The Exosome and Trans-Acting Small Interfering RNAs Regulate Cuticular Wax Biosynthesis during Arabidopsis Inflorescence Stem Development

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The primary aerial surfaces of land plants are covered with a cuticle, a protective layer composed of the cutin polyester matrix and cuticular waxes. Previously, we discovered a unique mechanism of regulating cuticular wax biosynthesis during Arabidopsis (Arabidopsis thaliana) stem elongation that involves ECERIFERUM7 (CER7), a core subunit of the exosome. Because loss-of-function mutations in CER7 result in reduced expression of the wax biosynthetic gene CER3, we proposed that CER7 is involved in degrading a messenger RNA encoding a CER3 repressor. To identify this putative repressor, we performed a cer7 suppressor screen that resulted in the isolation of the posttranscriptional gene-silencing components RNA-DEPENDENT RNA POLYMERASE1 and SUPPRESSOR OF GENE SILENCING3, indicating that small RNAs regulate CER3 expression. To establish the identity of the effector RNA species and determine whether these RNAs control CER3 transcript levels directly, we cloned additional genes identified in our suppressor screen and performed next-generation sequencing of small RNA populations that differentially accumulate in the cer7 mutant in comparison with the wild type. Our results demonstrate that the trans-acting small interfering RNA class of small RNAs are the effector molecules involved in direct silencing of CER3 and that the expression of five additional genes (EARLY RESPONSE TO DEHYDRATION1, AUXIN RESISTANT1, AXLIN RESISTANT1, a translation initiation factor SU1 family protein, and two genes of unknown function) is controlled by both CER7 and trans-acting small interfering RNAs.

Cell walls of the primary surface tissues of land plants are coated with a cuticle, a lipid structure that plays a critical role in plant protection from desiccation, serves as a barrier against pathogen and insect attack (Kunst and Samuels, 2003), and prevents organ fusions during development (Sieber et al., 2000). The cuticle is synthesized by epidermal cells and is composed of cutin and cuticular waxes. Cutin is a polyester rich in oxygenated fatty acids with chain lengths of 16 or 18 carbons and glycerol (Pollard et al., 2008), whereas waxes include predominantly aliphatic very-long-chain fatty acid-derived components together with variable amounts of secondary metabolites such as flavonoids and triterpenoids (Jetter et al., 2006; Pollard et al., 2008).

Forward genetic approaches using Arabidopsis (Arabidopsis thaliana) ceriferum (cer) mutants and reverse genetic approaches have been instrumental in the identification of genes involved in cuticular wax biosynthesis and the regulation as well as the isolation of genes encoding plasma membrane-localized proteins that are required for wax export (Kunst and Samuels, 2009; von Wettstein-Knowles, 2012). Despite significant advances in our knowledge of the wax biosynthetic and transport machinery, the regulation of wax deposition is still not well understood, and a relatively small number of genes involved in this process have been identified to date.

The first transcription factors reported to regulate wax accumulation during cuticle formation were WAX INDUCER1/SHINE1 (WIN1/SHN1) and its homologs SHN2/SHN3 and the Medicago truncatula transcription factor WXP1 (Aharoni et al., 2004; Broun et al., 2004; Zhang et al., 2005). Subsequent work demonstrated that the WIN1/SHN1 transcription factor activates cutin biosynthetic genes and only indirectly affects wax accumulation (Kannangara et al., 2007). Other transcription factors that control wax biosynthesis include MYB96 and MYB30, which activate cuticular wax biosynthesis under drought stress and in response to pathogen attack, respectively (Raffaele et al., 2008; Seo et al., 2011). Both of these transcription factors have been shown to bind the conserved sequences in the promoters of wax biosynthetic
genes. Furthermore, DECREASE WAX BIOSYNTHESIS (DEWAX), an APETALA2/Ethylene Responsive Factor-type transcriptional repressor, controls wax deposition during diurnal light/dark cycles via direct interaction with the promoters of wax-related genes. DEWAX also plays an important role in determining the total organ-specific wax load on Arabidopsis shoots (Go et al., 2014).

In addition to transcription factors, our work on the wax-deficient cer7 mutant resulted in the surprising discovery that CER7, a core subunit of the exosome, also controls wax biosynthesis in developing Arabidopsis inflorescence stems (Hooker et al., 2007). Inflorescence stems emerge at the transition from vegetative to reproductive development and grow rapidly, with most cell elongation occurring in the top 3 cm of stem below the apical meristem. Even though cell elongation in the stem decreases progressively from top to bottom and eventually stops completely, cuticular wax load and composition remain remarkably constant throughout stem development (Suh et al., 2005). A comparison of transcriptomes of rapidly expanding and nonexpanding stem cells revealed that wax-related genes are preferentially or exclusively expressed in rapidly elongating stem segments (Suh et al., 2005), but how their expression is temporally regulated in a polar top-to-bottom fashion in developing stems was not known.

Characterization of the cer7 mutant revealed that CER7 affects wax deposition in developing stems by positively regulating transcript levels of CER3, a key wax biosynthetic gene (Hooker et al., 2007). As expected of major genes required for wax production, CER3 transcript levels in wild-type stems are high at the stem apex and gradually decrease toward the stem base, concomitant with a progressive reduction in CER7 expression levels (Lam et al., 2012). Conversely, in the stem wax-deficient cer7 mutant, CER3 expression is equally low at both the top and bottom of the stem (Lam et al., 2012). To dissect the process of CER7-mediated regulation of CER3 expression, we performed a screen for second-site suppressors of cer7 and identified a series of wax restorer (war) mutants. Cloning of genes disrupted in the war3 and war4 suppressors demonstrated that they encode SUPPRESSOR OF GENE SILENCING3 (SGS3) and RNA-DEPENDENT RNA POLYMERASE1 (RDR1), proteins involved in posttranscriptional gene silencing (PTGS; Lam et al., 2012).

