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ORIGINAL RESEARCH

Posttranscriptional regulation of cellulose synthase genes by small RNAs derived from cellulose synthase antisense transcripts

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Abstract

Transcriptional regulatory mechanisms governing plant cell wall biosynthesis are incomplete. Expression programs that activate wall biosynthesis are well understood, but mechanisms that control the attenuation of gene expression networks remain elusive. Previous work has shown that small RNAs (sRNAs) derived from the *HvCESA6* (*Hordeum vulgare*, *Hv*) antisense transcripts are naturally produced and are capable of regulating aspects of wall biosynthesis. Here, we further test the hypothesis that *CESA*-derived sRNAs generated from *CESA* antisense transcripts are involved in the regulation of cellulose and broader cell wall biosynthesis. Antisense transcripts were detected for some but not all members of the *CESA* gene family in both barley and *Brachypodium distachyon*. Phylogenetic analysis indicates that antisense transcripts are detected for most primary cell wall CESA genes, suggesting a possible role in the transition from primary to secondary cell wall biosynthesis. Focusing on one antisense transcript, *HvCESA1* shows dynamic expression throughout development, is correlated with corresponding sRNAs over the same period and is anticorrelated with *HvCESA1* mRNA expression. To assess the broader impacts of *CESA*-derived sRNAs on the regulation of cell wall biosynthesis, transcript profiling was performed on barley tissues overexpressing *CESA*-derived sRNAs. Together, the data support the hypothesis that *CESA* antisense transcripts function through an RNA-induced silencing mechanism, to degrade *cis* transcripts, and may also trigger *trans*-acting silencing on related genes to alter the expression of cell wall gene networks.

KEYWORDS
antisense transcription, cellulose, small RNA

1 | INTRODUCTION

As young plant cells grow and divide, they produce thin and elastic primary cell walls (PCWs). When cell growth ceases, certain cell types will undergo cell wall thickening to form rigid secondary cell walls (SCWs). The major polysaccharide for both PCW and especially SCW is cellulose. Cellulose is made by plasma membrane resident glycosyltransferases (GTs) called cellulose synthases (CESAs). CESAs

synthesize individual β-(1,4)-linked glucan chains, which associate to form larger paracrystalline microfibrils. Individual CESA proteins interact to form large, rosette-shaped cellulose synthase complexes (CSCs) (Brown & Montezinos, 1976; Giddings et al., 1980; Herth, 1985; Kimura et al., 1999; Mueller & Brown, 1980). The exact number of CESA proteins in a given CSCs is unclear, but current models describe it as a hexamer of trimers that utilize at least three unique non-redundant CESA isoforms (Gonneau et al., 2014; Hill et al., 2014; Taylor et al., 2000, 2003). Additionally, PCW and SCW CSCs use different sets of CESAs. In *Arabidopsis thaliana*, for example, *AtCESA1*, *AtCESA3*, and *AtCESA6/2/5* are highly coexpressed and interact to form PCW CSCs (Persson et al., 2007) whereas *AtCESA4*, *AtCESA7*, and *AtCESA8* are highly coexpressed and form SCW CSCs (Brown et al., 2005; Persson et al., 2005). All plants examined to date have coexpressed orthologs of each of these *Arabidopsis CESA*s indicating conservation across plant lineages (Carroll & Specht, 2011). In *Hordeum vulgare* (barley), for example, *HvCESA1*, *HvCESA2*, and *HvCESA6* are coexpressed and comprise CSCs for PCWs, whereas *HvCESA4*, *HvCESA7*, and *HvCESA8* are for SCW CSCs (Burton et al., 2004).

PCW and SCW formation each require the concerted action of many additional GTs and cell wall-modifying enzymes. Hemicellulose and pectin GTs, needed for PCW formation, tend to be coexpressed with PCW *CESA*s, whereas GTs and lignin biosynthetic enzymes tend to be coexpressed with SCW *CESA*s (Brown et al., 2005; Mutwil et al., 2009; Persson et al., 2005). Thus, PCWs and SCWs are each synthesized by the products of specific gene networks. Importantly, as cells begin to cease cell growth, there is a transition from PCW to SCW gene networks. The factors that drive network transition are not fully understood but are beginning to come to light (Li et al., 2016; Watanabe et al., 2018).

As might be expected, the actions of hormones and transcription factors are major players in regulating cell wall gene networks. Auxin, abscisic acid, brassinosteroids, cytokinins, ethylene, and gibberellic acid have been shown to play various roles in SCW formation (Didi et al., 2015). Transcription factor (TF) networks have been identified as activators of primary (Saelim et al., 2019; Sakamoto et al., 2018) and secondary wall biosynthetic programs both naturally and in response biotic and abiotic stresses (Hussey et al., 2013; Ko et al., 2012; Ko et al., 2014; Kubo et al., 2005; McCarthy et al., 2009; Mitsuda et al., 2005, 2007; Nakano et al., 2015; Wang & Dixon, 2012; Yamaguchi & Demura, 2010; Zhang et al., 2018; Zhong et al., 2006, 2010; Zhong & Ye, 2014; Zhou et al., 2009). Although much is known about activation and upregulation of cell wall synthesizing components, the corresponding mechanisms that selectively downregulate the same gene networks are still unclear (Li et al., 2016; Wang & Dixon, 2012).

Previous work has demonstrated that the transition from PCW to SCW may be regulated in part at the posttranscriptional level by *CESA*-derived small RNAs (sRNAs) (Held et al., 2008). Here, we test the hypothesis that cell wall gene networks can be regulated by antisense RNA-derived sRNAs centered around the expression of *CESA* genes. A survey of barley and *Brachypodium distachyon CESA* (*BdCESA*) genes for additional antisense transcripts was performed. Antisense transcripts were detected for some but not all *HvCESA* genes, with a concentration on PCW *CESA*s. A developmental time course of one of these antisense transcripts (*HvCESA1*) and its corresponding sRNAs over time also showed a correlated relationship. This analysis was extended to the closely related grass, *Brachypodium*, to see if this phenomenon was unique to barley. Antisense RNAs were also detected for some but not all *BdCESA*s and were generally confined to direct barley orthologs, suggesting evolutionary conservation. Lastly, cell wall gene expression profiling was performed to examine the extent to which *CESA* sRNAs can impact the expression of cell wall gene networks. The data show close and distant targeting of cell wall-related genes moderated by sRNA mechanisms demonstrating the potential for broader cell wall gene network regulation.

