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ROS3, an RNA-binding protein required for DNA demethylation in Arabidopsis

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Abstract

DNA methylation is an important epigenetic mark for transcriptional gene silencing (TGS) in diverse organisms[1–6]. Recent studies suggest that the methylation status of a number of genes is dynamically regulated by methylation and demethylation[7–10]. In Arabidopsis, active DNA demethylation is mediated by the ROS1 (repressor of silencing 1) subfamily of 5-methylcytosine DNA glycosylases through a base excision repair pathway[8,10–13]. These demethylases play critical roles in erasing DNA methylation and preventing TGS of target genes[7,8,10]. However, it is not known how the demethylases are targeted to specific sequences. We report here the identification of ROS3, an essential regulator of DNA demethylation that contains an RNA recognition motif. Analysis of *ros3* mutant and *ros1ros3* double mutant suggests that ROS3 acts in the same genetic pathway as ROS1 to prevent DNA hypermethylation and TGS. Gel mobility shift assays and analysis of ROS3 immunoprecipitate from plant extracts showed that ROS3 binds to small RNAs in vitro and in vivo. Immunostaining shows that ROS3 and ROS1 proteins colocalize in discrete foci dispersed throughout the nucleus. These results demonstrate a critical role for ROS3 in preventing DNA hypermethylation and suggest that DNA demethylation by ROS1 may be guided by RNAs bound to ROS3.

We developed a sensitive assay system in Arabidopsis to genetically dissect active DNA demethylation[10,14]. The system consists of the *RD29A-LUC* transgene (firefly luciferase reporter driven by the stress-responsive *RD29A* promoter) and the non-allelic endogenous *RD29A* gene. The *RD29A* promoter is subjected to continuous siRNA-directed DNA methylation such that active DNA demethylation is required to keep the *RD29A* and *RD29A-LUC* genes transcriptionally active. In *ros1* mutants, the *RD29A* promoter for both the transgene and endogenous gene becomes hypermethylated and both genes are silenced[10]. In addition, the *35S-NPTII* transgene linked to *RD29A-LUC* is also silenced such that *ros1* mutant plants are sensitive to kanamycin. We isolated the *ros3* mutant from a T-DNA mutagenized population[15]. Like *ros1* mutations, *ros3* causes a substantial reduction in bioluminescence

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Author Contributions X.Z. did the cloning, mutant analysis, RNA binding and other experiments. J.Z. and A.K. contributed to mutant analysis. D.M., F.Z. and K.I. contributed to DNA methylation analysis. W.L. contributed to small RNA results. O.P. and C.S.P. contributed immunostaining data. J.K.Z. designed the project and wrote the paper.

emission (Fig. 1a and Supplementary Fig. 1a and 1b) as well as sensitivity to kanamycin (Fig. 1b). Genetic analysis indicated that the *ros3* mutation is recessive and affects a nuclear gene (data not shown).

Northern blot (Fig. 1c) and nuclear run-on (Fig. 1d) assays showed that the *RD29A-LUC* and *35S-NPTII* transgenes and the endogenous *RD29A* gene are repressed transcriptionally in *ros3* plants. Compared with the wild type, both the endogenous (Fig. 2a) and transgene (Fig. 2b) *RD29A* promoters in *ros3* are substantially more heavily methylated at CpG, CpNpG (N is A, T or C) and CpNpN sites (Supplementary Table 1). Southern blot analysis with methylation-sensitive restriction enzymes also indicated DNA hypermethylation in the *RD29A* promoters in the mutant (Supplementary Fig. 2a and 2b). Treatment with the cytosine methylation inhibitor 5-aza-2'-deoxycytidine increased *RD29A-LUC* expression in the *ros3* mutant to the wild type level (Supplementary Fig. 3). These results suggest that DNA hypermethylation is responsible for the TGS in *ros3* mutant plants.

