

Small-interfering RNAs from natural antisense transcripts derived from a cellulose synthase gene modulate cell wall biosynthesis in barley

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Small-interfering RNAs (siRNAs) from natural *cis*-antisense pairs derived from the 3'-coding region of the barley (*Hordeum vulgare*) *CesA6* cellulose synthase gene substantially increase in abundance during leaf elongation. Strand-specific RT-PCR confirmed the presence of an antisense transcript of *HvCesA6* that extends ≥ 1230 bp from the 3' end of the *CesA*-coding sequence. The increases in abundance of the *CesA6* antisense transcript and the 21-nt and 24-nt siRNAs derived from the transcript are coincident with the down-regulation of primary wall *CesAs*, several *Csl* genes, and *GT8* glycosyl transferase genes, and are correlated with the reduction in rates of cellulose and (1 \rightarrow 3),(1 \rightarrow 4)- β -D-glucan synthesis. Virus induced gene silencing using unique target sequences derived from *HvCesA* genes attenuated expression not only of the *HvCesA6* gene, but also of numerous nontarget *Csls* and the distantly related *GT8* genes and reduced the incorporation of D-¹⁴C-Glc into cellulose and into mixed-linkage (1 \rightarrow 3),(1 \rightarrow 4)- β -D-glucans of the developing leaves. Unique target sequences for *CslF* and *CslH* conversely silenced the same genes and lowered rates of cellulose and (1 \rightarrow 3),(1 \rightarrow 4)- β -D-glucan synthesis. Our results indicate that the expression of individual members of the *CesA/Csl* superfamily and glycosyl transferases share common regulatory control points, and siRNAs from natural *cis*-antisense pairs derived from the *CesA/Csl* superfamily could function in this global regulation of cell-wall synthesis.

CesA/Csl genes | *cis*-NAT pairs | RNA regulation | small RNAs | VIGS

Plant cell walls constrain the sizes and shapes of plant cells, establish cell architecture, and are the basis of plant form and stature (1, 2). They are composite structures of cellulose microfibrils interlaced with complex glycans embedded in a physiologically active pectin matrix. The primary wall is a dynamic structure that is expanded as new material is added to accommodate cell growth, whereas thick secondary walls are elaborated in certain cell types during differentiation. Although cellulose microfibrils are the principal scaffold in both primary and secondary walls, genes encoding cellulose synthases (*CesAs*) are differentially expressed and are coordinately regulated with the production of a great many gene products required for biogenesis of distinct sets of matrix polymers. Over 1,500 genes in Arabidopsis, rice, and maize are currently annotated to possess cell wall-related functions (<http://cellwall.genomics.purdue.edu>), and we estimate that over 1,000 genes of unknown function are involved in cell wall biogenesis (3). We still have much to learn about the specific functions of the *Csls*, the hundreds of glycosyl transferase genes and the many classes of other genes that function in wall biogenesis, but also to learn about the regulation of large networks responsible for wall biogenesis at specific stages of development.

In this work, we found naturally occurring small interfering RNAs (siRNAs) derived from the *HvCesA6* gene. At late stages of leaf elongation, the levels of these RNAs increase markedly coincident with a decrease in expression of the *CesAs*, *Csls*, and an evolutionarily distant *GT8* glycosyl transferase gene. The inverse

correlation with *CesA* expression during normal leaf growth suggests that they form natural *cis*-antisense pairs in this down-regulation and can act in *trans* on other related *Csl* transcripts. This down-regulation could constitute a key requisite for the transition to new wall biogenesis programs during differentiation.

Virus-induced gene silencing (VIGS) is a reverse genomic technique to activate a natural sequence-specific RNA degradation system (4), which has been used to silence *CesAs* in tobacco (*Nicotiana benthamiana*), resulting in swollen phenotypes and drastic decreases in cellulose content (5). VIGS based on Barley Stripe Mosaic virus (BSMV) has been shown to be an effective means for transient silencing in barley (*Hordeum vulgare*) and hexaploid wheat (*Triticum aestivum*) (6, 7), thus providing a means to explore gene function in grasses species, for which the number of cell wall mutants is quite limited (3). However, we describe that VIGS experiments targeted to conserved regions of barley *CesAs* resulted in enhanced reduction in transcript levels of not only the evolutionarily related *CesA* and *Csl* genes, but also of nontarget *GT8* genes. Our results, coupled with data from Arabidopsis and rice small RNA databases, indicate that the expression of individual members of the *CesA/Csl* superfamily and glycosyl transferases share common regulatory control points, and that small interfering RNAs from natural *cis*-antisense pairs derived from *CesAs* could function in global regulation.

