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RST1 and RIPR connect the cytosolic RNA exosome to the Ski complex in Arabidopsis

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The RNA exosome is a key 3′–5′ exoribonuclease with an evolutionarily conserved structure and function. Its cytosolic functions require the co-factors SKI7 and the Ski complex. Here we demonstrate by co-purification experiments that the ARM-repeat protein RESURRECTION1 (RST1) and RST1 INTERACTING PROTEIN (RIPR) connect the cytosolic Arabidopsis RNA exosome to the Ski complex. rst1 and ripr mutants accumulate RNA quality control siRNAs (rqc-siRNAs) produced by the post-transcriptional gene silencing (PTGS) machinery when mRNA degradation is compromised. The small RNA populations observed in rst1 and ripr mutants are also detected in mutants lacking the RRP45B/CER7 core exosome subunit. Thus, molecular and genetic evidence supports a physical and functional link between RST1, RIPR and the RNA exosome. Our data reveal the existence of additional cytosolic exosome co-factors besides the known Ski subunits. RST1 is not restricted to plants, as homologues with a similar domain architecture but unknown function exist in animals, including humans.

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The RNA exosome provides all eukaryotic cells with a key 3′–5′ exoribonucleolytic activity that participates in the maturation of various non-coding RNAs and in the degradation of both non-coding and coding RNAs (reviewed in refs. 1–3). The RNA exosome consists of a core complex composed of nine subunits (Exo9) to which the exoribonucleases RRP6 and DIS3/RRP44 differentially associate within the nucleolus, nucleoplasm or cytosol4–5. Whilst the overall structure and function of the RNA exosome is conserved, both the composition and enzymatic activities of exosome complexes vary among organisms. For example, most non-plant Exo9s including those in yeast and human are catalytically inactive6, whereas plant Exo9s have retained a phosphorylolytic activity originating from its prokaryotic ancestor7,8. This unique phosphorylolytic activity of plant Exo9 acts in combination with the hydrolytic activities provided by RRP6 and DIS39. Another exception among RNA exosomes is the association of human Exo9 with functionally distinct DIS3L and DIS3 proteins, only the latter of which is conserved in yeast and plants10,11.

In all eukaryotes investigated, the catalytic activities of the RNA exosome are modulated by cofactors termed activator–adapter or exosome targeting complexes. These complexes aid in the recognition of specific types of RNA substrates and couple exosome-mediated degradation to cellular processes, such as ribosome biogenesis or mitosis12–19. All exosome targeting complexes that have been characterized to date contain an RNA helicase from the MTR4/SKI2 family as a central component. In addition, exosome targeting complexes typically comprise RNA-binding proteins, non-canonical poly(A) polymerases or factors mediating protein–protein interactions. Most exosome targeting complexes described to date are nuclear. They include the TRAMP (TRF4-AIR1-MTR4 polyadenylonylation) complex20–22 in both baker’s yeast and humans, the human PAXT (polyA tail exosome targeting) complex23, the NEXT (nuclear exosome targeting) complexes that differ slightly in humans and plants24,25, and the MTREC (Mtr4-like 1 (Mtl1)-Red1-core) complex in fission yeast26,27. These MTR4 containing complexes assist the exosome in nuclear RNA surveillance by targeting various RNA substrates, including precursors of ribosomal and other non-coding RNAs, spurious transcripts generated by pervasive transcription and untimely, superfluous or misprocessed mRNAs21,24,25,27–32. In remarkable contrast to the diversity of nuclear exosome cofactors, a single conserved protein complex, the Superkiller (SKI) complex, is known to assist the exosome in the cytosol. The SKI complex consists of the MTR4-related RNA helicase SKI2, the tetracricopeptide-repeats protein SKI3 and two copies of the WD40-repeat protein SKI833–35. Association of the SKI complex with the exosome core complex requires an additional protein, SKI736. Recent data revealed the functional conservation of SKI7 across eukaryotes37. In mammals and plants, SKI7 is produced by alternative splicing from a single locus that encodes also the HSBI protein37–39. HSBI functions together with the G-protein Dom34/PELOTA in No-Stop decay by releasing ribosomes stalled on RNAs lacking a stop codon40–43. In the yeast Saccharomyces cerevisiae, Ski7 and Hsbi are closely related paralogs. Therefore, it was for long inferred that yeast Ski7 mediates the association of the exosome with the ribosome34,44. Recent data now challenge this view by showing that the Ski2–Ski3–Ski8 complex can directly bind to ribosomes, while Ski7 is associated with Exo945,45.

