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SGS3 and RDR6 interact and colocalize in cytoplasmic SGS3/RDR6-bodies

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ABSTRACT

Suppressor of gene silencing 3 (SGS3) is involved in RNA-dependent RNA polymerase 6 (RDR6)-dependent small-interfering RNA (siRNA) pathways in *Arabidopsis*. However, the roles of SGS3 in those pathways are unclear. Here, we show that SGS3 interacts and colocalizes with RDR6 in cytoplasmic granules. Interestingly, the granules containing SGS3 and RDR6 (named SGS3/RDR6-bodies) were distinct from the processing bodies where mRNAs are decayed and/or stored. Microscopic analyses and complementation experiments using SGS3-deletion mutants suggested that proper localization of SGS3 is important for its function. These results provide novel insights into RDR6-dependent siRNA formation in plants.

Structured summary:
MINT-7014710: *SGS3* (uniprotkb:Q9LDX1) and *RDR6* (uniprotkb:Q9SG02) *physically interact* (MI:0218) by *bimolecular fluorescence complementation* (MI:0809)
MINT-7014697: *RDR6* (uniprotkb:Q9SG02) and *SGS3* (uniprotkb:Q9LDX1) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

1. Introduction

RNA silencing is an essential gene regulatory system including mRNA cleavage, translational repression, DNA methylation and heterochromatin formation in eukaryotes. RNA silencing is mediated by 21–30 nt small RNAs, which are classified into microRNAs (miRNAs), small-interfering RNAs (siRNAs), and the recently discovered PIWI-interacting RNAs (piRNAs) on the basis of their biogenesis. These small RNAs induce gene silencing through a sequence-dependent manner.

In plants, some siRNAs are generated through the functions of endogenous RNA-dependent RNA polymerases (RDRs). RDRs synthesize double-stranded RNAs (dsRNAs), which are the substrates for siRNAs. RNA-dependent RNA polymerase 6 (RDR6) is a critical enzyme for the synthesis of dsRNAs in sense-transgene-mediated silencing, for the synthesis of *trans*-acting small-interfering RNA (ta-siRNAs), and for anti-virus silencing pathways. In these pathways, an additional component, suppressor of gene silencing 3 (SGS3), functions together with RDR6 [1]. SGS3 is a plant-specific protein containing three domains: zinc finger (ZF), rice gene X and SGS3 (XS) and coiled-coil (CC). Of these, the XS domain is involved in RNA binding and the CC domain is involved in homodimer formation [2,3]. Previous studies have suggested that SGS3 binds and stabilizes RNA templates to initiate RDR6-mediated dsRNA synthesis. However, the relationship between SGS3 and RDR6 remains obscure.

Here, we show that SGS3 and RDR6 colocalize in certain cytoplasmic granules and interact in these granules. Interestingly, the granules containing SGS3 and RDR6 (named SGS3/RDR6-bodies) were distinct from the processing-bodies (P-bodies) known as mRNA degradation and storage sites [4,5]. Furthermore, we analyzed the roles of SGS3 localization in SGS3 function using domain deletion mutants.

2. Materials and methods

2.1. Plasmids

Plasmids with the prefix pTA7002 [6] and pBIC vectors containing SGS3, SGS3ΔZF, SGS3ΔXS, SGS3ΔCC, RDR6, decapping protein

Abbreviations: AGO, argonaute; BiFC, bimolecular fluorescent complementation; DCL, dicer-like enzyme; DCP1, decapping protein 1; miRNA, microRNA; GFP, green fluorescent protein; P-body, processing body; RDR6, RNA-dependent RNA polymerase 6; SGS3, suppressor of gene silencing 3; siRNA, small-interfering RNA; YFP, yellow fluorescent protein; ta-siRNA, *trans*-acting small-interfering RNA

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1 (DCP1), dicer-like 4(DCL4) and nucleolin were used for agroinfiltration [7]. pIG vectors containing SGS3-FLAG, SGS3ΔZF-FLAG, SGS3ΔXS-FLAG and SGS3ΔCC-FLAG were used for transformation of *Arabidopsis*. See Table S1 for details of the plasmids.

