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A *Medicago truncatula rdr6* allele impairs transgene silencing and endogenous phased siRNA production but not development

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Summary

RNA-dependent RNA polymerase 6 (RDR6) and suppressor of gene silencing 3 (SGS3) act together in post-transcriptional transgene silencing mediated by small interfering RNAs (siRNAs) and in biogenesis of various endogenous siRNAs including the tasiARFs, known regulators of auxin responses and plant development. Legumes, the third major crop family worldwide, has been widely improved through transgenic approaches. Here, we isolated *rdr6* and *sgs3* mutants in the model legume *Medicago truncatula*. Two *sgs3* and one *rdr6* alleles led to strong developmental defects and impaired biogenesis of tasiARFs. In contrast, the *rdr6.1* homozygous plants produced sufficient amounts of tasiARFs to ensure proper development. High throughput sequencing of small RNAs from this specific mutant identified 354 potential MtRDR6 substrates, for which siRNA production was significantly reduced in the mutant. Among them, we found a large variety of novel phased loci corresponding to protein-encoding genes or transposable elements. Interestingly, measurement of *GFP* expression revealed that post-transcriptional transgene silencing was reduced in *rdr6.1* roots. Hence, this novel mis-sense mutation, affecting a highly conserved amino acid residue in plant RDR6s, may be an interesting tool both to analyse endogenous pha-siRNA functions and to improve transgene expression, at least in legume species.

Introduction

Transgenes can undergo sequence-homology-dependent silencing, leading to repression of their expression. These mechanisms result from the activation of 'genome defence' mechanisms, which naturally protect plants against viral or bacterial infection and limit the movement of transposable elements (Vaucheret, 2006). Transgenes, inserted in sense orientation or as inverted repeats, are recognized as 'genomic invaders' and trigger post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS). These processes are mediated by small interfering RNAs (siRNA) of predominantly 21–24 nucleotides (Brodersen and Voinnet, 2006; Parent *et al.*, 2012), which mediate either the degradation of complementary RNA for PTGS or lead to chromatin changes in related genomic sequences to prevent subsequent expression for TGS. Both transgenic and endogenous siRNAs are processed from long double stranded (ds) RNA precursors by RNase III proteins, called Dicer-like (DCLs). One siRNA strand is then selectively recruited into an argonaute (AGO)-containing complex to act on its DNA or RNA target, leading to TGS or PTGS, respectively.

In plants, endogenous siRNAs can be classified into several types: the heterochromatic siRNAs (hc-siRNA), the natural anti-sense transcript-derived siRNAs (nat-siRNA), the trans-acting siRNAs (ta-siRNA) and other phased secondary siRNAs (Axtell, 2013; Brodersen and Voinnet, 2006; Mallory and Vaucheret, 2010; Vaucheret, 2006; Vazquez, 2006; Xie and Qi, 2008; Zhai *et al.*, 2011). These categories differentiate from one another by the type of genomic loci from which they arise, components of their biosynthetic pathways, and their mode of action on targets. To summarize, hc-siRNAs mediate TGS at their target DNA loci, whereas other siRNAs mainly direct PTGS.

In contrast to microRNAs (miRNA), most siRNAs require for their biogenesis the action of an RNA-dependent RNA polymerase (RDR), an enzyme able to convert single-stranded RNA into dsRNA (Voinnet, 2008, 2009; Willmann *et al.*, 2011). In the plant model

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Arabidopsis thaliana, even though six different RDRs have been reported, only RDR1, RDR2 and RDR6 have been linked to RNA silencing (Wassenegger and Krczal, 2006; Willmann *et al.*, 2011). RDR6 was discovered in independent screens of suppressors of transgene silencing (Dalmay *et al.*, 2000; Elmayan *et al.*, 1998; Mourrain *et al.*, 2000) and of mutants with abnormal juvenile-to-adult transition (Peragine *et al.*, 2004). This protein acts in complex with SGS3, a XS domain containing protein, which binds and stabilizes the RDR6 substrates before their conversion into dsRNA (Elmayan *et al.*, 2009; Fukunaga and Doudna, 2009; Mourrain *et al.*, 2000; Peragine *et al.*, 2004; Yoshikawa *et al.*, 2005; Zhang and Trudeau, 2008). Among RDRs, RDR6 has the broadest diversity of functions and substrates. In sense-transgene PTGS (S-PTGS), the RDR6/ SGS3 complex has been associated to DNA methylation at the transcribed region of the silenced transgene (Jauvion *et al.*, 2012). It also mediates antiviral defences (García-Ruiz *et al.*, 2010; Wang *et al.*, 2010, 2011), RNA-dependent DNA methylation of transposable elements (Nuthikattu *et al.*, 2013; Panda and Slotkin, 2013; Pontier *et al.*, 2012) and is involved in the biogenesis of various endogenous siRNAs, at least nat-siRNAs and ta-siRNAs (Borsani *et al.*, 2005; Howell *et al.*, 2007; Song *et al.*, 2012a). The formation of ta-siRNAs is triggered by cleavage of primary non-protein-coding *TAS* transcripts through a miRNA/AGO complex (Allen and Howell, 2010; Allen *et al.*, 2005; Axtell *et al.*, 2006; Vazquez *et al.*, 2004). One of the resulting cleavage products is converted by RDR6 into a dsRNA and further processed by DCL4 into secondary ta-siRNAs, with a 21-nt increment. In *A. thaliana*, the *TAS1*, *TAS2* and *TAS4* transcripts are cleaved by 22-nt miRNAs associated to AGO1 (Peragine *et al.*, 2004) and the related ta-siRNAs regulate in *trans* PentatricoPeptide Repeat (PPR) proteins mRNAs for *TAS1*/*TAS2* or MYB transcription factors for *TAS4* (Allen *et al.*, 2005; Vazquez *et al.*, 2004; Yoshikawa *et al.*, 2005). In contrast, cleavage of *TAS3* requires a 21-nt miRNA, miR390 and the AGO7/ZIPPY protein (Hunter *et al.*, 2003; Montgomery *et al.*, 2008). Some *TAS3*-derived ta-siRNAs, called the tasiARFs, repress the expression of transcription factors from the Auxin Response Factor family (*ARF*; Allen and Howell, 2010; Williams *et al.*, 2005; Adenot *et al.*, 2006). This tasiARF/*ARF* regulatory node is essential for key auxin responses and therefore plant development and reproduction (Guilfoyle and Hagen, 2007; Hayashi, 2012). It is widely conserved across all land plants (Axtell *et al.*, 2006; Nogueira *et al.*, 2009; Talmor-Neiman *et al.*, 2006), and, to date, all developmental defects associated to RDR6 or SGS3 loss of function were linked to this regulatory node (Adenot *et al.*, 2006; Fahlgren *et al.*, 2006; Hunter *et al.*, 2006; Li *et al.*, 2005; Nagasaki *et al.*, 2007; Satoh *et al.*, 1999, 2003; Song *et al.*, 2012a; Timmermans *et al.*, 1998; Yan *et al.*, 2010; Yifhar *et al.*, 2012).