The *nrpd1a-1* mutation in the largest subunit of RNA polymerase IVa blocks the accumulation of 24-nt siRNAs corresponding to the *RD29A* promoter (data not shown). Analysis of *nrpd1aros3* double mutant showed that the *nrpd1a* mutation causes a significant increase in *RD29A-LUC* expression (Fig. 2c and 2d) and substantial decrease in CpG, CpNpG and CpNpN methylation at the transgene *RD29A* promoter (Fig. 2e) in the *ros3* mutant background. The release of TGS in *ros3* by *nrpd1a* suggests that the *RD29A* promoter siRNAs are the initial trigger of TGS in the *ros3* mutant. The levels of the *RD29A* promoter siRNAs do not differ substantially between *ros3* and the wild type (Supplementary Fig. 1c).

To analyze the genetic interaction between *ros3* and *ros1*, we crossed *ros1-1* with *ros3* and generated a *ros1ros3* double mutant. Analysis of cytosine methylation levels at the endogenous *RD29A* as well as transgenic *RD29A* promoters showed that the levels of CpG, CpNpG and asymmetric CpNpN methylation in *ros1ros3* are similar to those in the *ros1* single mutant (Fig. 2a and 2b, and Supplementary Table 1). DNA methylation at the intergenic 80, At1g23740389-0769, At4g5390602-0993 and At2g1699280-9430 loci is higher in *ros1* and *ros3* single mutants compared to the wild type, and the double mutant shows a methylation pattern similar to *ros1* (Supplementary Fig. 4a–d, and Supplementary Table 2). Therefore, the effects of the *ros1* and *ros3* mutations on the methylation levels are not additive, indicating that *ROS3* and *ROS1* function in the same genetic pathway to demethylate DNA.

The second and third pairs of true leaves in *ros3* are narrower and slightly lobed compared to those in the wild type (Supplementary Fig. 5a). Based on the leaf morphology and kanamycin sensitivity phenotypes, we mapped the *ros3* mutation to a genomic region on Chromosome V (Supplementary Fig. 5b). Sequencing of all annotated genes in this region revealed a deletion of 260 bp in *ros3* that includes the first intron and part of the second exon of AT5G58130 (Supplementary Fig. 5c and Supplementary 6). AT5G58130 encodes a protein with an N-terminal RNA recognition motif (RRM), a central COG5406 domain found in the nucleosome binding factor SPN and a C-terminal sequence with a secondary structure similar to those of RRMs (Supplementary Fig. 5c, Supplementary Fig. 7 and Supplementary 8). A wild-type genomic fragment of At5G58130 was found to complement the luminescence, leaf morphology (Supplementary Fig. 5d and 5e) and DNA hypermethylation (Fig. 2a and 2b) phenotypes of the *ros3* mutant, confirming that AT5G58130 is *ROS3*.

Expression of a translational fusion of yellow fluorescence protein (YFP) at the N terminus of ROS3 in onion epidermal cells or Arabidopsis showed a clear nuclear localization of the fusion protein (Supplementary Fig. 9a). Close inspection revealed that the fusion protein is concentrated in discrete foci in the nucleoplasm and is also present in the nucleolus (Supplementary Fig. 9b and 9c). Analysis of GUS reporter gene expression driven by the

ROS3 promoter suggested that *ROS3* is ubiquitously expressed in plant tissues (Supplementary Fig. 9d).

Because ROS3 contains an RRM domain, we tested whether it may bind to RNA. We expressed recombinant ROS3 or its truncated forms with an N-terminal histidine tag (Fig. 3a and 3b). Electrophoretic mobility-shift assays with single stranded and double stranded RNAs of different sizes (Supplementary Table 3) were carried out. ROS3 was able to bind a single stranded RNA of sequence b (corresponding to the RD29A promoter) that are either 21-, 24- or 26-nt, but it did not bind 21-, 24- or 26-nt single stranded RNA of sequence a (Fig. 3c). It also did not bind a 40-nt single stranded RNA (Fig. 3c). There was no binding (data not shown) to any double stranded RNA or any other single stranded RNA tested (Supplementary Table 3). Removal of the N-terminal RRM domain and the C-terminal region (Fig. 3c, lanes 4, 12 and 14; Fig. 3d, lane 4) or of the N-terminal RRM domain alone (Fig. 3d, lane 2) abolished binding to RNA (b). However, removal of the C-terminal region did not substantially affect RNA binding (Fig. 3d, lane 3). The binding to the 24-nt RNA (b) is ROS3 protein concentration-dependent, and is competed by excess unlabeled small RNA of the same sequence (Fig. 3e). These results show that ROS3 has the capacity to bind specific small RNAs and that the RRM domain is required for this binding. Although ROS3 could bind 21-, 24-and 26-nt single stranded RNAs of sequence b in vitro, RNA blot analysis showed that only an ~24-nt small RNA of sequence b is present in Arabidopsis (Supplementary Fig. 10a).

