ANALYSIS OF COTTON GENES FOR THE ANTIFUNGAL PROTEIN OSMOTIN

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Abstract

Fungal diseases in cotton, such as various types of root rot and wilt symptoms, have been known for over 100 years, causing destruction of a large percentage of the cotton crop and large economic loss throughout the cottonbelt. It is now well known that almost all plants naturally produce their own defensive proteins, called pathogenesis-related (PR) proteins, directed against pathogens such as fungi and bacteria. PR proteins called osmotins are made in response to fungal pathogen stress or osmotic stress (water deprivation or salt exposure). Osmotins are thought to actually form pores in fungal membranes, leading to osmotic rupture and destruction of the fungal cells. Tobacco and potato osmotin genes have been shown to have biological control regions for overproduction of osmotin when the plants are exposed to fungal pathogens or drought. Since osmotins have antifungal activity against most fungal species, it should be possible to use the osmotin gene as a novel defense gene effective against numerous pathogenic fungi. A cotton genomic library harbored in lambda phage was screened with a tobacco osmotin hybridization probe to isolate prospective osmotin genes, in order to study their regulation of gene expression. A cotton genomic clone containing a 16.0-kb DNA segment was found to encompass two osmotin genes. One gene is full-length with an open reading frame of 729 basepairs without any introns, and encodes a presumptive osmotin-like preprotein of 242 amino acids. The other gene is almost full-length, but lacks the 5'-flanking promoter region and eight codons of the N-terminal coding region. The open reading frames of the complete gene and its corresponding cDNA clone are identical in sequence, indicating that this osmotin gene is indeed expressed in cotton. The cDNA insert is nearly full-length, lacking codons for four N-terminal amino acids. The two presumptive cotton osmotin preproteins can definitely be identified as PR5 proteins from their similarities to the deduced amino acid sequences of osmotin-like PR5 preproteins. The presumptive preproteins have N-terminal signal sequences, and the mature forms of the proteins would likely be targeted for extracellular secretion as neutral isoforms. In addition to basal TATA and CAAT promoter elements, other prospective promoter/enhancer elements, such as two ethylene response elements with the canonical sequence AGCCGCC, implicated as being positive regulatory elements in the expression of a number of PR proteins, occur in the 5'-flanking sequence of the full-length gene.

Introduction

Plants produce their own defense proteins directed against disease-causing pathogens such as fungi, bacteria, and viruses (Stintzi et al., 1993; Kitajima and Sato, 1999). These proteins are called pathogenesis-related (PR) proteins, and are constitutively made by plants in low amounts, but accumulate in increased amounts after infection by a microbial pathogen. The PR superfamily of proteins currently includes 14 subfamilies (Buchanan et al., 2000). The PR proteins are synthesized as either basic vacuolar-targeted or neutral/acidic extracellular-targeted preproproteins (Stintzi et al., 1993). The neutral/acidic extracellular forms have N-terminal signal sequences for targeting into the secretory pathway, whereas the basic vacuolar forms also have C-terminal signal sequences for vacuolar targeting. For example, Melchers et al. (1993) determined that three tobacco vacuolar-targeted preproproteins (thaumatin-like protein, chitinase, and β-1,3-glucanase) contain N- and C-terminal signal sequences. Removal of the C-terminal propeptides resulted in extracellular secretion of the mature proteins.

PR5 proteins have antifungal activity against a broad spectrum of fungal pathogens (Hu and Reddy, 1997; Koiwa et al., 1997; Malehorn et al., 1994). One PR5 protein, called osmotin by Bressan and coworkers (Nelson et al., 1992; Singh et al., 1987), is produced during osmotic stress due to water deprivation or salt exposure in tobacco cells. The tobacco osmotin has antifungal activity, causing the release of intracellular materials and hyphal rupture of many types of fungal cells (Abad et al., 1996). Transgenic potato plants that constitutively overproduce either potato or tobacco osmotin delay development of disease symptoms after infection with the fungus *Phytophthora infestans* (Liu et al., 1994; Zhu et al., 1996). A proposed mechanism of PR5 protein activity is the formation of transmembrane pores in the fungal plasma membrane, leading to permeabilization and rupture of the membrane (Batalia et al., 1996). However, Yun et al. (1997; 1998) determined that spheroplasts of resistant fungal cells could be sensitive to one form of osmotin and resistant to another. This indicates that osmotin does not directly and non-specifically enter into the membrane, suggesting instead a receptor-protein complex. Osmotin may bind a plasma membrane receptor, such as a yeast Pir protein, subverting a fungal cell signal transduction pathway in its mechanism of action (Yun et al., 1998).

