Specific cDNA fragments corresponding to putative cellulose synthase genes (CesA) were inserted into potato virus X vectors for functional analysis in Nicotiana benthamiana by using virus-induced gene silencing. Plants infected with one group of cDNAs had much shorter internode lengths, small leaves, and a “dwarf” phenotype. Consistent with a loss of cell wall cellulose, abnormally large and in many cases spherical cells ballooned from the undersurfaces of leaves, particularly in regions adjacent to vascular tissues. Linkage analyses of wall polysaccharides prepared from infected leaves revealed a 25% decrease in cellulose content. Transcript levels for at least one member of the CesA cellulose synthase gene family were lower in infected plants. The decrease in cellulose content in cell walls was offset by an increase in homogalacturonan, in which the degree of esterification of carboxyl groups decreased from ~50 to ~33%. The results suggest that feedback loops interconnect the cellular machinery controlling cellulose and pectin biosynthesis. On the basis of the phenotypic features of the infected plants, changes in wall composition, and the reduced abundance of CesA mRNA, we concluded that the cDNA fragments silenced one or more cellulose synthase genes.

INTRODUCTION

Primary cell walls of higher plants are dynamic, extracytoplasmic structures that typically are deposited in dividing and growing cells. They provide strength and flexibility for the plant as a whole but also allow intercellular exchange of water, nutrients, phytohormones, and other small molecules. After the cessation of cell growth, wall deposition may continue, but the thickened wall is referred to as a secondary wall. Wall composition varies widely across the plant kingdom and also between tissues and cell types within a particular species. In dicotyledons, cellulosic microfibrils, variously associated with xyloglucans, glucomannans, and heteroxylans, are embedded in a matrix consisting primarily of pectic polysaccharides. Additional networks of structural proteins or glycoproteins are also present. Numerous models have been developed to depict the possible interactions of cellulose and other molecular networks within the wall (Carpita and Gibeaut, 1993; McCann and Roberts, 1994; Carpita, 1996; Fry, 1996; Cosgrove, 1999).

Despite the fundamental importance of cell walls in plant growth and development, a complete description of enzymes involved in their biosynthesis has not been obtained. Biochemical approaches have been frustrated by difficulties associated with the purification of membrane-bound polysaccharide synthases, by the inherent instability of many such enzymes, by possible changes in specificity during extraction, by potential losses of critical cofactors, and by probable requirements for a multienzyme complex and ancillary proteins (Gibeaut and Carpita, 1994; Kawagoe and Delmer, 1997; Delmer, 1999). Partially purified synthase preparations invariably have contained several proteins, and although some glycosyl transferases have been isolated (Edwards et al., 1999; Perrin et al., 1999), there are few if any reports of the purification and biochemical characterization of a polysaccharide synthase that can be linked unequivocally with the synthesis of the backbone of a cell wall polysaccharide in higher plants.

An important breakthrough in understanding wall synthesis in plants was made by Pear et al. (1996), who identified candidate genes for cellulose synthases by noting mRNAs that were highly abundant during secondary cell wall cellulose synthesis in cotton fibers and comparing their sequences with the sequences of bacterial cellulose synthases. The cotton genes were designated CelA1 and CelA2; however, to avoid confusion with fungal glucan hydrolase genes, they are now referred to as CesA-1 and CesA-2 (Delmer, 1999). Correction of the radial swelling, low-cellulose phenotype (rsw1) and the irregular xylem phenotype (irx3) of Arabidopsis by complementation with Arabidopsis genes of similar sequence (Arioli et al., 1998; Taylor
et al., 1999) added weight to the original conclusion that the cotton (Gossypium hirsutum) GhCesA-1 and GhCesA-2 gene products were indeed cellulose synthases (Pear et al., 1996). However, surprisingly large numbers of CesA and cellulose synthase–like (Csl) genes, some of which show only 30% sequence identity with the cotton GhCesA genes, have subsequently been identified in Arabidopsis (Cutler and Somerville, 1997; see http://cellwall.stanford.edu/tree. html). These genes might encode several different cellulose synthase isoenzymes that participate in primary or secondary wall synthesis or are responsible for cellulose deposition in different tissues. Some might also encode synthases that are required for the synthesis of noncellulosic wall polysaccharides, such as xyloglucans, mannans, xylans, or galactans. Many of these are structurally similar to cellulose, and the fundamental mechanisms required for their biosynthesis are probably conserved.

It therefore remains difficult to identify with confidence cellulose synthase or other polysaccharide synthase genes on the basis of their sequence similarity with the cotton GhCesA genes. At this early stage in the characterization of genes involved in wall synthesis in higher plants, a gene knockout system, which could be used to evaluate rapidly the effects of candidate genes on the synthesis of cell wall polysaccharides, would prove valuable in assigning functions to the wide array of cellulose synthase–like genes. One such system is virus-induced gene silencing (VIGS), which can be used to examine gene function in Nicotiana spp. In this system, genes or gene fragments of interest are inserted into a modified potato virus X (PVX; potexviral group) cDNA, and RNA transcripts are prepared in vitro for infection of N. benthamiana seedlings (Ruiz et al., 1998). Post-transcriptional gene silencing results in less mRNA for endogenously expressing plant genes that have a sequence similarity of ~80% or more with the sequences carried by the virus. In addition, cDNAs of only 300 to 500 bp are sufficient to effect silencing (Ruiz et al., 1998). Thus, full-length cDNAs or genes are not required, which is particularly advantageous for analysis of cellulose synthase–like genes, the mRNAs of which may be as long as 3.5 kb (Delmer, 1999).

The precise mechanism of silencing has not been defined, but it may involve the formation of double-stranded RNA (Waterhouse et al., 1998), and it appears to mimic certain characteristics of the plant's natural defenses against viral attack (Ratcliff et al., 1997; Baulcombe, 1999). The major advantage of the VIGS system relates to the relative speed with which the role of a gene product can be defined, compared with the slower antisense or sense suppression approaches in transgenic plants. In addition, the system can be used to knock out potentially lethal genes, because young seedlings are allowed to become established before the gene is introduced by way of the viral RNA vector (Ruiz et al., 1998).

