ANALYSIS OF A COTTON GENE CLUSTER FOR THE ANTIFUNGAL PROTEIN

OSMOTIN

Jeffery Roland Wilkinson, B.S., M.S.

Dissertation Prepared for the Degree of

DOCTOR OF PHILOSOPHY

UNIVERSITY OF NORTH TEXAS

December 2003

APPROVED:

Robert M. Pirtle, Major Professor
Kent D. Chapman, Committee Member
John E. Knesek, Committee Member
Gerard A. O’Donovan, Committee Member
Douglas D. Root, Committee Member
Art J. Goven, Chair of the Department of
Biological Sciences
Sandra L. Terrell, Interim Dean of the Robert B.
Toulouse School of Graduate Studies

Three overlapping genomic clones covering 29.0 kilobases of cotton DNA were found to encompass a cluster of two presumptive osmotin genes (*OSMI* and *OSMII*) and two osmotin pseudogenes (*OSMIII* and *OSMIV*). A segment of 16,007 basepairs of genomic DNA was sequenced from the overlapping genomic clones (GenBank Accessions AY303690 and AF304007). The two cotton osmotin genes were found to have open reading frames of 729 basepairs without any introns, and would encode presumptive osmotin preproteins of 242 amino acids. The open reading frames of the genes are identical in sequence to two corresponding cDNA clones (GenBank Accessions AF192271 and AY301283). The two cDNA inserts are almost full-length, since one lacks codons for the four N-terminal amino acids, and the other cDNA insert lacks the coding region for the 34 N-terminal amino acids. The cotton osmotin preproteins can be identified as PR5 proteins from their similarities to the deduced amino acid sequences of other plant osmotin PR5 preproteins. The preproteins would have N-terminal signal sequences of 24 amino acids, and the mature 24 kilodalton isoforms would likely be targeted for extracellular secretion. Prospective promoter elements, including two ethylene response elements, implicated as being positive regulatory elements in the expression of a number of PR-proteins, occur in the 5′-flanking regions. The mature osmotin proteins accumulate in cotton plants treated with the inducers ethephon and
hydrogen peroxide. Thus, the two cotton osmotin genes encode osmotin proteins. The coding regions of the two genes have been expressed and isolated as fusion polypeptides in a bacterial expression system. Binary constructs containing the open reading frames of the two osmotin genes under the control of the 35S CaMV promoter have been generated for eventual production of transgenic *Arabidopsis* and cotton plants for potential constitutive expression of the osmotin proteins for increased resistance against fungal pathogens.
ACKNOWLEDGMENTS

This research was supported in part by Agreement 01-897TX from Cotton Incorporated, overseen by Dr. Gay M. Jividen and Dr. Roy G. Cantrell, and by University of North Texas Organized Research Funds.

I thank Drs. Robert M. and Irma L. Pirtle for their support and encouragement throughout my study at the University of North Texas. I also thank Dr. Kent D. Chapman, Dr. John E. Knese, Dr. Gerard A. O’Donovan, and Dr. Douglas D. Root for serving on my dissertation committee.

I thank my parents, Dr. Dan L. and Sondra K. Wilkinson, my wife Dr. R. Kathleen M. Wilkinson, and my wonderful children, Ruby and Jack, for the stability, guidance, wisdom, and perspective they provide in my life.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td></td>
<td>LIST OF ILLUSTRATIONS</td>
<td>vii</td>
</tr>
<tr>
<td></td>
<td>Chapter 1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Chapter 2. MATERIALS AND METHODS</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Preparation of a Heterologous Radioactive Hybridization Probe for the Osmotin Gene</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Screening a Cotton cDNA Library for Putative Osmotin cDNA Clones</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Isolation of Phagemid DNAs Containing Osmotin cDNA Inserts</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>DNA Sequence Analysis of cDNA Clones pCcOSM47B and pCcOSM52A Encoding Cotton Osmotin Proteins</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Screening Cotton Genomic Libraries for Presumptive Clones Encompassing Osmotin Genes</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Mini-lysate Phage Preparations for Genomic Clones Containing Potential Cotton Osmotin Genes</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Screening Lambda Clones by Sequencing with cDNA Insert-Specific Primers</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Large-scale Phage Preparations of the Genomic Clone LCgOSM16B</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Physical Mapping of the Cotton Genomic Insert in LCgOSM16B DNA</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Subcloning and Sequencing of a SalI/BamHI Fragment of LCgOSM16B Encircling One Osmotin Gene and a Portion of a Second Osmotin Gene</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Preparation and Transformation of Electrocompetent <em>E. coli</em> DH5a Cells</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Sequencing of the XhoI/BamHI Fragment of pCgOSM16B Encompassing the Entire Open Reading Frame of the <em>OSM I</em> Gene</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Preparation of Homologous Radioactive Hybridization Probes for Osmotin Genes</td>
<td>38</td>
</tr>
</tbody>
</table>
Screening a Second Cotton Genomic Library for Putative Clones with Osmotin Genes ..................................................  41
Mini-lysate Phage Preparations for the Genomic Clones Harboring Potential Osmotin Genes ..................................................  43
Large-scale Phage Preparations for Genomic Clones LCgOSM12A and LCgOSM7B ..........................................................  43
Physical Mapping of the Cotton Genomic Inserts in the Clones LCgOSM12A and LCgOSM7B ..................................................  44
Subcloning and Sequencing of a 4.0-kb EcoRI Fragment from the Genomic Clone LCgOSM12A ..................................................  44
Subcloning and Sequence Analysis of 3.4-kb HindIII and 3.9-kb EcoRI Fragments from the Genomic Clone LCgOSM7B ..................  46
Design of an Anti-osmotin Antibody ..................................................  48
Expression of Fusion Osmotin Polypeptides in a Bacterial Expression System ..................................................  48
Small-Scale Culture for Induction and Detection of Osmotin Fusion Proteins ..................................................  50
Induction and Isolation of Osmotin Fusion Proteins in Large Scale Cultures ..................................................  53
Construction of Cassettes Containing the Osmotin Coding Regions and the 35S CaMV Promoter ..................................................  55
Construction of a Agrobacterium tumefaciens Binary Vector for Transformation of Arabidopsis and Cotton Plants for Constitutive Expression of the Osmotin Genes ..................................................  60
Preparation of Electrocompetent Agrobacterium tumefaciens LBA4404 Cells ..................................................  62
Plant Material ..................................................  66
Induction, Isolation, and RT-PCR of RNA from Cotton Plant Extracts ..................................................  67
Induction, Isolation and Western Blotting of Osmotin Proteins from Cotton Plants ..................................................  72
Genomic Blot Analysis ..................................................  74
Construction of Three-Dimensional Models of the OSMI and OSMII Proteins ..................................................  75

3. RESULTS ......................................................................................................  76

Isolation and DNA Sequence Analysis of Cotton Osmotin cDNA Clones ..................................................  76
Isolation and DNA Sequence Analysis of a Cotton Osmotin Gene Cluster with Two Presumptive Genes and Two Pseudogenes Harbored in Three Overlapping Genomic Clones ..................................................  90
Genomic Blot Hybridization ..................................................  106
Western Blot Analysis of Osmotin Proteins in Cotton Tissue Extracts .................................................. 109
Total RNA Extraction for Northern Blot Hybridization and RT-PCR
Analysis ..........................................................................................................................110
Osmotin Expression Induced by Ethephon Treatment........................................113
Construction of Plasmid Vectors for Expression of Osmotin Fusion Polypeptides in
a Bacterial Expression System................................................................................117
Expression of Cotton Fusion Osmotin Polypeptides in an E. coli Expression
System.........................................................................................................................121
Construction of Agrobacterium tumefaciens Binary Plasmid Vector Constructs
for Transformation of Arabidopsis and Cotton Plants for Constitutive Expression of
Cotton Osmotin Genes..................................................................................................123

4. DISCUSSION ......................................................................................................138

APPENDIX A: DNA Sequence of GenBank Accession AY303690 ................148
APPENDIX B: DNA Sequence of GenBank Accession AY304007.................154
REFERENCE LIST....................................................................................................161
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Table of the current classifications of pathogenesis-related proteins</td>
<td>5</td>
</tr>
</tbody>
</table>

vi
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 35S Cauliflower mosaic virus (CamV) cassette</td>
<td>59</td>
</tr>
<tr>
<td>2. Representative alkaline-blot hybridization of isolated osmotin-like cDNAs</td>
<td>77</td>
</tr>
<tr>
<td>3. Complete DNA sequence of the coding region and 3’-UTR for pCcOSM47B</td>
<td>81</td>
</tr>
<tr>
<td>4. Complete DNA sequence of the coding region and 3’-UTR for pCcOSM52A</td>
<td>83</td>
</tr>
<tr>
<td>5. Deduced amino acid sequence of the cotton osmotin preprotein for pCcOSM47B</td>
<td>85</td>
</tr>
<tr>
<td>6. Deduced amino acid sequence of the cotton osmotin preprotein for pCcOSM52A</td>
<td>86</td>
</tr>
<tr>
<td>7. Phylogenetic comparison of a number of the DNA sequences of plant osmotin gene and cDNA coding regions</td>
<td>88</td>
</tr>
<tr>
<td>8. Phylogenetic comparisons of predicted amino acid sequences for plant PR5 preproteins</td>
<td>89</td>
</tr>
<tr>
<td>9. Alignments of predicted amino acid sequences of several plant osmotin preproteins</td>
<td>91</td>
</tr>
<tr>
<td>10. Predicted three-dimensional structures of cotton osmotin proteins I and II compared to the crystal structure of tobacco PR-5d protein</td>
<td>93</td>
</tr>
<tr>
<td>11. Physical maps of three overlapping cotton genomic clones encompassing a cluster of two osmotin genes (OSMI and OSMII) and two osmotin pseudogenes (OSMIII and OSMIV)</td>
<td>97</td>
</tr>
<tr>
<td>12. Nucleotide sequence of a DNA segment encompassing only the open reading frame and the 5’- and 3’-untranslated regions of the cotton osmotin gene I (OSMI)</td>
<td>99</td>
</tr>
<tr>
<td>13. Nucleotide sequence of a DNA segment encompassing only the open reading frame and the 5’- and 3’-untranslated flanking regions of the cotton osmotin gene II (OSMII)</td>
<td>102</td>
</tr>
<tr>
<td>14. Genomic blot hybridization for osmotin</td>
<td>107</td>
</tr>
<tr>
<td>15. Western blot analysis of tissue extracts from cotton plants</td>
<td>111</td>
</tr>
</tbody>
</table>
16. RT-PCR of RNA extracted from cotton plant tissues ...........................................114
17. Western blot analysis of cotton plants treated with water as a control ..................115
18. Western blot analysis of cotton plants treated with ethephon to induce osmotin protein expression .................................................................118
19. Diagram of the Pinpoint™ Xa-3 vector (Redrawn from Promega catalog) showing the insertion site for the osmotin I and II PCR products ........................................120
20. Western blot analysis of biotinylated proteins from bacterial extracts ....................124
21. Western blot analysis of osmotin fusion proteins from bacterial extracts ..............126
22. Western blot analysis of purified osmotin proteins from large-scale induction of E. coli ...128
23. The plasmid map of pCambia 2301 vector (redrawn from CAMBIA web site) .......132
24. Agarose gel electrophoresis of the PCR products from Agrobacterium transformed with the plasmid pCambia-35S-OSMI .........................................................134
25. Agarose gel electrophoresis of the PCR products from Agrobacterium transformed with the plasmid pCambia-35S-OSMII ..........................................................136
CHAPTER 1

ANALYSIS OF A COTTON GENE CLUSTER FOR THE ANTIFUNGAL PROTEIN

OSMOTIN

Introduction

Fungal diseases in cotton, such as various types of root rot and wilt annually cause destruction of a large percentage of the cotton crop and result in large economic loss. Almost all plants naturally produce several classes of defense proteins, called pathogenesis-related (PR) proteins, in response to a wide range of environmental stressors. One of the families of PR proteins, designated the PR5 proteins, are directed specifically against pathogens such as fungi and bacteria (Singh et al., 1987, Stintzi et al., 1993; Van Loon and Van Strien, 1999). In particular, the protein osmotin is produced in response to pathogen stress or osmotic stress (Singh et al., 1989; Abad et al., 1996; Yun et al., 1998), and shows homology with thaumatin, a sweet-tasting protein from the African shrub *Thaumatococcus danielli* (van der Wel and Loeve, 1972). Transgenic plants engineered to overexpress osmotin genes from tobacco and potato have been shown by other laboratories to produce effective anti-fungal proteins against several crop pathogens (Zhu et al. 1996; Batalia et al. 1996).

