Supplementary Materials

***N*6-methyladenosine Reader ECT1 Mediates Seed Germination *via* the DAG2-ECT1-PHYB/RGA1 Regulatory Cascade in *Arabidopsis thaliana***

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**Supplementary Tables and Datasets are available online at (https://github.com/cma2015/Datasets/tree/ECT1\_paper).**

**Supplementary Table Legends**

**Supplementary Tab. 1** Cloning of plasmid constructs.

**Supplementary Tab. 2** Primers used for cloning of plasmid constructs.

**Supplementary Tab. 3** Primers for qPCR and genotyping used in this study.

**Supplementary Tab. 4** Probes for EMSA experiments.

**Supplementary Tab. 5** Information of reference genomes used in this study.

**Data S1** Identification of ECT1-binding sites from RIP-seq data.

**Data S2** Predicted upstream transcription factors of ECT1.

**Data S3** Predicted downstream transcription factors of ECT1.

**Data S4** Amino acid sequences of YTH domains and accession numbers of YTH domain proteins.

图表, 图示

中度可信度描述已自动生成**Supplementary Figures**

**Figure S1. Evolutionary analysis of YTH proteins. (A)** Number and classification of YTH proteins identified in 26 species. The evolutionary relationships of these YTH proteins were constructed using the TimeTree (https://timetree.org/). “\*” indicates proteins used as outgroups. **(B)** Evolutionary relationships of YTH proteins. Bootstrap values are represented by circles on the evolutionary branches. The background color of sequence names indicates the classification of YTH subfamilies, and the color of leaf nodes represents the taxonomic units of the species to which the sequences belong.

图示

描述已自动生成**Figure S2. RNA-binding ability of ECT10 and ECT11.** Left: Phylogenetic tree of YTH proteins in Arabidopsis and humans, with subfamilies experimentally confirmed as m6A readers highlighted with red circles. Right: EMSA. 5ʹ-biotin-labeled synthetic RNA with or without m6A modification motif *UGUAA* were incubated with GST-ECT10, GST-ECT11, or GST protein alone (negative control). Samples were analyzed using native polyacrylamide gel electrophoresis (PAGE). The gels were blotted onto nylon membranes, and the signals were detected by streptavidin-coupled horseradish peroxidase and electrochemiluminescence (ECL). FP, free probe.

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低可信度描述已自动生成**Figure S3. Sequence and structural analysis of ECT1. (A)** Multiple sequence alignment of the YTH domain of ECT proteins. The secondary structure elements of ECTs are indicated, and amino acids are colored according to the level of sequence conservation: red letters, similar residues; and red boxes, identical residues. Amino acids that form the methyl-interacting aromatic cage are marked with red stars. **(B)** A model of the YTH domain of the ECT1 protein generated by the homology modeling server SWISS-MODEL. Trp267, Trp324, and Trp329 represent the three conserved Trp residues in ECT1, with another highlighted region representing the m6A-modified adenosine.

图表

描述已自动生成**Figure S4. Identification of ECT1 overexpression line.** **(A)** Schematic diagram of the construction of the *p35S*:*ECT1-3xFLAG* transgenic overexpression vector. **(B)** ECT1-3xFLAG protein expression in the ECT1 overexpression line. Proteins were extracted from 4-day-old Col-0 and Col-0 p35S:ECT1-3xFLAG (line 8) seedlings and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis using an anti-FLAG antibody; anti-ACTIN was utilized as a loading control to detect ACTIN. Col-0 seeds served as a negative control. **(C)** Comparison of *ECT1* mRNA expression in 4-day-old Col-0 and Col-0 p35S:ECT1-3xFLAG (line 8) seedlings. *ACT7* served as the reference gene for normalization. The presented data represents the average (of three replicates) ± SD. Significant differences as determined by Student’s t-test, \*\*\*\*: *p* < 0.0001.



**Figure S5. Identification of the *ECT1* T-DNA insertion line. (A)** Diagrams of the T-DNA insertion sites within the ECT1 locus in the *ect1-1* line. The gray box denotes the UTR, the black box signifies the exon, the black line denotes the intron, and the blue box shows the YTH domain of ECT1. The arrows indicate the directions and positions of the primers used for the PCR-based genotyping in Figure B. **(B)** Polymerase chain reaction (PCR) for the detection of ECT1 coding sequence (CDS) and a partial T-DNA fragment in genomic DNA from Col-0 and *ect1-1* mutant plants. **(C)** Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis for the quantification of *ECT1* transcript levels in 4-day-old wild-type and *ect1-1* mutant seedlings. *ACT7* was used as the internal control. Values represent the means (of three replicates) ± SD. Significant differences as determined by Student’s t-test, \*\*\*\*: *p* < 0.0001.