PTGS by mRNA degradation or translational inhibition in plants is controlled by microRNAs (miRNAs) and trans-acting small interfering RNAs (ta siRNAs). TasiRNAs are 21-nucleotide-long, plant-specific class of endogenous small RNAs generated by cleavage of the TRANS-ACTING SI RNA (TAS) gene-derived transcripts. TasiRNA biogenesis is initiated by a miRNA-loaded RNA-induced silencing complex (Allen et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005) that makes the first cut in the TAS transcript at a specific target site, followed by the conversion of one of the two cleavage products into a double-stranded RNA (dsRNA) by SGS3 and RDR6 (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). The resulting dsRNA is then processed into 21-nucleotide tasiRNA duplexes by the RNAse III DICER-LIKE4 (DCL4; Dunoyer et al., 2005; Gasioulis et al., 2005; Hiraguri et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005) and methylated by the RNA methyltransferase HUA ENHANCER1 (HEN1) that protects them from degradation and 3′ end uridylation (Li et al., 2005; Yu et al., 2005). Finally, only the guide strands of the tasiRNA duplexes associate with ARGONAUTE1 (AGO1; Baumberger and Baulcombe, 2005) to carry out PTGS of complementary target RNAs.

SGS3 is a well-established component of the tasiRNA biogenesis pathway (Peragine et al., 2004; Yoshikawa et al., 2005), but RDR1 had not been reported previously to be involved in tasiRNA biogenesis or in the silencing of endogenous genes. RDR1 has only been implicated in antiviral defense for small interfering RNA (siRNA) synthesis targeted against viral RNAs (Yu et al., 2003; Garcia-Ruiz et al., 2010). Thus, upon the identification of RDR1 in our screen for suppressors of cer7 wax deficiency, it was not clear whether tasiRNAs, or another type of noncoding RNAs, control CER3 expression to influence wax deposition. The goal of this work was to establish the molecular identity of the effector noncoding RNA species and to determine whether these effector RNAs control CER3 transcript levels directly or indirectly by PTGS of a positive regulator of CER3. To accomplish this, we isolated additional components of the CER3-silencing machinery and performed next-generation sequencing (RNA-sequencing [RNA-seq]) of small RNA populations that differentially accumulate in the cer7 mutant in comparison with the wild type and the cer7 rdr1 and cer7 sgs3 suppressor lines. Small RNAs that uniquely accumulated in the cer7 mutant, but not in the wild type or the suppressors, were then used to find their cognate gene targets by sequence complementarity. Our results show that tasiRNAs are involved in CER3 silencing during stem wax deposition in the course of Arabidopsis inflorescence stem development and provide evidence that the silencing of CER3 by tasiRNAs is direct.

RESULTS
Identification of Additional Factors Required for CER7-Mediated CER3 Silencing

To identify proteins involved in the biogenesis of noncoding RNA effectors that control the expression of the CER3 gene during stem wax deposition and are negatively regulated by CER7, we carried out a genetic screen for mutations that abolish the cer7 wax-deficient phenotype. As reported previously, 99 lines were isolated from this suppressor screen. Seventy-seven lines were classified as group 1 war mutants with completely recovered wax loads on their inflorescence stems (Lam et al., 2012). These mutants fall into six complementation groups named war1 to war6. The identification of WAR3/RDR1 and WAR/SDS3 encouraged us to proceed with the cloning and
characterization of additional WAR genes, WAR5 and WAR6, to determine their role in CER3 silencing.

To identify WAR5, the war5-1 cer7-1 (Landsberg erecta [Ler] ecotype) mutant was crossed to cer7-3 (Columbia-0 [Col-0] ecotype) to generate a mapping population. Genetic analysis of the F2 progeny demonstrated a roughly 3:1 segregation ratio of the wax-deficient mutant to the waxy wild-type (1,590:516; \( \chi^2 = 0.279, P > 0.5 \)), implying that wax recovery was caused by a single recessive mutation. The war5-1 mutation was mapped to a 110-kb region on chromosome 3 bordered by the markers K7L4 and MDQ17 (Fig. 1A). Sequencing of several candidate genes in this area uncovered a G-to-A nucleotide change at the last position of the fifth intron (junction of the fifth intron and sixth exon) of At3g15390, expected to cause splicing defects during mRNA processing in the war5-1 mutant. Sequencing of the war5-2 allele, which was isolated from the same suppressor screen, revealed a C-to-T point mutation in At3g15390 at position 2,619 (from ATG of the genomic DNA), leading to a premature stop codon (Fig. 1B). At3g15390 encodes SILENCING DEFECTIVE5 (SDE5), a putative RNA-trafficking protein reported to be required for sense transgene PTGS and the production of tasiRNAs (Hernandez-Pinzon et al., 2007; Jauvion et al., 2010). Two transfer DNA (T-DNA) insertional alleles obtained from the T-DNA mutant collection (Alonso et al., 2003), sde5-5 (SALK_114489) and sde5-6 (SALK_115496; Fig. 1B; Supplemental Table S1), do not show signs of stem wax deficiency but exhibit slightly downward-curled leaf edges, as observed in previously reported null sde5 mutants (Jauvion et al., 2010). However, similar to the original war5 alleles isolated in our suppressor screen, when sde5-5 and sde5-6 were crossed into the cer7-3 background, the double mutants showed wild-type-like wax accumulation on stems (Fig. 1C). Gas chromatography/flame ionization detection (GC/FID) analysis of the stem wax of war5 cer7-1 alleles and sde5 cer7-3 double mutants confirmed wild-type wax loads (Fig. 1D). Wax composition profiles and CER3 expression levels in all war5 cer7-1 and sde5 cer7-3 double mutants were also similar to those in the wild type (Fig. 1, E and F). Taken together, these data indicate that SDE5 is WAR5. Therefore, war5-1 and war5-2 were renamed sde5-7 and sde5-8, respectively (Supplemental Table S1).

