

A Protein Complex Required for Polymerase V Transcripts and RNA-Directed DNA Methylation in *Arabidopsis*

Julie A. Law,¹ Israel Ausin,¹ Lianna M. Johnson,²
Ajay A. Vashisht,⁵ Jian-Kang Zhu,^{3,4}
James A. Wohlschlegel,^{5,*} and Steven E. Jacobsen^{1,6,*}

¹Department of Molecular Cell and Developmental Biology, University of California at Los Angeles, Los Angeles, CA 90095, USA

²Life Sciences Core Curriculum, University of California Los Angeles, Los Angeles, CA 90095, USA

³Center for Plant Stress Genomics Research, 4700 King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia

⁴Department of Botany and Plant Sciences, University of California, Riverside, Riverside, CA 92521, USA

⁵Department of Biological Chemistry, David Geffen School of Medicine at UCLA, Box 951737, BSRB-377A, 615 Charles E. Young Drive South, Los Angeles, CA 90095-1737

⁶Howard Hughes Medical Institute, University of California at Los Angeles, Los Angeles, California 90095, USA

Summary

DNA methylation is an epigenetic modification associated with gene silencing. In *Arabidopsis*, DNA methylation is established by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), which is targeted by small interfering RNAs through a pathway termed RNA-directed DNA methylation (RdDM) [1, 2]. Recently, RdDM was shown to require intergenic noncoding (IGN) transcripts that are dependent on the Pol V polymerase. These transcripts are proposed to function as scaffolds for the recruitment of downstream RdDM proteins, including DRM2, to loci that produce both siRNAs and IGN transcripts [3]. However, the mechanism(s) through which Pol V is targeted to specific genomic loci remains largely unknown. Through affinity purification of two known RdDM components, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) [4] and DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) [5, 6], we found that they copurify with each other and with a novel protein, RNA-DIRECTED DNA METHYLATION 1 (RDM1), forming a complex we term DDR. We also found that DRD1 copurified with Pol V subunits and that RDM1, like DRD1 [3] and DMS3 [7], is required for the production of Pol V-dependent transcripts. These results suggest that the DDR complex acts in RdDM at a step upstream of the recruitment or activation of Pol V.

Results and Discussion

DRD1 and DMS3 Copurify with Each Other and a Novel Protein, RDM1, as Well as with Pol V Subunits

To better understand the roles of DRD1, a putative chromatin remodeler, and DMS3, a protein with homology to the hinge region of structural maintenance of chromosome (SMC)

proteins, in RdDM, we generated transgenic *Arabidopsis* plants expressing epitope fusions of either DRD1 or DMS3 and used these plants to affinity purify complexes containing these proteins from flower extracts. Various epitope-tagged fusions of DRD1 and DMS3, driven by their endogenous promoters, were able to complement defects in DNA methylation observed in their respective mutant backgrounds at the *MEA-ISR* locus (Figure 1), demonstrating that these fusion proteins are functional *in vivo*. After affinity purification of DRD1-3xFlag-BLRP or DMS3-3xFlag-BLRP, copurifying proteins were identified through mass spectrometric (MS) analyses (Table 1; Table S1, available online). Peptides corresponding to DRD1, DMS3, and At3g22680 were by far the most abundant in both purifications (Table 1 and Table S1). Copurification of At3g22680 with both DRD1 and DMS3 suggested that At3g22680 may be a novel component of the RdDM pathway. Indeed, a mutation in At3g22680, termed RNA-DIRECTED DNA METHYLATION 1 (RDM1), was recently isolated from a genetic screen for proteins necessary for RdDM [8].

