RDR1 and SGS3, Components of RNA-Mediated Gene Silencing, Are Required for the Regulation of Cuticular Wax Biosynthesis in Developing Inflorescence Stems of Arabidopsis$^{1[W][OA]}$

Patricia Lam$^2$, Lifang Zhao$^2$, Heather E. McFarlane, Mytyl Aiga, Vivian Lam, Tanya S. Hooker, and Ljerka Kunst*

Department of Botany, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada

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 that involves the ECERIFERUM7 (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, RNA-DEPENDENT RNA POLYMERASE1 and SUPPRESSOR OF GENE SILENCING3, implicating RNA silencing in the control of cuticular wax deposition during inflorescence stem development in Arabidopsis (Arabidopsis thaliana).

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 protects the plant from nonstomatal water loss (Riederer and Schreiber, 2001), UV 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 signaling (Wang et al., 2011) and to have a role in preventing organ fusions during development by limiting the contact of neighboring epidermal cells (Sieber et al., 2000; Wang et al., 2011). The cuticle is composed of two types of lipids: cutin, a plant-specific polyester of 16- and 18-carbon-long (C16 and C18) 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 to C36 VLCFAs that serve as the precursors for wax compounds. This elongation process is catalyzed by the fatty acid elongase complex composed of four enzymes: a β-ketoacyl-CoA synthase, a β-ketoacyl-CoA reductase, a β-hydroxyacyl-CoA dehydratase, and an enoyl-CoA reductase (Millar et al., 1999; Zheng et al., 2005; Bach et al., 2009; Beaudoin et al., 2009). Following elongation, VLCFAs are processed by the enzymes of the acyl-reduction pathway, which yields primary alcohols and alkyl esters, and the decarbonylation pathway, which 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, ECERIFERUM4 (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 decarbonylation pathway with a known function is a cytochrome P450, designated MIDCHAIN ALKANE HYDROXYLASE1, responsible for the oxidation of alcanes 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. The 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 LEAF1 (CFL1) gene and demonstrated that it encodes a WW domain protein involved in cuticle development in Arabidopsis (Arabidopsis thaliana) and rice (Oryza sativa). They provided biochemical evidence that AtCFL1 interacts with HDG1, a class IV homeodomain-Leu zipper transcription factor, which regulates two cuticle development-related genes, BODYGUARD and FIDDLEHEAD. Other transcription factors known to regulate cuticle formation are WAX INDUCER1/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 RNase, 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 an 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 RNase, we proposed that it acts indirectly by degrading the mRNA specifying a repressor of CER3 transcription. A prediction of our model is that inactivation of this putative repressor would bypass the requirement of CER7 in wax biosynthesis. Therefore, we carried out a genetic screen for mutations that suppress the stem wax deficiency of cer7 in an 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 wax restorer (war) mutants with mutations in genes distinct from CER7. Here, we describe the cloning and characterization of the war3 and war4 suppressors of cer7. Surprisingly, WAR3 and WAR4 encode components of the RNA-silencing machinery, implicating RNA silencing in the control of cuticular wax deposition during inflorescence stem development in Arabidopsis.

RESULTS

The ProCER6: CER3 Transgene Complements the cer7-3 Wax Deficiency

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

As a negative control, we also introduced the ProCER3: CER3 transgene into cer7-3, but this construct failed to complement the cer7-3 phenotype and increased CER3 transcript was not detected (Fig. 1B). These data provide direct evidence that the cer7 phenotype is related to reduced CER3 transcription and that the CER3 promoter sequence is relevant to the CER7-mediated control of 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 cer7 sti double mutant seeds were mutagenized with ethyl methanesulfonate (M1 population). The stichel (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. 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, and CER3 transcript levels of each mutant. Ninety-nine of the putative suppressor lines displayed the sti trichomes, and a diagnostic PCR-based cleaved-amplified polymorphic sequence assay showed that they also carried the original cer7-1 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 decreases in aldehyde, alkane, secondary alcohol, and ketone levels, was restored to near wild-type composition 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 POLYMERASE1

Genetic analysis of the F2 progeny from a backcross of the 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-0 ecotype to create a mapping population. Thirty-five F2 plants exhibiting a waxy phenotype were used to

Figure 1. CER3, under the control of the CER6 promoter, can complement cer7-3. A, Stems of 5-week-old wild type (WT; Columbia-0), 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 ± so (n = 4). Statistically significant differences from cer7-3 (P < 0.05) are indicated by asterisks.

