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A YTHDF–PABP interaction is required for m\(^6\)A-mediated organogenesis in plants

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Abstract

N\(^6\)-methyladenosine (m\(^6\)A) in mRNA is key to eukaryotic gene regulation. Many m\(^6\)A functions involve RNA-binding proteins that recognize m\(^6\)A via a YTS21-B Homology (YTH) domain. YTH domain proteins contain long intrinsically disordered regions (IDRs) that may mediate phase separation and interaction with protein partners, but whose precise biochemical functions remain largely unknown. The Arabidopsis thaliana YTH domain proteins ECT2, ECT3, and ECT4 accelerate organogenesis through stimulation of cell division in organ primordia. Here, we use ECT2 to reveal molecular underpinnings of this function. We show that stimulation of leaf formation requires the long N-terminal IDR, and we identify two short IDR elements required for ECT2-mediated organogenesis. Of these two, a 19-amino acid region containing a tyrosine-rich motif conserved in both plant and metazoan YTHDF proteins is necessary for binding to the major cytoplasmic poly(A)-binding proteins PAB2, PAB4, and PAB8. Remarkably, overexpression of PAB4 in leaf primordia partially rescues the delayed leaf formation in ect2 ect3 ect4 mutants, suggesting that the ECT2-PAB2/4/8 interaction on target mRNAs of organogenesis-related genes may overcome limiting PAB concentrations in primordial cells.

Keywords Arabidopsis; ECT2; IDR; m\(^6\)A; PABP

Subject Categories Development; Plant Biology; RNA Biology

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Introduction

Methylation of internal adenosine in pre-mRNA to form N\(^6\)-methyladenosine (m\(^6\)A) is a key regulatory step in eukaryotic mRNA biogenesis and functionality. In both plants and animals, it may dictate distinct processing steps in the nucleus (Zheng et al., 2013; Haussmann et al., 2016; Lence et al., 2016; Roundtree et al., 2017; Tang et al., 2018; Pontier et al., 2019; Parker et al., 2020; Wei et al., 2021) and influence properties such as mRNA half-life and translatability in the cytoplasm (Sommer et al., 1978; Wang et al., 2014, 2015; Meyer et al., 2015; Herzog et al., 2017; Ke et al., 2017; Weng et al., 2018; Zaccara & Jaffrey, 2020). The principal readers of m\(^6\)A contain a YTS21-B homology (YTH) domain (Dominissini et al., 2012; Wang et al., 2014, 2015; Patil et al., 2018) that recognizes m\(^6\)A through a highly conserved aromatic cage (Luo & Tong, 2014; Theler et al., 2014; Xu et al., 2014, 2015; Zhu et al., 2014; Li et al., 2014b). YTH domains fall into two main phylogenetic groups, DC and DF (Patil et al., 2018). YTHDC proteins are often nuclear (Nayler et al., 2000; Zhang et al., 2010) and typically contain long intrinsically disordered regions (IDRs) in addition to the YTHDC domain (Patil et al., 2018). In some cases, YTHDC domains are part of multidomain proteins, such as the long isoform of the plant Cleavage and Polyadenylation Specificity Factor 30 (CPSF30) implicated in selection of poly(A) sites (Pontier et al., 2019; Hou et al., 2021; Song et al., 2021). YTHDF proteins are typically cytoplasmic (Zhang et al., 2010; Wang et al., 2014; Arribas-Hernández et al., 2018, 2021b; Kan et al., 2021; Worpenberg et al., 2021) and have a YTH domain placed at the C-terminus after an IDR of variable length (Meyer & Jaffrey, 2017). Plant YTHDF proteins are, for this reason, referred to as EVOLUTIONARILY CONSERVED C-TERMINAL REGION (ECT) (Ok et al., 2005), and higher plants encode an expanded family of YTHDF proteins with 11 in Arabidopsis thaliana (arabidopsis) compared to 3 in mammals, and 1 in Drosophila and yeast (Li et al., 2014a; Fray & Simpson, 2015).

In arabidopsis, the YTHDF proteins ECT2, ECT3, and ECT4 are specifically expressed in rapidly dividing primed stem cells in organ primordia (Arribas-Hernández et al., 2018; Arribas-Hernandez et al., 2020). ECT2-4 are necessary for the rapid division of these cells, such that organogenesis is delayed due to slow growth in ect2 ect3-1 ect4-2 (henceforth abbreviated tc234) mutants (Arribas-Hernández et al., 2018; Arribas-Hernandez et al., 2020). Similar developmental defects are observed in plants with hypomorphic mutations in m\(^6\)A methyltransferase components or in which such components are knocked down during post-embryonic development (Bodi et al., 2012; Shen et al., 2016; Ružička et al., 2017). ECT2 and ECT3 are the main m\(^6\)A effectors in rapidly dividing primordial cells, since ect2 ect3, but no other double mutant examined, exhibits clear delays in organogenesis (Arribas-Hernández et al., 2018; Flores-Téllez et al., 2023; Martínez-Pérez et al., 2023). Accordingly, the involvement of ECT4 in organogenesis is only revealed by exacerbation of the organogenesis defects in ect2 ect3 mutants upon additional inactivation of ECT4 (Arribas-Hernández et al., 2018; Arribas-Hernandez et al., 2020).
ECT2 binds directly to m^6^A deposited in DRACH or GGAU contexts in target mRNAs by the nuclear MTA/MTB adenosine methyltransferase complex (Parker et al., 2020; Arribas-Hernández et al., 2021a), and all described in vivo functions of ECT2 and ECT3 depend fully on intact m^6^A-binding pockets (Arribas-Hernández et al., 2018; Arribas-Hernandez et al., 2020; Martinez-Pérez et al., 2023). The genetic redundancy between ECT2 and ECT3 is reflected in their strongly overlapping sets of targets, for which they compete to bind in vivo (Arribas-Hernández et al., 2021b). In the absence of ECT2, ECT3, and ECT4, target mRNAs of ECT2/3 tend to be downregulated (Arribas-Hernández et al., 2021b), perhaps because of more rapid mRNA degradation, although indirect effects acting at the level of target mRNA transcription cannot be excluded at this point. Consistent with a stabilizing effect of ECT2/3 binding to m^6^A, increased mRNA degradation rates upon global inhibition of transcription have been observed for a few m^6^A targets in plants with post-embryonic methyltransferase knockdown (Anderson et al., 2018). Because sequence signatures consistent with endonucleolytic cleavage were detected in these transcripts (Anderson et al., 2018), it is possible that ECT2/3 binding to m^6^A in target mRNA exerts merely a passive, protective role via m^6^A-binding mediated by the YTH-domain. At the extreme, such a model would be compatible with ECT proteins relying nearly exclusively on their YTH domains for extreme, such a model would be compatible with ECT proteins relying nearly exclusively on their YTH domains for in vivo function, with the IDRs perhaps mainly there to drive sequestration into distinct biophysical phases in response to stress, as shown previously to be the case (Arribas-Hernández et al., 2018; Scute-naire et al., 2018). Several lines of evidence suggest, however, that the IDR plays a more active and specific role in the mechanism underlying m^6^A function. First, individual nucleotide resolution cross-linking-immunoprecipitation (iCLIP) experiments with ECT2 recovered sequence tags attributable to the IDR, suggesting a direct involvement of the IDR in RNA binding (Arribas-Hernández et al., 2021a). Second, systematic tests of the ability of arabidopsis ECT paralogs to substitute for ECT2-4 upon ectopic expression in leaf primordia identified three paralogs (ECT1, ECT9, and ECT11) with divergent function, and showed that the IDRs of these proteins are major contributors to the functional divergence (Flores-Téllez et al., 2023). It is an obvious possibility that the IDR mediates contacts to proteins relevant for mRNA translation and stability. Precedents for such a model come from studies of human YTHDF2 in which different regions of the N-terminal IDR interact with (i) the CNOT1 subunit of the CCR4-NOT complex (Du et al., 2016), (ii) the adaptor protein HRSP12, which acts as a bridge between YTHDF2 and the RNase P/RNR complex (Park et al., 2019) and (iii) the nonsense-mediated mRNA decay factor UPF1 (Boo et al., 2022). Thus, if a similar conceptual model for YTHDF function applies to plant ECT proteins, distinct IDR elements crucial for in vivo function may be defined. Here, we use ECT2 to address this basic question regarding YTHDF function in plants. We show that the first 410 amino acid residues (aa) in the N-terminal IDR are indispensable for function in vivo and, surprisingly, that this region is as important for RNA association in vivo as the m^6^A-binding pocket in the YTH domain. We also identify two distinct, ~20–40 aa elements that are required for the molecular functions of ECT2 in plant development. One of these elements has little, if any effect on RNA binding, but is required for association with the major cytoplasmic poly(A) binding proteins PAB2/4/8 in vivo and sufficient for PAB8 interaction in two-hybrid assays. The biological importance of the physical ECT2-PAB8 association is supported by the partial suppression of leaf formation defects in te234 mutants upon overexpression of PAB4 in leaf primordia. These observations support a model in which ECT2/3-PAB2/4/8 interaction on target mRNAs of organogenesis-related genes overcomes limiting concentrations of PABP in rapidly dividing primordial cells.

