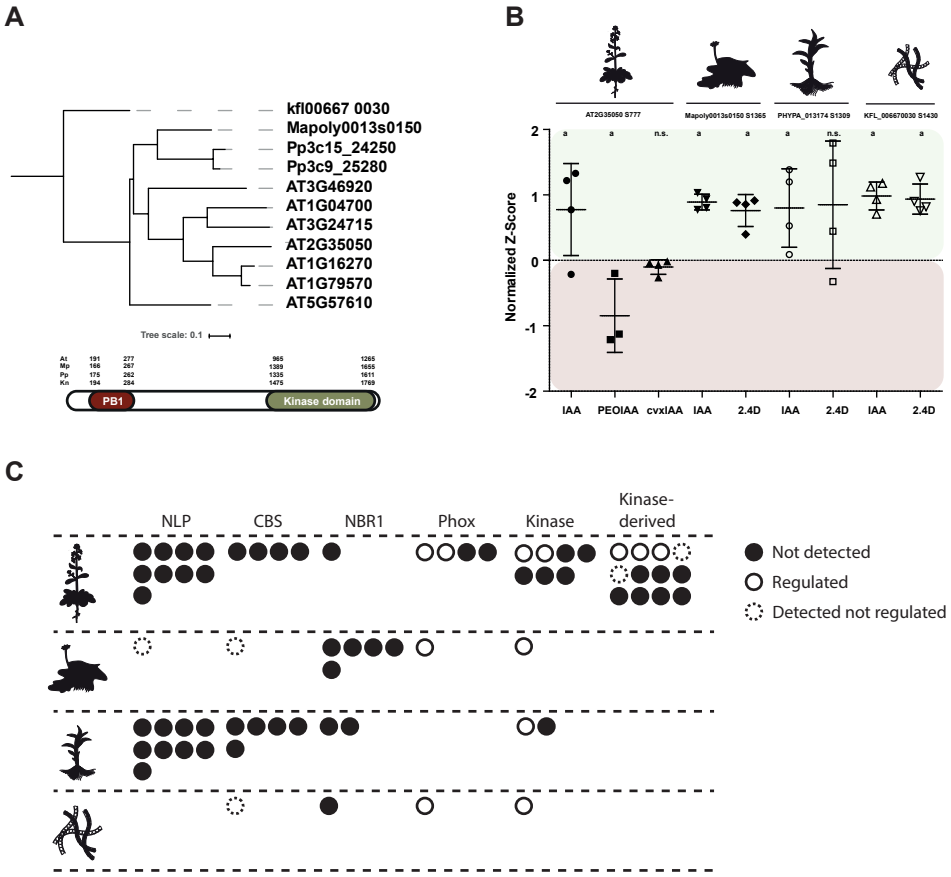


Figure 6: Evolutionary investigation into rapid auxin signalling. (A) Phylogenetic analysis of MARK. Schematic depicts MARK protein domain topology with annotated amino acid positions of the domains. For At the AT2G35050 was used and for Pp Phypa-013-174

was used. (B) Z-score normalized phosphopeptide intensity values of the identified MARK over all measured species. Letters a. depict significance (FDR≤0.05) while n.s. means not significant. (C) Schematic depiction of all PB1 containing proteins other than ARFs and Aux/IAAs in plant species. Number of circles represent the number of proteins in the corresponding species. Empty circles represent proteins found to be regulated in the phosphoproteomic analysis while dashed empty circles represent detected ones with localisation probability of ≥0.75 but not differentially regulated.

Phox mediates auxin-dependent plant growth

The PHOX group in Arabidopsis consists of 4 members while Mp and Kn contain a single copy and Pp has no PHOX representatives. The protein consists of multiple tetratricopeptide repeat (TPR) domains and a PB1 domain. The identified phosphosites fall outside of these domains yet consistently between a TPR and PB1 domain. Both domains are able to function as scaffolds to mediate protein-protein interactions. This might suggest that PHOX proteins function as adaptor proteins in larger protein signalling hubs. It was indeed found that they function as a myosin adapter proteins and localize to the membrane (Kurth et al. 2017). Mutant analysis, deleting all 4 copies, in Arabidopsis showed a reduction in root hair growth (Kurth et al. 2017). We conducted root growth assays on a quadruple phox mutant (also described as MadB 4KO; Kurth et al. 2017). Analysis of primary root growth after 2 days of auxin treatment did not show significant differences to wild-type (Figure S2). Although reduction in primary root growth is a hallmark for auxin sensitivity. Within our phosphoproteomic analysis we focused on rapid auxin responses and these effects might therefore not be visible upon long-term auxin treatments. Nevertheless, we also generated a knock-out line in Marchantia, since it has a reduced genomic complexity and allows to knock out the entire family with a single mutation. In Marchantia, hallmarks for auxin sensitivity are: reduced thallus growth and ectopic rhizoid formation (Figure 7A). Interestingly, the MpphoxKO mutant showed reduced thallus growth and ectopic rhizoid formation even in control conditions (Figure 7A). Exogenous auxin application caused a small but significant reduction in thallus area compared to MpTAK1 control (Figure 7B). These physiological changes along with the rapid phosphoproteome regulation show that PHOX proteins are required for auxin-dependent growth in Marchantia and suggest a role in rapid signalling.

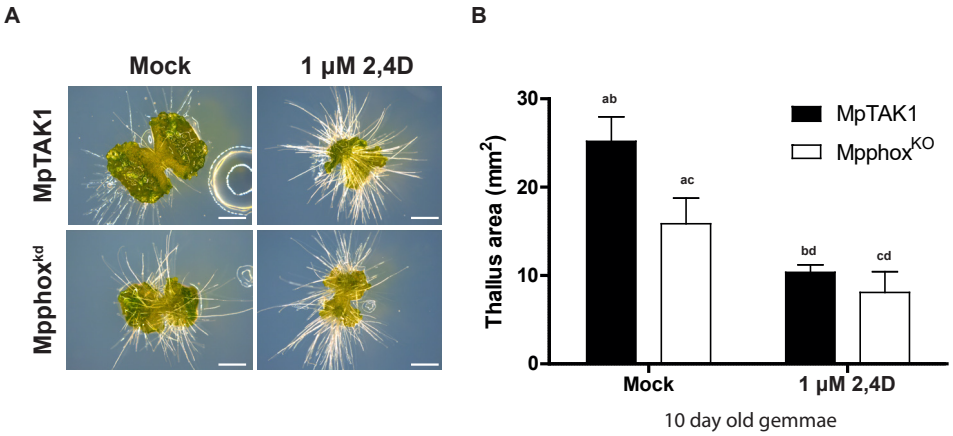


Figure 7: Phenotypic analysis of Mpphoxko implies involvement in auxin signalling. Gemmeas were grown for 10 days on media with or without the chemical auxin 2,4D. Ectopic rhizoid formation (A) and reduced thallus growth (B) implies involvement in auxin signalling. Images are representative of at least ≥10 gemmalings per condition. Bar graphs shows quantification of thallus area of at least ≥10 gemmalings per condition. Letters a, b, c and d in figure B represent statistical t-test significance ($p \leq 0.05$) between respective comparisons.

MpMARK is critical for auxin-dependent growth

We further focused our analysis on the involvement of MARK in auxin signalling. First we investigated the role of AtMARK in root growth. To that end we used a homozygous SALK insertion line. In both control and auxin -treated conditions, we did not detect differences in primary root growth between atmark mutant and wild-type (Figure S2). Since there are multiple family members in Arabidopsis, redundancy might mask functions in auxin-dependent growth. Marchantia has a single copy of MARK and we therefore addressed MpMARK function in this species. To generate a MpMARK deletion mutant, a 14.7 kb genomic region including promotor and coding region was targeted with CRISPR/CAS9 using two sgRNA's. This led to the isolation of a mutant with a deletion of the entire genomic region. To analyse auxin resistance, we extended our analysis using multiple auxin variants, NAA, IAA and 2,4D in a concentration gradient. We noted that already under control conditions, MpmarkKO has reduced thallus growth to about half the size of the Tak-1 (Figure 8A). Auxin application did not cause ectopic rhizoid formation, which indicates resistance to auxin in the mutant. Only at high auxin concentrations, effects were observed primarily with NAA (Figure 8C). We further observed that during prolonged growth under control conditions, the number of gemmae cups were significantly reduced compared to Tak-1 (Figure 8B).

Such phenotypes were also observed for MpTIR1 overexpression lines (Hirotaka Kato, personal communication). These results suggest partial auxin resistance and implies that MpMARK is involved in auxin response.