In addition to ta-siRNAs, large populations of phased 21-nt siRNAs have been reported in several plants (Howell *et al.*, 2007; Klevebring *et al.*, 2009; Rock, 2013; Song *et al.*, 2012b; Xia *et al.*, 2012; Yifhar *et al.*, 2012; Zhai *et al.*, 2011; Zhang *et al.*, 2012;). Their biogenesis is generally triggered by a 22-nt-miRNA-dependent cleavage of their precursors (Howell *et al.*, 2007; Zhai *et al.*, 2011). However, unlike ta-siRNAs, most pha-siRNAs derive from protein-coding genes and mediate PTGS of their own precursor or of related genes from the same family. Depending the species considered, pha-siRNA precursors have been reported to encode different types of proteins, like NBS-LRR proteins which are key mediators of defence responses (Li *et al.*, 2012; Shivaprasad *et al.*, 2012; Timmermans *et al.*, 1998; Zhai *et al.*, 2011) but also PPR proteins or MYB transcription factors (Howell *et al.*, 2007; Rock, 2013; Xia *et al.*, 2012; Zhang *et al.*, 2012). *Medicago truncatula* and soybean belong to legumes (Fabaceae), a main crop family. In these two plants, identification of phased 21-nt siRNAs in small RNA libraries revealed that the large majority derived from transcripts coding for NBS-LRR disease resistance genes, whose primary slicing was triggered by four different 22-nt miRNAs (Zhai *et al.*, 2011).

In *Lotus japonicus,* one legume species, *rel* mutants arose from a screen for mutants with abnormal leaf morphology (Yan *et al.*, 2010). *rel1*/*sgs3* and *rel3*/*ago7* plants are sterile and share flower and leaf defects, that were linked to impaired tasiARF production and mis-regulation of their *ARF* targets. In this study, we isolated and characterized *rdr6* and *sgs3* mutants in *M. truncatula* in order to obtain genetic backgrounds with reduced transgene silencing and also to further investigate RDR6/SGS3 functions in legumes. Two *sgs3* and one *rdr6* alleles showed a strong deregulation of tasiARF/*ARF* expression, associated with severe developmental defects. In contrast, a second *rdr6* allele, carrying a point mutation in a conserved amino acid residue of the RDR domain, was not significantly affected in tasiARF/*ARF* expression and did not lead to any obvious developmental. Analysis of the small RNA transcriptome of this *rdr6.1* mutant revealed that it was significantly affected in siRNA production from 354 potential RDR6 substrates, including known and novel phased loci. In addition, we showed that the *rdr6.1* genetic background, which does not affect development, reduced S-PTGS efficiency in transgenic roots carrying a GFP transgene.

Results

Isolation of *rdr6* and *sgs3* mutants in *Medicago truncatula*

To identify *RDR6* and *SGS3* genes in the model legume *M. truncatula*, tBLASTX analyses were performed on the *M. truncatula* genome v3.5 (Young *et al.*, 2011) using *A. thaliana SGS3* (Xie *et al.*, 2012) and *RDR6* cDNAs as queries. Among 22 *SGS3*-like genes in *M. truncatula*, all containing a characteristic XS domain, three were very similar to *A. thaliana* SGS3 (Figure S1, Table S1). In addition, a unique *RDR6* homologue was found (Table S1). According to quantitative RT-PCR experiments, *MtSGS3a*, *MtSGS3b* and *MtRDR6* genes were expressed in all organs tested with higher levels in roots, while *MtSGS3c* transcripts were barely detectable (Figure S2).

To isolate *rdr6* and *sgs3* mutants, we used TILLING in EMS-treated plants (Le Signor *et al.*, 2009) to search for mutations in regions coding for the conserved RNA-binding XS domain of *MtSGS3a* or the RNA-dependent-RNA polymerase (RDR) domain of *MtRDR6*. *MtSGS3a* was chosen because it was the closest homologue of *AtSGS3* (Table S1). For this gene, two potentially disruptive mutations were selected for further analyses (Figure 1a). The *sgs3a.1* mutation was located at position +1167 bp from the start codon at an intron–exon junction. The *sgs3a.2* mutation, +1421 bp from the start codon, led to the change of a highly conserved neutral glycine residue ($G_{425,}$ Figure S3) into a polar glutamine residue. For *MtRDR6*, out of nine alleles identified by TILLING, the only allele affecting a conserved residue in the RDR domain was *rdr6.1*. This mutation at position +2555 from the start codon (Figure 1b) led to the change of a highly conserved glycine residue ($G_{852,}$ Figure S4) into a serine polar amino acid. As expected, RT-qPCR experiments confirmed that *RDR6* transcript levels were not affected

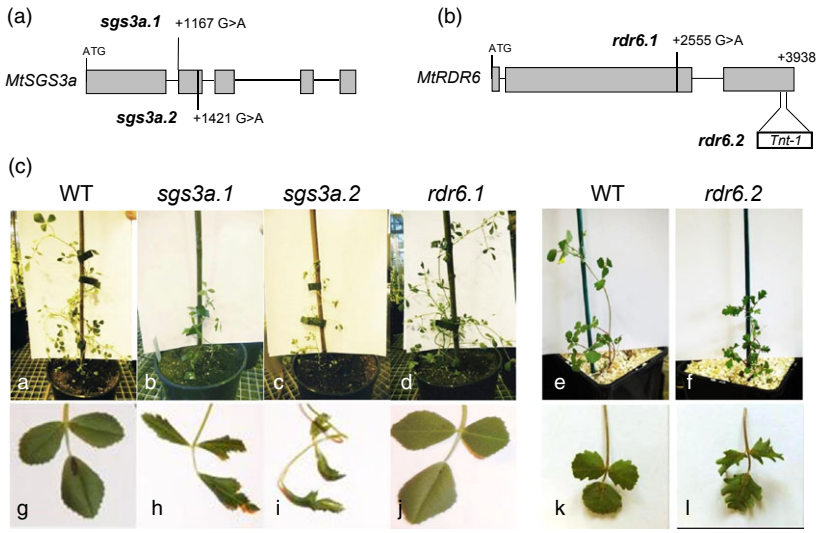

Figure 1 Mutations and phenotypes of the *sgs3a* and *rdr6* lines. (a and b) Schematic representations of *MtSGS3a* (a) and *MtRDR6* (b) genes with exons (grey boxes), introns (horizontal bars) and positions of the four mutations. For each point mutation (G > A) or insertion (*Tnt-1* transposon), the exact nucleotidic position from the start codon is indicated. (c) Adult plants (a–f) and compound adult leaves (g–l) from *sgs3a.1* (b, h), *sgs3a.2* (c, i), *rdr6.1* (d, j), *rdr6.2* (f, l) and related WT (A17genotype: a, g and R108 genotype: e, k) lines.

