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Comparison of cloned genes provides evidence for intergenomic exchange of DNA in the evolution of a tobacco glucan endo-1,3-β-glucosidase gene family

(plant defense hydrolases/*Nicotiana sylvestris*/*Nicotiana tomentosiformis*/gene conversion/homeologous recombination)

CHRISTOPH SPERISEN*, JOHN RYALS†, AND FREDERICK MEINS*‡

*Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland; and †CIBA-Geigy Agricultural Biotechnology Research Unit, P.O. Box 12257, Research Triangle Park, NC 27709

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ABSTRACT **Two genes for prepro glucan endo-1,3-β-glucosidase (1,3-β-glucanase; 1,3-β-D-glucan glucanohydrolase, EC 3.2.1.39) of tobacco were cloned and their sequences were compared with cDNA clones. Southern analysis indicates that the genomic clones represent genes derived from ancestral parents of tobacco similar to the present day species *Nicotiana sylvestris* and *Nicotiana tomentosiformis*, whereas the genes represented by two of the cDNA clones appear to be unique to tobacco. The coding sequences of the genomic clones and cDNA clones differed at <2.2% of the positions, indicating that the tobacco 1,3-β-glucanase gene family is highly conserved. Alternating blocks of sequence in the cDNA clones were identical to the coding sequence of the two genomic clones. These results and an analysis of evolutionary distances for nucleotide substitution are consistent with the hypothesis that the evolution of the tobacco 1,3-β-glucanase gene family has involved exchange of DNA between members of the tomentosiformis and sylvestris subgenomes by recombination or gene conversion.**

Glucan endo-1,3-β-glucosidases (1,3-β-glucanase; 1,3-β-D-glucan glucanohydrolase, EC 3.2.1.39) are abundant hydrolases implicated in the defense reaction of plants against fungal pathogens (1). These enzymes are regulated, at least in part, at the mRNA level by the plant hormones auxin, cytokinin, and ethylene and in response to infection by viral, bacterial, and fungal pathogens (2).

The basic isoforms of tobacco 1,3-β-glucanase are encoded by a small gene family with a total of at least four members derived from ancestral species of tobacco most closely related to the present day *Nicotiana sylvestris* and *Nicotiana tomentosiformis* (3–7). Nevertheless, the three classes of tobacco 1,3-β-glucanase cDNA clones differ at only 18 positions in 1055 bases of overlapping sequence (3). The mechanisms for maintaining sequence identity in members of the same gene family are poorly understood (8). Here, we report the isolation and partial characterization of two genomic clones containing the complete coding sequence of different basic isoforms of tobacco prepro-1,3-β-glucanase.§ Comparison of the sequences of the genomic and cDNA clones provides evidence that the high level of homology within the 1,3-β-glucanase gene family is maintained by intergenomic exchange of DNA between genes present in the sylvestris and tomentosiformis subgenomes of tobacco.

MATERIALS AND METHODS

Plant Material and Culture Methods. *Nicotiana tabacum* L. cv. Havana 425, *N. sylvestris*, and *N. tomentosiformis* plants were grown from seed in a greenhouse. The cloned cell line 275N was isolated from pith parenchyma tissue of Havana 425 tobacco on October 3, 1979 (9), and has been continuously subcultured on an agar-containing medium as described (10).

Isolation of DNA, Gel Electrophoresis, and Southern Blot Analysis. High molecular weight DNA was isolated from leaf and callus tissue by the method of Murray and Thompson (11). Gel electrophoresis in agarose gels and Southern blot analysis were essentially as described (3). DNA samples (5–10 μg) were digested to completion with the restriction endonucleases indicated, precipitated, dissolved in gel-loading buffer, electrophoresed on 0.8% agarose (Bio-Rad) or 4% NuSieve agarose (FMC) gels at 2 V/cm, and transferred to a Zeta-Probe nylon membrane (Bio-Rad). Prehybridization, hybridization, and washes were as recommended by the manufacturer. An additional wash was performed at 50°C in 0.1× SSC (1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0/0.1% $NaDodSO_4$). The hybridization probe was the insert of tobacco 1,3-β-glucanase cDNA clone pGL43 (3, 12) labeled by using a random-primer labeling kit (Boehringer Mannheim).