2.2. Agroinfiltration and Western blot analysis

Agroinfiltration and dexamethasone treatment were performed as described previously [8]. All *Agrobacterium* cultures were normalized at OD_{600} = 0.5. At 1 day post-infiltration (dpi), we sprayed 30 μM dexamethasone solution onto agroinfiltrated leaves, which had been infiltrated with pTA7002-derivatives. Microscopic analyses were performed as described previously [8]. See Supplementary materials for details on Western blot analysis.

2.3. Complementation of sgs3-13 and Northern blot analysis

The *Arabidopsis* T-DNA insertion line *sgs3-13* (SALK_039005) [9,10] was selected by PCR-based genotyping using the following sets of primers: LbaI and SGS3-90F, and SGS3-90F and SGS3-173R. See Table S1 for primer sequences. *sgs3-13* plants were transformed with a floral dipping method [11]. T1 transformants were selected on plates containing 25 μg/ml hygromycin. See Supplementary materials for details on Northern blot analysis of siR255.

3. Results

3.1. SGS3 colocalizes with RDR6 in a cytoplasmic granule

We first examined the subcellular localization of SGS3. SGS3–mCherry fusion protein (SGS3:mCherry) was expressed in the leaves of *Nicotiana benthamiana* by agroinfiltration. At 2 dpi, we observed SGS3:mCherry localization in certain granules in the cytoplasm, as previously reported (Fig. 1A and Fig. S1A) [12]. A previous study reported that RDR6 mainly localizes in the nucleus [13]. However, considering that SGS3 functions together with RDR6 [1], RDR6 should also localize in the same cytoplasmic granules. Therefore, we next examined the localization of RDR6. As shown in Fig. 1B and Fig. S1B, RDR6–green fluorescent protein (GFP) fusion protein (RDR6:GFP) was mainly localized to cytoplasmic granules, similar to SGS3:mCherry. We then coexpressed SGS3:mCherry with RDR6:GFP and observed that the green and red signals merged perfectly in the same cytoplasmic granules (Fig. 1C and Fig. S1C). Next, we examined whether the SGS3 granules are related to some well known organelles like chloroplasts, mitochondria or Golgi apparatus. The granules were not merged with such characterized organelles (Fig. S2).

3.2. SGS3 interacts with RDR6

To test the interaction between SGS3 and RDR6, we performed bimolecular fluorescent complementation (BiFC) analysis. We coexpressed yellow fluorescent protein $(YFP)^N$:SGS3, which has the N-terminal half of YFP at the N-terminus of SGS3, and YFP^C:RDR6, which has the C-terminal half of YFP fused to the N-terminus of RDR6, in *N. benthamiana* leaves. At 2 dpi, we detected BiFC signals in the cytoplasmic granules (Fig. 1D and Fig. S1D). As negative controls, we checked another three combinations (YFP^N plus YFP^C, YFP^N:SGS3 plus YFP^C, and YFP^N plus YFP^C:RDR6), and confirmed that no BiFC signals were detected. YFP^N plus YFP^C was shown in Fig. S1E. These results show that SGS3 and RDR6 specifically interact in the cytoplasmic granules.

