Running head: RNA silencing controls plant cuticular wax synthesis

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RDR1 and SGS3, components of RNA-mediated gene silencing, are required for regulation of cuticular wax biosynthesis in developing inflorescence stems of Arabidopsis

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ABSTRACT

The cuticle is a protective layer that coats the primary aerial surfaces of land plants, and mediates plant interactions with the environment. It is synthesized by epidermal cells and is composed of a cutin polyester matrix that is embedded and covered with cuticular waxes. Recently, we have discovered a novel regulatory mechanism of cuticular wax biosynthesis which involves the CER7 ribonuclease, a core subunit of the exosome. We hypothesized that at the onset of wax production, the CER7 ribonuclease degrades an mRNA specifying a repressor of CER3, a wax biosynthetic gene whose protein product is required for wax formation via the decarbonylation pathway. In the absence of this repressor, CER3 is expressed, leading to wax production. To identify the putative repressor of CER3 and to unravel the mechanism of CER7 mediated regulation of wax production, we performed a screen for suppressors of the cer7 mutant. Our screen resulted in the isolation of components of the RNA silencing machinery, RDR1 and SGS3, implicating RNA silencing in the control of cuticular wax deposition during inflorescence stem development in Arabidopsis.
INTRODUCTION

The acquisition of the cuticle, a hydrophobic structure that covers the surface of primary aerial plant tissues, represents one of the key evolutionary adaptations that allowed plants to successfully colonize land. The cuticle is synthesized by the epidermal cells and it protects the plant from non-stomatal water loss (Riederer and Schreiber, 2001), ultraviolet radiation (Reicosky and Hanover, 1978), pathogen invasion (Barthlott and Neinhuis, 1997), insect attack (Eigenbrode and Espelie, 1995), and other environmental stresses (Riederer, 2006). Additionally, the cuticle has been reported to mediate osmotic stress signalling (Wang et al., 2011), and have a role in preventing organ fusions during development by limiting the contact of neighbouring epidermal cells (Sieber et al., 2000; Wang et al., 2011). The cuticle is composed of 2 types of lipids: cutin, a plant-specific polyester of 16- and 18-carbon (C16 and C18) long hydroxy- and epoxy-fatty acids and glycerol (Nawrath, 2006; Pollard et al., 2008), and wax, a mixture of very long-chain fatty acids (VLCFAs) and their derivatives and variable amounts of triterpenoids and phenylpropanoids (Jetter et al., 2006; Nawrath, 2006). Wax compounds that are embedded within the cutin matrix are referred to as intracuticular waxes, whereas those that coat the surface of the cutin framework are referred to as epicuticular waxes.

Cuticular wax biosynthesis takes place in several cellular compartments and involves pathways for the synthesis of VLCFA wax precursors and their subsequent modification to diverse wax constituents. C16 and C18 fatty acids are made in the plastid of epidermal cells and are then exported to the endoplasmic reticulum (ER) where they are elongated to C24-C36 VLCFAs that serve as the precursors for wax compounds. This elongation process is catalyzed by the fatty acid elongase complex (FAE) comprised of four enzymes: a β-ketoacyl-CoA synthase (KCS), a β-ketoacyl-CoA reductase (KCR), a β-hydroxyacyl-CoA dehydratase (HCD), and an enoyl-CoA reductase (ECR) (Millar et al., 1999; Zheng et al., 2005; Bach et al., 2008; Beaudoin et al., 2009). Following elongation, VLCFAs are processed by the enzymes of the acyl-reduction pathway that yields primary alcohols and alkyl esters, and the decarbonylation pathway that produces aldehydes, alkanes, secondary alcohols and ketones (Samuels et al., 2008). The enzymes of the acyl-reduction pathway have been identified, and include a fatty acyl reductase CER4 that converts VLCFA-CoAs to primary alcohols (Rowland et al., 2006) and a bifunctional wax synthase/diacylglycerol acyltransferase WSD1 (Li et al., 2008) that generates wax esters. In contrast to the well-characterized acyl-reduction pathway, the only enzyme of the
The decarbonylation pathway with a known function is a cytochrome P450, designated mid-chain alkane hydroxylase 1 (MAH1), responsible for the oxidation of alkanes to secondary alcohols and ketones (Greer et al., 2007). Like the VLCFA elongation enzymes, all the characterized wax modification enzymes reside in the ER (Samuels et al., 2008).

Even though a number of key wax biosynthetic enzymes and their cellular compartmentations have been established, little is known about the regulation of wax biosynthesis. Regulation of wax production is affected by both developmental and environmental cues, but only a small number of genes involved in this process have been identified to date. Recently, Wu et al. (2011) reported the isolation of the CURLY FLAG LEAF 1 (CFL1) gene and demonstrated that it encodes a WW domain protein involved in cuticle development in Arabidopsis and rice. They provided biochemical evidence that AtCFL1 interacts with HDG1, a class IV homeodomain-leucine zipper transcription factor, which regulates two cuticle development-related genes, BODYGUARD (BDG) and FIDDLEHEAD (FDH). Other transcription factors known to regulate cuticle formation are WAX INDUCER1 (WIN1)/SHINE and its homologs, which primarily control cutin, and indirectly wax accumulation (Aharoni et al., 2004; Broun et al., 2004; Kannangara et al., 2007). The MYB96 transcription factor was shown to promote cuticular wax biosynthesis under drought conditions by binding directly to the conserved sequences in the promoters of wax biosynthetic genes and activating their transcription (Seo et al., 2011). As well, MYB30 was shown to activate the expression of wax biosynthetic genes in response to pathogen attack, but it remains to be determined to what extent this transcription factor participates in wax biosynthesis under normal conditions (Raffaele et al., 2008).

Besides direct activation of wax biosynthetic genes by transcription factors, our work on the wax-deficient cer7 mutant revealed that wax production in Arabidopsis stems is also controlled by the CER7 ribonuclease, a core subunit of the exosome that is responsible for the 3’ to 5’ degradation of RNA (Hooker et al., 2007). Functional characterization of the CER7 enzyme demonstrated that it positively regulates mRNA levels of CER3, a wax biosynthetic gene whose protein product is required for wax formation via the decarbonylation pathway (Hooker et al., 2007; Rowland et al., 2007). Based on analysis of cer3 mutants, CER3 is predicted to function at the start of the decarbonylation pathway, but the reaction that it catalyzes is still unknown (Rowland et al., 2007). Because CER7 is a ribonuclease, we proposed that it acts...
indirectly by degrading the mRNA specifying a repressor of \textit{CER3} transcription. A prediction of our model is that inactivation of this putative repressor would bypass the requirement of CER7 in wax biosynthesis. We therefore carried out a genetic screen for mutations that suppress the stem wax deficiency of \textit{cer7} in attempt to identify the putative repressor, as well as additional regulatory components downstream of CER7. Our screen resulted in the isolation of a series of \textit{wax restorer (war)} mutants with mutations in genes distinct from \textit{CER7}. Here we describe the cloning and characterization of the \textit{war3} and \textit{war4} suppressors of \textit{cer7}. Surprisingly, \textit{WAR3} and \textit{WAR4} encode components of RNA silencing machinery, implicating RNA silencing in the control of cuticular wax deposition during inflorescence stem development in Arabidopsis.