While the identities of the proteins in the DRD1 and DMS3 purifications were similar, the relative stoichiometries were quite different, suggesting that these proteins may be present in more than one complex. Affinity purification of DRD1 yielded DRD1, DMS3, and RDM1 at roughly similar levels (Table 1), suggesting that these three proteins may form a stable complex *in vivo*. Consistent with this notion, pair-wise interactions between DRD1, DMS3, and RDM1 were confirmed by coprecipitation experiments (Figure 2 and Figure S1A). Furthermore, these pair-wise interactions were found to be resistant to DNase and RNase treatment (Figures S1B and S1C) and were stable under high-salt conditions (Figures S1C and S1D), suggesting that the associations between these three proteins are stable and mediated by protein-protein interactions. The DRD1 purification also yielded peptides corresponding to many of the previously identified Pol V subunits, but not subunits specific to Pol II or Pol IV [9–12] (Table 1). However, the relative abundance of Pol V peptides was much lower than those of DMS3 and RDM1, which could reflect either a weak association of Pol V with DRD1 or a strong association of Pol V with a small fraction of DRD1. Nonetheless, an interaction between NRPE1, the largest subunit of Pol V, and DRD1 was confirmed by coimmunoprecipitation (Figure 2C).

Upon purification of DMS3, the relative abundance of DRD1 and RDM1 was significantly lower when compared to the DRD1 purification (Table 1), suggesting that DMS3 may only be interacting with DRD1 and RDM1 a portion of the time. There were also fewer peptides corresponding to the subunits of the Pol V polymerase in the DMS3 purification (Table 1). Although the interaction between DMS3 and NRPE1 was not confirmed by coimmunoprecipitation analysis, presumably because of sensitivity issues, peptides corresponding to Pol V subunits were detected in two independent DMS3 purifications. Together, these findings suggest that DRD1 and DMS3 may be present in multiple complexes, one of which contains DRD1, DMS3, and RDM1, and at least one other that contains DRD1, possibly DMS3, and subunits of the Pol V polymerase.

*Correspondence: jwohl@mednet.ucla.edu (J.A.W.), jacobsen@ucla.edu (S.E.J.)

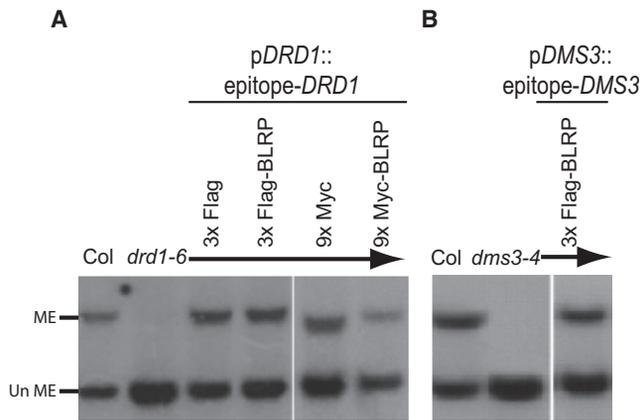


Figure 1. Complementation of Mutants with Epitope-Tagged DRD1 and DMS3

Analysis of DNA methylation at the *MEA-ISR* locus by Southern blotting after digestion of genomic DNA with the methylation-sensitive restriction enzyme, *MspI*. Bands representing methylation (ME) or a lack of methylation (un ME) are indicated. Digestion of genomic DNA extracted from wild-type plants of the Colombia (Col) ecotype serve as a positive control for DNA methylation levels. BLRP, biotin ligase recognition peptide. See also Tables S2 and S3.

(A) The loss of DNA methylation in the *drd1-6* mutant alone and the restoration of DNA methylation after transformation of this mutant with a transgene carrying the indicated Flag and Myc epitope-tagged fusion of the DRD1 gene under the control of its endogenous promoter (pDRD1).

(B) The loss of DNA methylation in the *dms3-4* mutant alone and the restoration of DNA methylation after transformation of this mutant with a transgene carrying the indicated Flag epitope-tagged fusion of the DMS3 gene under the control of its endogenous promoter (pDMS3). Complementation assays shown were conducted with tissue from T3 homozygous transgenic plant lines.