2 | METHODS

2.1 | Plant growth and tissue collection

Seeds of *H. vulgare* cv. Black Hulless were imbibed in aerated water for 24 h to stimulate germination. Imbibed seeds were transferred to moist vermiculite and placed in the dark at 28°C until hypocotyls emerged, generally 3–5 days. Seedlings were then transferred to autoclaved soil (Promix BX) supplemented with Osmocote (Scotts) 14-14-14 slow release fertilizer (1.8 g L^{-1}). Seedlings were grown in a Percival E36HOX growth chamber under high-intensity fluorescent lamps (450–700 μmol m^{-2} s^{-1}) programmed for a 16-h photoperiod (25°C day^{-1}, 20°C night).

B. distachyon seeds were imbibed in aerated water for 48 h to stimulate germination and then transferred to damp vermiculite and incubated at 22°C in the dark for 7 days to stimulate cotyledon growth. On Day 9, seedlings were transferred to autoclaved soil (Promix BX) supplemented with Osmocote (Scotts) 14-14-14 slow release fertilizer (1.8 g L^{-1}). Seedlings were grown in a Percival E36HOX growth chamber under high-intensity fluorescent lamps (180–200 μmol m^{-2} s^{-1}) programmed for a 20-h photoperiod (22°C constant). Third leaf tissue from five or more plants was excised, measured for length, and pooled in liquid nitrogen at 17, 19, 21, 24, and 27 days post imbibition (dpi).

2.2 | Preparation of barley and *Brachypodium* RNA

Pooled third leaf samples for both survey and time course experiments were pulverized using a mortar and pestle under liquid nitrogen and then homogenized under TRIzol® reagent (Invitrogen-Thermo/Fisher). Aliquots of each RNA sample were treated for DNA contamination using the TURBO DNA-free kit (Invitrogen-Thermo/Fisher) per the manufacturer's instructions for rigorous treatment. An aliquot of each RNA sample (0.5 μg) was separated on a single agarose gel (0.7% to 1%) and visualized with ethidium bromide dye to check for RNA degradation. Gels were imaged by a Chemidoc EQ camera (Bio-Rad)

using Quantity One software (Version 4.5.2 Build 070) to verify uniform RNA loadings. Gel images were analyzed using ImageJ (Version 1.49E). Semiquantitative time course measurements were normalized to the RNA loading, whereas qPCR measurements were normalized to actin housekeeping gene expression.

2.3 | Detect of antisense RNA transcripts

2.3.1 | GSP design for tagged, SS-RT-PCR

Gene-specific primers (GSPs) for antisense transcript detection for *HvCESA* and *BdCESA* gene families were designed using the OligoAnalyzer 3.1 software, as described previously (Held et al., 2008). Primers were verified for specificity by BLAST analysis against either the NCBI barley or *Brachypodium* transcript library. Each primer was pairwise aligned against every member of the corresponding *CESA* gene family to ensure specificity. To improve PCR strand specificity, a unique oligonucleotide tag was added to the 5′ end of each sense GSP for cDNA synthesis (Craggs et al., 2001). Either the *tag1* or *tag2* sequence was added to the 5′ end of each barley sense GSP, whereas the *tag2* sequence was added to the 5′ end of each *Brachypodium* sense GSP for cDNA synthesis (Table S1).

2.3.2 | Preparation of CESA antisense cDNA for family surveys

First-strand cDNAs for antisense transcripts of *HvCESAs and BdCESAs* were synthesized from 1.7 μg of DNase-treated total RNA extracted from barley (13 dpi) and *Brachypodium* third leaves (17 dpi) using the SuperScript III First-Strand Synthesis System (Invitrogen 18080-051), using tagged sense GSPs (Table S1). Control cDNAs were prepared as follows: *oligo dT*-primed (OdT) cDNA, no primer control (NPC) cDNA (cDNA reactions lacked a primer), and no reverse transcriptase (NRT) control cDNA (cDNA reactions lacked RT). cDNA reactions were then treated with RNase H to remove residual complementary RNA per the manufacturer's protocol and then diluted 10-fold with nuclease free water.

2.3.3 | Amplification of antisense transcripts

CESA family survey for antisense expression by tagged, SS-RT-PCR

First-strand cDNAs synthesized for the detection of each *HvCESA* antisense transcript were amplified by PCR using the *tag1* primer and the corresponding antisense GSP. For *BdCESA* antisense transcripts, first-strand cDNAs synthesized for the detection of each were amplified by PCR using the *tag2* primer and the corresponding antisense GSP (Table S1). *Oligo dT*-primed cDNA was also amplified individually with each pair of *HvCESA* sense and antisense GSPs, as controls for amplicon size and sense mRNA presence. To rule out nonspecific amplification by the tag primers (Tag controls), *oligo dT*-primed cDNAs were amplified with antisense GSPs and the *tag1* primer (for barley samples) or *tag2* primer (for *Brachypodium* samples). All PCR amplifications were assembled on ice in 25-μl reactions using 5 μl of 5× Green GoTaq buffer (Promega M3001), 0.5–1 μl of each primer (10 μM), 0.5 μl of dNTPs (10 μM each), 4 μl of diluted cDNA template, and 1.25 units of GoTaq polymerase. Cycling conditions for all reactions were optimized for melting temperature and extension time (Table S1). Barley PCR reactions were cycled with 2 min of activation at 95°C, followed by 35 cycles of 95°C for 1 min, optimized annealing temperature for 1 min, and 72°C for the optimized extension time. Final elongation was 72°C for 5 min. *Brachypodium* PCR reactions were cycled with 2 min of activation at 95°C, followed by 37 cycles of 95°C for 30 s, optimized annealing temperature for 30 s, and 72°C for the optimized extension time. Final elongation was 72°C for 5 min. At least three technical replicates were performed for each antisense cDNA sample. Experiments were performed with at least three biological replications.