The SKI complex is conserved in Arabidopsis thaliana46, but its physical association with the exosome core has not been investigated yet. An initial experiment to affinity-capture factors associated with the Arabidopsis exosome identified the homologue of DIS3 and two nuclear RNA helicases, AtMTR4 and its closely related homologue HEN247. In addition, Arabidopsis Exo9 systematically co-purified with a 1840 amino acid ARMS-repeat protein of unknown molecular function named RESURRECTION1 (RST1)25. RST1 was originally identified in a genetic screen for factors involved in the biosynthesis of epicuticular waxes47. Epicuticular waxes are a protective layer of aliphatic very long-chain (VLC) hydrocarbons that cover the outer surface of land plants48, rst1 mutants have less wax on floral stems than wild-type plants, and ~70% of the seeds produced by rst1 mutants are shrunk due to aborted embryogenesis49. The molecular function of RST1 remains unknown. Interestingly, one of the two RRP45 exosome core subunits encoded in the Arabidopsis genome, named RRP45B or CER7 (for ECERIFERUM 7) was also identified in a genetic screen aimed at identifying enzymes or regulators of wax biosynthesis49. The wax-deficient phenotype of rrp45b/cer7 mutants (cer7 from now on) is suppressed by mutations in genes encoding RNA silencing factors, such as RDR1, RDR6, AGO1, SGS3 and DCL450,51. This and the identification of small RNAs accumulating in cer7 mutants revealed that the wax deficiency observed in cer7 plants is due to post-transcriptional silencing of CER3 mRNA50,51, encoding a protein that together with the aldehyde decarbonylase CER1 catalyses the synthesis of VLC alkanes from VLC acyl-CoA52,53. These results demonstrated that the RNA exosome contributes to the degradation of the CER3 mRNA and that the wax-deficient phenotype of cer7 mutants is a consequence of the established link between RNA degradation and silencing pathways50,51,54. Indeed, in plants, the elimination of degradation intermediates such as uncapped or RISC-cleaved mRNAs by 3′–5′ and 5′–3′ exoribonucleases prevents that they trigger post-transcriptional silencing (PTGS)55–60, a mechanism required for the destruction of non-self RNAs originating from viruses or transgenes.

Here, we demonstrate by multiple reciprocal co-purification assays coupled to mass spectrometry analyses that the Arabidopsis exosome core complex Exo9 associates with the ARM-repeat protein RST1, SKI7, another protein that we named RIPR (for RST1 interacting protein) and the Ski complex. Our data show that RST1 and RIPR suppress the silencing of transgenes as well as the production of secondary siRNAs from endogenous exo- somal targets such as RISC-cleaved transcripts and certain endogenous mRNAs which are prone to PTGS. Those mRNAs include the CER3 mRNAs explaining that the rst1 and ripr mutants share the cer7 wax-deficiency phenotype. Taken together, our biochemical and genetic data establish RST1 and RIPR as cofactors of the cytoplasmic exosome and the Ski complex in plants.

Results

Wax deficiency in rst1 mutants is caused by CER3 silencing. To investigate whether the wax deficiency of rst1 plants is linked to compromised degradation of the CER3 mRNA as reported in cer7 mutants49, we compared the stems of wild-type and mutant plants grown under identical conditions. Due to the light reflecting properties of the wax crystals that cover the outer cuticle, stems of wild-type Arabidopsis plants appear whitish (or bluish in cold light, Fig. 1a). Consistent with previous reports, plants lacking the exosome core subunit RRP45A have whitish stems signifying intact wax biosynthesis49. By contrast, plants with T-DNAs inserted in the RRP45B/CER7 (AT3G60500) and RST1 (AT3G27670) loci have glossy green stems indicating wax deficiency47,49 (Fig. 1a). Gas chromatography followed by mass spectrometry (GC-MS) analysis of extracts obtained from the stem surface confirmed that the amounts of the VLC derivatives nonacosane, 15-nonacosanone and 1-octacosanol, three major components of epicuticular stem wax in Arabidopsis, were similarly reduced in cer7 and in rst1 mutants (Fig. 1b). Ectopic...
expression of RST1 fused to GFP in rst1-3 plants restored the biosynthesis of nonacosane, 15-noracosanone and 1-octacosanol and resulted in wild-type-like whitish stems (Fig. 1a, b). Previous studies established that the wax deficiency of cer7 mutants is due to post-transcriptional silencing of the mRNA encoding CER3\(^{49-51,54}\), a subunit of a VLC alkane-forming complex\(^{52,53}\). Indeed, RNA blots revealed a severe reduction of the CER3 mRNA and an accumulation of CER3-derived small RNAs in both cer7 and rst1 mutants (Fig. 1c). Mutating the PTGS factor SUPPRESSOR OF GENE SILENCING 3 (SGS3) in rst1-2 plants abolished the production of CER3-derived small RNAs, restored wild-type levels of the CER3 mRNA and allowed the production of epicuticular wax as demonstrated in the rst1 sgs3 double mutant (Fig. 1d). These results show that the wax-deficient phenotype of rst1 mutants is caused by silencing of the CER3 mRNA, as reported for cer7 mutants.