Here, we have used VIGS to show that one CesA homolog from Nicotiana spp silences an endogenous cellulose synthase gene and very probably encodes a cellulose synthase, whereas another Nicotiana spp cDNA, which is 80% identical with the first, produces a completely different phenotype. This emphasizes the need for development of discriminating systems for the functional analysis of polysaccharide synthase genes in wall synthesis in higher plants.

RESULTS

Isolation of cDNAs from N. tabacum

Three cDNA fragments corresponding to cellulose synthase (CesA) genes from N. tabacum were generated by polymerase chain reaction (PCR), and their positions in relation to the cellulose synthase gene GhCesA-1 from cotton (Pear et al., 1996) are shown in Figure 1. Although mRNAs encoding cellulose synthases from higher plants are up to 3.5 kb long (Pear et al., 1996), cDNA fragments of 300 to 500 bp are long enough to silence genes in the VIGS system (Ruíz et al., 1998). The cDNA fragment that is to be subjected to functional analysis in the VIGS system must therefore be given careful consideration. In the case of the cotton CesA gene, sequences encoding membrane-spanning regions, putative catalytic regions, and UDP-glucose binding regions of the enzyme have been identified, as have generally conserved and hypervariable regions (Pear et al., 1996; Delmer, 1999).

To isolate the N. tabacum homologs of the cotton GhCesA gene, PCR primers were designed so that the PCR products would start at the same point at their 3′ ends, just 3′ to the encoded QXXRW motif found in the homologous region (HR3) of all CesA genes. The positioning of the 5′ primers allowed the inclusion of different lengths of the adjacent plant-specific insertion regions (the conserved plant-specific CRP4 region and the hypervariable HVR2 regions) and, in the case of the longest cDNA, inclusion of the homologous region HR2 (Figure 1A; Pear et al., 1996; Delmer, 1999).

The three N. tabacum cDNAs so obtained were designated NtCesA-1a, NtCesA-1b, and NtCesA-2. They are 670, 377, and 485 bp long, respectively, and their nucleotide sequences have been lodged in the EMBL and GenBank databases with accession numbers AF233892 for NtCesA-1a and -1b and AF233893 for NtCesA-2. The nucleotide sequences of cDNAs NtCesA-1a and NtCesA-1b are identical where they overlap, and the cDNAs clearly represent fragments of the same gene. The 485-bp NtCesA-2 cDNA corresponds to a related but distinct gene; it shares 80% identity with the NtCesA-1a cDNA at the nucleotide level.

The sequences of the N. tabacum cDNAs are compared with the corresponding sequences of the cotton GhCesA-1 gene and the Arabidopsis AtCesA-1 (rsw1) gene in Figure 1B. Sequence alignments show that the NtCesA-1 and NtCesA-2 cDNAs fall into the CesA group of the CesA superfamily (C. Somerville and T. Richmond, http://cellwall.stanford.edu/tree.html).
**CesA Gene Family of Nicotiana spp**

High-stringency DNA gel blot analyses of genomic DNA from *N. tabacum* showed the presence of five or six fragments that hybridized with the NtCesA-1 and NtCesA-2 probes. Subsequent low-stringency screening of whole-plant cDNA libraries from *N. benthamiana* with a NtCesA-1a probe yielded cDNAs corresponding to four separate genes, which had nucleotide sequences ranging from 60 to 97% similar with respect to the NtCesA-1a sequence (data not shown). Each of the cDNA fragments represents a CesA gene (C. Somerville and T. Richmond, http://cellwall.stanford.edu/tree.html), and the cellulose synthases in Nicotiana spp are probably encoded by a gene family of at least five or six genes. The CesA-1a DNA sequences from the two Nicotiana spp were 97% identical.

**Phenotypes of Infected Plants**

Plants infected with either the PVX-NtCesA-1a or the PVX-NtCesA-1b construct were markedly shorter in stature than the control plants, which were infected with the PVX control construct (Figure 2A). The PVX-NtCesA-1a plants exhibited more severe symptoms than did the PVX-NtCesA-1b plants. The PVX-NtCesA-2 construct caused no obvious change in plant height (Figure 2A). Although the internode lengths of the PVX-NtCesA-1a and PVX-NtCesA-1b plants were dramatically shorter, the numbers of nodes on the main stems of all plants were approximately equal. The leaves from PVX-NtCesA-1a and PVX-NtCesA-1b plants were generally smaller than PVX control leaves (see Methods). Furthermore, chlorotic regions, a relatively crisp or crunchy texture, and the presence of numerous surface lumps, particularly on the underside or abaxial surfaces of the leaves, were evident (Figures 2B and 2C). Leaves from the PVX-NtCesA-2 plants were ~30% smaller than leaves of the PVX control plants and appeared somewhat softer in texture. The reasons for the different levels of severity of symptoms observed in the PVX-NtCesA-1a and PVX-NtCesA-1b plants (Figure 2) are unclear. In any event, the NtCesA-1a and NtCesA-1b plants eventually recovered, with normal leaf growth becoming apparent after ~4 months. These phenotypic characteristics were observed in at least five experiments that were conducted over 12 months at two locations.

**Anatomic Changes**

Scanning electron microscopy of the leaves from both the PVX-NtCesA-1a and the PVX-NtCesA-1b plants showed numerous clumps of expanded cells protruding from the abaxial surfaces of leaves, especially in regions adjacent to vascular tissues (Figure 3). In some cases, individual cells ballooned from the epidermis, and swollen cells could be detected in trichomes (Figures 3B, 3D, and 3F). These phenotypic effects were apparent not only on the abaxial surfaces of leaves (cf. Figures 3A and 3B) but also on the surfaces of stems (data not shown). Regions of apparently unperturbed epidermal surfaces were also present (Figure 3F). The leaf surfaces of the PVX control plants were generally smooth (Figures 3C and 3E). In PVX control plants, numerous intercellular airspaces were visible (Figure 3A), but these were much smaller in the PVX-NtCesA-1a and PVX-NtCesA-1b plants (Figure 3B).