**RESULTS**

\textit{ProCER6:CER3} transgene complements the \textit{cer7-3} wax deficiency

A key assumption in finding the target of the \textit{CER7} exosomal ribonuclease is that it acts on an mRNA encoding a repressor that binds the promoter of the \textit{CER3} gene to control its transcription during development. Presumably, the mRNA of this putative repressor is not degraded in the \textit{cer7} mutant, and the presence of the repressor inhibits \textit{CER3} transcription. Consequently, the \textit{CER3} protein and all the wax components downstream of \textit{CER3} in the wax biosynthetic pathway are not synthesized. To test our proposed model, we attempted to rescue the \textit{cer7} phenotype by expressing the \textit{CER3} coding region behind the epidermis-specific \textit{CER6} promoter (Millar et al., 1999) to which the predicted repressor should not bind. As expected, the transformants that received the \textit{ProCER6:CER3} transgene were waxy (Fig. 1A) and had restored \textit{CER3} transcript levels as detected by quantitative real time PCR (Fig. 1B).

As a negative control, we also introduced the \textit{ProCER3:CER3} transgene into \textit{cer7-3}, but this construct failed to complement the \textit{cer7-3} phenotype and increased \textit{CER3} transcript was not detected (Fig. 1B). These data provide direct evidence that the \textit{cer7} phenotype is related to reduced \textit{CER3} transcription, and that the \textit{CER3} promoter sequence is relevant to CER7-mediated control of \textit{CER3} transcript levels.
**war mutants suppress the wax-deficiency of cer7**

To search for the putative CER3 repressor and identify additional components involved in CER7-mediated regulation of cuticular wax biosynthesis, we performed a genetic screen for extragenic mutations that suppress the cer7 glossy (wax-deficient) stem phenotype (Fig. 2). For the initial screen, approximately 12,000 cer7sti double mutant seeds were mutagenized with EMS (M1 population). The sti mutation, which results in a single-pronged trichome (Ilgenfritz et al., 2003), was introduced into the cer7 background to rule out possible wild-type seed contamination. The M1 population was grown to maturity for bulk harvest of the M2 seeds. Visual inspection of the M2 population resulted in the identification of 824 putative cer7 suppressors with waxy inflorescence stems. These suppressors were named war mutants (for wax restorer).

The M3 progeny of all the putative suppressors were then subjected to more rigorous analyses to confirm the sti trichome phenotype and the presence of the original cer7-1 mutation, and to determine the wax load, wax composition, as well as CER3 transcript levels of each mutant. Ninety-nine of the putative suppressor lines displayed the sti trichomes, and a diagnostic PCR-based CAPS assay showed that they also carried the original cer-7-1 mutant allele. Thus the restored stem wax loads in these lines were due to mutations at sites distinct from the original cer7-1 mutation. The 99 lines retained after the secondary screen fell into two general groups: group 1 including plants with completely waxy, wild-type-looking stems, and group 2 including plants with waxy stem bases, but glossy tops. We decided to focus on suppressor lines from group 1 and selected 32 war lines with the highest wax loads for further analysis. Allelism tests and rough genetic mapping revealed that they fall into at least four complementation groups, war1 through war4 (Fig. 3).

Stem wax analyses showed that all four war mutants have considerably higher wax loads than the cer7-1 mutant (Fig. 3B). War1, war2 and war4 have 67%, 71% and 90% of wild-type wax levels, respectively, whereas war3 accumulates 10% greater than wild-type wax levels (Fig. 3B). Furthermore, the cer7-1 wax composition characterized by a decrease in aldehyde, alkane, secondary alcohol and ketone levels, was restored to near wild-type compositions in the war lines (Fig. 3C). All the war mutants were also analyzed for the expression of CER3. Quantitative real time PCR measurements demonstrated that CER3 transcript accumulation was mostly or completely restored to wild-type levels, and paralleled the restoration of wax loads in each
suppressor line (Fig. 3D). Here we report the cloning and characterization of genes disrupted in war3 and war4 mutants.

**WAR3** encodes *RNA-dependent RNA polymerase 1*

Genetic analysis of the F2 progeny from a backcross of war3-1 cer7-1 suppressor line to cer7-1 showed an approximately 3:1 segregation ratio of the glossy mutant to the waxy wild-type (620:232; $\chi^2 = 2.26; p>0.1$), indicating that wax restoration was due to a recessive mutation in a single nuclear gene. To map the war3-1 mutation, war3-1 cer7-1 in the Landsberg erecta (Ler) background, was crossed to cer7-3 in the Columbia ecotype to create a mapping population. Thirty-five F2 plants exhibiting a waxy phenotype were used to establish linkage of war3-1 to markers F3F19 and F20D23 on chromosome 1 (Fig. 4A).

The map position of war3-1 was further delineated to a 150 kb genomic region between markers T5E21 and F10B6I-5 using a population of 232 waxy individuals (Fig. 4A). Sequencing of several candidate genes in this region revealed a point mutation in the third exon of At1g14790 at position 3171 (G to A transition), which is predicted to cause a premature stop codon in the war3-1 mutant. At1g14790 was also sequenced in war3-2 and war3-3, two additional alleles of war3 found in the suppressor screen, and in both cases missense mutations were detected (Fig. 4B), confirming that WAR3 is indeed At1g14790. At1g14790 encodes RNA-dependent RNA polymerase 1 (Yu et al., 2003). RNA-dependent RNA polymerases convert single-stranded RNA (ssRNA) to double-stranded RNA (dsRNA) that serves as the substrate for DICER. In Arabidopsis, there are 6 known RDRs. While RDR2 and RDR6 have been shown to be involved in silencing of endogenous transcripts during development, RDR1 has not yet been demonstrated to play a role in this process (Dalmay et al., 2000; Mourrain et al., 2000; Xie et al., 2004). Instead, RDR1 has been reported to be involved in anti-viral defense, and shown to promote turnover of viral RNAs in infected plants (Yu et al., 2003). Four additional alleles of war3 were identified from the T-DNA insertional mutant collection (Alonso et al., 2003): SALK_109922, SALK_112300, SALK_125022, SALK_007638 (Fig. 4B). Single homozygous war3 mutants do not have a visible wax phenotype, or any other morphological phenotypes. However, when homozygous war3 T-DNA mutants were crossed into the cer7-3 background, double mutants showed wild-type wax accumulation on inflorescence stems (Supplemental Fig. S1, A and B), indicating that these war3 alleles were also able to suppress the cer7-related wax
deficiency. No other morphological phenotypes were detected in the war3 cer7 double mutants. To verify that the mutation identified in war3 is responsible for the wax restoration of cer7-1, the genomic and promoter region encompassing At1g14790 was transformed into the war3-1 cer7-1 double mutant. Resulting transformants had a wax-deficient glossy stems confirming that WAR3 is RDR1 (Supplemental Fig. S2). Therefore, the war3 alleles described here will be subsequently referred to as rdr1 (Supplemental Table S1).