图示

描述已自动生成**Figure S6. Complementation of the *ect1-1* mutant by the expression of *pECT1*:*ECT1-3*x*FLAG*.** **(A)** Schematic representation of the *pECT1*:*ECT1-3*x*FLAG* transgene. Arrows indicate the primers used for polymerase chain reaction (PCR) in (B). **(B)** PCR assay for the detection of *ECT1-3*x*FLAG* fragment in genomic DNA from Col-0 and *pECT1*:*ECT1-3*x*FLAG* expressing in *ect1-1* plants. **(C)** Representative images of plates used for quantification of seed germination in (D). **(D)** Analysis of germination percentages in Col-0 and five *pECT1*:*ECT1-3*x*FLAG*/*ect1-1* complementation lines treated with 0 or 50 μM GA3, in either white light or dark conditions. Data from three replicates were averaged. The bar graph represents the average germination rates from three trials, with error bars indicating standard deviation. Distinct letters denote statistically significant differences based on one-way ANOVA (Tukey’s Honestly Significant Difference), *p* < 0.05.

图表, 条形图

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**Figure S7. Complementation of the *ect1-1* mutant by the expression of *p35S*:*ECT1-3*x*FLAG*.** **(A)** Schematic representation of the *p35S*:*ECT1-3*x*FLAG* transgene. Arrows indicate the primers used for polymerase chain reaction (PCR) in (B). **(B)** PCR assay for the detection of *ECT1-3*x*FLAG* fragment in genomic DNA from Col-0 and *p35S*:*ECT1-3*x*FLAG* expressing in *ect1-1* plants. **(C)** Analysis of germination percentages in Col-0, ECT1OE-8, and three *p35S*:*ECT1-3*x*FLAG*/*ect1-1* complementation lines treated with 0 or 50 μM GA3, in either white light or dark conditions. Data from three replicates were averaged. The bar graph represents the average germination rates from three trials, with error bars indicating standard deviation. Distinct letters denote statistically significant differences based on one-way ANOVA (Tukey’s Honestly Significant Difference), *p* < 0.05.

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**Figure S8. The m6A reading protein function of ECT1 is required for seed germination under GA treatment.**

**(A)** RT-qPCR analysis for the quantification of *ECT1* transcript levels in 4-day-old Col-0, *ect1* (*SAIL\_319\_A08*), and muECT1#20(GFP-tagged ECT1 mutated variant (ECT1W267A, W324A, W329A) in the *ect1* (*SAIL\_319\_A08*) mutant background transgenic plants) seedlings. *ACT7* was used as the internal control. Values represent the means (of three replicates) ± SD. Significant differences as determined by Student’s t-test, \*\*\*\*: *p* < 0.0001. **(B)** Sequencing of *ECT1* gene in Col-0 and muECT1#20 seedlings. The mutation in muECT1#20 results in amino acid changes from Trp to Ala at positions 267, 324, and 329, due to a TGG to GCT base substitution. **(C)** Analysis of germination percentages in Col-0, *ect1* (*SAIL\_319\_A08*), and muECT1#20 with 0 or 50 μM GA3, in either white light or dark conditions. Data from three replicates were averaged. The bar graph represents the average germination rates from three trials, with error bars indicating standard deviation. Distinct letters denote statistically significant differences based on one-way ANOVA (Tukey’s Honestly Significant Difference), *p* < 0.05.

图形用户界面, 应用程序

描述已自动生成**Figure S9. ECT1 directly interacts with DCP5 within P-bodies. (A)** Subcellular localization of EYFP-ECT1 in *N*. *benthamiana* leaves treated with either mock or 50 µM GA3. **(B)** Y2H analysis reveals the interaction between ECT1 and DCP5 in yeast, as observed on selective media lacking tryptophan, leucine, and histidine. AD-DCP5 and BD-ECT1 were expressed in yeast. Yeast cells were cultured on CSM-L-T plates or CSM-L-T-H plates. **(C)** Colocalization of EYFP-ECT1 with mCherry-DCP5 following treatment with 50 µM GA3. EYFP-ECT1 was transiently co-expressed with mCherry-DCP5 in *N*. *benthamiana* leaves.

图表, 雷达图

描述已自动生成**Figure S10. Identification of upstream and downstream regulatory factors linked to ECT1. (A)** Diagram illustrating the putative upstream transcription factors of ECT1. **(B)** Diagram illustrating the downstream transcription factors targeted by ECT1.