**RST1 is a suppressor of transgene silencing.** Two independent genetic screens identified rst1 point mutations as suppressors of silencing. The first screen aimed to identify mutations suppressing the phenotype of MIM156 plants. MIM156 plants express an artificial non-coding RNA with an unchangeable miRNA 156 recognition site\(^{61}\). Ectopic expression of this miRNA target mimicry (MIM) construct reduces both levels and activity of endogenous miR156 and leads to a characteristic phenotype with spoon-shaped cotyledons, prematurely serrated rosette leaves and a reduced leaf initiation rate during vegetative growth (Fig. 2a). MIM156 rst1-4 plants were recovered from an EMS-treated population of MIM156 plants that had been visually screened for restoration of normal growth and development. MIM156 rst1-4 plants display the spoon-shaped cotyledons of the parental line, but wild-type-like leaf initiation rates and rosette leaf serration. We mapped the suppressor mutation by whole-genome sequencing to the RST1 gene and specifically the G3118A mutation causing a G706D amino acid change (Fig. 2b). Expressing a genomic RST1 construct in MIM156 rst1-4 plants restored the MIM156 phenotype confirming that the rst1-4 mutation was responsible for the suppressor effect (Fig. 2a). We then tested the accumulation of the CER3 mRNA and CER3-derived siRNAs in this novel rst1 allele. As compared with the T-DNA insertion alleles rst1-2 and rst1-3, rst1-4 mutants had residual levels of the full-length CER3 mRNA and lower levels of CER3-derived siRNAs, indicating that rst1-4 is a weak allele (Fig. 2c). Next, we analysed the accumulation of both the full-length MIM156 non-coding RNA and MIM156-derived siRNAs by RNA blots. This experiment revealed low levels of MIM156-derived siRNAs in the parental MIM156 line indicating that the MIM156 transcript is spontaneously targeted by PTGS, as often observed with highly expressed transgenes. Compared with the parental line, MIM156 rst1-4 plants had reduced levels of the full-length MIM156 transcript, but accumulated increased levels of MIM156-derived siRNAs (Fig. 2d). The increased accumulation of these siRNAs in the MIM156 rst1-4 suggests that RST1 restricts the production of MIM156-derived siRNAs, which prevents complete destruction of the full-length transcript by PTGS.
The second screen directly aimed at identifying factors affecting the post-transcriptional silencing of the 35Sprom:GUS transgene in the reporter line L1 jmj14-4. This screen identified rst1-5, a C4824T mutation in RST1 resulting in a truncation of the RST1 protein (Q1010*) (Fig. 2b). Compared with the L1 jmj14-4 parental line, L1 jmj14-4 rst1-5 plants had decreased levels of GUS mRNA and increased levels of GUS-derived siRNA (Fig. 2c). This result resembled the effects of rst1-4 on the accumulation of MIM156 transcript and MIM156-derived siRNAs (Fig. 2d). Backcrossing L1 jmj14-4 rst1-5 to wild-type yielded rst1-5 plants, which showed a pronounced accumulation of CER3-derived siRNA similar to rst1-2 and rst1-3 (Fig. 2c). To further demonstrate that rst1-5 enhances PTGS, we introduced the rst1-5 mutation into the well-established reporter lines 6b4 and Hc1 (Fig. 2f). These lines harbour the same 35S:GUS transgene as the L1 jmj14-4 line, but inserted at different locations in the Arabidopsis genome. In a wild-type background, line 6b4 does not trigger sense transgene PTGS (S-PTGS), while line Hc1 triggers S-PTGS in 20% of the population (Fig. 2f). In genetic backgrounds having impaired RNA degradation, both Hc1 and 6b4 lines trigger S-PTGS at increased frequencies, which provides a quantitative readout. 86% of the 6b4 rst1-5 plants and 100% of the Hc1 rst1-5 plants triggered silencing of the 35S:GUS reporter (Fig. 2f). This result confirmed that RST1 functions as a suppressor of S-PTGS comparable with other proteins involved in RNA degradation. Of note, the role of RST1 as S-PTGS suppressor is also supported by an independent study published during the reviewing process of our paper.

RST1 co-purifies with the exosome, SKI7 and RIPR. To examine the intracellular distribution of RST1, we used a rst1-3 mutant line expressing RST1 proteins fused to GFP at its N- or C-terminus. Both fusion proteins were functional as they rescued the developmental phenotype induced by a MIM156 transgene.

**Fig. 2** RST1 suppresses silencing of transgenes. **a** The rst1-4 mutation suppresses the developmental phenotype induced by a MIM156 transgene. **b** Diagram of the AT3G27670 gene encoding the RST1 protein. Boxes represent exons, lines represent introns. Triangles indicate the position of the T-DNA insertions in rst1-2 and rst1-3 lines. Vertical lines indicate the point mutations in rst1-4 and rst1-5. **c** rst1-4 is a weak allele. Accumulation of the CER3 mRNA (left) and CER3-derived siRNAs (right) in wild-type (WT), cer7-3 and the four rst1 alleles used in this study shown by RNA blots hybridised with a probe specific to CER3. The methylene blue stain of the membrane (MB) and hybridisation to U6 siRNA are shown as loading controls. **d** RNA blots showing the accumulation of the full-length MIM156 ncRNA and MIM156-derived siRNAs visualised by hybridisation with a probe specific to the IPS1 backbone of the MIM156 transgene. 7SL RNA and U6 siRNA are shown as loading controls. **e** RNA blots showing that the rst1-5 mutation results in reduced levels of the GUS mRNA and increased levels of GUS-derived siRNAs in the L1 jmj14-4 background. 25S rRNA and U6 siRNA are shown as loading controls. **f** The rst1-5 mutation increases S-PTGS frequency in both 6b4 and Hc1 reporter lines. The barplot shows the proportion of plants with silenced GUS expression in the indicated genotypes. The source data are available at [https://doi.org/10.6084/m9.figshare.c.4483406].
domain of the *Arabidopsis* SKI7 protein. Inspection of the peptides detected in the RST1 IP revealed the presence of peptides specific to the SKI7 splice isoform (Supplementary Fig. 1). This and the fact that SKI7 proteins are bound to yeast and human exosome complexes indicate that the AT5G10630 gene product which co-purified with RST1 is indeed SKI7 rather than HBS1. The three core proteins of the cytoplasmic Ski complex SKI2, SKI3 and SKI8 were not significantly enriched (Fig. 4a). Together, these IP results support the exclusively cytoplasmic localisation of RST1 and confirm its interaction with the Exo9 core complex. Furthermore, a protein of unknown function encoded by AT5G44150 and that we termed RIPR for RST1 INTERACTING PROTEIN was the most enriched protein in all RST1 IPs (Fig. 4a).