**war4 contains a mutation in SUPPRESSOR OF GENE SILENCING 3**

The unexpected finding that RDR1 is involved in regulation of stem wax deposition downstream of the CER7 ribonuclease prompted us to proceed with positional cloning of additional war suppressors to obtain more leads about the pathway involved. Genetic analysis of the F2 progeny from a backcross of war4-1 cer7-1 suppressor line to cer7-3 showed an approximately 3:1 segregation ratio of the glossy mutant to the waxy wild type (1951:641; $\chi^2 = 0.101; p>0.7$), indicating that wax restoration was due to a recessive mutation in a single nuclear gene. The approximate map position of war4 was determined using 22 F2 progeny from a war4-1 cer7-1 (Ler ecotype) cross to cer7-3 (Columbia ecotype) which localized the war4-1 mutation between markers CIW8 and NGA139 on chromosome 5 (Fig. 4C). Fine mapping was carried out using 641 F2 plants, and allowed us to narrow down the war4-1 mutation to a 100 kb region flanked by the markers K19M13 and MQM1, which contained 22 genes. Sequencing of candidate genes in this region revealed a C to T point mutation at position 454 in the first exon of At5g23570, predicted to cause a premature stop codon. Mutations in At5g23570 were also detected in four additional war4 alleles (Fig. 4D). At5g23570 encodes SUPPRESSOR OF GENE SILENCING 3 (SGS3), an RNA-binding protein that is required for post-transcriptional gene silencing (Mourrain et al., 2000) and trans-acting siRNA (ta-siRNA) production (Mourrain et al., 2000; Peragine et al., 2004). SGS3 is thought to bind and protect RNA from degradation before its conversion to dsRNA by an RDR (Yoshikawa et al., 2005). We obtained two T-DNA insertional war4 mutants from the T-DNA insertional mutant collection (Alonso et al., 2003), sgs3-13 (SALK_039005) and sgs3-14 (SALK_001394), which contain T-DNA insertions in the second intron and the first exon of At5g23570, respectively. The single sgs3 mutants do not exhibit stem wax deficiency, but as previously described for several other sgs3 alleles, sgs3-13 and sgs3-14 have slightly downward-curled leaf margins (Peragine et al., 2004). To test the
ability of sgs3-13 to suppress the cer7-caused stem wax deficiency like the war4-1 allele, we crossed it into the cer7-3 background. The resulting double mutant showed a waxy wild-type stem phenotype (Supplemental Fig. S1, A and B), and downward curled leaf margins further demonstrating that At5g23570 is WAR4. In addition, we introduced the SGS3 coding region under control of the cauliflower mosaic virus 35S promoter into the war4-1 cer7-1 double mutant and obtained glossy cer7-like T1 progeny, indicative of successful complementation (Supplemental Fig. S2). Thus, WAR4 is SGS3, and we renamed all the war4 alleles described here sgs3 (Fig. 4D; Supplemental Table S1).

**RDR1 and SGS3 are expressed throughout the plant**

Quantitative RT-PCR was used to assess expression levels of RDR1 and SGS3 in various organs. Aerial tissues were harvested from 4-6 week old plants, whereas seedling and roots were collected from 14-day-old plants. RDR1 and SGS3 expression was detected in all tissues (Fig. 5), but at varying levels. Expression patterns for RDR1 and SGS3 were very similar, with high expression levels found in seedlings, cauline leaves, rosette leaves and flowers. Moderate levels were detected in the stem top and base. Low levels of RDR1 and SGS3 expression were detected in roots and siliques.

To determine cell-type specific expression patterns of RDR1 and SGS3, we examined GUS activity in transgenic plants transformed with constructs in which the promoter region of RDR1 and SGS3 were fused to the GUS reporter gene (ProRDR1:GUS and ProSGS3:GUS, respectively). Cross-sections of the top of the stem show that both ProRDR1:GUS and ProSGS3:GUS are expressed in all stem tissues (Fig. 6, A and B).

In order to establish the subcellular localization of SGS3, a SGS3:YFP fusion protein under control of the 35S promoter was created (Pro35:SGS3:YFP) and expressed in transgenic sgs3-15 cer7-1 plants. The SGS3:YFP transgene was able to complement the waxy phenotype of sgs3-15 cer7-1, indicating that the SGS3:YFP fusion protein was functional. In developing stems, SGS3 was found to be localized to a reticulate structure typical of the ER (Fig. 6C, Supplemental Fig. S3 A to C). When leaves were examined, in addition to localization to the ER, SGS3 was also found to be present in the cytoplasm and in punctate structures, also termed cytoplasmic foci or granules, in agreement with previous reports (Fig. 6D, Supplemental Fig. S3, D to F) (Glick et al., 2008; Elmayan et al., 2009; Kumakura et al., 2009). The punctae observed
were not motile suggesting that they are not Golgi bodies, and did not co-localize with the hexyl rhodamine B stain, suggesting that they are not mitochondria (Supplemental Fig. S3 D to F). Because RDR6 was shown to interact with SGS3 and co-localize with SGS3 in similar puncta (Kumakura et al., 2009), we attempted to also determine the subcellular localization of RDR1. We expressed the \textit{RDR1:GFP} transgene under control of the native promoter, and transgenic \textit{rdr1-2 cer7-1} plants carrying \textit{ProRDR1:RDR1:GFP} were wax-deficient like the \textit{cer7-1} mutant, indicating that the RDR1:GFP fusion protein was functional. However, we were unable to detect strong fluorescent signal by confocal microscopy in any of the complemented lines. Low RDR1:GFP expression levels may be due to the weak RDR1 promoter.

\textbf{RDR1 and SGS3 are involved in regulation of \textit{CER3} expression in developing inflorescence stems}

Our suppressor screen resulted in the identification of several alleles of \textit{RDR1} and \textit{SGS3} suggesting that an RNA-based regulatory mechanism, possibly involving small RNAs, controls \textit{CER3} expression during cuticular wax deposition in developing inflorescence stems. During development, cuticular wax is synthesized predominantly at the top of the stem where the stem is actively elongating, and waxes are deposited evenly along the stem (Suh et al., 2005). This requires higher expression of wax biosynthetic genes, including \textit{CER3}, at the top of the stem than at the stem base.