by the mutation (Figure S5). To obtain an additional independent *rdr6* allele, we screened the *Tnt-1* insertion mutant collection (http://medicago-mutant.noble.org/mutant/) and identified the *rdr6.2* allele (NF17932), where the transposable element was inserted 123-bp upstream from the stop codon (position +3938). RT-PCR experiments using primers spanning the insertion site did not allowed to detect any transcript. However, specific primers before the insertion revealed the presence of a low-abundant 3′ truncated transcript, suggesting this line may not be a complete null mutant (Figure S5). Unlike heterozygous plants which had a wild-type phenotype, homozygous plants of the two *sgs3a* and the *rdr6.2* mutant lines exhibited severe developmental defects (Figure 1c): plants were sterile and very small, due to reduced shoot and root growth (Figure S6). In addition, leaves were narrower and down-curled, with highly serrated and even-lobed margins (Figure 1c). In contrast, homozygous *rdr6.1* plants had a wild-type developmental and growth phenotype (Figures 1c and S7).

Accumulation of *TAS3*-tasiARFs is impaired in *sgs3a* and *rdr6.2* mutants, but not in *rdr6.1*

In plant studied so far, the RDR6/SGS3 complex is involved in several siRNA biogenesis pathways, including ta-siRNAs, which are well-known developmental regulators (Adenot *et al.*, 2006; Fahlgren *et al.*, 2006; Hunter *et al.*, 2006; Li *et al.*, 2005; Song *et al.*, 2012b; Yan *et al.*, 2010). Among the four Arabidopsis *TAS* genes, only *TAS3* is conserved in *M. truncatula* (Jagadeeswaran *et al.*, 2009). By mining previous data of small RNA deep sequencing in this species (http://medicago.toulouse.inra.fr/MIRMED, Truchet *et al.*, 1985), we identified several siRNAs derived from the *MtTAS3* locus (Medtr2 g033380), including two tasiARFs (D7+ and D8+, Table S2). Those siRNAs are predicted to target *MtARF2* (Medtr8 g100050), *MtARF3* (Medtr4 g128910) and *MtARF4* (Medtr2 g093740) transcripts for PTGS. To monitor D7+ and D8+ tasiARF and *MtARF* accumulation in the mutants, we first performed quantitative RT-PCR experiments on leaf RNAs (Figure 2). In the *sgs3a.1, sgs3a.2* and *rdr6.2*, a drastic reduction in tasiARF levels was observed in comparison with their WT siblings (depletion ratio of 5.9, 20 and 5.5, respectively, Figure 2a). Consequently, at least one of the *MtARF* targets was up-regulated more than threefold in these mutants (Figure 2b). Impairment of tasiARF biogenesis was more drastic in *sgs3a.2* compared with *sgs3a.1*, and, as a consequence, we observed higher levels of *MtARF2*, *MtARF3* and *MtARF4* transcripts and more severe leaf morphological defects in this line. In contrast, only a slight decrease of tasiARF levels was observed in *rdr6.1* leaves (2.2-fold, Figure 2a), which was not followed by significant changes in *MtARF* expression (mutant/WT ratio from 1.23 to 1.47 according the gene, Figure 2b). In roots, neither tasiARF nor *MtARF* expression were affected by the *rdr6.1* mutation (Figure S8). As this hypomorphic allele did not significantly affect development, we decided to investigate it further in

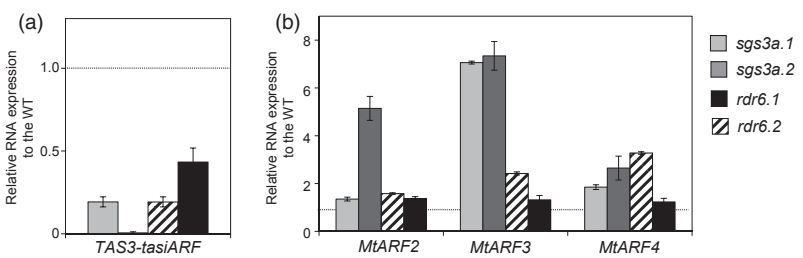

Figure 2 tasiARF and *MtARF* expression is significantly impaired in *sgs3a* and *rdr6.2* mutants, but not in *rdr6.1*. RNA levels of *TAS3*-tasiARF D7+ (a) and its *MtARF2*, *MtARF3*, *MtARF4* targets (b) were measured by real-time RT-PCR in leaves of *sgs3a.1* (pale grey boxes), *sgs3a.2* (dark grey boxes), *rdr6.2* (gridded boxes) and *rdr6.1* (black boxes) homozygous plants and their respective WT siblings. Transcript levels were normalized first with three reference genes (defined using Genorm software, see Experimental procedure) and calibrated to the values of the respective WT siblings (dashed line). Error bars represent standard deviations of three technical replicates, and one representative biological experiment out of three is shown.

term of endogenous siRNA populations and transgene silencing efficiency.

rdr6.1 plants are affected in endogenous siRNA production from 354 loci

Small RNA analyses in plants with impaired RDR6 or SGS3 functions have revealed that, besides *TAS* genes, RDR6 and SGS3 are involved in the biogenesis of secondary phased siRNAs (pha-siRNAs) from large sets of dsRNAs (Howell *et al.*, 2007; Li *et al.*, 2012; Song *et al.*, 2012b; Zheng *et al.*, 2010). In *A. thaliana*, for instance, Zheng *et al.* (2010) identified 200 novel RDR6 substrates. To investigate how the *rdr6.1* mutation may affect endogenous siRNA populations, we constructed small RNA libraries from plants of two *rdr6.1* siblings (*rdr6-A*, *rdr6-B*) and the corresponding WT siblings (WT-A and WT-B) and sequenced them by Solexa technology (Illumina). After removal of adaptors and other RNAs (rRNA, tRNA), more than 14-million reads were obtained per library, that were represented between 1 840 674 and 2 448 747 distinct small RNAs from 20- to 25-nt per library (Table S3). When focusing on the 21-nt RNA fraction, which includes ta-siRNAs and pha-siRNAs described so far in *M. truncatula* (Jagadeeswaran *et al.*, 2009; Zhai *et al.*, 2011), the minimal correlation between the biological replicates (A and B) was of 0.9877. These values allowed us to search for small RNAs with differential accumulation between *rdr6.1* and WT libraries. Statistical analyses were performed on small RNAs with more than 5 reads at least in both *rdr6.1* or both WT libraries. Comparison of normalized abundances (see Experimental procedure) revealed 436 differential small RNAs, among which 433 were significantly depleted in *rdr6.1* in comparison with WT libraries (Figure 3a; in red, *P* value <0.05; listed in Table S4). For all differential siRNAs tested, RT-qPCR experiments confirmed a reduced level in *rdr6.1* in comparison with WT plants. As expected, accumulation of these siRNAs was also strongly reduced in the three other lines, although the depletion ratio was generally higher in *rdr6* than *sgs3* mutants (Figure 3b).