The focus of this dissertation research has been the molecular cloning and analysis of three overlapping genomic clones encompassing a cluster of two actual cotton osmotin genes
and two osmotin pseudogenes. This research project is the initial step in the production of transgenic cotton plants genetically engineered to constitutively overproduce the antifungal protein osmotin so as to increase the resistance of the transgenic plants against fungal and bacterial pathogen invasion.

At least thirty different fungal pathogens are capable of infecting cotton, and these infections result in crop loss that can have a great economic importance (Watkins, 1981; Lyon and Becerra-LopezLavelle, 2000). The fungal infections that occur most frequently in Texas are cotton root rot (Phymatotrichum omnivorum), Verticillium wilt (Verticillium albo-atrum), and Fusarium wilt (Fusarium oxysporum Esp. vaxinfectum). Symptoms displayed by pathogen-infected plants include marginal chlorosis or necrosis in leaves, discoloration of the stem vascular bundles, decrease in photosynthesis, and an increase in respiration, contributing to a significant reduction of plant productivity and a loss of economic output (Hamptom et al., 1990; Paplomatas et al., 1992; Dong et al., 2003). Other diseases are known that also damage cotton, such as boll rot, seedling disease complex, leaf spot, powdery mildew, and rust, are caused by several different fungal species such as Rhizocotonia, Thielaviopsis, Glomerella, Phytophthora, Fusarium, Puccinia, and Pythium, and also result in reduced yield or total crop loss. In addition to damage from fungal infections, cotton plants can be damaged by various bacterial blights, such as that caused by Xanthomonas campestris pv. Malvacearum (Watkins, 1981; Carpenter and Johnson, 2001).

Although many cultural practices and different cotton cultivars are employed to manage these diseases, many fungal pathogens such as Verticillium dahliae are able to remain viable in
soil many years (Dong et al., 2003; Schnathorst, 1981). The use of current upland cultivars has had little to no benefit in protection from infection and preventing pathogen damage (Dong et al., 2003). Although some cultivars derived from *Gossypium barbadense* are more resistant to infection, none have shown a genetic resistance that prevents infection of the vascular system of the plant (Dubrey and Slater, 1997). Although pathogen resistance may not be a simple inherited trait, these cultivar differences are assumed to have a genetic basis, since cultivar differences that affect the rate or level of responses correlate with levels of resistance (Cui et al., 2000).

The inherited differences that may affect resistance to *Verticillium* and other pathogens involve a number of signal transduction pathways that are considered to be parts of a generalized defense mechanism. The response includes the hypersensitive reaction, which leads to the induction of numerous plant genes encoding defense proteins. These responses: 1) confine the pathogens by increasing the quantities of structural proteins that are incorporated into the extracellular matrix; 2) produce “antibiotic”-synthesizing enzymes of secondary metabolism; and 3) increase the production of soluble proteins for pathogen defense. In cotton, these responses include increased synthesis of antimicrobial phytoalexins; sesquiterpenoids such as gossypol (Brooks and Watson, 1991; Liu et al., 1999; Luo et al., 2001; Tan et al., 2000); cell-wall lignification due to the synthesis of hydroxyproline-rich glycoproteins and the deposition of lignin (Van Loon and Van Kamen, 1970; Vance et al., 1980); and the production of a broad family of proteins termed pathogenesis-related (PR) proteins (Linthorst, 1991; Stintzi et al., 1993; Kitajima and Sato, 1999).
The PR-proteins are constitutively made by plants in low amounts, but are induced and accumulate in increased amounts after pathogen stress (Singh et al., 1989). The PR proteins were originally classified into five major groups based upon their electrophoretic mobilities in native (nondenaturing) polyacrylamide gels (Stinzi et al., 1993). The number of PR proteins has increased as the protein superfamily has been further studied, and has been divided into 14 subfamilies (van Loon and van Strien, 1999). For a PR protein to be denoted as a bona fide PR protein, it must meet two necessary and sufficient criteria. Firstly, it must be induced upon pathogen exposure in plant tissues where it is not normally expressed. Secondly, the expression of the presumptive PR protein must occur for a minimum of two plant-pathogen interactions, and/or a single plant-pathogen interaction must be confirmed by two independent laboratories (van Loon and van Strien, 1999). Using these two criteria, van Loon and van Strien (1999) classified the PR proteins into 14 families, each with at least one representative member (Table 1).

As new PR proteins are discovered, they are denoted by numbers (starting with one) in the order in which they are described. It is common, though incorrect, to define newly isolated mRNAs as PR family members, but since PR proteins are by definition proteins, genomic or cDNA sequences technically should not be named as PR proteins until fully characterized (van Loon et al., 1994). Proteins that are homologous to PR proteins, but found in healthy tissues, are not classified as PR proteins until induced by a pathogen or chemical inducer. Thus, the term PR-like protein is used until the two criteria are met for each newly identified protein or DNA sequence (van Loon and van Strien, 1999).
<table>
<thead>
<tr>
<th>Family</th>
<th>Type</th>
<th>Member</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR-1</td>
<td>Tobacco</td>
<td>PR-1a</td>
<td>Unknown</td>
</tr>
<tr>
<td>PR-2</td>
<td>Tobacco</td>
<td>PR-2</td>
<td>β-1,3-glucanase</td>
</tr>
<tr>
<td>PR-3</td>
<td>Tobacco P, Q</td>
<td></td>
<td>chitinase type I, II, IV, V, VI,</td>
</tr>
<tr>
<td>PR-4</td>
<td>Tobacco 'R'</td>
<td></td>
<td>chitinase type I, II</td>
</tr>
<tr>
<td>PR-5</td>
<td>Tobacco S</td>
<td></td>
<td>thauatin-like</td>
</tr>
<tr>
<td>PR-6</td>
<td>Tomato Inhibitor</td>
<td>I</td>
<td>proteinase-inhibitor</td>
</tr>
<tr>
<td>PR-7</td>
<td>Tomato p69</td>
<td></td>
<td>endoproteinase</td>
</tr>
<tr>
<td>PR-8</td>
<td>Cucumber</td>
<td>Chitinase</td>
<td>chitinase type III</td>
</tr>
<tr>
<td>PR-9</td>
<td>Tobacco 'ligin-forming peroxidases'</td>
<td></td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PR-10</td>
<td>Parsley 'PR-1'</td>
<td></td>
<td>ribonuclease-like</td>
</tr>
<tr>
<td>PR-11</td>
<td>Tobacco class V</td>
<td>chitinase</td>
<td>Chitinase type I</td>
</tr>
<tr>
<td>PR-12</td>
<td>Radish Rs-AFP3</td>
<td></td>
<td>Defensin</td>
</tr>
<tr>
<td>PR-13</td>
<td><em>Arabidopsis</em></td>
<td>THI2.1</td>
<td>Thionin</td>
</tr>
<tr>
<td>PR-14</td>
<td>Barley LTP4</td>
<td></td>
<td>lipid-transfer protein</td>
</tr>
</tbody>
</table>

Table 1. The table above shows the current classification of the pathogenesis-related proteins, with a representative member of each of the 14 classes and their function (modified from van Loon and van Strien, 1999).
PR and PR-like proteins have been found in a wide range of species and tissue types (Singh et al., 1987; Roberts and Selitrennikoff, 1990; Rebmann et al., 1991; Huynh et al., 1992; Nelson et al., 1992; Fils-Lycaon et al., 1996; Tatersall et al., 1997; Anžlovar et al., 1998; Newton and Duman., 2000). The PR proteins are synthesized as three different types, either basic, neutral, or acidic preproteins (Stintzi et al., 1993). The neutral/acidic forms have N-terminal signal sequences for targeting into the extracellular secretory pathway. These PR proteins accumulate in the extracellular compartment, and are normally acidic proteins induced by pathogen stress, but not by wounding (Bol et al., 1990; Brederode et al., 1991). The basic forms have an additional C-terminal signal sequence for vacuolar targeting. Removal of the C-terminal propeptides from the basic forms results in extracellular secretion of the mature protein (Melchers et al., 1993).

PR-5 proteins are produced in response to phytopathogens and have antifungal activity against a broad spectrum of fungal pathogens (Hu and Reddy, 1997; Koiwa et al., 1997; Malehorn et al., 1994). One PR-5 protein, in addition to being produced during pathogen stress, is produced during osmotic stress in tobacco cells, and hence was called osmotin (Singh et al., 1987; Nelson et al., 1992). Similar to other PR proteins, PR-5 proteins were found to be produced as three distinct forms and are classified as three separate subgroups: acidic (PR-S), basic (osmotin), and neutral proteins (osmotin-like-protein, OLP) (Kim et al., 2002). Some confusion results in isolation protocols from the use of the terms osmotin I and osmotin II to describe osmotin solubility, with osmotin I being soluble in standard buffer and osmotin II only being soluble in the presence of detergents (Singh et al., 1985; Singh et al., 1989). To avoid
this confusion, the nomenclature has evolved to include species abbreviations and an identifier to show that the designation is descriptive of a thaumatin-like protein, an osmotin protein, or an osmotin-like protein. Using cotton (*Gossypium hirsutum*) as an example, the first osmotin protein described in the PR-5 family would be termed Ghosm1 (or Gholp1) if it were determined to be the neutral or acidic form, though some researchers use the tlp (thaumatin-like protein) abbreviation, due to osmotins similarity to thaumatin, to encompass all PR-5 proteins. Osmotins are grouped with the other PR proteins since they are produced during pathogen stress (van Loon et al., 1994). In addition, they are induced by the same elicitors, are targeted to the same areas, and have antifungal activity (Vigers et al., 1991).

Tobacco osmotin has been shown to have 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 when infected with the fungus *Phytophthora infestans* delay development of disease symptoms (Liu et al., 1994; Zhu et al., 1996). Although all three forms of osmotins (neutral, acidic, and basic) have been identified in tobacco, it is unclear if the various forms have any biological significance (Velazhahan et al., 1999). It has been demonstrated that in the early stages of infection, transgenic plants expressing the extracellular form of osmotin appear to have fewer disease symptoms than plants that express the vacuolar form of osmotin (Velazhahan et al., 1999).

The research done by the Bressan group at Purdue (Nelson et al., 1992; Raghothama et al., 1993; Liu et al., 1995; Abad et al., 1996; Raghothama et al., 1997) has provided the basic understanding of the structure and possible function of osmotins and their genes. The
characteristics and possible functions of osmotins have also been investigated in other species, such as potato (Pierpoint et al., 1987), tomato (King et al., 1988), and grape (Tattersall et al., 1997). Through these studies, it appears that the three-dimensional structure of PR-5 proteins is highly conserved and that the signal-transduction pathways for inducing the osmotin proteins seem also to be conserved (Velazhahan et al., 1999).

PR-5 proteins tend to be quite resistant to acidic or basic denaturation, temperature-induced denaturation, and proteolytic activity. This resistance to denaturation is attributed to the presence of 16 highly conserved cysteine residues that form eight disulfide bonds (Koiwa et al., 1994). These conserved cysteine residues are found in zeamatin and thaumatin, as well as other osmotin-like proteins, as from Arabidopsis thaliana (GenBank Accession Number X89008). Three conserved domains (I, II, and III) are found when examining the three-dimensional structures of thaumatin from Thaumatococcus daniellii, zeamatin from Zea mays, and an osmotin-like protein from Arabidopsis thaliana (de Vos et al., 1985; Ogata et al., 1992; Batalia et al., 1996; Koiwa et al., 1997). These three domains contain two basic structural motifs, a folded ß-sheet and ß-ribbons containing small loops and turns (van der Wel and Loeve, 1972). Only one of the eight conserved disulfide bonds is located outside domain I or II. Domains II and III are thought to be involved in binding to membranes receptors in fungal membranes.