Results and Discussion

Experimental system for assessment of ECT2 function in vivo

The te234 mutant displays a consistent 2-day delay in emergence of the first true leaves compared to wild type (Arribas-Hernández et al., 2018). This phenotype is easily scored at 9 days after germination (DAG) in which wild type has clearly visible, > 1 mm long true leaves while the first true leaves of te234 are barely visible and always < 0.5 mm in size (Fig 1A, left panel). This clear and fully penetrant phenotype is restored by the transgenic expression of ECT2-mCherry driven by the endogenous ECT2 promoter (Arribas-Hernández et al., 2018), or by the RPS5A/US7Y (At3g11940) promoter (Flores-Téllez et al., 2023) with activity in highly dividing meristematic cells (Weijers et al., 2001; Scarpin et al., 2023). Since te234 mutants are fertile and can be transformed with normal efficiency, the ability to complement the delayed leaf formation of te234 at 9 DAG constitutes a sensitive readout for ECT2 function in vivo. Previously we used this system to show that eight of the eleven arabidopsis YTHDF proteins have molecular functions sufficient to complement the leaf formation defects of te234 mutants when expressed in organ primordia (Flores-Téllez et al., 2023). Thus, we set out to use this robust system to test a series of IDR-deletion mutants in ECT2 to identify elements of functional importance (Fig 1A).

The N-terminal, but not the C-terminal, IDR is required for function

We first designed and cloned mCherry-tagged ECT2 transgenes devoid of either the first 410 amino acids (aa) in the large N-terminal IDR (ECT2^410-4-4-mCherry), or the last 56 aa in the shorter C-terminal IDR (ECT2_C^AC-mCherry) (Fig 1B, Dataset EV1). After transformation of te234 plants, functionality was measured as the complementation frequency in the first transgenic generation compared to the ECT2_C^AC-mCherry construct for which about 60% complementation frequency is routinely obtained (Arribas-Hernández et al., 2018). T-DNA silencing is the main reason that complementation frequencies do not reach 100% with a wild type construct (Flores-Téllez et al., 2023). Expression of the wild type and deletion mutants in te234 showed that ECT2AC was fully functional while ECT2^410 loss function (Fig 1B-D). We further subdivided the 410-aa DN deletion into two ~200-aa deletions, an N-terminal (AN1) and a C-terminal (AN2) half (Fig 1E, Dataset EV1). In both cases, the mutant proteins with only half IDR retained only residual activity (Fig 1F and G). Thus, the N-terminal IDR of ECT2, as well as its N-terminal and C-terminal halves individually, is essential for function in vivo.
The N-terminal IDR is required for full RNA-binding activity of ECT2 in vivo

We next used UV-cross-linking to assess whether the IDR is required for RNA association in vivo. We exposed entire seedlings to UV light, subjected ECT2WT-mCherry or ECT2DN-mCherry to immunoaffinity purification, and visualized the amount of cross-linked, immunoprecipitated (CLIPed) RNA by radiolabeling with polynucleotide kinase (PNK) after DNase treatment (Arribas-Hernández et al., 2021a). We also included the ECT2W464A-mCherry mutant with a lesion in the m6A-binding aromatic cage as a control that exhibits strongly decreased, yet not abolished RNA association in vivo (Arribas-Hernández et al., 2021a). These experiments showed that RNA association was clearly reduced in ECT2DN compared to wild type, to levels similar to those detected with the m6A-binding deficient mutant (Fig 1H). Thus, both IDR and YTH domains are necessary for full RNA-binding activity in vivo. This result has important implications for how specificity in YTHDF-target mRNA interactions may be achieved and is consistent with our previous analysis that uncovered CLIP-seq tags attributable to both YTH-domain and IDR parts of ECT2 (Arribas-Hernández et al., 2021a). We note that the present result does not allow an assessment of whether the requirement of the IDR for RNA association relies on direct RNA contacts, interaction with other RNA binding proteins, or a combination of the two. We also note that the result does not exclude important functions of the N-terminal IDR other than those implicated in RNA association.
Two distinct 30–40 aa regions in the N-terminal IDR, N3.2, and N8 are required for ECT2 function in vivo

Having established that the N-terminal IDR is required for ECT2 function, we next searched for smaller functional elements using a mutational approach. To design such mutants, we first analyzed sequence features of the N-terminal IDR with an eye toward the following two properties: (i) amino acid composition, as this gives clues to biophysical properties such as ability to engage in phase separation (Kato et al., 2012; Vernon et al., 2018) and (ii) conservation among ECT2 orthologs in dicotyledonous plants (Fig EV1A). Based on these features, we designed six smaller deletions in the N-terminal IDR of ECT2 (ΔN3-AN8), each 40–70 aa in size (Fig 2A, Dataset EV1) and assessed their functionality as above. The results showed that despite protein accumulation similar to ECT2WT, ECT2ΔN3, and ECT2ΔN8 gave significantly lower complementation frequencies than wild type (Fig 2B and C). In contrast, no significant reductions in complementation frequency were found for ECT2ΔN3, ECT2ΔN7 (Fig 2B and C), with ECT2ΔN6 even giving rise to slightly higher complementation frequencies than ECT2WT (Fig 2C). We previously verified that ECT2ΔN6 fulfills developmental functions similar to wild type ECT2, but exhibits reduced antiviral activity (Martínez-Pérez et al., 2023), underscoring the fact that our functional complementation assay based on leaf emergence identifies elements required for developmental functions of ECT2, not necessarily all of its functions. We further subdivided the N3 element into two 40-aa segments (N3.1 and N3.2) (Fig 2D and E, Dataset EV1) and found that ECT2ΔN3.2 was as defective as ECT2ΔN3, while ECT2ΔN3.1 retained wild type function (Fig 2F and G). We also combined ΔN3.2 and ΔN8 (Fig EV1B) and observed a largely additive effect (Martínez-Pérez et al., 2023) per type. Ponceau (Pon) staining is used as loading control.

We therefore used CRISPR-Cas9 to engineer an in-frame chromosomal deletion corresponding to ΔN3ΔN8 in the ect3-1/ect4-2 (de34) genetic background (Fig 2D and H). In addition to several out-of-frame deletion alleles conferring a full te234 phenotype in the de34 background, we also recovered an in-frame deletion, ect2-4, matching nearly perfectly the ΔN3ΔN2 deletion used in the transgenic approach (Figs 2D and EV1D). The resulting ect2-4/ect3-1/ect4-2 triple mutants expressed levels of the mutant ECT2-4 protein containing the N3.2 deletion comparable to ECT2WT protein levels in the de34 parental background (Fig 2I). Importantly, all ect2-4/ect3-1/ect4-2 homozygous lines reisolated after backcrossing of a Cas9-free ect2-4 homozygous individual to the de34 parental line exhibited a delay in leaf formation, such that leaf emergence was intermediate between de34 and te234 mutants (Fig 2J and K, Appendix Fig S1), consistent with partial loss of ECT2 function. We conclude that N3.2 is required for full ECT2 function in vivo.

ECT2ΔN3.2 has no obvious defect in RNA binding in vivo

To characterize molecular functions of N3.2, we first tested whether RNA binding in vivo was compromised in the ECT2ΔN3.2 protein using PNK labeling of RNA co-immunoprecipitated with ECT2ΔN3.2, mCherry and ECT2WT-mCherry after UV-crosslinking in vivo. This assay did not show reduced PNK labeling of RNA co-purified with ECT2ΔN3.2 compared to ECT2 wild type. Rather, labeling of...
cross-linked ECT2^{N3.2}-RNA complexes was more intense than of ECT2^{WT}-RNA complexes (Figs 3A and EV2A and B). Because ECT2-RNA complexes with or without the N-terminal IDR generated by proteolysis during immuno-purification are labeled with different efficiency, presumably due to different accessibility of 5' ends as a consequence of IDR-RNA contacts (Arribas-Hernandez et al., 2021a), we think it unwise to interpret this result more deeply than to conclude that ECT2^{D N3.2} retains RNA-binding capacity. We also note that this experiment does not provide insight into the identities of the co-purified mRNAs, and it remains possible that N3.2 is implicated in mRNA target selection. Nonetheless, taken together with the fact that ECT2/3/4 are major mediators of developmental m^A functions (Arribas-Hernandez & Brodersen, 2020), the indication of intact RNA-binding activity of ECT2^{D N3.2} suggests that m^A is unlikely to act only via protection against local endonucleolysis, as proposed previously (Anderson et al., 2018). This indication also led us to focus our attention on properties other than RNA binding to understand the function of N3.2.
**N3.2 is required for full interaction with the cytoplasmic PABPs, PAB2/4/8**