Mapping of the 433 depleted siRNAs on *M. truncatula* A17 genomic sequences allowed to identify 354 loci (whole predicted genes or transposable elements, 500-bp intergenic regions), that generated significantly less siRNAs in the *rdr6.1* mutant than in the related WT (package edger, FDR 5%). Depending on the locus, cumulated siRNA abundances were between 2.3- and 91-fold lower in *rdr6.1* than in WT libraries, with a mean depletion ratio of 6.8 (Table S5). Consistently with the RT-qPCR results (Figures 2 and S8), *MtTAS3* did not belong to these regions. Indeed, although *MtTAS3*-derived ta-siRNAs were clearly produced with a 21-nt increment (phasing score of 37.9), none of them were significantly depleted in the *rdr6.1* libraries in comparison with the WT ones (FDR 5%, Figure S9a and b). According to genome annotation, the selected loci corresponded to 240 protein-coding genes, 71 transposable elements (TE), 25 intergenic regions and 18 nonannotated regions (Table S5). Most putative RDR6 substrates identified by this approach encoded NBS-LRR disease resistance proteins (54%, Figure 4). However, we identified other protein-coding gene families, such as Penta/TetratricoPeptide Repeat (PPR/TPR)-rich proteins, GDSL lipases, haem peroxidases, proteolysis-associated proteins but also a large set of transposable elements (TE) (Figures 4 and S10). Analyses of small RNA phasing on each of the 354 loci revealed that 136 corresponded to phased loci (Table S5, phasing score higher than 10; see Experimental procedure). Among them, 56 were already listed as pha-siRNAs by Zhai *et al.* (2011) and 80 were novel, including 34 TEs and 32 NBS-LRR genes.

Post-transcriptional transgene silencing is reduced in roots of the *rdr6.1* mutant

In Arabidopsis, the first *rdr6* and *sgs3* mutants were identified in screens for suppression of transgene silencing (Dalmay *et al.*, 2000; Elmayan *et al.*, 1998; Mourrain *et al.*, 2000). Strong *rdr6* and *sgs3* alleles impaired both development and sense-transgene post-silencing (S-PTGS), while hypomorphic alleles led to normal phenotypes but were defective in S-PTGS (Adenot *et al.*, 2006). Such genetic backgrounds, useful to reduce transgene silencing, were not reported in legumes. We thus analysed S-PTGS efficiency in the *rdr6.1* genetic background using a GFP reporter transgene assay. The Pro_{RolD} promoter, highly active in legume transgenic roots (Elmayan and Tepfer, 1995), was used to avoid transcriptional silencing of the transgene and the *nptII* selection gene, whose expression was driven by the constitutive CaMV Pro_{35S} promoter. Northern blot experiments first confirmed that *GFP*-derived siRNAs accumulated at higher levels (48% more) in 'GFP−' versus 'GFP+' transgenic roots, confirming efficient S-PTGS in the set of 'GFP−' roots (Figure 5b). GFP measurements were then performed on more than 30 independent kanamycin-

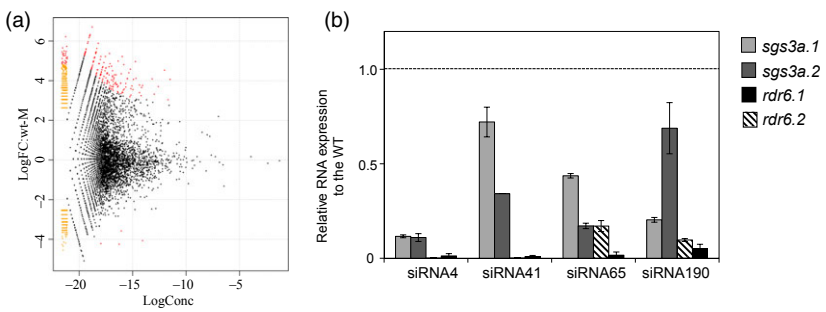

Figure 3 The *rdr6.1* mutant is depleted in large sets of phased and unphased endogenous siRNAs. (a) Ma-Plot representation of the log-fold change of each 21-nt RNA in function of their log normalized abundance. The 436 siRNAs identified as differentially accumulating between the two *rdr6.1* and the two related WT siblings (with a FDR of 5%) correspond to the red dots. (b) Confirmation of down-accumulation of four selected siRNAs (siRNA4, siRNA41, siRNA65 and siRNA190) in the *sgs3a* and *rdr6* mutants by real-time RT-PCR. Small RNA levels were normalized with three conserved miRNAs as references (see Experimental procedure) and calibrated with the related WT value. Error bars represent standard deviation of two technical replicates, and one representative biological experiment is shown out of three. Lines: *sgs3a.1* (pale grey boxes), *sgs3a.2* (dark grey boxes), *rdr6.2* (gridded boxes) and *rdr6.1* (black boxes).