Results

Detection of Barley *CesA6* Antisense Transcripts by Strand-Specific RT-PCR. Strand-specific RT-PCR using several different primers specific for the *HvCesA6* gene showed the presence of natural antisense transcripts of the *HvCesA6* gene (Fig. 1). First-strand cDNAs were synthesized by using either gene-specific sense PCR primers (which anneal only to antisense transcripts), or a gene-specific antisense PCR primer, or oligo dT (which anneal only to sense transcripts). PCR products were detected for short (191 bp) and medium (1,041 bp) length antisense transcripts, but not the long antisense transcript (3,131 bp) (Fig. 1B). Control sense transcripts for all three (short, medium, and long) products by PCR were detected. DNA sequencing of the medium length product confirmed that amplification was from an *HvCesA6* antisense transcript. The medium-length PCR product indicates that the natural *HvCesA6* antisense sequence extends at least 1,230 bp from the 3' end of the *HvCesA6* coding sequence.

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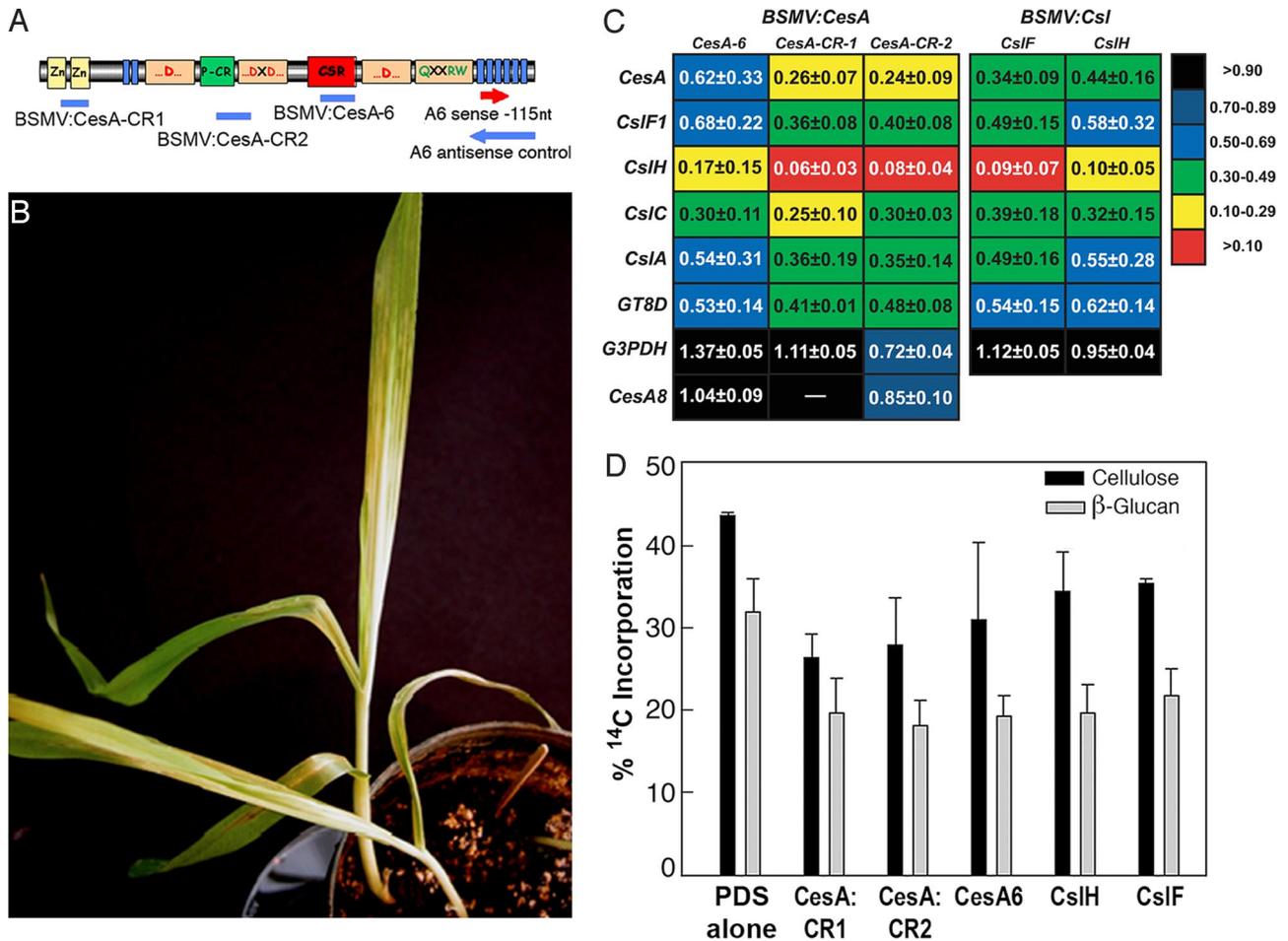