Construction and Screening of a Genomic Library. DNA (80 μg) prepared from pooled leaves of five different Havana 425 tobacco plants raised from the same batch of seed was digested to completion with the restriction endonuclease *Sac* I. The fragments were separated on a 0.8% agarose gel and the region corresponding to a size range of 3.5–5.5 kilobases (kb) was cut into six equal-sized, transverse strips. The DNA electroeluted from the strips that hybridized with the insert of tobacco 1,3-β-glucanase cDNA clone pGL43 was used to construct a library. In brief, DNA (0.2 μg) was mixed with 1 μg of *Sac* I-digested vector λOngC DNA (Stratagene) and ligated with T4 DNA ligase (Biofinex; Praroman, Switzerland). The ligated DNA was packaged and the phage were plated on the bacterial host VCS 257 (Stratagene). The library was screened by plaque hybridization using the insert of the tobacco 1,3-β-glucanase cDNA clone pGL43 (3, 11) as described (13).

Sequence Analysis. The inserts of λOngC DNA were subcloned into Bluescript plasmids (Stratagene) and sequenced on both strands by the dideoxynucleotide chain-termination method (14). Deoxynucleotide and amino acid sequences were analyzed by computer with Genetics Computer Group software (15).

Primer Extension. Total RNA was prepared from tobacco plants infected with *Phytophthora parasitica* var. *nicotianae* and primer extension was carried out as described (16). The oligonucleotide primers used were 5′-GTCAATGCTGCTG-

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‡To whom reprint requests should be addressed.

§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M60402 and M60403).

GCAAC-3′ for gene GLA and 5′-CTCAATGGTGCTG-GCAAC-3′ for gene GLB.

RESULTS

The Structure of Two Tobacco 1,3-β-Glucanase Genes. A genomic library was prepared from tobacco DNA enriched for 1,3-β-glucanase sequences. Nine positive clones were obtained in a screen of 6.5×10^4 recombinants. Based on restriction endonuclease analysis the clones fell into two groups giving *Sac* I fragments of 4.5 or 4.7 kb. The *Sac* I fragment of one representative clone in each group was subcloned and sequenced. The relevant nucleotide and deduced amino acid sequences of the two 1,3-β-glucanase genes, GLA and GLB, are shown in Fig. 1.

The starts of transcription were determined by primer extension using gene-specific oligonucleotides (data not shown). Several bands were obtained for each gene, indicating that there are multiple transcription start sites or that several additional 1,3-β-glucanase genes are represented in the mRNA population. Based on the intensity of the bands the major start site in both genes is 16 base pairs (bp) upstream of the first available initiation codon. The sequence around the putative initiation codon CATCATGTC is in fair agreement with the consensus for the start of translation for plant genes (17).

The structures of the two 1,3-β-glucanase genes are very similar (Fig. 1). After introducing 17 gaps, nucleotides are identical at 92.3% of the positions in the 5′ flanking sequences. The differences mainly result from insertions or deletions of up to 65 nucleotides. Included in the 5′ flanking regions are sequences with features common to many other eukaryotic genes. The sequence AATATAAATA beginning 35 nucleotides upstream from the transcription start sites of the GLA and GLB genes is similar in sequence and position to the TATA box (18). The sequence CCAATA 39 and 48 nucleotides upstream from the TATA sequences of the GLA and GLB genes, respectively, resembles the CAAT box (19).

The coding sequence of each gene is interrupted with an intron, 789 and 747 nucleotides long in GLA and GLB, respectively. After introducing 14 gaps, the introns are identical at 88.1% of the positions. The sequences at the exon/intron boundaries conform to the consensus splice sites deduced from other plant and animal genes (20). The exons of each gene encode a sequence of 370 amino acids. This preproform of 1,3-β-glucanase contains an N-terminal hydrophobic signal peptide of 32 amino acids and a C-terminal extension of 22 amino acids (3). Both the signal peptide and C-terminal extension are cleaved off during intracellular transport (3). The nucleotides in the exons differ at only 24 positions and result in 6 amino acid substitutions. Three amino acid changes are located in the signal peptides. The deduced amino acid sequence of the C-terminal extension of the two genes differs at one position.