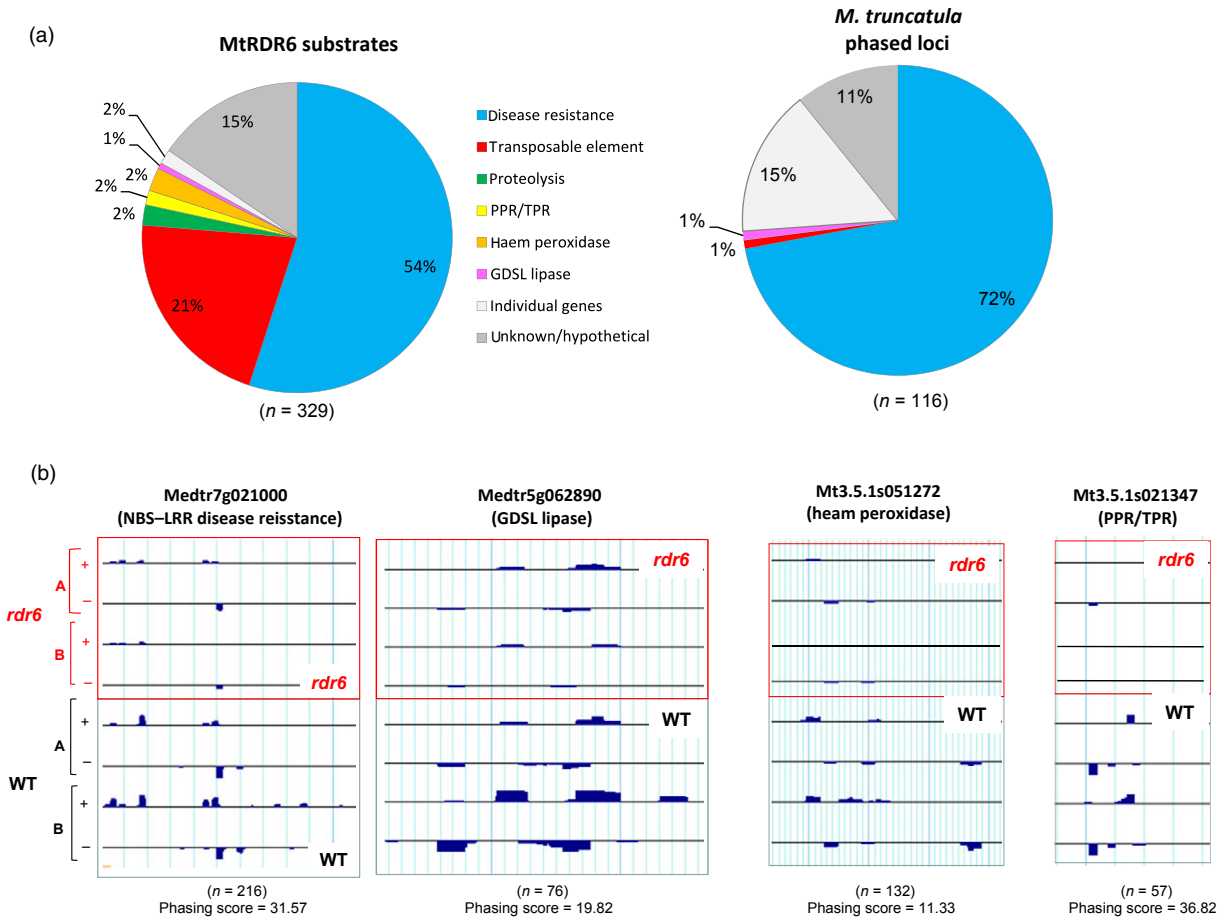

Figure 4 Characterization of the siRNA-producing substrates of MtRDR6. (a) Functional categories of the 329 annotated MtRDR6 substrates identified in the *rdr6.1* mutant (left) in comparison with the *M. truncatula* phased loci described by Zhai *et al.*, (2011) (right). PPR/TPR: Penta/TetratricoPeptide Repeat proteins, UK/hyp: unknown/ hypothetical proteins. (b) Examples of siRNA mapping on selected MtRDR6-dependent phased loci. Small RNAs from two independent *rdr6.1* (rdr6-A and B, in red) siblings and their related WT siblings (WT-A and B, in black) are shown. The 21-nt siRNAs (with perfect matches and with more than five reads in one library) are represented by blue boxes on + or − strands. Box height is proportional to siRNA abundance (from 5 to 10 reads as a maximal threshold). Below each graph indicates the total number (*n*) of distinct 21-nt siRNAs that mapped to the locus (whole gene) and their phasing score, calculated according to Howell *et al.*, (2007). Above each graph, the gene ID (v3.5.1; Yan *et al.*, 2010) and its functional category are indicated.

resistant transgenic roots of *rdr6.1* or WT sibling plants, in three biological experiments, and the proportions of 'GFP−' (no GFP signal) versus 'GFP+' (GFP signal higher than negative controls) were quantified. Interestingly, the proportion of 'GFP−' roots was significantly reduced in the mutant background (Figure 5a; 58.7% versus 75%, khi2 test, $\alpha < 0.01$), suggesting that S-PTGS efficiency was partly impaired in roots by the *rdr6.1* mutation. Northern blots experiments confirmed that GFP-derived siRNAs were reduced approximately threefold in *rdr6.1* roots in comparison with the WT roots (Figure 5c). Hence, this mutant background can serve to improve transgene expression in *Medicago truncatula*. Furthermore, related *RDR6* alleles in plants (at least in legumes) could be interesting tools to limit post-transcriptional gene silencing of transgenes without affecting development.

Discussion

The availability of genetic backgrounds with impaired transgene silencing and normal development may be a challenge for transgenic crop production. In this context, the RNA-dependent RNA polymerase RDR6 and its partner SGS3, which act together in sense-transgene PTGS in the model plant *Arabidopsis thaliana*, are good candidates. However, these proteins are also involved in the biogenesis of distinct endogenous siRNAs, including the trans-acting siRNAs, and play a variety of roles in development, auto-incompatibility or in responses to pathogens and abiotic stresses (Wassenegger and Krczal, 2006; Willmann *et al.*, 2011). In this work, we identified a specific *rdr6.1* mutation which partly stabilizes transgene expression, without altering development in the legume species *M. truncatula*. Interestingly, this allele yields plants strongly affected in production of a large set of endogenous siRNAs, including some of the recently described phasiRNAs (Zhai *et al.*, 2011).

In *A. thaliana*, *rdr6* and *sgs3* null mutants have pleiotropic developmental defects. Although these mutants lack all categories of ta-siRNAs, their phenotypes were mainly associated to the impairment of the tasiARF/*ARF* regulatory node and its impact on auxin responses. Indeed, *tas3* and *ago7* mutants, which are

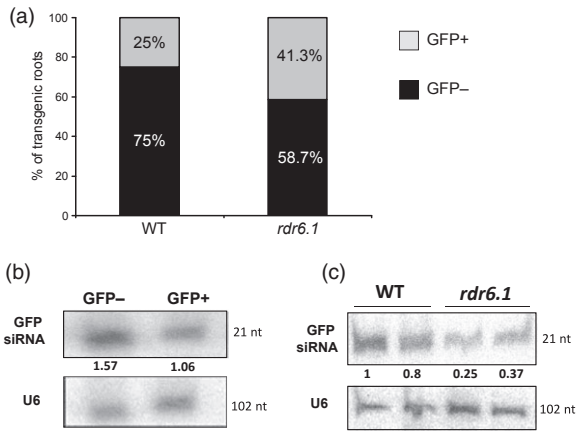

Figure 5 Transgene PTGS is reduced in *rdr6.1* roots. (a) Roots of *rdr6.1* and related WT siblings were transformed with a Pro_{RolD}::*GFP* Pro_{35S}::*nptII* construct using *A. rhizogenes*. GFP fluorescence was measured on more than tips (1 cm long) of 30 kanamycin-resistant roots per genotype in three biological experiments, and the proportion of GFP negative (GFP−) and GFP positive (GFP+) roots was determined. GFP− values were defined by measuring background from 30 roots transformed with the empty vector lacking the *GFP* transgene. (b) Levels of *GFP*-derived siRNAs from pools of 20 'GFP+' or 'GFP−' roots of the WT genotype were analysed by RNA gel blot experiments dedicated for small RNAs. A *GFP*-specific probe was used to monitor 21-nt *GFP*-derived siRNAs as well as a U6 snRNA probe for internal loading control. Values were obtained after normalization with U6 using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). (c) Levels of *GFP*-derived siRNAs in pools of 20 transgenic roots from WT or *rdr6.1* genetic backgrounds were analysed by RNA gel blot experiments as described above.