To determine whether ROS3 may bind small RNAs in vivo, ROS3 protein was immunoprecipitated from Arabidopsis extracts using anti-ROS3 antisera. The immunoprecipitate was fractionated on a polyacrylamide gel and putative ROS3-bound small RNAs of ~15–30 nt were extracted from the gel, cloned and sequenced. Out of 288 clones sequenced, 140 had inserts of 10–30 nt (Supplementary Table 4). Interestingly, the sequences are very rich in Gs. Two 25-nt sequences, RNA (c) (5'-GGGAGUCCGGAGACGUCGGCGGGGG-3') and RNA (d) (5'-UCGGGAGGGAAGCGGAUGGGGGCCG-3') were chosen for further analysis. These small RNAs correspond to a genomic region (intergenic between At3G41979 and At3TE58310) that appears to have higher DNA methylation in the *ros1dml2dml3* demethylase triple mutant than in the Columbia wild type control plants[9] (http://neomorph.salk.edu/epigenome.html). Both RNA (c) and RNA (d) could be detected as ~25 nt small RNAs in plants by Northern blot analysis (Supplementary Fig. 10b and 10c). ROS3 protein is able to bind RNA (c) and RNA (d) in vitro (Fig. 3f). Bisulfite sequencing analysis indicated higher CG, CNG and CNN methylation in *ros3* compared to the wild type in the genomic region corresponding to the small RNAs (Supplementary Fig. 11 and Supplementary Table 2). These results suggest that ROS3 binds to small RNAs in vivo, and the small RNAs may direct demethylation of target sequences. Further studies may reveal whether ROS3 is also capable of binding larger RNAs and what specific sequence features it recognizes.

We immunolocalized ROS3 and ROS1 proteins using antibodies recognizing the native proteins or epitope tags fused to ROS1 and ROS3 recombinant proteins. Immunolocalization in *Arabidopsis* leaf nuclei at interphase revealed that in wild-type plants ROS3 is localized in the nucleoplasm as well as nucleolus (Fig. 4a–c). The ROS3 signals appear as scattered speckle-like structures, ranging in number from 5 to more than 12 per nucleus (Fig. 4, and Supplementary Table 5). This pattern is consistent with the YFP-ROS3 result (Supplementary Fig. 9b and 9c). ROS1 was similarly found to be dispersed throughout the nucleoplasm and nucleolus, although the immunolocalization signals tended to appear somewhat more diffuse and smaller than ROS3 foci (Fig. 4a and 4b, and Supplementary Table 6). In *ros3* and *ros1* mutants, no significant signals could be obtained, which indicates that the antibodies are specific for ROS3 and ROS1, respectively (Fig. 4a). Moreover, epitope-tagged ROS3 and ROS1 proteins displayed interphase localization patterns very similar to the patterns observed

for the wild-type proteins (Supplementary Fig. 12). Simultaneous immunolocalization of cMyc-tagged ROS1 and native ROS3 revealed substantial colocalization of ROS1 and ROS3 (Fig. 4b and Supplementary Table 7).

ROS3 and ROS1 immunostaining was also carried out in *ros1* and *ros3* mutants, respectively. ROS1 localization was found to be severely disrupted in the *ros3* mutant background (Fig. 4c). The ROS3 interphase localization pattern was less changed in the *ros1* mutant, although ROS3 nucleolar localization was reduced in *ros1* (Fig. 4c). These results suggest that ROS1 and ROS3 are inter-dependent for their nuclear, and especially nucleolar, co-localization.

