Arabidopsis RNA-binding proteins controlling development

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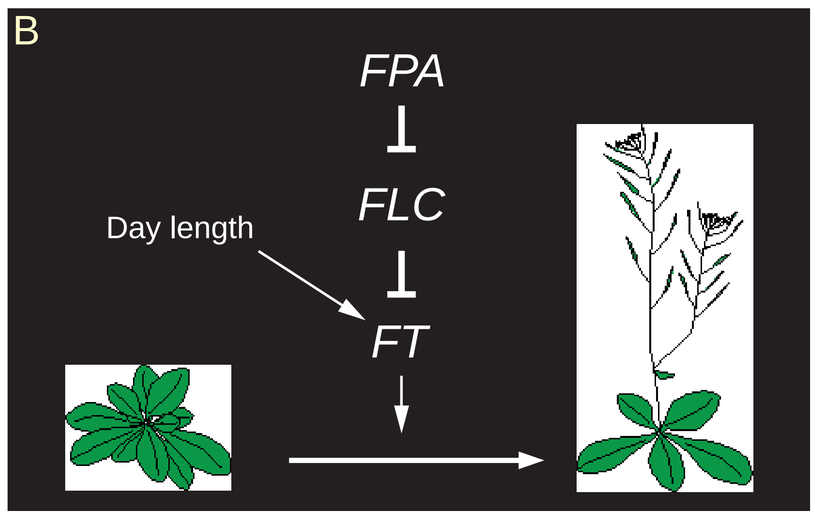
FPA, A RRM-CONTAINING PROTEIN INVOLVED IN REGULATION OF FLOWERING TIME

Like all living organisms, plants must sense and adapt appropriately to environmental changes in order to survive. The switch to flowering (reproductive growth) is a key step in their life cycle. Therefore, to maximise reproductive success, plants have developed mechanisms to adapt the time at which they flower. These mechanisms rely on complex and intricate networks of gene expression regulation.

Genetics allowed the isolation of many genes involved in controlling flowering time. Genes encoding RNA-binding or RNA-processing proteins feature prominently. However the mechanisms by which these proteins may regulate gene expression are poorly documented. By studying FPA, a poorly characterised RRM-containing protein promoting flowering, we aim to deepen our knowledge of the roles of RNA-binding proteins in regulating gene expression *in vivo*.

FPA encodes a protein comprising 3 RNA-recognition motifs (RRM) indicating a function in RNA processing. *FPA* promotes flowering: *fpa* mutants flower later than WT in both long and short days **(A)**. As genetically defined, the major function of *FPA* is the down-regulation of *FLC* expression **(B)**. *FLC* is a strong repressor of flowering: it down-regulates the expression of floral pathway integrators such as *FT*, thereby delaying flowering **(B)**. Its expression is regulated by several genetic pathways **(C)**. *FPA* belongs to the autonomous pathway. However, the mechanism used to down-regulate *FLC* expression as well as the direct RNA targets of FPA are unknown.

fpa mutant flowers late.
Both genotypes were grown alongside each other under the same conditions.



***FPA* regulates gene expression to promote flowering.**
See "*FPA*-regulated gene expression" panel for real-time RT-PCR data.

C

AUTONOMOUS

FCA: RRM, mRNA 3' end processing
FLD: LSD-like, histone methylase
FLK: KH domain
FPA: RRM
FVE: MSI1-like, histone modification
FY: Pfs2-like, mRNA 3' end processing
HAC1: Histone acetyltransferase
LD: Homeodomain, interacts with SUF4
PRP39-1: TPR repeats, Splicing factor
REF6: Jumonji/Zn finger
SKB1: Histone arginine methylase
U2AF35b: Splicing factor

PAF1c

EFS: SET domain, H3 K4 methyltransferase
ELF7: Paf1-like, PAF1 complex
ELF8: Ctr9-like, PAF1 complex
VIP3: WD repeats, PAF1 complex
VIP4: Leo1-like, PAF1 complex
VIP5: Rtf1-like, PAF1 complex

SWR1/SRCAPc

ARP6: Actin-related protein, SWR1/SRCAP complex
HTA8: Histone H2A.Z
HTA9: Histone H2A.Z
HTA11: Histone H2A.Z
PIE1: ISW1p-like, SWR1/SRCAP complex
SEF: SWC6-like, SWR1/SRCAP complex

