

# Trends in Plant Science

## Plant RNA Interactome Capture: Revealing the Plant RBPome

--Manuscript Draft--

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Abstract:	The application of RNA interactome capture to plants has enabled comprehensive determination of the plant RNA-binding proteome and identified novel families of RNA-binding proteins (RBPs). The technique is providing insight into the evolution of the eukaryotic repertoire of RBPs and will enhance prospects for engineering RBPs to improve crop traits.



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Dear Susanne,

Thank you very much for your constructive comments. We have tried to address all the comments made by you and the referees in the revised manuscript and the changes are detailed in the response to referees document submitted with the revised manuscript. We hope that these changes will satisfactorily address the referee's comments and look forward to hearing from you in due course. Thank you for your assistance,

Prof. Gail M Preston

Dear Susanne,

Thank you very much to you and the referees for your constructive comments. We have tried to address all the comments made in the revised manuscript and the specific changes made are detailed in the text below. We hope that the changes made will satisfactorily address these comments and look forward to hearing from you in due course. Thank you for your assistance.

### **Editorial comments**

1. Add a little historical perspective. I appreciate that it would be best to add at least a couple of relevant references when referring to seminal technology papers, but please see my next point regarding the number of references. You could however consider deleting some of the newer references that will now also be covered by Köster et al.

**This is an excellent point, which we have addressed in the beginning of the Spotlight. We have introduced a short historical section where we explain how techniques to identify RNA-binding proteins have evolved over time, from the 1950's to the present. We have also included citations for Pramanik et al, where they use UV cross-linking and chromatography on oligo(dT)-cellulose columns to study RNPs in alfalfa embryogenesis, and Singh et al, where they provide a historical perspective on the discovery of different families of RBPs.**

2. Just to add, that I disagree with the reviewer's point to delete panel B from the figure. Keeping our broad audience in mind, I think this panel is helpful. Again keeping our broad audience in mind, I see your spotlight as the nice broad introduction to a complex topic before pointing the reader to the more indepth coverage by Koester et al.

**We fully agree with this point of view and panel B has been retained in the final figure.**

3. Point the reader to the related review article for further reading (I have attached a copy for your perusal). Unfortunately you cannot just say "see review by Köster et al in the same issue", because your article will be published online well ahead of the printed issue and this would be confusing. Therefore the review will have to be added to the citation list as Köster, T. et al (2016) RNA-binding proteins revisited – the emerging arabidopsis mRNA interactome. Trends Plant Science 22. This brings the references up to the total of 12. So for any new reference to add, you would have to delete an existing one.

**We have highlighted the related review to the reader by adding the reference of Köster. et al/ at the end of the section 'RNA interactome capture' and also in the section 'RNA interactome capture reveals both known and novel proteins in the plant RBPome'. The revised article now includes 13 references, which is the limit you specified in the email (12 references as in any Spotlight + 1 additional reference to address the reviewer's comment).**

### **Referee Comments**

Reviewer #1: The authors have written an interesting short review that rightly brings attention to techniques that are being applied to gain a better understanding of the vital role that RNA-binding proteins play in plant physiology. As such it is both timely and of undoubted interest to the readers of TPS. However, I feel that it would certainly benefit from some improvements, as follows.

1. The opening sentence could wrongly imply that the systematic study of RBPs is a recent development, when in fact it goes back several decades. Indeed the components of the 'RNA interactome capture' technique also go back decades; for instance combining UV crosslinking with oligo(dT) chromatography is by no means a recent invention. For example, you could quote the 1996 study by Pramanik and Bewley, J Exp Bot 45, 1871, in which they used oligo(dT) chromatography to analyse mRNPs involved in alfalfa embryogenesis. They were not looking at the function of 'single or small subsets of RBPs' but rather were attempting to look at mRNP systematically. The same point applies to studies of animal mRNPs where the systematic analysis of RBPs associated with mRNAs goes back to the early 1990s and 1980s. Even back in the 1970s, scientists were using gradient ultracentrifugation to analyse mRNP complexes; just to quote one example, the Russian scientist Ajotkhozhin and others were using density gradients, gaining some early insights into the composition of plant mRNPs. In the context of animal cells, the discovery and indeed naming of the group of proteins called 'hnRNPs' was also the result of a systematic study of RNA-associated proteins. So my point is that the review lacks a certain historical perspective. A section could therefore be added I feel that places the 'RNA interactome capture' technique in proper context, and at least some of the early studies should be quoted and acknowledged.