Because *ROS1* transcript levels are reduced in mutants defective in DNA methylation[16], we tested whether *ros3* may prevent demethylation by reducing *ROS1* expression. *ROS1* mRNA level was reduced in *ago6-1*[17] as expected, but was increased in *ros3* (Fig. 4d). Similarly, *ROS3* mRNA level was decreased in *ago6-1* but increased in *ros1* (Fig. 4d). The results suggest that *ros3* does not reduce *ROS1* mRNA levels and that the DNA methylation status at some critical sites may be sensed to regulate the transcript levels of active DNA demethylation factors such as ROS1 and ROS3.

Our results suggest that ROS3 functions in the same DNA demethylation pathway with ROS1. ROS3 is capable of binding small RNAs in vitro and in vivo, suggesting that small RNAs and/or larger RNAs bound to ROS3 may guide sequence-specific DNA demethylation by ROS1. In plants, 24-nt siRNAs bound to Argonaute 4 (AGO4) and AGO6 can direct DNA methylation and TGS[5,6,17–21]. Recent studies suggest that piwi-interacting small RNAs function to direct de novo DNA methylation and silencing of retrotransposons in mammalian germ cells[22]. In mammalian cells, promoter-directed siRNAs can also induce TGS[23,24]. Interestingly, some promoter-directed small RNAs were recently found to cause gene activation in human cells[25,26]. Our work showing that a protein required for DNA demethylation is capable of binding to small RNAs contributes to understanding the mechanism of DNA demethylation and adds to the expanding role of small RNAs in gene regulation[20,21,27]. It also sets the stage for future work to determine