We next tested the possible importance of N3.2 in protein–protein interaction. We conducted immunoprecipitation-mass spectrometry (IP-MS) analyses of RNase-treated lysates prepared from plants expressing mCherry fusions of either ECT2\textsuperscript{WT}, ECT2\textsuperscript{DN}, ECT2\textsuperscript{DN3}, or ECT2\textsuperscript{DN3,2} (Appendix Fig S2). These analyses showed that while the full N-terminal IDR was required for association with almost a hundred proteins in vivo (Fig 3B and Dataset EV2), only 16 and 12
proteins showed significant depletion in ECT2AN3 and ECT2AN3.2 purifications, respectively, compared to ECT2WT. 9 of these proteins were common between the three datasets (Fig 3C-E and Dataset EV3). To further validate and narrow the number of interactors of possible functional significance, we completed four additional IP-MS analyses using hemagglutinin (HA)-tagged ECT2, Venus-tagged ECT3, and FLAG-TFP tagged ECT1 (Arribas-Hernández et al, 2018; Flores-Téllez et al, 2023) (Fig 3F-H, Appendix Fig S2, and Dataset EV2). Because ECT2 and ECT3 exhibit genetic redundancy in plant development (Arribas-Hernández et al, 2018; Arribas-Hernández et al, 2020), we reasoned that interactors necessary for ECT2 activity must co-purify with both proteins. On the other hand, ECT1 does not have the developmental function of ECT2/3 despite its strong similarity to ECT3 (55% amino acid identity) (Flores-Téllez et al, 2023). Therefore, such interactors may be absent from ECT1 purifications. The comparison between the proteins differentially enriched in each purification relative to mock IPs from non-transgenic plants showed that 20 candidates had a significant enrichment in HA-ECT2WT and ECT3-Venus fractions, and that 5 of them were not found in ECT1 pulldowns (Fig 3I and Dataset EV3). Remarkably, 3 of these proteins, the poly(A) binding proteins PAB2, PAB4, and PAB8, were also among the 9 N3.2-dependent interactors (Fig 3J and K) and are, therefore, outstanding candidates for functionally important interactors. Poly(A)-binding proteins (PABPs) associate with the poly(A) tail of mRNA to effect a multitude of functions related to mRNA translation and PAB2, PAB4, and PAB8 are the three most highly and broadly expressed paralogs among the eight cytoplasmic PABPs in arabidopsis (Belostotsky, 2003; Goss & Kleiman, 2013). Thus, we proceeded to characterize ECT2-PABP interactions in more detail.

**ECT2 function and PABP interaction rely on a small Tyr-rich region**

To reinforce the link between ECT2 function and PABP interaction, we further subdivided AN3.2 into two halves (Fig 4A, Dataset EV1) and used these smaller deletion mutants in the te234 complementation assay (Fig 1A). This effort narrowed the functionally important part to the region defined by AN3.2.2, a 19-amino acid region containing six tyrosine residues (Fig 4A and B). These six tyrosine residues are required for function because their mutation into alanine (ECT2Tyr→Ala) resulted in loss of ECT2 function comparable to that caused by the ECT2AN3 deletion mutant, despite protein accumulation comparable to ECT2WT (Fig 4C and D, Dataset EV1). We finally refined the refined ECT2AN3.2.2 mutant for IP-MS analysis that also showed reduced interaction with PAB2, PAB4, and PAB8 in

**Figure 4.** The Tyr-rich region in N3 is a deeply conserved YTHDF element required for ECT2 function and PABP binding.

A Graphical representations of the partition of AN3.2 into AN3.2.1 and AN3.2.2, of the mutations in ECT2Tyr→A, and of the –N12cct2 fragment used in (G). Corresponding protein sequences are listed in Dataset EV1. B Complementation assay of AN3.2.1 and AN3.2.2 ECT2 deletion mutants as in Fig 1C. Red lines with asterisks indicate significant differences according to pairwise Fisher exact test with Holm-adjusted P-values (**< 0.05, ***< 0.01, ****< 0.001). Black lines indicate no significant difference. C Complementation assay of ECT2Tyr→A as in (B). D Western blot analysis probed med mCherry antibodies (x-mCh) to show expression levels of wild-type ECT2 protein compared to ECT2Tyr→A protein in three independent lines (L1–L3) in the te234 background. te234 plants were used as a control. Ponceau staining is shown as loading control. E IP-MS analysis of the ECT2-interacting proteins lost upon deletion of N3.2.2 compared to ECT2WT as in Fig 3E–D. Statistical significance was calculated using empirical Bayes statistics with Benjamin–Hochberg adjusted P-values. The experiment was done once using biological triplicates. F GST retention assay. Lysates from plants expressing ECT2WT-mCherry and ECT2AN3.2-mCherry in the te234 background (input) were incubated with immobilized GST-PAB2 or GST protein. Lysate from nontransgenic plants was used as control. Retained proteins were eluted using glutathione and analyzed by western blot. Ponceau (Pon) staining is used as loading control for the input and to visualize GST-PAB2 and GST proteins in eluates. G In vitro binding assay with purified components. A 230-aa N-terminal fragment of ECT2 (~N12cct2) that includes the N3.2 region was incubated with immobilized GST-PAB2 or GST at a concentration of 10 or 25 μM, respectively. Retained proteins were eluted using glutathione and analyzed by SDS-PAGE and Coomassie staining. Bands running at 70 and 55 kDa in the purified GST-PAB2 fraction likely correspond to proteolytic fragments that were not fully separated from the 100 kDa fusion protein by gel filtration. H Principle of the bacterial two-hybrid system that uses the T25 and T38 fragments of Bordetella pertussis adenylate cyclase (CyaA), which reconstitute an active adenylate cyclase when fused to interacting polypeptides, resulting in cAMP production. The CAMP-CAP complex then facilitates induction of genes required for non-glucose sugar fermentation (e.g., the mal, xyl, or rbs operons). Inspired by the illustration in the original report on the CyaA reconstitution-based two-hybrid system (Karimova et al, 1998).

I Biological two-hybrid analysis of the PAB8-N3cct2 interaction. The pT25-PAB/pT38-N3.2cct2 plasmid combination, three different empty plasmid negative control combinations, and a positive control combination using T25/T38 fusions to the GCN4 homodimerization coiled-coil domain (GCN4Δ25), were transformed into the cyaa E. coli B7H101 strain and plated on MacConkey/maltose plates. Primary transformants of the different plasmid combinations were streaked on MacConkey/sugar plates as shown and grown at 31°C for 4 days with photographs recorded every 10 min. Incubation times until the pictures shown were glucose, 11 h; xylose, 52 h; ribose, 83 h; maltose, 41 h. Red colonies indicate sugar fermentation. Glucose serves as a control for non-cAMP-dependent fermentation. The experiment was repeated three times for ribose, xylose, and glucose and five times for maltose.

J Section of the full amino acid sequence alignment of YTHDF proteins from 24 species of land plants or 10 species of animals (Datasets EV5 and EV6, and a simplified version in Fig EV4), showing the N3.2.2 region within the 11 arabidopsis ECTs. Blue, ECT proteins that can functionally substitute ECT2; purple, ECT proteins that cannot functionally substitute ECT2 (Flores-Téllez et al, 2023). Phylogenetic clades -A, -B, -C, and -D are indicated for each protein. The dashed extension of N3.2 region of ECT2 identified ends with a Tyr that does not exactly align with a similarly located Tyr in the ECT4/5/6/7/10 paralogs due to short insertions in some of the proteins.

K Consensus amino acid sequences in the N3 region of the IDR of YTHDF proteins according to the alignment of the full-length sequences of a selection of proteins from 24 species of land plants (green) or 10 species of animals (red) (see Materials and Methods). For the consensus sequences of the DF-A clade alone (top), or the DF-A, -B, -C, and -D clades combined (bottom) are shown. For the consensus sequences include either all 10 animal species together (top) or only vertebrates (3 species, bottom). In all cases, the logos (Schneider & Stephens, 1990) were generated with the Weblogo tool (Crooks et al, 2004). Logos of individual DF-B, -C and -D plant clades can be found in Fig EV3B. An annotated and simplified representation of the alignment is shown in Fig EV4. All protein sequences with the name and taxonomy of all species used, and the full alignment, as provided are Datasets EV5 and EV6. Source data are available online for this figure.
Figure 4.
vivo (Fig 4E). Thus, loss of ECT2 function in mutants in the N3 region correlates closely with the loss of association with PAB2, PAB4, and PAB8, suggesting that the physical association between ECT2 and cytoplasmic PABPs is functionally important.