Zeamatin’s structural domains are similar to those of thaumatin (i.e. ß-barrels consisting of 11 ß-strands). The ß-barrel is somewhat flattened and has been referred to as a ß-sandwich. There is also a short arm consisting of one a-helix and three disulfide bonds (Batalia et al.,
Batalia et al. (1996), when examining the crystal structure of zeamatins, suggested that the protein might form a homodimer. This homodimer is similar to membrane channel pore-forming proteins that form cyclic aggregates (Velazhahan et al., 1999).

Roberts and Selitrennikoff (1990) proposed that one possible mechanism of PR-5 protein action may be through the formation of transmembrane pores in the fungal plasma membrane that could lead to permeabilization and rupture of the plasma membranes (Batalia et al., 1996). Roberts and Selitrennikoff (1990) suggested that this should be similar to the mechanisms of action of polypeptides such as melittin from bee venom (Mackler and Kreil, 1977), cecropins from the haemolymph of insects (Steiner et al., 1981), magainins from toad skin (Zasloff, 1987), and thionins from cereals (Okada and Yoshizumi, 1973; Bohlmann et al., 1988), as well as larger proteins like the bacteriocins such as colicin (Parker et al., 1989) and halocin (Torreblanca et al., 1989), and the vertebrate complement attack complex of the immune system (Bhakdi and Tranum-Jensen, 1987). Many of these polypeptides and proteins have amphipathic properties, and may act by binding to cells through a cationic region of the molecules, followed by insertion of a hydrophobic domain through the lipid bilayer of the membrane (Roberts and Selitrennikoff, 1990). However, the demonstration by Yun et al. (1997; 1998) that spheroplasts of resistant fungal cells could be sensitive to one osmotin isoform and resistant to another isoform may indicate that osmotins do not directly and non-specifically enter membranes, suggesting that osmotins may interact through a membrane receptor-protein complex. Osmotins may bind plasma membrane receptors (such as yeast PIR proteins) or regulatory elements of the mating pheromone system (STE proteins: STE4, STE18,
STE20, STE5, STE11, STE7, FUS3, KSS1, and STE12), subverting a fungal cell transduction pathway in their mechanism of action (Yun et al., 1997; 1998).

The pheromone receptors are known to have associated G proteins. Alterations in the associated G proteins have been shown to attenuate the effects of the osmotins (Yun et al., 1998). For example, mutations of SST2, a negative regulator of Gα proteins, result in supersensitivity to osmotins. The presence of osmotin is followed by a rapid phosphorylation of STE7, and this phosphorylation event precedes any changes in cell vitality or morphology, clearly demonstrating that osmotin subverts target cell signal transduction as part of its mechanism of action. (Yun et al., 1998). In this paper, it was also shown that a specificity exists between a particular PR-5 family protein and the plasma membrane of its target cell (Yun et al., 1997). In addition to the specificity of the PR-5 pheromone receptor interaction, cell wall components have also been shown to be important factors in determining resistance to osmotins. Among these identified components is a family of yeast cell wall-localized proteins, called the PIR proteins. Through the action of the PIR and STE proteins, osmotins have been shown to stimulate a mitogen-activated protein kinase (MAPK) signal system in yeast which induces changes in the cell wall (Yun et al., 1998). Of the many MAPK pathways, the best characterized MAPK pathway controls the mating of haploid yeast cells.

Mating of yeast haploid cells is triggered by the binding of pheromones to cell type-specific receptors on cells of the opposite mating type. The a-factor pheromone binds to an a-factor receptor (STE2, in MATa cells) and a-factor pheromone to a-factor receptor (STE3 in MATa cells). Once the signal is initiated, it is transmitted by a heterotrimeric guanosine
triphosphate-binding protein G protein (Dietzel and Kurjan, 1987; Miyajima et al., 1987; Whiteway et al., 1989; Yun et al., 1998). The G-protein β and γ subunits, through the STE20 protein kinase, stimulate MAP kinase modules (STE11, STE7, and FUS3/KSS1) whose components constitute a signal complex that is associated with the scaffolding protein STE5 (Teague et al., 1986; Elion et al., 1990; Rhodes et al., 1990; Leberer et al., 1992; Choi et al., 1994). The transcription of genes that are involved in the arrest of cell division, formation of projections, agglutination, and fusion of mating partners, and nuclear fusion are activated by STE12 (Dolan and Fields, 1990; Sprague and Thorner, 1992).

Although the MAPK pathway regulating pseudohyphal growth integrates nutritional cues, many signaling elements are identical to the mating pathway (Herskowitz, 1995). Upon nutrient deprivation, in particular nitrogen starvation, diploid cells respond by forming pseudohyphae chains of cells that resemble the hyphae of filamentous fungi (Gimeno et al., 1992). This formation of filamentous arrays is characteristic of invasive growth (Roberts and Fink, 1994). The formation of pseudohyphae and the filamentous arrays during invasive growth is regulated by STE20, STE11, STE7, FUS3, KSS1, and STE12 kinase modules, which are the elements shared with the mating-specific MAPK pathway (Liu et al., 1993; Roberts and Fink, 1994; Mösch et al., 1996; Cook et al., 1997; Madhani et al., 1997).

Further evidence of the activation of signal transduction cascades by plant antifungal proteins transmitted via G-proteins is seen by the abilities of G protein inhibitors to block the cytotoxic effect of plant defensin proteins (Thevissen et al., 1996). Although the evidence for osmotins acting through these MAPK cascades is compelling, mutations in genes for pheromone
receptors and G proteins do not lead to blockage of invasive growth or pseudohyphal development. Also, there is no competition between the osmotins and pheromones for common receptors, indicating other molecular components must exist in the signaling pathways (Yun et al., 1998). Although the exact mechanisms of action of osmotins remain unknown, osmotin-mediated signaling through the MAPK pathway does function to produce changes in the fungal cell wall that facilitates osmotin and other PR proteins access to the plasma membrane, resulting in cell death.

The 5'-flanking regions of several PR protein genes have been shown to have positive promoter/enhancer regulatory elements for inducible gene expression (Lam and Chua, 1989; Thomas, 1993; Kawagoe et al., 1994; Hatton et al., 1995; Ohme-Takaj and Shinshi, 1995; Sessa et al., 1995; Guilfoyle, 1997; Zhou et al., 1997; Kitajima et al., 1998; Xu et al., 1998; Nawrath and Métraux, 1999). For example, the tobacco osmotin gene characterized by the Bressan group (Nelson et al., 1992; Raghothama et al., 1993; Liu et al., 1995; 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. These signals have also been shown to be involved in the expression of other PR protein genes (Boller et al., 1983; Guo et al., 2000; Reymond, 2001; Seo et al., 2001). For example, the tobacco osmotin gene has an AT-1 box-like sequence (5'-AATTATTTTATG-3'), two ethylene-responsive elements (AGC boxes), and an abscisic acid-responsive element containing a G-box motif (Liu et al., 1995). These motifs function to increase osmotin expression when threatened with fungal pathogens or osmotic stress. Similar promoter elements that activate gene expression when
challenged with fungal pathogens have also been found in osmotin genes from potato (Zhu et al., 1995).

Although no PR-5 protein sequences have been reported in cotton, chitinase (PR-1) and β-glucanase (PR-2) proteins and their genes have been analyzed. The chitinase and β-glucanase genes have many similar promoter elements found in the chitinase and β-glucanase genes of tobacco (Hudspeth et al., 1996; Levorsan and Chlan, 1996; 1997; Chlan and Bourgeois, 2001). The chitinase and β-glucanase protein structures also have many analogous structural features across various plant species. It is likely that the cotton PR-5 proteins and their genes may be highly similar to previously studied osmotin proteins and genes.

In an effort to improve the overall resistance of cotton plants to fungal pathogens, it may be possible to use osmotin genes as a defense mechanism in transgenic plants overexpressing the antifungal protein. For this research, it is necessary to identify the PR-5 protein genes in the cotton genome and to assess the efficacy of the osmotin gene products against common cotton fungal pathogens. Additionally, in an effort to understand the regulation of the osmotin genes in cotton, it will be important to investigate the targeting of the native PR proteins, as well as the environmental signals that induce their production. Even though a number of promoter elements have been identified, the overall regulation of expression of the PR-protein gene superfamily is still not well understood. This dissertation describes the characterization of a family of cotton osmotin genes and cDNAs as a first step in analyzing the osmotin gene family in environmental/hormonal-specific, tissue-specific, developmental-specific gene-expression, chromosomal arrangement, gene organization, and regulation of expression of this largely
uncharacterized gene family. This dissertation describes the first PR-5 proteins characterized in cotton, the first PR-5 pseudogenes reported, and the first cluster of PR genes. The research work also involved the construction of recombinant vectors containing the coding regions and N-terminal targeting domains for the *OSMI* and *OSMII* genes for the overexpression of the osmotin proteins in *Arabidopsis* and cotton plants. The expression and isolation of osmotin fusion proteins using a bacterial expression system was also done in this work. The research presented increases our understanding of the complex responses of plants in pathogen interactions, and should ultimately lead to the production of transgenic cotton plants with increased resistance to fungal pathogens.
CHAPTER 2

MATERIALS AND METHODS FOR ANALYSIS OF THE COTTON OSMOTIN GENE FAMILY

Preparation of a Heterologous Radioactive Hybridization Probe for the Osmotin Gene

Dr. Ray Bressan of Purdue University, West Lafayette, IN (Nelson et al., 1992) provided a heterologous tobacco osmotin gene subcloned into pTZ18, a pGEM vector (Promega). This plasmid DNA was electroporated into E. coli strain DH5α, amplified as described in Sambrook and Russell (2001), and further purified by high performance liquid chromatography (HPLC) (Merion and Warren, 1989). An 884-bp HindIII/SalI fragment was isolated on a 1.0% LE agarose gel and purified by QIAquick gel extraction column (QIAGEN). Briefly, three volumes of QG buffer (QIAGEN) were added to one volume of gel, determined from the weight of the gel slice. The gel slice was incubated at 50°C, vortexing every 2-3 min., for a total of 10 min. or until the gel slice was completely dissolved. The mixture was then applied to the QIAquick column and bound to the membrane by centrifugation for 1 min. in a tabletop microfuge. After the flow-through was discarded, the column was washed by adding 750 µl of PE buffer (QIAGEN) to the column and centrifugation for one minute. To dry the column, it was centrifuged for an additional minute after the flow-through had been discarded. The DNA was eluted by pipetteing 30 µl of H2O directly onto the column, incubating it for 1 min., and centrifuging for 1 min. The purity and concentration of the isolated 884-bp
HindIII/SalI fragment was determined by electrophoresis on a 0.8% agarose gel. The yield was estimated by comparison with standard marker bands with known sizes and amounts of DNA. Using the Prime-a-Gene Labeling System (Promega), Dr. Irma Pirtle of this laboratory used this fragment as template to generate radioactively labeled random-primed DNA fragments (Feinberg and Vogelstein, 1983) for use as hybridization probe to screen cotton complementary DNA (cDNA) and genomic libraries.

**Screening a Cotton cDNA Library for Putative Osmotin cDNA Clones**

A cotton cDNA library, generated from mRNA of 48-hour dark-grown cotyledons (*Gossypium hirsutum* cv. Delta Pine 62) and harbored in Stratagene UniZAP lambda vector, was kindly provided by Dr. R.N. Trelease of Arizona State University (Ni and Trelease, 1991). This cDNA library was screened by the plaque-hybridization procedure (Benton and Davis, 1977) to identify positive clones encoding presumptive osmotin-like proteins. This work was done with Dr. David Yoder and Dr. Robert Pirtle of our laboratory. Plaques of the 48-hour cotyledon cDNA library were screened using the host bacterial strain *E.coli* XL-Blue MRF (Stratagene). The cDNA library was diluted in SM buffer (100 mM NaCl, 10 mM MgSO4, 50 mM Tris-HCl (pH 7.5), and 0.01% w/v gelatin). Ten microliter aliquots of the diluted library were used to inoculate XL1-Blue MRF bacteria that had been centrifuged and resuspended in 200 µl of 10 mM MgSO4 to an A600 of 0.5. The cultures were incubated for 30 min. at 37°C with shaking. Top agarose (100 ml of NZY broth (85 mM NaCl, 10 mM MgSO4, 0.5%( w/v) yeast extract, 1% (w/v) casein hydrolysate (pH 7.5)) with 7 gm of LE agarose, autoclaved) and
the inoculated XL1-Blue MRF cells were mixed at 55°C, plated on NZY bottom agar plates (100 ml NZY broth and 15 gm of Difco agar), and incubated overnight at 37°C. The plates with lysed bacterial lawns were placed overnight at 4°C. The plates were dry-lifted using positively-charged nylon membranes (Hybond-N+, Amersham). The nylon membrane filter replicates were placed plaque-side up for 2 min. on Whatman 3MM® paper saturated with denaturing solution (1.5 M NaCl and 0.5 M NaOH) and twice for 2 min. on Whatman 3MM® paper saturated with neutralizing solution (1.5 M NaCl and 1 M Tris-HCl (pH 7.3)). The membranes were then soaked in rinsing solution (0.2 M Tris-HCl (pH 7.5), 2X SSC (1X SSC is 150 mM NaCl and 15 mM Na citrate, pH 7.0)), and finally blotted on Whatman 3MM® paper. The nylon membranes were dried at 65°C for 30 minutes in a Blue M oven and then for 30 to 60 min. at 80°C in a vacuum oven to ensure fixation of the DNA to the positively-charged nylon membranes.