**Transcriptional Activity of *N. benthamiana* CesA Genes**

In attempts to examine the effects of VIGS on the endogenous amounts of mRNA for the NbCesA-1 and NbCesA-2 genes, we isolated total RNA from young leaves, stems, roots, and flowers of uninfected control plants for RNA gel blot analysis. In all cases, hybridization signals were very low, and comparisons between signal intensities were difficult. More sensitive, gene-specific, reverse transcription (RT)-PCR confirmed that transcripts for both genes were present in low amounts in all tissues tested from *N. benthamiana* wild-type plants (data not shown). In the VIGS plants, RNA gel blot hybridization analyses indicated that accumulation of the recombinant viral transcript was high in all plants and in all tissues (data not shown). This demonstrated that the virus was spreading through the infected plants and that high amounts of NbCesA-1 and NbCesA-2 mRNA were being transcribed from the PVX vectors. However, the high amounts of viral transcripts, which carried the NtCesA inserts, precluded use of NbCesA cDNAs as probes to monitor endogenous NbCesA mRNA levels in infected plants. Similarly, oligonucleotides from the NtCesA-1 sequence could not be used in more sensitive RT-PCR procedures (Frohman et al., 1988; Burton et al., 1999) to determine whether VIGS treatment had decreased the abundance of endogenous NbCesA-1 or NbCesA-2 mRNA transcripts in leaf extracts.

**Examining the effects of VIGS on CesA-1 mRNA levels** therefore required a cDNA encoding a region outside the NtCesA-1a sequence. Using anchor-ligated PCR, we amplified a 399-bp cDNA from *N. benthamiana* leaf RNA preparations. The 399-bp fragment included a 99-bp sequence at its 3’ end that was 98% identical with the 99-bp sequence at the 5’ end of the NtCesA-1a cDNA and 100% identical at the amino acid sequence level. This overlap confirmed that the amplified 399-bp fragment corresponded exactly to the NtCesA-1a gene. The 399-bp cDNA fragment extended beyond the 5’ end of NtCesA-1a by 300 bp, and the sequence of this 5’ region of the cDNA fragment could therefore be used to design primers for RT-PCR (Figure 1A).

Quantitative RT-PCR was realized by adjusting the number of cycles during the PCR reaction until easily detectable but submaximal amounts of DNA were amplified. The amounts of amplified DNA were subsequently quantitated from a digital camera image of the gel. Care was taken to
Figure 1. Alignments of Isolated cDNAs against Plant CesA Genes.

(A) Positions and lengths of the three cDNAs (NtCesA-1a, NtCesA-1b, and NtCesA-2) from N. tabacum are shown in relation to the regions of plant CesA genes, as described by Delmer (1999). CRP denotes conserved plant-specific insertions, HVR denotes hypervariable plant-specific insertions, HR denotes homologous regions of all CesA genes, and NC denotes a region with no obvious conservation (Delmer, 1999). The three conserved aspartic acid residues (D) are indicated, together with the conserved QXXRW motif that is believed to be located at the catalytic site (Delmer, 1999). Note that the cDNAs start at different points at their 5’ ends but finish at the same point at their 3’ ends. The position of the fragment amplified during reverse transcription–PCR for estimation of mRNA abundance is also shown.

(B) Nucleotide sequence alignments of the N. tabacum cDNAs NtCesA-1a, NtCesA-1b, and NtCesA-2 with the corresponding sequences of CesA genes from Arabidopsis (AtCesA-1) (Arioli et al., 1998) and G. hirsutum (GhCesA-1) (Pear et al., 1996). The regions HR2, HVR2, CRP4, and HR3 are as described in (A). The primers used for nested, anchor-ligated PCR of the region immediately 5’ to the NtCesA-1a cDNA fragment are
ensure that any apparent decreases in CesA-1 mRNA abundance did not result simply from an overall decrease in nuclear transcription attributable to the PVX infection in VIGS plants. Control RT-PCR amplifications were therefore performed using primers for mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme of the glycolytic pathway, because GAPDH mRNA abundance should be a reasonable measure of the relative metabolic activity of the cells.

Reductions in RT-PCR products amplified with both GAPDH and NtCesA-1 primers were observed in the PVX-NtCesA-1a and PVX-NtCesA-1b plants (Figures 4A and 4B). For the PVX-NtCesA-1 plants, the RT-PCR experiments indicated that GAPDH mRNA abundance was ~66% of that observed in PVX control plants and that the NtCesA-1 mRNA decreased to ~43% of that observed in the PVX control plants (Table 1).

**Linkage Analysis and Major Polysaccharides in the Cell Walls**

The linkage compositions of wall polysaccharides from the PVX control plants and from plants infected with cDNA fragments for putative cellulose synthase genes are compared in Table 2. Several different wall preparations were analyzed during the course of this work, and although absolute values differed between experiments, the relative changes in individual polysaccharides were always similar. Methylation analyses showed a complex linkage composition that was consistent with walls classified in the type I group of Carpita and Gibeaut (1993), which includes an arabinoxylloglucan typical of solanaceous plants (Hayashi, 1989) (Table 2). The major changes associated with VIGS were decreases of ~22% in 4-Glc residues and larger increases in 4-GalA residues in walls from PVX-NtCesA-1a and PVX-NtCesA-1b plants (Table 2). Walls from the PVX-NtCesA-2 plants had compositions similar to those from control plants, although 4-Manp residues appear to be slightly increased in PVX-NtCesA-2 plants (Table 2).

The most abundant polysaccharide types in the wall preparations were subsequently deduced from the linkage compositions shown in Table 2, basing calculations on the totals for individual glycosyl residues that are characteristic of well-defined wall polysaccharides. These calculations embody certain assumptions about polysaccharide structures but are widely used as good indicators of the contents of specific polysaccharide types in plant cell walls (Bacic et al., 1988; Shea et al., 1989; Gorshkova et al., 1996; Nunan et al., 1998). As shown in Figure 5, the cellulose contents in walls from PVX-NtCesA-1a and PVX-NtCesA-1b plants were ~25% less than those in walls from PVX control and PVX-NtCesA-2 plants. The lower cellulose contents of these walls (Figure 5) reflected the decreased amounts of 4-Glcp in the linkage analyses (Table 2) but were offset by increases in homogalacturonan (Figure 5), which reflected the increased 4-GalpA amounts in linkage analyses (Table 2). The greater 4-Manp concentrations in PVX-NtCesA-2 plants (Table 2) suggest that walls of the latter might have a higher glucomannan content (Figure 5). Amounts of other polysaccharides in the wall preparations remained approximately similar, which suggests that the increase in homogalacturonan content in walls with lower cellulose content is not simply a result of generally increased carbon flow to other wall polysaccharides; if that were the case, then increases in all wall components would be expected.