The WAR6 gene was mapped to chromosome 3 between markers T6H20 and F18B3 using 20 waxy individuals from the F2 population of a war6-4 cer7-1 (Ler background) × cer7-3 (Col-0 background) cross (Fig. 1G). Instead of continuing with fine mapping, we examined the genes in the region of interest for candidates that are involved in RNA silencing, as all the previously identified WAR genes encode proteins required for siRNA biogenesis (Lam et al., 2012). One of the candidate genes in this region is At3g49500, which encodes RDR6, one of the six RNA-dependent RNA polymerases in the Arabidopsis genome. RDR6 was shown to colocalize with SGS3 in cytoplasmic SGS3/RDR6 granules (Kumakura et al., 2009) and functions together with SGS3 to convert single-stranded RNA into dsRNA during tasiRNA formation (Peragine et al., 2004; Vazquez et al., 2004; Yoshikawa et al., 2005). Sequencing of the RDR6 genomic region in the war6-4 mutant revealed a G-to-A mutation that introduced a stop codon in the second exon of RDR6. Four additional war6 alleles from our suppressor screen were also sequenced, and all contained missense mutations in RDR6 (Fig. 1H), confirming that WAR6 is RDR6. We obtained two additional war6 alleles, rdr6-11 and rdr6-12, which contain a C-to-T nonsense mutation at nucleotide 805 and a 7-bp deletion between nucleotides 997 and 1,003 of RDR6, respectively (Peragine et al., 2004). Single homozygous rdr6 mutants do not show a visible wax phenotype, but, as reported previously, rdr6-11 and rdr6-12 exhibit strong downward-curled leaf margins (Peragine et al., 2004). When these two mutants were crossed into the cer7-3 background, the double mutants exhibited near-wild-type stem wax loads and composition (Fig. 1, I–K), confirming that these rdr6 alleles were also able to suppress the cer7 wax deficiency. As expected, real-time PCR demonstrated substantially increased CER3 transcript levels in all war6 cer7-1 alleles and rdr6 cer7-3 double mutants (Fig. 1L). Therefore, we renamed all war6 alleles rdr6 (Supplemental Table S2).

The fact that WAR5 and WAR6 encode two additional tasiRNA biosynthetic proteins suggests that tasiRNAs are the noncoding effector RNA molecules involved in CER3 silencing. Surprisingly, mutations in either RDR1 or RDR6 can suppress cer7 wax deficiency, suggesting that they have nonredundant roles in the conversion of single-stranded RNA to dsRNA during tasiRNA formation. To determine whether this is indeed the case, rdr1-7 cer7-3 was crossed with rdr6-12 cer7-3 to create the rdr1-7 rdr6-12 cer7-3 triple mutant. If RDR1 and RDR6 functioned redundantly, we would expect to detect increased CER3 transcript accumulation, leading to higher wax load on the stems of the triple mutant. Visual inspection and GC/FID analysis of rdr1-7 rdr6-12 cer7-3 stem wax demonstrated that the wax load and composition (Fig. 2, A–C) in triple mutants were comparable to those of the double mutants. CER3 transcript levels were also unchanged in the triple mutant (Fig. 2D). Thus, RDR1 and RDR6 act nonredundantly in the production of tasiRNAs that control wax accumulation on Arabidopsis stems.

DCL4, AGO1, and HEN1 Proteins Are Also Required for the Regulation of CER3 Expression during Stem Wax Deposition

In addition to RDRs, which generate dsRNAs, all small RNA biosynthetic pathways require DCL endonucleases that slice dsRNAs into 20- to 25-nucleotide RNA duplexes, AGO proteins that bind small RNAs and are responsible for small RNA-guided cleavage of target mRNAs, and the HEN1 protein, an RNA methyltransferase that introduces a methyl group on DCL-generated small RNAs to protect them from degradation and uridylation (Chen et al., 2002; Boutet et al., 2003; Li et al., 2004). Single homozygous rdr6 alleles exhibited near-wild-type stem wax loads and composition (Fig. 1, I–K), confirming that these rdr6 alleles were also able to suppress the cer7 wax deficiency. As expected, real-time PCR demonstrated substantially increased CER3 transcript levels in all war6 cer7-1 alleles and rdr6 cer7-3 double mutants (Fig. 1L). Therefore, we renamed all war6 alleles rdr6 (Supplemental Table S2).

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Figure 1. Identification of SDE5 and RDR6 as additional factors required for CER7-mediated CER3 silencing. A, Schematic representation of the chromosomal location of war3 as determined by fine mapping. The markers used for mapping and the number of recombinants are indicated. B, Schematic representation of the SDE5 gene structure. The 5' and 3' untranslated regions are shown.
and stem wax restoration, as observed in double mutants. We generated double mutants by crossing homozygous ago mutants with the corresponding cer7 allele according to ecotype, and their stem wax accumulation was evaluated by visual inspection, followed by gas chromatography analysis. The only double mutant that had waxy stems and near-wild-type wax load and composition was ago1-11 cer7-1 (Fig. 3, A–E). Quantitative PCR analysis confirmed that CER3 transcript levels were also restored to wild-type levels in the ago1-11 cer7-1 mutant (Fig. 3F). These results demonstrate that AGO1 is the AGO protein required for the production of tasiRNAs involved in the regulation of CER3 expression during stem wax deposition.

If HEN1 is also required for the formation of the tasiRNAs involved in CER3 silencing in Arabidopsis inflorescence stems, as we suspected, then a hen1 cer7 double mutant should have a waxy stem phenotype. To test this, the viable, weak hen1-8 allele of HEN1 (Yu et al., 2010) was initially crossed with cer7-3, and hen1-8 cer7-3 double mutants were identified by PCR genotyping. Besides the delayed flowering and morphological defects characteristic of hen1 mutants, hen1-8 cer7-3 double mutants were visibly wax deficient (Supplemental Fig. S2, A and B). However, GC-FID measurements of hen1-8 cer7-3 stem wax accumulation demonstrated that the stem wax load was partially restored and was more than 2-fold greater than that of the cer7-3 mutant (Supplemental Fig. S2C). We suspect that only partial restoration was observed due to the weak nature of the hen1-8 allele used in this experiment. Therefore, we obtained a strong hen1

To investigate which of the 10 AGOs in Arabidopsis is involved in regulating CER3 expression, we obtained T-DNA insertion mutants for all AGO genes, except for AGO1, for which we acquired an ethyl methanesulfonate-generated mutant, ago1-11 (Supplemental Table S4; Alonso et al., 2003; Kidner and Martienssen, 2005). Double mutants were generated by crossing homozygous ago mutants with the corresponding cer7 allele according to ecotype, and their stem wax accumulation was evaluated by visual inspection, followed by gas chromatography analysis. The only double mutant that had waxy stems and near-wild-type wax load and composition was ago1-11 cer7-1 (Fig. 3, A–E). Quantitative PCR analysis confirmed that CER3 transcript levels were also restored to wild-type levels in the ago1-11 cer7-1 mutant (Fig. 3F). These results demonstrate that AGO1 is the AGO protein required for the production of tasiRNAs involved in the regulation of CER3 expression during stem wax deposition.