specifically impaired in the biogenesis of *TAS3*-derived siRNAs, showed phenotypes similar to *rdr6* and *sgs3* mutants (Adenot *et al.*, 2006). In rice, mutations in orthologous genes for *RDR6*, *AGO7* and *DCL4,* all involved in tasiARF production, led to a strong shoot phenotype (Nagasaki *et al.*, 2007). In *L. japonicus*, a model legume, leaf and reproductive defects of the *rel1/sgs3* and *rel3/ago7* mutants were also linked to an impaired biogenesis of tasiARFs (Yan *et al.*, 2010). In this work, we identified three TILLING and one *Tnt-1* insertion mutants in the *MtSGS3a* and *MtRDR6* genes *in M. truncatula*. Three of these recessive mutations—*sgs3a.1, sgs3a.2* and *rdr6.2*— yielded highly similar phenotypes, ie small and sterile plants, whose leaves were narrower, down-curled and with an abnormal lobed shape. As expected, molecular analyses revealed reduced levels of tasiARFs and increased levels of their *ARF* mRNA targets in these lines. Although we cannot exclude that additional endogenous siRNAs participate to their phenotypes, these data strongly suggest that, like in other species, most developmental defects observed in the *sgs3a* and *rdr6.2* mutants are associated to this perturbation of the tasiARF/*ARF* regulatory node. Interestingly, the phenotypes we observed in *M. truncatula* slightly differed from the *rel* mutants in *L. japonicus*. Indeed, these mutants had no growth defect and their compound leaves often lacked one or two leaflets (Yan *et al.*, 2010). These differences between the two legumes could be linked to specific effects of the mutations analysed. Alternatively, some RDR6/SGS3 substrates may also be species-specific, as already proposed to explain the unexpected lack of shoot apical meristem formation during embryogenesis in the rice *rdr6/shootless2* mutant (Nagasaki *et al.*, 2007; Satoh *et al.*, 1999, 2003).

In contrast to the other alleles, the *rdr6.1* mis-sense mutation did not led to any obvious developmental defect. RT-PCR experiments showed that tasiARF levels, barely affected in leaves and unchanged in roots, were sufficient to ensure proper expression of *MtARFs* during development. Although this mutation appeared not to be crucial for RDR6 action on *TAS3* transcripts, homozygous *rdr6.1* plants were significantly impaired in the accumulation of 433 siRNAs, which derive from 354 distinct loci. According to these molecular data, *rdr6.1* may be considered as a hypomorphic mutant. However, siRNA depletion ratio varied from 2.3 to 91 depending on the locus considered, suggesting that the mutation did not affect all substrates with the same efficiency and that this particular amino acid substitution may affect substrate selection by RDR6. Among the depleted siRNAs identified, a large set mapped to the so-called phased loci, previously reported in *M. truncatula* thanks a genome-wide small RNA analysis (Zhai *et al.*, 2011). According to these authors, production of secondary siRNAs from these loci depends on the initial binding of a 22-nt miRNA and cleavage by AGO1. In contrast, the cleavage of *TAS3* transcript is mediated by a 21-nt miR390/AGO7 complex, and this latter protein co-localizes with SGS3 and RDR6 into cytoplasmic siRNA bodies (Jouannet *et al.*, 2012; Kumakura *et al.*, 2009). Although less probable, another explanation of the *rdr6.1* molecular phenotype may be that the amino acid substitution at a conserved position differentially impacts the recruitment of AGO1- or AGO7-processed precursors into siRNA bodies.

In *A. thaliana*, more than 200 RDR6 substrates have been reported with a great variety of functions, including mRNAs, intergenic RNAs and transposons (Zheng *et al.*, 2010). Here, we identified 354 potential loci that produce siRNAs in an RDR6-dependent manner in *M. truncatula*. Some of them encode proteins that function in gene expression and nucleic acid-based biological functions (e.g. translation, RNA processing. . .), including members of the large PPR/TPR gene families (Howell *et al.*, 2007; Rock, 2013; Xia *et al.*, 2012; Zhang *et al.*, 2012). We also identified novel RDR6 substrates coding for enzymes, like haem peroxidases, lipases or peptidases. Recent analyses revealed that, unlike in *A. thaliana* or rice, legumes and *Solanaceae* accumulate large sets of phased siRNAs from NBS-LRR disease resistance genes (Li *et al.*, 2012; Zhai *et al.*, 2011). In agreement with these data, a large proportion of the 354 selected loci belonged to this gene family, thus confirming that MtRDR6 is involved in NBS-LRR-derived pha-siRNA biogenesis and reinforcing the hypothesis that 21-nt secondary siRNA-mediated PTGS may be a key regulatory mechanism of biotic interactions in legumes. However, the WT-like phenotype of *rdr6.1* plants suggests that except for ta-siRNAs, the various endogenous siRNAs produced in an RDR6-dependent manner have no major implication in *M. truncatula* development and growth.

Unexpectedly, around 20% (71/354) of the putative MtRDR6 substrates were transposable elements. This result is surprising, as very few TE were identified in the phased loci previously listed by Zhai *et al.* (2011). In *A. thaliana*, Zheng *et al.* (2010) reported that a large proportion of RDR6-dependent dsRNAs arose out of TEs but most were generally not associated to siRNA production. Here, we cannot rule out that some of the TE-derived siRNAs depleted in *rdr6.1* may in fact derive from paralogous loci silenced by PTGS that contain a TE-related exon. Nevertheless, our results strongly suggest that several TEs are substrates of MtRDR6 for production of secondary siRNAs. These data are in agreement with recent studies indicating that 21-nt siRNAs processed from

RDR6-dependent dsRNAs may be involved in RNA-dependent DNA methylation of TEs, although their direct function in transcriptional silencing is still under debate (Law and Jacobsen, 2010; Nuthikattu *et al.*, 2013; Panda and Slotkin, 2013; Pontier *et al.*, 2012; Stroud *et al.*, 2013). Finally, unlike in rice or Arabidopsis where most RDR6-dependent dsRNAs are sliced by DCL4 into phased siRNAs (Song *et al.*, 2012b; Xie *et al.*, 2005), 62% of the potential RDR6 substrates detected in this study were not predicted as phased loci. Although some phasing scores may be impacted by the presence of introns in genomic regions or by small RNAs that match to paralogs and may disrupt the phasing pattern, the proportion of RDR6 substrates leading to unphased siRNAs seems to be relatively high in *M. truncatula*.