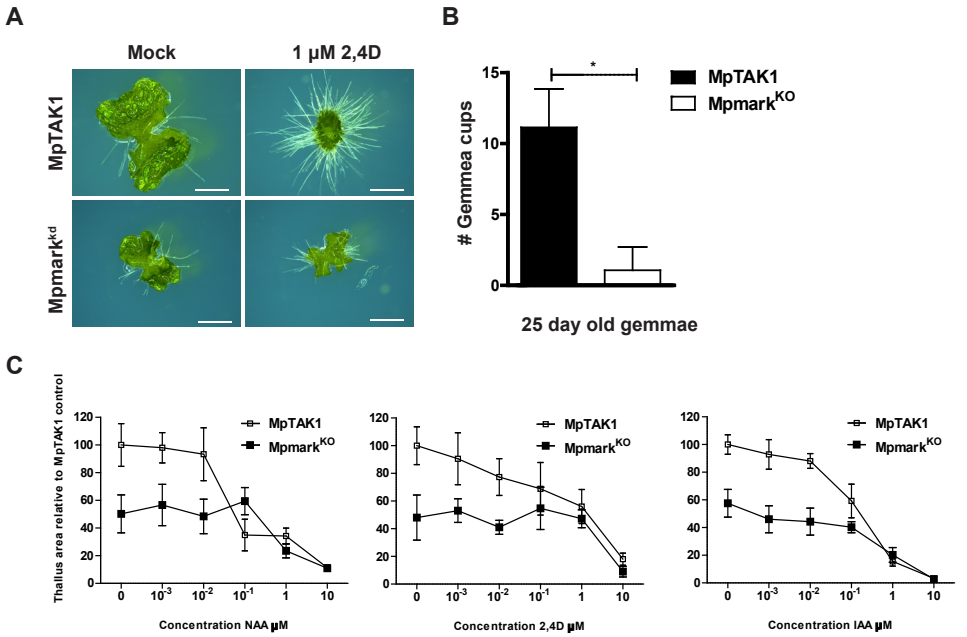


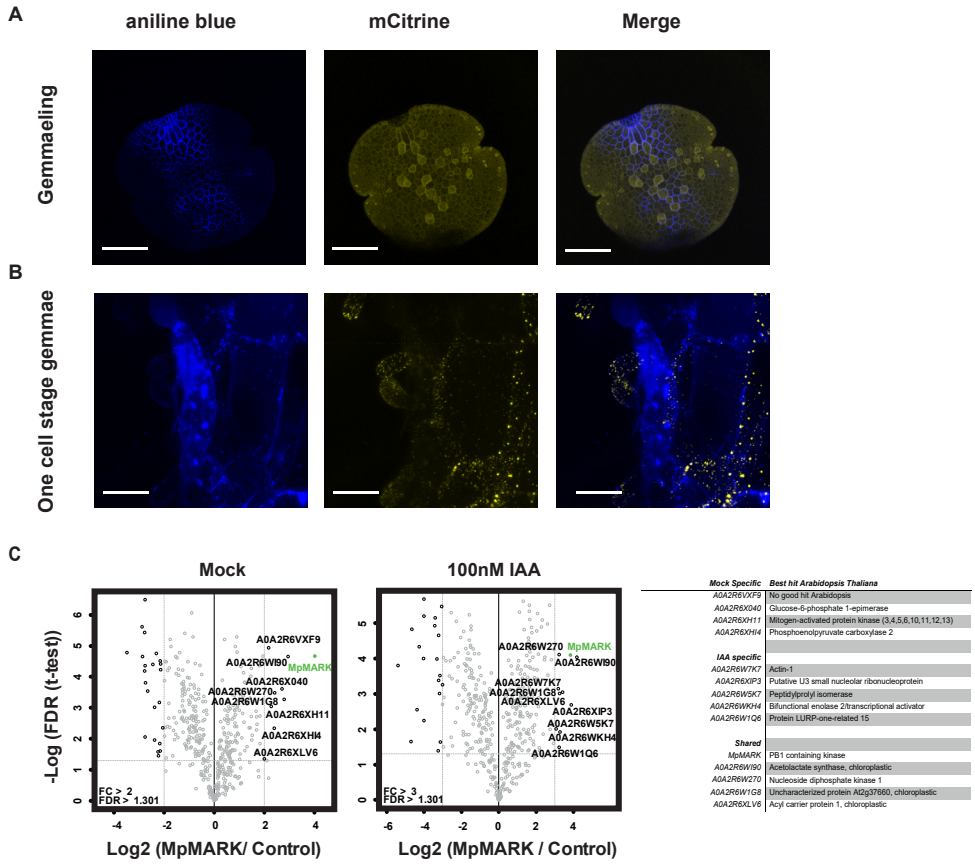
Figure 8: Phenotypic analysis of MpmarkKO. (A) 10 day old gemmalings grown on normal plates or plates supplement with auxin show auxin resistance of MpMARK. (B) Quantification of number of gemmacups per plant after 25 days of growth on normal media. Star indicates t-test significance ($p \leq 0.05$) of at least ≥8 gemmalings. (C) MpMARK shows concentration dependent resistance to auxin variants. Data shows measurements of at least ≥10 gemmalings per treatment.

Identification of MpMARK protein interactions

To gain further insights into MpMARK function, we complemented the MpmarkKO with a translational fusion of MpMARK fused to mCitrine. Localisation analysis in young gemmalings showed ubiquitous expression (Figure 9A). However, at single-cell stages of gemma development, we found MpMARK to primarily be localized in puncta close to the plasma membrane (Figure 9B). Similar puncta have been observed for other PB1 domain-containing proteins, such as AtARF7 and 19 (Powers et al. 2019), and this may mean that MpMARK oligomerizes via its PB1 domain.

To identify potential regulators and substrates of MpMARK, we performed AP-MS/MS on

MpMARK-Citrine both under control conditions and after 2 minutes of auxin treatment. The affinity purification led to a modest but significant enrichment of MpMARK (Figure 9C). Nevertheless, the Marchantia MPK type A/B homologue (MpMPK) was found to interact with MpMARK under control conditions, while no MpMPK peptides were found upon auxin treatment (Figure 9C) (Kazuya Ichimura et al. 2002). This observation suggests that MpMPK associates with MpMARK under non auxin conditions. We further identified a nucleoside diphosphate kinase (NDPK) (Figure 9C). Classically, these conserved group of kinases convert nucleoside diphosphates (NDPs) into nucleoside triphosphates. However, these enzymes been identified as “moonlighting” histidine kinases in animal and plants (Z. Lu and Hunter 2018). The NDPK1 homologue in Arabidopsis (NDPK2), was shown to interact with MPK3 and MPK6 (Moon et al. 2003) and is probably involved in auxin transport (Choi et al. 2005). The observation that a MpMARK interactor is involved in auxin transport further strengthens MpMARK involvement in auxin signalling.



Mock Specific	Best hit Arabidopsis Thaliana
A0A2R6VXF9	No good hit Arabidopsis
A0A2R6X040	Glucose-6-phosphate 1-epimerase
A0A2R6XH11	Mitogen-activated protein kinase (3,4,5,6,10,11,12,13)
A0A2R6XHI4	Phosphoenolpyruvate carboxylase 2
IAA specific	
A0A2R6W7K7	Actin-1
A0A2R6XIP3	Putative U3 small nucleolar ribonucleoprotein
A0A2R6W5K7	Peptidylprolyl isomerase
A0A2R6WKH4	Bifunctional enolase 2/transcriptional activator
A0A2R6W1Q6	Protein LURP-one-related 15
Shared	
MpMARK	PB1 containing kinase
A0A2R6WI90	Acetolactate synthase, chloroplastic
A0A2R6W270	Nucleoside diphosphate kinase 1
A0A2R6W1G8	Uncharacterized protein At2g37660, chloroplastic
A0A2R6XLV6	Acyl carrier protein 1, chloroplastic

Figure 9: MpMARK localises in distinct puncta. (A) Confocal microscopy of young gemmaling show ubiquitous expression of pMpMARK:MpMARK-mCitrine. Within gemma cups MpMARK localizes in distinct puncta closely to the plasma membrane (B). AP-MS/MS analysis on mock and auxin treated 10 day old gemmalings identify MpMPK as an interactor of MpMARK under mock conditions (C). Volcano plots show fold changes (FC, x-axis) and significance (FDR, y-axis). Proteins passing the threshold of FDR 0.05 and specific fold change are marked and have their name displayed. Table shows best hit of Mp proteins with Arabidopsis.