The 5'-flanking regions of several PR protein genes have been shown to have positive promoter/enhancer regulatory elements for inducible gene expression (Guilfoyle, 1997). The tobacco osmotin gene characterized by the Bressan group (Nelson et al., 1992; Raghothama et al., 1993; Raghothama et al., 1997) is up-regulated by an array of hormonal and environmental signals, such as fungal infection, drought, salinity, wounding, cold, ethylene, and abscisic acid. The tobacco gene has several cis-acting elements, including two ethylene-responsive elements (GCC boxes) and an abscisic acid-responsive element containing a G-box motif, that increase osmotin expression when the tobacco plant is exposed to fungal pathogens or osmotic stress. Similar promoter elements that activate gene expression when threatened with fungal pathogens have also been found in osmotin genes from potato (Zhu et al., 1995). However, the overall regulation of expression of the PR protein gene family is still not well understood. In this report, we have characterized two cotton genes and a cDNA clone for osmotin, in order to study the chromosomal arrangement, gene organization, and regulation of expression of this largely uncharacterized gene family.

Methods and Materials

Isolation of Cotton Genomic and cDNA Clones for Osmotin

A heterologous tobacco osmotin gene subcloned into a pGEM vector (Promega) was provided by Dr. Ray Bressan of Purdue University, West Lafayette, IN (Nelson et al., 1992). An 884-bp *Hin*dIII/*Sal*I fragment was purified from the tobacco genomic subclone as template to generate radioactively-labeled random-primed DNA fragments (Feinberg and Vogelstein, 1983) for use as hybridization probe to screen cotton cDNA and genomic libraries. A cotton cDNA library, generated from mRNA of 48-hour dark-grown cotyledons (*Gossypium hirsutum* cv. Delta Pine 62) and harbored in the Stratagene UniZAP lambda vector, was kindly provided by Dr. R.N. Trelease of Arizona State University (Ni and Trelease, 1991). The cDNA library was screened by the plaque-hybridization procedure (Benton and Davis, 1977) to identify positive clones encoding presumptive osmotin-like proteins. Prehybridization was done for 4 hours at 55°C in a solution containing 6X SSC (1X SSC is 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0), 5X Denhardt's reagent, 0.5% SDS, and denatured sheared salmon sperm DNA (100 µg/ml). Subsequently, hybridization was done overnight at 55°C using a solution containing 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA (pH 7.5), 2.5 mM sodium pyrophosphate (pH 8.0), denatured

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sheared salmon sperm DNA (100 µg/ml), and ^{32}P-labeled probe. After hybridization, the membranes were rinsed once at room temperature with 2X SSC for 5 min., once with 2X SSC, 0.1%SDS at 55°C for 30 min., and twice with 1X SSC, 0.1% SDS at 55°C for 30 min. The cloned cDNA fragments were biologically excised into the Bluescript SK(-) phagemid vector in vivo with proteins from ExAssist Interference-Resistant helper phage and *Escherichia coli* SOLR strain (Stratagene). A cDNA clone designated pCcOSM47 was selected for DNA sequence analysis.

A cotton (*Gossypium hirsutum*, cv. Acala SJ-5) genomic library contained in the lambda vector EMBL3, generously provided by Dr. David M. Anderson of Phytogen Seeds, Placentia CA (Grula et al., 1995), was screened to isolate presumptive clones encompassing osmotin-like genes by the plaque hybridization procedure (Benton and Davis, 1977). This genomic library was screened by hybridization with the heterologous tobacco DNA probe, as was done for the cDNA library. In order to identify the genomic clones corresponding to the cDNA clone pCcOSM47, a number of phage DNAs were isolated by a minilysate procedure and subjected to cycle sequencing (Fan et al., 1996) with oligonucleotide primers designed for the osmotin cDNA coding region. One genomic clone designated LCgOSM16B was selected for further analysis, since its preliminary sequence indicated a high degree of identity with the cDNA sequence.