To our knowledge, except in *A. thaliana*, all *rdr6* or *sgs3* mutants described so far in plants were severely impaired in development, due to the deregulation of the tasiARF/*ARF* node and the consequent effects on auxin responses. In *A. thaliana*, Adenot *et al.* (2006) identified hypomorphic *rdr6* and *sgs3* mutants with a wild-type phenotype and normal tasiARF biogenesis. As sense-transgene PTGS was affected by these alleles, these authors suggested that this regulatory mechanism may be more sensitive than development to perturbations in RDR6/SGS3 functions. In *M. truncatula,* we observed that, in addition to the depletion of numerous endogenous phased and unphased siRNAs, S-PTGS of a *GFP* transgene was partly reduced in transgenic roots of *rdr6.1* homozygous plants in comparison with WT siblings. Interestingly, the *rdr6.1* point mutation leads to the substitution of a glycine residue inside the RDR domain (G_{852}), which is conserved in all plant RDR6 proteins analysed. To our knowledge, none of the *rdr6* mutants described so far in other species was affected at this conserved position. This novel genetic background could thus become an interesting tool to stabilize transgene expression in *M. truncatula* and other crops.

Experimental procedure

Plant material and growth conditions

Medicago truncatula (*Jemalong A17* genetic background) TILLING mutants for *MtRDR6* and *MtSGS3a* were obtained from the *RevGen*UK platform (John Innes Centre, Norwich, UK, http://revgenuk.jic.ac.uk/TILLING.htm). Mutations, potentially deleterious or untolerated for protein function, were predicted by CODDLE (http://www.proweb.org/coddle/) and SIFT (Zheng *et al.*, 2010). The *rdr6.2* insertion line (NF17932, *R108* genetic background) was obtained by screening the *Tnt-1* insertion mutant collection, generated at the Samuel Roberts Noble Foundation (http://medicago-mutant.noble.org/mutant/). All experiments were performed on plants genotypes after two successive backcrosses with the respective WT line. Segregation analysis of the developmental phenotypes in *sgs3a* and *rdr6.2* mutants was 3:1 ($n > 25$; khi2 test, $P < 0.05$), indicative of single locus recessive mutations. Genotyping was done by PCR on genomic DNA extracted following (Song *et al.*, 2012b), using gene-specific primers (Table S6) designed with the Primer3 software (http://frodo.wi.mit.edu/primer3/). Seeds were scarified in concentrated sulphuric acid during 3 min, rinsed four times with distilled water and surface sterilized for 20 min in bleach (12% (v/v) sodium hypochlorite). After washings, they were sown on 1% agar plates, stratified for 6 days at 4 °C and kept overnight at 24 °C in the dark. Germinated seedlings were transferred in perlite/sand (3/1, v/v) pots, irrigated with the nitrogen-rich nutritive solution (Soluplant 18.6.26, Duclos International, Lunel Viel, Montpellier, France), in a growth chamber at 24 °C under long-day conditions (16 h light at 150 μE light intensity). For each analysis, three independent biological replicates were performed, including at least 30 plants per genotype and condition, and a Kruskall–Wallis test was used to determine significant differences (*$\alpha < 0.05$, **$\alpha < 0.01$, ***$\alpha < 0.001$).

Bioinformatic analyses

A. thaliana RDR1 to *RDR6* and *SGS3* cDNA sequences (TAIR, http://www.arabidopsis.org/) were used with tBLASTX to query the following genomes: *M. truncatula* (Mt 3.5.1, Ng and Henikoff, 2003; soybean Glyma1.181 (http://www.plantgdb.org/GmGDB/), *Lotus japonicus* (Miyakogusa.jp 2.5, Kumakura *et al.*, 2009) and rice OsGDB187 (www.plantgdb.org/OsGDB/). RDR6 and SGS3 sequences from other species came from GenBank at NCBI (www.ncbi.nlm.nih.gov/). Conserved protein domains were examined using the Conserved Domains software (PROSITE) at NCBI and the Pfam protein families database (http://pfam.wustl.edu/). Sequence alignments were made using the ClustalW program (http://www.ebi.ac.uk/clustalw/). SiRNAs derived from the *MtTAS3* locus (Medtr2 g033380, Sato *et al.*, 2008) were searched by BLASTN in the *M. truncatula* small RNA libraries (Lelandais-Brière *et al.*, 2009).

Small RNA libraries construction, sequencing and analysis

Small RNA-enriched fractions were extracted from four-day-old seedlings of two independent *rdr6.1* siblings (rdr6-A and B) and their corresponding WT siblings (WT-A and B) using the 'mirVana™ miRNA Isolation' kit (Ambion Life technology, www.lifetechnologies.com/). *rdr6.1* and the related WT siblings were obtained after two backcrosses and a subsequent self-fertilization of independent homozygous plants. Small RNA libraries were constructed from 1 μg of RNAs using the 'TruSeq small RNA Sample prep' kit (Illumina, https://www.illumina.com/) and sequenced using the TruSeq SBS Kit v5 on a Genome Analyzer IIx (Illumina). Sequences have been deposited at the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE39421). The raw data processing and rRNA/tRNA filtering were performed as previously described (Robinson *et al.*, 2010). To search for miRNAs, a BLASTN search was performed on the miRBase v17 database (http://www.mirbase.org/). Identification of small RNAs with differential accumulation between WT and *rdr6.1* libraries was performed on all 21-nt RNAs, having more than 5 reads in at least two duplicated libraries. We used the package 'edgeR' (Robinson *et al.*, 2010) to compare read abundances, after normalization with 21-nt RNAs total read numbers of each library. This method is based on modelling data with negative binomial distributions, using empirical Bayes estimations and exact tests to assess statistical significance. As two replicates were available for each genotype, the common estimation of variance parameter was used, and False Discovery Rate (FDR) multiple testing adjustments were performed (Benjamini and Hochberg, 1995). A BLASTN analysis was performed on *M. truncatula* genome (v3.5.1; Jouannet *et al.*, 2012) plus additional unpublished contigs shown in Table S7 (J. Gouzy unpublished data) to identify genomic loci that matched perfectly to the *rdr6-1* depleted siRNA and to localize additional siRNAs covering these loci. SiRNA positions and abundances on selected RDR6-dependent loci were visualized by Genome viewer. To identify the putative MtRDR6 substrates, cumulated siRNA abundances from each genomic region (com-

plete gene or 500-bp intergenic region) were statistically compared between the *rdr6.1* and WT libraries, using the same tests as for individual siRNAs. In order to detect siRNA phasing, the method developed by (Howell *et al.*, 2007) was used, consisting in computing a phasing score for each position on the genomic loci, with phase cycles of 21 nucleotides in length. According to the scores obtained with the known *M. truncatula TAS3* (Moreno *et al.*, 2013) and phased loci (Zhai *et al.*, 2011), loci with a phasing score ≥ 10 were considered as phased.