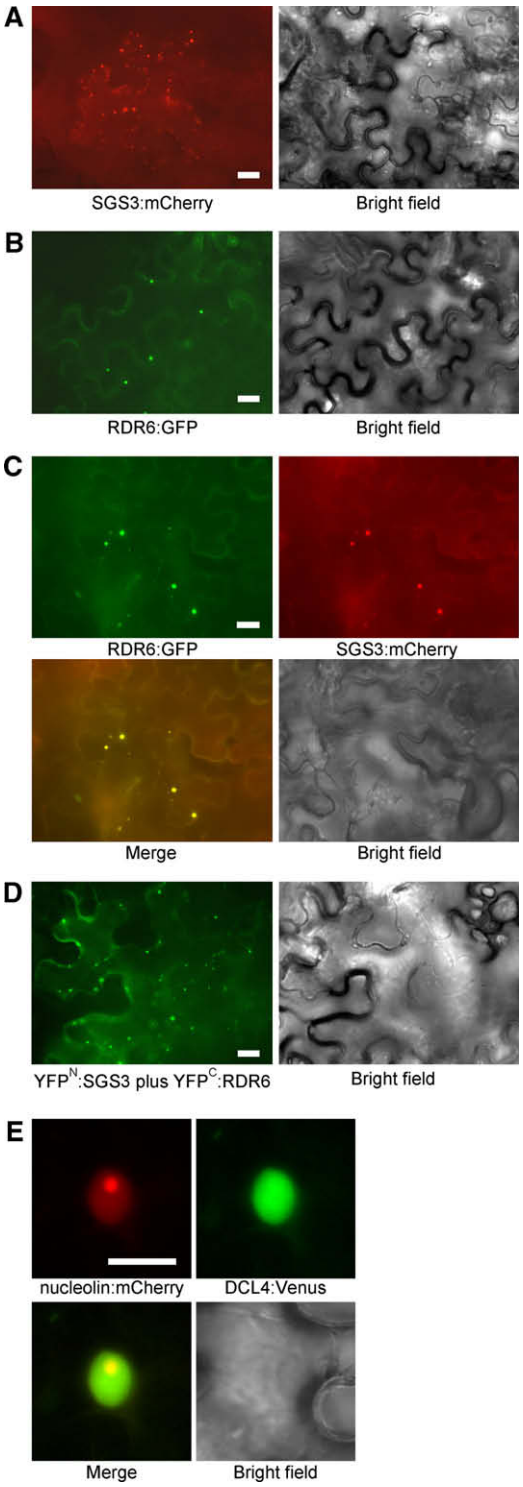

Fig. 1. (A) Left: SGS3:mCherry localized in cytoplasmic granules. (B) Left: RDR6:GFP localized in cytoplasmic granules. (C) SGS3:mCherry and RDR6:GFP colocalized in cytoplasmic granules. Left: green channel image (RDR6:GFP); right: red channel image (SGS3:mCherry); bottom left: the merged image. (D) Left: BiFC image from YFP^N:SGS3 and YFP^C:RDR6. (E) Left: nucleolin:mCherry; right: DCL4:Venus; bottom left: the merged image. Bars represent 20 μm. The right-hand pictures in (A, B, and D) and right bottom pictures in (C and F) show bright field images.

3.3. DCL4 localizes to nuclei but not in cytoplasmic granules

Dicer-like 4 (DCL4) cleaves dsRNAs synthesized by RDR6 into siRNAs, and is indispensable for siRNA pathways in which RDR6

is involved [14]. A previous paper reported that DCL4 localizes in the nucleus by using a partial N-terminal region of DCL4 [15]. Based on SGS3 and RDR6 localization data, we hypothesized that full-length DCL4 localizes not only in the nucleus, but also to SGS3/RDR6-bodies. To test this, we generated a full-length DCL4–Venus fusion construct (DCL4:Venus) and expressed that in *N. benthamiana* leaves together with a nuclear marker, nucleolin:mCherry. At 2 dpi, we observed DCL4:Venus localization only in nuclei (Fig. 1E) and failed to detect cytoplasmic granules.

3.4. SGS3–RDR6 granules are different from P-bodies

Processing-bodies (P-bodies) are well characterised cytoplasmic granules. We and other groups have shown the existence of P-bodies in plants by characterizing *Arabidopsis* decapping enzymes, AtDCP1 and AtDCP2, which make certain cytoplasmic granules [4,5,16]. In addition, we have recently shown that *Arabidopsis* argonaute (AGO)1, which is involved in miRNA, TAS1/TAS2-ta-siRNA, sense-transgene silencing and anti-virus silencing pathways [17,18], also localized in P-bodies [Fujioka et al., unpublished data]. Because SGS3, RDR6 and AGO1 function together in some silencing pathways, we tested whether the granules containing SGS3 and RDR6 are in fact P-bodies. We first co-expressed either SGS3:GFP or RDR6:GFP together with DCP1:mCherry, which is a plant P-body marker. Interestingly, granules containing SGS3 and RDR6 did not overlap with those containing DCP1 (Fig. 2A and B). We then co-expressed YFP^N:SGS3 and YFP^C:RDR6 together with DCP1:mCherry and observed that BiFC fluorescence of YFP^N:SGS3 and YFP^C:RDR6 did not overlap with the fluorescence of DCP1:mCherry (Fig. 2C). These results indicate that granules consisting of SGS3 and RDR6 (hereafter referred to as SGS3/RDR6-bodies) are different from P-bodies. It should be noted that some SGS3/RDR6-bodies were located adjacent to P-bodies (Fig. 2), suggesting that SGS3/RDR6-bodies may have some functional relationships with P-bodies.