To confirm the decreases in cellulose in walls of PVX-NtCesA-1a and PVX-NtCesA-1b plants indicated by methylation analyses (Table 2), we applied the acetetic acid/nitric acid procedure for estimating crystalline cellulose (Updegraff, 1969) to the same wall preparations. The crystalline cellulose content of walls from five individual PVX-NtCesA-1a plants, four PVX-NtCesA-1b plants, four PVX-NtCesA-2 plants, and five PVX control plants are summarized in Table 3. Averaging ~38%, the crystalline cellulose content of walls from PVX control and PVX-NtCesA-2 plants (Table 3) were only slightly less than the values of 41 to 43% for 4-Glcp residues estimated by methylation analysis (Table 2). However, the average crystalline cellulose content of walls from PVX-NtCesA-1a and PVX-NtCesA-1b plants were ~16 and 23% lower than those of PVX control plants (Table 3).

**Degree of Esterification of Pectic Polysaccharides**

From the results shown in Table 2, the amounts of esterified t-GalpA and 4-GalpA residues can be calculated to decrease from ~50% in PVX control plants to ~33% in walls from the PVX-NtCesA-1a and PVX-NtCesA-1b plants. Thus, the degree of esterification of pectic polysaccharides decreased by ~35% in the PVX-NtCesA-1 plants.

To further examine the lesser esterification of pectic polysaccharides in walls of the PVX-NtCesA-1a plants as well as
to locate the Ca\(^{2+}\)-pectate, we stained leaf sections of those plants with NiCl\(_2\)/Na\(_2\)S, which binds to deesterified polygalacturonates, presumably in regions of Ca\(^{2+}\) cross-linking (Varner and Taylor, 1989). Low amounts of staining were detected in PVX control (data not shown) and PVX–NtCesA-2 leaves (Figure 6A). Increased staining intensity indicated that increased amounts of polygalacturonate material were associated with walls of the PVX–NtCesA-1a and PVX–
NtCesA-1b plants, particularly where cells ballooned from the lower epidermis of the leaves (Figures 6B, 6C, and 6D).

Protein and Amino Acid Composition

Although some variation in protein content (10 to 15% [w/w]) was observed between cell walls from the PVX control and the PVX–NtCesA plants, the differences were small. Amino acid analyses showed that glycine (~10% [mol/mol]), alanine (8% [mol/mol]), and lysine (10% [mol/mol]) were the most abundant amino acids; the wall-associated proteins also contained ~6% (mol/mol) proline and 2% (mol/mol) hydroxyproline residues. No significant differences were observed in amino acid compositions of wall-associated proteins.

DISCUSSION

The functions of three cDNAs corresponding to putative cellulose synthase genes from Nicotiana spp were analyzed with the VIGS system. In this system, endogenous plant genes can be silenced by high expression of homologous DNA fragments carried in the genome of the infecting virus (Kumagai et al., 1995; Kjemtrup et al., 1998; Ruiz et al., 1998). Thus, if any of the cDNAs represented part of a cellulose synthase gene, then endogenous expression of that gene would be diminished and the cellulose content in the cell walls would be expected to decrease.

Although the sequences of NtCesA-1 and NtCesA-2 were 80% identical where they overlapped, infection of N. benthamiana seedlings with RNA carrying the NtCesA-1 or NtCesA-2 sequences produced dramatically different effects (Figure 2). Thus, growth of the PVX–NtCesA-1a and PVX–NtCesA-1b plants was severely inhibited after infection compared with those plants infected with PVX–NtCesA-2 and the PVX control. The stunted growth patterns of plants infected with PVX–NtCesA-1a and PVX–NtCesA-1b were highly reproducible and were characterized not only by much shorter internode lengths but also by the presence of smaller leaves, which were both “lumpy” in form (Figures 2B and 2C) and “crisp” in texture.

Examination of the leaves from the PVX–NtCesA-1a and PVX–NtCesA-1b plants by light and electron microscopy showed extensive disruption of the surfaces of infected leaves. (A) Shown left to right are a PVX control plant, a PVX–NtCesA-2 plant, a PVX–NtCesA-1b plant, and a PVX–NtCesA-1a plant. The severe stunting of the PVX–NtCesA-1 plants is evident.

(B) Shown left to right are the abaxial surfaces of fully expanded leaves from PVX control, PVX–NtCesA-1b, and PVX–NtCesA-1a plants. The interveinal regions of PVX control leaves are relatively smooth.

(C) Shown is the abaxial surface of the PVX–NtCesA-1b leaf. A pronounced “lumpy” appearance is evident. The texture of leaves from PVX–NtCesA-1 plants was very “crisp” compared with controls.

Figure 2. Appearance of Plants Infected with PVX Transcripts.
Figure 3. Scanning Electron Micrographs of Leaves from PVX-Infected Plants.

(A) A PVX control leaf, showing the relatively smooth epidermal surface and trichomes of the adaxial leaf surface. Numerous airspaces in the spongy and palisade mesophyll are visible in the cross-section of the leaf.

(B) A leaf section from a PVX-NtCesA-1b plant, showing the abaxial surface distortions and cells ballooning out from the epidermis. Swollen cells can also be observed on trichomes. The mesophyll appears to have much smaller airspaces.

(C) and (D) Abaxial surface views of PVX control and PVX-NtCesA-1b leaves, respectively.

(E) and (F) Higher magnification views of PVX control and PVX-NtCesA-1b leaves, respectively.