HvCESA1 *antisense time course analysis*

First-strand cDNAs synthesized using the *HvA1-sense-tag1* GSP were used as templates for PCR following the same assembly as the initial survey above. Cycling conditions for PCR reactions using *HvA1-antisense-1* GSP and *tag1* primer included 2 min of activation at 95°C, followed by 34 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 45 s. Final elongation was 72°C for 5 min. Antisense transcript cycling conditions were optimized to terminate amplifications during the mid-/late-log phase so that semiquantitative densitometry could be performed. Three replicates of equal volumes of antisense PCR products for each time point were separated by agarose gel electrophoresis. Gels were imaged by a Chemidoc EQ camera using Quantity One software (Version 4.5.2 Build 070). Gel images were analyzed using ImageJ (Version 1.49E). Background subtraction was performed with a rolling ball radius of 50.0 pixels. Densitometry was performed and then normalized to the densitometry results from the RNA loading gel for each time point.

To confirm the semiquantitative time course analysis, a quantitative PCR (qPCR) approach was adapted for tagged, strand-specific amplification. For qPCR, first-strand cDNAs were synthesized using the SuperScript III kit (Invitrogen) with both *HvA1-sense-tag2* and *Actin-R* GSPs and 3.5 μg of DNA-free, total RNA for each time point. NRT, NPC, and *oligo dT*-primed cDNAs were again generated as negative controls. qPCR reactions were assembled in quadruplicate using the iTaq Universal SYBR green supermix (Bio-Rad) according to manufacturer's instructions. Reactions were thermocycled on a Rotor Gene-Q instrument (Qiagen). For the detection of *HvA1* antisense transcripts, qPCR was performed using *tag2* and *HvA1-antisense* primers and cycling conditions included 2 min of activation at 95°C, followed by 45 cycles of 95°C for 15 s, 52°C for 30 s, and 72°C for 30 s. For the detection of *actin* as a housekeeping gene control, *Actin-F* and *Actin-R* primers were used and cycling conditions included 2 min of activation at 95°C, followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. A final melt analysis was performed for each amplification to confirm single product detection. Antisense PCR products were further separated by agarose gel electrophoresis

and extracted for DNA sequencing to confirm expected product size and specificity. Cycle threshold (C_T) values were used to determine relative expression values for each time point as described previously (Held et al., 2008).

2.3.4 | Characterization of amplicons

Equal volumes of each PCR product for each sample and control reaction were separated by agarose gel electrophoresis with ethidium bromide staining. Antisense amplification products were excised and purified with the Zymoclean Gel DNA Recovery Kit (Zymo) and cloned into the *pGEM T-Easy* vector kit (Promega). Clones were fully sequenced and confirmed as the targeted sequence. Inclusion of *tag* sequences confirmed that cDNA samples were primed by *sense-tag1* or *sense-tag2* GSPs and thus could only be derived from endogenous antisense transcript templates.

2.4 | Ribonuclease protection assays

2.4.1 | Design of *HvCESA1* RPA probes

A 400-base pair region inside the sequence of the *HvCESA1* antisense was amplified by RT-PCR from an *oligo dT*-primed cDNA using 5′TAAG CGCCCAGCTTTCAA and 5′GATACCTCCAATGACCCAGAAC oligonucleotide primers and GoTaq Green polymerase (Promega). The PCR product was cloned into the *pGEM T-Easy* vector (Promega). α-^{32}P-UTP-radiolabeled (Perkin Elmer Health Sciences) probes were prepared from linearized plasmid templates (*SpeI* or *NcoI*) having 5′ overhangs from either T7 or SP6 RNA polymerase using the MAXIscript Kit (Ambion) to produce the *HvCESA1* antisense-targeting (466 nucleotides [nt]) and *HvCESA1* sense-targeting (506 nt) riboprobes, respectively. A 61-nt portion of the *HvCESA1* sense-targeting riboprobe and an 82-nt portion of the *HvCESA1* antisense-targeting riboprobe were derived from the *pGEM T-Easy* vector, so empty vector (EV) probes were similarly prepared for both as negative controls.

2.4.2 | *HvCESA1* time course RPA assay

Ribonuclease protection assays (RPAs) were performed by using the RPA III kit (Ambion) according to manufacturer's instructions and as previously described (Held et al., 2008). Labeled sense and antisense riboprobes targeting *HvCESA1* and control probes were gel purified by 5% PAGE containing 8 M urea in 1× TBE buffer per kit instructions and hybridized with 10- to 20-μg 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 and then stopped with inactivation buffer (Ambion), and protected fragments were precipitated by using 10-μg yeast RNA as a carrier. The protected fragments were separated by 12.5% PAGE containing 8 M urea in 1× TBE buffer. γ-^{32}ATP (Perkin Elmer Health Sciences) end-labeled Decade Marker (Ambion), prepared per manufacturer's protocol, served as the size standard. Autoradiograms of RPA gels were uniformly scanned at 600-dpi grayscale in a lossless format. The intensity of bands in the 21- to 24-nt range were analyzed using ImageJ (Version 1.49E).