the relationship between small RNAs directing DNA methylation and TGS and those directing DNA demethylation and gene activation. Although our data suggest that ROS3 with its bound RNAs may guide sequence specific demethylation, it is also possible that ROS3 may suppress DNA methylation by sequestering RNAs that guide DNA methylation, or by promoting ROS1 protein stability or nuclear localization.

METHODS SUMMARY

The *ros3* mutant was isolated from a mutagenized population of *RD29A-LUC* plants by luminescence imaging[15]. Gene silencing phenotypes of the mutant were examined by RNA blot analysis and nuclear run-on assays. DNA methylation levels were assessed by bisulfite sequencing of genomic DNA or Southern blot analysis. The *ros3* mutant was mapped and cloned following previously published strategy[10]. The subcellular localization of ROS3 protein was determined by confocal imaging of cells or plants expressing YFP-ROS3 (driven by the 35S promoter). The sub-nuclear co-localization of ROS3 and ROS1 proteins was examined by immunostaining using antibodies against the wild type protein or epitope tags. Bacterially expressed recombinant ROS3 protein and its truncated forms were purified and used for gel mobility shift assays to examine RNA binding in vitro. To test in vivo RNA binding by ROS3, ROS3 protein was immunoprecipitated from plant extracts. Small RNAs from the immunoprecipitate were identified by cloning and sequencing.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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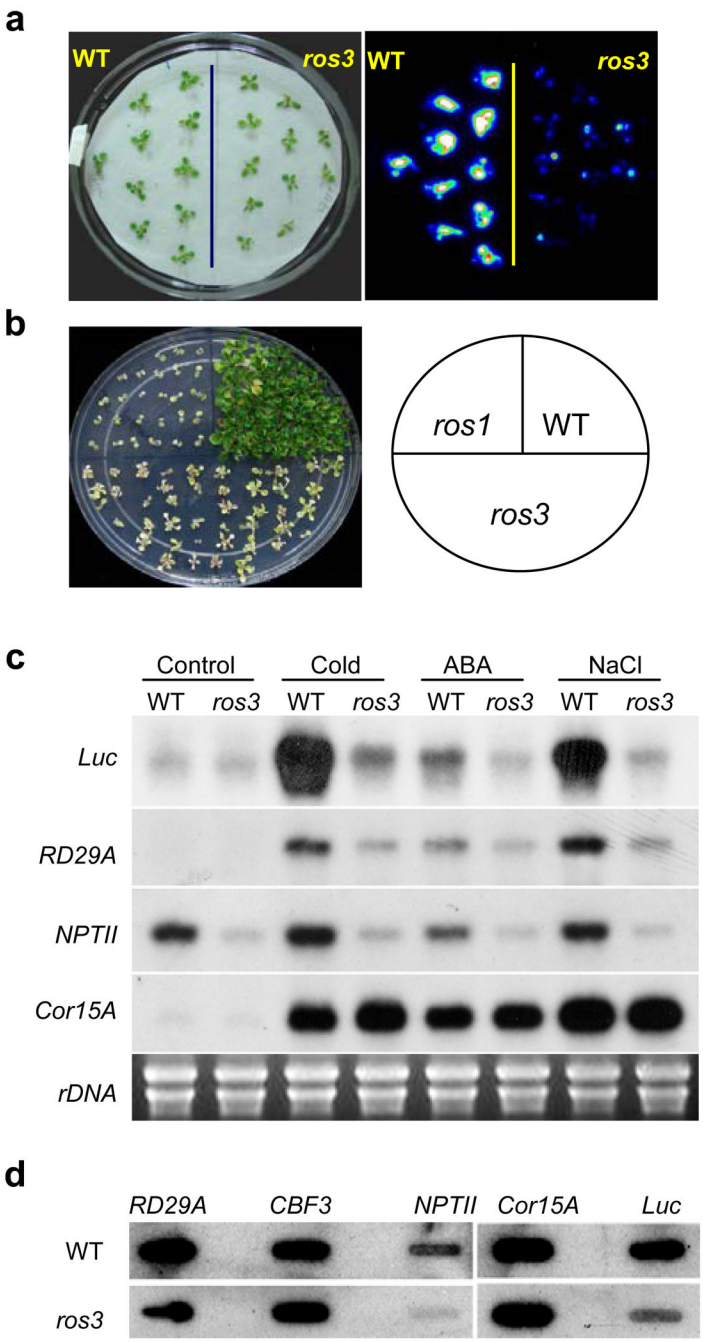