To determine if \textit{CER3} transcription is developmentally regulated in Arabidopsis inflorescence stems, and investigate whether RNA silencing is involved in modulating \textit{CER3} expression, we monitored \textit{CER3} transcript levels in elongating stems by real-time PCR. As expected, \textit{CER3} transcript levels were considerably greater at the stem top than at the base of wild-type stems (Fig. 7). As shown previously, \textit{cer7-1} mutant plants displayed reduced \textit{CER3} transcript accumulation (Hooker et al., 2007) that did not significantly differ between the stem top and stem base. By contrast, an introduction of \textit{rdr1-2} or \textit{sgs3-15} mutations in the \textit{cer7-1} background resulted in a major surge in \textit{CER3} transcript accumulation, with the \textit{CER3} transcript levels reaching several fold greater levels than those detected in the wild-type stem top and stem base (Fig. 7). These data indicate that RDR1 and SGS3, implicated in small RNA biogenesis, are necessary for down-regulation of \textit{CER3} during development of Arabidopsis inflorescence stems.
DISCUSSION

We previously proposed a novel mechanism of regulating cuticular wax biosynthesis in developing Arabidopsis inflorescence stems, which involves the CER7 exosomal ribonuclease (Hooker et al., 2007). We hypothesized that CER7 controls the transcription of CER3, a key wax biosynthetic gene, via degradation of an mRNA encoding a negative regulator of CER3. To test this model, we expressed the CER3 transgene in the cer7-3 mutant using the epidermis-specific CER6 promoter, which is not affected by the same negative regulator as CER3, and successfully complemented the cer7-3 stem wax phenotype.

To identify the proposed negative regulator and other factors required for CER7-mediated control of CER3 expression, we performed a screen for suppressors of cer7-1, which restore cer7-related stem wax deficiency to wild-type wax levels. We isolated four classes of suppressors designated war1 to war4. In this study, we characterized war3 and war4, and the genes disrupted by these mutations. WAR3 encodes RNA-DEPENDENT RNA POLYMERASE 1 (RDR1), one of the six RDR proteins described in Arabidopsis. RDR proteins have been found in diverse eukaryotes, and are considered to be core members of the RNA silencing machinery. They catalyze the conversion of a single-stranded RNA template into double-stranded RNA (dsRNA), which serves as a substrate for dicer-like enzymes in the production of a type of small RNAs termed small interfering RNAs (siRNAs). It is well documented that RDR2 and RDR6 participate in siRNA mediated gene silencing in Arabidopsis (Peragine et al., 2004; Vazquez et al., 2004; Xie and Qi, 2008), but evidence for such a role for RDR1 is currently lacking, as it has only been reported to be involved in antiviral defense by promoting turnover of viral RNAs in infected plants (Yu et al., 2003). Moreover, Yu et al. (2003) reported that RDR1 expression in leaves is only induced upon viral infection, however we observed that RDR1 was constitutively expressed in most tissues at varying levels, consistent with expression patterns from the At-TAX tiling microarray experiments (Laubinger et al., 2008).

Map-based cloning of WAR4 revealed that it encodes SUPPRESSOR OF GENE SILENCING 3 (SGS3), a plant-specific protein suggested to bind and stabilize RNA template to initiate RDR-catalyzed dsRNA synthesis. SGS3 is essential for the synthesis of dsRNA in transgene silencing, virus silencing and the synthesis of trans-acting siRNAs involved in the regulation of gene expression during normal plant development (Peragine et al., 2004), and has been shown to directly interact with RDR6 in cytoplasmic punctae (Kumakura et al., 2009).
The identification of RDR1 and SGS3 in our screen for the *cer7-1* suppressors demonstrates that in addition to RDR6, RDR1 function also requires participation of SGS3. Furthermore, even though RDR1 has not been reported to be involved in endogenous gene silencing, based on our results it seems reasonable to speculate that RDR1 and SGS3 are involved in the production of an as yet uncharacterized small RNA species that directly or indirectly mediates transcriptional gene silencing of *CER3* to control wax deposition over the length of the stem. At the top of the stem where the stem is actively growing, wax biosynthetic genes are highly expressed (Suh et al., 2005). Conversely, at the base of the stem where growth has terminated, the expression of wax biosynthetic genes is reduced. As expected, in the wild type we found higher levels of the *CER3* transcript in the stem top compared to the stem base (Fig. 7). In the *cer7-1* mutant, *CER3* expression is significantly decreased, with *CER3* transcript levels being similarly low in both the top and bottom of the stem, which results in the wax-deficient phenotype. In contrast to the *cer7-1* mutant, *CER3* transcript levels in *rdr1-2 cer7-1* and *sgs3-15 cer7-1* double mutants are considerably higher in both the top and the stem base than *CER3* levels detected in the wild-type (Fig. 7), resulting in restoration of stem wax loads.

The simplest model that integrates all our findings is presented in Fig. 8. Small RNA precursors are known targets of the exosomal RNA ribonucleases (Chekanova et al., 2007). We hypothesize that in the wild-type stem tops where *CER7* is highly expressed (Supplemental Fig. S4), and the CER7 activity is presumably high, this exosomal ribonuclease degrades a precursor of a small RNA species that acts as a repressor of *CER3* expression. This results in enhanced *CER3* transcription and wax production via the decarbonylation pathway. *CER7* expression progressively decreases from the top towards the base of the stem (Supplemental Fig. S4), causing a gradual increase in small RNA accumulation. This is associated with down-regulation of *CER3* expression in the epidermal cells and cessation of wax production at the stem base. In the *cer7* mutant, where the CER7 exosomal subunit is not functional, buildup of small RNA causes CER3 silencing and stem wax deficiency. The biogenesis of small RNA precursors involved in silencing of *CER3* requires RDR1 and SGS3 activities. In the absence of RDR1 or SGS3 in the *rdr1 cer7* or *sgs3 cer7* double mutant, respectively, the small RNA species responsible for *CER3* repression will not be generated, abolishing the need for CER7 in wax biosynthesis.
In an attempt to verify this model and identify the potential small RNA species that represses *CER3* expression, we identified 33 small RNAs that map to the region upstream of *CER3* (Arabidopsis Small RNA Project 2010; http://asrp.cgrb.oregonstate.edu/). However, none of these RNAs map to the fragment of the *CER3* promoter that was used in our previous experiments to demonstrate that CER7 is required for transcription of the *CER3* gene during stem wax deposition (Hooker et al., 2007). This suggests that the regulation of *CER3* expression by small RNAs may be indirect and could involve another component, perhaps a positive regulator of *CER3* transcription, which is controlled by post-transcriptional gene silencing (PTGS). In this scenario, in wild type stem tops, the precursor of the small RNA repressor may be degraded by CER7 allowing the putative positive regulator to activate *CER3* transcription. At the bottom of the stem where the *CER7* activity is lower, the small RNA repressor may silence the positive regulator of *CER3*, causing down-regulation of *CER3* expression. In the *cer7* mutant, there may be a large accumulation of the small RNA repressor throughout the stem, silencing a positive regulator of *CER3* and resulting in very low levels of *CER3* transcription. In the *rdr1 cer7* or *sgs3 cer7* double mutants that lack the small RNA repressor, the putative positive regulator of *CER3* would be continuously expressed causing high levels of *CER3* transcription and wax biosynthesis.