2.5 | Custom cell wall microarray analysis

2.5.1 | Viral inoculation of barley plants

Plant inoculations were carried out as described previously (Held et al., 2008; Holzberg et al., 2002). Third leaf tissues from plants visibly demonstrating photobleaching were harvested 7 to 13 days after inoculation, with maximal photobleaching at about 8 days after inoculation. Senescent tissue was trimmed from the leaf tip if present, followed by snap freezing in liquid nitrogen. Frozen virus-induced gene silencing (VIGS)-infected tissues were pulverized using a mortar and pestle under liquid nitrogen and then combined with TRIzol® reagent (Invitrogen, Carlsbad CA). RNA was then prepared per the TRIzol® protocol.

2.5.2 | Construction of custom microarray

A custom, single-channel, Agilent (Santa Clara, CA) microarray based on the 8 × 16K architecture was designed to identify genes regulated in response to CESA silencing enriched in sequences involved in cell wall biosynthesis, stress response, and RNA regulation. Each slide contained eight arrays, with approximately 16K probes per array (Wolber et al., 2006). A total of 3778 60-mer probes were selected from a list of candidate genes by the Agilent eArray service, with four technical replications of each probe per array. EV-treated samples and *HvCESA* silencing (*HvCESA*-CR2)-treated samples were prepared and prescreened for silencing of *HvCESA6* transcript levels via qPCR prior to microarray analysis to confirm a *HvCESA* family silenced state as described earlier (Held et al., 2008).

2.5.3 | Microarray hybridization and data extraction

VIGS-treated barley RNA samples were verified for quality by a Bioanalyzer 2100 instrument and hybridized to the custom 8 × 16K microarray per the manufacturer's protocol (Agilent). Sixteen total samples were hybridized, one per array, with six BSMV-EV-treated samples (negative control) and 10 BSMV-*HvCESA*-CR2-treated samples. Hybridized arrays were imaged with an Agilent Technologies Scanner G2505B, and signals were extracted using the Agilent Feature Extraction Tool (Version 10.7.3.1 using protocol GE1_107_Sep09).

2.5.4 | Processing of microarray data

Extracted microarray data was processed using the limma package from Bioconductor. Backgrounds were corrected using the normexp method

with a +50 offset (Ritchie et al., 2007). Arrays were normalized between each other using the quantile method. All signals within 110% of the 95th percentile of the negative controls for six or more arrays were ignored. Signals from replicate probes for each array were then averaged and used to identify differentially expressed genes (adjusted $p < .05$).

2.6 | Collection of *BdCESA* sRNA sequences

Brachypodium sRNASeq dataset OBD02 (GSM1266844) (Jeong et al., 2013) hosted at mpss.danforthcenter.org was queried (Nakano et al., 2006) using selected *BdCESA* nucleotide sequences. All sRNAs matching *BdCESAs* were BLASTed against the *Brachypodium* genome to ensure specificity to only *BdCESA* genes (E-value cutoff of 1E-10), and any sequences with alternate targets were omitted.

3 | RESULTS

3.1 | Antisense transcripts are detected for multiple barley *CESA*s

Tagged, strand-specific RT-PCR (SS-RT-PCR) (Craggs et al., 2001; Li et al., 2013) was used to survey the barley *CESA* gene family for antisense transcripts in barley third leaves (Burton et al., 2004; Held et al., 2008). The presence of antisense RNAs were tested for *HvCESA1* (MLOC_55153.1), *HvCESA2* (MLOC_62778; AK366571), *HvCESA4* (MLOC_66568.3), *HvCESA5/7* (MLOC_43749; AK365079), *HvCESA6* (MLOC_64555.1), and *HvCESA8* (MLOC_68431.4). *HvCESA3* (MLOC_61930.2) was omitted from this study because its expression did not cluster with either primary or secondary wall expression (Burton et al., 2004). To ensure antisense strand specificity, a tag sequence (*tag1*) was added to the 5′ end of each barley gene-specific cDNA synthesis primer (Craggs et al., 2001) (Figure 1a). Antisense transcripts were detected for *HvCESA1*, *HvCESA4*, and *HvCESA6*, with lengths of 913, 966, and 898 nucleotides, respectively (Figure 1b). DNA sequencing confirmed that the antisense transcripts were complementary to the corresponding exonic sequence with no introns or indels. Further, all three amplicons included the *tag1* sequence on the correct end of the transcript, confirming that the PCR product was the direct product of an antisense transcript. Control, sense amplicons of the same sizes (minus the length of the tag) were detected for each *HvCESA* and showed much brighter bands, despite being cycled under the same conditions, indicating that their relative quantity is very high compared with corresponding antisense transcripts. No antisense transcripts were observed for the remaining *HvCESAs* (Figure 1b).

3.2 | Expression of *HvCESA1* antisense and sense transcripts anticorrelate during leaf growth

HvCESA1 antisense transcripts were monitored during barley third leaf development as previously described for *HvCESA6* (Held et al., 2008) using the tagged SS-RT-PCR method. Untagged SS-RT-