Because our previous purifications of *Arabidopsis* exosome complexes with tagged RRP41 as bait were analysed using an older and less sensitive mass spectrometer, we repeated the experiment using the same experimental settings as we used for the RST1 IPs (6 IPs, Fig. 4b; Supplementary Data 2). This new experiment confirmed the previously reported co-purification of the conserved exoribonuclease RRP44 and the two nuclear RNA helicases MTR4 and HEN2 with Exo9 and reproduced the co-purification of RST1. In addition, the new experiment revealed a significant enrichment of both RIPR and SKI7 in the RRP41 IPs.

Peptides specific to the alternative subunit RRP45A were present in the RRP41 IPs, but absent when RST1 was used as bait, suggesting that RST1 may preferentially interact with CER7 (aka RRP45B)-containing exosome complexes. To test this hypothesis, we stably expressed GFP-tagged RRP45A and RRP45B/CER7 in *Arabidopsis*. Indeed, both RST1 and SKI7 were significantly enriched with CER7 as bait (Fig. 5; Supplementary Data 3). RRP45A co-purified with the nucleoplasmic RNA helicase HEN2 with Exo9 and reproduced the co-purification of RST1. In addition, the new experiment revealed the physical association of RIPR with both RST1 and SKI7 in the RRP41 IPs.

Because both RRP41 IPs were significantly enriched with CER7 as bait (Fig. 5; Supplementary Data 3), RRP45A co-purified with the nucleoplasmic RNA helicase HEN2 with Exo9 and reproduced the co-purification of RST1. However, this new experiment did not reveal the association of RIPR with both RST1 and SKI7 in the RRP41 IPs. The difference in the interaction of CER7 and RRP45A cannot be explained by different intracellular localisation of the baits, because both CER7-GFP and RRP45A-GFP were present in the nuclear and cytoplasmic compartments (Supplementary Fig. 2).

In order to confirm the physical association of RIPR with RST1, we used RIPR with GFP-tags at either the N-terminal or C-terminal ends as bait in co-purification experiments (4 IPs, Fig. 6; Supplementary Data 4). RST1 was the most enriched protein in RIPR IPs. The nine subunits of the exosome were also detected, but were less enriched than in the IPs with RST1 as bait (compare Figs. 4, 6). By contrast, SKI7 as well as the three components of the Ski complex SKI2, SKI3 and SKI8 were amongst the most significantly enriched proteins co-purifying with RIPR (Fig. 6).

Altogether, the multiple reciprocal IPs confirm the interaction of RST1 with CER7-containing exosome core complexes and identify SKI7 and RIPR as additional binding partners of both RST1 and Exo9. Furthermore, our data indicate that RIPR binds Exo9 and SKI7, while RIPR binds to RST1-SKI7 and the Ski complex.

**Loss of RIPR function phenocopies *rst1* mutants.** RIPR is conserved amongst flowering plants but has no clear sequence homologues in mosses, green algae or outside of the green lineage. RIPR is a 356 amino acid protein that lacks obvious functional domains and motifs or sequence homologies to known proteins. Confocal microscopy of *Arabidopsis* roots stably expressing RIPR-GFP fusion proteins revealed a diffuse cytoplasmic distribution (Fig. 7a) similar to the intracellular distribution of RST1
mRNA-derived small RNAs accumulate in cer7, ripr and rst1. The association of RST1 and RIPR with the exosome complex and the fact that loss of RST1 or RIPR phenocopies the cer7 mutation suggested that both RST1 and RIPR are involved in the exosome-mediated degradation of the CER3 mRNA before it can become a template for the production of CER3-derived small RNAs. To identify other common targets of RST1, RIPR and the exosome complex, we analysed small RNA libraries prepared from wild-type plants and from cer7, rst1 and ripr mutants (Fig. 8a). This analysis identified more than 300 mRNAs that gave rise to small RNAs in cer7 (Supplementary Data 5), including five of the six mRNAs that were previously shown to undergo silencing in absence of the RRP45B/CER7 exosome subunit51. Many of the loci that generate small RNAs in cer7 mutants have previously been shown to produce siRNAs in sk2 single mutants, sk2 xrn4 double mutants or in the decapping mutants dcp2 and vcs57,58,60, and/or are known or predicted targets of miRNAs (Supplementary Data 5). Because these siRNAs are produced from protein-coding genes in RNA degradation mutants, they have been termed ct-siRNAs (coding-transcript siRNAs) or rqc-siRNAs (RNA quality control siRNAs)57-58,60. About one-third of the loci that generate small RNAs in cer7 mutants have previously been shown to produce siRNAs in sk2 single mutants, sk2 xrn4 double mutants or in the decapping mutants dcp2 and vcs57,58,60, and/or are known or predicted targets of miRNAs (Supplementary Data 5). Because these siRNAs are produced from protein-coding genes in RNA degradation mutants, they have been termed ct-siRNAs (coding-transcript siRNAs) or rqc-siRNAs (RNA quality control siRNAs)57-58,60. About one-third of the loci that generate rqc-siRNA in cer7 mutants produced significant amounts of rqc-siRNAs in rst1 and ripr mutants as well, while only very few loci were specifically observed in only ripr or rst1 (Fig. 8b). The observation of quasi identical populations of small RNAs in rst1 and ripr and the fact that almost all loci affected by rst1 or ripr are also affected in cer7 strongly support the conclusion that RST1 and RIPR are required for the degradation of at least a subset of cytoplasmic exosome targets.