The 3′ untranslated region of the GLA gene extends 201 and 237 nucleotides beginning with the termination codon to two putative polyadenylylation signals, AAGAAA and ATTAAT (21). The corresponding 3′ untranslated region of the GLB gene is 24 nucleotides longer and differs at 13 positions. The difference in length is partially due to a 27-bp sequence in GLA, which is repeated in GLB. After introducing 11 gaps, the 3′ flanking sequences of the two genes are identical at 92.7% of the positions.

Southern Blot Analyses. Southern blot analysis indicates that the 1,3-β-glucanase gene family of tobacco has its origin in ancestral species related to *N. sylvestris* and *N. tomentosiformis* (3). To determine the origin of GLA and GLB, DNA from the two genomic clones and the three *Nicotiana* species was digested with *Sac* I and *Hin*dIII, and Southern analysis was performed with the cDNA clone pGL43 used as a probe for the second exon. Both restriction endonucleases cut outside the second exon and are expected to give hybridizing restriction fragments of 4476 and 1620 bp, respectively. Both predicted fragments were found in digests of tobacco DNA, confirming that the clones GLA and GLB represent 1,3-β-glucanase genes in the tobacco genome (Fig. 2). The 4.5-kb fragment was identical in size to the strongly hybridizing fragments obtained with DNA from *N. sylvestris* and GLA. The 1.6-kb fragment was identical in size to the strongly hybridizing fragments obtained with DNA from *N. tomentosiformis* and GLB. These results indicate that gene GLA is derived from *N. sylvestris* and gene GLB is derived from *N. tomentosiformis*.

Comparison of cDNA and Genomic Clones. To find out if genes GLA and GLB are expressed, we compared their coding sequences with the sequences of the partial cDNA clones pGL31, pGL36, and pGL43 (3). The 582-bp cDNA pGL36 was identical to GLA, suggesting that pGL36 and GLA represent the same expressed gene. The cDNAs pGL31 and pGL43, however, differed in sequence from both GLA and GLB (Fig. 3). The cDNA pGL31 is identical to the GLA gene from the 5′ end of the sequence to position 572 and, with the exception of a single base difference, from position 723 to the 3′ end of the sequence. The sequence between these regions is identical to the GLB gene, which differs in the comparable region of the GLA gene at 8 positions. The cDNA clone pGL43 shows a similar pattern but in an inverse order. The sequence of the cDNA clone pGL43 is identical to the GLB gene from the 5′ end to position 643 and from position 852 to position 1097, and the sequence between these regions is identical to the GLA gene. In contrast to these alternating blocks of identical sequences, the nucleotide differences between GLA and GLB are distributed rather uniformly. The simplest explanation for these patterns appears to be that DNA has been exchanged between 1,3-β-glucanase genes.

Origin of Recombinant Sequences. In principle, exchange of DNA sequences in 1,3-β-glucanase genes could have occurred in the cell line used as a source of the cDNA clones or during the evolution of tobacco and its progenitor species. The cell line 275N used to prepare the cDNA library had been propagated serially in culture for >10 years. Cultured plant cells are known to undergo rapid mitotic recombination (22), raising the possibility that the cDNA clones pGL31 and pGL43 represent recombinant genes unique to cell line 275N. To test this hypothesis, we digested the cloned DNAs and DNA prepared from the cell line and tobacco leaves with *Alu* I and *Ssp* I to generate unique fragments with each of the cloned DNAs. The *Alu* I/*Ssp* I fragments predicted from the sequences and the fragments found by Southern analysis using pGL43 as a hybridization probe for the second exon are shown in Fig. 4. Mixing experiments show that DNA from the cell line and DNA from tobacco leaves have identical restriction fragments (Fig. 4*B*). Moreover, all of the fragments obtained with GLA, GLB, pGL31, and pGL43 were present in genomic DNA obtained from tobacco leaves (Fig. 4*C*). These results provide strong evidence that the genes represented by pGL31 and pGL43 did not arise by recombination in cell culture.