Gel Filtration Profiles of DRD1, DMS3, RDM1, and NRPE1

To further characterize the associations between DRD1, DMS3, RDM1, and Pol V, we generated protein extracts from F1 flowers resulting from a cross between 9xMyc-DRD1 and DMS3-3xFlag-BLRP transgenic plants and analyzed these extracts by gel filtration followed by western blotting. This analysis, like the MS analysis, supports the notion that DRD1 and DMS3 are probably present in multiple protein complexes. With the use of a Superose 6 column, DRD1 eluted as a broad

Table 1. Mass Spectrometric Analyses of DRD1 and DMS3 Affinity Purifications

DRD1 Purification					
Protein	Spectra	Unique Peptides	% Coverage	NSAF	% of DRD1
DRD1	245	50	38.6	2.29E-03	1.00
At3g22680	48	11	52.8	2.45E-03	1.07
DMS3	115	30	48.3	2.28E-03	0.99
NRPE1*	45	35	20.2	1.89E-04	0.08
NRPE2	20	10	9.1	1.42E-04	0.06
NRPE3A	14	9	32	3.65E-04	0.16
NRPE3B	5	4	16	1.30E-04	0.06
NRPE5*	3	2	12.6	1.12E-04	0.05
NRPE7*	2	2	11.8	9.34E-05	0.04
NRPE9A	3	3	34.2	2.19E-04	0.10
DMS3 Purification					
Protein	Spectra	Unique Peptides	% Coverage	NSAF	% of DMS3
DMS3	2804	98	87.1	3.45E-02	1.00
At3g22680	184	20	60.1	5.84E-03	0.1691
DRD1	94	39	41.3	5.48E-04	0.0159
NRPE1*	4	4	3.3	1.05E-05	0.0003
NRPE2	5	4	5	2.21E-05	0.0006
NRPE3A	4	4	13.8	6.49E-05	0.0019

Proteins copurifying with DRD1 (upper) or DMS3 (lower) are indicated, and approximate stoichiometry is shown as the percentage of DRD or DMS3 using NSAF values [23]. *Polymerase subunits that are specific to (or highly enriched in) Pol V over Pol II and Pol IV complexes [10]. The other subunits are shared with Pol II, Pol IV, or both polymerases [10]. See also Table S1.

high-molecular-weight peak that coeluted with the peak of endogenous NRPE1 and a small portion of the total DMS3 protein (Figure 3). These findings are consistent with the presence of Pol V peptides in the DRD1 purification. In addition, the fact that a smaller proportion of DMS3 coeluted with NRPE1 than was observed for DRD1 is consistent with the identification of fewer Pol V peptides in the DMS3 purification than in the DRD1 purification. In addition to its coelution with NRPE1, DRD1 is also present in lower-molecular-weight fractions, where the majority of DMS3 and RDM1 coelute around 440KDa (Figure 3), suggesting that DRD1 associates with Pol V in a complex that is largely separate from its association with DMS3 and RDM1. This finding is also consistent with the

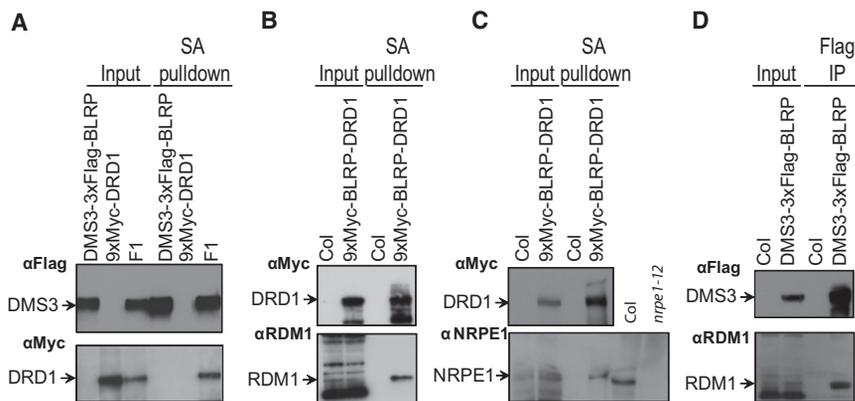


Figure 2. Characterization of DDR Complex Components

(A–D) Streptavidin (SA) pull-down and coimmunoprecipitation assays confirming interactions from mass spectrometric analyses. The BLRP tag is biotinylated in vivo, allowing interaction with streptavidin. Input lanes confirm expression of the epitope fusion proteins and the endogenous NRPE1 or RDM1 proteins in the parental lines indicated above each lane. F1 represents a cross between the two parental lines. Because these F1 lines only possess a single copy of each transgene, they exhibit lower expression levels as compared to the parental lines. SA pull-down lanes show coprecipitation of (A) DRD1 with DMS3, (B) DRD1 with RDM1, and (C) DRD1 with NRPE1, and Flag coimmunoprecipitation lanes show (D) DMS3 with RDM1. In (C), protein extracts from Col and *nrpe1-12* plants are included to confirm the identity of the coprecipitating band. For each western blot, the antibody used is indicated (upper left). See also Figure S1.