Prehybridization in a hybridization oven (Techne Hybridizer HB-1D) of the nylon membrane replicas was done for 4 hours at 55°C in a hybridization tube containing 40 mls of 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) (Sambrook and Russell, 2001). 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 sheared salmon sperm DNA (100 µg/ml), and 32P-labeled probe (Anderson and Young, 1985; Ausubel et al., 1987; Sambrook and Russell, 2001). Denhardt’s reagent (1X) is 0.02% (w/v) Ficoll (Type
400), 0.02% (w/v) polyvinylpyrrolidone, and 0.02% (w/v) bovine serum albumin (fraction V).

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 nylon replicas were dried at 65°C and placed in autoradiography using Kodak X-OMAT® film. Nylon membrane replicas that exhibited strong positive hybridization signals with the ³²P-labeled probe derived from the heterologous 884-bp HindIII/SalI tobacco osmotin probe were subjected to several rounds of plaque purification. These plaques were picked by removing small agar plugs from the top agar/NZY bottom agar plates and soaking the plugs overnight at 4°C in 1 ml of SM buffer (100 mM NaCl, 10 mM MgSO₄, 50 mM Tris-HCl (pH 7.5), and 0.01% w/v gelatin) containing a few drops of chloroform. The phages were repeatedly plated at decreasing concentrations until plaque purity was achieved (ie., until all plaques derived from the original plaque hybridized or “lit up”).

**Isolation of Phagemid DNAs Containing Osmotin cDNA Inserts**

Phagemid pBluescript SK(-) clones containing putative cotton osmotin cDNA inserts were excised in vivo using Stratagene ExAssist Interference-Resistant helper phage with the *E. coli* SOLR strain (Stratagene) as host. This work was done in conjunction with Dr. David Yoder of our laboratory. DNA was isolated by a mini-plasmid preparation (Titus, 1991) to isolate cDNA inserts from the phagemid vectors, and subsequently digested with the restriction endonucleases *Kpn*I and *Sac*I (Promega). The digested phagemid DNAs were fractionated on a 1% LE agarose gel, alkaline-blotted to positively-charged nylon membrane, and hybridized
with a $^{32}$P-labeled heterologous tobacco probe (as described above). The nylon membrane replicas were then placed in autoradiography with Kodak X-OMAT® X-ray film. Several intensely hybridizing bands were detected, and the corresponding phagemids were isolated by using the Wizard Miniprep kit (Promega). The cDNA inserts were then subjected to thermal cycle sequencing using the M13-40 and M13-50 universal sequencing primers. Two of the phagemids DNAs, designated pCcOSM47B and pCcOSM52A, were then isolated by large-scale plasmid preparation (Sambrook and Russell, 2001) and purified by HPLC (Merion and Warren, 1989) for use in double-stranded DNA sequencing.

**DNA Sequence Analysis of cDNA Clones pCcOSM47B and pCcOSM52A Encoding Cotton Osmotin Proteins**

The plasmid cDNA clones designated pCcOSM47B and pCcOSM52A encoding putative osmotin cDNAs were sequenced on both strands using a primer-based approach by terminator cycle sequencing (Fan et al., 1996) with the ThermoSequenase Radiolabeled Terminator Cycle Sequencing Kit (US Biochemicals) and [$a-^{33}$P]-labeled dideoxynucleoside triphosphates (Amersham). Oligodeoxynucleotide primers for use in the sequencing reactions were designed using DNASIS® software version 2.1 (Hitachi). The resulting primers, ranging in $T_m$ values from 55°C to 65°C, were synthesized by Biosynthesis (Lewisville, Texas). Using the GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer), the templates were denatured at 95°C for five min. for one cycle, followed by 60 cycles in which the template DNAs were denatured at 95°C for 30 seconds, the primer annealed at five degrees below the
primer Tₘs for 30 seconds, and the primers were extended for one min. at 72°C. After 60 cycles, this was followed by a final extension of 30 seconds for one cycle at 72°C. The reaction mixtures were maintained at 4°C upon completion of the reactions. The reactions were terminated with the addition of 4.5 µl of stop solution consisting of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. This work was done in collaboration with Dr. David Yoder of our laboratory.

The resulting sequencing reactions were analyzed manually on 0.45-mm thick denaturing 6% and 8% acrylamide gels (49% urea (w/v), 29:1 acrylamide/bis-acrylamide) using 1X glycerol-tolerant gel buffer (20 mM Tris, 7 mM taurine, and 0.1 mM Na₂EDTA) at 1400 V, 75 amps, and 90 watts. Upon completion of electrophoresis, sequencing gels were soaked in 3 L of 10% acetic acid and 12% methanol for 15 min. to remove excess urea and the gel was gently transferred to a pre-wetted 3MM® backing sheet. The sequence gels mounted on the 3MM® paper were dried under a constant vacuum using a Model 483 slab dryer (Bio-Rad) for 30 minutes at 60°C, followed by 3-4 hours at 80°C. The dried gels were then placed in autoradiography directly on Kodak X-OMAT® film for 24 to 36 hours. The sequence tracts from the gel autoradiograms were read manually, and then analyzed using DNASIS® software version 2.1. The extent of identities of the putative cotton osmotin cDNAs to other osmotin cDNA/gene sequences were determined using DNA and protein sequences downloaded from the GenBank database. The cDNA sequence for pCcOSM47B was assigned the GenBank Accession number AF192271, and the cDNA sequence for pCcOSM52A was assigned the GenBank Accession number AY301283.
Screening Cotton Genomic Libraries for Presumptive Clones Encompassing Osmotin Genes

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 of Benton and Davis, (1977) using the heterologous tobacco DNA probe described earlier. This aspect of the work was done in collaboration with Dr. David Yoder and Dr. Robert Pirtle of our laboratory. A 50 ml culture of the lambda-sensitive host bacterial strain *E. coli* K802 in 2 X NZYC broth (171 mM NaCl, 18.2 mM MgCl$_2$·6H$_2$O, 2% (w/v) NZ amine, 0.2% (w/v) casamino acids, 1% (w/v) yeast extract) was grown overnight in a shaker incubator at 37°C. Approximately 1 x 10$^7$ plaques of EMBL3 lambda phage containing the cotton genomic library were screened in order to over-represent the entire cotton genome (3-4 fold). The 50 ml culture of the bacterial host cells was pelleted and then resuspended in 15 ml of 10 mM MgSO$_4$. The resuspended host cells were then infected with sufficient phage to achieve a multiplicity of infection (M.O.I. = number of phage/number of bacteria) of 0.1, in order to achieve a plaque density of about 3 x 10$^5$ plaque-forming units (p.f.u.) per plate. The infected cultures were incubated for 15 min. at 37°C. The infected host cells were mixed with four ml of top agarose (0.7% agar) (48°C), and spread evenly on NZY bottom agar plates (0.5% yeast extract, 1% NZY amine, 0.5% NaCl and 0.02M MgSO$_4$, in 1.5% agar). The recombinant phages were incubated on the bacterial lawn at 37°C overnight. The phage growth resulted in lysed plaques with an approximate diameter of 1.5 mm covering the entire plate (about 15,000-18,000 plaques/plate). Prior to making nylon
membrane replicas, the plates were chilled to 4°C. Each plate was overlaid with a pre-wetted (in 1 M NaCl) Hybond N+ nylon membrane (Amersham). After four minutes, the membranes were removed and successively placed on a stack (3-4) of Whatman 3MM® papers saturated with denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 7 min., then placed on two successive stacks of Whatman 3MM® papers (3-4) saturated with neutralizing solution (0.5M NaCl, 0.1 M Tris-HCl, pH 7.3) for 5 min. each, and the filters were immersed briefly in rinsing solution (2xSSC, 0.2 mM Tris-HCl, pH 7.5). The membrane filter replicas were then dried at 37°C and baked in a vacuum oven at 80°C for two hours.

Prehybridization in a hybridization oven (Techne Hybridizer HB-1D) of the positively-charged nylon replicas was done for 4 hours at 55°C in a hybridization tube containing 40 ml of 6X SSC (1X SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 5X Denhardt’s reagent, 0.5% SDS, and denatured sheared salmon sperm DNA (100 µg/ml) (Sambrook and Russell, 2001). 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 sheared salmon sperm DNA (100 µg/ml), and 32P-labeled probe (Anderson and Young, 1985; Ausubel et al., 1987; Sambrook and Russell, 2001). 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 nylon membrane replicas were dried at 37°C and placed in autoradiography using Kodak X-OMAT® film with a lanthanide intensifying screen at -80°C. Plaques exhibiting intense positive signals were subjected to successive rounds of plaque
puriﬁcation until all plaques on a single plate hybridized to the labeled probe, indicative of plaque purity. To identify lambda genomic clones that corresponded to the cDNA insert in the clone pCcOSM47B, the DNAs from several phage clones were isolated by a mini-lysate procedure described below.

Mini-lysate Phage Preparations for Genomic Clones Containing Potential Cotton Osmotin Genes

Agar plugs containing phage plaques (with M.O.I. of 0.2 to 0.4) that gave strong positive hybridization signals were soaked in 1 ml SM buffer (20 mM Tris-HCl (pH 7.5), 20 mM MgSO$_4$·7H$_2$O, 100 mM NaCl and 2% gelatin) at 4°C overnight. Aliquots of the mixtures were mixed with 200 µl portions of an *E. coli* K802 culture (in 10 mM MgSO$_4$) for 30 minutes at 37°C to adhere the phages. The bacterial cultures were then transferred to 50 ml of LB broth (170 mM NaCl, 1% (w/v) bactotryptone, and 0.5% yeast extract, pH 7.5) and incubated overnight at 37°C. To complete lysis, the cultures were incubated at 37°C for 15 min. with 1 ml of chloroform. The lysates without the chloroform were decanted into 50 ml conical siliconized polypropylene tubes and centrifuged in a Sorvall table top centrifuge for 15 min. at 3,000 rpm (2,500 xg) using a swinging bucket rotor. For storage, the supernatants were transferred to clean 50 ml conical tubes and a few drops of chloroform were added before placing the tubes at 4°C. The DNAs were extracted from 15 ml portions of each lysate by a mini-lysate preparation (Tiemeier et al., 1977; Sambrook and Russell, 2001).
Cellular debris was removed by centrifugation at 7,500 rpm (6,600xg) in a SA-600 rotor for 10 minutes at 22°C in a preparative centrifuge (Sorvall RC-5C). The supernatants were digested with DNase I (1 mg/ml) and RNase A (1 mg/ml) for 40 min. at 37°C. The supernatants were placed on ice and incubated for one hr after the addition of one volume of a 20% PEG (polyethylene glycol) and 2 M NaCl solution for precipitation of the phage. Phage particles were recovered by centrifugation at 16,800 xg for 20 minutes at 4°C in a Sorvall centrifuge. The resulting pellets were collected and resuspended in 400 µl of STE buffer (20 mM Tris-HCl, 250 mM NaCl, and 1 mM Na₂EDTA, pH 7.5). Any remaining cellular debris was removed by centrifugation at maximum rpm for 2 min. at 4°C in an Eppendorf microfuge.