The Tyr-rich region mediates direct ECT2–PAB8 interaction and is conserved in plant and metazoan YTHDF proteins

We next sought to define three principal properties of the ECT2-PAB interaction: (i) whether the interaction is direct, (ii) whether the N3.2 peptide is sufficient for interaction, and (iii) whether a motif within N3.2 is conserved across more YTHDF proteins. We first expressed and purified PAB8 fused to glutathione-S-transferase (GST) and conducted in vitro binding assays using a GST-PAB8 affinity column with dilute total lysates prepared from seedlings of either nontransgenic plants, plants expressing ECT2WT-mCherry or ECT2N3.2-mCherry. ECT2WT-mCherry was retained by GST-PAB8, but not by control columns loaded with equimolar quantities of GST (Fig 4F). Importantly, ECT2N3.2-mCherry bound much less efficiently to GST-PAB8 than ECT2WT-mCherry (Fig 4F). This result is consistent with a direct and specific PAB8-ECT2 interaction mediated by the Tyr-rich N3.2.2 region, but does not rule out “bridging” by an unknown factor present in the total lysate. Thus, we conducted in vitro binding assays with heterologously expressed GST-PAB8 and a 230-aa N-terminal fragment of ECT2 that includes the N3.2 region. This fragment corresponds approximately to the N1 region analyzed in Fig 1 and is, consequently, referred to as ~N1ECT2. The binding assays showed that at a concentration of 10 μM, ~N1ECT2 bound specifically to GST-PAB8 (Fig 4G), indicating that the interaction is direct. At 2.5 μM concentration, considerably less ~N1ECT2 was bound by GST-PAB8 (Fig 4G), indicating that the ECT2-PAB8 interaction has relatively low affinity. Finally, to test for direct interaction between N3.2ECT2 and PAB8 using orthogonal methodology, we turned to a bacterial two-hybrid system that relies on reconstitution of adenylate cyclase activity in E. coli adenylate cyclase (cyu−) mutants when interacting proteins are fused the T18 and T25 fragments of the Bordetella pertussis adenylate cyclase. This allows expression of genes requiring the cAMP-dependent global transcription factor, catabolite activator protein (CAP, Fig 4H). Such genes include the xyl, rbs, and mal operons required for metabolism of the non-glucose sugars xylose, ribose, and maltose, respectively (Emmer et al, 1970; Kolb et al, 1993). The CAP-dependent expression of these genes can be detected by growth on indicator plates containing either X-Gal for blue/white screening (lacZ expression) or MacKonkey/sugar for indication of the ability to ferment the sugar in question by appearance of pink colonies (Karimova et al, 1998). Co-expression of T18-N3.2ECT2 and T25-PAB8, but not of the negative controls (T18-N3.2ECT2 + T25, T18 + T25-PAB8, T18 + T25) reconstituted CAP production as seen by CAP-CAP-dependent fermentation of the three different sugars, and a high frequency of blue colonies among primary transformants screened on LB/X-Gal plates (Figs 4I and EV3A). These results indicate that N3.2ECT2 and PAB8 interact directly in bacteria. Taken together, the results of the series of ECT2 IP-MS experiments, the GST-PAB8 retention assay, the in vitro ~N1ECT2-GST-PAB8 binding assay, and the N3.2ECT2-PAB8 bacterial two-hybrid interaction strongly indicate that ECT2 binds directly to PABPs and that the Tyr-rich N3.2 region is necessary and sufficient for this interaction.

Because an interaction between human YTHDF3 and cytoplasmic PABP (PABC1) has been reported (Zhang et al, 2019), and because ECT2 function is provided by ectopic expression of not only 8 of the 11 Arabidopsis ECTs but also a liverwort YTHDF protein separated by ~450 million years of evolution from ECT2 (Flores-Téllez et al, 2023), we next studied plant and metazoan YTHDF sequences to see if an amino acid motif within the N3.2/N3.2.2 region shows signs of conservation. The Tyr-rich element in N3.2.2 stood out as the most clearly conserved motif across plant YTHDF proteins (Fig 4J and K), and different variants of this motif were even more prominent within the different phylogenetic clades of plant YTHDF proteins (Figs 4J and K, EV3B and EV4). Interestingly, 7 of the 8 Arabidopsis YTHDF proteins with ECT2-like function featured the Tyr-rich motif, while none of the 3 proteins (ECT1/9/11) unable to provide the ECT2-like growth-promoting function did so (Flores-Téllez et al, 2023) (Figs 4J and EV4). For ECT3, the only Arabidopsis YTHDF protein with ECT2-like function that did not have a clear Tyr-rich motif in the N3.2 region (Fig 4J), a similar motif is present elsewhere in its N-terminal IDR. These observations further strengthen the causal link between PABP interaction mediated by the Tyr-rich motif and growth-promoting activity of plant YTHDF proteins. Remarkably, a similar Tyr-rich motif was also present in vertebrate and even metazoan YTHDF proteins more broadly (Figs 4K, EV3B and EV4). These observations suggest that the deeply conserved Tyr-rich motif is a novel short linear motif (SLiM) that mediates interaction with cytoplasmic PABP. The significance of a conserved biochemical function of YTHDF proteins linked to PABP across animals and plants underscores the importance of future in-depth characterization of the interaction between the Tyr-rich SLiM in YTHDFs and PABP, including by structural means.

We note that in addition to the human YTHDF3-PABC1 interaction, previous reports have indicated YTHDF-PABP associations. ECTs and PAB2/4/8 have been found to co-purify with stress granule markers even in the absence of stress (Kosmacz et al, 2019), and a recent report also found an association between ECT2 and PAB2 to be mediated by a 400-aa N-terminal IDR fragment of ECT2 (Song et al, 2023). Although two 200-aa fragments corresponding to N- and C-terminal halves of the IDR were sufficient for PAB2 interaction in qualitative in vitro binding assays, the region necessary for interaction was not more accurately defined (Song et al, 2023), precluding conclusive experiments on the importance of the ECT2-PAB2 interaction to be conducted. Nonetheless, because the C-terminal half of the IDR does not include N3.2, the observations by (Song et al, 2023) raise the possibility that regions of ECT2 other than N3.2 may also be implicated in PABP binding, perhaps akin to the interaction between mammalian Paip1 and PABP mediated by distinct PAM1 and PAM2 motifs (Roy et al, 2002). Such a scenario would be consistent with our IP-MS and GST-PAB8 binding data that show significant reduction, but not ablation, of ECT2-PABP interaction upon deletion of N3.2 and/or N3.2.2.

Increased PAB4 dosage is sufficient to partially suppress leaf formation defects in te234 mutants

We next sought genetic evidence to corroborate the functional importance of the ECT-PABP interaction. Since PAB2, PAB4, and PAB8 are largely functionally redundant in strict genetic terms during vegetative growth, and pab2 pab4 pab8 triple mutants are
embryonically lethal (Dufresne et al., 2008; Gallie, 2017; Zhao et al., 2019), we could not employ standard knockout analyses. Nonetheless, physically interacting components of biological systems often show dosage dependence, such that the importance of their interaction can be revealed genetically through either non-allelic non-complementation (Kidd et al., 1999), synthetic lethal-type genetic interaction, or overexpression suppression (van Leeuwen et al., 2017).

**ect2** and **pab2** did not interact genetically (Fig EV5A), and indeed, given the number of loci involved (**ECT2*/ECT3*/ECT4*/-PAB2*/PAB4*/PAB8*) and the clear phenotype of **te234** mutants, the most straightforward genetic test is the effect of PABP overexpression in organ primordia of **te234** mutants. Thus, we expressed C-terminally Venus-tagged PAB4 in **te234** mutants under the control of the strong **US7Y*/RPS5A* promoter specific for rapidly dividing cells (Weijers et al., 2001) (Fig 5A). After 9 days of growth, a clear, if partial, rescue of the delayed leaf emergence was visible in **PAB4-Venus**-expressing **te234** mutants compared to **te234** seedlings without the **PAB4** transgene (Fig 5B–D), and partial suppression of the defects in the size and shape of the leaves could also be appreciated at later stages (Fig 5B). Importantly, when the same **US7Y**:PAB4-

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Figure 5. Overexpression of PAB4 partially suppresses defective leaf formation caused by loss of ECT2/3/4 function.

A Western blot analysis showing expression of the **US7Yp**:PAB4-Venus-OCSt (PAB4-Venus) transgene in **te234** plants. Notice the integrity of the fusion protein. Ponceau (Pon.) staining is used as loading control.

B Partial suppression of the delay in leaf formation (top) and defective leaf morphology (bottom) of **te234** plants in two independent transgenic lines (L1, L2) overexpressing PAB-Venus in actively dividing cells. DAG, days after germination. The partial suppression.

C Morphological appearance and Venus fluorescence in the first true leaves of a 10-day-old T2 seedling expressing PAB4-Venus in the **te234** background (left) compared to a sibling without PAB4-Venus expression (right) due to segregation of the single-insertion transgene in **T2**.

D Box plot showing the quantification of the size of leaves of 10-day-old T2 seedlings of two independent **te234** PAB4-Venus lines. Boxes represent the interquartile range (25th–75th percentile) of the values, with the central line indicating the median. The whiskers indicate 1.5-fold the interquartile range. Because the lines have single-locus T-DNA insertions, the transgene segregates in the T2 population and the leaf size of sibling plants with or without the transgene (+/– Venus) growing side by side can be measured. Significance was assessed using Student’s t-test (**P < 0.0001**).

E Left side, phenotypes at 9 DAG of Col-0 WT and **te234** seedlings used as female parents in crosses **te234** mutants with or without the **US7Yp**:PAB4-Venus transgene, as described in the scheme (middle). Right side, phenotypes and Venus fluorescence at 9 DAG of F1 of the indicated genotypes. Seedlings represented on the left and right sides of the panel were grown in parallel and are directly comparable.