**Discussed in the answer to the Editor comment 1.**

2. As well as providing detail in relation to the technique, it would be beneficial to provide a critical analysis of the technique. Like all techniques it has its limitations. For example, it is clear that not all RNA-binding proteins are efficiently UV-crosslinked. Additionally, some RBPs interact with mRNAs in a very transient manner and might therefore be under-represented. Oligo(dT) capture itself has its problems; non-specific binding can occur, and some mRNPs have no, or very short poly(A) tails.

**This is a good point. Therefore, we have included a small paragraph about the limitations of RNA interactome capture at the end of the 'RNA interactome capture section'.**

3. Lines 67-68 The concept of 'enigmRBPs' is intriguing and could perhaps be discussed in more detail? it might also be worth quoting animal studies that also show the association of metabolic enzymes with mRNAs. For example there is a lot of evidence that GAPDH binds mRNAs.

**Due to word count limitation, we cannot develop this point much further. However, we have added a couple of examples of enigmRBPs described in both yeast and humans.**

4. Most proteins, including RBPs, are multifunctional, indeed several bind DNA as well as RNA. Others might be involved in multiple posttranscriptional processes; famously the oncogenic mammalian splice factor SRSF1 is involved in both splicing

and translation. Similar layers of functional complexity occur in the plant context; it might be worth discussing this point.

**We agree that this is an extremely interesting point. However, due to word count limits, further discussion of this is beyond this Spotlight.**

5. Lines 90-92 the idea that RBPs could be of interest to plant breeders to increase yield or resistance to stress is not a novel concept; this has been proposed in many papers and review articles which should be quoted, not just reference 9, but also e.g. Ciuzan et al 2015, *Physiol Plant.* 153:1-11; Lorkovic 2009 *Trends Plant Sci.* 14:229-36 etc.

**We agree that the idea that RBPs could be of interest to plant breeders to increase yield or resistance to stress is not a novel concept. However, since RNA interactome capture has enabled the discovery of many (putative) novel plant RBPs, these could be manipulated to increase yield or resistance to stress. Thus, what is novel here is the scope of RBPs that can be manipulated for crop improvement. Unfortunately, due to reference number limitation we cannot cite more papers in which this strategy is proposed. We have slightly amended the wording of this section to make it clearer that the idea that RBPs are of interest to plant breeders is not a novel idea.**

6. Line 94. Modification of RNA-binding activity (reference 10) - could details be provided here of the type of modification.

**Again, this is an interesting point that we would like to address. Unfortunately, because of word count limitation and because this is not the main focus of the Spotlight, we cannot address it further.**

7. Figure 1 panel B; I am not persuaded that panel B really adds much useful information.

**As discussed in the answer to the Editor comment 3, we would like to keep panel B for the broad audience.**

Reviewer #2: This is timely and well written manuscript on important topics in RNA biology. I have minor comments which need to be fixed before final acceptance.

1. On page 2 references for that data describing RBPome in listed organisms should be included

**That is a very good point. Unfortunately, due to the limited number of references (12 references in the whole Spotlight + 1 extra reference), we cannot include the citation of every paper where they apply interactome capture to the listed organisms.**

2. In figure 1, part A, step 7 authors included Bioanalyzer as an RNA analysis method. This is of course used, but it does not provide any idea about the composition or function of analysed RNA samples. It is used just for checking quality of RNA samples. Therefore, I suggest to remove this.