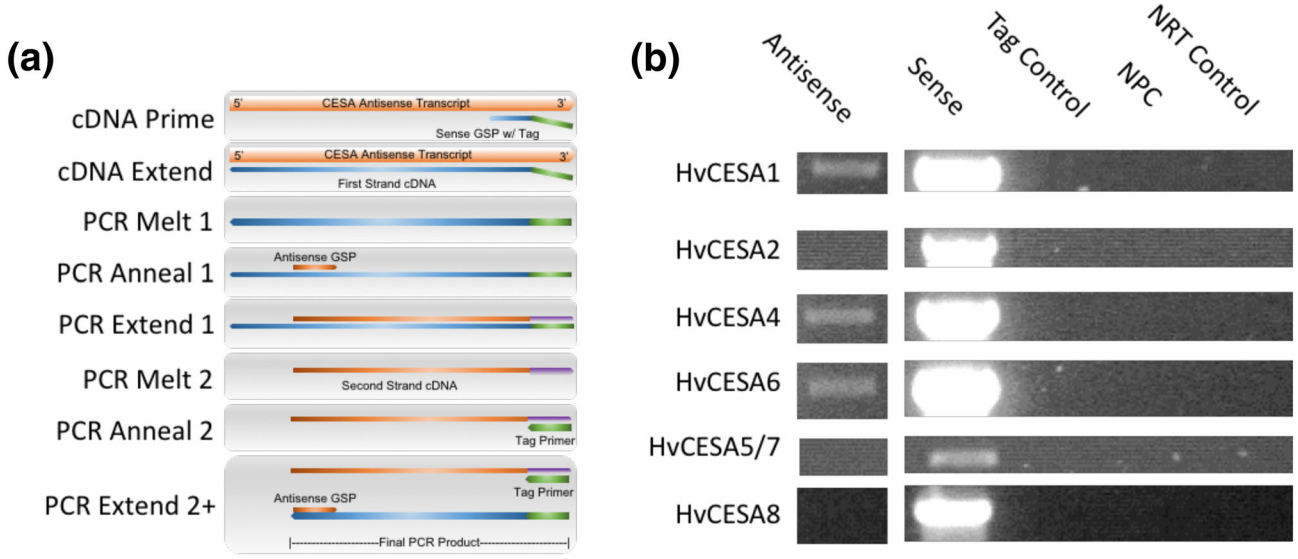

FIGURE 1 A survey of the *HvCESA* family for antisense transcripts. (a) Schematic representation of tagged, SS-RT-PCR for antisense transcript detection. First-strand cDNA synthesis uses a sense gene-specific primer (GSP) that is reverse complementary only to putative antisense transcripts. To minimize PCR artifacts, a unique tag is added to the 5′ end of the sense GSP for first-strand cDNA synthesis. Tagged cDNA is amplified with an antisense GSP and the tag primer. Thus, only bona fide antisense transcripts will be amplified. (b) Tagged, SS-RT-PCR of barley third leaf RNA for the detection of *HvCESA* antisense transcripts. PCR was performed with *antisense GSPs* and *tag* primer for Antisense, Tag control, no primer control (NPC), and no RT (NRT) control samples. Sense transcripts were amplified using both antisense and (untagged) sense GSPs from *oligo dT*-primed cDNA. Identity of the tagged, antisense PCR products was confirmed by DNA sequencing. See Table S1 for individual primer sequences

PCR was used to track the *HvCESA1* sense transcript levels. The quantity of *HvCESA1* antisense transcript was lowest on Day 10 and then increased to a maximum on Days 15 and 16 by a factor of ~2.5–4.5 (Figures 2b and S1A). Over the same time period, *HvCESA1* sense signal was highest on Days 10 to 13 and then fell by approximately half on Days 14 to 16 (Figure 2b). A qPCR approach was also adapted for the detection of antisense transcripts. This approach used two GSPs for the simultaneous synthesis of cDNAs for both antisense transcripts and internal housekeeping gene controls. In general, an increase across third leaf development between Days 13 and 16 was observed, although the relative increase in expression was smaller than detected by the semiquantitative approach (Figure S2B). Nonetheless, the accumulation of *HvCESA1* antisense transcripts, coupled with the decrease of *HvCESA1* sense transcripts were similar to those previously observed for *HvCESA6* (Held et al., 2008).

3.3 | *HvCESA1* sRNAs also accumulate over development

RPAs were performed to examine the presence and abundance of *CESA*-derived sRNAs over the same time period. Antisense *HvCESA1* sRNAs (~21–24 nt) were identified via a RPA using a sense RNA riboprobe (Figures 3 and S2). The sense probe was designed to be internal to the known antisense region of *HvCESA1* (Figure S3), so only antisense sRNAs within the *HvCESA1* antisense transcript would be detected. The signal intensity of the *HvCESA1* sRNAs varied over