**CONCLUSION**

We have uncovered a novel mechanism of regulating cuticular wax biosynthesis during stem elongation, which involves the exosome and RNA mediated gene silencing. Such an intricate system of regulation may be utilized by the plant to control metabolism during cuticle development as a great amount of energy is expended by epidermal cells to generate cuticular lipids. RNA silencing of *CER3* expression requires SGS3 and RDR1, providing evidence that RDR1 plays a role in gene regulation in addition to its role in antiviral defense. Identifying other components involved in this process, the RNA species responsible, and its target are important objectives for future research.
MATERIALS AND METHODS

Plant Material and Growth Conditions

cer7-1 sti and cer7-3 are in the Ler genetic background and the Columbia-0 genetic background, respectively. T-DNA insertion lines rdr1-1, rdr1-5 (SALK_109922), rdr1-6 (SALK_112300), rdr1-7 (SALK_125022), rdr1-8 (SALK_007638), sgs3-13 (SALK_039005) and sgs3-14 (SALK_001394) are in the Columbia-0 genetic background and were obtained from the ABRC (www.arabidopsis.org). Seeds were germinated on AT-agar plates (Somerville and Ogren, 1982) for 7-10 days and transplanted to soil (Sunshine Mix 4, SunGro). All plants were grown at 20°C under continuous light (90-110 µE m⁻² s⁻¹ of photosynthetically active radiation) in an environmental chamber.

Molecular complementation of cer7 with the CER3 transgene

The 1899bp CER3 coding region was excised from the plasmid pESC-TRP:ProGAL1:CER3 (P. Lam unpublished results) using BamHI and NheI. This fragment was cloned into the plasmid pBluescriptII:ProCER6 (P. Lam, unpublished results) into the corresponding restriction enzyme sites to generate pBluescriptII:ProCER6:CER3. The ProCER6-CER3 fragment was then excised using XhoI and SstI and cloned into pRD400 (Datla et al., 1992) that was excised with SalI and SstI (SalI and XhoI form compatible ends). The resulting plasmid, pRD400:ProCER6:CER3 was transformed into Agrobacterium tumefaciens strain GV3101, pMP90 (Koncz and Schell, 1986) via electroporation. Cer7-3 plants were transformed using the floral dip method (Clough and Bent, 1998).

Mutagenesis of cer7-1 sti

Approximately 12,000 cer7-1 sti seeds were soaked in a solution of 0.1M Na₃PO₄, 5% DMSO and 100mM Ethyl Methanesulphonate (EMS) for 5 hours. After mutagenesis, the seeds were washed twice with 100mM Na₂S₂O₃ and then twice with distilled water for 15 minutes per wash. Seeds were allowed to dry overnight before planting directly in soil in 64 total pots. Plants were grown until maturity and M₂ seeds were harvested collectively from each pot, yielding 64 batches. In the primary screen, M₂ seeds from each of the 64 batches were grown up and scored for a waxy stem phenotype. Plants that did not have a waxy phenotype were discarded. Those...
plants that were waxy were grown to maturity and seeds were harvested individually. These plants were then subjected to a secondary screen to confirm that they do have a waxy stem, the *stichel* trichome and that the *cer7-1* mutation was still present.

**Genotyping**

DNA was extracted according to Berendzen et al. (2005). To genotype *cer7-1*, dCAPS primers *cer7-1_AflIII-F* and *cer7-1_AflIII-R* were used to amplify a 210bp fragment. The PCR product was then digested with AflIII and run on a 1.5% agarose gel. The mutation in *cer7-1* allows for the cleavage of the PCR product after AflIII digestion resulting in an 185bp and a 25bp product. T-DNA insertion lines were genotyped using LBb1.3 and gene specific primers as listed in Supplemental Table S3.

**Cuticular wax extraction and analysis**

Cuticular waxes were extracted from 4-6 week old Arabidopsis stems. Stems were immersed for 30 seconds in chloroform containing 10µg n-tetracosane which was used as an internal standard. After extraction, samples were blown down under a gentle stream of nitrogen and redissolved in 10µL *N,O*-bis(trimethylsilyl) trifluoroacetamide (Sigma) and 10µL pyridine (Fluka). Samples were derivatized for 90 minutes at 80°C. After derivatization, excess BSTFA and pyridine were removed by blowing down under nitrogen and samples were dissolved in 30µL of chloroform. Gas-liquid chromatography was performed in the samples using a HP 6890 series gas chromatograph equipped with flame ionization detection and a 30m HP-1 column with helium as the carrier gas. GC was carried out with temperature-programmed on-column injection and oven temperature set at 50°C for 2 min, raised by 40°C min⁻¹ to 200°C, held for 2 min at 200°C, raised by 3°C min⁻¹ to 320°C, and held for 30 min at 320°C.

Quantification of wax loads were determined by comparing the flame ionization detector peak areas to the internal standard. Stem surface area was calculated by photographing stems prior to wax extraction, measuring the number of pixels, converting them to cm², and multiplying by π.
Quantitative RT-PCR
RNA was extracted from plant tissue using TRIzol (Invitrogen) as per manufacturer’s protocol. RNA quantification was performed using a NanoDrop 8000 (Thermo Scientific). 500ng of total RNA was treated with DNaseI (Fermentas) and then used for first strand cDNA synthesis using iScript RT supermix (Bio-Rad). Quantitative RT-PCR was performed using gene-specific primer sets from Supplemental Table S3, in 20µL reactions using iQ SYBR green supermix (Bio-Rad) and run on the iQ5 real-time PCR detection system (Bio-Rad). Data were analyzed using the Pfaffl method (Pfaffl, 2001), and control samples were normalized to 1. Statistical significance was measured with a student’s T-test.

Positional cloning of suppressor lines
To map the position of suppressor lines, each suppressor line was crossed to cer7-3 and grown to the F2 generation. DNA from leaves was collected on FTA cards (Whatman), and 30-40 plants with the wildtype waxy stem phenotype (plants homozygous for the suppressor mutation) were subjected to PCR using simple sequence length polymorphism (SSLP) markers to determine linkage. To further pinpoint the location of each suppressor loci, over 1000 plants were screened with SSLP markers until a narrow interval was found.