3.5. The XS and CC domains are important for the localization of SGS3

SGS3 has three domains referred to as ZF, XS, and CC (Fig. 3A). To investigate which domains are important for the localization of SGS3, we made a wild-type SGS3 construct and three deletion constructs, SGS3ΔZF, SGS3ΔXS, and SGS3ΔCC, which have respective deletions in the ZF, XS, and CC domains, fused with GFP at their C termini (named as SGS3:GFP, SGS3ΔZF:GFP, SGS3ΔXS:GFP, and SGS3ΔCC:GFP, respectively). Typical localization patterns of these constructs and parental SGS3:GFP are shown in Fig. 3B–E and the observed number of granules is shown in Fig. 3F. We verified the expression of these proteins by Western blot analysis (Fig. 3G). The numbers of granules of SGS3ΔXS:GFP or SGS3ΔCC:GFP were greatly decreased compared with that of SGS3:GFP and SGS3ΔZF:GFP. In addition, granules containing SGS3ΔXS:GFP and SGS3ΔCC:GFP showed different shapes compared with those containing SGS3:GFP; almost all SGS3ΔXS:GFP granules and some SGS3ΔCC:GFP granules localized together and formed large granules (Fig. 3D and E). These results indicate that the XS and the CC domains are important for proper SGS3 localization.

3.6. The ZF, XS, and CC domains are important for SGS3 function

To examine the importance of ZF, XS, and CC domains for SGS3 function in planta, we introduced wild-type SGS3 or the deletion constructs of SGS3 attached with a FLAG epitope under control of the native SGS3 promoter and terminator into the *sgs3-13* mutant [9] (Fig. 4A). Leaves of *sgs3-13* plants curled downward and were narrower than those of Col-0 plants as reported previously [9] (Fig. 4B). T1 plants of the *sgs3-13* mutant transformed with SGS3:FLAG (SGS3 in *sgs3-13*) showed round leaf phenotype like Col-0 plants. This result indicated that the *sgs3-13* mutant was fully complemented by our parental SGS3:FLAG transgene. On the other hand, *sgs3-13* mutants transformed with either SGS3ΔZF:FLAG, SGS3ΔXS:FLAG or SGS3ΔCC:FLAG (SGS3ΔZF in *sgs3-13*, SGS3ΔXS in *sgs3-13* or SGS3ΔCC in *sgs3-13*), all T1 plants showed narrow leaf phenotypes like the *sgs3-13* plants (Fig. 4B). These results suggest that the ZF, XS, and CC domains are all important for SGS3 function in planta. Indeed, a ta-siRNA, siR255, of each mutant (SGS3ΔZF in *sgs3-13*, SGS3ΔXS in *sgs3-13* or SGS3ΔCC in *sgs3-13*) was not restored although that of SGS3 in *sgs3-13* was restored (Fig. 4C).