To remove phage proteins, one volume portions of phenol (saturated with TE (10 mM Tris-HCl (pH 7.5), 1 mM Na₂EDTA) were added to the resuspended solutions, vortexed thoroughly, and centrifuged in a microfuge for 5 min. at room temperature. The aqueous phases (upper) were transferred to new 1.5 ml siliconized microfuge tubes and 0.5 volumes of phenol/chloroform:isoamyl alcohol (24:1) were added, vortexed, and then centrifuged for 5 min. at room temperature.

The aqueous phases (upper) were transferred to new 1.5 ml tubes and one volume portions of chloroform: isoamyl alcohol (24:1) were added, vortexed, and then centrifuged for 5 min. at room temperature. The aqueous phases (upper) were transferred to new 1.5 ml tubes and the DNAs precipitated by adding two volumes of cold 95% ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.2) and chilling overnight at -20°C. After warming the solutions up to room temperature, the DNAs were pelleted by centrifugation at 4°C for 30 minutes in the
microfuge. The pellets were washed with 70% ethanol and vacuum-dried before dissolving in 50 µl per sample of TE buffer. The phage DNAs were digested with several restriction endonucleases, the fragments resolved on 0.8% LE agarose gels, alkaline-blotted, and hybridized.

Prehybridization was done for 4 hours at 55°C in a solution containing 6X SSC (1X SSC is 150 mM NaCl 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 sheared salmon sperm DNA (100 µg/ml), and 32P-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 nylon replicas were dried at 37°C and placed in autoradiography using Kodak X-OMAT® film with an intensifying screen at -80°C. Those lambda clones that gave intense positive signals were selected for DNA sequencing (Fan et al., 1996).

Screening Lambda Clones by Sequencing with cDNA Insert-Specific Primers

Using the oligonucleotide primer designated 29F3 (5’-TATTCAGCCAGGGTGTTTGG-3’), designed specifically for sequencing a unique stretch of the coding regions of the osmotin cDNA inserts, lambda clones harboring putative osmotin genes were subjected to terminator cycle sequencing (Fan et al. 1996) using the
ThermoSequenase Radiolabeled Terminator Cycle Sequencing Kit (USBiochemicals) and \([a-^{33}\text{P}]\)-labeled dideoxynucleoside triphosphates (Amersham). The reaction mixtures were then denatured for 5 min. at 95°C in a thermal cycler. The reactions then ran for 35 to 40 cycles with the following regimen: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C, and polymerization for 30 seconds at 72°C. After the final cycle, the reactions were maintained at 72°C for 30 seconds and then cooled to 4°C (Innis and Gelfand, 1990). One genomic clone, designated LCgOSM16B, was selected for further sequence analysis, since its preliminary sequence indicated tentative sequence identity with the cDNA insert of the cDNA clone pCcOSM47B.

**Large-scale Phage Preparations of the Genomic Clone LCgOSM16B**

Using the stored lysate containing the phage LCgOSM16B, 50 ml of LB broth was inoculated with pre-incubated bacterial suspensions and the diluted phage to achieve an M.O.I. of 0.2 to 0.4. The growth of the lambda phage was monitored each day by titering the phage with *E. coli* K802 on NZY top agarose and NZY bottom agar plates. The titered phages were continually amplified to obtain greater than 1 x 10^{10} plaque-forming units (p.f.u.). Once the titers were sufficiently high, several 2 L Erlenmeyer flasks, each with 500 ml of LB broth, were inoculated with pre-incubated phage and *E. coli* K802 to achieve a M.O.I. of 0.2 to 0.4), and these cultures were incubated overnight at 37°C. After the overnight incubation, 8 ml of chloroform were added to each flask, and the cultures were shaken an additional 15 min. at 37°C to achieve complete cell lysis. The lysates, but not the chloroform layers, were transferred
to fresh sterile 2 L flasks. RNase A (10 mg/ml, heat-treated) and DNase I (5 mg/ml) were added to final concentrations of 1 µg/ml each and the lysates were incubated at room temperature for 30 minutes. Subsequently, NaCl was added to a final concentration of 1M and the flasks were shaken at room temperature until the NaCl was completely dissolved (approximately 15 min.).

To precipitate the chromosomal DNA and other cellular debris, the flasks were stored at 4°C for two hours. The lysate solutions were then transferred to 500 ml polypropylene centrifuge bottles and then centrifuged in a Sorvall GS-3 rotor for 30 min. at 5,200 x g (6,000 rpm) at 4°C. The supernatants were transferred to sterile 2 L flasks with stir bars. Polyethylene glycol (PEG 8000) was added to a final concentration of 10% (w/v) to each flask. The lysates were stirred for two hours to fully dissolve the PEG. The flasks were stored overnight at 4°C to precipitate the phage particles. The precipitated phage were transferred to 500 ml centrifuge bottles and centrifuged at 5,200 x g (6,000 rpm) at 4°C for 30 min. in the Sorvall GS-3 rotor. The supernatants were decanted and discarded, leaving the precipitated phage pellets in the centrifuge bottles. SM buffer (4 ml) was added to suspend each pellet, which was then transferred to another one of the centrifuge bottles. The first bottle was rinsed with an additional 2 ml of SM buffer and then transferred to the second centrifuge bottle, and so forth. The pellets were finally resuspended and transferred to 15 ml polypropylene tubes (Falcon). After centrifuging for 15 min in a Sorvall table top centrifuge at 3,200 rpm (2,500 xg), the supernatants were transferred to clean tubes. The transferred supernatants were centrifuged several times until clarified of any particulate matter.
The phage solutions, in 7.4 ml portions, were transferred into pre-weighed 10.4 ml polycarbonate Oak Ridge ultracentrifuge tubes (Nalgene). To determine the amount of CsCl to add to each tube, the tubes were re-weighed and the net weight of the solution was multiplied by the factor 0.71. After adding the calculated amount of CsCl, the tubes were balanced using CsCl dissolved in SM buffer (71% CsCl by weight). The balanced Oak Ridge tubes were loaded into a Beckman Ti 75 angled rotor, and pre-chilled to 4°C for ultracentrifugation in a Beckman L-65 ultracentrifuge. Ultracentrifugation of the tubes was done for 18-24 hours at 220,000 xg under a vacuum (50 microns or less). A siliconized Pasteur pipette was used to remove an opaque band from each tube containing the phage particles. The recovered phage were dialyzed at 4°C in dialysis bags (2.5 cm dry flat width, 12,000 daltons molecular cut-off, Fisher), and 1 L of dialysis buffer (100 mM Tris-HCl (pH 8.0) and 0.3 M NaCl) with continuous stirring in a 1 L glass cylinder. The dialysis tubing was previously prepared by boiling cut pieces of tubing in 1 L of 215 mM EDTA and 70 mM sodium bicarbonate, washing with sterile water, and storage in 70% ethanol at -20°C. The dialysis buffer solution was replaced every eight hours with fresh buffer at 4°C. After 24 hours of dialysis, the phage solution was transferred to 50 ml round bottom polypropylene centrifuge tubes. To remove the phage protein coat, the solution was extracted twice with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). The organic phases were back-extracted with an equal volume of sterile distilled water and then combined with the original aqueous phases. The phage DNA was then precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes
of 95% cold ethanol at -70°C for one hour. The yield of the purified LCgOSM16B phage DNA was determined to be about 1.7 mg by an A260 measurement.

Physical Mapping of the Cotton Genomic Insert in LCgOSM16B DNA

The DNA from the genomic clone LCgOSM16B was digested with numerous single and double restriction endonuclease digestions for physical mapping by agarose gel electrophoresis and alkaline blotting (Reed and Mann, 1985). All the restriction enzyme digestions were in accordance with the manufacturer’s instructions. Restriction digests were typically performed in a volume of 20 µl. The reaction mixture contained 2.0 µl of the appropriate 10X buffer, 1 to 4 µg of DNA, 10-20 units of restriction enzyme (or enzymes), and sufficient H2O up to volume. The digests were incubated at the appropriate temperature for 2-16 hours, depending on the stability of each enzyme. The LCgOSM16B DNA fragments were electrophoresed on both 0.8% and 2.0% agarose gels. To determine the sizes of very small restriction fragments (less than 1.0 kb), a 4.0% agarose gel consisting of NuSieve:LE agarose (3:1) was used. The restriction digests were mixed with 6X loading dye (0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll 400, 10 mM Tris-HCl (pH 7.5), and 50 mM EDTA (pH 8.0)) and loaded into the gel. The gels were electrophoresed at 2.3 volts/cm in a horizontal gel apparatus using TAE buffer (400 mM Tris-acetate and 2 mM EDTA, pH 8.5). The resolved fragments were transferred to positively-charged nylon membranes in 0.4M NaOH, dried at 37°C, and then hybridized using the heterologous tobacco osmotin probe.
Prehybridization in a hybridization oven (Techne Hybridizer HB-1D) of the positively-charged nylon replicas was done for 4 hours at 60°C in a hybridization tube containing 40 ml of 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) (Sambrook and Russell, 2001). Hybridization was done overnight at 60°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 sheared salmon sperm DNA (100 µg/ml), and 32P-labeled probe (Anderson and Young, 1985; Ausubel et al., 1987; Sambrook and Russell, 2001). After hybridization, the membranes were rinsed once at room temperature with 2X SSC for 5 min., once with 2X SSC, 0.1% SDS at 60°C for 30 min., and twice with 1X SSC, 0.1% SDS at 60°C for 30 min. The nylon replicas were dried at 37°C and placed in autoradiography using Kodak X-OMAT® film with an intensifying screen at -80°C. The position of the putative osmotin transcription unit was localized within the cotton DNA segment in the LCgOSM16B lambda clone by constructing the physical map.

**Subcloning and Sequencing of a SalI/BamHI Fragment of LCgOSM16B Encompassing One Osmotin Gene and a Portion of a Second Osmotin Gene**

Based upon the physical map of the genomic clone LCgOSM16B, a 7.2-kb SalI/BamHI fragment was deduced to encompass the entire open reading frame of a cotton osmotin gene (designated the OSMI gene). This 7.2-kb fragment was selected for isolation and subsequent subcloning into the SalI/BamHI sites of the plasmid vector pUC19. The
LCgOSM16B phage DNA and pUC19 vector DNA were both doubly digested with the restriction endonucleases SaI and BamHI. A small amount of the digest was electrophoresed on a 0.8% agarose gel to ascertain the completeness of the digest and to estimate the amount of the 7.2-kb fragment. The vector DNA and lambda DNA digests were then phenol/chloroform (1:1)-extracted and then precipitated in 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol for 30 min. at -80°C. The DNA was pelleted by centrifugation at 4°C at 12,000 rpm (11,750 xg) for 15 min., washed three times with 70% ethanol, vacuum dried, and resuspended in minimal TE buffer to dissolve the pellets. The 7.2-kb SaI/BamHI fragment of LCgOSM16B and the linearized pUC19 vector DNA were purified by electrophoresis on a 0.8% agarose gel and excised from the gel. The slices were then put in dialysis bags (2.5 cm dry flat width, 12,000 MW cut-off, Fisher) with 1X E buffer (40 mM Tris-acetate, 2 mM Na₂EDTA, pH 8.0), tied on both ends, and placed in a horizontal electrophoresis apparatus in E buffer. The DNA was extracted from the agarose slice by electroelution at 4-5 V/cm for 2 to 3 hours, with a brief 2 to 3 min. reversal of electrophoretic polarity at the end of the run (Sambrook and Russell, 2001). The resulting eluates from the dialysis bags were frozen and lyophilized. The purity and concentration of the 7.2-kb SaI/BamHI fragment of LCgOSM16B and the linearized pUC19 vector DNA were determined by electrophoresis on a 0.8% agarose test gel and comparison of the ethidium bromide-stained bands with those of standard marker bands with known sizes and amounts of DNA.
The 7.2-kb SalI/BamHI fragment of LCgOSM16B was subcloned into the SalI/BamHI sites of the pUC19 vector as follows. To concentrate the eluted DNAs, the 7.2-kb SalI/BamHI DNA fragment and the vector DNA eluates were first mixed at a 3:1 ratio (insert:vector), and 1 µl of 5S carrier RNA (10 µg/µl) added. Then the mixture was phenol/chloroform (1:1)-extracted and precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol for 30 min. at -80°C. The DNA was pelleted by centrifugation at 4°C at 12,000 rpm (11,750 xg) for 15 min., washed three times with 70% ethanol, vacuum dried, and resuspended in 7 µl of distilled water. The DNA was mixed with 2 µl of 5X DNA ligase buffer (250 mM Tris-HCl (pH 7.6), 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000) and 1.0 µl (1 unit) of T4 DNA ligase. The ligation mixture was incubated at 22°C for 3 hours.