Molecular complementation of suppressor lines and subcellular localization of RDR1 and SGS3
A 5252bp DNA fragment containing 1754bp of the upstream region of RDR1 and the coding region minus the STOP codon was amplified from WT Col plants with primers RDR1p-attB1 and RDR1-attB2_noSTOP using Phusion polymerase (Finnzymes). Gateway adapters were added using the adapter protocol (Invitrogen). This 5252bp fragment was cloned into pDONR221 using BP Clonase II (Invitrogen) to create pDONR221:ProRDR1:RDR1ΔSTOP and was sequenced to confirm that no mutations were introduced during PCR. The fragment was then recombined into the destination vector pGWB4 (Nakagawa et al., 2007) using LR Clonase II (Invitrogen) to generate pGWB4:ProRDR1:RDR1:GFP.

To generate SGS3:YFP for sub-cellular localization analysis, the coding sequence of SGS3 (At5g23570) was obtained from leaf cDNA using primers SGS3-attB1 and SGS3-attB2_noSTOP with Phusion polymerase (Finnzymes). The PCR product was introduced into the
pDONR207 entry vector using BP Clonase II (Invitrogen). Sequencing was performed to confirm error free inserts which were then transferred to the binary vectors pEarleyGate104 (Earley et al., 2006) using LR Clonase II (Invitrogen).

These constructs were introduced into *rdr1-2 cer7-1* and *sgs3-15 cer7-1* plants via *Agrobacterium*-mediated transformation as described above.

Spinning disk confocal microscopy was performed on a Perkin Elmer Ultraview VoX Spinning Disk Confocal mounted on a Leica DMI6000 inverted microscope. GFP and YFP were detected using a 488nm laser and 528/38-nm emission filters. For ER staining, stems and leaves of transgenic *sgs3-15 cer7-1* plants expressing SGS3-YFP were immersed in hexyl rhodamine B solution (1.6 µM) for 10 to 30 min. Hexyl rhodamine B was excited with a 561nm laser line and a 600-nm long-pass emission filter. Acquired images were processed using Volocity (Improvision) and ImageJ.

**RDR1 and SGS3 promoter:GUS fusions and GUS Activity Assay**

To generate *ProRDR1:GUS*, a 1754bp region upstream of the *RDR1* initiation codon was amplified from genomic DNA using the primers RDR1pro_EcoRI-F and RDR1_XbaI-R with Phusion polymerase (Finnzymes). The PCR product was digested with EcoRI and XbaI and cloned into the corresponding restriction enzyme sites of pBluescriptIISK(+) (Stratagene). After confirmation that no errors were induced from PCR, the *ProRDR1* region was excised using SalI and BamHI and cloned into the corresponding sites of pBI101 (Clontech) to generate *pBI101:ProRDR1:GUS*. To generate *ProSGS3p:GUS*, a 2177-bp long region containing 2141 bps immediately upstream of the *SGS3* translation start site and 36 bps downstream of the *SGS3* translation start site was amplified from genomic DNA using gene specific primers SGS3pro-attB1 and SGS3pro-attB2 with Phusion polymerase (Finnzymes). The obtained fragment was introduced to pDONR207 entry vector, sequenced to confirm accuracy and transferred into the pMDC163 destination vector.

Stems from transgenic plants containing the *ProRDR1p:GUS* and *ProSGS3p:GUS* constructs were removed and immersed in GUS staining buffer containing 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 100 mM Na$_3$HPO$_4$, 100 mM NaH$_2$PO$_4$, 0.2% Triton-X-100, and 1 mM 5-bromo-4-chloro-3-indoly1-β-D-glucuronide (X-Gluc) for 1-2 h at 37°
C. Stems were then cleared of chlorophyll by overnight incubation in 75% ethanol. Stained and cleared samples were examined by compound light microscopy.

**Accession Numbers**
Sequence data from this article can be obtained from the Arabidopsis Genome Initiative database under the following accession numbers: CER7 (At3g60500), CER3 (At5g57800), RDR1 (At1g14790), SGS3 (At5g23570).

**Supplemental Data**
The following materials are available in the online version of this article.

**Supplemental Figure S1.** Wax levels are restored in rdr1-7 cer7-3 and sgs3-13 cer7-3 double mutants.

**Supplemental Figure S2.** The RDR1 and SGS3 transgene can complement war3-1 cer7-1 and war4-1 cer7-1, respectively.

**Supplemental Figure S3.** Co-localization of SGS3:YFP labeled network and the ER network stained by hexyl rhodamine B.

**Supplemental Figure S4.** Quantitative RT-PCR of CER7 expression levels in the top 3cm and the bottom 3cm of a 10cm stem, as well as the epidermis.

**Supplemental Table S1.** Nomenclature and description of the rdr1 alleles.

**Supplemental Table S2.** Nomenclature and description of the sgs3 alleles.

**Supplemental Table S3.** Primers used in this study

**Acknowledgments**
We thank the Salk Institute for Genomic Analysis Laboratory for providing sequence-indexed Arabidopsis T-DNA insertion mutants, the Bioimaging Facility at the University of British Columbia for help with microscopy, Jonathan Griffiths and Tegan Haslam for helpful discussions and critical evaluation of the manuscript, and Donald Yung for technical assistance.

**Author Contributions**
P.L., T.S.H., and L.K. designed research. P.L., L.Z., H.E.M., M.A., V.L., and T.S.H. performed experiments. P.L. and L.K. wrote the article.
Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A (2004) The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. Plant Cell 16: 2463–2480

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653 –657

Bach L, Michaelson LV, Haslam R, Bellec Y, Gissot L, Marion J, Da Costa M, Boutin J-P, Miquel M, Tellier F, et al (2008) The very-long-chain hydroxy fatty acyl-CoA dehydratase PASTICCINO2 is essential and limiting for plant development. Proc Natl Acad Sci USA 105: 14727–14731

Barthlott W, Neinhuis C (1997) Purity of the sacred lotus, or escape from contamination in biological surfaces. Planta 202: 1–8

Beaudoin F, Wu X, Li F, Haslam RP, Markham JE, Zheng H, Napier JA, Kunst L (2009) Functional characterization of the Arabidopsis β-ketoacyl-coenzyme A reductase candidates of the fatty acid elongase. Plant Physiol 150: 1174–1191

Berendzen K, Searle I, Ravenscroft D, Koncz C, Batschauer A, Coupland G, Somssich IE, Ulker B (2005) A rapid and versatile combined DNA/RNA extraction protocol and its application to the analysis of a novel DNA marker set polymorphic between Arabidopsis thaliana ecotypes Col-0 and Landsberg erecta. Plant Methods. doi: doi:10.1186/1746-4811-1-4

Broun P, Poindexter P, Osborne E, Jiang C-Z, Riechmann JL (2004) WIN1, a transcriptional activator of epidermal wax accumulation in Arabidopsis. Proc Natl Acad Sci USA 101: 4706–4711