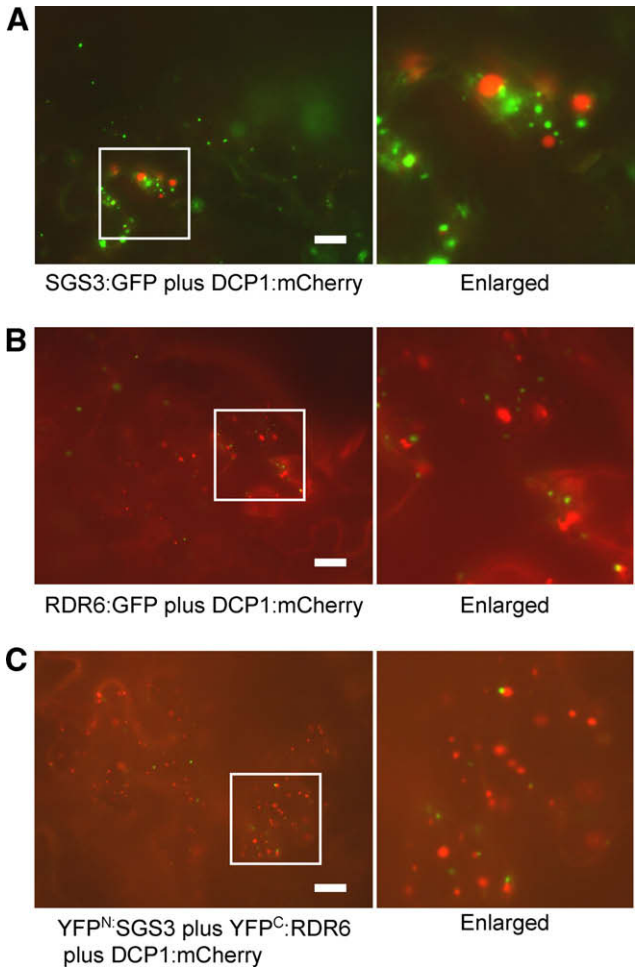

Fig. 2. Co-expression of SGS3:GFP and DCP1:mCherry (A), RDR6:GFP and DCP1:mCherry (B), and YFP^N:SGS3, YFP^C:RDR6 and DCP1:mCherry (C). The insets are enlarged to show more detailed localization on the right. Bars represent 20 μm.

4. Discussion

Based on available cellular localization data [4,5,15] and Figs. 1 and 2, we present a three-step model for RDR6-mediated siRNA pathways in plants (Fig. 5), in which the TAS1/TAS2-ta-siRNA pathway is described as an example. This model includes the following three steps: RDR6-mediated dsRNA synthesis in SGS3/RDR6-bodies, DCL4-mediated dsRNA cleavage in the nucleus, and AGO-mediated RNA cleavage in P-bodies.

Previously, Glick et al. showed SGS3 localization in cytoplasmic granules [12] while Elmayan et al. did not report such cytoplasmic granules but diffused cytoplasmic localization of SGS3 [2]. Our SGS3 localization data supported Glick's data and we could clear this contradictory situation.