Discussion
This study identifies RST1 and RIPR as two previously unknown cofactors which support the function of the cytoplasmic RNA exosome in Arabidopsis. Three lines of evidence back our conclusion. Firstly, RST1 and RIPR are physically associated with the

Fig. 6 RIPR co-immunoprecipitates RST1, SKI7 and the Ski complex. The Volcano plot shows the enrichment of proteins co-purified with GFP-tagged RIPR as compared with control IPs. Y- and X-axis display adjusted p-value and fold change, respectively. The dashed line indicates the threshold above which proteins are significantly enriched. The source data are available in Supplementary Data 4.

Fig. 5 RST1 and RIPR are bound to CER7-containing exosomes. Volcano plots show the enrichment of proteins co-purified with GFP-tagged RRP45B/CER7 (a) or RRP45A (b) as compared with control IPs. Y- and X-axis display adjusted p-value and fold change, respectively. The dashed line indicates the threshold above which proteins are significantly enriched (adjP<0.05). The source data are available in Supplementary Data 3.
exosome core complex and the Ski complex, respectively, both of which act together in the degradation of cytoplasmic RNA. Secondly, both RST1 and RIPR suppress the silencing of transgenic reporters similar to almost all known main RNA degradation factors including proteins involved in decapping, the 5′-to-3′ exoribonuclease DIS3L2/SOV and the cytoplasmic exosome together with the 5′-to-3′ exoribonuclease and can therefore be regarded as a "sov" homologue, Col-0 does not accumulate rqc-siRNAs (except siRNA from the AT2G01008 locus)66, perhaps because most of its RNA substrates can also be degraded by the cytoplasmic 5′-3′ exoribonuclease XRNX, the 3′-5′ exoribonuclease DIS3L2/sov and the cytoplasmic exosome together with the Ski complex ensure the rapid elimination of mRNAs after their degradation has been initiated by decapping, deadenylation or RISC-mediated cleavage, thus preventing the production of rqc-siRNAs. Vice versa, accumulation of rqc-siRNAs indicates impaired RNA degradation. The fact that similar rqc-siRNA profiles are observed in cer7, rst1 and ripr mutants indicates that cer7, RST1 and RIPR contribute to the degradation of an overlapping set of mRNA targets.

Due to a natural variation, the Col-0 accession that is used as wild-type here and in most other studies investigating RNA degradation in plants lacks a fully functional DIS3L2/SOV 3′-5′ exoribonuclease and can therefore be regarded as a "sov" mutant66,68. Compared with plants expressing a functional SOV homologue, Col-0 does not accumulate rqc-siRNAs (except siRNAs derived from the AT2G01008 mRNA)66, perhaps because most of its RNA substrates can also be degraded by the cytoplasmic exosome. Therefore, we cannot exclude that the
accumulation of rqc-siRNAs in cer7, rst1 and ripr is only observed because these mutants simultaneously lack SOV. However, the fact that the wax-deficient phenotype caused by the production of CER3-derived siRNAs is also observed in Landsberg and C24 accessions, both of which possess a functional SOV protein, implies that SOV and the cytoplasmic RNA exosome are not fully redundant.

It is important to note that both the loci concerned and the levels of siRNAs from a given loci vary among plants of the same genotype and grown under identical conditions. Not each of the loci that give rise to rqc-siRNA undergoes silencing, i.e., full suppression of its expression. Yet, PTGS is obviously common among plants of the same genotype and grown under identical conditions. Not each of the loci that give rise to rqc-siRNA undergoes silencing, i.e., full suppression of its expression. Yet, PTGS is obviously common.

The source data are available in Supplementary Data 5. It is therefore tempting to speculate that SOV and the cytoplasmic RNA exosome are not fully redundant.

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In Arabidopsis and closely related species, two genes encode the exosome core subunit RRP45. Arabidopsis RRP45A and RRP45B/CER7 share 88% identity over their first 300 amino acids. Both subunits are located in cytosolic and nuclear compartiments, and are at least in stable transformants, similarly enriched in nucleoli. Interestingly, both RST1 and RIPR only associated with RRP45B/CER7-containing exosomes, while no peptide of RST1 was detected in any of the 14 experiments that we performed using RRP45A as bait. Instead, RRP45A-containing exosomes preferentially co-purified with the nuclear RNA helicase HEN2, in line with previous results obtained using HEN2 as bait.

Our data also have important implications on the physical organisation of the cytoplasmic RNA exosome and the Ski complex. In Arabidopsis and closely related species, two genes encode the exosome core subunit RRP45. Arabidopsis RRP45A and RRP45B/CER7 share 88% identity over their first 300 amino acids. Both subunits are located in cytosolic and nuclear compartiments, and are at least in stable transformants, similarly enriched in nucleoli. Interestingly, both RST1 and RIPR only associated with RRP45B/CER7-containing exosomes, while no peptide of RST1 was detected in any of the 14 experiments that we performed using RRP45A as bait. Instead, RRP45A-containing exosomes preferentially co-purified with the nuclear RNA helicase HEN2, in line with previous results obtained using HEN2 as bait.