MARK kinases mediate fast auxin phosphoresponse

To identify substrates of MpMARK and AtMARK, we employed phosphoproteomic analysis on MpmarkKO mutant thallus and Atmark mutant root tips, as well as their wild-type controls, both under control conditions and following a 2-minute auxin. To obtain potential substrates of MpMARK and AtMARK, we identified phosphopeptides that were differentially abundant between wild-type and mutant plants. The differentially regulated phosphopeptides only belonging to wildtype (Tak-1 and Col-0) should represent potential substrates (Figure 10A). From this, we made an overlay between the substrate set of Arabidopsis and Marchantia to investigate potential conserved regulation by MARK kinases (Figure 10A). Motif analysis of the potential substrate sets identified a proline directed serine kinase motif, which suggests the involvement of a MARK-dependent MAPK signalling cascade (Figure 10B). Within the conserved substrate set, we identified two MAPKKK's, MAP3K epsilon protein kinase 2 (MAP3KE2) and YODA (YDA) to be potentially regulated by MARK (Supplemental table 1). Strikingly, we also identified the transmembrane kinase receptor TMK1 to be differentially phosphorylated in both species (Figure 10C) (Supplemental table 1). The identified phosphosites fall within the cytoplasmic domain and were only identified upon auxin treatment (Figure 10C). Although the role of these residues in TMK1 regulation are unknown, this result suggests that TMK1 acts downstream of MARK, both in Arabidopsis and in Marchantia. We further identified the nucleoside diphosphate kinase NDPK2 in the potential conserved substrate set (Supplemental table 1). MpNDPK1 was already identified as an interactor of MpMARK. The identification of MARK-dependent NDPK phospho-regulation further strengthens this observation. The identified phospho-regulated site is conserved between Arabidopsis and Marchantia. It was shown in mice that phosphorylation of this site causes enzyme inactivation (Onyenwoke et al. 2012). In both species, the phosphosite site was only found under control conditions (Supplemental table 2). In summary, our analysis on MARK mutants revealed that in both Arabidopsis and Marchantia, MARK kinases mediate a common regulatory pathway in response to auxin.

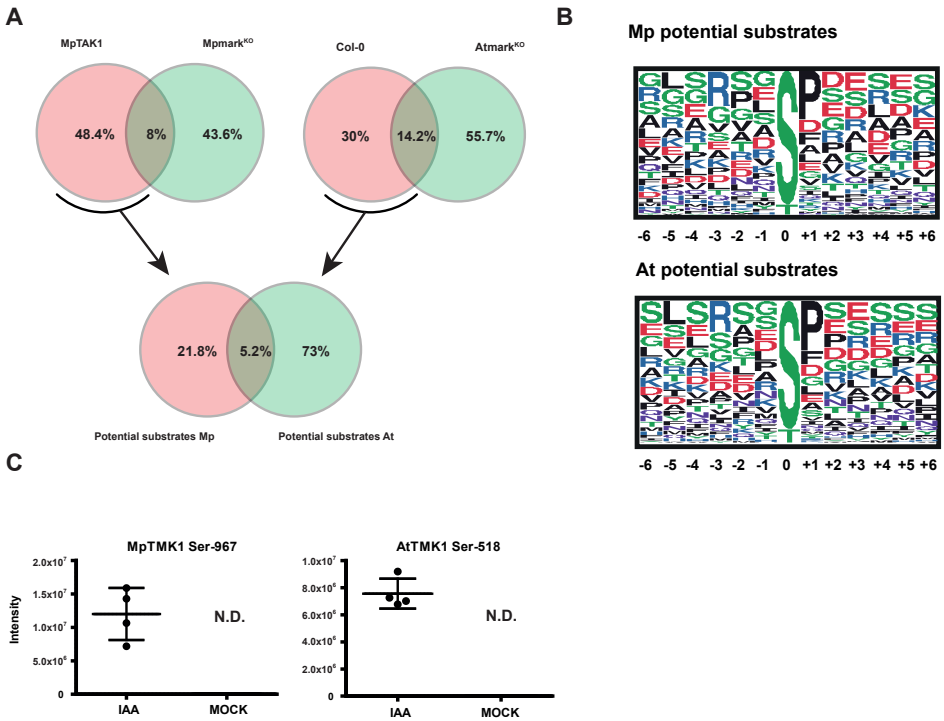


Figure 10: Phosphoproteomic analysis on MARK mutant. (A) Comparisons of differentially regulated phosphopeptides, (FDR≤0.05, or specific to one condition) revealed conserved potential substrates of MARK. (B) Motif analysis on differentially regulated phosphopeptides revealed serine proline directed kinase motif. (C) Phosphopeptides identified belonging to At or MpTMK. Blots show raw peptide intensities. N.D. means not detected

DISCUSSION

The transcriptional response to auxin has been elucidated in significant detail (Weijers and Wagner 2016). However, auxin can also elicit rapid physiological responses, and the mechanisms underlying such fast responses are poorly understood. To shed more light on this regulation, we employed phosphoproteomic analysis on Arabidopsis root tips that had been treated with auxin for as little as 2 minutes. Our analysis revealed that auxin can elicit a rapid change in phosphorylation status of many proteins. This observation suggests a dual mode of auxin action; one through gene regulation and one by rapid auxin-dependent phosphorylation. In the phytohormone, field such a dual mode is not unprecedented. In abscisic acid (ABA) signalling the PYR/PYL/RCAR family regulates both transcriptional responses as well as rapid ABA phosphorylation-

dependent stress responses. Of interest is that the same receptor family mediates both responses. The PYR/PYL/RCAR proteins associate with the PP2C phosphatase and inhibits its activity, thus allowing activation of SnRK2 kinases and phosphorylation of ABF transcription factors (Yoshida, Mogami, and Yamaguchi-Shinozaki 2015). On the other hand, SnRK2 ABA-dependent effects have also been described on stomatal regulation through inhibiting potassium ion influx (Joshi-Saha, Valon, and Leung 2011). In analogy, rapid auxin-dependent root growth responses depend on TIR1 (Fendrych et al. 2018; Dindas et al. 2018) . This suggests that TIR1 action is also dual, triggering both a relatively slow transcriptional response and a fast non-transcriptional response. To investigate TIR1 dependency we employed phosphoproteomic analysis on the recently developed ccvTIR-cvxIAA pair. This analysis showed that phospho-regulation observed with IAA is not dependent on TIR1. This raises the interesting possibility that the phosphorylation response is mechanistically distinct from the response driving fast growth responses. To further dissect TIR1 action in non-transcriptional responses, we preformed AP-MS/MS on TIR1 and AFB1. We observed that RPL24B probably interacts with TIR1/AFB1 in an auxin-dependent manner. Translational control by auxin through TIR1 would be an attractive avenue to investigate, yet such a mechanism probably does not explain the speed by which root growth is modulated by auxin.

We further extended our phosphoproteomic analysis on evolutionary distant green plant species. This led to the surprising observation that all investigated species have a similar rapid phosphorylation-dependent auxin response. As an example, the same PB1-containing MAPKKK (MARK) undergoes differential regulation over all measured species in response to both natural and synthetic auxins. The fact that both 2,4-D and IAA can trigger this response provides information about the flexibility of the auxin binding site on the yet unknown receptor for this response. PB1-dependent signalling has been well established in animal systems leading to activation of, amongst others, MPK signalling cascades (Moscat et al. 2006). Indeed, in phosphoproteomic analysis on mpk mutants, MARK was identified as an indirect substrate (Rayapuram et al. 2018). We further identified in Marchantia that MpMARK interacts with MPK, an interaction that appears to be lost upon auxin treatment. In light of classical MPK signalling, this would suggest that MARK, belonging to the MAPKKK group, activates MPK by phosphorylation. We however did not significantly identify MPK6 activation. In animal cells, the MAPK ERK5, lacking a PB1 domain, requires the PB1 domain of MEK5 for interaction showing that PB1-PB1 interactions are not an absolute requirement for PB1 signalling modules (Nakamura et al. 2006). In our phosphoproteomic screen in Mpmark

and Atmark mutants, we identified other MAPKKK's (MAP3KE2 and YDA) as potential conserved substrates. Perhaps MARK provides a docking station for MPK signalling modules which after auxin perception releases the module to translocate to specific cellular compartments. Such a mechanism would propose MARK to be an allosteric modulator in a way similar to the kinase suppressor of RAS (KSR) in animal systems (Morrison 2001; Langeberg and Scott 2015). MARK, like KSR, contains an N-terminal interaction domain and a C-terminal kinase domain. As a pseudokinase, KSR does not directly phosphorylate substrates, but provides a docking station for scaffolding of signalling modules (Langeberg and Scott 2015). We unfortunately did not identify such interactions in our AP-MS/MS on MpMARK. The specific "puncta" localisation of MpMARK however suggests an assembly of signalling modules as such puncta has been observed in Wnt signalling (Schwarz-Romond et al. 2005).

Other than MPK, we identified NDPK to be a potential substrate and interactor of MARK in both Arabidopsis and Marchantia. In Arabidopsis, NDPK2 may activate MPK (Moon et al. 2003). This further corroborates the existence of a MAPK signalling cascade. It was further shown that NDPK2 is probably involved in auxin transport (Choi et al. 2005). In animal systems, NDPK homologues are important moonlighting kinases involved in cell proliferation and differentiation (Z. Lu and Hunter 2018). NDPK regulates G-protein signalling by providing GTP and histidine phosphotransferase activity (Cuello et al. 2003). It is known that auxin induces rapid GTP loading of ROPs which then activate downstream events such as decreased PIN internalisation (Jones et al. 2014). The identified conserved phosphosite was shown in animal systems to reduce NDPK activity (Onyenwoke et al. 2012). It will be interesting to address if MARK regulates GTP availability through NDPK activity regulation.