**We agree with this point of view, thus we removed Bioanalyser from the figure. Accordingly, we have also removed it from the figure legend.**

### **Other changes**

**1- We found and corrected a mistake in the manuscript. The number of core RBPs identified by the Marondedze *et al*, Reichel *et al* and Zhang *et al* papers is 79 and not 78 as previously written.**

# Plant RNA Interactome Capture: Revealing the Plant RBPome

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**Key words:** RNA-binding protein, RNA-binding domain, RBPome, UV crosslinking

**The application of RNA interactome capture to plants has enabled comprehensive determination of the plant RNA-binding proteome and identified novel families of RNA-binding proteins (RBPs). The technique is providing insight into the evolution of the eukaryotic repertoire of RBPs and will enhance prospects for engineering RBPs to improve crop traits.**

## RNA interactome capture

The study of RNA-protein complexes started in the 1950's with the visualization of transcripts associated with proteins following transcription by RNA polymerase II. Since then, many experimental studies have aimed to characterize ribonucleoprotein (RNP) complexes from diverse organisms using different techniques (see Singh *et al.* [1] for a review) leading to the biochemical characterization of a number of RNA-binding proteins (RBPs). For instance, Pramanik and Bewley used a combination of UV crosslinking and chromatography on oligo(dT)-cellulose columns to study RNPs in alfalfa embryogenesis [2]. Other studies purified RNP complexes using velocity sedimentation, gel filtration or formaldehyde fixation and isopycnic centrifugation.

31 In the recent years, the combination of these techniques with high-throughput  
32 technologies has boosted the discovery of RBPs. Thus, the use of fluorescent RNA  
33 probes coupled to microarrays or immobilized RNA probes coupled to mass  
34 spectrometry has enabled identification of hundreds of RBPs. Computational  
35 analyses have also been used to identify RBPs based on protein homology and  
36 identification of known RNA-binding domains (RBDs). While valuable to catalogue  
37 RBPs harbouring classical RNA-binding domains, these studies failed to identify  
38 unorthodox RBPs that lack similarity with known RBPs. Moreover, none of these  
39 approaches has succeeded in determining the full range of RBPs that are active  
40 under different cellular states.

41  
42 In 2012 Castello *et al.* and Baltz *et al.*, described a technique termed RNA  
43 interactome capture that enabled comprehensive identification of the human RNA-  
44 binding proteome (RBPome) [3–5]. This technique offers the unprecedented  
45 opportunity to study the dynamic behaviour of RBPs under different cellular  
46 conditions since it enables global identification of active RBPs *in vivo*. However,  
47 because RNA interactome capture is based on the pull-down of poly(A) RNAs  
48 crosslinked to RBPs, it possesses some limitations that should be considered. For  
49 instance, some RBPs might be under-represented due to low efficiency of UV  
50 crosslinking, very transient/weak interaction with RNAs, or binding to non-  
51 polyadenylated RNAs (e.g. most of the mature organelle RNAs) rather than poly(A)  
52 RNAs. The application of this method has rapidly expanded to several human cell  
53 lines; and organisms including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*,  
54 *Drosophila melanogaster*, and the parasites *Trypanosoma brucei*, *Leishmania*  
55 *donovani* and *Plasmodium falciparum*. Therefore, RNA interactome capture has  
56 made an important contribution to the RNA field by enabling systematic identification  
57 of RBPomes in different biological systems.

58  
59 Recent studies by Marondedze *et al.* (2016), Reichel *et al.* (2016) and Zhang *et al.*  
60 (2016) [6–8] have provided the first experimental evidence that RNA interactome



capture can be applied to plants (Figure 1), enabling the determination, for the first time, of the plant RBPome [9].

## **RNA interactome capture reveals both known and novel proteins in the plant RBPome**

Unsurprisingly, all three studies identified a number of known RBPs or proteins harbouring known RBDs, thus affirming the robustness of the technique. They also identified *in silico* predicted RBPs, demonstrating that they indeed bind RNA *in vivo*. A subset of the RBPs identified have orthologs in other organisms including yeast, mouse or humans, and thus are likely to represent conserved or core RBPs. These core proteins are normally associated with translation or splicing.