Fig. 3. VIGS of the *CesA* gene superfamily in barley. (A) Schematic indicating the protein domains encoded by *CesA* mRNAs (28). Blue bands indicate the regions of the *CesA* cloned into the VIGS constructs used in these experiments. VIGS fragments were designed to silence the *CesA* gene family as well as the *HvCesA6* gene. Red and blue arrows indicate the directional sequence used for sense and antisense riboprobe synthesis, respectively. (B) Onset of silencing is detected by photobleaching as a result of the cosilencing of phytoene desaturase. (C) Silencing of *CesAs*, *CslHs*, and *CslFs* by VIGS results in a global reduction in the expression of genes involved in cell wall biosynthesis. Expressions of *CesA6*, *Csl*, *GT-8D*, and *G3PDH* genes in plants infected with respective VIGS constructs, assayed by comparative qRT-PCR, are the mean \pm SD of 3–5 biological replicates, are relative to plants infected with empty vector-PDS control plants, and are normalized to *ubiquitin10* gene expression. (D) Incorporation of D- 14 C-Glc into cellulose and β -glucan are a percentage of the total radioactivity of each cell wall polymer \pm SD of 3 biological replicates.

“class-specific region” (CSR; ref 8) of an *HvCesA6* gene, the closest ortholog of the Arabidopsis and rice primary wall *CesA1*.

The first leaf of barley plants was infected with the *CesA* silencing constructs and grown until the onset of VIGS. All VIGS plants, including controls, were coinoculated with a *phytoene desaturase* silencing construct (BSMV:PDS) to serve as a visual marker of gene silencing. Plants inoculated with VIGS constructs began to show strong photobleaching because of silencing of the *phytoene desaturase* ~6–7 days postinoculation in both the second and third leaf, and the broad sectors showing photobleaching were taken for analyses of expression and cell wall synthesis (Fig. 3B). The growth of the second and third leaves was similar after inoculation and systemic infection by any of the BSMV constructs, although a slight decrease was observed during the latter days of growth compared to those of uninoculated or mock-inoculated control plants [see supporting information (SI) Fig. S1].

VIGS of *CesAs* Reduces the Expression of Target Genes as well as Other Cell Wall Biosynthetic Genes. Transcript abundance was measured by comparative qRT-PCR by using SYBR green (7). Compared to BSMV:PDS control plants, the expression of *CesA* genes were substantially reduced in BSMV/*CesA* infected plants (Fig. 3C). Further, VIGS constructs targeting the entire *CesA* gene family

(*CesA*-CR1 and *CesA*-CR2) showed a more pronounced reduction in total *CesA* expression than did the *CesA*-6 construct, which targets a sequence unique to the *HvCesA6* isoform (Fig. 3C).

To assess the effects of *CesA* VIGS in barley on the expression of related, nontarget genes, we performed comparative qRT-PCR. We found that the expression levels of several *Csl* genes were also significantly reduced in *CesA* VIGS plants (Fig. 3C). Further, the expression of the distantly related but coordinately regulated *GT8* genes was also reduced, suggesting that the reductions are not a result of cross-suppression (Fig. 2D), but rather the expression of several related and unrelated cell wall biosynthetic gene families are linked with that of cellulose synthase. A secondary wall *CesA8* gene whose expression is not linked with that of the primary wall genes (Fig. 2D), was relatively unaffected by VIGS (Fig. 3C). Also, expression of a metabolic housekeeping gene exhibited either slightly reduced or increased expression relative to BSMV:PDS controls, but differences were much smaller than those observed for cell wall-related genes (Fig. 3C).