Chekanova JA, Gregory BD, Reverdatto SV, Chen H, Kumar R, Hooker T, Yazaki J, Li P, Skiba N, Peng Q, et al (2007) Genome-wide high-resolution mapping of exosome substrates reveals hidden features in the Arabidopsis transcriptome. Cell 131: 1340–1353

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743
Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC (2000) An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell 101: 543–553

Datla RSS, Hammerlindl JK, Panchuk B, Pelcher LE, Keller W (1992) Modified binary plant transformation vectors with the wild-type gene encoding NPTII. Gene 122: 383–384

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J 45: 616–629

Eigenbrode SD, Espelie KE (1995) Effects of plant epicuticular lipids on insect herbivores. Ann Rev Ent 40: 171–194

Elmayan T, Adenot X, Gissot L, Laussergues D, Gy I, Vaucheret H (2009) A neomorphic sgs3 allele stabilizing miRNA cleavage products reveals that SGS3 acts as a homodimer. FEBS J 276: 835–844

Glick E, Zrachya A, Levy Y, Mett A, Gidoni D, Belausov E, Citovsky V, Gafni Y (2008) Interaction with host SGS3 is required for suppression of RNA silencing by tomato yellow leaf curl virus V2 protein. Proc Natl Acad Sci USA 105: 157–161

Greer S, Wen M, Bird D, Wu X, Samuels L, Kunst L, Jetter R (2007) The cytochrome P450 enzyme CYP96A15 is the midchain alkane hydroxylase responsible for formation of secondary alcohols and ketones in stem cuticular wax of Arabidopsis. Plant Physiol 145: 653–667

Hooker TS, Lam P, Zheng H, Kunst L (2007) A core subunit of the RNA-processing/degrading exosome specifically influences cuticular wax biosynthesis in Arabidopsis. Plant Cell 19: 904–913

Ilgenfritz H, Bouyer D, Schnittger A, Mathur J, Kirik V, Schwab B, Chua N-H, Jürgens G, Hülskamp M (2003) The Arabidopsis STICHEL gene is a regulator of trichome branch number and encodes a novel protein. Plant Physiol 131: 643 –655

Jetter R, Kunst L, Samuels L (2006) Composition of plant cuticular waxes. In M Riederer, C Muller, eds, Biology of the Plant Cuticle, Vol 23. Blackwell Publishing Ltd, Oxford, pp 145-181
Kannangara R, Branigan C, Liu Y, Penfield T, Rao V, Mouille G, Hofte H, Pauly M, Riechmann JL, Broun P (2007) The transcription factor WIN1/SHN1 regulates cutin biosynthesis in Arabidopsis thaliana. Plant Cell 19: 1278–1294

Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Mol Gen Genet 204: 383–396

Kumakura N, Takeda A, Fujioka Y, Motose H, Takano R, Watanabe Y (2009) SGS3 and RDR6 interact and colocalize in cytoplasmic SGS3/RDR6-bodies. FEBS Lett 583: 1261–1266

Laubinger S, Zeller G, Henz SR, Sachsenberg T, Widmer CK, Naouar N, Vuylsteke M, Scholkpof B, Ratsch G, Weigel D (2008) At-TAX: a whole genome tiling array resource for developmental expression analysis and transcript identification in Arabidopsis thaliana. Genome Biol 9: R112

Li F, Wu X, Lam P, Bird D, Zheng H, Samuels L, Jetter R, Kunst L (2008) Identification of the wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase WSD1 required for stem wax ester biosynthesis in Arabidopsis. Plant Physiol 148: 97–107

Millar AA, Clemens S, Zachgo S, Giblin EM, Taylor DC, Kunst L (1999) CUT1, an Arabidopsis gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. Plant Cell 11: 825–838

Mourrain P, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel J-B, Jouette D, Lacombe A-M, Nikic S, Picault N, et al (2000) Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell 101: 533–542

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T (2007) Development of series of gateway binary vectors, pGW Bs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng 104: 34–41

Nawrath C (2006) Unraveling the complex network of cuticular structure and function. Curr Opin Plant Biol 9: 281–287

Peragine A, Yoshikawa M, Wu G, Albrecht HL, Poethig RS (2004) SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. Gene Dev 18: 2368–2379
Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 29: 2002–2007

Pollard M, Beisson F, Li Y, Ohlrogge JB (2008) Building lipid barriers: biosynthesis of cutin and suberin. Trends Plant Sci 13: 236–246

Raffaele S, Vailleau F, Léger A, Joubès J, Miersch O, Huard C, Blée E, Mongrand S, Domergue F, Roby D (2008) A MYB transcription factor regulates very-long-chain fatty acid biosynthesis for activation of the hypersensitive cell death response in Arabidopsis. Plant Cell 20: 752–767

Reicosky DA, Hanover JW (1978) Physiological effects of surface waxes: I. light reflectance for glaucous and nonglaucous Picea pungens. Plant Physiol 62: 101–104

Riederer M (2006) Introduction: biology of the plant cuticle. In M Riederer, C Muller, eds, Biology of the Plant Cuticle, Vol 23. Blackwell Publishing Ltd, Oxford, pp 1-10

Riederer M, Schreiber L (2001) Protecting against water loss: analysis of the barrier properties of plant cuticles. J Exp Bot 52: 2023–2032

Rowland O, Lee R, Franke R, Schreiber L, Kunst L (2007) The CER3 wax biosynthetic gene from Arabidopsis thaliana is allelic to WAX2/YRE/FLP1. FEBS Lett 581: 3538–3544

Rowland O, Zheng H, Hepworth SR, Lam P, Jetter R, Kunst L (2006) CER4 encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis. Plant Physiol 142: 866–877

Samuels L, Kunst L, Jetter R (2008) Sealing plant surfaces: cuticular wax formation by epidermal cells. Annu Rev Plant Biol 59: 683–707

Seo PJ, Lee SB, Suh MC, Park M-J, Go YS, Park C-M (2011) The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in Arabidopsis. Plant Cell 23: 1138–1152

Sieber P, Schorderet M, Ryser U, Buchala A, Kolattukudy P, Metraux J-P, Nawrath C (2000) Transgenic Arabidopsis plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell 12: 721–738

Somerville CR, Ogren WL (1982) Isolation of photorespiratory mutants of Arabidopsis. In Methods in Chloroplast Molecular Biology. Elsevier, New York, pp 129–139
Suh MC, Samuels AL, Jetter R, Kunst L, Pollard M, Ohlrogge J, Beisson F (2005) Cuticular lipid composition, surface structure, and gene expression in Arabidopsis stem epidermis. Plant Physiol 139: 1649 –1665