Source data are available online for this figure.
Venus transgene was expressed in te234 heterozygous and te234 homozygous backgrounds, acceleration of leaf formation was only observed in the te234 homozygous background (Fig 5E and Appendix Fig S1J), demonstrating that PAB4 overexpression in leaf primordia does not accelerate leaf formation when ECT2–ECT3/ECT4 are expressed. Thus, increased PAB4 dosage is sufficient to alleviate the delayed leaf formation resulting from the simultaneous knockout of ECT2, ECT3 and ECT4, supporting the functional relevance of the interaction between these essential families of RNA-binding proteins.

We note that PAB2, PAB4, and PAB8 mRNAs are all high-confidence m6A–ECT2/3 targets (Shen et al., 2016; Parker et al., 2020; Arribas-Hernández et al., 2021b) (Fig EV3B). It is, therefore, possible that their expression may be lower in ect2 ect3 ect4 mutants than in wild type, and that such a difference in expression might contribute to the slow leaf formation and explain the partial phenotypic rescue by PAB4 overexpression. We consulted our previously published transcriptome analyses to address this possibility (Arribas-Hernández et al., 2021b). In entire root tips, no significant differences in PAB2/4/8 mRNA expression were observed between ect2 ect3 ect4 mutants and wild type (Fig EV3C). However, in ECT2-expressing root protoplasts selected by fluorescence-assisted cell sorting, a 3-fold downregulation of PAB4 and slightly less than twofold downregulation of PAB8 mRNAs were in fact observed in cells devoid of ECT2/3/4 function compared to ECT2-expressing controls (Fig EV3C). Thus, we cannot entirely exclude the possibility that simple downregulation of PAB4 mRNA in te234 mutants underlies the suppression of slow leaf formation by PAB4 overexpression. We disfavor this possibility because all single homozygous knockout mutants in PAB2/4/8 genes have normal developmental phenotypes (Gallie, 2017; Zhao et al., 2019), suggesting that PABP function in vivo is not excessively sensitive to dosage. In addition, the argument in favor of functional importance of the physical association of ECT2 and PAB2/4/8 relies not only on the suppression of te234 mutant phenotypes by PAB4 overexpression in organ primordia but also on the tight correlation between loss of ECT2 function and loss of PAB2/4/8 association.

Based on the ECT2–PAB2/4 interaction, and the observation that some ECT2 mRNA targets have shorter half-lives in the absence of ECT2/3/4, it was recently proposed that m6A–ECT2/3 generally acts to stabilize mRNA targets via PAB2/4 interaction (Song et al., 2023). However, this proposition is not supported by evidence, because no efforts were made to specifically disable the ECT2–PAB2/4 interaction, or to restore PAB2/4 binding to ECT2/3/4 mRNA targets in the absence of m6A or ECT2/3/4. Thus, while clearly demonstrating the interaction between ECT2 and PAB2/4, the work by (Song et al., 2023) does not allow conclusions to be drawn on the functional importance of this interaction. In contrast, the combination of (i) the clear correlation between partial loss of ECT2 function and PAB2/4/8 interaction in the ECT2ΔN1–ECT2ΔN3.2, ECT2ΔN3.2–ECT2ΔN3.2 series of mutants, (ii) the PAB2/4/8 association with ECT3, but not with ECT1, and (iii) the partial suppression of the leaf formation phenotype of te234 mutants by PAB4 overexpression shown here, strongly indicates that the ECT–PABP interaction indeed is of functional importance for the m6A–ECT-mediated rapid cellular proliferation in leaf primordia (Arribas-Hernandez et al., 2020). While our data clearly does not disprove the model for ECT–PABP function at all mRNA targets suggested by (Song et al., 2023), we propose a rather more cautious interpretation of the evidence at hand. We suggest that the PABP concentration in rapidly dividing primordial cells is limiting, and that at least a subset of ECT2/3/4 target mRNAs relevant for leaf formation requires the ECT–PABP interaction to compete efficiently for PABP binding with other mRNAs. Increasing the cytoplasmic PABP concentration by PAB4 overexpression would, therefore, alleviate the requirement for m6A–ECT2/3/4 to ensure sufficient PABP binding to this subset of target mRNAs. Because in early vertebrate embryogenesis, mRNAs with short poly(A) tails are recently shown to compete less efficiently for limiting amounts of PABP than those with longer poly(A) tails (Xiang & Bartel, 2021), we suggest that the ECT2/3/4 targets relevant for organogenesis are likely to be found among mRNAs with short poly(A) tails. We also note that a requirement for cytoplasmic PABP for viral translation and infectivity, as demonstrated in the case of Turnip Mosaic Virus (Dufresne et al., 2008), may be part of the explanation why metabolically active, primordial cells would evolve to function with limiting PABP concentrations and a requirement for some mRNAs to use the m6A–ECT axis to compete efficiently for limiting amounts of PABP. Nonetheless, other models to explain the importance of the ECT–PABP interaction cannot be excluded at this point. For example, rather than stabilizing PABP binding to m6A–ECT2/3 target mRNAs, the ECT–PABP interaction might increase the rate of exchange of nuclear and cytoplasmic PABP (Mangus et al., 2003) to accelerate formation of translation-competent mRNPs upon export into the cytoplasm.

Materials and Methods

Plant material and growth conditions

All the plant lines used in this study are in the A. thaliana Columbia-0 (Col-0) ecotype. The following mutant and transgenic lines have been previously described: ect2-3, ect2-1 ect3-1 ect4-2 (te234), ect3-1 ect4-2 (de34), ect2-1 ECT2-mCherry, ect2-1 ECT2W464A, Cherry, ect2-1 HA-ECT2, ect2-3 ECT3-Venus, ect4-2 ECT4-Venus (Arribas-Hernández et al., 2018), ect1-2 ECT1-FLAG-TFP (Flores-Téllez et al., 2023), and pab2-1 (Dufresne et al., 2008). Seeds were sterilized by immersion in 70% EtOH for 2 min followed by incubation in 1.5% NaOCl, 0.05% Tween-20 for 10 min, and immediately washed twice H2O. The seeds were spread on plates containing Murashige & Skoog (MS) medium (4.1 g/l MS salt, 10 g/l sucrose, 8 g/l Bacto agar). Plates were stratified in darkness at 4°C for 2–5 days before transfer to Aralab incubators at 21°C, with a light intensity of 120 μmol/m² and 16 h light/8 h dark photoperiod. Seedlings for UV-cross-linking and IP were grown on vertical plates. When necessary, seedlings were transferred to soil after 10 days of in vitro growth and maintained in Percival incubators with identical settings.

Construction of ECT2 deletion mutants and PAB4-Venus overexpressers

To introduce deletions or point mutations in ECT2p:ECT2-mCherry–ECT2t and to generate US7p:PAB4-Venus–OCSt transgenes, we employed the scar-free USER cloning method (Bitinaite & Nichols, 2009) to piece together PCR-amplified DNA fragments in all cases.
except for the 6xTyr-to-Ala mutation, for which appropriate dsDNA (Dataset EV4) for USER cloning was directly synthesized (Integrated DNA Technologies, gBlocks). As template for PCR, we used plasmids containing wild type ECT2p:ECT2-mCherry (genomic DNA) (Arribas-Hernández et al., 2018) for ECT2-mCherry constructs, GreenGate pGGA012 for the US7/RYPSSA promoter, GreenGate pGGFP05 for the OCS terminator (Lampropoulos et al., 2013), and ECT3p:ECT3-Venus (Arribas-Hernández et al., 2018) for Venus. The genomic sequence of PAB4 (At2g23350) was amplified from Col-0 WT genomic DNA, prepared as previously described (Arribas-Hernández et al., 2016). The fragments were amplified with dU-substituted primers and KAPA Hifi Hotstart Uracil+ ReadyMix (Bitinaite & Nichols, 2009), and inserted in the pCAMBIA3300-U vector (pCAMBIA3300 with a double PacI USER cassette inserted between the PstI-XmaI sites at the multiple cloning site) (Nour-Eldin et al., 2006). The resulting plasmids were checked by restriction digestion analysis and sequencing before transformation into ect2-1 ect3-1 ect4-2 mutant plants via Agrobacterium-mediated floral dip (Clough & Bent, 1998) using the GV3101 strain (Koncz & Schell, 1986). The sequences of primers used for every fragment of each construct are detailed in Dataset EV4.

**Construction of the ect2-4 ect3-1 ect4-2 mutant**

To generate an in-frame deletion mutant at the endogenous ECT2 locus, we used the pKIR1.1 CRISPR-Cas9 system (Tsutsui & Higashiyama, 2017). Two plasmids for sgRNA expression (pKIR1.1-ect2-N3.2A and pKIR1.1-ect2-N3.2B) were generated by ligation of oligonucleotides targeting the sequences ATGGAGGCGTTGATGTTCATGG and ACTTTTGTGAGGTACCAATGC and inserted in the pCAMBIA3300-U vector (pCAMBIA3300 with a double PacI USER cassette inserted between the PstI-XmaI sites at the multiple cloning site) (Nour-Eldin et al., 2006). The resulting plasmids were checked by restriction digestion analysis and sequencing before transformation into ect2-1 ect3-1 ect4-2 mutant plants via Agrobacterium-mediated floral dip (Clough & Bent, 1998) using the GV3101 strain (Koncz & Schell, 1986). The sequences of primers used for every fragment of each construct are detailed in Dataset EV4.