To distinguish between DNA exchange events in tobacco and in the ancestral parents of tobacco, we compared the *Alu* I/*Ssp* I fragments obtained with DNA prepared from leaves of tobacco, *N. sylvestris* and *N. tomentosiformis* (Fig. 4*C*). The 201- and 648-bp fragments of GLA were present in *N. sylvestris* DNA but not in *N. tomentosiformis* DNA, confirming that this gene is of *N. sylvestris* origin. Similarly, the 178- and 227-bp fragments of GLB were found in *N. tomentosiformis* DNA but not in *N. sylvestris* DNA. In this case, two additional fragments of 206 and 311 bp were not found in DNA from either parental species. There was, however, a fragment in *N. tomentosiformis* DNA equal to the sum of these two GLB fragments. This is

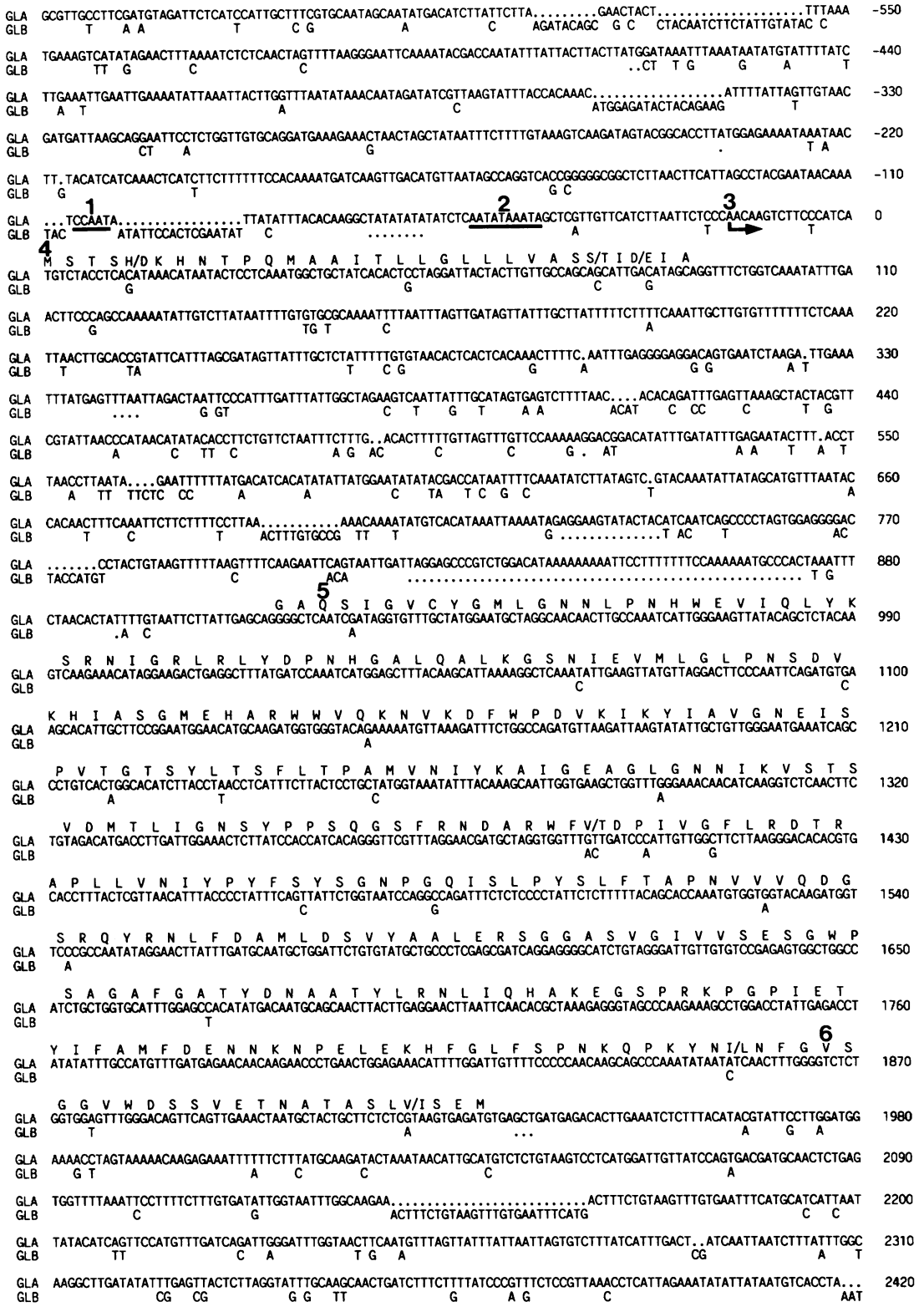

FIG. 1. Partial nucleotide sequences of tobacco 1,3-β-glucanase genomic clones GLA and GLB. Slashes between symbols in the deduced amino acid sequence indicate that the two clones encode different amino acids at that position. Differences in sequence between GLA and GLB are indicated below the main sequence. 1, CAAT box underlined; 2, TATA box underlined; 3, start of transcription indicated with an arrow; 4, first amino acid of the preproenzyme; 5, first amino acid of the proenzyme; 6, first amino acid of the C-terminal extension. The putative polyadenylylation signals AAGAAA and ATTAAT start at positions 2132 and 2195, respectively.

consistent with the hypothesis that GLB is of *N. tomentosiformis* origin but that a new restriction site was generated in tobacco.

The cDNA clone pGL43 gave two *Alu* I/*Ssp* I fragments, a 648-bp fragment found in *N. sylvestris* DNA and a 227-bp fragment found in *N. tomentosiformis* DNA, indicating that the gene represented by pGL43 is unique to tobacco. The cDNA clone pGL31 gave a 178-bp fragment unique to *N. tomentosiformis* DNA and a 470-bp fragment present in DNA of both ancestral species. The 470-bp fragment was cleaved into two fragments of 184 and 286 bp when digested with *Hae* III (data not shown). The latter of these fragments was unique