Figure 1. The ros3 mutation causes transcriptional gene silencing

a, Stress-induced expression of the *RD29A-LUC* transgene in wild type (WT) and *ros3* mutant plants after treatment with 300 mM NaCl for 5 hr. **b**, Like *ros1*, the *ros3* mutant plants are sensitive to kanamycin. **c**, Northern blots showing that *ros3* reduces the transcript levels of *LUC*, *NPTII* and endogenous *RD29A*, but not of the control, *COR15A*. Plants were either untreated (Control) or treated with cold (4°C) for 24 hr, 100 μM ABA for 3 hr, or 300 mM NaCl for 5 hr. **d**, Nuclear run-on assay showing the pre-mRNA levels of *LUC*, *NPTII* and *RD29A* genes in wild-type and *ros3*. *COR15A* and *CBF3* were used as controls.

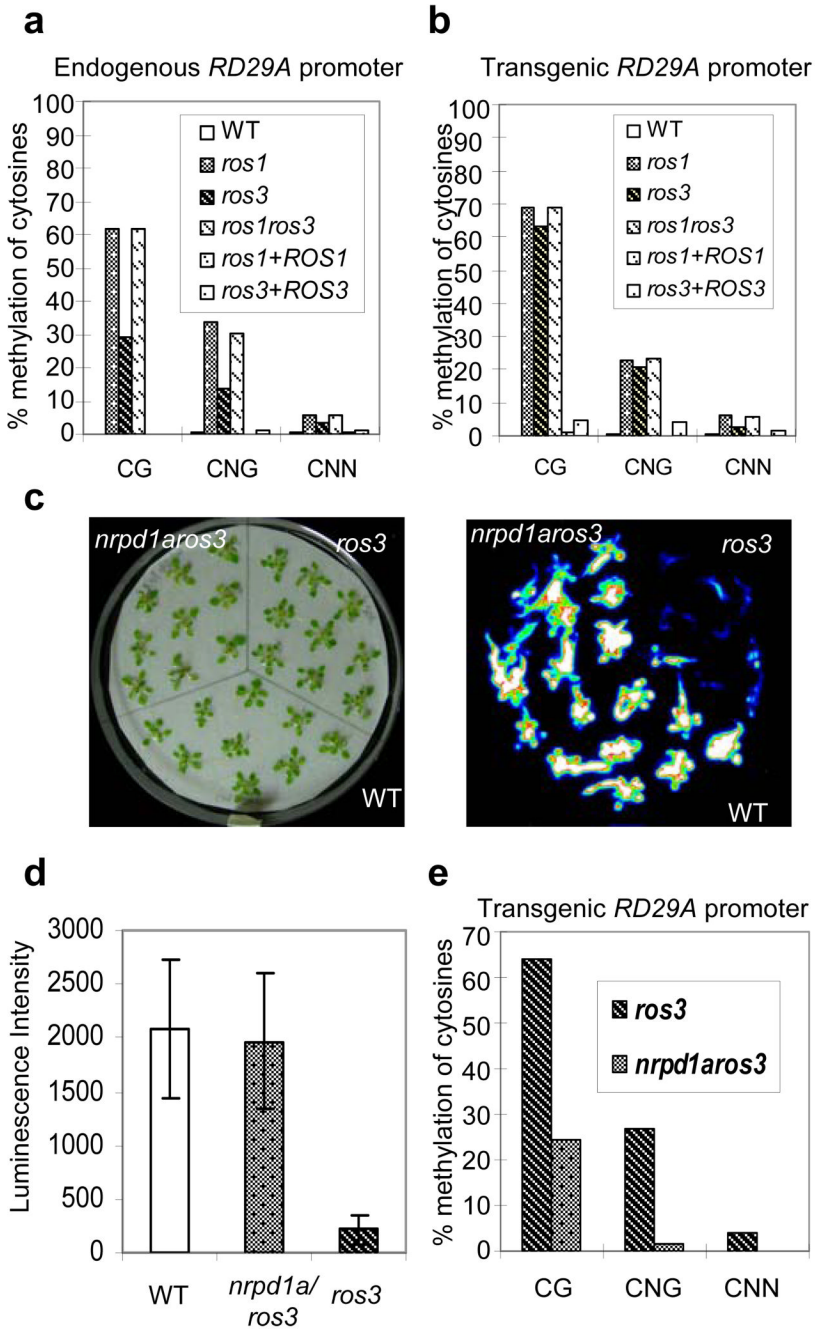

Figure 2. DNA hypermethylation in ros3 and suppression of ros3 by nrpd1a

a and **b** Bisulfite sequencing analysis of promoter methylation status of the endogenous *RD29A* (**a**) and *RD29A-LUC* transgene (**b**) in WT, *ros1*, *ros3*, *ros1ros3*, *ros1* complemented with wild type *ROS1* transgene, and *ros3* complemented with wild type *ROS3* transgene. **c**, Suppression of *ros3* by the *nrpd1a-1* mutation. Seedlings of WT, *ros3* and *nrpd1aros3* double mutant grown in MS medium for three weeks were transferred to a filter paper soaked with 300 mM NaCl for 5 hr. Left, picture of the seedlings; right, luminescence image. **d**, Quantification of luminescence in (**c**). Error bars represent standard deviation (n=20). **e**, DNA methylation status at the transgene *RD29A* promoter in *ros3* and *nrpd1aros3* plants.

