Functional Analysis of β-Glucan Synthase Genes Using Virus-Induced Gene Silencing (VIGS).

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Introduction

Despite the importance of cell walls in the growth and development of higher plants, a complete description of enzymes involved in their biosynthesis has not been obtained. Few, if any, cell wall polysaccharide synthases have been purified or characterized due to difficulties associated with the purification of membrane-bound polysaccharide synthases, the inherent instability of many such enzymes, the probable requirements for a multienzyme complex and for the participation of ancillary proteins (Gibeaut and Carpita, 1993; Delmer, 1999). However, Pear *et al.* (1996) have identified candidate genes for cellulose synthases (*GhCesA-1* and *GhCesA-2*) by comparing sequences of genes that were expressed during secondary cell wall cellulose synthesis in cotton fibres, with sequences of bacterial cellulose synthases. Correction of a low-cellulose phenotype of *Arabidopsis (AtCesA-1)* by complementation with an *Arabidopsis* gene of similar sequence (Arioli *et al.*, 1998), suggested that the cotton *GhCesA-1* and *GhCesA-2* gene products were indeed cellulose synthases (Pear *et al.*, 1996). Large numbers of *CesA* and 'cellulose-synthase-like' genes *(Csl),* some of which are only 30% identical with the cotton *CesA* genes, have since been identified in *Arabidopsis* (Cutler and Somerville, 1997). These genes could encode different cellulose synthase isoenzymes, or they could encode synthases that are required for the synthesis of non-cellulosic wall polysaccharides, such as xyloglucans, mannans, xylans or galactans.

To rapidly evaluate the effects of candidate genes on the synthesis of cell wall polysaccharides and to assign functions to putative 'cellulose-synthase-like' genes, it would be useful to have a gene 'knock-out' system. One such system is virus-induced gene silencing (VIGS), where genes or gene fragments of interest are inserted into a modified potato X potexvirus (PVX) cDNA, and RNA transcripts are prepared *in vitro* for infection of *Nicotiana benthamiana* seedlings (Ruiz et al., 1998). Post-transcriptional gene silencing results in reduced mRNA levels of endogenously expressing plant genes that have a sequence similarity of about 80% or more with the sequences carried by the virus. Furthermore, cDNAs of only 300-500 bp are sufficient to effect silencing (Ruiz *et al.*, 1998).

We have used VIGS to show that while one *CesA* homologue from *Nicotiana* silences endogenous cellulose synthase genes and very probably encodes a cellulose synthase, another *Nicotiana* cDNA, which is 81.5% identical with the first, produces a completely different phenotype. Similarly, a *CesA* homologue from barley also silences cellulose synthase genes in *Nicotiana*.

Materials and Methods

Fragments of putative glucan synthase cDNAs from tobacco, designated NtCesA-1a, NtCesA-1b and NtCesA-2, were amplified from 2 μl aliquots of a cDNA mixture using primer

combinations based on cotton *CesA* sequences (Pear et al., 1996) and sequences of cDNAs encoding putative glucan synthases from barley (R.A. Burton and G.B. Fincher, unpublished data). Products were cloned into the *Eco*RV site of pBluescript pBS(SK+) and their identity verified by nucleotide sequence analysis. The cDNAs were excised from pBluescript and ligated into the PVX vector pP2C2S (Baulcombe et al., 1995). Infectious RNA molecules were produced by *in vitro* transcription of the DNA constructs and were rubbed onto the second leaves of 4- to 5-week-old *Nicotiana benthamiana* seedlings in the presence of a small amount of carborundum powder (Ruiz *et al.*, 1998). Plants were grown in a glasshouse at 24˚C, under a 16h/8h light/dark cycle for 3-12 weeks.

For scanning electron microscopy, leaf or stem tissues were mounted on aluminium stubs, frozen in liquid N_2 slush and fractured using a scalpel blade. Surfaces were coated with platinum and observed using a scanning electron microscope.

For the preparation of cell walls, leaves were harvested 22-112 d post-inoculation. After grinding the leaves in buffer with a mortar and pestle, the "crude cell wall" preparation was recovered by centrifugation. Subsequent washing procedures were used to remove cytoplasmic components from the cell wall preparation. All procedures were performed at room temperature.

Wall preparations were methylated as described by McConville *et al.* (1990). Derivatives were separated and analyzed in a gas chromatograph linked to a mass spectrometer. Identification of the derivatives and deduction of the glycosidic linkages were based on published mass spectra and the elution order in relation to standards. Polysaccharide compositions of the samples were estimated by procedures based upon the structures of well-characterized wall polysaccharides from *Nicotiana plumbaginifolia* (Sims and Bacic 1995; Carpita and Gibeaut, 1993).

Results

Plants infected with the PVX-NtCesA-1 constructs were markedly shorter in stature than the control plants, which were infected with the PVX-Control construct. The PVX-NtCesA-2 construct caused no obvious change in plant height. Scanning electron microscopy of the leaves from the PVX-NtCesA-1 plants showed numerous clumps of expanded cells protruding from the undersurfaces of leaves, especially in regions adjacent to vascular tissues. In some cases, individual cells ballooned out from the epidermis and swollen cells could be detected in trichomes. These phenotypic effects were also evident on the surfaces of stems. Regions of apparently unperturbed epidermal surfaces were also present.

Cellulose contents of walls from PVX-NtCesA-1 plants were approximately 25% lower than those in walls from PVX-Control and PVX-NtCesA-2 plants. The lower cellulose contents were offset by increases in homogalacturonan. The degree of esterification of pectic polysaccharides decreased by approximately 35% in the PVX-NtCesA-1 plants. Amounts of other polysaccharides in the walls remained approximately similar.

Discussion

The stunted growth patterns of plants infected with PVX-NtCesA-1 were highly reproducible. Not only were greatly reduced internode lengths observed, but leaves were also smaller, and were both "lumpy" in form and "crisp" in texture. The swelling of cells in plants infected

with PVX-NtCesA-1 was consistent with a temporary loss of cell wall strength or rigidity which allows uncontrolled cell growth, and which might be expected if virus-induced silencing of cellulose synthase genes were occurring.

To further investigate the possibility that the swelling of cells in plants infected with PVX-NtCesA-1 was attributable to a reduction in cellulose content, cell walls were isolated from infected leaves for analysis. The cellulose contents of walls isolated from PVX-NtCesA-1 leaves were about 25% lower than the cellulose content in walls of the PVX-Control plants. The loss of cellulose in walls of the VIGS plants was accompanied by a 45% increase in levels of pectic polysaccharides and the degree of esterification of pectic polysaccharides decreased from about 50% in walls of control plants to about 33% in walls of infected plants.

Thus, it may be concluded that the *Nicotiana* cDNA fragments silenced cellulose synthesis in the VIGS plants. An homologous cDNA from barley produced similar phenotypic effects and it is concluded that it too silenced cellulose synthesis.

Acknowledgments

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