Bars in (A) and (B) = 200 μm; bars in (C) and (D) = 1 mm; bars in (E) and (F) = 100 μm.
leaves, particularly on their undersurfaces and in the vicinity of vascular bundles. The latter effect presumably reflected the spread of infection across the leaves as the virus moved through the vascular system (Santa Cruz et al., 1998). Groups of cells protruded from the normally smooth epidermis (Figure 3), and in some cases, individual cells ballooned from epidermal surfaces. Compared with control plants, cells in the spongy and palisade mesophyll were tightly packed (Figure 3). The swelling of cells in plants infected with PVX–NtCesA-1a and PVX–NtCesA-1b was consistent with a temporary loss of cell wall strength or rigidity that allowed abnormal cell expansion, which might be expected if virus-induced silencing of cellulose synthase genes was occurring. Indeed, the VIGS plants observed here shared common symptoms with the radial swelling (rsw1) mutants of Arabidopsis (Arioli et al., 1998), in which cellulose synthesis is disrupted by a point mutation in a cellulose synthase gene.

To further investigate the possibility that the swelling of cells in plants infected with PVX–NtCesA-1a or PVX–NtCesA-1b was attributable to a decrease in cellulose content, we isolated cell walls from infected leaves for analysis. The cellulose content of walls isolated from PVX–NtCesA-1a and PVX–NtCesA-1b leaves was ~25% less than that in walls of the PVX control plants, as measured by methylation analysis (Figure 5). The lower cellulose content of walls from PVX–NtCesA-1a and PVX–NtCesA-1b plants was confirmed by the acetic acid/nitric acid procedure for estimating crystalline cellulose (Table 3).

The loss of cellulose in walls of the PVX–NtCesA-1a and PVX–NtCesA-1b plants was accompanied by a 45% increase in homogalacturonan (Figure 5). Furthermore, the degree of esterification of pectic polysaccharides decreased from ~50% in walls of control plants to ~33% in walls of plants infected with PVX–NtCesA-1a or PVX–NtCesA-1b. That pectin esterification was less in the infected plants than in the control plants was confirmed by NiCl2/Na2S staining of tissue sections (Figure 6). Cell walls were also isolated by microdissection from the characteristic lumps that were observed on leaves of the PVX–NtCesA-1a and PVX–NtCesA-1b plants, although yields of these walls were low and replication of experiments was difficult. Nevertheless, their cellulose content was reduced by 50 to 75% compared with walls from the PVX control plants (data not shown).

The increase in pectin content of walls, together with much less esterification of the pectic polysaccharides (Figure 5), suggested that plants infected with the PVX–NtCesA-1a and PVX–NtCesA-1b constructs specifically compensated for the decreased cellulose content of walls through the deposition of additional pectic polysaccharides. Furthermore, the presence of longer sections of pectic polysaccharides containing deesterified galacturonosyl residues would allow the formation of more extensive Ca2+-bridged junction zones (Rees, 1977; Powell et al., 1982; Brett and Waldron, 1990). These junction zones could strengthen the pectin network in walls weakened by the loss of cellulose. A comparison can be made here with cell walls of plants that have been adapted to grow in the presence of high concentrations of 2,6-dichlorobenzonitrile (DCB), a herbicide that specifically inhibits cellulose synthesis (Shedletzky et al., 1992). Walls of DCB-adapted cells have not only low cellulose contents but also greatly increased proportions of pectic polysaccharides with less than usual esterification (Shedletzky et al., 1992; Wells et al., 1994). Whether or not the lower cellulose contents of walls from the Arabidopsis mutants rsw1 and irx3 are also compensated by increases in deesterified pectic polysaccharides has not been reported (Arioli et al., 1998; Taylor et al., 1999).

Associated with the reduction in cellulose content of the cell walls in the PVX–NtCesA-1a plants was an apparent

Table 1. Estimation by RT-PCR of mRNA Abundance in Leaves of PVX Control and PVX–NtCesA-1 Plants

<table>
<thead>
<tr>
<th>mRNA</th>
<th>PVX Control</th>
<th>PVX–NtCesA-1</th>
<th>Percentage of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>141 ± 4</td>
<td>93 ± 15</td>
<td>66</td>
</tr>
<tr>
<td>NbCesA-1</td>
<td>75 ± 3</td>
<td>33 ± 11</td>
<td>43</td>
</tr>
</tbody>
</table>

*Values indicate averages of relative intensities of bands seen in Figure 4. Standard errors are shown.*
decrease in NtCesA-1 mRNA in the infected leaves (Figure 4 and Table 1). Although these RT-PCR results must be interpreted cautiously (Table 1), they provide prima facie evidence that infection with the PVX–NtCesA-1 constructs results in a marked decrease in CesA-1 mRNA in PVX–NtCesA-1 plants and that the decrease is greater than that observed for mRNA encoding the cellular “housekeeping” enzyme, GAPDH (Figure 4 and Table 1).

On the basis of these results, we conclude that the NtCesA-1a and NtCesA-1b cDNAs from N. tabacum have silenced a
Figure 5. Polysaccharide Compositions of Cell Walls Isolated from Leaves of PVX-Infected Plants.

The major differences in polysaccharide compositions are the decreased cellulose and increased homogalacturonan contents of walls from the PVX-NtCesA-1a and PVX-NtCesA-1b plants. A small increase in galactoglucomannan is apparent in walls from the PVX-NtCesA-2 leaves. Specific polysaccharide content was determined as described in Methods from data in Table 2. Error bars show standard errors. RGI, rhamnogalacturonan 1.

Although we can conclude that the NtCesA-1 cDNA corresponds to a cellulose synthase gene, at this stage the data do not allow us to rule out a role for NtCesA-2 in cellulose synthesis. Five or six genes in Nicotiana spp have a high degree of sequence similarity with the NtCesA-1 gene. The NtCesA-2 gene could indeed encode a cellulose synthase, but if the NtCesA-2 isoenzyme expressed in leaf tissue at very low amounts at the time of VIGS was only one of several cellulose synthases involved in wall synthesis, no obvious phenotypic effect might be observed. This raises another point about the use of VIGS and the interpretation of VIGS data with genes that are members of multigene families. The optimal strategy for VIGS as a means of assigning function to a particular member of a gene family would have two stages. First, use of cDNA fragments corresponding to the most highly conserved domains of the gene would reveal the collective functions of the multigene family. Having established a role for the gene family in the trait of interest, VIGS vectors based on the least-conserved regions of the gene could subsequently be used to assign a function to the individual gene or to subsets of the gene family.