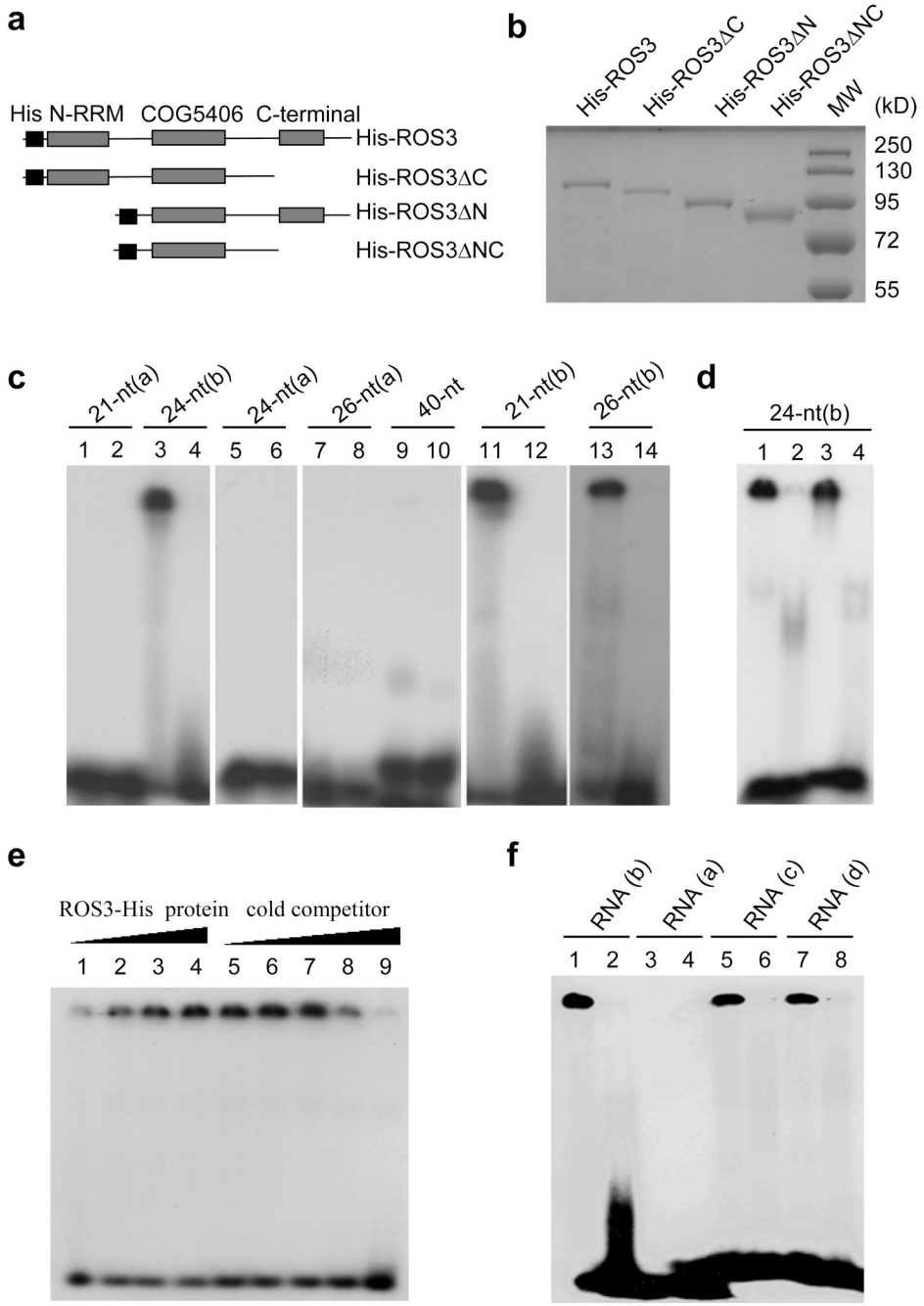

Figure 3. ROS3 binds small RNAs

a, Diagram of ROS3 and its truncated mutant forms. **b**, Coomassie stained SDS-PAGE gel showing the recombinant proteins used for RNA binding assays. **c**, ROS3 but not ROS3ΔNC binds 21-,24- and 26-nt single stranded RNA of sequence b. Lanes 1, 3, 5, 7, 9, 11 and 13, ROS3; lanes 2, 4, 6, 8, 10, 12 and 14, ROS3ΔNC. **d**, ROS3ΔC but not ROS3ΔN or ROS3ΔNC binds 24-nt single stranded RNA of sequence b. Lane 1, ROS3; lane 2, ROS3ΔN; lane 3, ROS3ΔC; lane 4, ROS3ΔNC. **e**, Protein concentration-dependent binding to 24-nt single stranded RNA (b) and competition by unlabeled small RNA. Lanes 1–4, increasing binding to 24-nt (b) small RNA by increasing ROS3 protein concentration; lanes 5–9, competition by increasing amount of cold 24-nt RNA (b). **f**, ROS3 binds 25-nt RNA (c) and RNA (d) in vitro.

RNAs of sequences b and a are used as controls. Lanes 1, 3, 5 and 7, ROS3; lanes 2, 4, 6 and 8, ROS3ΔN.