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Figure 1. (Continued.)

regions 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 positions and types of the mutations in sde5 mutant alleles are also shown. C. Stems of 6-week-old Ler wild-type (Ler WT), cer7-1, two war5 cer7-1 double mutant, Columbia-0 (Col-0) wild-type, cer7-3, and two sde5 cer7-3 double mutant plants showing the suppression of the cer7 wax-deficient phenotype in the double mutants as indicated by whitish stems. D. Stem wax loads of war5 cer7-1 and sde5 cer7-3 double mutants compared with their corresponding wild type and the cer7 mutants. Values represent means ± sd (n = 3). Statistically significant differences between samples are indicated by different letters at P < 0.01 using one-way ANOVA and Tukey’s test. E. Stem wax composition of war5-1 cer7-1 and sde5-5 cer7-3 double mutants compared with their corresponding wild type and the cer7 mutants. Wax compositions for all double mutants are restored to near-wild-type like ratios of major wax components. L, Quantiﬁcation of tasiRNAs controlling CER3 family members that are involved in the production of stem wax deposition. We reasoned that secondary mutations in the tasiRNA biogenesis pathway downstream of cer7 that abolish tasiRNA production should produce near-wild-type CER3 transcript accumulation and stem wax restoration, as observed in rdr1 cer7 and sgs3 cer7 double mutants. We generated double mutants of dcl1 cer7 to dcl4 cer7 to determine which of the four DCL family members can rescue the cer7 stem wax deﬁciency. Of the four double mutants, dcl4-2 cer7-3 alone displayed glucaceous, waxy wild-type-looki...
Figure 2. RDR1, RDR6, and DCL4 are all required for CER7-mediated CER3 silencing. A, Stems of 6-week-old Col-0 wild-type (WT), cer7-3 mutant, rdr1-7 cer7-3 and rdr6-12 cer7-3 double mutant, and rdr1-7 rdr6-12 cer7-3 triple mutant plants showing the suppression of the cer7-3 wax-deficient phenotype in both the double and triple mutants as indicated by whitish stems.
allele, hen1-1, and crossed it with cer7-1 to generate the hen1-1 cer7-1 double mutant. The hen1-1 cer7-1 double mutant exhibited stunted and delayed growth phenotypes of the hen1-1 mutant and produced very short inflorescence stems compared with the wild type (Fig. 3, G and H). Wax analysis revealed that the wax load was restored to wild-type levels in hen1-1 cer7-1 (Fig. 3I). Although total wax levels were restored in the double mutant, stem wax composition was intermediate between the wild type and cer7-1, with higher primary alcohol and wax ester levels and lower than wild-type levels of alkanes, secondary alcohols, and ketones compared with the wild type (Fig. 3J).

Full restoration of the wild-type wax load by hen1-1 in the cer7-1 background and partial rescue of the cer7-3 wax deficiency by the introduction of the hen1-8 mutation are consistent with HEN1 being a conserved component of the tasiRNAs biogenesis pathway required for CER3 gene silencing.

Is DRB4 Required for CER3 Silencing?

TasiRNA production also involves the DOUBLE-STRANDED RNA-BINDING DOMAIN4 (DRB4) protein that interacts with DCL4 in vivo and was shown to be required in vitro for DCL4-cleaving activity (Nakazawa et al., 2007; Fukudome et al., 2011). To determine if DRB4 is essential for DCL4 function in CER3 silencing, we generated a drb4-1 cer7-3 double mutant using a previously characterized drb4-1 allele (SALK_000736) that carries a T-DNA insert between the start of transcription and the first ATG of the DRB4 gene and lacks detectable DRB4 mRNA (Adenot et al., 2006; Nakazawa et al., 2007). The drb4-1 cer7-3 homozygous plants germinated and developed up to the cotyledon stage, but their development was arrested and they eventually died without forming true leaves (Supplemental Fig. S3), similar to the dcl4-10 cer7-3 double mutant (Supplemental Fig. S1B). The inability of both double mutants to develop past the cotyledon stage supports the hypothesis that, in the absence of CER7, inactivation of either DCL4 or DRB4 activity cannot be substituted by other members of the DCL or DRB families, resulting in a block in the tasiRNA biosynthetic pathway and seedling lethality.

CER7 Disruption Causes the Accumulation of Small RNAs and the Repression of Their Target Genes, Including CER3

The identification of genes mutated in the war5 and war6 suppressors of cer7 as SDE5 and RDR6, respectively, and the verification that mutations in DCL4, AGO1, and HEN1 genes can rescue the cer7 wax deficiency demonstrate that a functional tasiRNA pathway is required for CER3 silencing during stem wax deposition in the course of Arabidopsis inflorescence stem development. To provide further evidence for the involvement of tasiRNA in CER3 silencing, and to determine whether these effector RNAs influence CER3 expression directly or indirectly via PTGS of a positive regulator of CER3, small RNA populations were prepared from wild-type, cer7, cer7 rdr1, and cer7 sgs3 stems and subjected to next-generation sequencing (RNA-seq). Our rationale was that if the silencing of a gene by small RNAs were direct, then the absence of the CER7 exosomal subunit in the cer7 mutant would result in an overabundance of the effector small RNAs and consequent down-regulation of their targets, including CER3.