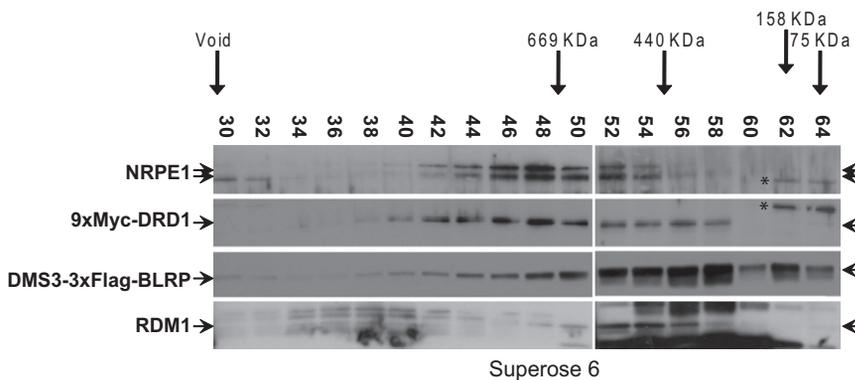


Figure 3. Gel Filtration of Copurifying Proteins
The elution profiles of NRPE1, RDM1, 9xMyc-DRD1, and DMS3-3xFlag-BLRP on a Superose6 column were detected with antibodies against endogenous NRPE1, endogenous RDM1, and either the Myc or the Flag epitope, respectively. Fraction numbers and sizing standards are indicated. In fractions 62 and 64, nonspecific background bands are marked by an asterisk (*). See also Figure S2.

presence of two distinct peaks of DRD1 after gel filtration with a superdex 200 column (Figure S2A), which gives better resolution of lower-molecular-weight complexes. Finally, DMS3 is also present in a slower eluting peak, the approximate size predicted for a DMS3 monomer (Figure 3 and Figure S2B).

Together the elution profiles of these proteins are in general agreement with the coprecipitation data and the MS analyses, demonstrating that a portion of DRD1, DMS3, and RDM1 coelute as a complex around 440 KDa and that DRD1, and DMS3 to a lesser extent, coelute with NRPE1 in higher-molecular-weight associations. However, the stoichiometry of the complex containing DRD1, DMS3, and RDM1 appears to differ between the MS and gel-filtration techniques. This difference could be attributed to the different sample preparation procedures used for the two techniques, with only the most stable interactions withstanding the more lengthy affinity purification procedure.

RDM1 Is Required for the Production of Pol V-Dependent Transcripts and DNA Methylation

Copurification of peptides corresponding to RDM1 with DRD1 and DMS3 suggests that this protein may also be required for RdDM. *RDM1* encodes an ~18 KDa protein of unknown function, and a crystal structure of RDM1 revealed that this protein has a unique fold [13]. To assess the role of this protein in RdDM, we analyzed the level of DNA methylation at the *MEA-ISR* locus by Southern blotting in a *ros1-1 rdm1-1* mutant background that was isolated from a *ros1* suppressor screen [8]. DNA methylation was lost in the *ros1-1 rdm1-1* mutant to a similar extent as observed for *nrpe1-12*, *drd1-6*, and *dms3-4*, demonstrating that RDM1 is required for RdDM at this locus (Figure 4A). Extensive analysis of DNA methylation at several other loci using several *rdm1* alleles, including an allele in a wild-type background, showed similarly strong losses of methylation demonstrating a general role of RDM1 in RNA-directed DNA methylation [8].