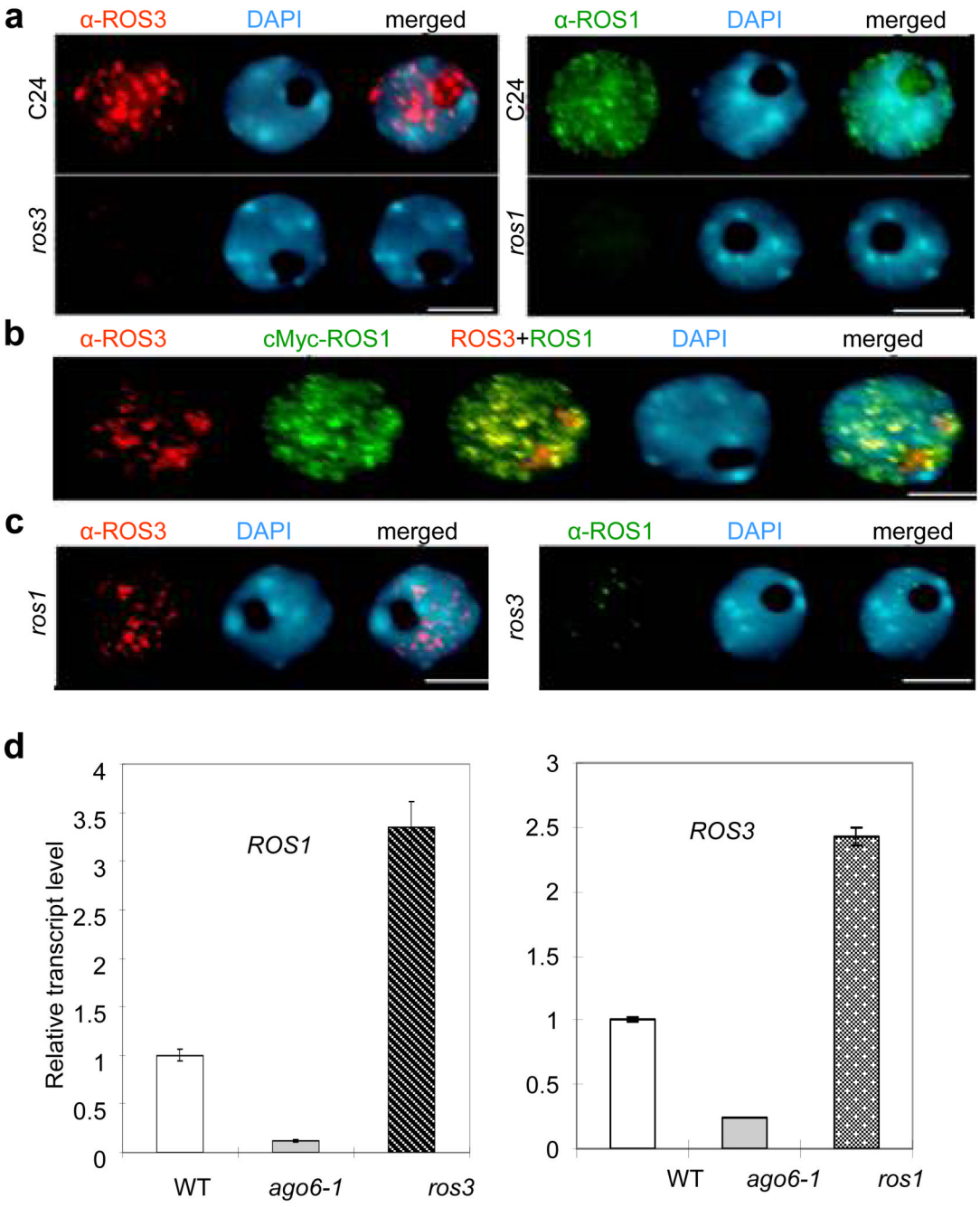

Figure 4. Co-localization of ROS3 with ROS1 in the nucleus of Arabidopsis mesophyll cells and assay of ROS1 and ROS3 mRNA levels

a, Localization of ROS3 and ROS1 by their respective antibodies. **b**, Dual immunolocalization of ROS3 and ROS1 with use of anti-ROS3 and anti-Myc. **c**, ROS1 localization in *ros3* mutant and ROS3 localization in *ros1* showing their inter-dependence for appropriate localization. Size bars correspond to 5 μm. **d**, Quantitative RT-PCR assay of relative *ROS1* and *ROS3* transcript levels in the various genotypes.