As expected, the predominant small RNA species detected in all genotypes were 21 and 24 nucleotides in length, and no major changes in the relative proportion of these different classes of small RNAs were detected, an indication that mutations in cer7, cer7 rdr1, or cer7 sgs3 do not affect the overall biogenesis of small RNAs (Supplemental Fig. S4). Because the high degree of complementarity between the tasiRNAs and their mRNA targets allows confident predictions of tasiRNA target genes, we used the small RNA sequences that uniquely indicated different targets at P < 0.01 using a one-way ANOVA and Tukey’s test. C. Stem wax composition of the drb4-1 rdr6-12 cer7-3 triple mutants compared with the wild type and the cer7-3 mutant. Wax composition for triple mutants is restored to wild-type-like ratios of major wax components.

Figure 2. (Continued.)
B, Stem wax loads of rdr1-7 cer7-3 and rdr6-12 cer7-3 double and rdr1-7 rdr6-12 cer7-3 triple mutants compared with the wild type and the cer7-3 mutant. Values represent means ± sd (n = 3). Statistically significant differences between samples are indicated by different letters at P < 0.01 using a one-way ANOVA and Tukey’s test. E, Stems of 6-week-old Col-0 wild-type, cer7-3 mutant, and dcl4-2 cer7-3 double mutant plants showing the suppression of the cer7-3 wax-deficient phenotype in the double mutant as indicated by whitish stems. F, Stem wax loads of the dcl4-2 cer7-3 double mutant compared with the wild type and the cer7-3 mutant. Values represent means ± sd (n = 3). Statistically significant differences between samples are indicated by different letters at P < 0.01 using a one-way ANOVA and Tukey’s test. G, Stem wax composition of the dcl4-2 cer7-3 double mutant compared with the wild type and the cer7-3 mutant. Wax composition is restored to wild-type-like ratios of major wax components. H, Quantitative real-time PCR showing that the CER3 transcript level is recovered to 100% of the wild-type level in the dcl4-2 cer7-3 double mutant. ACTIN2 was used as an internal control, and control samples were normalized to 1. Values represent means ± sd (n = 3).
Figure 3. The cer7-1 ago1-11 and hen1-1 cer7-1 double mutants have a wild-type-like stem wax phenotype. A, Morphology of the cer7-1 mutant (left) compared with the cer7-1 ago1-11 double mutant (right). B, Closeup of the cer7-1 ago1-11 double mutant. The double mutant is dwarf and sterile, with short broad leaves like the ago1-11 single mutant, and has a waxy stem. C, Stems of 6-week-old cer7-1 (top) and cer7-1 ago1-11 (bottom) plants showing that the cer7-1 ago1-11 double mutant has a restored stem wax phenotype. D, Wax analysis of stems of the wild type (WT), cer7-1, and the ago1-11 cer7-1 double mutant shows that wax levels are restored to that of the wild type. Values represent means ± SE (n = 4). Statistically significant differences between samples are indicated by different letters at P < 0.01 using a one-way ANOVA and Tukey’s test. E, The levels of all wax components are restored back to wild-type levels in the ago1-11 cer7-1 double mutant. F, Quantitative real-time PCR showing the expression levels of CER3 in the hen1-1 cer7-1 and ago1-11 cer7-1 double mutants. Even though wax loads are restored to that of the wild type in these double mutants, CER3 transcript levels are only partially restored. ACTIN2 was used as an internal control, and control samples were normalized to 1. Values represent means ± SD (n = 4). G and H, hen1-1 mutants are characterized by delayed flowering, reduced organ size, and curly pointed leaves compared with the wild type. These phenotypes are also apparent in the hen1-1 cer7-1 double mutant. I, Wax analysis of hen1-1 cer7-1 shows that wax levels are like that of the wild type, indicating that hen1-1 can suppress the cer7-1 wax-deficient phenotype. Values represent means ± SE (n = 4). Statistically significant differences between samples are indicated by different letters at P < 0.01 using a one-way ANOVA and Tukey’s test. J, Wax composition of the hen1-1 cer7-1 double mutant is similar to that of the wild type and cer7-1. Even though the overall wax loads are like that of the wild type, the hen1-1 cer7-1 mutant has increased levels of primary alcohols and esters and decreased levels of aldehydes, like the cer7-1 mutant, but similar levels of alkanes, secondary alcohols, and ketones to the wild type.
This analysis revealed that small RNAs that accumulate to the highest levels in the cer7 background correspond to six genes, including CER3 (At5g57800; Fig. 4A). To validate this result, we carried out quantitative real-time PCR analysis and confirmed that the expression of all six genes, identified as targets of the highest accumulating small RNAs, was reduced dramatically in the cer7 mutant (Fig. 5). In addition, using quantitative PCR, we wanted to confirm that the small RNAs that were found to be abundant by RNA-seq were indeed accumulating to high levels in the cer7 mutant background. We selected two small RNAs that targeted CER3: siRNA-1 (5′-UGCAUGUAACAGAUCAGGCU-3′) and siRNA-2 (5′-AACAGAUUGAUCACGAAUGGC-3′). Stem-loop reverse transcription (RT)-PCR confirmed that the expression of both siRNAs was dramatically increased in cer7 (Supplemental Fig. S6). Collectively, these data demonstrate that small RNAs that accumulate in the cer7 mutant in the absence of CER7 are indeed effector molecules involved in direct silencing of the identified genes and that the expression of CER3 and the other five identified genes is controlled by both CER7 and tasiRNAs.

Among the small RNA reads from the cer7 genotype, a considerably higher proportion of the total reads aligned to the CER3 gene than observed in the wild type (approximately 20% versus approximately 1%; Fig. 4B). Since CER3 expression was found to be controlled by small RNAs, we were interested in determining the areas of complementarity between the small RNAs and their CER3 target. All the reads that mapped to the CER3 gene from cer7 and the wild type, therefore, were aligned with CER3, and the density of reads at each position of the gene was plotted (Fig. 4C). In the cer7 mutant background, most reads show complementarity to the first 1,000 bp in three distinct regions, as indicated by the peaks in the line graph, in addition to two other areas at around 1,200 and 2,200 bp. In the wild type, the plotted densities show a single peak at around 500 bp that corresponds to the first peak in cer7. The additional small RNAs that exhibit sequence identity with CER3 and that were found exclusively in the cer7 background are likely substrates of the exosome and are degraded in the wild type to allow for the expression of CER3.