The phenotypic differences observed here in plants infected with the closely related (80% sequence identity) PVX--NtCesA-1 and PVX--NtCesA-2 constructs serve to emphasize that sequence comparisons alone do not allow an unequivocal identification of cellulose synthase genes. Neither does it allow identification of genes encoding other polysaccharide synthases that participate in wall synthesis in higher plants. This point can be illustrated by comparing nucleotide sequence identities of the cellulose synthase genes for which proof-of-function is available. The N. tabacum NtCesA-1a sequence used here is 69 and 72% identical with corresponding regions of the CesA cellulose synthase genes of cotton (GhCesA; Pear et al., 1996) and Arabidopsis (AtCesA-1; Arioli et al., 1998), respectively.

These values are much less than the 80% similarity with the N. tabacum NtCesA-2 sequence, which, although it did not appear to silence cellulose synthesis in the current VIGS experiments, still might participate in cellulose synthesis in secondary walls or in other tissues at other times.

More likely, proof-of-function for the many Csi genes that have been identified in plants (Cutler and Somerville, 1997) will have to be demonstrated on an individual basis. The VIGS system offers a relatively quick and simple method for screening large numbers of gene fragments for biological function, especially when gross morphological changes are associated with gene silencing (Figures 2 and 3).

Another question that should be addressed relates to the relative reduction of cellulose content in plants infected with the PVX--NtCesA-1a and PVX--NtCesA-1b constructs. Why is cellulose content of the walls reduced by only 25%, and why is NbCesA mRNA apparently reduced by a similar amount? First, preexisting walls would contain normal amounts of cellulose; however, if cellular activity, including wall synthesis, were stopped very rapidly after infection, then large decreases in final cellulose content of the walls would not be expected. An additional explanation is that the magnitude of the effect on wall composition depends on the developmental age of the cell at the time of its infection. The virus is transported through the phloem from the point of in-
to be monitored in situ would allow changes in specific components of the cell wall. Cells will require the availability of antibodies or stains that remain to be demonstrated. Answers to the specific questions regarding viral infection patterns and VIGS in infected higher plants can be tailored to changing demands during normal growth and development.

**METHODS**

**Isolation of cDNAs**

Total RNA was extracted from tobacco (Nicotiana tabacum) suspension-cultured cells at mid-log phase by using the phenol-quandine isothiocyanate procedure (Trizol; Gibco BRL, Gaithersburg, MD), and single-stranded cDNA was prepared from 2 μg of total RNA with Superscript II reverse transcriptase (Gibco BRL) and the TRACE primer (Frohman et al., 1988). Fragments of putative glucan synthase cDNAs from tobacco, designated NtCesA-1a, NtCesA-1b, and NtCesA-2, were amplified by polymerase chain reaction (PCR) from 2-μL aliquots of the cDNA reaction mixture with primer combinations based on cotton GhCesA sequences (GhCesA-1, GenBank accession number US8283; GhCesA-2, GenBank accession number US8284; Pear et al., 1996) and sequences of cDNAs encoding putative glucan synthases from barley (R.A. Burton and G.B. Fincher, unpublished data), as follows: NtCesA-1a, 5’ primer 5’-CTTGATGGCATCAGGGCCAG-3’ and 3’ primer 5’-CATAGCCATACCAGGGGAC-3’; NtCesA-1b, 5’ primer 5’-GAGCTTAGAGAAGAGATTTGG-3’ and 3’ primer 5’-CATAGCCATACCAGGGGAC-3’; and NtCesA-2, 5’ primer 5’-GTCAGACAGTGATTATG-3’ and NtCesA-2, 3’ primer 5’-CATAGCCATACCAGGGGAC-3’.

The PCR program involved 35 cycles at 94°C for 40 sec, 49°C for 40 sec, and 72°C for 90 sec in a reaction mixture containing a standard PCR buffer with 200 μM deoxynucleotide triphosphates and 10% DMSO. Products were cloned into the EcoRV site of pBlueScript SK+ (Stratagene, La Jolla, CA), and their identity was verified by nucleotide sequence analysis (Sanger et al., 1977).

**DNA Constructs and Seeding Infection**

The cDNAs were excised from pBlueScript SK+ and ligated into the potato virus X (PVX) vector pP2C2S, as described previously.
show enlarged epidermal cells enriched in calcium pectate, which is
Leaves from the PVX control plants stain in a similar fashion. Staining for calcium pectate. A faint outline of epidermal cells is visible.

An increase in staining intensity from brown to black is indicative of increasing concentrations of calcium pectate. Bar in Figure 6A = 100 μm.

Figure 6. Staining Abaxial Leaf Surfaces for Calcium Pectate by Using NiCl2/Na2S.

(A) Surface view of a leaf from a PVX–NtCesA-2 plant that shows little staining for calcium pectate. A faint outline of epidermal cells is visible. Leaves from the PVX control plants stain in a similar fashion.

(B) to (D) Lumps on leaves of plants infected with PVX–NtCesA-1a show enlarged epidermal cells enriched in calcium pectate, which is concentrated in the walls around the swollen cells. The enlarged cells of PVX–NtCesA-1a leaves show various degrees of staining, presumably because the onset of gene silencing occurs at different stages of development. The surface lumps are those seen in Figures 3B and 3D. An increase in staining intensity from brown to black is indicative of increasing concentrations of calcium pectate. Bar in (D) = 100 μm for (A) to (D).

(Baulcombe et al., 1995). The constructs were all in the sense orientation and were designated PVX–NtCesA-1a, PVX–NtCesA-1b, and PVX–NtCesA-2. The control constructs consisted of either a near-full-length cDNA encoding the green fluorescent protein (Haseloff et al., 1997), designated PVX-GFP (Ruiz et al., 1998), or a cDNA encoding β-glucuronidase (Jefferson et al., 1987), designated PVX-GUS (Chapman et al., 1992). The latter constructs are hereafter referred to as PVX control.