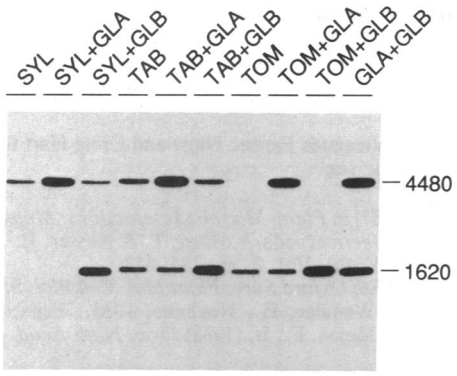

FIG. 2. Southern analysis of 1,3-β-glucanase genomic clones and DNA prepared from leaves of tobacco (TAB), *N. sylvestris* (SYL), and *N. tomentosiformis* (TOM). DNA in the combinations indicated was digested with *Sac* I and *Hin*dIII. The hybridization probe was the insert of 1,3-β-glucanase cDNA clone pGL43. Numbers on the right are bp.

to *N. sylvestris* DNA. Therefore, it appears that pGL31, like pGL43, represents a gene unique to tobacco.

DISCUSSION

The present study shows that tobacco 1,3-β-glucanase is encoded by at least four different DNA sequences. These coding sequences differ by <2.2% of the positions, indicating that the 1,3-β-glucanase gene family is highly conserved. In contrast, the 1,3-β-glucanase coding sequences diverge by 19.2% in 1065 bp from the coding sequence of the closely related species *Nicotiana plumbaginifolia* (23) and by 47% in 351 bp from the monocot barley (24). Similar findings have been reported for the gene family encoding ribulose-bisphosphate carboxylase small subunit (rbcS), which shows an intraspecific sequence divergence of <10% and an interspecific divergence of >23% (25–28).

Several mechanisms have been proposed for maintaining uniformity of amino acid sequences encoded by small gene families. If sequence homogeneity results from selection, then there should be high ratios of silent to nonsilent nucleotide substitutions in pairwise comparisons among different sequences encoding the mature proteins (25). Pichersky *et al.* (25) found ratios ranging from 7:1 to 33:1 in different members of the tomato rbcS family. We found a value of 3:1 in a comparison of GLA and GLB. This supports the hypothesis that selection had been at least in part responsible for the high intraspecific sequence identity of the tobacco 1,3-β-glucanase family.