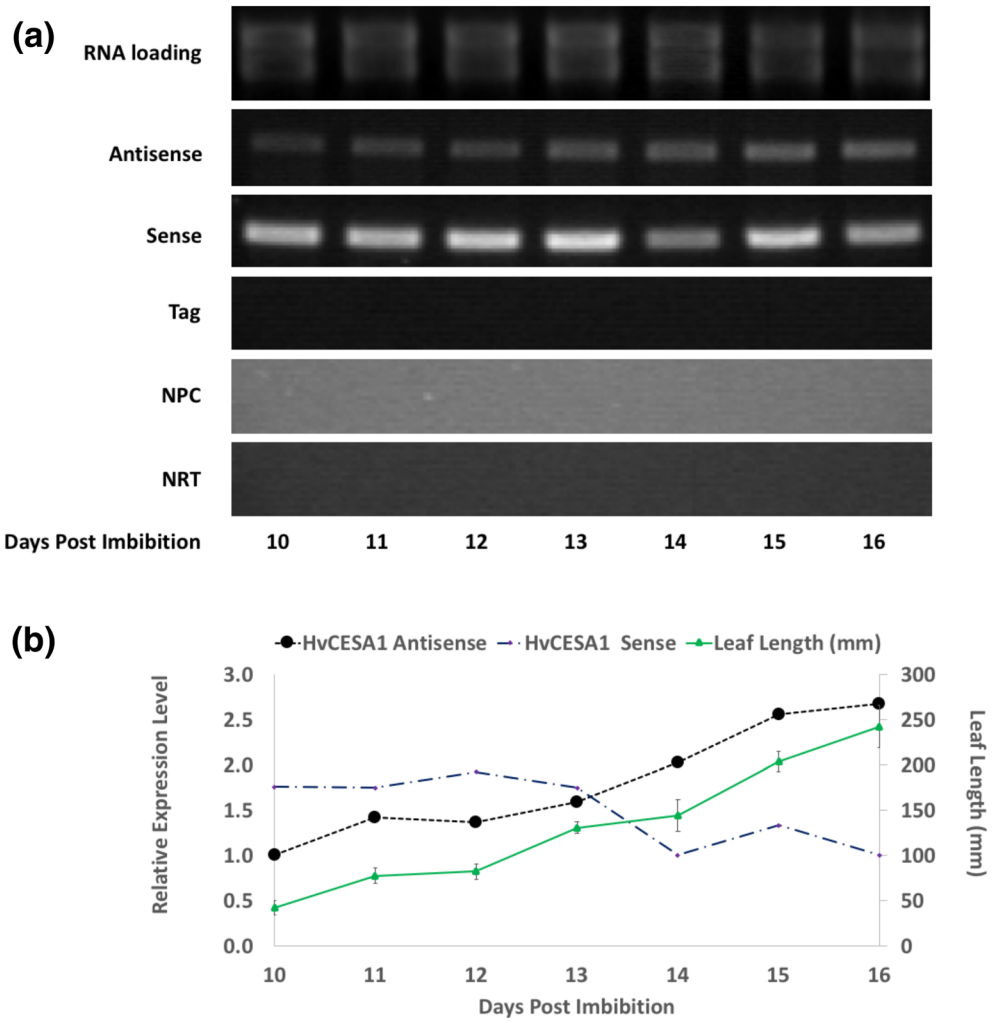

FIGURE 2 *HvCESA1* antisense expression during leaf development. (a) Semiquantitative, tagged SS-RT-PCR was performed to estimate changes in *HvCESA1* antisense transcripts over third leaf development (10–16 days post imbibition). First-strand cDNAs were prepared using either *HvA1-sense-tag1* GSP, *oligo dT*, or no primer at all (NPC). The *tag1* and *HvA1-antisense-1* primers were used for PCR amplification of the antisense from *HvA1-sense-tag1* GSP primed cDNA, NPC cDNA, and NRT cDNA samples. For sense amplification, *HvA1-sense* and *HvA1-antisense-1* were used with *oligo dT*-primed cDNAs. To rule out nonspecific amplification by the tag primer, *oligo dT*-primed cDNA was amplified using *tag1* and *HvA1-antisense-1* primers (Tag). PCR products were confirmed by DNA sequencing. (b) Gel densitometry was performed to estimate *HvCESA1* sense and antisense transcript abundances. Data were normalized to RNA loadings and expressed relative to the first day of collection (=1). Values are representative of multiple technical replicates ($n \geq 3$). Overlaid are the average leaf blade lengths (mm) ± SD ($n \geq 3$)

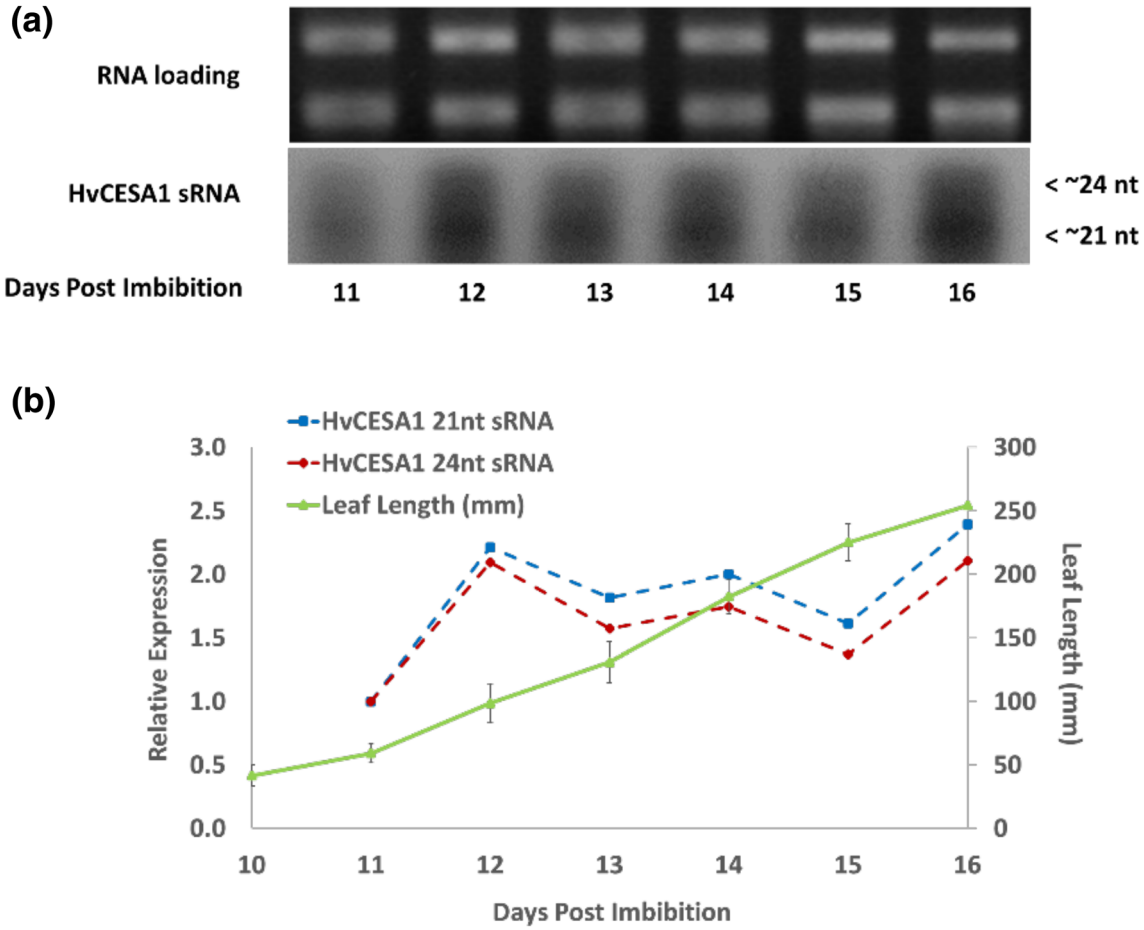

FIGURE 3 Detection of *HvCESA1* sRNAs by ribonuclease protection assay. (a) Ribonuclease protection assays were performed to detect *HvCESA1*-derived sRNAs across barley leaf development (11–16 dpi). A sense RNA probe was used to specifically protect *HvCESA1* antisense RNAs. *HvCESA1* sRNAs (~21–24 nt) were detected with size estimation by Decade Ladder (Ambion). (b) Densitometry was performed to evaluate the change in *HvCESA1* derived sRNA abundances. Data were normalized to RNA loadings and are expressed relative to the first day of collection (=1). Values are representative of multiple technical replicates ($n \geq 3$). Overlaid are the average leaf blade lengths (mm) ± SD ($n \geq 3$)

time, showing an overall increase in intensity from Days 11 to 16. The overall dynamic increase of the signal was by a factor of ~2.5 for bands in the 21- to 24-nt sRNA range (Figure 3), a trend similar to that of the antisense transcripts and to *HvCESA6* sRNAs previously observed (Held et al., 2008).