Besides MARK and NDPK, we identified multiple other PB1-containing proteins in our datasets. Amongst others the PHOX proteins also showed conserved differential regulation. This group was shown to be involved in Myosin XI-K related processes (Kurth et al. 2017). Of interest is that in an interatomic screen both AtMARK and PHOX2(CLMP1) were retrieved as interaction partner of spindle checkpoint protein MAD2 (Van Leene et al. 2010). This might suggest a PB1-PB1 interaction between MARK and PHOX proteins. Further, in a recent auxin shotgun proteomic screen, a kinase-derived PB1 protein was identified and the mutant showed defects in lateral root formation (Pu et al. 2019). These findings underscore the probable involvement of PB1-containing proteins in auxin signalling other than AuxIAAs and ARFs. How these PB1 proteins are involved in auxin signalling should be subject of future investigations.

Although potential novel auxin signal effector proteins were identified in our phosphoproteomic screen, an unanswered question remains how auxin is perceived in this pathway. Fendrych et.al. (2018) and Dindas et.al. (2018) provided evidence for a role of TIR1 in rapid auxin processes, yet we show rapid phosphorylation changes to be independent of TIR1. In our mark mutant analysis, we did however identify auxin-dependent phosphorylation of TMK1. Together with ABP1, this transmembrane kinase was proposed to act as a non-genomic auxin perception module (Xu et al. 2014). Yet recent insights questioned the biological significance of ABP1. At least in our analysis, ABP1 is perhaps not a likely receptor since we identified MpTMK1 to be differentially phosphorylated while Marchantia does not have a ABP1 ortholog (Kato et al. 2015). This raises the question: Could TMK1 be the sole auxin receptor? Recently it was shown that the kinase domain of TMK1 can phosphorylate non-canocial Aux/IAAs after increased auxin level and cleavage, yet whether TMK1 directly binds auxin was not explored (Cao et al. 2019).

In summary, through phosphoproteomic analysis we revealed that auxin can mediate rapid phosphorylation changes. It appeared that these effects are evolutionarily conserved and that probable PB1-dependent signalling cascades are at the core of these responses. Future research should delineate how exactly PB1 domain-containing proteins are responsible for such rapid auxin signalling.

MATERIAL AND METHODS

Plant material and growth

Arabidopsis seeds were surfaced sterilized and dry seeds were grown on half strength Murashige and Skoog (MS) plates. Plates were vertically grown in a grow chamber at 22◦C in standard long day lighting (16h:8h light:dark). Five days after germination, root tips were treated and directly harvested on liquid nitrogen. For auxin resistance measurements, seedlings were grown vertically for 5 days, transferred to plates containing the indicated concentrations of auxin and grown for additional 2 days before recording growth.

Marchantia polymorpha Tak-1 was used as wild-type in this study. Marchantia plants were grown on half strength Gamborg B5 medium. Physcomitrella patens and Klebsormidum nitens were grown and propagated on BCD medium plates. Plants were grown for 10 days and treated with auxin, harvested and directly frozen in liquid nitrogen. For mutant analysis in Marchantia, gemmalings were grown on half strength Gamborg B5 medium supplemented with or without auxin.

Microscopy and image analysis

Marchantia gemmalings were imaged using a Leica epifluorescence microscope. For confocal imaging, a Leica SP5 was used equipped with an Argon laser. YFP was excited with a 514 nm laser, and emission was collected between 520-550 nm. Images were analysed using ImageJ v1.52a.

Phosphopeptide enrichment

For phosphopeptide enrichment, magnetic $Ti4^{+}$-IMAC (MagResyn) were used as described in chapter 4 and according to manufactures protocol. Enrichments were perforemed in biological quadruplicate.

mCitrine and Venus pulldowns

For pulldowns on Citrine and Venus translational fusions, ground plant material was lysed in mild lysis buffer (50 mM Tris pH 7.5, 150mM NaCl, 2mM MgCl2, 0.2mM EDTA, 1xCPI, 0.5mM DTT, 0.2% NP40 and 1mg/ml DNAse) and mildly sonicated using a Bioruptor (Diagenode). After lysate clearance, supernatant was submitted to enrichment using GFP-Trap agarose beads (Chromotek) for 45 minutes at 4◦C while gently rotating. Beads

were subsequently washed twice in lysis buffer, twice in lysis buffer without detergent and trice in 50mM Ammoniumbicarbonate (ABC)(Sigma) for 2 min at 2000xg at 4◦C. After a final wash, bead-precipitated proteins were alkylated using 50mM Acrylamide (Sigma). Precipitated proteins were next submitted to on-bead trypsin digestion using 0.35µg trypsin (Roche) per reaction. After overnight incubation at 25◦C peptides were desalted and concentrated using C18 Stagetips.

Phylogenetic analysis

Phylogentic analysis on PB1 domain-containing proteins was performed as described in Mutte et.al. 2018 using Arabidopsis thaliana, Physcomitrella patens, Amborella trichopoda, Marchantia polymorpha and Klebsormidium nitens proteomes (Mutte et al. 2018).

Mass spectrometry and data analysis

After stagetip processing, peptides were applied to online nanoLC-MS/MS using a 120 min acetonitrile gradient from 8-50% for phospoproteomics while a 60 min acetonitrile gradient from 8-50% was used for AP measurements. Spectra were recorded on a LTQ-XL mass spectrometer (Thermo Scientific) according to Wendrich et.al. 2017 (Wendrich et al. 2017). For phosphoproteome analysis on mark mutants, a Q-exactive HFX (Thermo Scientific) was used with 60 min acetonitrile gradient from 8-50%. Data analysis of spectra was done in MaxQuant software package according to Wendrich et.al. 2017 (Wendrich et al. 2017) with the addition of phosphorylation as a variable modification. For Marchantia polymorpha, Physcomitrella patens and Klebsromidium nitens, the latest uniprot proteome FASTA files were used. Filtering of phosphodatasets was done in Perseus in a hybrid filtering approach as described by (Nikonorova et al. 2018). Motif enrichment was done on pre-aligned peptide sequences using Motif-X (Schwartz and Gygi 2005; Chou and Schwartz 2011). Data visualization was performed in Adobe Illustrator and R.

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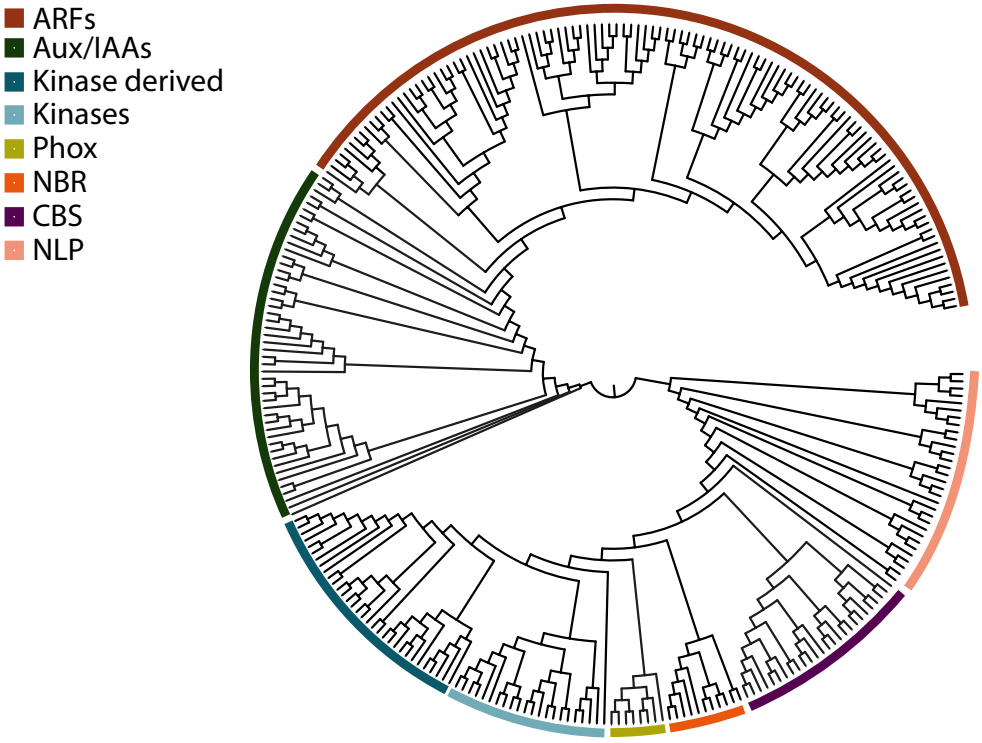
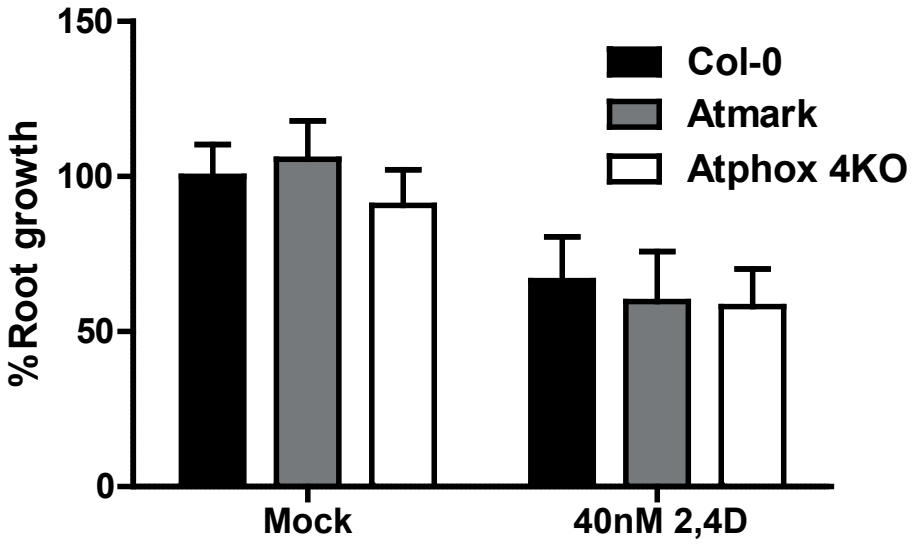
SUPPLEMENTARY INFORMATION