DISCUSSION

The transition from vegetative to reproductive growth in Arabidopsis is marked by the initiation and rapid elongation of inflorescence stems. During stem growth, the maximal rate of cell elongation occurs in the top segment near the apical meristem and decreases sharply toward the base, with no elongation detected below 7 cm from the top (Suh et al., 2005). Quantitative analysis of cuticular lipid deposition demonstrated that, in the course of stem elongation and after the elongation has ceased, stem wax load and composition remain constant. Thus, the biosynthesis and deposition of wax constituents on the surface of expanding epidermal cells is closely matched to surface area expansion. Our work on the cer7 mutant and cer7 suppressors provides evidence that CER7, a core subunit of the exosome, and tasiRNAs govern wax deposition on the surface of elongating Arabidopsis stems by controlling the expression of the wax biosynthetic gene CER3.

TasiRNAs Regulate Cuticular Wax Biosynthesis in Developing Inflorescence Stems

We showed previously that the CER7 subunit of the exosome controls wax biosynthesis in the epidermis of developing Arabidopsis stems by positively regulating transcript levels of the wax biosynthetic gene CER3 (Hooker et al., 2007). To characterize the mechanism of CER7-mediated regulation of wax production and determine the target of CER7, we performed a screen for suppressors of the cer7 mutant, which resulted in the identification of PTGS proteins, SGS3 and RDR1. Based on this information, we hypothesized that small RNAs, most likely tasiRNAs, mediate wax deposition in elongating stems and that the levels of these small RNAs are determined by CER7 activity (Lam et al., 2012). While SGS3 is an established component of the tasiRNA biosynthetic pathway, RDR1 has not been reported to be involved in the silencing of endogenous genes. To provide additional evidence for the involvement of tasiRNAs in regulating stem wax deposition, we investigated the cer7 suppressors war5 and war6. We demonstrate that the WAR5 and WAR6 genes encode SDE5 and RDR6, additional components of the tasiRNA biogenesis pathway. Based on sequence similarity with a mammalian mRNA transport protein, SDE5 has been proposed to be involved in the RNA nucleocytoplasmic trafficking required for tasiRNA formation (Hernandez-Pinzon et al., 2007; Jauvion et al., 2010), but the exact role of the SDE5 protein has not been determined. RDR6, on the other hand, is one of six RNA-dependent RNA polymerases in Arabidopsis, and it is well documented that RDR6 is required for the production of dsRNA during the biogenesis of tasiRNAs involved in the silencing of endogenous gene transcripts (Peragine et al., 2004; Vazquez et al., 2004; Xie and Qi, 2008).

Surprisingly, our cer7 suppressor screen resulted in the identification of two RDR proteins, RDR1 and RDR6, mutations in which rescue the cer7 wax-deficient phenotype. Construction of the rdr1 rdr6 cer7 triple mutant and quantitative analysis of its stem wax load revealed that the wax load on the surface of the triple mutant is similar to that measured on stems of the rdr1 cer7 and rdr6 cer7 double mutants. These results support two conclusions: (1) RDR1 functions not only in PTGS of virus-derived RNAs, as demonstrated previously (Yu et al., 2003; Garcia-Ruiz et al., 2010), but also in the production of tasiRNAs involved in endogenous gene regulation in plants; and (2) RDR1 and RDR6 play nonredundant roles in tasiRNA biosynthesis. Why two different RDR enzymes are required for this process, and how their respective roles in the formation of dsRNA differ, remain to be determined.

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We further investigated which of the AGO and DCL isoforms are responsible for CER3 silencing and whether HEN1 activity is also required. The results of our reverse genetics experiments showing that hallmark players of tasiRNA formation, AGO1 and DCL4, as well as HEN1, participate in the regulation of CER3 expression are consistent with the conclusion that tasiRNAs are the small RNA class controlling this process.

CER3 Silencing by TasiRNAs Is Direct

We previously proposed that small RNA buildup in the stem epidermis in the absence of CER7-dependent exosomal activity might abolish CER3 expression directly or indirectly by PTGS of a positive transcriptional regulator of CER3. We reasoned that if CER3 silencing is direct, then a large proportion of the tasiRNAs that accumulate in the cer7 mutant relative to the wild type would show complementarity to CER3 mRNA. RNA-seq of small RNA populations from developing Arabidopsis stems revealed that this is indeed the case. This experiment identified five other genes with sequence identity to highly abundant tasiRNAs, whose expression was dramatically down-regulated in the cer7 mutant, similar to CER3. The expression of these genes is also positively regulated by CER7 and negatively regulated by tasiRNAs, as shown by quantitative real-time PCR analysis. Two of the highly repressed genes identified are...

Figure 4. RNA-seq analysis. A, Heat map showing the top 20 differentially expressed genes. B, Among the small RNA reads in the cer7 libraries, a significant proportion of the total reads align to the CER3 gene compared with the wild-type (WT) libraries. C, Line graph representing the relative density of all the reads that align to CER3 and locations along the CER3 gene where the reads align. The cer7 libraries are shown as solid lines and the wild-type libraries as dashed lines.
in *cer7* encode proteins of unknown function, whereas *SUI1* (At5g54940) codes for a translation initiation factor, *AUX1* (At2g38120) for a well-studied auxin influx transporter, and *ERD14* (At1g76180) for a dehydrin protein that accumulates in response to dehydration, cold, salt stress, and abscisic acid. *AUX1* is known to play a role in the root gravitropic response, root hair development, and leaf phyllotaxy (Péret et al., 2012). *AUX1* function in developing stems has not been investigated, even though the *AUX1* gene is highly expressed in the stem. Similarly, *ERD14* transcripts were detected in stems, cauline leaves, roots, and flowers, but the biochemical function and physiological roles of *ERD14* and other dehydrin family members are not fully understood (Kiyosue et al., 1994). Whether *AUX1*, *ERD14*, *SUI1*, and the two genes of unknown function whose expression is also controlled by CER7 and tasiRNAs have unique roles during stem development unrelated to wax biosynthesis.