3.4 | Antisense transcripts are detected for multiple *BdCESAs*

RNA pools from rapidly growing *Brachypodium* third leaves were assayed using tagged, SS-RT-PCR for *BdCESA1* (Bradi2g34240), *BdCESA2* (Bradi1g04597), *BdCESA4* (Bradi2g49912), *BdCESA5* (Bradi1g02510), *BdCESA6* (Bradi1g53207), *BdCESA7* (Bradi3g28350), *BdCESA8* (Bradi1g54250), and *BdCESA9* (Bradi4g30540) antisense RNA transcripts (see Table S1 for primers). *BdCESA3* (Bradi1g29060) and *BdCESA11* (Bradi1g36740) were not examined, as they each are missing specific motifs characteristic of CESAs (Handakumbura et al., 2013).

PCR amplification of the antisense cDNAs yielded antisense amplicons for *BdCESA1*, *BdCESA4*, *BdCESA6*, and *BdCESA8*, with lengths of 1059, 1078, 1107, and 1009 base pairs respectively (Figure 4). Multiple sequence alignment of *BdCESA1* and *BdCESA8* with barley *CESA*s showed that antisense transcripts were detected for orthologous PCW *CESA*s (Figure S4). DNA sequencing of each antisense amplicon confirmed that all transcripts were complementary and exonic (no introns or indels) and that all four amplicons included the *tag2* primer from cDNA synthesis again indicating that SS-RT-PCR products could only have come from endogenous antisense RNA transcripts. Control sense amplicons of the same sizes were detected for each *BdCESAs* and showed much brighter bands despite being cycled under the same conditions (Figure 4). Similar to barley, the relative quantity of *BdCESA* antisense transcripts is low compared with the sense mRNAs. No antisense transcripts for the remaining *BdCESAs* were detected despite the presence of the control sense amplicons.

To evaluate the presence of *BdCESA* sRNAs, sRNASeq databases were queried. Third leaf tissue datasets were not available,

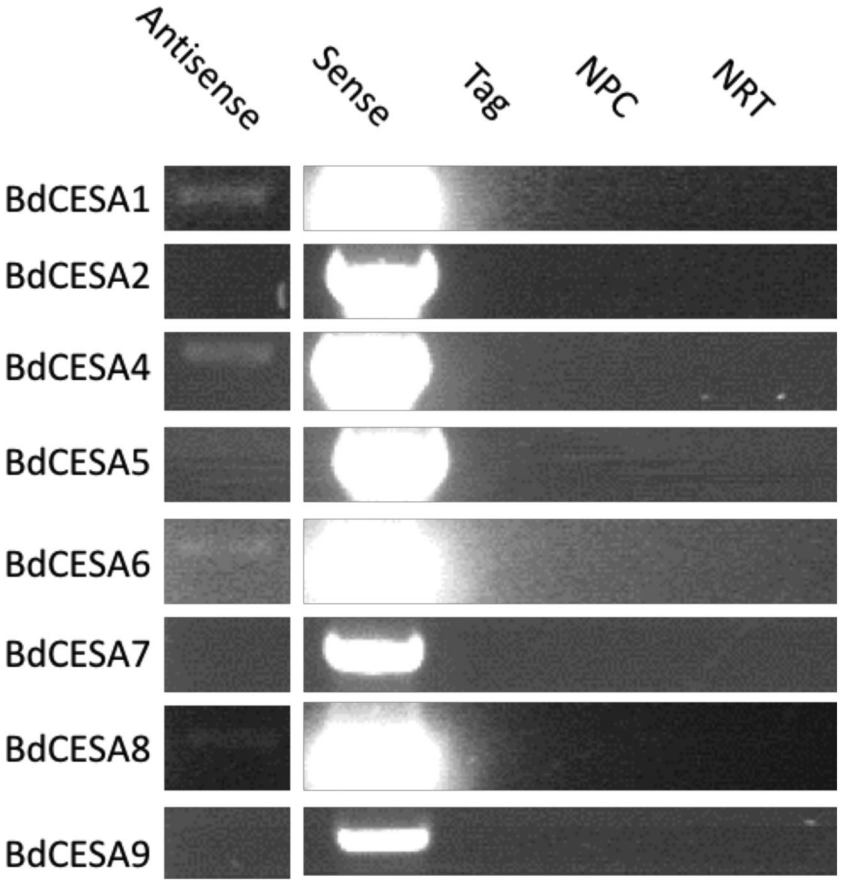

FIGURE 4 A survey of the *BdCESA* family for antisense transcripts. Tagged SS-RT-PCR was performed to detect antisense transcripts in *Brachypodium*. First-strand cDNA was prepared using either tagged, sense GSPs for *BdCESA1*, *BdCESA2*, *BdCESA4*–*BdCESA9* (Antisense; NRT), *oligo dT* (Sense; Tag), or no primer at all (NPC). Corresponding untagged antisense GSPs and the *tag2* primer were used for amplification of the Antisense, Tag, NPC, and NRT samples. For the sense amplification, untagged sense and antisense GSPs were used with *oligo dT*-primed cDNAs. PCR products were confirmed by DNA sequencing. See Table S1 for individual primer sequences

but similar tissue from 6-week-old leaf and stem was considered comparable. sRNASeq data showed sRNA populations that matched each of the *BdCESAs* (Table S2). *BdCESA1*, *BdCESA4*, and *BdCESA8*, which produce antisense transcripts (Figure 4), had elevated sRNA counts compared with the other *BdCESAs*, although *BdCESA6*, which also produced antisense transcripts, had a lower count (Table S2). *BdCESAs* not associated with antisense transcripts, generally had lower counts, with the lone exception of *BdCESA5*. The source of *BdCESA5*-derived sRNAs is unclear, but they are apparently generated independent of antisense transcripts. In general, *BdCESAs* that expressed antisense transcripts had elevated sRNA counts compared with those where antisense transcripts were not detected.