Supplemental figure 1: Phylogenetic tree of PB1-containing proteins in plant species. A clear distinction between the ARF/Aux/IAA clade can be made showing a further subdivision of kinase derived, kinase, Phox, NBR, CBS and NLP clades.



Supplemental figure 2: Auxin root growth assay on Atmark and Atphox 4KO lines reveal no classical auxin dependent root growth inhibition.

Supplemental table 1: Top regulated proteins under IAA conditions

Protein	Gene	Position	Log2(FC)
AT4G25890	RPP3B	106	5,71
AT5G07350	Tudor1	975	5,26
AT5G52400	CYP715A1	477	5,13
AT1G01100	RPP1A	102	5,05
AT2G37340	RS2Z33	219	4,58
AT2G27710	RPP2A	105	4,24
AT3G53420	PIP2-1	280	4,18
AT5G61780	Tudor2	971	4,17
AT5G52400	CYP715A1	482	4,11
AT5G52400	CYP715A1	474	4,11
AT1G70770	F5A18.5	87	4,00
AT1G80180	F18B13.26	105	3,99
AT5G52400	CYP715A1	473	3,98
AT5G25780	TIF3B1	710	3,50
AT1G70770	F5A18.5	112	3,49
AT4G35100	PIP2-7	273	3,48
AT3G01390	VHA-G1	3	3,42
AT3G10530	F18K10.11	22	3,41
AT4G35100	PIP2-7	276	3,40
AT5G63220	At5g63220	84	3,16
AT2G46020	BRM	2056	3,06
AT1G64790	ILA	1872	3,05
AT3G53420	PIP2-1	281	2,98
AT5G14240	F18O22_30	255	2,97
AT1G80180	F18B13.26	98	2,96
AT4G30160	VLN4	806	2,91
AT1G52380	NUP50A	114	2,85
AT3G18480	CASP	589	2,79
AT1G16970	KU70	553	2,79
AT5G39570	At5g39570	357	2,78
AT3G53500	RS2Z32	205	2,73
AT3G53500	RS2Z32	207	2,73
AT3G05760	At3g05760	4	2,60
AT1G68070	T23K23.8	143	2,52
AT1G32130	IWS1	193	2,46
AT2G37340	RS2Z33	211	2,43
AT4G13510	AMT1-1	492	2,42
AT3G49601	At3g49601	398	2,35
AT1G03910	CTN	450	2,34
AT2G25720	At2g25720	50	2,34
AT5G37710	At5g37710	426	2,31

AT5G56000	HSP90-3	219	2,26
AT1G18150	MPK8	539	2,25
AT1G04780	At1g04780	435	2,20
AT5G57370	At5g57370	98	2,20
AT4G30440	GAE1	12	2,18
AT3G53180	At3g53180	404	2,18
AT4G02510	TOC159	589	2,16
AT1G64780	AMT1-2	472	2,16
AT3G06390	CASPL1D2	22	-0,47
AT1G35580	CINV1	11	-0,57
AT3G10660	CPK1	129	-0,86
AT5G63550	At5g63550	15	-0,90
AT5G46570	BSK2	25	-0,91
AT1G61590	T25B24.6	55	-1,14
AT2G45140	PVA12	164	-1,17
AT4G33400	F17M5.160	58	-1,17
AT4G08390	SAPX	236	-1,23
AT1G32400	TOM2A	260	-1,28
AT1G53310	PPC1	11	-1,29
AT1G70180	At1g70180	194	-1,30
AT3G49590	T9C5.180	404	-1,36
AT1G18950	At1g18950	432	-1,47
AT5G17330	GAD1	8	-1,48
AT3G13860	HSP60-like 2	2	-1,49
AT5G61960	ML1	426	-1,53
AT1G27090	At1g27090	4	-1,70
AT5G54430	PHOS32	21	-1,75
AT5G03280	EIN2	924	-2,00
AT3G62900	At3g62900	1314	-2,12
AT1G24764	MAP70.2	309	-2,21
AT5G49890	CLC-C	27	-2,35
AT4G25500	RS40	182	-2,35
AT3G49190	F2K15.50	21	-2,48
AT1G35580	CINV1	70	-5,65
AT3G53710	AGD6	188	-6,04

Supplemental table 2. Table shows conserved potential substrate of MpMARK and At-MARK.

Protein	Gene	Name	Position At	Position Mp	Group At	Group Mp
AT1G31870	F5M6.12	Uncharacterized protein	200	285	MOCK Specific	MOCK Specific
AT1G63700	YDA	Protein kinase superfamily protein	204	386	MOCK Specific	MOCK Specific
AT1G63700	YDA	Protein kinase superfamily protein	204	763	MOCK Specific	MOCK Specific
AT4G01290	At4g01290	Chorismate synthase	89	851	MOCK Specific	MOCK Specific
AT4G34340	TAF8	TBP-associated factor 8	248	246	MOCK Specific	MOCK Specific
AT5G63310	NDPK2	nucleoside diphosphate kinase 2	199	207	MOCK Specific	MOCK Specific
AT2G20190	CLASP	CLIP-associated protein	1116	527	IAA Specific	MOCK Specific
AT2G36620	RPL24B;RPL24A	ribosomal protein L24	84	28	IAA Specific	MOCK Specific
AT3G07980	MAP3KE2	mitogen-activated protein kinase kinase kinase 6	611	953	IAA Specific	MOCK Specific
AT3G07980	MAP3KE2	mitogen-activated protein kinase kinase kinase 6	606	953	IAA Specific	MOCK Specific
AT3G16310	NUP35	mitotic phosphoprotein N\' end (MPPN) family protein	148	103	IAA Specific	MOCK Specific
AT4G03550	CALS12	glucan synthase-like 5	1024	1085	IAA Specific	MOCK Specific
AT5G41950	At5g41950	Protein HLB1	168	509	IAA Specific	MOCK Specific
AT5G41950	At5g41950	Protein HLB1	214	509	1,03	MOCK Specific
AT3G50370	At3g50370	Uncharacterized protein	187	2215	0,97	MOCK Specific
AT4G03550	CALS12	glucan synthase-like 5	1053	1085	0,89	MOCK Specific

AT1G11480	At1g11480	eukaryotic translation initiation factor-related	535	978	0,72	MOCK Specific
AT3G52930	F8J2_100	Aldolase superfamily protein	350	349	0,64	MOCK Specific
AT1G18190	GC2	golgin candidate 2	43	222	0,57	MOCK Specific
AT1G21630	At1g21630	Calcium-binding EF hand family protein	1115	855	0,56	MOCK Specific
AT1G31870	F5M6.12	Uncharacterized protein	204	285	-0,31	MOCK Specific
AT1G12360	KEU	Sec1/munc18-like (SM) proteins superfamily	4	556	-0,69	MOCK Specific
AT3G50370	At3g50370	Uncharacterized protein	1445	2215	-0,94	MOCK Specific
AT2G07360	At2g07360	SH3 domain-containing protein	1125	55	-0,97	MOCK Specific
AT1G21630	At1g21630	Calcium-binding EF hand family protein	1002	855	-1,73	MOCK Specific
AT3G07020	UGT80A2	UDP-Glycosyltransferase superfamily protein	145	64	MOCK Specific	IAA Specific
AT4G17330	G2484-1	G2484-1 protein	760	915	MOCK Specific	IAA Specific
AT4G17890	AGD8	ARF-GAP domain 8	318	365	MOCK Specific	IAA Specific
AT1G59870	ABCG36	ABC-2 and Plant PDR ABC-type transporter family protein	63	887	IAA Specific	IAA Specific
AT1G66150	TMK1	Receptor-like kinase (TMK1)	518	967	IAA Specific	IAA Specific
AT5G57610	At5g57610	MpMARK	220	676	0,62	IAA Specific
AT5G57610	At5g57610	MpMARK	220	779	0,62	IAA Specific
AT1G43690	At1g43690	ubiquitin interaction motif-containing protein	73	40	-1,12	IAA Specific
AT1G43690	At1g43690	ubiquitin interaction motif-containing protein	73	77	-1,12	IAA Specific
AT5G57610	At5g57610	MpMARK	220	461	0,62	5,21

