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,³ Jian-Kang Zhu,³,⁴ James A. Wohlschlegel,⁵,⁶ and Steven E. Jacobsen¹,⁶,∗
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 Abdulrahman 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 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.
The loss of DNA methylation in the gene under the control of its endogenous promoter (pDRD1). The loss of DNA methylation after transformation of this mutant with a transgene.

BLRP, biotin ligase recognition peptide. See also Tables S2 and S3.

With the use of a Superose 6 column, DRD1 eluted as a broad and DMS3 are probably present in multiple protein complexes. 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 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 1. Complementation of Mutants with Epitope-Tagged DRD1 and DMS3

Analysis of DNA methylation at the MIA-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.

Figures 2. Characterization of DDR Complex Components

(A–D) Streptavidin (SA) pull-down and coimmunopurification 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 lines show copurification 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.

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

<table>
<thead>
<tr>
<th>Protein</th>
<th>Spectra</th>
<th>Unique Peptides</th>
<th>% Coverage</th>
<th>NSAF</th>
<th>% of DMS3 DRD1 Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRD1</td>
<td>245</td>
<td>50</td>
<td>38.6</td>
<td>2.28E-03</td>
<td>1.00</td>
</tr>
<tr>
<td>3x Flag</td>
<td>48</td>
<td>11</td>
<td>52.8</td>
<td>2.45E-03</td>
<td>1.07</td>
</tr>
<tr>
<td>3x Flag</td>
<td>115</td>
<td>30</td>
<td>48.3</td>
<td>2.28E-03</td>
<td>0.99</td>
</tr>
<tr>
<td>3x Flag</td>
<td>45</td>
<td>35</td>
<td>20.2</td>
<td>1.89E-04</td>
<td>0.08</td>
</tr>
<tr>
<td>3x Flag</td>
<td>20</td>
<td>10</td>
<td>9.1</td>
<td>1.42E-04</td>
<td>0.06</td>
</tr>
<tr>
<td>3x Flag</td>
<td>14</td>
<td>9</td>
<td>32</td>
<td>3.65E-04</td>
<td>0.16</td>
</tr>
<tr>
<td>3x Flag</td>
<td>5</td>
<td>4</td>
<td>16</td>
<td>1.30E-04</td>
<td>0.06</td>
</tr>
<tr>
<td>3x Flag</td>
<td>3</td>
<td>2</td>
<td>12.6</td>
<td>1.12E-04</td>
<td>0.05</td>
</tr>
<tr>
<td>3x Flag</td>
<td>2</td>
<td>2</td>
<td>11.8</td>
<td>9.34E-05</td>
<td>0.04</td>
</tr>
<tr>
<td>3x Flag</td>
<td>3</td>
<td>3</td>
<td>34.2</td>
<td>2.19E-04</td>
<td>0.10</td>
</tr>
</tbody>
</table>

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.
DMS3, is present 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 DRD1, 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 F18F14 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 JP4456 and JP4457 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 ACTIN 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 based on the pEarleyGate vectors [14], as described in [15], which contains the BirA gene under the control of an ACTIN 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

Southern Blotting

Complementation of the epitranscript associated DRD1 and DMS3 proteins, as well as the effect of a mutation in the RDM1 gene on DNA methylation, were
transcript. Error bars represent the SD among at least three biological samples of the indicated genetic backgrounds after normalization to the level of an internal control.

(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 IGN transcript in the indicated loci in the indicated genetic backgrounds after normalization to the level of an ACTIN locus. 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 T2 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 peptatin, 1 mM PMSF, and 1 protease inhibitor cocktail tablet [Roche, 14696200]]. The tissue was further homogenized by bouncing 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.

Affinity Pull-down and pull-down assays testing the 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, 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) for 2.5 hr at 4 C with rotation. The beads were then washed twice for 5 ml of LB and then distributed evenly between 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) for 2.5 hr at 4 C with rotation. The beads were then washed twice for 5 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 8% SDS-PAGE gels (Figures S1D and S1E), respectively, 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 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 1:10000 as secondary antibody, and goat anti-rabbit IgG horseradish peroxidase (Thermo Scientific, 31460) was used at a dilution of 1:50000 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 RP2132).

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) 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 8% 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 the salt 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 μl of either streptavidin agarose (50% slurry, Sigma A2220) or M2 Flag agarose beads (50% slurry, Sigma 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 μl 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 13,200rpm. The supernatants were then filtered through a 2 micron filter and 500 μl were loaded onto either a Superdex 200 10/300 GL column (GE healthcare, 17-5172-01) or a Superdex 6 10/300 GL column (GE Healthcare, 17-5172-01) and 250 μl fractions were collected. For the Superdex 6 column, 45 μl 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 μl 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 μl from every other fraction and DMS3-3xFlag-BLRP was detected in 10 μl 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 RNA was mixed with 2 μl Turbo DNase (Ambion) were added and samples incubated for 2 hr at 37°C. RNA was then cleaned up with the RNAeasy Mini Kit (QIAGEN). Purified RNA was then eluted with 62 μl DEPC-treated H2O, to which 7 μl of 10X Turbo buffer and 1 μl 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 μl of RNA was mixed with 2 μl of dNTPs (2.5 mM each) and 1 μl 12.5 uM primer 1 in a final volume of 11 μl. This was heated to 65°C for 5 min and cooled on ice for 1 min. Fourteen microliters of a mix containing 2.5 μl Platinum Taq buffer (minus MgCl2), 2 μl 50 mM MgCl2, 1 μl 0.1 M DTT, 0.3 μl RNaseOUT, 0.3 μl Platinum Taq (Invitrogen), 0.3 μl SuperScriptIII (Invitrogen), and 0.25 μl 10 μM Taqman probe was added to each sample, and incubation continued for 2 hr at 37°C. 1 min. 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 replicates were done and standard errors determined.

The primers are as follows: ACTIN primer 1, JP2453; ACTIN primer 2, JP2452; ACTIN probe, TTTCCTCATGTTAGATGGAGAAAT; IGNS primer 1, JP6606; IGNS primer 2, JP6607; IGNS probe, TGACACAGGTTAATATCGCC GG; MEA-ISF primer 1, JP3734; MEA-ISF primer 2, JP3734; and MEA-ISF probe, TTGGGGCCGAATAACAGCAAGTCC.

Supplemental Information

Supplemental Information includes two figures and three tables and can be found with this article online at doi:10.1016/j.cub.2010.03.082.

Acknowledgments

We thank T. LaGrange for providing the NRPE1 antibody and members of the Jacobsen laboratory for helpful discussion. Jacobsen lab research was supported by U.S. National Institutes of Health (NIH) grant GM60398. J.A.L. was supported by a postdoctoral fellowship from the Ministerio de Educacion y Cultura. J.A.L. was supported by the National Research Service Award SF32GM820453. Wohlschlegel lab research was supported by University of California, Los Angeles Jonsson Cancer Center. S.E.J. is an investigator of the Howard Hughes Medical Institute. S.E.J., J.A.W., J.A.L., and I.A. designed the experiments; J.A.L., I.A., M.J.M., and A.A.V. performed the experiments; J.K.Z. provided the rost-1 rdmt-1 mutant allele and the RDM1 antibody; J.A.L. wrote the paper.

Received: December 27, 2009
Revised: March 19, 2010
Accepted: March 27, 2010
Published online: April 21, 2010

References