Preparation and Transformation of Electrocompetent E. coli DH5a cells

To prepare electrocompetent cells for transformation, 200 µl of an E. coli DH5a glycerol stock was inoculated into 50 ml of LB medium (Luria-Bertani medium; 10 grams of NaCl, 10 grams of Bacto-tryptone, and 5 grams of yeast extract per liter), and grown overnight at 37°C at 250 rpm in a New Brunswick shaker/incubator. Fifteen ml of the overnight culture was inoculated into several 500 ml cultures in 2 L flasks. The large cultures were grown overnight in 2 L flasks at 37°C with shaking. The next day, the bacterial cells were harvested by centrifugation in a Sorvall GS-3 rotor at 5,500 rpm (2,790 xg) for 15 min. at 4°C. After removal of the supernatants, the cells were resuspended in one volume of sterile ice-cold water.
The bacterial cells were again resuspended and then re-centrifuged in the Sorvall GS-3 rotor at 5,500 rpm (2,790 xg) for 15 min. at 4°C. The cells were resuspended twice in 0.5 volumes of ice-cold water, and once in 0.02 volumes of ice-cold water, with centrifugation to re-pellet the cells between each resuspension. The final resuspension was in 0.003 volumes of cold filter-sterilized 10% glycerol. The electrocompetent *E. coli* DH5a cells were aliquoted into Eppendorf tubes and stored at -80°C.

To transform the electrocompetent *E. coli* DH5a cells, 1-2 ng of the recombinant plasmid DNA from the ligation of the 7.2-kb *SalI/BamHI* fragment of LCgOSM16B and the linearized pUC19 vector DNA was mixed with 40 µl of the electrocompetent cells in a disposable BTX® electroporation cuvette with a 1 mm gap. After chilling on ice for 1 min., the plasmid was transformed into the *E. coli* cells by electroporation in a BTX® Electro Cell Manipulator® (ECM 395) set in the high voltage mode (3kV) with a 1.5 kV set charge with a 5-6 millisec pulse length. Immediately following the electric pulse, 1 ml of LB media was added to the cuvette and the suspension gently mixed by pipetting. The cell mixture was then transferred into a sterile Falcon tube and incubated for 30 to 60 min. at 37°C with shaking at 225 rpm. The transformed cells were plated in 200 µl aliquots on LB-ampicillin (50 µg/ml) plates. The plates had previously been prepared with 4 µl of IPTG (isopropyl thio-β-D-galactoside, 200 mg/ml) and 40 µl of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 20 mg/ml) with incubation overnight at 37°C. Bacterial colonies containing the recombinant plasmids were selected based on their white appearance (non-recombinant colonies being blue) and re-streaked on fresh LB-ampicillin (50 µg/ml) plates containing IPTG and X-gal for colony
purification and to serve as master plates. The master plates were incubated at 37°C overnight and then stored at 4°C.

To screen for recombinant cells, selected colonies from the master plates were inoculated into 25 ml LB-ampicillin (50 µg/ml) medium and grown overnight at 37°C with shaking. The DNAs from the cultures were isolated using a Wizard mini-plasmid preparation kit (Promega). Five to ten ml of each culture were pelleted by centrifugation at 3,200 rpm for 10 minutes in a table top centrifuge (Sorvall). The supernatant was discarded and the inner walls of the tubes were briefly blotted dry. The cells were resuspended in 200 µl of cell resuspension solution (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, and RNase A (100 µg/ml)) and transferred to a 1.5 ml siliconized microfuge tubes. The cells were mixed with 200 µl of cell lysis solution (0.2 M NaOH and 1% SDS) and incubated for 3 to 5 min. before 200 µl of neutralizing solution (1.32 M potassium acetate) was added and mixed.

The bacterial DNA and cellular debris were pelleted by centrifugation at 14,000 rpm for 10 minutes at room temperature in a tabletop microfuge. The supernatants were transferred to Wizard® Minipreps Columns (Promega) with 3 cc syringe barrels, attached to a vacuum manifold (Promega), containing 1 ml of Wizard® Minipreps DNA Purification Resin. The lysates and resin mixtures were vacuum-eluted from the columns, and then washed with 2 ml of column wash solution (80 mM potassium acetate, 8.3 mM Tris-HCl (pH 7.5), 40 mM EDTA, and 55% ethanol), and dried for 30 seconds by vacuum. After the syringe barrels were removed, the minicolumns were placed in clean microfuge tubes and centrifuged for 2 min. at 10,000 xg to remove any residual column wash solution. The minicolumns were transferred to
1.5 ml microfuge tubes and 50 µl of sterile H₂O was added to each tube. After one min. of incubation at room temperature, the columns were centrifuged for 20 seconds to elute the DNA samples. The isolated plasmid DNAs were doubly digested with the restriction endonucleases SalI and BamHI, electrophoresed on 0.8% LE agarose gels, transferred to positively charged Hybond N+ nylon membranes (Amersham) by alkaline blot hybridization, and hybridized with the heterologous tobacco probe as described previously to determine subclones containing the desired insert. The resulting recombinant subclone was designated pCgOSM16B.

Both strands of the 7.2-kb cotton insert in pCgOSM16B were sequenced, using a primer-based approach, with primers either specific to the plasmid subclone pCcOSM47 or unique to the plasmid subclone pCgOSM16B. These primers were designed using DNASIS software as described earlier. The genomic DNA insert in pCgOSM16B was subjected to DNA sequencing on an Applied Biosystems Model 377XL DNA Sequencer by Lone Star Labs, Houston, Texas. 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 (Fan et al., 1996).

**Sequencing of the XhoI/BamHI Fragment of pCgOSM16B Encompassing the Entire Open Reading Frame of the OSMI Gene.**

To further confirm its DNA sequence, the plasmid pCgOSM16B was doubly digested with the restriction endonucleases XhoI and BamHI, electrophoresed on a 0.8% LE agarose
gel, and the 5.5-kb cotton genomic segment (containing the \textit{OSMI} gene) and a 5.0-kb segment (containing the pUC19 vector and the \textit{OSMII} gene) were purified using the QIAquick Gel Extraction Kit (QIAGEN). Both strands of the pCgOSM16B fragments were sequenced, using a primer-based approach, with the designed primers specific to either the subclone pCcOSM47 or the subclone pCgOSM16B. The genomic DNA insert in pCgOSM16B was subjected to DNA sequencing on an Applied Biosystems Model 377XL DNA Sequencer by Lone Star Labs (Houston, Texas), as described above. Terminator cycle sequencing was done manually as necessary to confirm difficult regions with compression effects or other sequence anomalies (Fan et al., 1996). Analyses of the DNA and deduced amino sequences were done with DNASIS version 2.1 software (Hitachi). The genomic sequence in the EMBL3 lambda vector containing the complete open reading frame for the \textit{OSMI} gene and the partial open reading frame for the \textit{OSMII} gene has been given the GenBank Accession Number AF304007.

Based upon the sequencing data, the 5.5-kb \textit{Xhol}/\textit{BamHI} fragment from the pCgOSM16B subclone was selected for subcloning. This 5.5-kb \textit{Xhol}/\textit{BamHI} fragment from the pCgOSM16B subclone hybridized to the homologous cotton osmotin probe and was selected for isolation and subsequent cloning into the multiple cloning site of the plasmid vector pGEM\textsuperscript{®}7Zf(+). The pCgOSM16B DNA and vector pGEM\textsuperscript{®}7Zf(+) DNA were both digested to completion with the restriction endonucleases \textit{Xhol} and \textit{BamHI}. Due to the incompatibility of the endonuclease digestion conditions, the double digestion was performed in two steps. Briefly, the DNA was first digested with \textit{Xhol}, followed by precipitation of the DNA and resuspension before digestion with \textit{BamHI}. The 5.5-kb fragment from the pCgOSM16B clone
and the linearized pGEM®7Zf(+) vector DNA were electrophoresed on a 0.8% agarose gel and extracted using the QIAquick Gel Extraction Kit (QIAGEN). Three volumes of QG buffer (QIAGEN) were added to one volume of gel, determined from the weight of the gel slice. The gel slice was incubated at 50°C, vortexing every 2-3 min., for a total of 10 min. or until the gel slice was completely dissolved. The mixture was then applied to the QIAquick column, allowing the DNA to bind to the membrane. The column was placed in a microfuge tube and centrifuged for 1 min. using a tabletop microcentrifuge. After the flow-through was discarded, the column was washed by adding 750 µl of PE buffer (QIAGEN) to the column and centrifuged for 1 min. The column was centrifuged for an additional minute after the flow-through had been discarded for drying. The DNA was then eluted by pipetting 30 µl of sterile distilled water directly into the column, incubation for 1 min., and centrifugation for 1 min. The purity and concentrations of the isolated 5.5-kb fragment from the pCgOSM16B clone and the linearized pGEM®7Zf(+) vector were checked by electrophoresis on a 0.8% agarose gel, and the yields were estimated by comparison with standard marker bands with known sizes and amounts of DNA. The 5.5-kb fragment from the pCgOSM16B clone was subcloned into the XhoI/BamHI sites of the pGEM®7Zf(+) vector. For ligation, the 5.5-kb fragment and pGEM®7Zf(+) vector DNA were mixed in a 3:1 ratio (insert:vector). Two µl of 5x ligase buffer (250 mM Tris-HCl (pH 7.6), 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000), and 1.0 µl (1 unit) T4 DNA ligase was added to 9 µl of the DNA mixture. The ligation mixture was incubated at 22°C for 4 hours.
To transform the electrocompetent *E. coli* DH5a cells, 1-2 ng of the recombinant plasmid DNA from the ligation of the 5.5-kb fragment from the pCgOSM16B clone and the linearized pGEM<sup>®</sup>7Zf(+) vector were mixed with 40 µl of the electrocompetent cells in a disposable BTX<sup>®</sup> electroporation cuvette with a 1 mm gap. After chilling on ice for 1 min., the plasmid DNA was electroporated into the *E. coli* cells using a BTX<sup>®</sup> Electro Cell Manipulator<sup>®</sup> (ECM 395) set in the high voltage mode at 3 kV with a 1.5 kV set charge having a 5-6 msec pulse length. The electroporated transformed cells were plated as described previously. The plasmid DNAs were selected as described previously. The isolated plasmid DNAs were doubly digested with the restriction endonucleases *Xho*I and *BamH*I. The DNA was electrophoresed on 0.8% LE agarose gels and the subclone yielding the appropriate sized fragments was designated pCgOSM16BI-*Xho*I/*BamH*I. Both strands of the cotton insert pCgOSM16BI-*Xho*I/*BamH*I were sequenced, using a primer-based approach, with primers either specifically designed for pCgOSM16B or pCgOSM16BI-*Xho*I/*BamH*I. The genomic DNA insert in pCgOSM16BI-*Xho*I/*BamH*I was subjected to DNA sequencing by Lone Star Labs (Houston, Texas) and analyzed with DNASIS software as described previously.

**Preparation of Homologous Radioactive Hybridization Probes for the Osmotin Genes**

Two homologous probes were prepared from pCgOSM16B DNA. The first was from an 884-bp *EcoRV* fragment containing the open reading frame of an osmotin gene (*OSMI*) found within the 5.5-kb *Xho*I/*BamH*I fragment of the pCgOSM16B plasmid. The second was from a 591-bp polymerase chain reaction (PCR) fragment amplified from the 3’-flanking region...
of a partial osmotin gene (**OSMII**) found in the *SalI/XhoI* fragment of the pCgOSM16B plasmid. The pCgOSM16B DNA was digested with the restriction endonuclease *EcoRV* and the DNA fragments were separated on a 1.0% LE agarose gel. A 884-bp *EcoRV* fragment was located in the 1.0% LE agarose gel and extracted from the gel using the QIAquick Gel Extraction Kit (QIAGEN) as described earlier. The DNA was then eluted by pipetting 30 µl of sterile distilled water directly into the column, incubation for 1 minute, and centrifugation for 1 minute. This fragment was then used by Dr. Irma Pirtle of our laboratory to generate $^{32}$P-labeled DNA fragments by the random priming method (Feinberg and Vogelstein, 1983) using the Prime-a-Gene Labeling System (Promega), for use as hybridization probe.