VIGS of *CesAs* Lowers Rates of Cellulose and β -Glucan Synthesis. Incorporation of 14 C-D-Glc into cellulose was substantially lower after silencing with BSMV/*CesA*-PDS constructs than with BSMV:PDS alone (Fig. 3D). Rates of cellulose synthesis were more

strongly suppressed in plants in which the entire *CesA* gene family was targeted for silencing than when the single *CesA6* gene alone was silenced, consistent with expression analyses. Despite the effort to specifically silence *CesA*, the incorporation of ^{14}C -D-Glc into β -glucan was also markedly lowered in the *CesA* silenced plants (Fig. 3D). The expression of *HvCslF6* gene, whose ortholog in rice was previously demonstrated to encode a factor involved in mixed-linkage β -glucan biosynthesis (9), was also reduced in these plants, possibly explaining the reductions in β -glucan biosynthesis (Fig. 3D). Plants silenced with unique targets from the *CslF* and *CslH* gene families also had reduced incorporation of D- ^{14}C -Glc into leaf cellulose and mixed-linkage β -glucan (Fig. 3D).

Isoxaben and 2,6-Dichlorobenzonitrile Alter Cellulose Biosynthesis via a Different Mechanism than VIGS. Two specific inhibitors of cellulose biosynthesis, 2,6-dichlorobenzonitrile (DCB) and isoxaben, inhibited cellulose synthesis within 3–6 h after spraying to levels similar to those exhibited by the VIGS plants (Fig. S2A). Comparative quantitative PCR was performed under the same conditions used for the VIGS plants. In contrast to *CesA* or *Csl* VIGS, the expression of *CesA* genes was relatively unaltered, and *CslFs*, *CslHs*, and *CslCs* were instead up-regulated by DCB or isoxaben (Fig. S2B). These data indicate that the alterations in expression of *Csl* and *GT8* genes as a result of *CesA* VIGS are not a result of the reduction of cellulose biosynthesis.

Detection of Antisense siRNAs for the *CesA* Gene Family by Ribonuclease Protection Assay. The experiments with DCB and isoxaben inhibition of cellulose synthesis showed that lowering cellulose content does not induce broad suppression of *CesA* and *Csl* transcription (Fig. S2B). Although there are no published reports of transitive silencing of endogenous genes, we hypothesized that the VIGS of *CesA* genes had spread to other nontarget regions by the generation of secondary siRNAs not part of the original VIGS target. Ribonuclease protection assays using the “*HvCesA6* sense probe” (214 nt) protected fragments of approximately 21 nt and 24 nt in barley leaf and coleoptile RNA, and the amounts of these were enhanced in leaves infected with BSMV:PDS-*CesA*-CR1 and BSMV:PDS-*CesA*-CR2, suggesting that the production of the naturally occurring siRNA(s) was enhanced by *CesA* VIGS (Fig. 4A). A negative control probe consisting of only the vector portion of the probe (“vector probe”, 99 nt) did not protect any of these fragments, demonstrating that the small RNAs are naturally occurring and arise from a *CesA* antisense transcript (nat-siRNA). Treatment of barley plants with DCB or isoxaben did not alter levels of either the 21-nt or 24-nt nat-siRNAs (Fig. 4B).

Discussion

Whereas we are beginning to learn how certain transcription factors specify developmental programs and positively regulate their gene networks, we know very little about how negative regulation of previous programs is coordinated with up-regulation of a new network. Transcriptional switches for programs involving different cell wall gene networks involving a switch from primary to secondary wall include both NAC-domain (10) and Myb transcription factors (11). The lack of secondary wall formation but not cellular specification when these and other NAC-domain containing proteins are repressed and the induction of ectopic secondary walls in overexpressing lines provide strong evidence for control of cell wall gene networks (10). In all eukaryotes, microRNAs (miRNAs), made from noncoding stem-loop transcripts, are processed to form 20–24-nt RNAs that target coding transcripts for elimination (12). From the studies conducted thus far, a majority of the targets of miRNAs are transcription factors, including NAC and Myb proteins (13). Regulation of leaf identity by an *APETALA2*-like gene, called *Glossy15*, maintains juvenile identity, whereas the miRNA miR172 down-regulates *glossy15* expression to promote transition