FRIGIDA

FES1: CCCH-type Zinc FInger
FRI: Coiled coil domains
FRL1: Related to FRI and FRL2
FRL2: Coiled coil domains
SUF4: C2H2-type Zinc Finger

OTHER

ABH1: CBC80, mRNA cap-binding protein
ART1: Unmapped
ELF5: NpW38-binding protein-like
ESD4: SUMO-specific protease
HOS1: Ubiquitin E3 ligase
HUA2: RPR domain, pre-mRNA splicing
MBD9: Methyl-CpG-binding domains
SAR1: NUP160-like, nucleoporin
SAR3: NUP96-like, nucleoporin

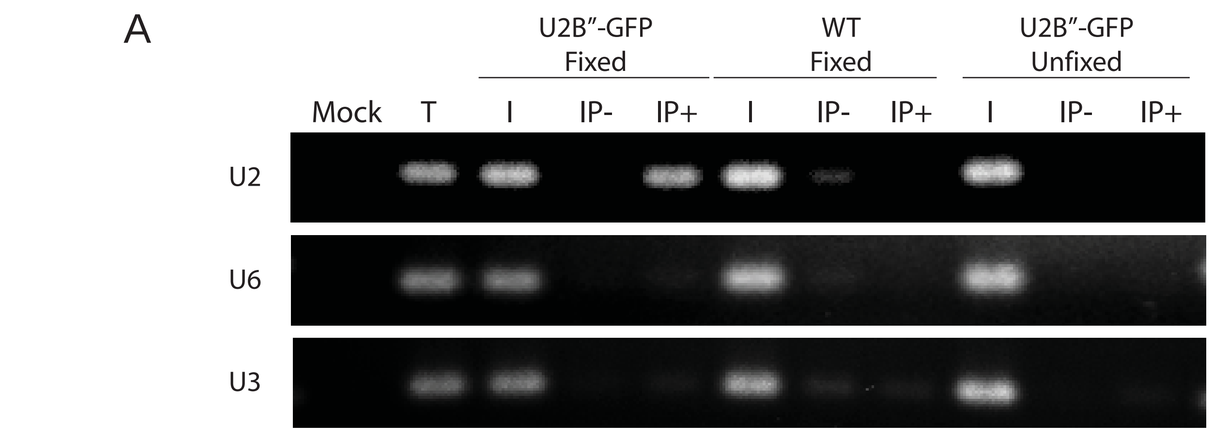
FLC expression is controlled by different genetics pathways.
Pathways promoting *FLC* expression are highlighted in green, those repressing in red. Genes encoding RNA-processing factors are highlighted in orange.

RIP: RNA IMMUNOPRECIPITATION TO IDENTIFY DIRECT RNA TARGETS OF FPA

To characterize the direct RNA targets of FPA *in vivo*, we are adapting to *Arabidopsis* two powerful techniques: RNA Immunoprecipitation (RIP, see below) and Cross-Linking and Immuno-Precipitation (CLIP).

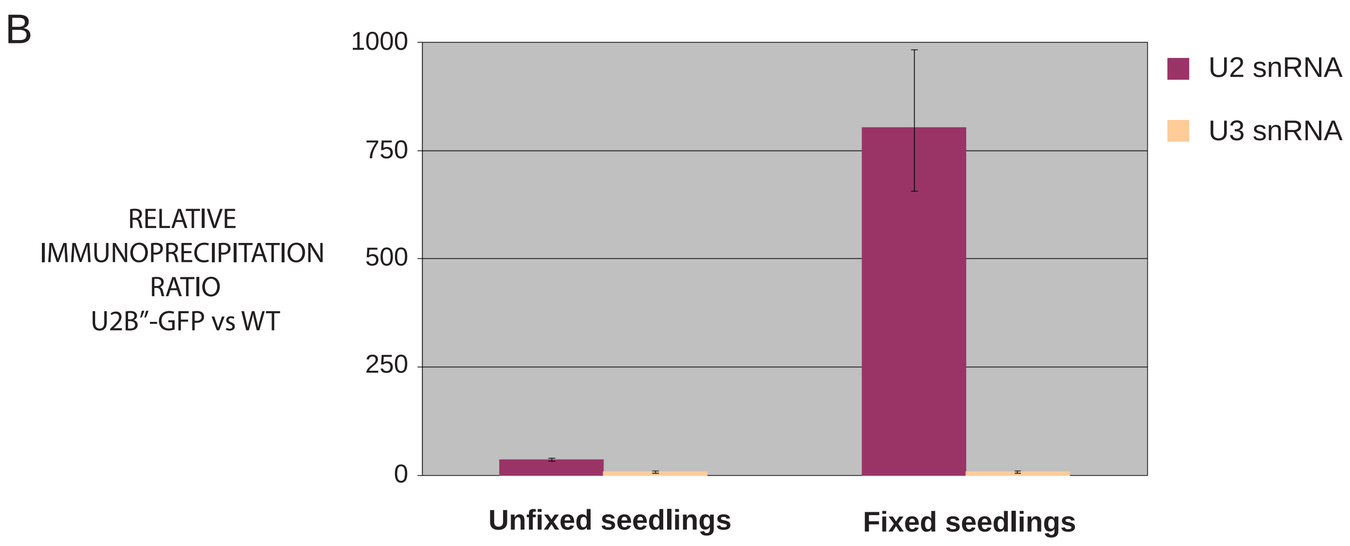
In RIP, 14-days old seedlings are treated with formaldehyde to fix RNA-protein interactions *in vivo*. Nuclear extracts are immunoprecipitated with specific antibodies under stringent conditions to disrupt unfixed interactions and immunoprecipitated RNA are recovered and analysed by RT-PCR or using DNA microarrays (see "FPA-regulated gene expression" panel).