The three studies identified 79 RBPs as the core RBPome active in the different *Arabidopsis* materials tested, including cell suspension cultures and leaves [6]; etiolated seedlings [7], and leaf mesophyll protoplasts [8]. The remaining candidate RBPs could represent developmental, tissue or cell-specific RBPs, although further studies are required to confirm these results [9]. Interestingly, these studies also identified many proteins with no orthologs in other organisms, no known RBDs, or for which RNA-binding activity was unprecedented. These include the photoreceptors phytochrome A and phototropin1, aquaporins, metabolic enzymes with functions in glycolysis and the citric acid cycle, and enzymes involved in cell redox homeostasis and photosynthesis. Some of these proteins could, therefore, represent 'enigmRBPs' exclusive to plants that possess moonlighting functions in RNA regulation or metabolism [10]. Examples of enigmRBPs such as phosphoglycerate kinase and thioredoxin have been described for other organisms including yeast or human cells [10]. Other proteins identified as putative RNA-binding proteins, which are more widely distributed across eukaryotes, include proteins involved in cytoskeleton and membrane transport, which have been proposed to be involved in RNA transport around the cell and from cell-to-cell.

## **Perspectives and future directions**

The large number of plant RBPs discovered through the application of RNA interactome capture reveals the critical role of RBPs in a cellular context and highlights the importance of RBP-mediated regulation for plant development and physiology. Concordant with the results obtained for other organisms, the subsets of active RBPs are expected to vary depending on cell type, tissue, developmental stage or environmental conditions [10,11]. Therefore, application of RNA interactome capture to different tissues and in different conditions will provide new insights into the dynamic behaviour of plant RBPomes and will help uncover the cues that govern RBP activity, fields that currently remain to be explored. Additionally, with further optimization to capture non-poly(A) RNAs, this technique could be used to identify organellar RBPs as well as the RBPomes of plants and symbionts or pathogens functioning during symbiosis and infection, respectively.

Although all three studies to date have focused on *Arabidopsis*, future studies will expand our knowledge of plant RBPs in crop species, potentially identifying RBPs with critical roles in agricultural traits such as yield, disease resistance or drought tolerance. Because RBPs can regulate the expression of several different genes simultaneously and in response to environmental conditions, RBPs could be of particular interest for plant breeders as targets to increase yield or resistance to stresses. Furthermore, RBPs often follow a modular design with different RBDs and auxiliary domains and some studies have successfully engineered RBPs to modify their RNA-binding activity [12]. Thus, by engineering the specificity, affinity or versatility of a single RBP it may be possible to affect the expression of multiple genes simultaneously with minimal genetic modification.

One of the major impacts of the discovery of the plant RBPome comes to light when put into a wider evolutionary context. Until now, only the RBPomes of *Plasmodium*, *Trypanosoma*, *Leishmania*, yeast, worms, flies, mouse and humans have been described, omitting a crucial group of eukaryotic organisms. Hence, the discovery of plant RBPomes opens the possibility of bridging the evolutionary gap and understanding how RBPomes were shaped during evolution and when and how

individual RBPs evolved. Intriguingly, Reichel *et al.* identified potential novel RBDs in plants such as DUF1296 (Domain of Unknown Function 1296). Experimental validation and structural analysis of these novel RBDs will provide new insights into the molecular basis of RNA-protein interactions, and it will be of interest to determine whether these plant-specific RBDs are linked to plant-specific traits.

The adaptation of RNA interactome capture to the model plant *Arabidopsis thaliana* will also encourage further studies in plants involving UV crosslinking approaches such as crosslinking, immunoprecipitation and sequencing (CLIP-seq) and its variants [13], to identify the RNAs bound by known and newly identified RBPs. This will enable comprehensive determination of RBP-RNA networks occurring in plants and their significance for plant growth, development and responses to environmental conditions.

### **Concluding remarks**

In summary, the studies conducted by Marondedze *et al.*, Reichel *et al.* and Zhang *et al.* have provided evidence, for the first time, that RNA interactome capture can be successfully applied to plants. This technique enables system-wide identification of active RBPs and their dynamic regulation in different cell types, tissues, developmental stages and environmental conditions. Additionally, application of RNA interactome capture to plant species represents a promising tool to understand how RBPs and RBPomes were shaped during evolution and a unique opportunity for understanding and engineering RBPs for crop improvement.