AT1G29400	ML5	MEI2-like protein 5	789	740	0,95	1,52
AT1G67230	CRWN1	little nuclei1	865	1102	0,51	1,28
AT3G50370	At3g50370	Uncharacterized protein	187	528	0,97	1,09
AT3G50370	At3g50370	Uncharacterized protein	1445	528	-0,94	1,09
AT3G50370	At3g50370	Uncharacterized protein	187	909	0,97	1,03
AT3G50370	At3g50370	Uncharacterized protein	1445	909	-0,94	1,03
AT1G32400	TOM2A	tobamovirus multiplication 2A	196	229	0,61	0,94
AT3G01360	T22N4.1	Family of unknown function (DUF716)	294	281	MOCK Specific	0,87
AT5G57610	At5g57610	MpMARK	220	1267	0,62	0,85
AT3G01360	T22N4.1	Family of unknown function (DUF716)	294	313	MOCK Specific	0,57
AT1G65440	GTB1	global transcription factor group B1	1403	142	IAA Specific	0,57
AT4G15802	HSBP	heat shock factor binding protein	6	139	-0,89	0,49
AT5G57610	At5g57610	MpMARK	220	707	0,62	-0,49
AT4G01290	At4g01290	Chorismate synthase	89	245	MOCK Specific	-0,70
AT3G14172	At3g14172	Uncharacterized protein	330	590	0,92	-0,77
AT1G21170	SEC5B	Exocyst complex component SEC5	179	52	1,26	-0,82
AT1G21170	SEC5B	Exocyst complex component SEC5	179	48	1,26	-0,82
AT4G17890	AGD8	ARF-GAP domain 8	318	322	MOCK Specific	-0,83
AT4G14200	At4g14200	Pentatricopeptide repeat (PPR) superfamily protein	688	73	IAA Specific	-0,84

AT1G68020	TPS6	UDP-Glycosyl-transferase / trehalose-phosp-hatase family protein	17	861	IAA Specific	-0,89
AT3G03050	CSLD3	cellulose synt-hase-like D3	755	14	-1,58	-0,91
AT1G59870	ABCG36	ABC-2 and Plant PDR ABC-type transporter fa-mily protein	63	99	IAA Specific	-0,94
AT3G53390	At3g53390	Transducin/ WD40 repeat-like superfamily protein	107	99	0,53	-1,19
AT5G41380	At5g41380	CCT motif family protein	119	150	MOCK Specific	-1,23
AT2G39130	At2g39130	Transmembrane amino acid trans-porter family protein	9	87	MOCK Specific	-1,27
AT2G39130	At2g39130	Transmembrane amino acid trans-porter family protein	6	87	MOCK Specific	-1,27
AT2G39130	At2g39130	Transmembrane amino acid trans-porter family protein	92	87	MOCK Specific	-1,27
AT3G50370	At3g50370	Uncharacterized protein	187	516	0,97	-1,28
AT3G50370	At3g50370	Uncharacterized protein	1445	516	-0,94	-1,28
AT1G48410	AGO1	Stabilizer of iron transporter SufD / Polynucleotidyl transferase	1001	223	-0,82	-1,75

Chapter 6

General discussion and future perspectives

Mark Roosjen

The sessile life of plants requires complex signalling to achieve growth and development in response to changing environmental conditions. Auxin is a phytohormone that controls many aspects of plant development and growth, and is important for developmental plasticity (reviewed in Weijers and Wagner 2016). In the past decades, a prominent auxin signaling pathway has been uncovered. This pathway eventually leads to transcriptional changes. The core effectors of the nuclear auxin pathway (NAP) consist of only three components, the SCFTIR1/AFB auxin receptor complex, the Aux/IAA co-regulators and the ultimate effectors: the auxin response transcription factors (ARFs). With the implication of auxin in diverse growth and developmental processes, it is clear that specificity must be determined at some step within this simple pathway. Although combinatorial interactions between ARFs and Aux/IAAs, different affinities amongst components, and different cellular expression patterns might determine specificity to some extent, much of the functional diversification is likely centred around ARF protein functioning.

Protein-centred ARF research

Chapter 2 provides a detailed overview of ARF function. Although the core components of the NAP have been identified for some time and ARF function has been described in various plant processes, many questions remain. For example, ARFs are generally found in large gene families – with 23 members in Arabidopsis – but are these ARFs indeed all involved in auxin-dependent transcription? Another question relates to the classification within the ARF family. Based on protein sequence homology and transactivation assays, ARFs have been classified as activators or repressors. Yet it is unclear if repressors cannot activate transcription or vice versa. It is than pertinent to ask whether we should look for novel functionalities in different plant processes, or whether we should put our efforts in delineating how ARFs function exactly.

Understanding ARF function requires a protein-centred strategy. Indeed, the resolving the crystal structure of their DBD and PB1 domains has provided the basis for DNA recognition and PB1 mediated homo- hetero-dimerization and oligomerization (Boer et

al. 2014; Korasick et al. 2015; Nanao et al. 2014). Now that the DBD and PB1 domains have been resolved structurally and characterized biochemically, it would be interesting to see how a full length ARF protein behaves in biochemical assays. As discussed in Chapter 2, the middle region of ARFs is predicted to be intrinsically disordered (ID). Such a domain provides an excellent platform for ARF protein regulation by post translational modifications and may attract specific co-factors. At least for one atypical ARF, ARF3, this ID was shown to mediate auxin-dependent ARF3 interaction with INDEHISCENT (IND) (Simonini et al. 2018). That co-factors are important for transcription factor functioning can be, for example, inferred from the Drosphila Hox transcription factors. Co-factor interaction can modulate Hox TF to gain novel DNA binding properties (Slattery et al. 2011).

Thus far, identification of ARF co-factors in specific plant cellular processes has been described in a scattered fashion. A holistic approach as performed by Smazcniak et.al. 2012 on MADS box TFs has not yet been published for ARF proteins. In Chapter 3, we employed unbiased AP-MS/MS approaches to resolve the ARF interactome. Conventional approaches proved to be cumbersome, which is likely caused by weak- or transient interactions. It is possible that most interactions occur through the intrinsically disordered MR. Indeed, interaction between ARF5 with the BRM chromatin remodeller is mediated by the MR (Wu et al. 2015). In our case, no interacting partners were detected using conventional immunoprecipitation strategies. Although protein interactions with intrinsically disordered domains are specific, they are usually transient, which may explain this result. To circumvent this issue, and improve detection of transient interactions, we employed proximity labelling based on BioID. Using this approach, we identified TPL, HDA interactions and ARF hetero-dimerization (ARF5 and ARF2; Chapter 3). This confirms recently published data on ARF2-TPL interaction (Choi, Seo, and Cho 2018). Choi et.al. 2018 showed that through the EAR and RLFGV co-repressor motifs, located within the middle region of ARF2, TPL can bind directly. Many class B ARFs contain either of these two motifs in their middle region, offering an explanation for how they can perform repressive activity without interaction through Aux/IAAs (Vernoux

et al. 2011; Choi, Seo, and Cho 2018). Since class B ARFs have scarce interactions with Aux/IAAs (Vernoux et al. 2011) but heterodimerize with class A ARFs and interact with TPL, it is interesting to ask how auxin can regulate this process. A model where repressive ARFs, such as ARF1 and ARF2, can repress class A activity regardless of auxin would demand constant inactivation of class A activity. Since this is not favourable, ARF-ARF affinities, ARF-DNA affinities for the same binding motifs, and class A: class B stoichiometry within cells or tissue types are probably important determinants. Investigation of such a model is not trivial in Arabidopsis due to redundancies among the ARFs. Indeed, using the simpler Marchantia polymorpha model system, containing only single copies of the ARF classes, it was shown that MpARF class B compete with the MpARF class A in a dose-dependent manner (Kato et.al. under revision 2019).

The current schematic depictions NAP in many auxin reviews and studies (including Chapter 2) should be revised since these only hold true for class A ARF activity. On the other hand, one can ask, as stated before, whether class B and C ARFs should be named auxin response factors, since auxin does apparently not directly control these ARFs through Aux/IAA action.