We have already developed RIP in *Arabidopsis* using the previously characterized interaction between U2B" and U2 snRNA **(A)**. Seedlings overexpressing U2B" fused to GFP (U2B"-GFP Fixed lanes) were used for nuclear extracts preparation. Immunoprecipitation was performed with commercial GFP antibodies and RT-PCR was used to detect U2, U6 or U3 snRNA. Only U2 was immunoprecipitated by anti-GFP antibodies, revealing the specificity of our RIP assay. Specificity was further demonstrated by the requirement for U2B"-GFP expression (WT fixed lanes) and formaldehyde fixation (U2B"-GFP Unfixed lanes).



RT-PCR detection of U2, U6 and U3 snRNA upon RIP of U2B"-GFP
See text above for details.

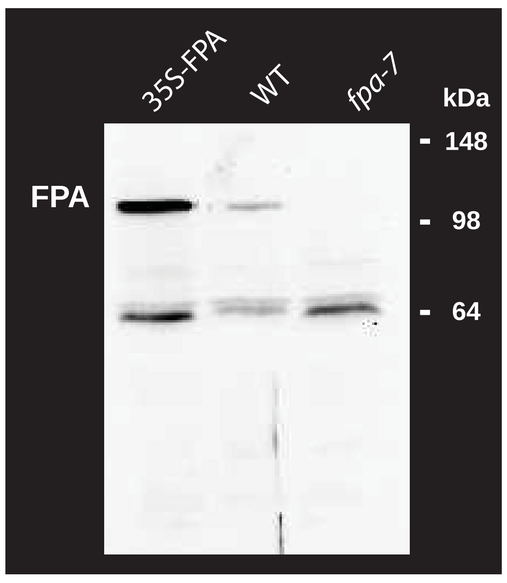
To obtain greater precision, RIP samples were further analysed by real-time RT-PCR **(B)**. The relative immunoprecipitation ratios of U2 and U3 snRNA between IP+ and IP- reactions with U2B"-GFP Fixed or Unfixed seedlings were calculated and expressed relative to reactions with WT Fixed seedlings using the 2-DeltaDeltaCt method. This analysis confirmed the specificity of our RIP assay. We are currently working out more stringent conditions to decrease the immunoprecipitation levels of unspecific and unfixed RNAs (data not shown).



Quantitative RT-PCR analysis of U2 and U3 snRNA from samples described in (A).
See text above for details. Each real-time PCR reaction was performed in triplicates.

We are currently performing RIP assays to identify direct RNA targets of FPA. We already raised antibodies against FPA and purified them by affinity (C). In the absence of known direct targets of FPA, RNA immunoprecipitated by anti-FPA antibodies will be detected using Affymetrix GeneChip® Arabidopsis Tiling 1.0R Array (see "FPA-regulated gene expression" panel).

C



Western blot detection of FPA in total *Arabidopsis* protein extracts
Genotypes are indicated on top of the figure.