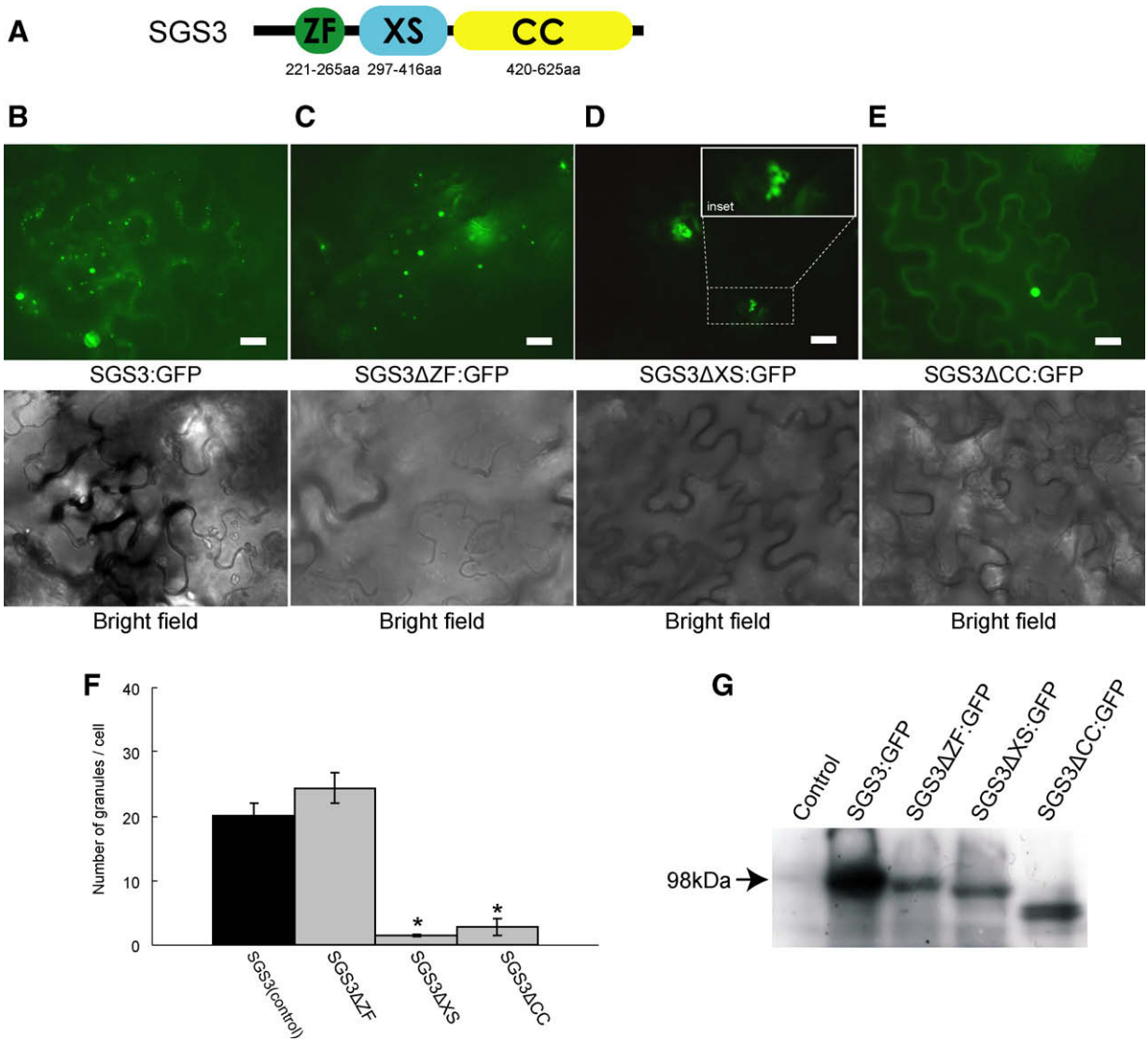

Fig. 3. Localization of SGS3 deletion mutants. (A) Diagram showing the domains of SGS3. Localization of (B) wild-type SGS3:GFP, (C) SGS3ΔZF:GFP, (D) SGS3ΔXS:GFP, and (E) SGS3ΔCC:GFP. Bottom pictures show bright field images. Bars represent 20 μm. (F) The average number of granules per a cell. Forty-five cells in total were examined. Infiltration experiments were done three times and 15 cells were examined in each experiment. Data are shown as mean values ± S.E.M. Asterisks show $P < 0.01$. (G) Western blot analysis of SGS3:GFP, SGS3ΔZF:GFP, SGS3ΔXS:GFP, and SGS3ΔCC:GFP.

We showed that SGS3 interacts with RDR6 (Fig. 1). Genetic experiments have shown that SGS3 stabilizes RDR6 template RNAs [1]. A recent paper reported that SGS3 binds dsRNAs containing 5′-overhangs [3]. Considering the SGS3–RDR6 interaction (Fig. 1) together with these previous data, we propose that SGS3 not only stabilizes RNAs, but also recruits them to RDR6 to initiate dsRNA synthesis in SGS3/RDR6-bodies. In this scenario, we can explain why SGS3 does not enhance RDR6 activity in vitro, although SGS3 is essential to generate RDR6-dependent siRNAs in planta [1]. We sometimes observed SGS3/RDR6-bodies adjacent to P-bodies (Fig. 2). Based on this observation, we predict that, at least in the case of ta-siRNAs and secondary nat-siRNAs [19,20], RDR6 templates cleaved by AGO in P-bodies directly transfer into SGS3/RDR6-bodies.