### How Do CER7 and TasiRNAs Mediate Stem Wax Deposition?

Endogenous small RNAs have been implicated in PTGS of genes controlling diverse aspects of plant development, but in most reported cases the effector molecules are miRNAs, not tasiRNAs (Baulcombe, 2005). To date, tasiRNAs have been shown to affect the expression of only a small number of genes, including those encoding pentatricopeptide-repeat proteins, putative MYB transcription factors, proteins of unknown function, and members of the ARF family of auxin-related transcription factors. Furthermore, the only tasiRNA with a well-defined biological role is tasi-ARF, which targets ARF3 and ARF4 genes to establish leaf polarity in Arabidopsis and controls the juvenile-to-adult transition in leaves (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005; Williams et al., 2005; Yoshikawa et al., 2005; Adenot et al., 2006; Hunter et al., 2006). Thus, our discovery that the *CER3* expression required for wax biosynthesis in developing inflorescence stems is regulated by tasiRNA is intriguing.
Even though additional examples of tasiRNA-regulated genes and processes are likely to emerge over time, it is clear that tasiRNAs are used infrequently in endogenous gene regulation in comparison with miRNAs. Moreover, since both types of small RNAs can function in PTGS, and tasiRNAs have more complex biogenesis than miRNAs, it is not obvious why tasiRNAs would ever be used for specific processes over miRNAs to control gene expression. Because the major difference between these two types of small RNAs is that tasiRNAs appear to have longer range movement than miRNAs (Dunoyer et al., 2010), it has been suggested that processes that require the formation of target gene expression gradients across organs will use tasiRNAs. For example, such gradients have been demonstrated to be responsible for the dorsoventral leaf patterning in Arabidopsis and maize (Zea mays; Chitwood et al., 2009; Nogueira et al., 2009; Schwab et al., 2009).

We currently have no evidence that this difference in mobility between tasiRNAs and miRNAs is relevant to stem cuticular wax deposition. Our results show that rapid wax deposition in the apical region with progressive attenuation toward the stem base that results in a constant wax load along the elongating florescence stem does involve a gradient of CER3 expression. We propose that this CER3 expression gradient is established by the gradual basipetal decrease of CER7-dependent exosomal activity, which in turn results in increased CER3 silencing by tasiRNAs. Indeed, we have detected a basipetal decline in CER7 transcript accumulation in developing Arabidopsis stems (Lam et al., 2012). However, how this temporal change in CER7 expression impacts CER3-specific tasiRNA abundance remains to be investigated.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) mutant lines cer7-7 stich and henl-1 are in the Ler genetic background, and cer7-3 and henl-8 are in the Col-0 genetic background. T-DNA insertion lines acls-5 (SALK_114489), acl6-5 (SALK_115496), nbl-11 (CS242265), nbl-12 (CS242266), acl2-1, acl3-1, and acl6-2 (CS86954), acl4-10 (CS66075), acl6-1 (SALK_003706), and all ago mutants listed in Supplemental Table S4 are in the Col-0 genetic background, whereas acl1-9 is in the Wassilewskija genetic background. All the T-DNA lines were obtained from the Arabidopsis Biological Resource Center (www.Arabidopsis.org). Seeds were germinated on AT-agar plates (Somerville and Ogren, 1982) and grown in soil (Sunshine Mix 4; SunGro) at 20°C under continuous light (90–120 μE m⁻² s⁻¹ photosynthetically active radiation).

Positional Cloning of Suppressor Mutations

To map the mutated genes in suppressor lines, each suppressor line (Ler ecotype) was crossed to cer7-3 (Col-0 ecotype), and genomic DNA from leaves of 40 to 50 F₂ plants with the wild-type waxy stem phenotype (plants homozygous for the suppressor mutation) was collected on FTA cards (Whatman) and held for 1 min at 200°C. After derivatization, excess N,O-bis(trimethylsilyl) trifluoroacetamide (Sigma) and 10 μL of pyridine (Fluka) were added to the samples and incubated at room temperature for 5 min. One microliter of each sample was injected and analyzed using a 2:1 split. To further identify the location of each suppressor mutation, 1,000 plants were screened with simple sequence length polymorphism markers until a narrow interval was obtained.

Genotyping

DNA was extracted according to Berendzen et al. (2005). To genotype henl-8, primers henl-2_F and henl-2_R were used to amplify a 585-bp PCR product. The mutation in henl-8 allows for the cleavage of the PCR product after HpaI digestion. To genotype ago1-11, primers ago1-11_F and ago1-11_R were used to amplify a 385-bp fragment. The PCR product was then subjected to EdlI digestion, which yields an extra band for the mutant allele. T-DNA insertion lines were genotyped using LbI and the gene-specific primers listed in Supplemental Table S6.

Cuticular Wax Extraction and Analysis

Cuticular waxes were extracted from 4- to 6-week-old Arabidopsis stems. Stems were immersed for 30 s in chloroform containing 10 μg of n-tetraacane (C24 alkane), which was used as an internal standard. After extraction, samples were blown down under nitrogen and redissolved in 10 μL of N,O-bis(trimethylsilyl) trifluoroacetamide (Sigma) and 10 μL of pyridine (Fluka). Samples were derivatized for 1 h 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 90 μL of chloroform. Wax analyses were performed on an Agilent 7890A gas chromatograph equipped with a flame ionization detector and an HP-1 methyl silicone column. Gas chromatography was carried out with oven temperature set at 50°C for 2 min and then raised by 4°C min⁻¹ to 200°C, held for 1 min at 200°C, raised by 5°C min⁻¹ to 320°C, and held for 15 min. One microliter of each sample was injected and analyzed using a 2:1 split. Quantitative determination of wax composition was carried out by comparing their flame ionization detector peak areas with that of the internal standard. Stem surface area was calculated by photographing stems prior to wax extraction, measuring the number of pixels, converting the values 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). One microgram of total RNA was treated with DNase I (Fermentas) and then used for first-strand complementary DNA synthesis using iScript RT supermix (Bio-Rad). Quantitative RT-PCR was performed in 20-μL reactions using iQ SYBR Green (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.