Physical Mapping and Genomic Blot Hybridization

The DNA from the genomic clone LCgOSM16B was digested with numerous restriction endonucleases for analysis by agarose gel electrophoresis and alkaline blot hybridization (Reed and Mann, 1985) to identify an appropriate fragment encompassing the hybridizing region. A 7.2-kb *Sal*I/*Bam*HI fragment was selected for subcloning into the plasmid vector pUC19 and the resulting recombinant plasmid was designated pCgOSM16B. Cotton genomic DNA (cv. Paymaster HS26) was purified using the procedure of Paterson et al. (1993). The genomic DNA was digested with several restriction endonucleases and the fragments resolved by electrophoresis on 0.8% agarose gels. The genomic DNA fragments were analyzed by alkaline blot hybridization with a homologous cotton DNA probe derived from an 884-bp *Eco*RV fragment of pCgOSM16B. The hybridization conditions with the cotton probe were the same as described above for the heterologous tobacco probe, except that the stringency was increased by raising the temperature to 60°C.

DNA Sequence Analysis

Both DNA strands of the 1,048-bp cotton cDNA insert in the plasmid pCcOSM47 and the 7.2-kb *Sal*I/*Bam*HI cotton genomic segment in pCgOSM16B were sequenced, using a primer-based approach. The cDNA insert was manually sequenced using terminator cycle sequencing (Fan et al., 1996) with ThermoSequenase and [α-^{33}P]-labeled dideoxynucleoside triphosphates (Amersham). The genomic DNA insert in pCgOSM16B was subjected to DNA sequencing on an Applied Biosystems Model 377XL DNA Sequencer by Lone Star Labs, Houston, TX. The sequencing reactions were done according to manufacturer protocols by terminator cycle sequencing with BigDye™ using 10% DMSO as denaturant. Terminator cycle sequencing was done manually to confirm difficult regions with compression effects or other sequence anomalies. Analyses of the DNA and deduced amino acid sequences were done with DNASIS software (Hitachi). The genomic sequence has been deposited in GenBank with Accession Number AF304007 and the cDNA sequence has been assigned GenBank Accession Number AF192271.

Results and Discussion

The cotton genomic insert in the lambda clone LCgOSM16B was determined to be 16.0 kb by physical mapping. Alkaline blot hybridization of cotton genomic DNA was done with the homologous 884-bp *Eco*RV fragment purified from pCgOSM16B to confirm that restriction fragments derived from the cloned osmotin genes truly occur in cotton genomic DNA, and also to estimate the number of similar osmotin genes in the cotton genome. For example, 5.5-kb *Xho*I/*Bam*HI and 0.9-kb *Eco*RV genomic fragments directly correspond with identically-sized restriction fragments generated by *Xho*I/*Bam*HI and *Eco*RV digests of the cloned cotton DNA segment of LCgOSM16B containing the full-length gene. Other hybridizing fragments in the *Xho*I/*Bam*HI and *Eco*RV digests of cotton DNA must be derived from several similar osmotin genes, since the hybridization was under relatively stringent conditions. Thus, there seem to be at least three or more actual osmotin genes in the allotetraploid cotton genome.

The full-length cotton osmotin gene has a wide variety of potential promoter elements in its 5'-flanking region that could bind an assortment of transcription factors for activation of its expression in different environmental situations, perhaps even through synergistic effects (Guilfoyle, 1997; Thomas, 1993). A prospective cap site for the 5'-end of the mature osmotin mRNA occurs 24 bp before the ATG initiation codon. Putative TATA and CAAT basal promoter elements occur 49 and 104 bp, respectively, upstream from the initiation codon. Two presumptive ethylene response elements (GCC boxes) with the sequence AGCCGCC occur 63 and 127 residues upstream from the initiation codon. Ethylene-response elements occur in promoters of numerous PR protein genes, and many of these genes have two or more GCC boxes (Guilfoyle, 1997). Kitajima et al. (1998) showed that the GCC elements of a tobacco osmotin-like PR5 protein are constitutive in roots and cultured cells, and are inducible by ethylene in tobacco leaves. The Bressan group (Nelson et al., 1992; Raghothama et al., 1993; Raghothama et al., 1997) has extensively studied the promoter region of a tobacco osmotin PR5 protein, which has two GCC boxes in a 140-bp minimum promoter region. These elements are required for ethylene responsiveness but are not sufficient for maximal ethylene-induced gene activation (Raghothama et al., 1997).