Because RDM1 copurified with components of RdDM known to affect the accumulation of IGN transcripts [3, 7], we tested the hypothesis that RDM1 would also be required for wild-type levels of such transcripts. We used quantitative reverse-transcriptase PCR (RT-PCR) to assess the levels of the IGN5 transcript in a *ros1-1 rdm1-1* mutant and found them to be reduced to a similar level as observed in *drd1-6*, *dms3-4*, and *nrpe1-12* mutants (Figure 4B). A previously unidentified Pol V-dependent transcript corresponding to the *MEA-ISR* locus was also found to depend on DRD1, DMS3, NRPE1, and RDM1 (Figure 4C). Thus, all the major proteins copurifying with DRD1 and DMS3 are required for the accumulation of Pol V-dependent IGN transcripts.

Our findings demonstrate that in addition to other associations, DRD1, DMS3, and RDM1 form a complex that we term DRD1-DMS3-RDM1 (DDR) and that RDM1, like DRD1 and DMS3, is required for the accumulation of Pol V-dependent transcripts. Furthermore, we show that DRD1 associates with many subunits of the Pol V complex. Together, these findings provide further insight into the mechanism through which intergenic transcripts are produced by Pol V. Because Pol V subunits copurify with DRD1, and DMS3 to a lesser extent, and because both DRD1 and DMS3 are required for the association of the NRPE1 subunit of Pol V with chromatin [3, 7], we propose that the DDR complex assists in the recruitment or activation of Pol V, after which DRD1, which contains a chromatin remodeling domain, may be important for the initiation or elongation of IGN transcripts by remodeling chromatin ahead of the Pol V polymerase.

Experimental Procedures

Generation of Gateway Entry Clones

Genomic fragments containing the promoter and genomic DNA corresponding to either the *DRD1* or the *DMS3* locus were amplified from the F16F14 BAC (ABRC) or genomic DNA isolated from the Col ecotype, respectively, by PCR with the following primers (Table S2): JP4003 and JP4004 for DRD1 and JP5446 and JP5447 for DMS3. PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen) per the manufacturer's instructions. For DRD1 and DMS3, carboxy-terminal 3xFlag and 3xFlag-BLRP tags (Table S3) were inserted into a 3' Asc I site in the pENTR/D-TOPO vector. For DRD1, amino-terminal 9xMyc and 9xMyc-BLRP tags were inserted into a Nco I restriction site engineered into the DRD1 genomic sequence upstream of the start codon through Quikchange Site-Directed Mutagenesis (Stratagene) per the manufacturer's instructions with the primers JP4430 and JP4431.

Generation of Gateway Destination Clones and Selection of Transgenic Arabidopsis Plants

The described pENTR/D constructs were digested with the Mlu I restriction enzyme and then recombined into a modified gateway destination vector based on the pEarleyGate vectors [14], as described in [15], which contains the BirA gene under the control of an *ACT1N* promoter and a gene conferring resistance to the BASTA herbicide, per the manufacturer's instructions (Invitrogen). BirA recognizes a lysine residue in the BLRP tag and catalyzes the addition of a biotin moiety onto this residue, which is recognized by streptavidin. These DNA constructs were then transformed into the AGLO strain of *Agrobacterium* by electroporation. *Arabidopsis* plants carrying the *drd1-6* mutant allele or the *dms3-4* mutant allele were transformed with DRD1 or DMS3 epitope-tagged constructs, respectively, as described in [16]. Transformed plants were BASTA selected and scored for single inserts of the transgene by segregation analysis. Protein expression and biotinylation were assessed by western blotting with antibodies against the Flag or Myc epitope or with streptavidin.

Southern Blotting

Complementation of the epitope-tagged DRD1 and DMS3 proteins, as well as the effect of a mutation in the *RDM1* gene on DNA methylation, were