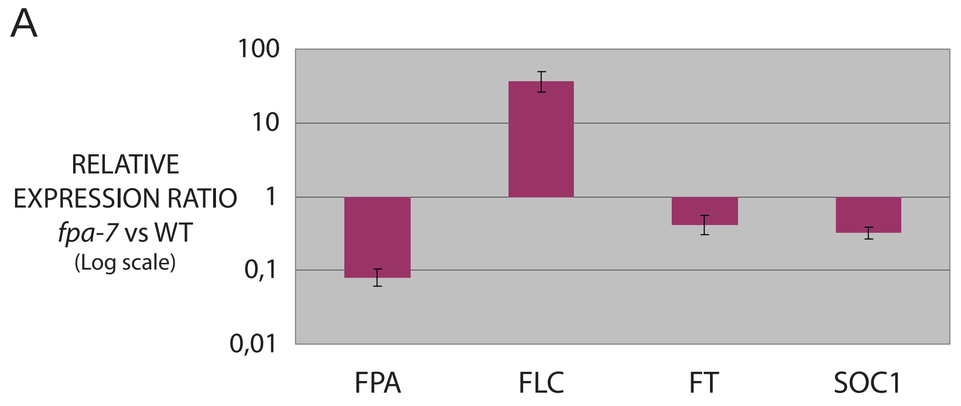
FPA-REGULATED GENE EXPRESSION

To widen our knowledge of *FPA*-regulated gene expression, mRNA abundances in Col0 (WT) and *fpa-7* will be compared using Affymetrix GeneChip® Arabidopsis Tiling 1.0R Array.

This DNA microarray consists of probes (25 base pairs) tiled through the complete non-repetitive *Arabidopsis* genome at an average of 35 base pair resolution, therefore leaving a gap of only 10 base pairs between probes.

It allows to detect changes in gene expression (for annotated and unannotated transcription units) as well as in RNA processing events.

RNA samples are prepared from 14 days-old seedlings (in biological triplicates for each genotype) using TRIzol. We could already confirm by quantitative RT-PCR that *fpa-7* mutation affects the expression of known FPA-regulated genes **(A)**.



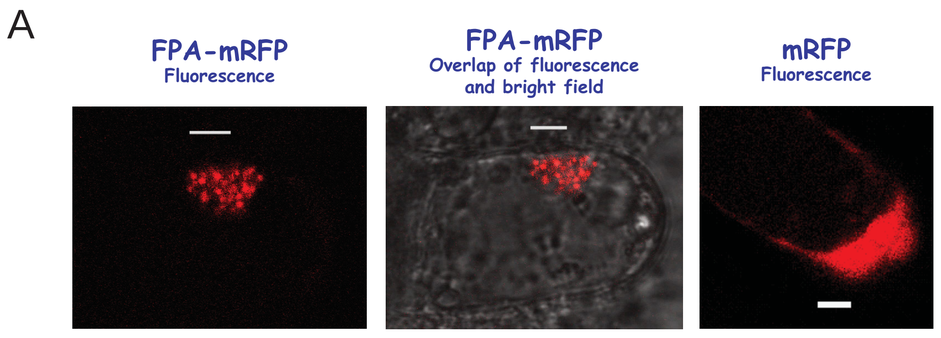
Real-time RT-PCR analysis of gene expression in *fpa-7* vs WT seedlings.
Real-time PCR data were analysed with the 2-DeltaDeltaCt method. For each genotype, three biological replicates were used to isolate total RNA using TRIzol. Each real-time PCR reaction was performed three times.

FPA IS A NUCLEAR PROTEIN ENRICHED IN SPECKLES.

Our preliminary analysis of purified nuclei from *Arabidopsis* suspension cells indicated FPA to be a nuclear protein (data not shown).

We confirmed this finding by localization of FPA-mRFP fusion protein expressed in *Arabidopsis* suspension cells using confocal microscopy (A).

Moreover, compared to mRFP (right panel), FPA appears to be concentrated in nuclear speckles. We are currently performing co-localization with other nuclear proteins to identify these FPA-containing structures.



***In vivo* localization of FPA fused to mRFP by confocal microscopy.**
FPA-mRPF was expressed in *Arabidopsis* cells maintained in culture.

CONCLUSIONS AND PERSPECTIVES

The characterization of FPA *in vivo* RNA targets by RIP and CLIP, together with assays aiming to reveal the function of FPA, will shed new light on the direct role of this RNA-binding protein in gene expression regulation.

Moreover RIP and CLIP will add invaluable tools towards the characterization of the roles and functions of uncharacterized RNA-binding proteins in *Arabidopsis thaliana*.

ACKNOWLEDGEMENTS

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