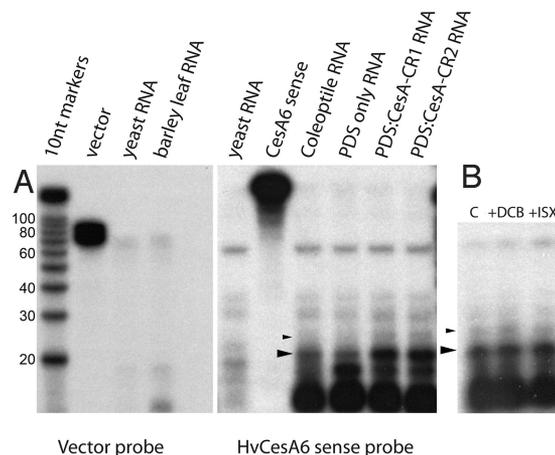


Fig. 4. Detection of *CesA* antisense siRNAs by ribonuclease protection assay. (A) Ribonuclease protection assays performed with the short *HvCesA6* sense probe were hybridized with 10 μg yeast RNA (negative control), A6-sense (probe-only, no RNase control), coleoptile RNA (10 μg), PDS-only (10 μg Vector-PDS VIGS RNA), PDS-*CesA*-CR1 (10 μg *CesA*-CR1 VIGS RNA), and PDS-*CesA*-CR2 (10 μg *CesA*-CR2 VIGS RNA). The vector-only probe [vector; probe-only controls without RNase were hybridized with 10- μg yeast RNA (negative control) and barley leaf (10- μg barley third leaf RNA). (B) Protection assays were also performed with the short *HvCesA6* sense probe hybridized with barley third leaf RNA (10 μg each) treated with Mock buffer (C), 100- μM DCB (+DCB), or 1- μM isoxaben (+ISX). Decade markers (Ambion) were electrophoresed as size standards and are to scale for both (A) and (B). Large arrowheads denote 21-nt RPA fragments, and the small arrowheads denote the 24-nt RPA fragments. Marker lanes are in 10-nt increments beginning with 20 nt.

to the adult state (14), a transition that involves drastic changes in wall architecture.

As cellulose is a major scaffold polymer in plant cell walls, it should not be surprising that coordinate expression of an entire gene network is regulated around the expression of specific cellulose synthases. Multiple *CesAs*, each thought to interact within heterotrimeric cellulose synthase complexes specific to primary or secondary walls (15), are coordinately expressed in maize, rice, barley, and poplar (16). Expression profiling of *Arabidopsis* during the transition from primary wall synthesis to vascular differentiation reveal several cell wall-related genes are coexpressed with the secondary wall cellulose synthases (17). The finding that VIGS of the *CesA/Csl* targets also silences *GT8* (Fig. 3C) indicates that coordinately up-regulated genes at one stage of development are coordinately down-regulated at another. Evidence for this comes directly from Milioni *et al.* (18) who showed by cDNA-AFLP analysis that induction of tracheary element formation in isolated *Zinnia* cells is accompanied by staged appearance and disappearance of several hundreds of genes. The expression of secondary cell wall-associated *HvCesA8* gene remained relatively high during the elongation phase of the third leaf, and the significant sequence identity in much of the 3' coding region of the gene makes it unlikely to be immune to silencing by either *CesA* nat-siRNAs or VIGS. However, the expanding leaf contains a spectrum of cell types making primary and secondary walls throughout elongation. Secondary wall cellulose synthesis is confined primarily to tracheids, fiber cells, and epidermal cells, and these cells are isolated from the virus, whose movement in vascular tissue is generally confined to the phloem.

A role for regulation of downstream transcripts of the individual members of the gene network by natural antisense siRNAs (nat-siRNAs) in this regulation is emerging (19). Our data indicate that the down-regulation of *CesA/Csls* involves nat-siRNAs generated by the cleavage of dsRNA natural antisense precursors. Curiously, the same antisense RNAs are enhanced by VIGS. Although the predominant *CesA* nat-siRNA is 21 nt, both 21-nt and 24-nt

nat-siRNA species were detected. Borsani *et al.* (20) observed a 24-nt nat-siRNA that sets the position for the phased cleavage of 21-nt small RNAs in response to salt stress, and more recently, nat-siRNAs are implicated in bacterial resistance in Arabidopsis (21). This study reports a potential role of siRNAs in a natural developmental program. Bidirectional transcription from *cis*-NAT pairs to form dsRNAs that are processed into 21-nt siRNAs has now been observed in *Drosophila* (22, 23) and mouse (24).