Real-time RT-PCR analyses

Total RNAs from organs of adult plants were isolated with TRIZol reagent (Invitrogen) and RNA quality and concentration was controlled using a Nanodrop ND1000 spectrophotometer (Thermo Fischer Scientific, www.thermofisher.com/). For RT-PCR, total RNA was treated with RNase-free DNase I (Thermo Scientific Molecular Biology, http://www.thermoscientificbio.com/). For mRNA quantification, 1 μg of RNAs was reverse-transcribed using the SuperScript II Reverse Transcriptase (Invitrogen, www.invitrogen.com/) following manufacturer's recommendations and an oligo-$d(T)_{18}$ as primer. Real-time PCRs were performed using the SYBR Green I master kit on a LightCycler 480 apparatus (Roche Diagnostics, www.roche-diagnostics.fr/), as described in (Plet *et al.*, 2011). *MtACTIN11*, *MtRBP1* and *MtH3L* were selected as reference genes using the Genorm software (http://medgen.ugent.be/~jvdesomp/genorm/; Vandesompele *et al.*, 2002), and transcript levels were normalized using the mean expression of the three reference genes. For small RNA quantification, real-time RT-PCR analyses were performed using the 'miScript reverse transcription' and the 'miRScript SYBR Green PCR' kits (Qiagen), following the manufacturer's recommendations. PCR were performed using a universal primer provided in the kit and specific primers corresponding to the exact sequence of the small RNAs studied (Table S6). As references, we used three miRNAs, whose biogenesis is not dependent of RDR6 activity: mtr-miR164a, mtr-miR167a and mtr-miR396b (sequences in Table S6). In all experiments, three independent biological replicates were performed, with three technical replicates per sample.

Obtention of *Medicago truncatula* transgenic roots and analysis of *GFP* expression

To obtain *M. truncatula* 'composite' plants with transgenic roots, we followed the protocol of (Boisson-Dernier *et al.*, 2001). A Pro_{RolD}::*GFP* Pro_{35S}::*nptII* construct in pXK7S2D vector (Karimi *et al.*, 2007) was introduced by electroporation into the *Agrobacterium rhizogenes* ARqua1 strain (Smr-derivative strain of A4T) and used for transformation of *M. truncatula* seedlings. An empty pXK7S2D vector (without *GFP*) was used as a negative control. Briefly, 2 weeks after inoculation, plants that developed kanamycin-resistant roots (expressing the *nptII* gene) were transferred onto growth pouch papers (Mega international, http://www.mega-international.com/) on Fahraeus medium (Truchet *et al.*, 1985) with 1.5% Bacto-Agar (Gibco-Life technology, http://fr-fr.invitrogen.com/) to allow root growth for three additional weeks in a growth chamber as described above. GFP fluorescence level in independent transgenic roots was quantified using ImageJ (Schneider *et al.*, 2012) on 1-cm root tip images obtained using a fluorescent microscope (Leica DMLB). For statistical analysis, we used a khi2 test with $n > 30$ root tips per construct and genotype in three independent biological experiments. *GFP* expression in pools of 20 transgenic roots was confirmed by RT-PCR experiments as described above, using *GFP* primers (Table S6). To analyse the accumulation of *GFP*-derived siRNAs in transgenic roots, small RNA gel blots were carried out as described in (Kumakura *et al.*, 2009). The *GFP* probe was amplified by PCR using *GFP* primers and labelled in the presence of 5 μL of (α^{32}P)dCTP (2000 Ci/mMole) using the 'Prime-a-Gene' Labeling System (Promega, www.promega.com/). A U6 snoRNA probe (Table S6) end-labelled by (γ-32P) ATP using the T4 Polynucleotide kinase (Thermo Scientific Molecular Biology, http://www.thermoscientificbio.com/) was used as loading control. Hybridation signal intensity was determined using ImageJ (Schneider *et al.*, 2012).

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Author contributions

P.B-S and EH performed the majority of the experiments. CL did genotyping and generated plant mutant material. Search for RDR6 and SGS3 orthologs, selection of the TILLING mutants, small RNA libraries construction and analysis were performed by CLB and PBS. Identification of the *rdr6.2* allele by a reverse screen was performed by JW at the Noble foundation and the *Tnt-1* insertional mutant collection was generated by KSM. ES and JG performed bioinformatic analyses, CR and ACC applied statistical analyses and phasing criteria on small RNA data. CLB, FF and MC designed experiments and with the help of PBS and CH wrote the manuscript.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Phylogenetic analysis of SGS3-like proteins in *M. truncatula* and *A. thaliana*.
Figure S2 *MtSGS3* and *MtRDR6* expression in organs.
Figure S3 The *sgs3a.2* mutation affects a conserved G_{425} aminoacid residue inside the XS domain of the MtSGS3a protein.
Figure S4 The *rdr6.1* mutation affects a highly conserved G_{852} aminoacid residue of MtRDR6.
Figure S5 Analysis of *RDR6* mRNA accumulation in *rdr6* mutants.
Figure S6 Shoot and root biomasses are severely impaired in the *sgs3a* mutant lines.
Figure S7 Shoot and root biomasses are not affected in homozygous *rdr6.1* mutant plants.
Figure S8 The *rdr6.1* mutation does not affect tasiARF accumulation and *MtARF* expression in roots.
Figure S9 Accumulation of the *TAS3*-derived siRNAs is not significantly impaired in the *rdr6.1* mutant.
Figure S10 Examples of three novel RDR6-dependent phased loci identified.
Table S1 Accessions of *MtRDR6* and Mt*SGS3a, b* and *c* genes and protein similarities with their Arabidopsis orthologues.
Table S2 List of *TAS3*-ta-siRNAs identified in small RNA libraries from *M. truncatula* root and nodules.
Table S3 Global analysis of *rdr6.1* and WT small RNA libraries.
Table S4 List of the 21-nt siRNAs depleted in the *rdr6.1* libraries.
Table S5 List of putative RDR6-dependent siRNA-producing loci identified.
Table S6 List of primers.
Table S7 Sequences of additional *M. truncatula* genes.