The observation that RST1 is amongst the most enriched proteins captured with either RRP41 or CER7 as bait suggests that RST1 is associated with the exosome core complex. The strong enrichment of both RIPR and SKI7 with RST1 as bait and the

Expressed ones. In addition, certain mRNAs prone to RNA silencing may be cleaved by off-targeted RISC, or are perhaps substrates of other endonucleases. Alternatively, secondary structures or strong association to proteins may impede degradation by at least one of the otherwise largely redundant 5′–3′ and 3′–5′ degradation pathways and could explain why some mRNAs are more likely to become substrate for RNA-dependent RNA polymerases than mRNAs which are efficiently degraded from both directions. Yet, about 30% of the rqc-siRNAs generating loci in rst1 and ripr are known or predicted miRNA targets (Supplementary Data 5). Hence, at least for those, the initial substrate for RDR6-dependent siRNA production could be a RISC-cleaved mRNA fragment. Since miRNAs and AGO1, the main effectors of RISC, are associated with polysomes, 5′ cleavage products that could be generated by RISC on polysomes resemble truncated mRNAs without a stop codon and without a polyA tail. Therefore, we can presume that RST1 and RIPR, together with the Ski complex and the RNA exosome, participate in the elimination of no-stop RNA. The notion that 5′ RISC-cleaved fragments that fail to be degraded by RST1–RIPR-SKI and the exosome become a substrate for the production of small RNAs fits well with the observation that the full-length cleavage fragments of only 10–20% of the Arabidopsis miRNA targets can be detected in the non-stop decay mutant pelota. Of note, this study and ours used the Col-0 accession, which does not express a functional SOV/DIS3L2. Therefore, the respective contribution of the exosome and the SOV/DIS3L2 pathways to prevent the production of siRNAs from 5′ fragments of RISC-cleaved mRNAs remains to be specifically addressed.

Our data also have important implications on the physical organisation of the cytoplasmic RNA exosome and the Ski complex. In Arabidopsis and closely related species, two genes encode the exosome core subunit RRP45. Arabidopsis RRP45A and RRP45B/CER7 share 88% identity over their first 300 amino acids. Both subunits are located in cytosolic and nuclear compartiments, and are at least in stable transformants, similarly enriched in nucleoli. Interestingly, both RST1 and RIPR only associated with RRP45B/CER7-containing exosomes, while no peptide of RST1 was detected in any of the 14 experiments that we performed using RRP45A as bait. Instead, RRP45A-containing exosomes preferentially co-purified with the nuclear RNA helicase HEN2, in line with previous results obtained using HEN2 as bait. Our results indicate that RST1 preferentially associates with the CER7-containing version of the Arabidopsis exosome. Compared with RRP45A, CER7 possesses an extra C-terminal domain of 135 amino acids, which may be important for the recruitment of RST1. However, a previous study suggested that these extra amino acids are dispensable for the function of CER7 in the degradation of the CER3 mRNA. Moreover, the ectopic expression of RRP45 in cer7 mutants rescued their wax-deficient phenotype. A possible explanation is that the cer7-3 allele might be a knockdown rather than a knockout mutant and still express residual amounts of CER7, which may be sufficient to promote the degradation of CER3 mRNAs when elevated levels of RRP45-containing exosomes take over other functions such as nuclear RNA surveillance. We can also speculate that overexpression of RRP45A allows a weak interaction with RST1 that is below the detection level in our IPs with RRP45A as bait. An alternative scenario could be that the physical interaction between RST1 and Exo9 is not essentially required for the function of both proteins in the turnover of the CER3 mRNA.

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observation that the Ski complex purifies mainly with RIPR suggests that RST1 and RIPR link the exosome to the Ski complex in plants. Future experiments will address the possibility that RIPR may be required to link the Ski complex with the core exosome while RST1 could stabilise the binding of Exo9 and SKI7. Other interesting possibilities are that RST1 and/or RIPR affect the recognition of target RNAs or the recruitment of the exosome to ribosomes. Yet, we find only a few ribosomal proteins enriched in individual IPs (Supplementary Data 1–4, or explore the interactive volcano plots available on figshare [https://doi.org/10.6084/m9.figshare.c.4483406]). Hence, we do not detect the association of the exosome to the ribosome that was observed in yeast45,46. Whether this has a technical basis or truly reflects a poor association of Exo9–RST1–RIPR–Ski complex with ribosomes remains to be investigated.

Interestingly, a recent study in yeast identified Ska1 as a protein that impedes the association of the yeast Ski–Exo complex with the ribosome45. Similar to RIPR in Arabidopsis, Ska1 affinity captured the Ski complex. Unlike RST1 or RIPR, the Ska–Ski complex is not required for the degradation of coding or non-coding RNAs and, instead, a specific function of the elimination of RNAs devoid of ribosomes such as 3′ UTRs or long non-coding RNAs. Apparently, overexpressing of Ska1 outcompetes the association of the Ski complex with ribosomes, suggesting that the association the Ski complex with either Ska1 or the ribosome is mutually exclusive45. Of note, sequence homologues of Ska1 seem to be restricted to S. cerevisiae and some closely related fungi, although proteins with similar functions may exist in other species.