Our data indicate that the generation of dsRNA by VIGS of the *CesA* genes interferes with a natural antisense control mechanism. The v-siRNAs generated by the RNAi silencing complex could serve as primers for RdRP to generate secondary siRNAs that might account for apparent cross-suppression observed here, noting that RdRP can transcribe bidirectionally in plants (25). However, we found that antisense siRNAs were naturally present in developing leaf and coleoptile tissues, opening the possibility for the production of other multiple small RNAs each having separate subsets of genes to control cell wall biosynthesis via transitivity (26, 27). Transitive silencing by VIGS is thought to only occur in transgenes (25), but this has not been exhaustively examined with a broad range of genes. Whereas our data demonstrate a role for *CesA* derived nat-siRNAs in the coordinated transition from primary to secondary cell wall expression programs, other functions may exist as well. For instance, *CesA* small RNAs might mediate the balanced coexpression of the multiple isoforms of *CesA* associated with primary or secondary cellulose formation. Such a mechanism could explain the difficulties associated with attempts to increase protein levels by overexpression of *CesAs* (28).

From examination of rice tiling arrays, 23.8% of rice genes exhibit antisense expression (29). Massively parallel signature sequencing (MPSS) of both rice and Arabidopsis transcripts show a considerable number of sequence tags derived from the noncoding strand of *CesA* and *Csl* genes, indicating natural antisense transcription in both grasses and dicots. Both MPSS and 454 sequencing have also detected unique small RNAs targeting *CesA* genes (<http://mpss.udel.edu>; <http://sundarlab.ucdavis.edu/>), and Lu *et al.* (30) also report a miRNA that derives from a natural *cis*-antisense pair involving *CesA*. Further, a related GT-2 family member encoding hyaluronan synthase has been demonstrated to be regulated by natural antisense expression, suggesting a conserved mechanism for regulation of Family 2 glycosyl transferases (31).

Henz *et al.* (19) found that 7.4% of Arabidopsis genes comprise overlapping natural *cis*-antisense transcripts (*cis*-NATs) and that frequency of anti-correlated expression is higher than expected in a small number of *cis*-NAT pairs. We examined the occurrence of *cis*-NATs among the *CesA*, *Csl*, and *GT8* genes of Arabidopsis and rice and compared their occurrence with the frequency of antisense RNAs found in above databases. Six of the ten Arabidopsis *CesA* genes have overlapping pairs of genes, including five of the six *CesA* genes shown to be coordinately expressed during both primary wall and secondary wall formation (Table S1). Only *AtCesA6* had no overlapping gene. In general, the *cis*-NAT pairs also exhibited a higher hit frequency of antisense RNAs, with several annotated as having sequence identities with other *AtCesAs*, including *AtCesA6* and *AtCslD3*.

In rice, a single *cis*-NAT was found among the ten annotated *CesAs* (Table S2). The *Csl* genes existed in far fewer *cis*-NAT pairs, but with the exception of *CslC*, all had at least 1 pair (Tables S3 and S4). We find significant sequence identity among *CesA* and *Csl* genes of rice, including 4 runs of >20 identical nucleotides between a *CesA* and a *CslF* (Fig. S3), and numerous additional stretches of identity with only 1–3 mismatches among many *CesA*-*Csl* combinations, including *CslA*, that is most distant from *CesA*. With few exceptions, the *Csls* have small RNAs annotated in MPSS tag collections. Notably, a *CslD5* is a *cis*-NAT pair with *GATL-5*, a Group C *GT8*. Of the 5 groups of *GT8* genes, no *cis*-NAT pairs are observed for *GT8-A* or *GT8-E*, but are represented well in Groups B–D, including the *GT8-C* (*GATL5*) with *CslD3*, and *GT8-D* with

a nuclear transport factor (Table S3). It will be a challenge to assemble the pathways for the spread of silencing by developmentally regulated *trans*-acting nat-siRNAs, but there is a growing body of public data to support its existence (32).

We were unable to detect siRNAs for the barley *GT8* gene by ribonuclease-protection assay, suggesting that the coordinated reduction, both natural and VIGS-induced, occurs via another mechanism (Fig. S4). Also, we did not detect 21-nt *HvCesA6* nat-siRNAs upstream of the VIGS site suggesting that these nat-siRNAs are confined to the 3'-coding region of the *CesA6* gene (Fig. S4). However, we observed protected bands of 24 nt and 30 nt, the significance of these is not yet understood.