### **Acknowledgements**

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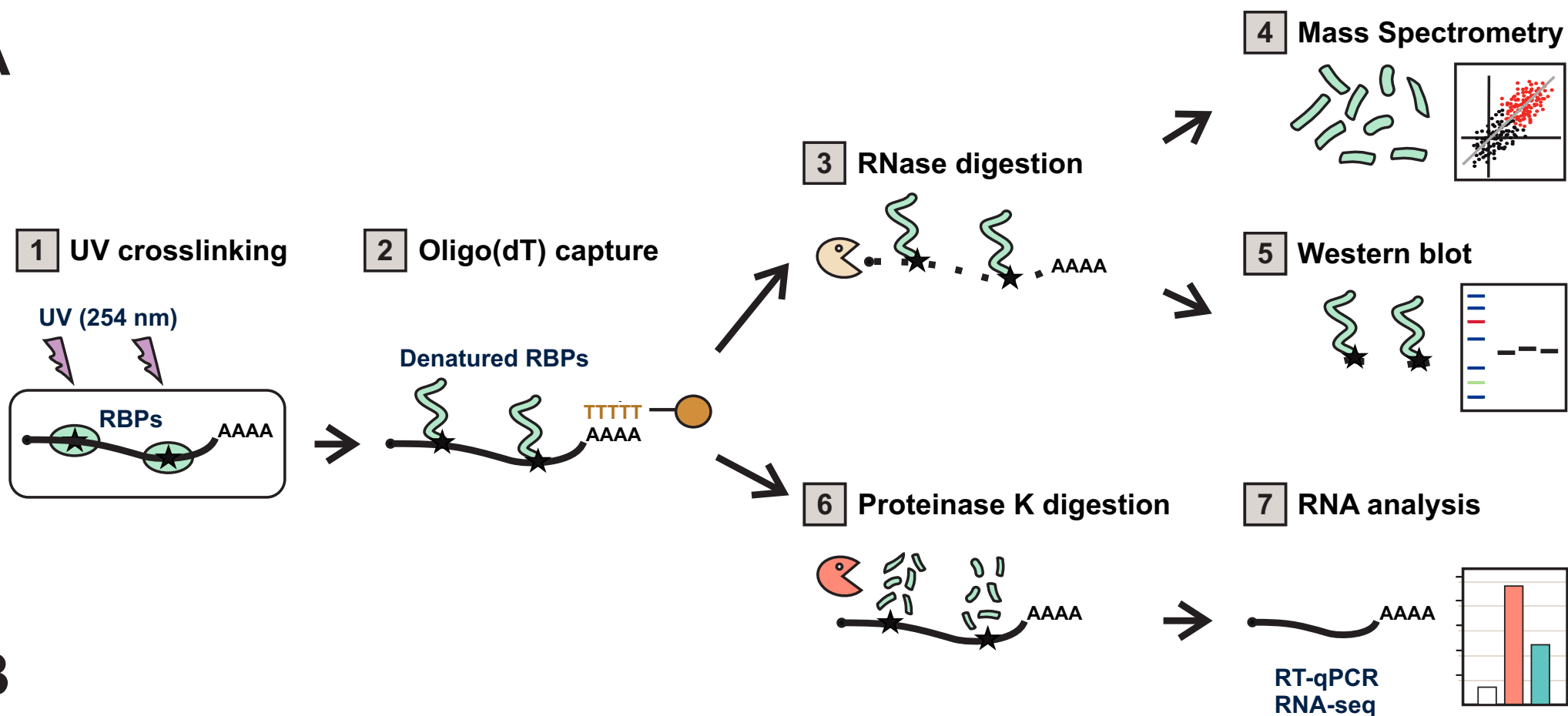
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**Figure 1. Overview of RNA interactome capture.**

- A. Cells are irradiated with UV light at 254 nm to promote crosslinking between RNAs and proteins that are in intimate contact (1). Next, cells are lysed and mRNAs pulled-down using oligo(dT) magnetic beads (2). After stringent washes, the RNA-protein complexes are recovered and can be analysed using different techniques. Firstly, RNA can be enzymatically digested (3) and the proteins quantitatively analysed by Mass Spectrometry (4) or Western blotting (5). Alternatively, the protein fraction can be enzymatically digested (6) and the RNA analysed by RT-qPCR or RNA sequencing (7).
- B. Schematic representation depicting the phylogenetic relationships between the organisms for which RBPomes have been identified by applying RNA interactome capture.

**A**



**B**