**Screening for te234 complementation**

Primary transformants (T1s) of te234 carrying wild type, deletion, or point mutation variants of ECT2-mCherry were selected on 15-cm diameter MSagar plates supplemented with glufosinate ammonium 7.5 mg/l (Sigma) to select plants with the transgene and ampicillin 10 mg/l to restrict agrobacterial growth. Nine days after germination, primary transformants were binned into one of three categories according to the size(s) of the true first leaves: full complementation (s ≥ 1 mm), partial complementation (0.5 mm < s < 1 mm), or no complementation (s < 0.5 mm). The complementation percentages were then calculated as the number of seedlings in each complementation category divided by the total number of transformants.

**Statistical analyses**

Fisher’s exact test was employed to determine the statistical significance of the observed differences among the T1 complementation categories, and the Holm–Bonferroni method was used to account for multiple testing. Student’s t-test was used to assess the significance of difference in leaf size between te234 mutants carrying or not the US7yp:PAB4-Venus-OCSt transgene and between CRISPR-generated ect2-4 ect3-1 ect4-2 mutants compared to the parental de34 (ect3-4 ect4-2) and the triple knockout te234 (ect2-1 ect3-1 ect4-2).

**ECT2-IDR analyses**

To assess conservation among dicot ECT2 homologs, we performed a protein alignment using CLUSTALW and transformed it into a conservation score (Livingstone & Barton, 1993). The geneIDs used for the alignment are the following: Helianthus annuus (A0A251VQ55), Lupinus angustifolius (LOC109348290), Vitis vinifera (ASC6V5), Solanum tuberosum (M1C7V3), Theobroma cacao (A0A061G5W6), Beta vulgaris subsp. vulgaris (LOC104888716), Corchorus capsularis (A0A1R3EW9), Brassica rapa (LOC103859518), Phaseolus angularis (A0A0L9Y19Y), Medicago truncatula (G7L842), Daucus carota subsp. sativus (A0A164SV78), Manihot esculenta (A0A2C9VJN4), Phaseolus vulgaris (V7CQK6), Glycine max (I1KSN3), Populus trichocarpa (B9GX7X), Gossypium raimondii (A0A0D2P5E4), and Trifolium pratense (A0A2K3P0Z0). The prediction of protein disorder was performed with the webserver IUPred2A in default mode (Mesáros et al., 2018).
Conservation analyses of the N3 region

We retrieved the full-length amino acid sequences of YTHDF proteins of 24 species of land plants (1 liverwort, 1 moss, 2 lycophytes, 4 ferns, 2 gymnosperms, 3 early angiosperms, 4 monocots, and 7 dicots) and 10 animals (1 cnidarian, 1 mollusk, 1 rotifer, 2 arthropods, 1 echinoderm, 1 amphioxus, and 3 vertebrates), and aligned them using MUSCLE (Edgar, 2004a, 2004b) with default parameters. Despite the inherent difficulty of aligning IDRs, this first alignment showed that the N3.2.2 motif is present in most but not all sequences of YTHDF proteins of plants and animals, albeit with a high proportion of insertions and shuffling of short sequences with similar amino acid composition inside the region. We then manually removed proteins from not flowering plants for which the N3 motif was not apparent (that was, e.g., the case for all the plant DF-F proteins analyzed; Flores-Téllez et al., 2023), but left the complete sets for angiosperm DF-A/B/C/D regardless of their degree of conservation. This manually curated list of sequences (Dataset EV5) was used to repeat the alignment (Dataset EV6). From the improved alignment, we obtained the consensus N3 sequence using WebLogo (Crooks et al., 2004) with default parameters. Additionally, we used Jalview (Waterhouse et al., 2009) to extract subsets of sequences (aligned within the full set context) to obtain clade-specific consensus N3 motifs in the same manner. In all cases, we collapsed the gaps caused by short and nonconserved insertions in three or less sequences for the smaller sets (vertebrates and individual plant DF-clades), or with quality scores (Blosum62) below a certain threshold for the larger ones (Q > 5 for animals, and Q > 50 for plants and for all sequences together). Because such adjustments generated differences in the length of the consensus sequences, we manually adjusted the width of different regions for illustration purposes, to reflect the original alignment of key residues. Finally, we hid all proteins in two species of angiosperms (Citrus sinensis and Setaria italica) and left only one paralog for recent gene duplications with quasi-identical sequences in plants to provide a representation of the alignment with a reasonable number of sequences (Fig EV4).

Western blotting

One hundred to three hundred milligram of tissue ground to a fine powder under liquid N2 was mixed with 5× (v/w) IP buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM MgCl2, and 0.1% Nonidet P40) freshly supplemented with protease inhibitor (Roche Complete tablets) and 1 mM DTT. The lysate was mixed immediately by vigorous shaking and spun at 13,000 g for 10 min. 4× LDS sample buffer (277.8 mM Tris–HCl pH 6.8, 44.4% (v/v) glycerol, 4.4% LDS, and 0.02% bromophenol blue) was mixed with the protein samples to a concentration of 1× LDS. The samples were denatured at 75°C for 10 min before being run on a 4–20% Criterion™ TGX™ Precast gel and in 1× Tris-glycine, 0.1% SDS at 90–120 V for ~1 h on ice. Using wet transfer, proteins were blotted onto an Amersham Protran Premium nitrocellulose membrane (GE Healthcare Life Sciences) in cold 1× Tris-glycine, 20% EtOH transfer buffer at 80 V for 1 h on ice. Following transfer, the membrane was blocked in 5% skimmed milk, 0.05% Tween-20, PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) for 30 min. After blocking, membranes were incubated with an anti-ECT2 antibody (Arribas-Hernández et al., 2018) or commercially available antibodies against mCherry (Abcam ab183628, 1:2,000 dilution) or Venus (Siegen AB2166-100; 1:1,000 dilution). The following day, membranes were washed in PBS-T (PBS buffer with Tween-20; Bio-Rad) to a total concentration of 0.05% and developed using HRP-coupled goat-anti-rabbit or, as appropriate, goat-anti-mouse secondary antibodies (Sigma) and chemiluminescence detection, as described (Arribas-Hernández et al., 2018).

Immunoprecipitation for mass spectrometry

The following genetic backgrounds were used for immunoprecipitation of ECT proteins. All ECT2-mCherry versions were expressed in ect2-1 ect3-1 ect4-2 mutants, HA-ECT2 was expressed in ect2-1, ECT3-FLAG-VENUS was expressed in ect3-2, and ECT1-FLAG-TFP was expressed in ect1-2. In all cases, 10-day old seedling tissue grown on MS-agar in Aralea chambers under a 16 h light (120 µmol/m²)/8 h darkness regime at 21°C was used for the immunopurifications.

To immunoprecipitate ECT proteins, ~300 µg of liquid nitrogen-ground tissue powder from 9- to 10-day old seedlings were added to 1 ml of IP buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM MgCl2, and 0.1% Nonidet P40) with the following additives: 5 mM DTT, 2 mM AEBSF (Sigma), 40 µg/ml RNase A, and 1.5 tablet/10 ml of complete protease inhibitor (Roche). Sigma plant protease inhibitor (Sigma P9599) was also added to IPs (200 µl/10 ml of IP buffer) of ect2-1 HA-ECT2 and ect2-1 ECT2-mCherry. Immediately after vigorous vortexing, the lysates were centrifuged at 4°C for 10 min (~13,000 g), and the supernatants were passed through hydrophilic 0.45 µm filters for incubation with 12.5 µl of washed beads (RFP-TRAP®_A (Chromotek, rta-20) for all ECT2-mCherry variants, GFP-TRAP®_A (Chromotek, gta-20) for ECT3-Venus, anti-HA affinity matrix (Roche, 3F10, Ref 11815016001) for HA-ECT2 and anti-FLAG M2 affinity resin (Sigma, A2220) for ECT1-FLAG TFP). After 1 h of slow rotation at 4°C, the beads were washed three times (5 min each) with ice-cold IP-buffer supplemented only with 4 mM DTT. To remove detergent and excess salt, we performed two additional washes with ice-cold 1× PBS. After the final wash, the purifications were either eluted using 100 µg/ml FLAG peptide (ECT1-FLAG-TFP) or the supernatant was removed from the beads (all ECT2-mCherry variants, ECT3-Venus, HA-ECT2). In any case, samples were kept at -80°C until tryptic digestion for mass spectrometry. The purifications were done in three replicates for both the transgenic lines expressing tagged ECTs, or nontransgenic plants for mock IPs.