Sequence homogeneity could also be maintained by DNA exchange resulting from recombination or gene conversion. These mechanisms would generate alternating blocks of sequence between different members of the same family (8). Our results indicate that such mechanisms have been important in the evolution of tobacco 1,3-β-glucanase. The gene represented by pGL31 shows alternating blocks identical to corresponding regions of GLA and GLB. The gene represented by pGL43 exhibits a similar pattern of alternating blocks, but in an inverse order. In contrast, the nucleotide differences between the GLA and GLB genes are distributed rather uniformly. These findings strongly suggest that DNA exchange occurred between different 1,3-β-glucanase genes. Similar evidence for DNA exchange within the rbcS gene family has been described for pea (29) and tomato (27).

Based on the limited number of clones examined, DNA exchange could have occurred between any combination of cloned and other as yet unidentified 1,3-β-glucanase genes. The exchange partners and products of exchange cannot be established by comparing the sequence of genes in a family. Nevertheless, analysis of the evolutionary distances for nucleotide substitutions (30) and pattern of restriction fragments obtained with cloned and genomic DNA suggests a plausible working hypothesis for the direction of DNA exchange.

Assuming that nucleotide substitutions occur at random, the evolutionary distance between genes undergoing recombination must be greater than the distance between the products of recombination and either parental gene. Moreover, the sum of the distances between a recombinant and each parental gene should equal the distance between the parental genes. The evolutionary distances for nucleotide substitutions in the overlapping coding region of cDNA and genomic clones of tobacco

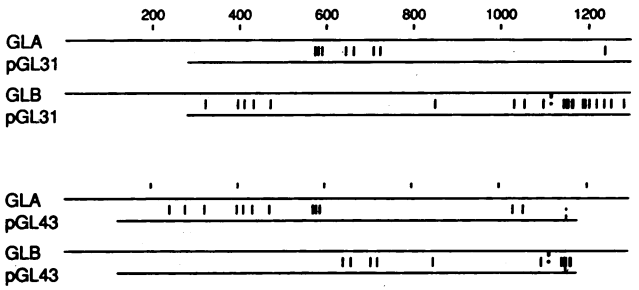

FIG. 3. Comparison of coding and 3′ untranslated sequences of genomic and cDNA clones for 1,3-β-glucanase. Horizontal lines, sequences; long vertical lines, nucleotide differences; short vertical lines, gaps between the compared sequences.