Like the *Csls*, the absence of a *cis*-NAT pair does not preclude the detection of small RNAs in the MPSS screen, with MPSS tags detected in almost all of them, including sequence identities among the rice *GAUT* and *GATL* genes of *GT8* (Table S4). How these antisense sequences are generated in the genes that do not comprise a *cis*-NAT pair is not known, but 2 possibilities are that noncoding antisense RNAs are driven by cryptic promoters or *trans*-acting small RNAs cleaved from related *cis*-NAT pairs spread silencing. Precedence for this type of cascade mechanism has been established in Arabidopsis initiated by a miRNA (33). A *GT8* ortholog to Arabidopsis *GAUT1* has no *cis*-NAT partner but has three hits in the MPSS database, including a sequence that matches 80 others.

Plants form cell walls that are dynamic structures of cellulose, crosslinking glycans, and various matrix polysaccharides, proteins, and phenolic substances that are reconstructed during embryogenesis, cell enlargement, and cell specification and differentiation. These transitions require not only a positive regulation of new cell wall gene networks, but also the coordinated silencing of the previous networks. siRNAs generated from antisense transcripts of *CesAs* could represent an initiator of a cascade of *cis*- and *trans*-acting silencers of cell-wall gene networks integral to differentiation.

Methods

Plant Growth Conditions—*Hordeum vulgare* cv. Black Hulless plants were grown ≤ 15 d as described (7), with VIGS inoculations at 6 or 7 d. Specific conditions are described in *SI Methods*. Coleoptiles were from caryopses soaked and germinated in wet filter paper and incubated 2.5 d in darkness, as described (34).

Extraction of RNA and First-Strand cDNA Synthesis. Total barley leaf and coleoptile RNA was extracted from 100–250 mg tissue by using TriZol reagent (Invitrogen) following the manufacturer's protocol, quantified by A_{260} . Before RT-PCR, genomic DNA contamination was removed from total RNA (10 μ g) by Turbo DNA-free DNase treatment (Ambion). The RNA samples were assayed for quality and quantity by agarose gel electrophoresis. First strand cDNA was synthesized from total RNA (1 μ g per reaction) by using iScript first strand cDNA synthesis kit (Bio-Rad) and subsequently diluted (1:5) with nuclease-free water.

Detection of Antisense Transcripts by Strand-Specific RT-PCR. First-strand cDNA was synthesized from 1.7 μ g total RNA extracted from late-expanded barley third leaves with the SuperScript III kit (Invitrogen), by using oligo dT as well as the gene-specific primers representative of short, medium, and long sense and antisense primers and subjected to PCR by using the primer-pair combinations and cycle conditions described in *SI Methods*.

Detection of siRNAs by Ribonuclease Protection Assays (RPA). Primers were designed for cloning a 465-bp fragment from the 3'-end of the *HvCesA6* coding region, and the fragment was amplified by RT-PCR from cDNA prepared from barley third-leaf RNA. The PCR product was sequenced and then cloned into the pGEM-T easy vector kit (Promega). α - 32 P-UTP (Amersham) radiolabeled probes were prepared from linearized plasmid templates having 5' overhanging ends, with either T7 or SP6 RNA polymerase by using the MAXIScript Kit (Ambion) to produce the *HvCesA6* antisense riboprobe (522 nt) and the *HvCesA6* sense riboprobe (214 nt), respectively. Vector sense probe (99 nt) representing the pGEM-T easy vector was prepared as a negative control. Primer design is described in *SI Methods*.

Ribonuclease protection assays were performed by using the RPAIII kit (Ambion). Labeled riboprobes were gel-purified and hybridized with 10–20 μ g total RNA from either barley, yeast, or mouse for 16–18 h at 42 °C. Reaction mixtures were digested with RNase A/T1 (1:100) for 30 min at 37 °C, then stopped with inactivation buffer (Ambion), and protected fragments were precipitated by

using 10- μ g yeast RNA as a carrier. The protected fragments were separated by 12.5% PAGE. The protected HvCesA6 nat-siRNAs were reproduced across ≥ 3 independent protection assays.