The second probe was produced by amplifying a 591-bp region in the 3’-untranslated region (UTR) of the *OSMII* gene. The 591-bp fragment was amplified by the polymerase chain reaction (PCR) using primers designed specifically to amplify this intergenic region, including unique restriction sites that would allow the PCR product to be subcloned directly into the multiple cloning site of the pGEM®7Zf(+) vector. The forward primer 5’-CTCGAATTTCCACACACATCATCTTCACC-3’, was designated OSMPRBF and contains an *EcoRI* restriction site (underlined). The reverse amplimer, 5’-CTCAAGCTTTACAACCGAAACCAAGCAC-3,’ was designated OSMPRBRV and contains a *HindIII* restriction site (underlined). The PCR amplification mixture (100 µl volume) contained 1 µg of pCgOSM16B DNA template, 10X High Fidelity Buffer (600 mM Tris-SO$_4$ (pH 8.9), 180 mM (NH$_4$)$_2$SO$_4$; Invitrogen), 10 mM dNTPs, 0.2 nmole of the forward and reverse primers, and 10 units of Platinum High Fidelity *Taq* DNA polymerase (Invitrogen). The
reaction mixture was then placed in a thermal cycler and the template denatured for 5 min. at 94°C. The reaction then ran for 5 cycles, with a denaturing regimen for 30 seconds at 94°C, an annealing regimen for 30 seconds at 55°C, and a polymerization regimen for 30 seconds at 72°C. This was followed by 30 cycles, with denaturation for 30 seconds at 94°C, annealing for 30 seconds at 70°C, and polymerization for 30 seconds at 72°C. After the final cycle, the reaction was maintained at 72°C for 7 min., and then chilled to 4°C (Innis and Gelfand, 1990).

The reaction mixtures were extracted using a QIAquick PCR Extraction Kit (QIAGEN). Briefly, five volumes of PB buffer (QIAGEN) was added to the PCR reaction mixture. The mixture was applied to a QIAquick column, allowing the DNA to bind to the matrix. The column was placed in a microfuge tube and centrifuged for 30 to 60 seconds. The flow-through was discarded, and the column was washed with 750 µl of PE buffer (QIAGEN), centrifuged for 30 to 60 seconds, and then spin-dried for 1 min. at maximum rpm after the flow-through had been discarded. The DNA was then eluted by addition of 30 µl of sterile water, incubation for one min., and centrifugation for one min.

The 591-bp PCR fragment was subcloned into the EcoRI and HindIII sites of the pGEM®7Zf(+) vector. For ligation, the purified PCR DNA and the pGEM®7Zf(+) vector DNA were doubly digested with the EcoRI and HindIII restriction endonucleases and small DNA fragments removed by using the QIAquick PCR Purification Kit (QIAGEN). The 591-bp PCR product derived from pCgOSM16B was ligated into the EcoRI and HindIII sites of the pGEM®7Zf(+) vector by mixing the DNA at a 3:1 ratio (insert:vector). The DNA (7 µl) was mixed with 2 µl of 5X DNA ligase buffer (250 mM Tris-HCl (pH 7.6), 50 mM MgCl₂, 5
mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000) and 1.0 µl (1 unit) of T4 DNA ligase (Invitrogen). The ligation mixture was incubated at 4°C for 16 hours.

Electrocompetent *E. coli* DH5α cells were transformed by electroporation as described earlier using 1-2 ng of the recombinant plasmid DNA from the ligation of the pCgOSM16B PCR product and the pGEM®7Zf(+) vector DNA. To screen for recombinant cells, colonies from master plates were selected as described previously. The plasmid DNA was isolated by a Wizard® Minipreps kit as described before. The isolated plasmid DNAs were doubly digested with the restriction endonucleases *EcoR*I and *Hind*III, electrophoresed on 0.8% LE agarose gels, and sequenced by terminator cycle sequencing (Fan et al., 1996). The resulting recombinant subclone was designated p3’PCR. Using the Prime-a-Gene Labeling System (Promega), Dr. Irma Pirtle of our laboratory used the PCR fragment as template to generate radioactively labeled random-primed DNA fragments (Feinberg and Vogelstein, 1983) to be used as a hybridization probe to screen a second cotton genomic library to isolate overlapping clones by chromosominal walking.

**Screening a Second Cotton Genomic Library for Putative Clones with Osmotin Genes**

A second cotton (*Gossypium hirsutum* cv. Acala SJ-3) genomic library contained in the lambda FIXII vector (Stratagene), generously provided by Dr. Thea Wilkins (Univ. of California at Davis), was screened, using the *E. coli* host strain XL1 Blue MRA, to isolate overlapping genomic clones containing the entire second osmotin gene, by the plaque hybridization procedure (Benton and Davis, 1977). This genomic library was screened by
hybridization using the two homologous cotton DNA probes prepared from the genomic subclone pCgOSM16B. A 50 ml culture of the lambda-sensitive host bacterial strain *E. coli* XL1 Blue MRA in 2xNZYC broth (171 mM NaCl, 18.2 mM MgCl$_2$·6H$_2$O, 2% (w/v) NZ amine, 0.2% (w/v) casamino acids, 1% (w/v) yeast extract) was grown overnight in a shaker incubator at 37°C. Approximately 1 x 10$^7$ pfu of the cotton genomic library in the lambda FIXII vector (Stratagene) were screened in order to over-represent the entire cotton genome. The 50 ml culture of the bacterial host cells was pelleted and then resuspended in 15 ml of 10 mM MgSO$_4$. The resuspended host cells were then infected with sufficient phage (M.O.I. of 0.1) in order to achieve a plaque density of about 3 x 10$^5$ plaque-forming units (pfu) per plate. The infected cultures were incubated for 15 min. at 37°C to adhere the phage. The infected host cells were mixed with four ml of top agarose (48°C) and spread evenly on NZY bottom agar plates (0.5% yeast extract, 1% NZY amine, 0.5% NaCl and 0.02 M MgSO$_4$). The recombinant phages were incubated on the bacterial lawn at 37°C overnight. The phage growth resulted in lysed plaques (with an approximate diameter of 1.5 mm) covering the entire plate. Prior to making membrane replicas, the plates were cooled to 4°C. Each plate was overlaid with a pre-wetted (1 M NaCl) Hybond N$^+$ nylon membrane (Amersham). After four min., the membranes were removed and successively transferred to denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 7 min., neutralizing solution (0.5 M NaCl, 0.1 M Tris-HCl, pH 7.3) for 2 x 5 min, and briefly in rinsing solution (2xSSC, 0.2 mM Tris-HCl, pH 7.5). The membranes were then dried at 37°C and vacuum baked at 80°C for two hours.
Prehybridization and hybridization of this genomic library was done as described earlier. Plaques exhibiting intense positive signals were subjected to successive rounds of plaque purification, using only the homologous coding region probe, until all plaques on a single plate hybridized to the labeled probe, indicative of plaque purity. To identify lambda clones that corresponded to the physical map of LCgOSM16B and the pCgOSM16B subclone, the DNAs from several phage clones were isolated by the mini-lysate procedure described earlier.

**Mini-lysate Phage Preparations for the Genomic Clones Harboring Potential Osmotin Genes**

Two genomic clones, designated LCgOSM12A and LCgOSM7B, that gave intense positive signals and had DNA fragments of appropriate sizes to overlap with the genomic clone LCgOSM16B from the first genomic library were selected for large scale phage preparations.

**Large-scale Phage Preparations for Genomic Clones LCgOSM12A and LCgOSM7B**

From the stored lysates for the clones LCgOSM12A and LCgOSM7B, 50 ml of LB broth was inoculated with pre-incubated suspensions, grown, and isolated as described earlier. The yields of the purified LCgOSM12A and LCgOSM7B phage DNAs were determined by A$_{260}$ measurements.
Physical Mapping of the Cotton Genomic Inserts in the Clones LCgOSM12A and LCgOSM7B

The DNAs from the genomic clones LCgOSM12A and LCgOSM7B were digested with numerous restriction endonucleases for analysis by agarose gel electrophoresis and alkaline blotting (Reed et al., 1985) for physical mapping. The physical mapping procedures were essentially the same as those described previously for the genomic clone LCgOSM16B. The positions of the OSMI and OSMII genes were localized within the LCgOSM12A and LCgOSM7B genomic clones, and the extent of the overlaps between the cotton genomic segments in three lambda clones was determined by physical mapping and DNA sequence analysis.

Subcloning and Sequencing of a 4.0-kb EcoRI Fragment from the Genomic Clone LCgOSM12A

Based upon the physical mapping data, a 4.0-kb EcoRI fragment was selected that encompassed the entire coding and flanking regions of the second putative osmotin gene (OSMII). This 4.0-kb EcoRI fragment from the LCgOSM12A clone, which hybridized to the homologous cotton osmotin probe, was selected for isolation and subsequent cloning into the multiple cloning site of the plasmid vector pUC19. The LCgOSM12A and pUC19 vector DNA were both digested to completion with the restriction endonuclease EcoRI. The linearized vector was dephosphorylated by treatment with calf intestine alkaline phosphatase (CIAP) in order to reduce vector self-ligation. The linearized vector (~8 μg in 80 μl H2O) was mixed with
10 µl of 10X CIAP buffer (500 mM Tris-HCl (pH 8.5), 1 mM EDTA), 1 µl (0.25 units/µl) 
CIAP, and sterile distilled water up to a final volume of 100 µl. The reaction mixture was 
incubated for one hour at 37°C, followed by extraction with phenol and chloroform and 
precipitation with ethanol.

The 4.0-kb EcoRI fragment from the LCgOSM12A genomic clone and the linearized 
dephosphorylated pUC18 vector were electrophoresed on a 0.8% agarose gel and the 
fragments extracted using the QIAquick Gel Extraction Kit (QIAGEN) as described above. 
The purity and concentrations of the isolated 4.0-kb EcoRI fragment and the linearized pUC18 
vector DNA were determined by electrophoresis on a 0.8% agarose gel. The yields were 
estimated by comparison with standard marker bands with known sizes and amounts of DNA. 
The 4.0-kb EcoRI fragment was then ligated into the EcoRI site of the dephosphorylated 
pUC18 vector as described previously, with incubation at 22°C for 4 hours. The recombinant 
plasmid designated pCgOSM12A was electroporated into electrocompetent E. coli DH5a 
cells. White colonies containing the recombinant plasmids were selected and re-streaked on 
fresh LB-ampicillin (50 µg/ml) plates containing IPTG and X-gal to ensure that each colony 
represented a single recombinant and to serve as master plates.

To screen for recombinant cells, selected colonies from the master plates were 
inoculated into 25 ml LB-ampicillin (50 µg/ml) and grown overnight at 37°C with shaking. 
DNAs from the cultures were isolated using the Wizard® Minipreps Kit (Promega). The 
isolated plasmid DNAs were digested with multiple restriction endonucleases and 
electrophoresed on 0.8% LE agarose gels. The subclone yielding the appropriate sized
fragments was designated pCgOSM12A (pOSMII). Both strands of the cotton insert pCgOSM12A were sequenced, using a primer-based approach, with primers specifically designed for the subclones pCgOSM16B and pCgOSM12A.