图示

中度可信度描述已自动生成**Figure S11. Identification of the *DAG2* T-DNA insertion line. (A)** Diagrams of the T-DNA insertion sites within the *DAG2* locus in the *dag2-2* line. The white box denotes the UTR, the black box signifies the exon, and the line denotes the intron. The arrows indicate the directions and positions of the primers used for the PCR-based genotyping in Figure B. **(B)** Polymerase chain reaction (PCR) assay for the detection of *DAG2* coding sequence (CDS) and a partial T-DNA fragment in genomic DNA (gDNA) from Col-0 and *dag2-2* mutant plants. **(C)** Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis for the quantification of *DAG2* transcript levels in 4-day-old wild-type and *dag2-2* mutant seedlings. *ACT7* was used as an internal control. Values represent the means (of three replicates) ± SD. Significant differences as determined by Student’s t-test, \*\*\*\*: *p* < 0.0001. **(D)** Representative images of plates used for the quantification of seed germination in (E). **(E)** Analysis of germination percentages in Col-0 and *dag2-2* mutants treated with 0 or 50 μM GA3, in either white light or dark conditions. Data from three replicates were averaged. The bar graph represents the average germination rates from three trials, with error bars indicating standard deviation. Significant differences as determined by Student’s t-test, \*: *p* < 0.05.

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**Figure S12. DAG2 interacts with *ECT1* promoter. (A)** Schematic representation of the *ECT1* promoter. The promoter fragment is used for (B). **(B)** EMSA. Biotin-labeled *ECT1* −811…−828 or *ECT1* −101…−119 promoter fragments with a wild-type *AAAAG* motif were incubated with GST-DAG2 or GST alone (negative control). Samples were analyzed using native polyacrylamide gel electrophoresis (PAGE). The gel was blotted onto a nylon membrane, and the signals were detected by streptavidin-coupled horseradish peroxidase and electrochemiluminescence (ECL). FP, free probe. Cold, excess of the DNA fragment, but unlabeled. In the experimental groups containing “cold” during the process, “cold” is added first to allow it to fully bind to GST-DAG2, and then the tested promoter is added 15 min later.

图示

描述已自动生成**Figure S13. DAG2 interacts with the *DAG2* promoter. (A)** Schematic representation of the *DAG2* promoter. The promoter fragment is used for (B). **(B)** EMSA. Biotin-labeled *DAG2* −1486…−1504 or *DAG2* −500…−518 promoter fragments with a wild-type *AAAAG* motif were incubated with GST-DAG2 or GST alone (negative control). Samples were analyzed using native polyacrylamide gel electrophoresis (PAGE). The gel was blotted onto a nylon membrane, and the signals were detected by streptavidin-coupled horseradish peroxidase and electrochemiluminescence (ECL). FP, free probe. Cold, excess of the DNA fragment, but unlabeled. In the experimental groups containing “cold” during the process, “cold” is added first to allow it to fully bind to GST-DAG2, and then the tested promoter is added 15 min later.

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描述已自动生成**Figure S14. DAG2 interacts with the *PHYB* promoter. (A)** Schematic representation of the *PHYB* promoter. The promoter fragment is used for (B). **(B)** EMSA. Biotin-labeled *PHYB* −1295…−1312 or *PHYB* −303…−320 promoter fragments with a wild-type *AAAAG* motif were incubated with GST-DAG2 or GST alone (negative control). Samples were analyzed using native polyacrylamide gel electrophoresis (PAGE). The gel was blotted onto a nylon membrane, and the signals were detected by streptavidin-coupled horseradish peroxidase and electrochemiluminescence (ECL). FP, free probe. Cold, excess of the DNA fragment, but unlabeled. In the experimental groups containing “cold” during the process, “cold” is added first to allow it to fully bind to GST-DAG2, and then the tested promoter is added 15 min later.

图表

描述已自动生成**Figure S15. ECT1 regulates ABA signaling-related genes.** Comparison of *PP2A*, *ABF3*,and *ABI4* mRNA expression in germinating Col-0 and *ect1-1* seeds (Seeds were kept in the dark at 22 °C for one hour and then exposed to red light for 5 minutes to induce germination. Following the red-light treatment, the seeds were returned to darkness for 12 hours). *ACT7* served as the reference gene for normalization. The presented data represents the average (of three replicates) ± SD. Statistical significance was assessed using Student’s t-test, ns: not significant, \*\*: *p* < 0.01, \*\*\*\*: *p* < 0.0001.