Non-transcriptional auxin signaling

Although the NAP has been extensively researched, it is interesting to study whether there are other (rapid) auxin-signalling cascades. Especially with the recent observations done by Fendrych et.al. (2018) and Dindas et.al. (2018), it appears that there are distinct auxin-dependent mechanisms at play in controlling either rapid or slow auxin growth responses (Fendrych et al. 2018; Dindas et al. 2018). TIR1 acts in both, and thus has a dual mode. This is similar to absisic acid (ABA) signalling. Within ABA signalling, the PYR/PYL/RCAR receptor family can mediate both rapid, through phosphorylation, and slow, through gene expression, responses (Klingler, Batelli, and Zhu 2010).

Although auxin-mediated phosphorylation-dependent processes have been described for polar auxin transport (Armengot, Marquès-Bueno, and Jaillais 2016) and the modulation of ARFs (Cho et al. 2014), a role in rapid auxin responses has not been

reported. In Chapter 5, we revealed that within 2 minutes after auxin treatment, numerous changes in protein-phosphorylation occur. We further showed that such changes also happen in evolutionarily distant species. Our identified rapid auxin dependent phospho-regulation showed minimal overlap with specific TIR1 activation by using the cvxIAA-ccvTIR1 pair (Uchida et al. 2018). We further showed that in the charophytic green alga Klebsormidium nitens, rapid auxin-dependent phosphorylation signalling occurs. Since this species does not have a copy of TIR1 (Mutte et al. 2018), the observed phosphorylation must be mediated by a different receptor. A potential non-genomic auxin receptor is ABP1, however, in Marchantia polymorpha no copy of ABP1 is present (Kato et al. 2015). Instead, we identified differential phosphorylation of TMK1 in response to auxin in both A. thaliana and M. polymorpha. Could TMK1 then be the sole auxin receptor? Recently, it was shown that auxin promotes the cleavage of TMK1, causing nuclear gene expression through phosphorylating non-canonical IAAs (Cao et al. 2019). It was further shown that auxin induces TMK1 nanoclustering (Pan et al. 2019). Such nanoclusters are important signalling platforms as has been shown in eukaryotic cells. Although this indirectly implicates TMK1 as an important auxin signalling module, future research should show whether TMK1 is responsible for the rapid non-genomic auxin signalling.

Oligomerization-dependent signalling: analogies drawn

The Phox-Bem1 (PB1) domain has a central role in auxin signalling. Within the NAP it mediates ARF-Aux/IAA interactions and possible homo-dimerization and oligomerization (Korasick et al. 2014; Nanao et al. 2014). The PB1 domain is not specific to plants, but is widespread amongst eukaryotic species. In other organisms, the PB1 serves as an important scaffolding domain in diverse cellular signalling processes (Moscat et al. 2006). In Chapter 5, rapid non-canonical auxin signalling was investigated through applying phosphoproteomics. With this analysis we identified an evolutionarily conserved PB1-containing kinase protein (MARK), as well as other PB1-containing proteins possibly involved in auxin signalling. MpMARK showed phase-like separation and was observed in cytoplasmic and membrane-localized puncta in single

cell stages of Marchantia polymorpha.. This observation suggests PB1-dependent clustering of signalling modules. Such clustering allows rapid switch-like behaviour as has been described for Dishevelled (Dvl) in Wnt signalling (Chong and Forman-Kay 2016). Within Wnt signalling the multidomain protein Dvl family are signal transducers which localize to cytoplasmic puncta (Schwarz-Romond et al. 2005). Dvl contains an oligomerization DIX domain which is responsible for puncta formation and mutants without DIX domain cannot activate Wnt signalling pathway (Smalley et al. 2005). Overexpression of Dvl causes formation of puncta and Wnt-independent signalling. The spontaneous and rapid formation of these puncta suggest rapid switching between on and off states of Wnt signalling (Sear 2007). Although we cannot directly link MARK to rapid auxin signalling, a rapid switch-like behaviour would explain the rapid and reversible auxin effects on root growth.

MARK belongs to the B4 group of MAPKKKs ((Kazuya Ichimura et al.) et al. 2002). This classification suggests involvement of MARK in MAPK signalling cascades. Indeed, in phosphoproteomic analysis on mpk6 and mpk3 mutants, MARK was identified to be differentially phosphorylated (Rayapuram et al. 2018). In our interactome analysis on MpMARK, we found the Marchantia polymorpha homolog of Arabidopsis MPKs to interact with MpMARK. This finding suggests a PB1-independent MPK cascade. In animal systems, the extracellular-signal-regulated kinases (ERKs) belonging to the MPK family respond to growth factors. Within these kinase cascades, PB1 domain-containing kinases organize the signalling modules. However, ERK5 lacking a PB1 domain, requires the PB1 domain of MEK5 for interaction. Although this shows that PB1-PB1 interactions are not necessary for signalling modules per se, PB1-PB1 interactions allow the formation of distinct PB1 protein complexes as observed for αPKC, Par-6 and p62 (Moscat et al. 2006). Since we identified numerous other PB1 domain containing proteins in our phosphoproteomics screen, it would be interesting to investigate how these relate to auxin signaling. To date, PB1-dependent signalling cascades, other than the NAP, have not been described in plants. It is imaginable that plants also contain oligomerization dependent processes. Indeed it was recently shown

that polarly localized SOSEKI (SOK) proteins require their DIX-like domain, like Dvl, to localize to specific cellular corners (Yoshida et al. 2019).

Advancing the field of plant proteomics

Throughout this thesis, mass spectrometry-based techniques were used to unravel auxin-dependent processes. In Chapter 3 and Chapter 5, technical integration and optimization is described, showing different possibilities within plant proteomics. In comparison to other proteomics fields, the field of plant proteomics is lagging behind. This is exemplified by frequent use of gel-based shotgun proteomics in numerous studies. Although such techniques can provide insights into plant protein-based processes, newer techniques will provide deeper insight due to better reproducibility and greater proteome coverage. Although there seems to be a slow adaptation towards better techniques, within this section state of the art proteomic principles will be discussed that might even further advance the field of plant proteomics.

Interpreting “omics” data from whole tissue is not trivial. Responses in different cell types or cellular heterogeneity can obscure biologically significant effects. Therefore single cell, or cell type-specific approaches have been developed to be able to detect processes within certain cells or cell types. Within plant proteomics, this can be achieved by using fluorescence activated cell sorting (FACS) (Petricka et al. 2012). This approach is heavily dependent on the successful cell separation based on fluorescent signals. Hence, tagging the cells or organelles would provide a better alternative. Such an approach has been used to study cardiac tissue in Xenopus utilizing INTACT (isolation of nuclei tagged in specific cell types)(Amin et al. 2014). Originally developed for Arabidopsis (Deal and Henikoff 2010), this system uses biotin ligase to specifically biotinylate the nuclear envelope, which allows subsequent enrichment of biotinylated nuclei. In this fashion nuclear proteomes could be resolved. In a similar manner, the recently developed yeast based TurboID and miniTurboID allow protein complex and proteome wide analysis through rapid biotinylation of interacting proteins (Branon et al. 2018). If expressed using cell type-specific promotors, these enzymes could

allow to resolve temporal and time resolved cell specific proteomes. Indeed, TurboID was employed in investigating stomatal development in Arabidopsis (Mair et al. 2019). Here, TurboID was expressed with a nuclear localisation signal using cell type specific promotor (FAMA) and compared to constitutively expressed TurboID, allowing enrichment of guard cell-specific nuclear proteomes (Mair et al. 2019). Through this approach, lowly abundant transcription factors were identified, which demonstrates the possibility to define cell-type specific proteomes using proximity labeling techniques. In another fashion, proteins can be tagged during translation through incorporation of so-called unnatural amino acids (UAAs). These UAAs can have reactive chemical handles, which allows their enrichment though click-chemistry. Through expansion of the genetic code using orthogonal aminoacyl-tRNA synthetase/tRNAxxx (aaRS/tRNA) and specific UAAs, cell type-specific proteomes were enriched in Drosophila (Elliott et al. 2016). This approach was recently extended in mice, wherein specific cells in the mouse brain were investigated (Krogager et al. 2018). In light of phytohormone research, such an approach would allow to extract hormone specific responsive cells when the orthogonal aaRS/tRNA cassette is placed under specific promotors such as DR5 for auxin.

Besides the usage in cell type-specific proteomes, proximity labeling and genetic code expansion have been used in interactomic analysis (this thesis and (A. Smits et al. 2016)). Other than the MS based identification and comprehensive mapping of protein-protein interactions in AP-MS/MS procedures, recent innovations can provide more information about structural topology and protein complex stoichiometry. In AP-MS/MS studies it is usual not trivial to prioritize interactions. Although computer-based methods can predict the most important interactions, stoichiometric insight into protein complexes will provide a more direct measure of importance (A. H. Smits and Vermeulen 2016). To quantitatively determine stoichiometric data from AP-MS/MS procedures, isotopically labeled reference peptides can be used or, in a label free manner, using the intensity based absolute quantification algorithm (IBAQ)(Wepf et al. 2009; A. H. Smits et al. 2013). The latter has been successfully applied in numerous studies, showing for example protein complex composition changes of the PRC2 complex during stem

cell differentiation (Kloet et al. 2016). Within plant proteomics, such approaches have not yet been applied to prioritize interactors or to follow changes in protein complex composition. However, it would allow to monitor protein complex changes and provide insights in protein complex dynamics in the context of development and or hormone response.