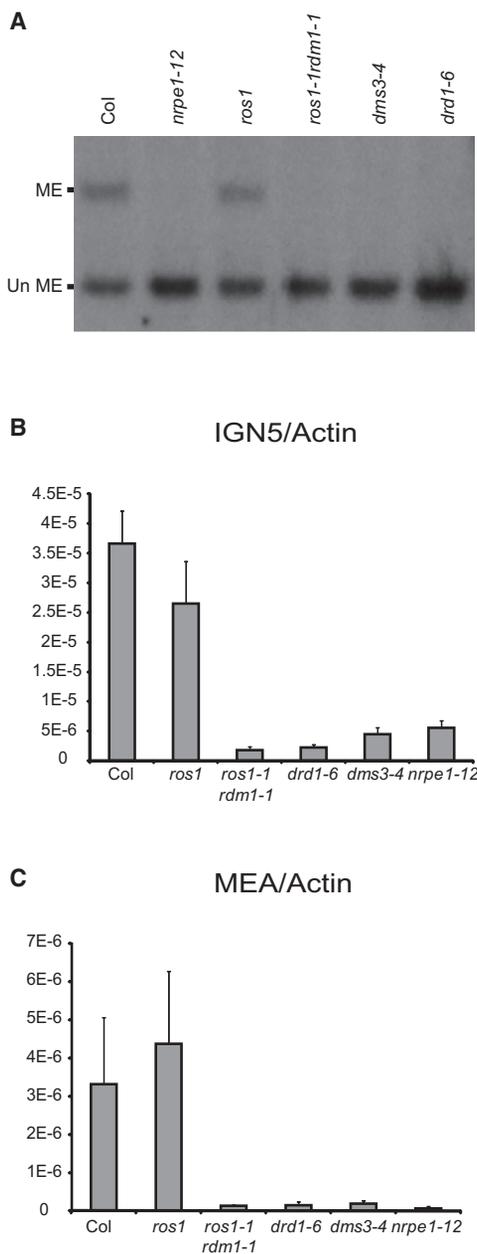


Figure 4. DNA Methylation and IGN Transcript Defects in a *ros1 rdm1* Mutant

(A) Southern-blot analysis as described in Figure 1 with DNA from wild-type Col plants or from the indicated mutant plants.

(B and C) Quantitative RT-PCR analysis of the abundance of Pol V-dependent transcripts corresponding to the (B) *IGN5* and (C) *MEA-ISR* loci in the indicated genetic backgrounds after normalization to the level of an *ACTIN* transcript. Error bars represent the SD among at least three biological replicates.

assessed by Southern blotting with a probe specific of the *MEA-ISR* locus as previously described [15].

Affinity Purification

Approximately 8 g of flower tissue collected from 3xFlag and 3xFlag-BLRP-DRD1 or 3xFlag-BLRP-DMS3 transgenic T_4 plants, or from Col plants as a negative control, were ground to a fine powder with a mortar and pestle in liquid nitrogen and suspended in 45 ml of lysis buffer (LB: 50 mM Tris [pH 7.6], 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, 0.1% NP-40, 0.5 mM DTT, 1 μg/μl pepstatin, 1 mM PMSF, and 1 protease inhibitor cocktail tablet

[Roche, 14696200]). The tissue was further homogenized by douncing and then centrifuged in an SS34 rotor for 25 min at 12,500 rpm. One hundred and twenty-five microliters of Dynabeads (M-270 Epoxy, Invitrogen, 143.01) that had been conjugated with Flag antibody (Sigma F 3165) according to the manufacturer's instructions were added to the supernatant for the DRD1 purification and 600 μl of 50% slurry Flag agarose beads for the DMS3 purification. After incubation at 4°C with rotation for 2.5 hr, the Flag beads were washed twice for 5 min with 40 ml of LB and then 5 times for 5 min with 1 ml of LB. Proteins were then eluted from the Flag beads by competition with 150 μl of 100 μg/ml of 3xFlag peptide (Sigma, F 4799) five times at room temperature.

Mass Spectrometry

The eluted protein complexes were precipitated by the addition of trichloroacetic acid and then digested by the sequential addition of lys-C and trypsin proteases as previously described [17]. The digested peptide samples were then fractionated online with sequential strong-cation exchange and reversed-phase chromatography and eluted directly into a LTQ-Orbitrap mass spectrometer (ThermoFisher) where MS/MS spectra were collected [18, 19]. Data analysis was performed with the SEQUEST and DTASelect2 algorithms, and peptide identifications were filtered with a false-positive rate of less than 5% as estimated by a decoy database strategy [20–22]. Normalized spectral abundance factor (NSAF) values were calculated as described in [23].