RIPR seems to be conserved in flowering plants but is absent from the genomes of mosses and green algae, suggesting a relative recent evolutionary origin. By contrast, RST1 is deeply conserved in the green lineage. Moreover, a single ARM-repeat protein comprising the same domain of unknown function DUF3037 (IPR022542) as RST1 is conserved in humans, across all metazoa and in ancient amoebozoa such as Dictostelium, but is apparently absent from modern fungi (PTHR16212 protein family). The human DUF3037 protein KIAA1797 was named Focadhesin, because its GFP fusion protein has been detected in focal adhesion points of astrocytoma cells71. Interestingly, a recently generated high-throughput data set monitoring the migration of proteins in sucrose gradients with or without RNase treatment detected Focadhesin as a putative component of an RNA-dependent complex22 [http://r-deep.dkfz.de/]. More work is needed to fully understand the molecular function of Focadhesin. It will be interesting to investigate whether Focadhesin is also associated with the function of the RNA exosome in animals.

Methods

Plant material. Plants were grown on soil or in vitro on the Murashige and Skoog medium supplemented with 0.5% sucrose at 20 °C in 16 h light and 8 h darkness. All plants were of the Col-0 accession, which serves as wild-type in all experiments. The T-DNA insertion lines ccr-7-2 (Salk_030100), ccr-7-3 (GK_099202), ripr45a (GK_685802), ripr3-3 (Salk_036905) and rsl1-3 (Salk_129800) have been described in refs. 49,50 and 61, respectively. The rsl1-5 and rsl1-4 alleles are EMS alleles identified during this study. Starting point for the identification of rsl-5 was the EMS mutagenesis of the line L1 jm14-4 line, in which PTGS of the SSSprom:GUS transgene inserted at the L1 locus is partially impaired by the jm14-4 mutation62. The rsl1-4 mutant was identified following EMS treatment of MIMI56 EMS mutagenesis of seeds was performed as described in ref. 73. Mutations were identified by mapping-by-sequencing performed using pooled F2 plants exhibiting the phenotype of interest. Sequencing libraries prepared with the Illumina TruSeq DNA Sample Preparation Kit were 10-plexed (Illumina adapters Set A) in a low-cell lane and sequenced on an Illumina HiSeq2000 instrument to obtain at least tenfold genome coverage. The SHOREmap technique was used to identify SNPs and mapping intervals. The EMS mutants were back-crossed to Col-0 to remove the MIMI56 transgene (rsl1-4) or the jm14-4 mutation and the L1 SSSprom:GUS reporter (rsl1-5). Presence or absence of the transgenes and mutations were confirmed by PCR genotyping.

PTGS analysis. Hc1 ripr-1, Hc1 ripr(ins1) and b4d ripr-5 plants were obtained by crosses. S-PTGS frequencies were assessed by GUS activity assays. Briefly, 0.5–1 g of soluble proteins extracts from inflorescences leaves were incubated with 150 µl of 2 mM 4-methyl-umbelliferyl-β-D-glucuronide, 29 mM NaHPO4, 21 mM Na2HPO4, 10 mM EDTA. Fluorescence was determined at 15 s intervals for 30 min at 37 °C with a Fluoroflucor Ascent 2.6. The GUS activity corresponds to the slope of the curve. Typically, GUS activity in non-silenced b4d and Hc1 is >500 FLUO min−1 µg−1. Plants are considered silenced if GUS activity is <30 FLUO min−1 µg−1.

CRISPR-Cas9 editing of ATSG44150. The target site at position +179 from the ATG of the ATSG44150 gene was selected for the CRISPR plant webtool [http://www.genome.arizona.edu/crispr/CRISPRSearch.html]. No off-targets were predicted for the guide RNA TCACTAGCATGCTTAAATCGA targeting the complementary strand at Chr7:17764907-17764927. Hundred pimocoles of the oligonucleotides 5′-ATTGTCATGCGCATGTTTG-3′ and 5′-AACCTC ATGGTTATGCGCTT-3′ containing the intoxicate was transfected to Arabidopsis E. coli cells (Invitrogen). The correct insertion of the guide RNA in the vector was confirmed by Sanger sequencing before plasmids were introduced in Agrobacterium tumefaciens strain GV3101 for the transformation of Col-0 plants by floral dip. pkII.1.T-DNA confers a red fluorescence protein expressed under the seed-specific OLE01 promoter. Fluorescent T1 seeds were selected for outgrowth. Two independent T2 plants homozygous for the insertion of a single T or C at position +179 were further characterised.

Expression of GFP-tagged fusion proteins. RPP41-GFP lines were made with constructs comprising the genomic sequence of RPP41 including 1000 bp upstream of the translation start site and were previously described in ref. 10. All other GFP fusion proteins were expressed from the UBQUITIN 10 promoter. C-termifinal fusion constructs contained the genomic sequence of the respective gene including the 5′ UTR, but lacking the Stop codon. For N-terminal fusions, the genomic sequences without the 5′ UTR, but including the 3′ UTR were used. All sequences were amplified from genomic DNA, cloned into pENTR1a (Invitrogen) and transferred to pUBC-GFP and pUBN-GFP destination vectors75, respectively, using Gateway® recombines. Expression vectors were transformed into Agrobacterium tumefaciens [http://proline.fruitproteomics.fr/]. The protein identification was validated using the following settings: Mascot pretty rank ≤ 1, FDR ≤ 1% for PSM scores, FDR ≤ 1% for protein set scores. The total number of MS/MS fragmentation spectra was used to quantify each protein from at least two independent biological replicates. If not specified otherwise, biological replicates consisted of plants of the same genotype grown at different dates and in different growth chambers.
For the statistical analysis of the co-immunoprecipitation data, we compared the data collected from multiple experiments for each bait against a set of 20 control experiments. Each experiment (v1.1.2 and v1.1.3) used two biological replicates that were calculated according to the DESeq2 normalisation method (i.e., median of the data collected from multiple experiments for each bait against a set of 20 biological replicates, RST1-GFP, three replicates GFP-RST1 and in 1 RFP sample. Control IPs included four biological replicates of Col-0 incubated with GFP antibodies, six IPs from four biological replicates of GFP-expressing plants treated with GFP antibodies and ten IPs performed with anti-HA antibodies in three biological replicates with high RST1-GFP signal replicates GFP-RST1 and in 1 RFP sample. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [http://proteomecentral.proteomexchange.org] via the PRIDE partner repository [10] with the data set identifier PXD0013435.