Isolation of RNA for RNA-seq

A total of 300 mg of tissue from the top 5 cm of Arabidopsis wild-type Col-0, cer7-3, rdr1-7 cer7-3, nbl-1 cer7-3, and sog5-13 cer7-3 stems was collected and frozen in liquid nitrogen. Tissues were ground to a fine powder with a pestle. Five milliliters of TRIzol (Invitrogen) was added to each sample and incubated at room temperature for 5 min. One milliliter of chloroform was added, and samples were mixed by shaking and incubated at room temperature for 2 to 3 min. Samples were centrifuged at 3,220g at 4°C for 30 min. After centrifugation, 3.5 mL of the top phase was transferred to a fresh tube containing 5 mL of isopropanol. RNA was precipitated at room temperature for 10 min before centrifugation at 3,220g at 4°C for 30 min. The pellet was washed with 75% (v/v) ethanol and centrifuged at 3,220g at 4°C for 15 min. The pellet was dried at room temperature for 10 min, and the RNA was suspended in 50 μL of water. To help dissolve the pellet, RNA was placed at 60°C for 10 min. The concentration and purity of RNA were determined using a NanoDrop 8000 (Thermo Scientific).

Small RNA Extraction and Library Construction

Twenty to 25 μg of purified total RNA was denatured in 2× small RNA loading dye (80% [v/v] formamide, 0.1% [v/v] xylene, and 0.1% [v/v] Bromphenol Blue) at 70°C for 10 min. RNA was then separated on a 15% (w/v) polyacrylamide gel run in 0.5× Tris/borate/EDTA buffer. Small RNAs approximately 15 to 40 nucleotides in length were excised from the gel using a clear razor blade. RNA was recovered by breaking the gel fragments in 0.4 M NaCl and incubating them overnight at 4°C, then precipitating with 1 μL of glycerol, 1/10th volume of 3 M NaOAc (pH 5.2), and 2 volumes of ethanol at −20°C for 6 h. The RNA was centrifuged at 12,000g at 4°C for 15 min, washed with 70% (v/v) ethanol, and air dried. The pellet was dissolved in 6 μL of diethyl pyrocarbonate-treated water. Small RNA library construction was done using the Illumina TruSeq Small RNA Sample Prep Kit as per the manufacturer’s protocol.

Bioinformatic Analysis of Small RNAs

The quality of the libraries was initially assessed using FastQC; all samples had high overall quality. Sequence data (in fastq format) were processed as follows using a
combination of publicly available tools and custom UNIX scripts. The Illumina adapters were removed from the raw sequences using the fastx-clip tool from the FASTX-Toolkit (version 0.0.13; Blankenberg et al., 2010), and reads with length shorter than five nucleotides were discarded. Sequence alignment was carried out using the BOWTIE aligner (version 0.12.8; Langmead et al., 2009) against the latest Arabidopsis reference genome from The Arabidopsis Information Resource (TAIR10) using the default parameters of the r-alignment mode. All 10 libraries reported alignment in more than 98% of all reads. After using a custom Perl script to convert the TAIR10 genome annotations from GFF to GTF format, counts of alignments to genome features were determined using the htseq-count tool from the HTSeq package (version 0.5.3p9; Anders and Huber, 2010) under the intersection-nonempty mode. The number of single-mapping reads that overlap each annotated genome feature from the TAIR10 annotations release (Lamesch et al., 2012) was counted and used as input for the statistical analysis of differential expression.

Differential expression analysis of RNA-seq read alignment counts was performed using the software package edgeR (Robinson et al., 2010; McCarthy et al., 2012) from the Bioconductor project (Genteman et al., 2004). A general linear model approach was used to locate features that differed between any of the four groups with the following criteria: fold change ≥ 2 between at least two groups, P < 0.01, and false discovery rate < 0.01.

To include reads that did not map to previously annotated features, an analysis of differential expression was also carried out on counts of unique sequence reads, taken from all 10 libraries combined. This was done using a combination of the fastx-collapser tool from the FASTX-Toolkit and a custom Perl script to output a file of unique sequences and counts for each library. Analysis of differential expression was carried out as above; it was found that all top differentially expressed (DE) sequences mapped to the top DE genes, with the majority aligning to the CER3 gene. The reads from the wild-type and cer7 libraries were aligned to the CER3 gene using megablast (Zhang et al., 2000), and the density of reads at each position of the gene was plotted using R (CRAN).

Sequence data from this article can be obtained from the Arabidopsis Genome Initiative database under the following accession numbers: CER7 (At3g50500), CER3 (At5g21150), AGO10 (At5g43810), and HEN1 (At4g20910). All 10 libraries were aligned to the Arabidopsis (TAIR10) using the default parameters of the n-alignment mode. All 10 libraries from each ecotype were counted and used as input for the statistical analysis of differential expression.

Analysis of differential expression was also carried out on counts of unique sequences and counts for each library. Analysis of differential expression was carried out as above; it was found that all top differentially expressed (DE) sequences mapped to the top DE genes, with the majority aligning to the CER3 gene. The reads from the wild-type and cer7 libraries were aligned to the CER3 gene using megablast (Zhang et al., 2000), and the density of reads at each position of the gene was plotted using R (CRAN).

The following supplemental materials are available.

**Supplemental Figure S1.** DCL4 is required for CER7-mediated CER3 silencing.

**Supplemental Figure S2.** The hen1-8 cer7-3 double mutant has a wax-deficient phenotype.

**Supplemental Figure S3.** DRB4 is required for CER7-mediated CER3 silencing.

**Supplemental Figure S4.** Small RNA profile of all mutant lines.

**Supplemental Figure S5.** Top 100 DE genes.

**Supplemental Figure S6.** Detection of siRNAs by quantitative PCR

**Supplemental Table S1.** Nomenclature and description of the sde5 alleles.

**Supplemental Table S2.** Nomenclature and description of the nrd6 alleles.

**Supplemental Table S3.** Nomenclature and description of the dcl4 alleles.

**Supplemental Table S4.** Nomenclature and description of the ago alleles.

**Supplemental Table S5.** Top 100 differentially expressed small RNAs between cer7 and the wild type.

**Supplemental Table S6.** Primers used in this study.

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