Information into stoichiometric abundances of protein complexes, together with topological interaction surfaces provides valuable insight into protein complex structure. Recently, , a technique called in planta quantitative cross-linking coupled mass spectrometry (IPQCX–MS) was developed, in which proteins in Arabidopsis are crosslinked using a holistic approach (Liu et al. 2018). An azide tag within the crosslinker was used to specifically enrich for crosslinked peptides (Liu et al. 2018). Such techniques are promising, yet only work for abundant proteins since these will be the major species in the crude extract. To allow structural topological analysis of specific protein complexes, xIP-MS was developed wherein conventional AP-MS/MS procedures were supplemented with crosslinking approaches (Makowski et al. 2016). A prerequisite for this approach is that interactions should be able to sustain the AP procedure and is therefore limited to stable protein complexes.

In summary, many new avenues can be explored in the field of plant proteomics to further our understanding of plant developmental and phytohormone responses. It is clear that the technological innovations made in the field of proteomics provide an excellent basis for integration into the field of plant proteomics. This is exemplified by the recent integration of TurboID into Arabidopsis (Arora et al. 2019; Kim et al. 2019; Mair et al. 2019). Making proteomics more accessible by developing standardized protocols and providing accessible expertise would aid in progressing plant proteomics and standardizing proteomics as a technique in many studies.

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English summary

The plant hormone auxin profoundly affects many aspects of plant growth and development. Since its discovery, the pathway leading to alteration in gene expression has been well documented. Strikingly, this nuclear auxin pathway (NAP) is short and consist of only three dedicated components, the SCF TIR1/AFB auxin receptor complex, AUX/IAA co-repressors and the auxin transcription factors (ARFs). Due to the simplicity of this pathway, a major question in the auxin field is how specificity is determined. In *Chapter 1*, I provide an introduction of the NAP, other physiological effects elicited by auxin, and how proteomic approaches might shed more light on auxin signalling.

In *Chapter 2*, we provide a deeper insight into ARFs by dissecting and highlighting current views on ARF functioning. Since ARFs are the direct output of the NAP, we reason that the specificity within auxin signalling must be controlled by ARFs. Numerous aspects of ARF functioning may contribute to specificity. This can be deducted from specific cellular expression of ARFs, the promotor architecture where ARFs bind to, the combinatorial interactions amongst the NAP components and the functioning of ARF domains. We highlight that, although the DBD and PB1 domains of ARFs have been structurally and to some extent functionally resolved, the specificity in ARF functioning might reside in the middle region. From predictions, it appears that the middle region is intrinsically disordered. This might provide a signalling hub for ARF functioning. Intrinsically disordered regions have no structure but can provide platforms for co-factor interactions.

That ARF co-factors are important for auxin output has been reported in a scattered fashion and is not clearly documented. We therefore, in *Chapter 3*, investigated which co-factors interact with ARFs. We utilized an unbiased quantitative affinity purification mass spectrometry approach to decipher the ARF interactome. Initial strategies utilizing conventional AP-MS/MS proved to be too cumbersome to retrieve ARF co-factors. We reasoned that the co-factors are probably too transient to survive the affinity purification procedure, and utilized crosslinkers to “freeze” and maintain the interactions during purification procedure. Optimisation of this strategy proved to be too cumbersome to be applied in a holistic ARF interactomic approach. Eventually, we integrated and optimized proximity labelling using BioID. This strategy tags neighbouring proteins with a biotin group within the cell allowing to capture “interacting” proteins of ARFs. Through this approach, we identified ARF-ARF and ARF-TPL interactions.

Besides the regulation of the NAP by auxin other non-NAP effects have been described. Auxin can for example elicit rapid membrane depolarisation and Ca^{2+} spiking. Other reports also implicated kinase cascades in auxin dependent processes. To dissect this, we employed (phospho)proteomic techniques. It can be generally noted that the field

of plant proteomics is not as advanced as the field of animal proteomics. Therefore in *Chapter 4*, we first integrated and optimized sample preparation techniques for shotgun and phosphoproteomic techniques in plant. We interrogated single-vessel-based approaches and offline stagetip-based peptide fractionation techniques to gain deeper and reproducible proteomes. Our results show that simple and cost-effective strategies can be employed to generate high-quality proteome coverage for plant research. Further efforts were undertaken to compare phosphopeptide enrichment strategies. This revealed that metal based phosphopeptide methods outperform metal-oxide based methods.

In *Chapter 5*, we employed the optimized phosphopeptide enrichment procedure to dissect whether fast auxin response is mediated by phosphorylation dependent processes. Our results show that auxin can elicit rapid phosphorylation changes within 2 minutes and that these responses are TIR1-independent. Physiological responses to auxin are widespread in the plant kingdom, and can also be detected in algae that lack NAP components. We therefore further asked if the fast phosphorylation response may be part of a more ancient auxin response system. Our analysis revealed a deep evolutionarily conserved auxin response, and identified a PB1 domain-containing MAPKKK to mediate auxin-dependent regulation.

Eventually, in *Chapter 6* we conclude this thesis and discuss the implications and context of our results. We further provide perspectives for future plant proteomic studies.

At first I would like to thank everyone that has helped me during the duration of my PhD. Although many people write 2-3 pages of acknowledgement I will keep it short.

I would like to thank my supervisor Prof. Dr. Dolf Weijers. Thanks Dolf for your support, discussions and time. I think already early on in my PhD we discovered that an academic career is not well suited for me. Fortunately you gave me the opportunity to continue as a technician in the group. I would like to thank you even more for that.

I would further like to thank Willy van den Berg and Simon Lindhoud for our fun times in purifying a lot of proteins that eventually/unfortunately didn't end up in this thesis.

Also Sjef Boeren thank you for all your help and guidance in proteomics. Your help has been pivotal for many parts of this thesis.

I would further like to thank all members of the Biochemistry department. Especially Laura van Egmond who is always helpful with all the matters of bureaucracy.

To my paranymphs Simon Lindhoud and Jan Willem Borst, thank you for putting yourself on display.

A further thank you to all the students, Lizzie, Tom, Monique, Thomas and Isabel for your help with the research.

Also a big thank you to my family, especially my father and mother, who have been always supportive throughout my educations.

At last a big thank you to my wife and daughter for your love and support.

Mark Roosjen was born on the 7th of June 1991 in Leeuwarden, The Netherlands. He went to High School in Leeuwarden, where he graduated at Piter Jelles Aldlan. In 2008 he started a bachelor in Law soon discovering that he didn't agree with many rules. He therefore went to study forensic sciences and eventually switched to a bachelor in biotechnology. He finished his bachelor with a thesis project of a year focusing on β-hemoglobinopathies at the Murdoch Children's Research Institute in Melbourne, Australia supervised by dr. Jim Vadolas. He further pursued his studies with a MSc. in biological chemistry at the Wageningen University. He performed his master thesis in the lab of Prof.dr. Michiel Vermeulen and Prof.dr. Jan van Hest developing a tagless protein enrichment procedure for quantitative mass spectrometry. Intrigued by mass spectrometry he went to conduct a PhD in the lab of Prof.dr. Dolf Weijers from which the results are presented in this thesis. Mark will continue as a technician in the lab of Prof.dr. Dolf Weijers focusing on proteomics and polarity proteins.

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The research presented in this thesis was performed at the Laboratory of Biochemistry, Wageningen University, and was financially supported by a VICI grant from the Netherlands Organization for Scientific Research (NWO; 865.14.001).

Cover design: “Vissersjongen” Harmen Roosjen 22-03-1940 and Mark Roosjen
Layout design: Tatyana Radoeva and Mark Roosjen
Printed by: GVO drukkers & vormgevers, Ede (NL) || gvo.nl

Propositions

1. A deeply conserved, rapid phosphorylation-based auxin response mediates plant growth.
   (this thesis)

2. Combinatorial approaches have to be employed to excavate the protein interactome, because conventional quantitative AP-MS/MS approaches only scratch its surface.
   (this thesis)

3. With nature in sharp decline, scientific research must focus on the prevention of food and water shortages.

4. In order to provide effective teaching for graduate students, graduate schools should employ personal teaching plans that are tailored to the needs of the student.

5. Graphic design must be integrated in the science curriculum.

6. In light of sustainability, governmental funds should be made available to convert fuel-based cars into electric vehicles.

7. Universities around the world should issue uniform diplomas for better and quicker integration of refugees in society.

Propositions belonging to the thesis, entitled
" Proteomics perspective on auxin biology"

Mark Roosjen
Wageningen, 30 March 2020