Infectious RNA molecules were produced by in vitro transcription of the DNA constructs, as described previously (Chapman et al., 1992). The infectious RNA was rubbed onto the second leaves of 4- to 5-week-old N. benthamiana seedlings in the presence of a small amount of carborundum powder (Ruiz et al., 1998). Plants were grown in a greenhouse at 24°C under a 16-hr photoperiod for an additional 3 to 12 weeks.

Transcript Levels and Reverse Transcription–PCR

To isolate an NbCesA-1 cDNA immediately upstream from the NtCesA-1a sequence, we extracted total RNA from N. benthamiana leaves by the phenol–guanidine isothiocyanate procedure already described. A gene-specific antisense oligonucleotide (5'-CTTGATCCACCGAAGCAGGAAG-3') was used to generate a single-stranded cDNA product with Superscript II reverse transcriptase. After a 35-μl oligonucleotide anchor was ligated to the 5' ends of the single-stranded CDNs with T4 RNA ligase (New England Biolabs, Beverly, MA), double-stranded CDNs were obtained by nested PCR with a second NbCesA-1-specific oligonucleotide as the 3' primer (5'-CTTATGCTTTGGGCTTAAAGGAGG-3') and an oligonucleotide complementary to the anchor as the 5' primer. The longest cDNA products were ligated into the pGEM T-Easy vector system (Promega) and sequenced by using a DNA sequencer (model 373; Applied Biosystems, Foster City, CA). The sequence of a 399-bp NbCesA-1 cDNA fragment thus obtained has been entered in the EMBL and GenBank databases under the accession number AF233891.

For reverse transcription (RT)-PCR, total RNA was extracted from N. benthamiana leaves, as described above. The quality and concentration of RNA preparations were accurately determined with a UV-visible spectrophotometer (model Cary 50BIO; Varian, Walnut Creek, CA) and using the Cary WinUV RNA–DNA estimation software. Samples of total RNA (1 μg) were used in a first-strand cDNA synthesis reaction with the reagents supplied in the Thermoscript RT-PCR System (Gibco BRL), according to the manufacturer’s instructions. The RNA samples were primed with oligo(dT)20, and the reverse transcription reaction was performed at 52°C for 1 hr in a final volume of 50 μL. Samples from each reaction (2 μL) were used in a 50-μL PCR mixture containing the single-stranded cDNA template, Taq polymerase buffer (Gibco BRL), 0.2 mM dNTPs, 1.5 units of Taq polymerase (Gibco BRL), and each glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide at 1 μM or each N. benthamiana oligonucleotide at 2 μM. Amplification of the extended NbCesA-1 sequence was performed for 30 cycles with the oligonucleotides 5'-TGCCATGCTGACCTGGTGTGCAGTG-3' and 5'-TACGGGTGGCATATGCCATCCC-3' at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec to yield a 211-bp product. This product was purified from agarose gels by using the BRESAcleave DNA purification kit (Geneworks, Adelaide, Australia) and sequenced on the DNA sequencer to confirm its identity.

GAPDH cDNA was amplified by using the two oligonucleotides GAPDH5 (5'-CAGGAACCTCAGGATATCCC-3') and GAPDH3 (5'-
respectively). The PCR cycles each consisted of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min. The 550-bp product was purified from an agarose gel and sequenced to confirm its identity.

To quantitate PCR band intensities, we scanned digital camera images of the gels and assigned individual bands a relative intensity value by using Gel-Pro Analyzer version 2.0 software (Media Cybernetics, Atlanta, GA). Preliminary experiments in which PCR amplifications were performed for 27, 30, 32, and 35 cycles showed that the intensities of these bands remained well below the maximal intensities, which were observed after 35 cycles.

Microscopy

For scanning electron microscopy, leaf tissues were mounted on aluminum stubs with O.C.T. compound (Agar Scientific, Stansted, UK) and frozen in liquid N₂ slush. Tissues were fractured with a scalpel blade, sputter-coated with platinum, and observed with a scanning electron microscope (model XL30 FEG; Philips, FEI Co., Eindhoven, The Netherlands) fitted with a cryostage (CT1500 HF; Oxford Instruments, Abingdon, Oxford, UK).

To locate polygalacturonate in cell walls, we fixed sections of leaves in 80% ethanol, rinsed them in water, stained them with 15 μL of 0.1% toluidine blue (Sigma; type 1-A) in 0.5 mL of 0.5 M sodium acetate buffer, pH 6.5, containing 30 mM ascorbic acid, were added to the samples. The samples were chilled to 4°C, washed three times with water, and dried. Although water-soluble components of the walls would have been lost during isolation, we expected that little if any cellulose, pectin, or other wall polysaccharides would be lost under these conditions.

Preparation of Cell Walls

Samples were taken 22 to 112 days after inoculation. Counting upward from the inoculated leaf, we harvested leaves (3 to 6 g fresh weight) from positions 4 to 6 for PVX control and PVX-NtCesA-2 plants and from positions 2 to 13 for PVX-NtCesA-1 plants. Midrib veins were removed. Three volumes of ice-cold 50 mM Mes buffer, pH 5.5, containing 30 mM ascorbic acid, were added to the samples. After grinding the sample with a mortar and pestle, we added Triton X-100 to 1.5% (v/v), and the “crude cell wall” preparation was recovered by centrifugation. Subsequent washing procedures were designed to remove cytoplasmic components from the cell wall preparation; each wash involved vigorous mixing, recovery of the walls by centrifugation, and filtration of supernatants through glass fiber filters (GF/A; Whatman, Maidstone, UK). All procedures were performed at room temperature. The crude wall preparation was extracted twice with 100 mM NaCl containing 1.5% (v/v) Triton X-100, three times with 100 mM NaCl, twice with H₂O, three times with methanol, and twice more with H₂O. Attempts to dissolve the starch in the wall preparations by stirring overnight in DMSO were ineffective. The DMSO-treated material was washed twice in H₂O and re-suspended in 20 mL of H₂O, and 100 units of porcine α-amylase (Sigma; type 1-A) in 0.5 mL of 0.5 M sodium acetate buffer, pH 6.5, were added. The samples were kept at 22°C for 3 hr with constant shaking. Starch degradation was monitored by iodine/iodide staining. After this treatment, the preparations still contained adherent cytoplasmic material, which was probably proteinaceous in nature. Preparations were therefore extracted twice with 1.5% sodium dodecyl sulfate at 60°C, washed three times in H₂O, and freeze-dried. Although water-soluble components of the walls would have been lost during isolation, we expected that little if any cellulose, pectin, or other wall polysaccharides would be lost under these conditions.