Vazquez F, Vaucheret H, Rajagopalan R, Lepers C, Gasciolli V, Mallory AC, Hilbert J-L, Bartel DP, Crété P (2004) Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. Mol Cell 16: 69–79

Wang Z-Y, Xiong L, Li W, Zhu J-K, Zhu J (2011) The plant cuticle is required for osmotic stress regulation of abscisic acid biosynthesis and osmotic stress tolerance in Arabidopsis. Plant Cell 23: 1971 –1984

Wu R, Li S, He S, Waßmann F, Yu C, Qin G, Schreiber L, Qu L-J, Gu H (2011) CFL1, a WW domain protein, regulates cuticle development by modulating the function of HDG1, a class IV homeodomain transcription factor, in rice and Arabidopsis. Plant Cell 23: 3392 –3411

Xie Z, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, Jacobsen SE, Carrington JC (2004) Genetic and functional diversification of small RNA pathways in plants. PLoS Biol 2: 642–652

Xie Z, Qi X (2008) Diverse small RNA-directed silencing pathways in plants. BBA-Gene Regul Mech 1779: 720–724

Yoshikawa M, Peragine A, Park MY, Poethig RS (2005) A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. Gene Dev 19: 2164 –2175

Yu D, Fan B, MacFarlane SA, Chen Z (2003) Analysis of the involvement of an inducible Arabidopsis RNA-dependent RNA polymerase in antiviral defense. Mol Plant Microbe Interact 16: 206–216

Zheng H, Rowland O, Kunst L (2005) Disruptions of the Arabidopsis enoyl-CoA reductase gene reveal an essential role for very-long-chain fatty acid synthesis in cell expansion during plant morphogenesis. Plant Cell 17: 1467–1481
FIGURE LEGENDS

Figure 1. CER3, under control of the CER6 promoter, can complement cer7-3.
A, Stems from 5-week-old wild-type (Columbia), cer7-3, and cer7-3 transformed with the ProCER6:CER3 transgene showing restored wax in the transgenic plant. B, Quantitative RT-PCR showing that CER3 expression levels are restored to wild-type levels in plants carrying the ProCER6:CER3 transgene. ACTIN2 was used as an internal control and control samples were normalized to 1. Values represent means ± SD (n=4). Statistically significant differences from cer7-3 (p<0.05) are indicated by *.

Figure 2. Summary of the suppressor screen.

Figure 3. Analysis of war mutants.
A, Stems of 6-week-old wild-type (Ler), cer7-1 and the 4 war mutants showing the suppression of the cer7-1 wax-deficient phenotype in the war mutants as indicated by glaucous stems. B, Stem wax loads of war1 to war4 compared to wild-type and cer7-1. Values represent means ± SD (n=3). Statistically significant differences between samples (p<0.05) are indicated by *. C, Stem wax composition of war1 to war4 compared to wild-type and cer7-1. Wax compositions for all war mutants are restored to near wild-type-like ratios of major wax components. D, Quantitative RT-PCR showing that CER3 transcript levels are restored to wild-type levels in the war mutants. ACTIN2 was used as an internal control and control samples were normalized to 1. Values represent means ± SD (n=4). Statistically significant differences between samples (p<0.05) are indicated by *.

Figure 4. Positional cloning of war3 and war4, and RDR1 and SGS3 gene structures.
A, Schematic representation of the chromosomal location of war3 as determined by fine mapping. The markers used for mapping and number of recombinants are indicated. B, Schematic representation of the RDR1 gene structure. 5’ and 3’ UTRs are indicated as gray boxes, exons as white boxes and introns as black lines. The translational start site is represented by the bent arrow. The position and types of the mutations in rdr1 mutant alleles are also shown. C, Schematic representation of the chromosomal location of war4 as determined by fine
mapping. The markers used for mapping and the number of recombinants are indicated. D, Schematic representation of the SGS3 gene structure and the position and types of mutations in sgs3 alleles. 5’ and 3’ UTRs are indicated as gray boxes, exons as white boxes and introns as black lines. The translational start site is represented by the bent arrow.

**Figure 5.** Expression analysis of RDR1 and SGS3 in different organs and tissues of wild-type Arabidopsis (Columbia) as determined by quantitative RT-PCR. ACTIN2 was used as an internal control and control samples were normalized to 1. Values represent means ± SD (n=4).

**Figure 6.** Expression of RDR1 and SGS3.
A and B, Tissue-specific expression of ProRDR1:GUS and ProSGS3:GUS in Arabidopsis stems. A stem of a 4-week-old transgenic plant expressing ProRDR1:GUS (A) or ProSGS3:GUS (B) was stained for GUS activity. A cross-section from the top 3cm of the stem is shown. Scale bar = 0.1mm. C and D, Localization of SGS3 by confocal microscopy. In stems SGS3:YFP is localized to the ER (C). In leaves, SGS3:YFP is localized to the cytoplasm and to puncta (D). Images are Z-projections of confocal stacks. Scale bar = 10 µm.

**Figure 7.** CER3 expression levels in the top 3cm and the bottom 3cm of a 10cm stem as measured by quantitative RT-PCR. ACTIN2 was used as an internal control and control samples were normalized to 1. Values represent means ± SD (n=4) and statistically significant differences (p<0.05) are indicated by *.

**Figure 8.** Model illustrating the role of RDR1 and SGS3, components of RNA silencing, in regulating cuticular wax biosynthesis at the top of the stem.
A, In the wild-type, the precursor of the smRNA that regulates expression of CER3 is degraded by CER7, therefore CER3 is expressed and cuticular wax production ensues. B, In the cer7 mutant, the smRNA precursor is not degraded and is used for the production of a smRNA species by a pathway which involves RDR1 and SGS3. smRNA functions to silence CER3, leading to decreased cuticular wax biosynthesis. C, In either rdr1 or sgs3 suppressors of cer7, the smRNA species responsible for CER3 silencing will not be synthesized, resulting in CER3 expression and wax production in the absence of CER7 activity.
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12,000 mutagenized lines

Primary Screen:
Screen for waxy stems in M₂

824 mutants

Secondary Screen in M₃:
• genotyping
• sti trichome
• wax analysis
• qPCR

99 mutants

Group 1
completely waxy stems - 77 mutants

Group 2
waxy stem bases - 22 mutants

Complementation Crosses and Rough Mapping

Select 4 mutants for Fine Mapping

Figure 2. Summary of the suppressor screen.
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Note: All experiments were performed in triplicate and analyzed using one-way ANOVA followed by Tukey’s multiple comparison test.
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(C) Schematic representation of the chromosomal location of war4 as determined by fine mapping. The markers used for mapping and the number of recombinants are indicated.  
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C, In either rdr1 or sgs3 suppressors of cer7, the smRNA species responsible for CER3 silencing will not be synthesized from CER3. Thus, CER3 expression and wax production in the absence of CER7 activity.