We detected DCL4 exclusively in the nucleus and failed to observe granules containing DCL4 in the cytoplasm (Fig. 1). Different localizations for DCL4 and either SGS3 or RDR6 (Fig. 1) suggests that dsRNAs synthesized by RDR6 should be transported into the nucleus. Interestingly, DRB4, which interacts with DCL4 [15] and involves DCL4-mediated ta-siRNA generation [21], also localizes in the nucleus [15], and nuclear localization of DRB4-targetting viral suppressor, *Cauliflower mosaic virus* P6 is essential for P6 suppressor activity [22]. Moreover, in *dcl4* mutants, other DCLs, which localize in the nucleus [15,23], can cleave DCL4 substrates [14]. These data further support the idea that dsRNAs synthesized by RDR6 are transported into the nucleus.

SGS3 contains three domains: ZF, XS, and CC domains (Fig. 3A). Of these, we showed that XS and CC domains are required for normal SGS3 localization (Fig. 3B–F) and are essential for SGS3 function in planta (Fig. 4B and C). Recent papers have suggested that the XS and CC domains are involved in RNA-binding and SGS3–SGS3 interactions, respectively [2] and [Kumakura et al., unpublished data]. Collectively, RNA binding activity and SGS3 homodimer formation should be important for proper SGS3 localization and SGS3 function in planta. Recently, we identified that P-bodies move on actin filaments in plants [Fujioka et al., unpublished data]. It will be interesting to determine whether SGS3/RDR6-bodies also move on actin filaments. Comparison of the localization patterns of