Three potential basic-region helix-loop-helix (bHLH) or E box motifs with the consensus sequence CANNTG occur 93, 223, and 486 residues upstream of the initiation codon of the cotton gene. The E box motif has been shown to be a seed-specific positive element for expression of the French bean β-phaseolin gene (Kawagoe et al., 1994). An H-box or AC-element (CCTACC(N_7)CT(N_4)A) in reverse orientation occurs 831 residues upstream from the ATG initiation codon. H-box motifs occur in the promoter region of the bean phenylalanine ammonia-lyase 2 (*PAL2*) gene (Hatton et al., 1995) and may be important in developmental-specific gene activation. There are four prospective GATA motifs with the sequence GATAA at residues 307, 411, 607, and 737 upstream from the initiation codon. GATA elements occur in the promoter regions of many light-regulated genes as well as some promoters, like the CaMV35S RNA promoter, that are light-unresponsive (reviewed in Guilfoyle, 1997). The promoter region of the tobacco osmotin gene (Nelson et al., 1992; Raghothama et al., 1993; Raghothama et al., 1997) also has G-box-like sequences, H-box, and other promoter elements that are activated by various environmental and hormonal cues.

Alignment of the deduced amino acid sequences for the cotton osmotin preproteins and other osmotin preproteins indicates that there is a 93% identity between the two putative osmotin proteins described in this report, a 72% identity between the cotton and chickpea proteins, a 66% identity between the cotton and tobacco preproteins, about 55% identities with the thaumatin and maize preproproteins, and only a 44% similarity with the *Arabidopsis* preprotein. The presumptive cotton osmotin preprotein encoded by the full-length gene would have 242 amino acids, and has a predicted N-terminal signal peptide cleavage site, giving rise to a mature, neutral osmotin isoform of 218 amino acids with an isoelectric point of 7.2. Most likely, this cotton osmotin would be destined for extracellular secretion to fulfill its pathogenic function.

The three-dimensional structures of thaumatin (de Vos et al., 1985), maize zeamatin (Batalia et al., 1996) and tobacco protein Pr-5d (Koiwa et al., 1997; GenBank Locus 2981950) have been determined and are highly homologous (Kitajima and Sato, 1999). The three proteins have an extensive β-strand network called a β-sandwich, and a second structural motif composed of small turns and loops stabilized by eight disulfide bonds, comprised of apparently 16 invariant Cys residues in all three proteins. de Vos et al. (1985) observed that the turn and loop domain maintained by the disulfide bonds occurs in a number of other proteins such as toxins, agglutinins, and cytotoxins that interact with membrane-bound receptors, and speculated that this turn/loop motif could be important for binding to membrane receptors. The two putative cotton osmotins have the 16 invariant Cys residues and have predicted β-strand structures similar to those occurring in thaumatin, zeamatin, and tobacco protein Pr-5, and hence may be able to interact with fungal membrane receptor proteins.

Summary

The two cotton osmotin genes are among the first osmotin genes analyzed. Since the open reading frame of one gene and its cognate cDNA are identical, that gene most likely is expressed in cotton plants to produce a presumptive osmotin preprotein. In particular, the putative preprotein from this gene has a characteristic N-terminal signal sequence and lacks a C-terminal propeptide for vacuolar targeting, and so the mature form of cotton osmotin would probably be targeted for extracellular secretion as a neutral isoform with an isoelectric point of 7.2. The putative mature cotton osmotin proteins may adopt the β-sandwich conformation described for thaumatin (de Vos et al., 1985), zeamatin (Batalia et al., 1996), and tobacco protein Pr-5d (Kitajima and Sato, 1999), since they have the 16 Cys residues and predicted β-sheet domains that occur in the tertiary structures of the three crystallized proteins. In this report, we have determined the structure and genomic arrangement of two cotton osmotin genes and have identified the potential promoter/enhancer elements in one of the genes, as a first step in analyzing environmental/hormonal-specific, tissue-specific, and developmental-specific osmotin gene expression. We are interested in the regulatory expression patterns of the osmotin gene family in response to fungal and bacterial invasion. We hope to produce sufficient amounts of cotton osmotin in a bacterial or baculovirus expression system to study the mechanism of action of this osmotin isoform on fungal membranes or on signal transduction pathways, and assay its effectiveness against fungal pathogens. Our goal is to genetically engineer cotton osmotin genes to constitutively overproduce the antifungal protein osmotin in transgenic cotton plants as a natural defense against fungal infections.

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