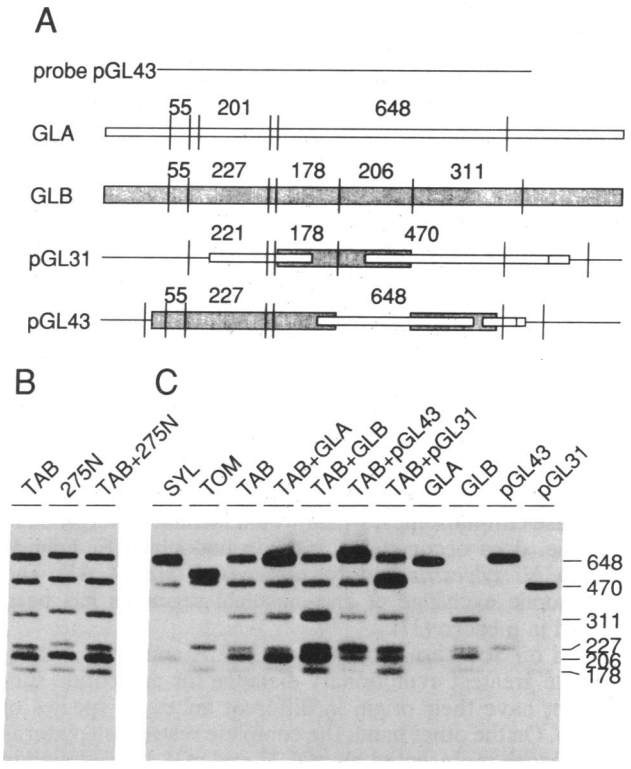

FIG. 4. Southern analysis of 1,3-β-glucanase clones and DNA prepared from cell line 275N and from leaves of tobacco (TAB), *N. sylvestris* (SYL), and *N. tomentosiformis* (TOM). (*A*) Partial restriction map showing expected lengths of *Alu* I/*Ssp* I restriction fragments in exon 2 and part of the 3′ untranslated sequence. Open bar, sequence identical to GLA; shaded bar, sequence identical to GLB; overlapping shaded and open bars, sequences identical in GLA and GLB; horizontal line, vector sequence; vertical lines, restriction sites. Size of restriction fragments is in bp. (*B* and *C*) Southern blots of the DNAs indicated digested with *Alu* I/*Ssp* I and hybridized with a pGL43 probe. Numbers on the right are bp.

Table 1. Evolutionary distances for nucleotide substitution in the overlapping coding regions of tobacco 1,3-β-glucanase clones

Comparison	Sequence length, bp*	Distance ± SE ($\times 10^3$)†
GLA vs. GLB	1111	20 ± 4.3
GLA vs. pGL31	829	9.7 ± 3.4
GLA vs. pGL43	987	13.3 ± 3.7
GLB vs. pGL31	829	10.9 ± 3.7
GLB vs. pGL43	987	6.1 ± 2.5
pGL31 vs. pGL43	829	13.4 ± 4.1

*Overlapping coding region.
†Evolutionary distance per nucleotide site calculated as described (30).

1,3-β-glucanase are shown in Table 1. The largest distance was between GLA and GLB. The sum of the distances between pGL31 and GLA and between pGL31 and GLB (20.6×10^{-3}) as well as the sum of the comparable distances for pGL43 (19.4×10^{-3}) were equal to the distance between GLA and GLB (20.0×10^{-3}). Therefore, it appears that the genes represented by pGL31 and pGL43 resulted from the exchange of DNA between GLA and GLB or their close relatives. At present, we cannot establish whether these products of exchange resulted from a single event or from multiple events. The fact that pGL31 and pGL43 have roughly reciprocal patterns of GLA and GLB sequences favors a single event model.

By analogy to other eukaryotic gene families (8), it is likely that genes encoding 1,3-β-glucanase in tobacco have undergone duplication to generate multiple copies. The genes GLA and GLB, which we believe exchanged DNA, are still present in the tobacco genome. This indicates that exchange occurred between some copies of the genes leaving other copies intact.

Tobacco is an amphiploid species (31). Its genome consists of tomentosiformis (T) and sylvestris (S) subgenomes derived from the ancestral species by sexual hybridization and subsequent chromosomal duplication (31). Therefore, in principle, intergenomic as well as intragenomic DNA exchange could play a role in the evolution of tobacco gene families. Analysis of crosses of tobacco with *N. tomentosiformis* and *N. sylvestris* (32, 33), Southern blot analysis with probes for specific genes (3, 34), comparison of DNA sequences (35, 36), and studies of single-copy DNA homology (7) indicate that the T and S subgenomes and their respective ancestral genomes are very similar at the chromosome and DNA levels. Therefore, the S and T subgenomes are highly conserved and evolving independently in tobacco at roughly the same rates as the ancestral genomes. This implies that intergenomic exchange of DNA is likely to be a rare event. Nevertheless, homologous pairing of metaphase chromosomes, which would facilitate intergenomic exchange, does occur at low rates in undoubled F_1 hybrids between *N. sylvestris* and *N. tomentosiformis* (4, 31), and intergenomic exchange of chromosomal segments has been reported in tobacco (37).

Based on Southern blot analysis, GLA and GLB, which show the greatest evolutionary distance for nucleotide substitution, have their origin in different ancestral species of tobacco. On the other hand, the complete restriction patterns for the genes represented by pGL31 and pGL43 are found in tobacco but not in either ancestral species. This indicates that these genes arose after the sexual hybridization that gave rise to modern tobacco. If, as we propose, GLA and GLB underwent DNA exchange to generate the genes represented by pGL31 and pGL43, then our results favor an intergenomic exchange of DNA, either by homeologous recombination at the same locus or by recombination between different loci in the S and T subgenomes. A more rigorous test of this hypothesis will depend on sequencing additional 1,3-β-glucanase genes of tobacco and its ancestral species.

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