SA Pull-Downs and Coimmunoprecipitation Analysis

From the indicated plant lines, 0.5 g of flower tissue was ground in liquid nitrogen with 2.5 ml of LB and spun in microfuge tubes for 10 min at 4°C at 13,200 rpm. The supernatants were incubated with 100 μl of streptavidin agarose (50% slurry Upstate, 16-126) or with M2 Flag agarose (50% slurry, Sigma A2220) for 2.5 hr at 4°C with rotation. After washing the beads five times with 1 ml of LB for 5 min each, the beads were resuspended in 50 μl of SDS-PAGE loading buffer and boiled for 5 min. Thirty microliters of input and bead eluate were run on 8% (Figure 2A) or 4%–12% (Figures 2B–2D) SDS-PAGE gels, and the various proteins were detected by western blotting. Flag westerns were carried out with the ANTI-FLAG M2 monoclonal antibody-peroxidase conjugate (Sigma A 8592) at a dilution of 1:5000. Myc westerns used the c-Myc 9E10 mouse monoclonal antibody (Santa Cruz Biotechnology, sc-40) at a dilution of 1:5000 as the primary antibody, and goat anti-mouse IgG horseradish peroxidase (Thermo Scientific, 31430) was used at a dilution of 1:5000 as the secondary antibody. For NRPE1, an antibody to the endogenous protein initially described in [24] was used at a dilution of 1:1000 as the primary antibody, and goat anti-rabbit IgG horseradish peroxidase (Thermo Scientific, 31460) was used at a dilution of 1:5000 as the secondary antibody. For RDM1, an antibody to the endogenous protein was used at a dilution of 1:3000 as the primary antibody, and goat anti-rabbit IgG horseradish peroxidase (Thermo Scientific, 31460) was used at a dilution of 1:25000 as the secondary antibody. All westerns were developed with ECL Plus Western Blotting Detection System (GE healthcare RPN2132).

Salt Stability and DNase and RNase Treatment

Coimmunoprecipitation and pull-down assays testing the salt stability of the protein associations were conducted as above with the following alterations: 1.5 g of the indicated tissue was ground in 7.5 ml of LB, centrifuged as above, and incubated with 300 μl of either streptavidin agarose (50% slurry Upstate, 16-126) (Figure S1D) or M2 Flag agarose beads (50% slurry, Sigma A2220) (Figure S1E) for 2.5 hr at 4°C with rotation. The beads were then washed once with 10 ml of LB and then distributed evenly between three microfuge tubes. One aliquot of beads was washed an additional 5 times with 1 ml of LB for 5 min each, another aliquot with LB supplemented with NaCl to a final concentration of 300 mM, and another with LB supplemented with NaCl to a final concentration of 500 mM. The beads were then resuspended in 50 μl of SDS-PAGE loading buffer and boiled for 5 min. Ten or twelve microliters of input and bead eluate were run on 4%–12% SDS-PAGE gels (Figures S1D and S1E), respectively, and the various proteins were detected by western blotting as above. Coimmunoprecipitation and pull-down assays testing stability of the protein associations upon DNase and RNase treatment were conducted with the following alterations: 1.5 g of the indicated tissue was ground in 7.5 ml of LB, then split into three 15 ml conical tubes. Thirty microliters of TURBO DNase (Ambion #AM2239) was added to one tube, 30 μl of RNase, DNase-free (Roche #11 119 915 001) was added to another, and 30 μl of buffer was added to the third tube. Tubes were rotated at 4°C for 30 min, and 250 μl of each extract were

removed to assess the DNase and RNase efficiency after phenol:chloroform extraction and isopropanol precipitation (data not shown). The remaining extract was centrifuged as indicated above and incubated with 100 μ l of either streptavidin agarose (50% slurry Upstate, 16-126) (Figure S1B) or M2 Flag agarose beads (50% slurry, Sigma A2220) (Figure S1C) for 2.5 hr at 4°C with rotation. The beads were then washed five times with 1 ml of LB for 5 min each and then resuspended in 50 μ l of SDS-PAGE loading buffer and boiled for 5 min. Ten microliters of input and bead eluates were run on 4%–12% SDS-PAGE gels (Figures S1B and S1C), and the various proteins were detected by western blotting as above.