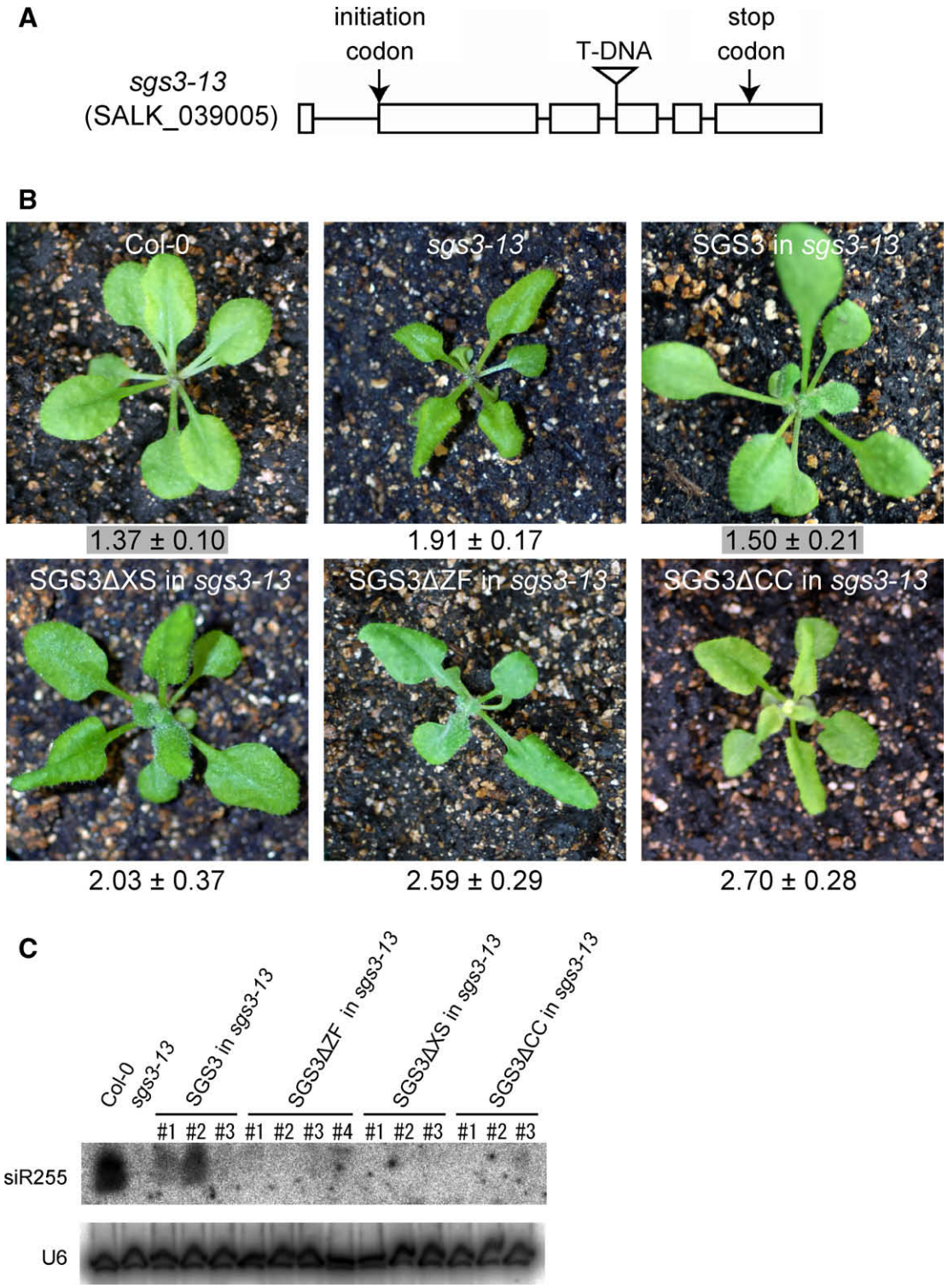

Fig. 4. (A) The T-DNA insertion site of *sgs3-13* (salk_039005). (B) Col-0 wild-type plants, *sgs3-13* mutant and *sgs3-13* mutants transformed with SGS3, SGS3ΔXS, SGS3ΔZF or SGS3ΔCC. Pictures were taken 20 days after planting. The average length and width of the sixth rosette leaves of individual plants were measured at 25 days after planting. The numbers under the photographs show the average length divided by the width (nine independent lines, respectively). Data are shown as mean values ± S.E.M. Values for which $P < 0.05$ in comparison with *sgs3-13* (control) are highlighted in gray. (C) Northern blot analysis of a ta-siRNA, siR255, and U6. The blots were probed with DNA olingonucleotides complementary to siR255 and U6, respectively.

wild-type SGS3 with the densely localized patterns in XS- and CC-deletion mutants may suggest that SGS3/RDR6-bodies also move throughout the cell, and that these domains are involved in the movement of SGS3/RDR6-bodies. On the other hand, the ZF domain is dispensable for RNA binding activity [3] and for normal SGS3 localization, but is still required for SGS3 function in planta (Fig. 4B). Thus, the roles of the ZF domain remain obscure. The ZF domain may be involved in the SGS3–RDR6 interaction.

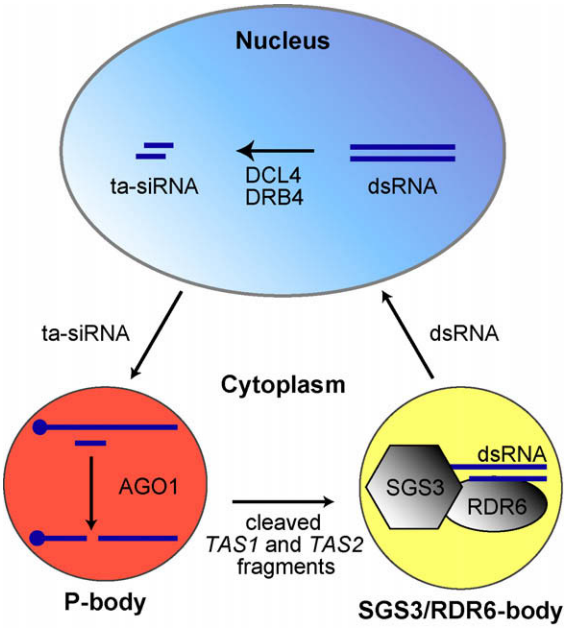

Fig. 5. Three-step model for plant siRNA pathways including AGO-mediated cleavage in P-bodies, RDR6-mediated dsRNA synthesis in SGS3/RDR6-bodies, and DCL4-mediated dsRNA cleavage in the nucleus.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.03.055.

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