3.5 | Broad gene expression changes are observed by increasing *CESA* sRNAs

Previous work has shown silencing *HvCESA* genes by VIGS caused significant and direct reductions in *CESA* gene expression and also caused indirect reductions in other cell wall biosynthetic genes (Held et al., 2008). That is because VIGS of CESA genes stimulates the production of naturally abundant *CESA* sRNAs which have the potential to regulate cell wall biosynthesis in *trans*. The original study only examined a small subset of cell wall biosynthesis genes (Held et al., 2008); therefore, to more broadly examine the effects cause by over production of *HvCESA* sRNAs on cell wall gene networks, a microarray study of *CESA* VIGS-treated barley tissues was performed to compare the expression patterns of EV-treated samples and *HvCESA*-silenced (*HvCESA*-CR2) samples. The results from the microarray indicate that 91 probes showed significant values (adj. $p \leq .05$), with a distribution of annotated functions (Table 1). A total of 70 probes showed downregulated expression, whereas 21 probes showed upregulated expression (Table S3). One of the significantly downregulated genes was *HvCESA6*, a major target of the VIGS construct, confirming that silencing had indeed taken place (Held et al., 2008). Approximately 43 of the probes are specific to genes annotated for cell wall modification activity, cell wall structural proteins, GT activity, and glycosylhydrolase activity, suggesting the potential for broader regulatory control on cell wall gene networks via *trans*-acting effects (Allen et al., 2005; Vazquez et al., 2004). If *CESA*-derived sRNAs are used to help in the PCW to SCW transition, one might expect a concomitant drop in expression of genes annotated for PCW biosynthesis. Although there are outliers on both sides, many downregulated genes from this list are predicted to function in PCW biosynthesis (especially CW glycoproteins) and numerous upregulated genes are predicted to function in SCW biosynthesis (particularly lignification) as would be expected (Table S2). Altogether, these data support the potential for broader cell wall gene network regulation via *CESA*-derived sRNAs.

TABLE 1 Distribution of gene annotations affected by virus-induced gene silencing (VIGS) of cellulose synthases (CESAs) in barley

Protein function	Number
Cell wall-modifying proteins	16
Transcription factor	16
Cell wall structural proteins	12
Glycosyltransferase	8
Glycosylhydrolases	7
Stress response	6
Cytoskeleton	4
Lignin biosynthesis	4
Metabolism	4
Promoter binding	3
Transport	3
Ribosomal	3
Epigenetic modulator	2
Photosynthesis	2
Unknown	1

Note: Protein functional groupings (protein function) are listed for genes significantly upregulated or downregulated by CESA VIGS as determined by microarray analysis. Number corresponds to the number of individual genes affected for each protein function category. A complete list of significantly upregulated and downregulated genes and their functional groups is presented in Table S3.

4 | DISCUSSION

Plant cell walls are composed of complex networks of cellulose, various hemicelluloses, pectin, lignin, and glycoproteins. The amounts and proportions of these polymers vary greatly among plant cell types and across plant development. The ability of plant cells to generate wall types tailored for specific physiological roles and the ability to change wall polysaccharide biosynthesis upon various external stimuli (e.g., biotic/abiotic stresses) requires complex, multi-level regulatory control. Gene expression networks for polymer biosynthesis are coregulated to facilitate coordinated polymer deposition, but they also need to allow flexibility to selectively respond various stresses.

Here, we provide further evidence that posttranscriptional regulation is employed to selectively attenuate the expression of CESA genes and that this regulation has the potential to broadly affect the expression of other cell wall biosynthetic genes. We also show that *CESA* antisense transcripts were not restricted to barley, as they also occur in *Brachypodium*. The detection of *CESA* antisense transcripts in another plant species suggests that they might be common in all higher plants. Further, antisense transcripts were detected for several orthologous PCW *CESA*s (Figure S4) and therefore may represent an evolutionary conserved regulatory mechanism for limiting the expression of PCW *CESA*s.

Although much is known about activation and repression of SCW gene networks, relatively little is known regarding the repression of PCW networks (Li et al., 2016; Wang & Dixon, 2012). Between barley and *Brachypodium*, a total of seven antisense transcripts were detected. Five of these antisense transcripts are produced from PCW *CESA* genes, with the lone SCW exceptions being *HvCESA4* and *BdCESA4* for barley and *Brachypodium*, respectively (Figure S4). Although the significance of *HvCESA4* and *BdCESA4* SCW antisense transcripts is not fully understood at present, the data support our previous hypothesis that posttranscriptional sRNA regulation is important for the transition from the PCW to SCW gene network (Held et al., 2008).

Future work directed at detecting antisense transcripts in *Arabidopsis* is in progress. Moving this research into a more tractable genomic model will help shed light on the mechanisms of sRNA biogenesis. Using an inducible SCW system in *Arabidopsis* (Pesquet et al., 2010; Zuo et al., 2000) should help further clarify the roles of *CESA* sRNAs and their putative involvement in mediating the transition from PCW to SCW biogenesis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with the work described in this manuscript.

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