Figure 2. Summary of the suppressor screen.
establish the 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 3,171 (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 POLYMERASE1 (RDR1; Yu et al., 2003). RDRs convert single-stranded RNA to double-stranded (ds) RNA that serves as the substrate for DICER. In Arabidopsis, there are six known RDRs. While RDR2 and RDR6 have been shown to be involved in the 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 antiviral defense and shown to promote the 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, and 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 cer7 double mutant. Resulting transformants had 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).

Figure 3. Analysis of war mutants. A, Stems of 6-week-old wild type (WT; Ler), cer7-1, and four war mutant plants 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 with the wild type and cer7-1. Values represent means ± sd (n = 3). Statistically significant differences between samples (P < 0.05) are indicated by asterisks. C, Stem wax composition of war1 to war4 compared with the 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.

war4 Contains a Mutation in SUPPRESSOR OF GENE SILENCING3

The unexpected finding that RDR1 is involved in the regulation of stem wax deposition downstream of the CER7 exoribonuclease 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 the war4-1 cer7-1 (Ler ecotype) suppressor line to cer7-3 (Columbia-0 ecotype), showed an approximately 3:1 segregation ratio of the glossy mutant to the waxy wild type (1,951:641; $\chi^2 = 0.101$, $P > 0.7$), 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 cross to cer7-3 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 SILENCING3 (SGS3), an RNA-binding protein that is required for post-transcriptional gene silencing (Mourrain et al., 2000) and trans-acting small interfering RNA (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 described previously 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 the 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 S2).

**RDR1 and SGS3 Are Expressed throughout the Plant**

Quantitative reverse transcription (RT)-PCR was used to assess the expression levels of RDR1 and SGS3 in various organs. Aerial tissues were harvested from 4- to 6-week-old plants, whereas seedlings and roots were collected from 14-d-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 or SGS3 was fused to the GUS reporter gene (ProRDR1:GUS or 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, an SGS3:yellow fluorescent protein (YFP) fusion protein under the control of the 35S promoter was created (Pro35S: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 and in punctate structures, also termed cytoplasmic foci or granules, in agreement with previous reports (Fig. 6D; Supplemental Fig. S3, D–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 colocalize with the hexyl rhodamine B stain, suggesting that they are not mitochondria (Supplemental Fig. S3, D–F). Because RDR6 was shown to interact with SGS3 and colocalize with SGS3 in similar punctae (Kumakura et al., 2009), we attempted to also determine the subcellular localization of RDR1. We expressed the RDR1:GFP transgene under the control of the native promoter, and transgenic rdr1-2 cer7-1 plants carrying ProRDR1:RDR1:GFP were wax deficient like the 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.

**RDR1 and SGS3 Are Involved in the Regulation of CER3 Expression in Developing Inflorescence Stems**

Our suppressor screen resulted in the identification of several alleles of RDR1 and SGS3, suggesting that an RNA-based regulatory mechanism, possibly involving small RNAs, controls 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 CER3, at the top of the stem than at the stem base.

To determine if CER3 transcription is developmentally regulated in Arabidopsis inflorescence stems, and to investigate whether RNA silencing is involved in modulating CER3 expression, we monitored CER3 transcript levels in elongating stems by real-time PCR. As expected, CER3 transcript levels were considerably greater at the stem top than at the base of wild-type stems (Fig. 7). As shown previously, cer7-1 mutant plants displayed reduced CER3 transcript accumulation (Hooker et al., 2007) that did not significantly differ between the stem top and stem base. By contrast, introduction of the rdr1-2 or sgs3-15 mutation in the cer7-1 background resulted in a major surge in CER3 transcript levels at the stem top, suggesting that RNA silencing is involved in CER3 regulation.
transcript accumulation, with the CER3 transcript reaching severalfold 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 the down-regulation of CER3 during the 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 RNase (Hooker et al., 2007). We hypothesized that CER7 controls the transcription of CER3, a key wax biosynthetic gene, via the 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, and the genes disrupted by these mutations. WAR3 encodes 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 dsRNA, which serves as a substrate for DICER-like enzymes in the production of a type of small RNAs termed 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 the 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 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 it 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 the 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 with 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 the restoration of stem wax loads.