Mass spectrometry

Purifications were prepared for mass spectrometry by trypsin/LysC digestion and C18 stage tipping. Beads containing purified proteins were resuspended in 20 µl lysis buffer (6 M guanidium chloride, 10 mM Tris[2-carboxyethyl]phosphine, 40 mM 2-chloroacetamide (CAA), 50 mM HEPES pH 8.5). Samples were sonicated with three 30" on ~30" off cycles using BioRuptor Pico and diluted 1:3 by adding 40 µl of digestion buffer (10% acetonitrile, 50 mM HEPES pH 8.5). 2 µl of LysC (50 ng/µl) were then added to the samples and incubated at 37°C for 3–4 h, while shaking at ~1,500 rpm. Subsequently, samples were diluted 1:10 by adding 140 µl more of digestion buffer and 1 µl of trypsin (50 ng/µl) was added, then briefly vortexed, and incubated at 37°C.
overnight while shaking at ~1,500 rpm. The reaction was stopped by adding 2% trifluoro acetic acid (1:1, 1% final concentration), ie. 200 µl after which samples were vortexed and spun down for 1' at 2,000 g. 200 µl pipette tips were packed with two C18 filters (Empore 2215. 66883-U). The C18 filters were activated by adding: 30 µl MeOH, 30 µl Buffer B (80% acetonitrile, 0.1% Formic Acid), 2× 30 µl Buffer A’ (3% acetonitrile, 1% trifluoroacetic acid). At this point, samples were loaded onto the StageTip with 50 µl aliquots at a time and spun through at ~1,200 g. The tips were then washed twice with 100 µl of Buffer A (0.1% FA) and eluted with 2× 30 µl Buffer B’ (40% I, 0.1% FA) into a clean 500 µl Protein LoBind Eppendorf tube. The eluted samples were speedvaced for ~60 min at 60°C and resuspended in 12 µl Buffer A (2% 1% TFA) containing iRT peptides. Finally, 1.5 µl were used for concentration measurements by Nanodrop. Non-targeted mass spectrometry analysis was performed on a quadrupole Orbitrap benchtop mass spectrometer, QExactive (Thermo Scientific), equipped with an Easy nano-LC 1000 system (ThermoFisher Scientific). 500 ng of peptide mixture from each sample was analyzed by online nano-scale liquid chromatography tandem mass spectrometry (LC-MS/MS) in turn. Peptides were separated on a 50 cm C18-column (Thermo EasySpray ES904/Thermo EasySpray ES903) using an EASY-nLC 1200 system (Thermo Scientific). The column temperature was maintained at 45°C. Buffer A consisted of 0.1% formic acid in water, and buffer B of 80% acetonitrile, 0.1% formic acid. The flow rate of the gradient was kept at 250 nl/min, and started at 10% Buffer B, going to 23% buffer B in 85 min. This was followed by a 30’ step going to 38% buffer B, increasing to 60% Buffer B in 10 min, and finally ramping up to 95% buffer B in 5 min, holding it for 10 min. to wash the column. The Q Exactive Classic instrument (Thermo Scientific, Bremen, Germany) was run in data dependent acquisition mode using a top 10 Higher-energy Collisional Dissociation (HCD)-MS/MS method with the following settings. Scan range was limited to 350–1,750 m/z. Full scan resolution was set to 70,000 m/z, with an AGC target of 3e6 and a maximum injection time (IT) value of 20 ms. Peptides were fragmented with a normalized collision energy of 25, having a dynamic exclusion of 60 s, excluding unassigned ions and those with a charge state of 1. MS/MS resolution was set at 17,500 m/z, with an AGC target of 1e6 and a maximum IT of 60 ms.

**Analysis of IP-MS data**

All raw LC-MS/MS data files were processed together using Proteome Discoverer version 2.4 (Thermo) with the use of label-free quantification (LFQ) in both the processing and consensus steps. In the processing step, oxidation (M), protein N-termini acetylation, cysteine carbamidomethyl set as static modification. All results were filtered with percolator using a 1% false discovery rate (FDR), and Minora feature detector was used for quantitation. SequestHT was used as database, matching spectra against the TAIR database. Two different methods of normalization were employed to infer the differentially enriched proteins between samples. When comparing ECT2-mCherry mutants to full length ECT2-mCherry, we made use of total sum normalization in addition to variance stabilizing normalization (VSN) (Huber et al, 2002). However, when analyzing the differential abundance of proteins co-purified with ECTs compared to non-transgenic plants (mock IP), we decided to do only VSN normalization to prevent inflation of the background in the non-transgenic mock samples that contain very little protein overall. The VSN normalization method, implemented using the ‘normalizeVSN’ function from the limma package, was employed to transform the abundance values and stabilize the variance across the samples. To identify differentially abundant proteins between samples, we made use of the R package limma (Smyth, 2004). First, a linear model was constructed using a design matrix and a contrast matrix to specify the contrasts between samples. Fold changes and P-values were then calculated based on the linear model. We used the fitted linear model as an input to treat and topTreat which performed moderated t-statistics and log-odds estimation using empirical Bayes statistics. P-values were adjusted for multiple testing using the Benjamini-Hochberg adjustment method (Benjamini & Hochberg, 1995).

**Silver staining**

10–20% of the samples containing immunoprecipitated ECTs for mass-spectrometry were subjected to SDS-PAGE in 4–20% Criterion™ TGX™ Precast Midi Protein gels (Bio-Rad) for visual assessment. After electrophoresis at 90 V, the gels were fixed with a solution of 30% EtOH, 10% AcOH for 20 min, and subsequently rinsed in a stepwise manner: 10 min in 20% EtOH, 10 min in 10% EtOH, and 2× 20 min in H2O. The gels were then submerged in sensitizing solution (0.02 M Na2S2O3) for 1 min, rinsed two times with water and incubated in 0.1% AgNO3 for 1 h. After two additional washes with the gels, the were developed with freshly made developing solution (0.04% formalin, 2% Na2CO3) until the signal intensity was adequate, at which point the development was quenched with 2% AcOH.

**Analysis of RNA association in vivo by cross-linking-immunoprecipitation-PNK labeling**

Cross-link, immunoprecipitation and PNK-labeling of ECT2-mCherry mutants were performed as previously described (Arribas-Hernández et al, 2021a). Briefly, 12-day-old seedlings were irradiated with 2,000 ml/cm² of 254 nm-UV light on ice. The tissue was then ground in liquid nitrogen, and lysates containing ECT2-mCherry RNPs were prepared by resuspending 1 g of ground tissue in 1.5 ml of iCLIP buffer (0.25% sodium deoxycholate, 0.25 lgepal, 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% SDS, 5 mM DTT, 4 mM MgCl2) supplemented with protease inhibitors (Roche cOmplete 1 tablet/10 ml, 4 mM PMSF and 1/30 (v/v) Sigma Plant Protease Inhibitor). After homogenization, the lysates were cleared by centrifugation for 10 min at max. speed, passed through a 0.45 µm filter and incubated with 20 µl RFP-TRAP®_A (Chromotek) beads for 1 h at 4°C under slow rotation. After removal of the supernatant, the beads were washed four times with iCLIP wash buffer (0.5% sodium deoxycholate, 2 M urea, 0.5% lgepal, 1% SDS, 50 mM Tris–HCl pH 7.5, 500 mM NaCl, 2 mM DTT, 4 mM MgCl2) and two times with PNK wash buffer (20 mM Tris–HCl, 10 mM MgCl2, 0.2% Tween-20). Beads were then resuspended in 100 µl PNK wash buffer and subjected to DNase and RNase digestion by addition of 2 µl Turbo DNase (Thermo Fisher) and 5 µl of RNase l (Ambion) (1:500 dilution) and stirring at 1100 rpm for 10 min at 37°C in an Eppendorf Thermomixer. Beads were then washed once with PNK wash buffer, twice with high-salt buffer (50 mM Tris–HCl pH 7.4, 1 M NaCl, 1 mM EDTA, 1% lgepal, 0.1% SDS, 0.5% sodium
deoxycholate), and twice again with PNK wash buffer. The RNA cross-linked to ECT2 on the beads was radioactively labeled at the 5’ end by PNK-mediated phosphorylation using γ-32P-ATP (20’ at 37°C). After three washes in PNK wash buffer, beads were resuspended in 2× LDS buffer (Thermo Fisher), incubated at 95°C for 10’, and the denatured RNP complexes were then subjected to SDS-PAGE, blotted onto a nitrocellulose membrane (Protran BA-85), and detected by autoradiography.

**Fluorescence microscopy**

Whole seedlings and leaves were imaged employing a Leica MZ16 F stereomicroscope equipped with a Sony a6000 camera.

**Purification of proteins**

Using the primer set MT545-MT546, PAB8 (AT1G49760) was amplified from cDNA obtained from oligo(dT)-primed reverse transcription of DNase-treated total RNA from Col-0 WT. The resulting PCR product was ligated in frame downstream of a glutathione S-transferase (GST) tag in pGEX-4T1 by restriction cloning. A cDNA fragment encoding the first 230 amino acids of ECT2 (~N1ECT2) was amplified from an ECT2 cDNA construct fused to mCherry (Flore-Télez et al., 2023) and ligated in-frame downstream of His6-SUMO in pET-24-derived vector containing His6-SUMO (Twist Bioscience).