Measurement of Gene Expression by qRT-PCR. Primer sets were designed within consensus regions to measure the expression of as many gene family members as possible in uninfected and VIGS-treated plants (Table S5). Reactions using the iTaq SYBR reagent kit (Bio-Rad) were performed in quadruplicate. Standard curves covering 3 orders of magnitude, and no-template controls were performed in triplicate for each primer set. Relative gene expression of VIGS plants and controls from 3–5 separate third-leaf samples was assessed by qRT-PCR using *ubiquitin10* expression as the normalizing target and *BSMVV-PDS* (*BSMVV/PDS4as*) as a calibrator (35). *Ubiquitin10* was deemed optimal after survey of 4 housekeeping gene candidates (data not shown; primers are listed in Table S5). Thermocycle conditions are described in *SI Methods*.

Design and Construction of BSMV Vectors for *CesA/CsI* VIGS. Targets for the specific suppression of the barley *CesA*, *CsIF*, and *CsIH* gene families were first designed by aligning full-length rice cDNA sequences of each gene family member using Clustal W (36). Targets were selected based on at least 85% nucleotide similarity and at least one 21-nt stretch of 100% nucleotide identity, and the lack of these criteria in nontarget genes. Potential VIGS target sequences garnered from rice were then aligned with available barley *CesA* and *CsI* EST sequences to identify the homologous regions, verify desired specificity, and design primers for cloning (as described in *SI Methods*).

For in vitro transcription of VIGS plasmids, capped viral RNAs were prepared from linearized plasmids by using the mMessage mMachine T7 in vitro transcription kit (Ambion). Plasmid linearization and in vitro transcription was monitored by agarose gel electrophoresis.

Viral Inoculation of Barley Plants. Plant inoculations were carried out as described previously (7). Briefly, in vitro transcripts of the *pBSMV α* , *pBSMV β* , *pBSMV γ -PDSas*, and the appropriate experimental gene silencing construct (either *BSMV γ* , *BSMV γ -CsIH-as*, *BSMV γ -CsIF-s*, *BSMV γ -CesA-CR1-s*, *BSMV γ -CesA-CR2-s*, or *BSMV γ -CesA-6-as*) were combined in a 1:1:0.5:0.5 ratio by volume,

respectively. For plant inoculations, 3 μ l of the mixture of α , β , γ -PDS, and respective γ -RNA transcripts were added to 22.5 μ l of FES (37) and applied to 6–7-day-old seedlings by rub-inoculating the first leaf of each plant 2–3 times. The γ -PDSas transcripts were coinoculated with all plants to serve as a visual marker for gene silencing, evident in the third leaves 6–7 d later.

In vivo Labeling of Barley Leaves with [14 C]-Glucose and Cell-Wall Characterization. For the developmental time courses and experiments with cellulose synthesis inhibitors, plants were pulse-labeled with 14 CO₂, liberated by adding a few drops of 1 M H₂SO₄ to a dish containing 10 μ Ci per plant of NaH¹⁴CO₃ (aq) (2.04 GBq mmol⁻¹; MP Biomedicals) in a sealed Plexiglas chamber. The plants were incubated in the presence of the radiolabel under fluorescent lighting for 1 h. The third leaves for each treatment were pooled and harvested into liquid nitrogen. For inhibitor experiments, barley seedlings were grown 11 d in a growth chamber, as described above, and 12 plants each were sprayed (\approx 5 ml/plant) with 0.1% (vol/vol) Tween 20 (aq) and 0.1% (vol/vol) DMSO containing either no inhibitor (mock), 1 μ M isoxaben, or 100 μ M DCB. Plants were incubated under fluorescent lights for 3–6 h at ambient temp before labeling as described above.

For VIGS experiments, expanding third leaves of plants (3–5 each) were harvested under 0.01% (vol/vol) Tween 20 (aq) and pulse labeled in vivo with D-[U- 14 C]-glucose (Amersham Pharmacia) for 2–3 h at ambient temp under fluorescent lights. Labeled leaf tissue was collected into liquid nitrogen from which cell walls were prepared as described earlier (38).

Cellulose and (1 \rightarrow 3),(1 \rightarrow 4)- β -Glucan Determination. The ability to incorporate radiolabeled carbon into cellulose from isolated walls (38) was assayed by acetic-nitric hydrolysis (39), and β -glucan was determined after hydrolysis with a *B. subtilis* endo- β -D-glucanase, which specifically cleaves a β -(1 \rightarrow 4)-linkage, only if it is preceded by a β -(1 \rightarrow 3) linkage (40), as described previously (41). Both methods are described in detail in *SI Methods*.

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