The simplest model that integrates all our findings is presented in Figure 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 RNase 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 decarboxylation pathway. CER7 expression progressively decreases from the top toward the base of the stem (Supplemental Fig. S4), causing a gradual increase in small RNA accumulation. This is associated with the down-regulation of CER3 expression in the epidermal cells and the cessation of wax production at the stem base. In the cer7 mutant, where the CER7 exosomal subunit is not functional, the buildup of small RNAs causes CER3 silencing and stem wax deficiency. The biogenesis of small RNA precursors involved in the 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 posttranscriptional gene silencing. 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 the 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 and 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

Arabidopsis (Arabidopsis thaliana) 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), rd17 (SALK_125022), rdr8-8 (SALK_007638), sgs3-13 (SALK_09005), and sgs3-14 (SALK_001394) are in the Columbia-0 genetic background and were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). Seeds were germinated on AT-agar plates (Somerville and Ogren, 1982) for 7 to 10 d 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 photosynthetically active radiation) in an environmental chamber.

Molecular Complementation of cer7 with the CER3 Transgene

The 1,899-bp CER3 coding region was excised from the plasmid pESC-TRP::ProGAL1::CER3 (P. Lam, unpublished data) using BamHI and Nhel. This fragment was cloned into the plasmid pBluescriptII::ProCER6 (P. Lam, unpublished data) into the corresponding restriction enzyme sites to generate pBluescriptII::ProCER6::CER3. The ProCER6::CER3 fragment was then excised using XhoI and Sall and cloned into pBluD400 (Dašla et al., 1992), which was excised with Sall and Sall (XhoI and XhoI form compatible ends). The resulting plasmid, pBluD400::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 0.1 M Na₂PO₄, 5% dimethyl sulfoxide, and 100 mM ethyl methanesulfonate for 5 h. After mutagenesis, the seeds were washed twice with 100 mM Na₂SO₄ and then twice with distilled water for 15 min 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 did have a waxy stem, the sti trichome, and that the cer7-1 mutation was still present.

Genotyping

DNA was extracted according to Berendzen et al. (2005). To genotype cer7-1, derived cleaved-amplified polymorphic sequence primers cer7-1/AlfI F and cer7-1/AlfI R were used to amplify a 210-bp fragment. The PCR product was then digested with AlfI and run on a 1.5% agarose gel. The mutation in cer7-1 allows for cleavage of the PCR product after AlfI digestion, resulting in 185- and 25-bp products. 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- to 6-week-old Arabidopsis stems. Stems were immersed for 30 min in chloroform containing 10 μg of n-tetracosane, which was used as an internal standard. After extraction, samples were blown down under a gentle stream of nitrogen and redissolved in 10 μL of N,O-bis(trimethylsilyl) trifluoroacetamide (Sigma) and 10 μL of pyridine (Fluka). Samples were derivatized for 90 min at 80°C. After derivatization, excess N,O-bis(trimethylsilyl) trifluoroacetamide and pyridine were removed by blowing down under nitrogen, and samples were dissolved in 30 μL of chloriform. Gas-liquid chromatography was performed in the samples using a HP 6890 series gas chromatograph equipped with flame ionization detection and a 30 m HP-1 column with helium as the carrier gas. Gas chromatography 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 waxes was determined by comparing the flame ionization detector peak areas with 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 the manufacturer’s protocol. RNA quantification was performed using a NanoDrop 8000 (Thermo Scientific). Five hundred nanograms of total RNA was treated with DNase (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 20-μL 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 method of Pfaffl (2001), and control samples were normalized to 1. Statistical significance was measured with Student’s t test.

Positional Cloning of Suppressor Lines

To map the positions 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 to 40 plants with the wild-type waxy stem
Supplemental Data

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

Supplemental Figure S1. Wax levels are restored in rdr1-7 cer7-1 and sgs3-13 cer7-3 double mutants.

Supplemental Figure S2. The RDR1 and SGS3 transgenes can complement var3-1 cer7-1 and var4-1 cer7-1, respectively.

Supplemental Figure S3. Co-localization of the 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 3 cm and the bottom 3 cm of a 10-cm 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.

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LITERATURE CITED


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