For heterologous protein expression, the plasmids encoding His-SUMO--~N1ECT2 and GST-PAB8 were transformed into E. coli BL21 (DE3 7tRNA) codon plus, and expression was induced at OD600 approximate of 0.6 by the addition of 0.5 mM IPTG. Cells were grown at 37°C in LB medium supplemented with 35 μg/ml kanamycin in the case of His6-SUMO--~N1ECT2 and 100 μg/ml ampicillin in the case of GST-PAB8. Induced cells were grown at 18°C overnight before collection by centrifugation. Cell pellets with induced expression of GST-PAB8 were resuspended in 1× phosphate-buffered saline (PBS; 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM KCI, and pH 7.3), while pellets with induced expression of His-SUMO--~N1ECT2 were resuspended in 20 mM Tris–HCl (pH 8), 10 mM imidazole, and 300 mM NaCl, respectively. Both resuspension buffers were supplemented with 1 mM DTT and EDTA-free protease inhibitor (cOmplete; Roche). Cells were lysed using a French press (20,000 psi 1×), and GST-PAB8 lysates were supplemented with 1% Triton-X-100. Crude lysate was cleared by centrifugation at 30,000 g for 30 min at 4°C and filtered through a 0.45-μm membrane. The cleared lysate containing GST-PAB8 was incubated on glutathione-coupled Sepharose 4 Fast Flow beads (GE Healthcare, lot 10258061) for 1 h at 4°C and washed extensively in 1×PBS. Proteins were eluted from glutathione beads by the addition of elution buffer (10 mM glutathione, 50 mM Tris–HCl pH 8, and 300 mM NaCl). His-SUMO--~N1ECT2 was purified on Ni²⁺-NTA resin by incubation for 1 h at 4°C after the beads were washed in wash buffer (20 mM Tris–HCl pH 8, 20 mM imidazole, 200 mM NaCl), and the bound protein was eluted in elution buffer (300 mM imidazole, 20 mM Tris–HCl pH 8, 300 mM NaCl). The elution buffers of GST-PAB8, GST, and His-SUMO--~N1ECT2 were dialyzed (ZellyTrans/ROTH T2; MWCO 6000–8000, 25 mm) into 20 mM Tris–HCl (pH 8), 200 mM NaCl, 1 mM 2-mercaptoethanol at 4°C overnight. His6-SUMO--~N1ECT2 was further purified on a HiLoad Superdex™ 200 10/300 GL prep grade column (GE Healthcare) connected to an HPLC ÄKTA Purifier system (GE Healthcare). The system was initially washed and equilibrated with 1.5 column volume (CV) of milliQ water and 2 CV of filtered and degassed running buffer (20 mM Tris–HCl (pH 8), 200 mM NaCl and 1 mM 2-mercaptoethanol). Eluates were monitored at A280 and purity checked by SDS-page analysis. Clean fractions were collected and purified His6-tagged SUMO protease 1 (ULP1) was used to cleave off the tag. Subsequently, Ni²⁺-NTA resin was used to bind the protease and impurities bound to the Ni²⁺-NTA resin in the first affinity purification, and ~N1ECT2 was collected in the flowthrough.

**In vitro binding assay with purified components**

Glutathione-coupled Sepharose 4 Fast Flow beads (GE Healthcare) were equilibrated in binding buffer (20 mM Tris–HCl (pH 8), 200 mM NaCl and 1 mM 2-mercaptoethanol). Once equilibrated, 100 pmol of GST and GST-PAB8 were immobilized on a bed volume of 10 μl beads by incubation with rotation at room temperature for 30 min. Following immobilization, the beads were washed with 100 μl of ice-cold binding buffer. Glycerol was added to untagged ECT21-230 to a final concentration of 1% (v/v). Binding reactions with immobilized GST and GST-PAB8 were carried out in 10 μl volume for 2 h at room temperature with rotation, either with a 10× molar excess (1,000 pmol, 10 μM) or 2.5× molar excess (250 pmol, 2.5 μM) of ~N1ECT2. Beads were washed twice in 500 μl binding buffer, and the proteins were eluted in elution buffer (10 mM glutathione, 50 mM Tris–HCl pH 8, and 300 mM NaCl). After addition of 4×LDS buffer and incubation at 75°C for 5 min, samples were analyzed by SDS-PAGE followed by InstantBlue (Sigma) staining.

**GST retention assay with total lysates**

A bed volume of 10 μl glutathione-coupled Sepharose beads (GE Healthcare) was used to immobilize 1,000 pmol of GST and GST-PAB8, respectively, as described above. Cell extracts were prepared from 11-day-old Col-0 seedlings and seedlings expressing ECT2-mCherry or ECT2N3.2,2-mCherry in the to234 background. For each cell extract, a total of 800 mg ground tissue was homogenized in 3,200 μl binding buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 10% glycerol, 5 mM MgCl₂, 0.1% NP40, supplemented with 4 mM DTT and 2× EDTA-free Roche complete protease inhibitor). After centrifugation for 10 min at 4°C at max. speed, the cell extract was filtered through a 0.45 μm filter and an aliquot was saved as input. 1 μl of cell extract was then added to 1,000 pmol of immobilized GST or GST-PAB8 protein and samples were rotated for 1.5 h at 4°C. Samples were then transferred onto a BioSpin Chromatography column (Biorad) and washed three times with 800 μl of cold binding buffer. Proteins were eluted in 35 μl of elution buffer (10 mM glutathione, 50 mM Tris–HCl pH 8, 300 mM NaCl). After addition of 4×LDS buffer, eluates were incubated at 75°C for 5 min and used for western blot with anti-mCherry antibody as described above.

**Bacterial two-hybrid assay**

Bacterial two-hybrid assays were performed using the BACTH System kit (Euromedex). To clone PAB8 into pKNT25 (generating a PAB8-T25 fusion), full-length PAB8 was PCR amplified using primers MT556 and MT557 (Dataset EV4) and the GST-PAB8
plasmid as a template. The N3.2ECT2-encoding fragment was PCR amplified using primers MT564 and MT565 (Dataset EV4) and a plasmid containing ECT2-mCherry cDNA as template (Flores-Téllez et al., 2023), and cloned into pUT18 to generate an N3.2ECT2-T18 fusion. PCR reactions were performed with Phusion High-fidelity DNA polymerase (NEB) according to manufacturer’s instructions. The purified PAB8 PCR product and pKNT25 vector were digested with HindIII and BamHI, while the purified N3.2ECT2 PCR fragment and pUT18 vector were digested with BamHI and SacI. Fragments were then gel-purified using the GeneJET Gel Extraction kit (Thermo). Ligations were carried out with T4 DNA ligase (Thermo) for 1 h at room temperature. 5 μl of the ligation mixture were transformed into XL1-blue electro-competent cells, plasmid DNA was isolated from single colonies using the GeneJET Plasmid Miniprep kit (Thermo), and verified by restriction digest and Sanger sequencing.

For the complementation assay, BTH101 cells were cotransformed with 20 ng of each recombinant plasmid (expressing T25 and T18 fusion proteins, GCN4 coiled-coil positive control fusion plasmids are part of the BACTH System kit, Euromedex) and plated on LB plates with respective antibiotics (Amp, Kan), IPTG (0.5 mM) and X-Gal (40 μg/ml). Additionally, cells were plated on MacConkey/maltose plates with respective antibiotics (MacConkey: Difco MacConkey agar base, ref 281810; maltose: Sigma, M-5885). Plates were incubated at 30°C, and reconstitution of adenylate cyclase activity was monitored by formation of blue colonies on LB/X-gal plates and pink colonies on MacConkey/maltose plates. Primary transformants obtained on MacConkey/maltose plates were restreaked on MacConkey/maltose, MacConkey/ribose (D-ribose: Sigma, R9629), MacConkey/xylene (D-xylene: BDH Chemicals Ltd, Poole, UK, product no. 30590) and MacConkey/glucose (D-glucose, Serva 22720.01), and grown at 31°C for 4 days with photographs taken every 10 min using an automated camera set-up (ReShape Biotech, Copenhagen, Denmark).

Availability of new materials

Seeds of arabidopsis mutants and transgenic lines generated in this study have been submitted to the Nottingham Arabidopsis Stock Centre.

Data availability

The mass spectrometry datasets produced in this study are available in the database of Proteomics Identifications (PRIDE) under project accession number PXD045801 (http://www.ebi.ac.uk/pride/archive/projects/PXD045801).

Expanded View for this article is available online.

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Author contributions

Peter Brodersen: Conceptualization; data curation; formal analysis; supervision; funding acquisition; methodology; writing – original draft; project administration; writing – review and editing. Mathias Due Tankmar: Data curation; formal analysis; investigation; visualization; methodology; writing – review and editing. Marlene Reichel: Formal analysis; validation; investigation; methodology; writing – review and editing. Laura Arribas-Hernandez: Conceptualization; formal analysis; supervision; validation; investigation; visualization; methodology; writing – review and editing.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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