Carboxyl Reduction of Uronic Acids

Wall preparations were subjected to the carboxyl reduction method for analysis of uronic acids and their esters, essentially as described by Kim and Carpita (1992). Approximately 6 mg of wall material was suspended in 5 mL of 0.5 M imidazole–HCl buffer, pH 7.0. Suspensions were chilled, 1.0 mL of 100 mg/mL NaBD₄ (NaB[H]₄) was added three times at 20-min intervals, and the mixture was left overnight at room temperature. Glacial acetic acid (0.5 mL) was added to destroy excess reductant. The suspensions were dialyzed against H₂O and freeze-dried. The dried preparations were re-suspended in 1.0 mL of H₂O, to which 0.2 mL of 0.2 M Mes buffer, pH 4.7, and 0.4 mL of 500 mg/mL 1-cyclohexyl-2-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate was added for 3 hr at room temperature. The samples were chilled to 4°C, 1.0 mL of 4 M imidazole buffer, pH 7.0, was added, and the samples were divided into two equal parts. For the second reduction, 1.0 mL of either NaBH₄ or NaBD₄, both at 70 mg/mL, was added, and samples were incubated for 16 hr at 4°C. Excess reductant was destroyed with 0.5 mL of acetic acid, and the samples were dialyzed and freeze-dried.

Methylation Analysis of Cell Walls

Wall preparations were methylated by the NaOH method of Ciucanu and Kerek (1984), as described by McConville et al. (1990). Carboxyl-reduced wall material was suspended in 100 to 200 μL of DMSO by using sonication, and 250 μL of a slurry of freshly prepared DMSO-NaOH, made by vigorously mixing three NaOH pellets (~1 g) per milliliter of DMSO, was added. Two 50-μL portions of methyl iodide were added 20 min apart, followed by a third addition of 100 μL. After a further 20 min, the reaction mixture was quenched with 4 mL of water. The methylated derivatives were partitioned into 1 mL of chloroform and washed three times with water before drying under a stream of nitrogen. All samples were methylated twice. Methylated polysaccharides were hydrolyzed at 120°C for 2 hr in 1.0 mL of 2.5 M trifluoroacetic acid containing 100 nmol of myo-inositol. Samples were dried under a stream of nitrogen. The partially methylated monosaccharides were dissolved in 50 μL of 2 M NH₄OH and reduced with 50 μL of 30 mg/mL NaBD₄ in 2 M NH₄OH. After 2.5 hr, excess reductant was destroyed with 20 μL of glacial acetic acid. The samples were dried under a stream of nitrogen, redissolved in 5% (v/v) acetic acid in methanol and evaporated to dryness (twice), redissolved in methanol, and dried.

Acetylation of the partially methylated alditols was performed in 0.5 mL of acetic anhydride at 100°C for 2.5 hr. The anhydride was destroyed with 2.0 mL of H₂O, and the partially methylated alditol acetate derivatives were partitioned into 1.0 mL of dichloromethane, washed three times with water, and dried.

The derivatives were separated and analyzed in a gas chromatograph (model 6890; Hewlett-Packard) linked to a mass spectrometer (model 5973), using a 25 m × 0.22 mm i.d. BPX70 column (SGE, Melbourne, Australia). Identification of the derivatives and deduction
of the glycosidic linkages were based on published mass spectra (Carpita and Shea, 1989) and the elution order in relation to standards. The degree of esterification of uronic acid groups was calculated by the relative proportions of diagnostic fragments in the NaBH₄- and NaBD₄-reduced samples (mass-to-charge ratios [m/z] of 205 and 207 for terminal nonreducing hexosyl derivatives; m/z 233 and 235 for 4- and 2,4-linked hexose derivatives, respectively; and m/z 305 and 307 for 3,4-hexose derivatives). The mole percentage of composition of the samples was calculated by normalizing the total ion chromatogram peak areas to the molecular masses of the corresponding derivatives. Methylation analyses were performed in duplicate, and the results were averaged.

**Calculation of the Polysaccharide Composition of Cell Walls**

To estimate polysaccharide compositions of the samples, we added together the proportions of selected partially methylated alditol acetate derivatives. These calculations were based on the structures of well-characterized wall polysaccharides from N. plumbaginifolia, as described by Sims and Bacic (1995), and from information in Carpita and Gibeaut (1993), as follows:

1. **Rhamnogalacturonan I (RG I)**: This compound consists of a repeating motif with 4-GalA; the branched residues are not included.

2. **Galacturonan**: Three 4-Glc units for every two 4,6-Glc branched units, each 2-Xyl branch terminates with t-Araf, and the remaining 4,6-Glc units are branched with t-Xyl.

3. **Galactoglucomannan**: Two 4-Man + 4,6-Man + 4,6-Man; this assumes a repeating motif of the mannosyl units with 4-Glc, and branches terminate with t-Gal; traces of 2-Gal indicate some disaccharide branches.

4. **Cellulose**: 4-Glc − 1.5 (4,6-Glc) − (4-Man + 4,6-Man); the 4-Glc not assigned to arabinogalactan or galactoglucomannan is assigned to cellulose.

5. **Arabinogalactan**: 6-Gal + 3-Gal; the traces of 3,6-Gal indicate the presence of relatively unbranched type II arabinogalactan.

6. **Xylan**: 4-Xyl + 2 (2,4-Xyl); this assumes that the branched residues are t-Gal.

**Determination of Crystalline Cellulose**

Between 10 and 30 mg of wall material from leaf preparations was placed in 10-ml plastic screw-cap vials with 4.0 ml of acetic acid:nitric acid:water (8:1:2 v/v) reagent (Updegraff, 1969). The vials were placed in a boiling water bath for 2.5 hr and regularly mixed to break up clumps of wall material. After acid digestion of the wall components, the insoluble crystalline cellulose was washed five times with water and then freeze-dried.

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