Gel Filtration

From the indicated plant lines, 0.3 g of flower tissue were ground in liquid nitrogen with 1.8 ml of LB and spun in microfuge tubes for 10 min at 4°C at 13,200rpm. The supernatants were transferred to new tubes and spun again for 10 min at 4°C at 13,200rpm. The supernatants were then filtered through a .2 micron filter and 500 μ l were loaded onto either a Superdex 200 10/300GL column (GE healthcare, 17-5175-01) column or a Superose 6 10/300 GL column (GE Healthcare, 17-5172-01) and 250 μ l fractions were collected. For the Superose 6 column, 45 μ l of every other fraction were run on a 4%–12% SDS-PAGE and probed for NRPE1, RDM1 and 9xMyc-DRD1 using the antibodies and dilutions outlined above. For DMS3-3xFlag-BLRP, 10 μ l of the same fractions were run on an 8% SDS-PAGE gel and detected using the Flag antibody described above. For the Superdex 200 columns, DRD1-3xFlag-BLRP was detected in 45 μ l from every other fraction and DMS3-3xFlag-BLRP was detected in 10 μ l of each fraction using the Flag antibody. Each column was calibrated prior to use with the Gel Filtration Calibration kit HMW (GE Healthcare, 28-4038-42).

Detection of Pol-V Dependent Transcripts by RT-PCR

RNA was isolated from approximately 0.2 g of flowers or seedlings by grinding in liquid nitrogen and 1 ml of TRIzol Reagent (Invitrogen). RNA was then extracted by using 0.2 ml of chloroform and precipitated with 0.5 ml isopropanol. The RNA pellet was washed with 1 ml 75% ethanol and resuspended in 88 μ l DEPC-treated H₂O. Ten microliters of 10 \times Turbo buffer and 2 μ l Turbo DNase (Ambion) were added and samples incubated for 2 hr at 37°C. RNA was then cleaned up with the RNeasy Mini Kit (QIAGEN). Purified RNA was then eluted with 62 μ l DEPC-treated H₂O, to which 7 μ l of 10 \times Turbo buffer and 1 μ l of Turbo DNase was added. Samples were incubated for another 2 hr at 37°C and DNase was removed with DNase inactivation beads.

Absence of DNA contamination was determined by PCR with no reverse transcriptase added to the reaction. RT-PCR was performed as follows: 1 μ g RNA was mixed with 2 μ l of dNTPs (2.5 mM each) and 1 μ l 12.5 uM primer 1 in a final volume of 11 μ l. This was heated to 65°C for 5 min and cooled on ice for 1 min. Fourteen microliters of a mix containing 2.5 μ l Platinum Taq buffer (minus MgCl₂), 2 μ l 50 mM MgCl₂, 1 μ l 0.1 M DTT, 0.3 μ l RNaseOUT, 0.3 μ l Platinum Taq (Invitrogen), 0.3 μ l SuperScriptIII (Invitrogen), and 0.25 μ l 10 μ M Taqman probe was added to each sample, and incubation continued for 30 min at 55°C, followed by 15 min at 70°C. After the addition of primer 2, the qPCR was started (2 min 95°C; 40 cycles of 15 s at 95°C, 1 min at 60°C). Quantities were determined from a standard curve and results are shown normalized to *ACTIN*. At least three biological replicas were done and standard errors determined.

The primers are as follows: *ACTIN* primer 1, JP2453; *ACTIN* primer 2, JP2452; *ACTIN* probe, TTTTCCTAGTTGAGATGGGAATT; *IGN5* primer 1, JP6606; *IGN5* primer 2, JP6607; *IGN5* probe, TGACCACGGTTAAATGGCGG; *MEA-ISR* primer 1, JP3734; *MEA-ISR* primer 2, JP3734; and *MEA-ISR* probe, TTGGGCCGAATAACAGCAAGTCC.

Supplemental Information

Supplemental Information includes two figures and three tables and can be found with this article online at doi:10.1016/j.cub.2010.03.062.

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