Epicuticular wax analysis. For each sample, three stem sections of 6 cm were immersed for 30 s in 10 ml of chloroform. Extracts were dried under N2 gas, dissolved in 150 µl of chloroform, transferred in an insert and again dried under N2 gas. Extracts were derivatized in a mix of BSTFA [74] pyridine (> 99.5%, Sigma) /pyridine (> 99.5%, Sigma) (50/50, V/V) (1 h at 80 °C with shaking at 300 rpm) before BSTFA-pyridine extracts were evaporated under N2 gas. The samples were dissolved in chloroform containing a mix of nine alkanes (C10–C12–C15–C18–C19–C22–C28–C32–C36) as internal standards. Derivatized silylated samples were analysed by GC-MS (436-GC, Bruker; column 30 m, 0.25-mm, 0.25 µm; HP-5 MS) with He carrier gas inlet pressure programmed for constant flow of 1 ml/min and mass spectrometric detector (SCION), 70 eV, mass to charge ratio 50–800. GC was carried out with temperature-programmed injection at 50 °C over 2 min. The temperature was increased by 40 °C/min to 200 °C, held for 1 min at 200 °C, further increased by 3 °C/min to 320 °C and held for 15 min at 320 °C. Injector temperature was set to 230 °C and 270 °C, respectively. Peaks in the chromatogram were identified based on their mass spectra and retention indices. Mass spectra detected by GC-MS were compared with the spectra of known compounds stored in the National Institute Standard and Technology (NIST) and in the Golm Metabolome databases. Nonacosane, 15-nonenocosenone and 1- octacosanol were identified with match values of 933, 865 and 952, respectively. Mass spectrometric detector peak areas were used for relative quantification with octacosane as internal standard.

RNA extraction and northern blots. RNA was extracted from the top 3 cm of inflorescence stems or from flowers with TRI-reagent (MRC) following the manufac-
turers instructions. After precipitation with 0.8 vol isopropanol for 1–3 h at –80 °C, RNAs were collected by centrifugation (30 min 16,000 × g, 4 °C), washed twice with 70% EtOH, dissolved in water to 2 mM EDTA. Five fractions corresponding to 130–180 bp products were excised and eluted overnight in water. After EtOH precipitation, size and concentration of the fractions were checked with an Agilent 2100 Bioanalyzer (Agilent Technologies). The fraction of 140–150 bp containing the 21–22 nt small RNAs of interest was sequenced on a HiSeq 4000 sequencer (single-end mode 1 × 50 bp).

Sequence reads were trimmed from 3′-adapters and low-quality bases (q < 30) using cutadap v1.18. Reads were aligned without mismatches to the Arabidopsis TAIR10 genome using ShortStack v3.5.8.97 in unique mode (–u). Counts of 21 nt and 22 nt reads were extracted and annotated against TAIR10. Differential expression analysis was performed with DESeq2. The data obtained from the two alleles of cer7, rts1 and ripr were analysed together. Downstream analysis and data visualisation were done with R. Only loci with log2FC > 1 and an adjusted p-value of <0.01 were considered. PhasiRNA were identified with the help of ShortStack’s phasing score (score > 5). Potential miRNA target miRNAs were predicted using the psRNATarget webserver at [http://plantgrn.noble.org/pdRNA4target](http://plantgrn.noble.org/pdRNA4target).

Statistical analysis. For the statistical analysis of proteomic and sequencing data, we used negative-binomial models based on the edgeR and DEseq2 packages, respectively, which calculate the fold change and adjusted p-values with a two-sided Wald test.

Gel and blot images. Uncropped blots, gels and stem images are provided in Supplementary Fig. 5 and at [https://doi.org/10.6084/m9.figshare.c.4483406].

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The small RNA-seq and mass spectrometry proteomics raw data that support the findings of this study have been deposited to the NCBI Gene Expression Omnibus (GEO) database, accession code GSE129736, and to the ProteomeXchange Consortium via the PRIDE[10] partner repository with the data set identifier PXD0013435, respectively. Full resolution versions of all images, the wax analysis data, the processed small RNA-seq data and interactive volcano plots are available at figshare.

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Microscopy. Plants were grown on MS agar plates supplemented with 0.5% sucrose. Roots from 10-day-old seedlings were excised, placed with water under a coverslip and examined with a ZEISS LSM 780 confocal microscope. The linear expressing IRF-PAB2 was a kind gift of C. Bousquet-Antonelli.


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Author contributions


Additional information

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