Subcloning and Sequence Analysis of 3.4-kb HindIII and 3.9-kb EcoRI Fragments from the Genomic Clone LCgOSM7B

Based upon the physical map, 3.4-kb HindIII and 3.9-kb EcoRI fragments from the genomic clone LCgOSM7B were selected that encompassed the entire coding and flanking regions of a third putative osmotin gene designated OSMIII. The 3.4-kb HindIII fragment from the LCgOSM7B clone was selected for subcloning into the HindIII site of the plasmid vector pUC18. The LCgOSM7B DNA and pUC18 vector DNA were both digested to completion with the restriction endonuclease HindIII, and the pUC18 vector was also dephosphorylated with calf intestine alkaline phosphatase (CIAP) prior to electrophoresis on a 0.8% agarose gel and extraction using the QIAquick Gel Extraction Kit (QIAGEN). The linearized vector was dephosphorylated by treatment with calf intestine alkaline phosphatase as described earlier. To concentrate the 3.4-kb HindIII fragment and the pUC18 vector, the two DNAs were first mixed at a 1:1 ratio (insert:vector) with 2 µl of 5S carrier RNA (10 µg/µl) and precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol for 30 minutes at -80°C and the DNAs were pelleted as usual. DNA ligation was basically as described previously with incubation at 22°C for 16 hours.
Electrocompetent *E. coli* DH5a cells were electroporated with 1-2 ng of the recombinant plasmid DNA from the ligation of the 3.4-kb pCgOSM7B fragment and the pUC19 vector DNA. The transformed cells were plated in 200 µl aliquots on LB-ampicillin (50 µg/ml) plates that were prepared with 4 µl of IPTG (200 mg/ml) and 40 µl of X-gal (20 mg/ml) and incubated overnight at 37°C. The white colonies containing the recombinant plasmids were re-streaked on fresh LB-ampicillin (50 µg/ml) plates containing IPTG and X-gal to ensure that each colony represented a single recombinant and to serve as master plates. The master plates were incubated at 37°C overnight and then stored at 4°C. DNAs from the cultures were isolated using the Wizard® Minipreps Kit (Promega). The isolated plasmid DNAs were digested with multiple restriction endonucleases and electrophoresed on 0.8% LE agarose gels. The resulting recombinant subclone was designated pCgOSM7B-*HindIII*. Both strands of the cotton insert in pCgOSM7B-*HindIII* were sequenced, using a primer-based approach, with primers designed specifically for the pCgOSM16B, pCgOSM12A or pCgOSM7B-*HindIII* plasmids. The genomic DNA insert in the pCgOSM7B-*HindIII* plasmid was then sequenced.

A 3.9-kb *EcoRI* fragment from LCgOSM7B was isolated, and ligated into the *EcoRI* site of pUC19 (the subclone being designated pCgOSM7B-*EcoRI*) and then sequenced in a similar manner by Ms. Kimberly Spradling of our laboratory. Analyses of the DNA and deduced amino sequences were done with DNASIS version 2.1 software (Hitachi).
Design of an Anti-osmotin Antibody

The deduced amino acid sequences derived from the osmotin cDNA sequences in the clones pCcOSM47B and pCcOSM52A and the osmotin genes designated *OSMI* and *OSMII* in the clones LCgOSM16B and LCgOSM12A were examined using the software packages DNASIS version 2.1 (Hitachi) and Antheprot V5.0 (http://pbil.icp.fr/ANTHEPROT) to determine appropriate polypeptide sequences to be used for the design of a polyclonal anti-osmotin antibody. The last 18 amino acids of the C-terminal end of the OSMI polypeptide were selected for the experimental design. The 18-mer oligopeptide and the polyclonal antibody preparation in rabbits were commercially prepared by Biosynthesis Inc. (Lewisville, Texas).

Expression of Fusion Osmotin Polypeptides in a Bacterial Expression System

The PinPoint™ Xa protein purification system (Promega) was used to express the presumptive fusion osmotin polypeptides in an *E. coli* expression system. The system is designed for the production and purification of fusion proteins that are biotinylated *in vivo*. To produce and purify putative fusion osmotin proteins, the two presumptive osmotin genes were amplified by PCR. Primers were designed to amplify the coding regions without the 5’- and 3’-untranslated regions (UTRs) and the N-terminal targeting domains. The forward amplimer for both the *OSMI* and *OSMII* genes was 5’-TTAAAGCTTTTTGAAAATCCGCAATGAG-3’ with a *Hind*III site (underlined). The designed reverse amplimer for the *OSMI* gene was 5’-AAAAGATCTAAGGCAGATTAACTAGACC-3’ and the reverse amplimer for the
The XhoI/BamHI fragment from the subclone pCgOSM16B was used as template for the *OSMI* gene and the plasmid pCgOSM12A was used as template for the *OSMII* gene. The PCR amplification mixture contained 1 µg of DNA template, 10X High Fidelity Buffer (600 mM Tris-SO₄ (pH 8.9), 180 mM (NH₄)₂SO₄; Invitrogen), 10 mM dNTPs, 0.2 nmole of the forward and reverse primers, and 10 units of Platinum High Fidelity *Taq* DNA polymerase (Invitrogen) and enough sterile distilled water for a final volume of 100 µl. The reaction mixtures were then placed in a Perkin Elmer 2400 thermal cycler and denatured for 5 min. at 94°C. The reactions then ran for 5 cycles, denaturing for 30 seconds at 94°C, annealing for 30 seconds at 58°C, and polymerizing for 30 seconds at 72°C. This was followed by a regimen of 30 cycles with denaturation for 30 seconds at 94°C, annealing for 30 seconds at 65°C, and polymerization for 30 seconds at 72°C. After the final cycle, the reactions were maintained at 72°C for seven min. and then cooled to 4°C (Innis and Gelfand, 1990). Primers and free nucleotides were removed from the PCR reaction mixtures using a QIAquick PCR Extraction Kit (QIAGEN), and the purified PCR products were then analyzed on a 0.8% agarose gel to confirm their sizes.

The two PCR products and the PinPoint™ Xa-3 vector DNA were doubly digested with *Hind*III and *Bg*II and the products separated by electrophoresis on 0.8% LE agarose gels. The bands containing the 700-bp *OSMI* and *OSMII* coding regions and the linearized 3.3-kb Xa-3 vector were extracted using the QIAquick Gel Extraction Kit (QIAGEN). The
purified PCR products and the linearized PinPoint™ Xa-3 vector were ligated by mixing the DNAs at a 3:1 ratio (insert:vector) as described earlier. *E. coli* DH5a cells were electroporated as usual, the transformed cells were plated as described before, the plasmid DNA was isolated as usual, and the DNAs were sequenced as described previously. The isolated plasmid DNAs were doubly digested with the restriction endonucleases *Hind*III and *Bgl*II and then electrophoresed on 0.8% LE agarose gels. Clones yielding the appropriate sized fragments were selected for sequencing and designated PinPoint-Xa-3-OSMI #4 and PinPoint-Xa-3-OSMII #86. Once the vector sequences containing the *OSMI* and *OSMII* constructs were confirmed by DNA sequencing, small-scale cultures and induction of the osmotin fusion proteins were done.

**Small-Scale Culture for Induction and Detection of Osmotin Fusion Proteins**

Following the manufacturer’s protocols (Promega), individual cultures of *E. coli* DH5a cells containing either the PinPoint-Xa-3-*OSMI* #4 construct, the PinPoint-Xa-3-*OSMII* #86 construct, the PinPoint™ Xa control vector, or the PinPoint™ Xa-3 vector were grown overnight at 37°C in 25 ml of LB-ampicillin (100 µg/ml) medium containing biotin (2 µM). An additional culture of *E. coli* DH5a cells containing no vector was also grown overnight at 37°C in 25 ml of LB medium with biotin (2 µM). The overnight cultures were diluted 1:100 in 25 ml of fresh LB or LB-ampicillin medium (100 µg/ml) with biotin (2 µM) and grown for one hour at 37°C in a shaker/incubator. To induce protein expression, IPTG was added to a final concentration of 100 µM and the cultures were incubated for 4-5 hours at 37°C with 225 rpm
in a shaker/incubator. Two 100 µl aliquots of each culture were placed in 1.5 ml microfuge
tubes and centrifuged for 5 min. with maximum rpm. The supernatants were discarded and the
pellets were resuspended in 50 µl of either 0.1 M sodium citrate (pH 5.0) or 1X sample buffer.
The 1X sample buffer contained 25% (v/v) stacking gel 4X buffer (500 mM Tris base and
0.4% SDS), 2% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.0025% bromophenol blue
(BPB) (Promega Technical Manual 028). Samples that were resuspended in 1X sample buffer
were heat-treated for 5 minutes at 100°C and stored at -70°C.

The isolated protein samples were separated on a polyacrylamide gel using a
discontinuous buffer system (Laemmli, 1970). This SDS-PAGE (sodium dodecysulfate-
polyacrylamide gel electrophoresis) system had two sections, an upper portion (stacking gel)
which contained 5% acrylamide/bis-acrylamide (29:1), 0.15 M Tris-HCl (pH 6.8), 0.1% SDS,
0.1% ammonium persulfate (APS), and 0.12% TEMED (N,N,N’,N’-tetraethylethylenediamine),
and a lower (separating) portion containing 15% acrylamide/bis-acrylamide (29:1), 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate (APS),
and 0.04% TEMED. Before electrophoresis on the 5% stacking/15% separating SDS-
polyacrylamide gel, 10 µl of 6X Sample Buffer (350 mM Tris-HCl (pH 6.8), 30% glycerol,
10% SDS, 9.3% DTT, 0.012% BPB) were added to the protein samples suspended in 0.1 M
sodium citrate (pH 5.0) buffer. All samples were heat-treated at 100°C for 5 min. and cooled
to room temperature before loading. A total of 40 µl of each sample was loaded per well and
separated at 30 mA per gel using 1X Running Buffer (25 mM Trizma base, 250 mM glycine,
0.1% (w/v) SDS) in a BioRad Mini-Protean II apparatus.
After the proteins had been separated by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane by electroblotting using the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad). Following the manufacturer’s protocol, the separating portion of the gel, a nitrocellulose membrane, two fiber filter pads, and two filter papers cut to the size of the gel were soaked for 45 min. in Transfer Buffer (48 mM Tris-HCl, 39 mM glycine, 20% (v/v) methanol, pH 9.2). The gel and membrane were arranged according to the manufacturer’s protocol and protein transfer achieved overnight at 70-90 mA using a Model 250/2.5 BioRad power supply.

Following transfer, the nitrocellulose membrane was tested for the presence of biotinylated proteins. The nitrocellulose membrane was incubated in TBST (1 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% TWEEN® 20) for 30 min. at room temperature with gentle shaking. The membrane was then incubated, using a 1:5,000 dilution of streptavidin-alkaline phosphatase in 1X TBST buffer, for 30 minutes at room temperature with gentle shaking. Finally, the membrane was washed twice with 1X TBST buffer for 5 min. with gentle shaking and then washed once briefly in distilled water. Protein bands were visualized by incubating the membrane with Western Blue® Stabililized Substrate for Alkaline Phosphatase (Promega).

Upon confirmation of the presence of the appropriate sized biotinylated protein, the membrane was tested for the presence of osmotin proteins using the polyclonal anti-osmotin antibody. The membrane was blocked with 5% powdered milk-TBS (20 mM Tris-HCl, 150 mM NaCl) by shaking for two hours at room temperature. The membrane was washed twice
with TBST for 10 min. with gentle shaking and subsequently incubated with the anti-osmotin antibody (from Biosynthesis, Inc.) at a 1:1,000 dilution in 5% powdered milk-TBST for one hour at room temperature. The membrane was washed twice with TBST for 10 min. with gentle shaking and then incubated with a 1:1,500 dilution of the secondary antibody (Anti-rabbit Ig, Horseradish Peroxidase-Linked Whole Antibody from donkey, Amersham). The excess secondary antibodies were removed by washing the membrane two times for 10 min. with TBST. The bound antibodies were then visualized by incubating the membrane with a 1:1 mixture of ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech) for one min. and by exposing the membranes to Kodak X-OMAT® X-Ray film.

**Induction and Isolation of Osmotin Fusion Proteins in Large Scale Cultures**

To attain sufficient quantities of the biotinylated osmotin fusion proteins for purification using the SoftLink™ Soft Release Avidin Resin (Promega), 500 ml cultures of the transformed cells containing the plasmids PinPoint-Xa-3-OSMI #4 and PinPoint-Xa-3-OSMII #86 were grown. Initially, 50 ml cultures of transformants with the PinPoint-Xa-3-OSMI #4 and PinPoint-Xa-3-OSMII #86 constructs were grown in LB-ampicillin (100 µg/ml) with biotin (2 µM) overnight at 37°C in a shaker/incubator. Using a 1:100 dilution of the overnight cultures, 500 ml of fresh LB-ampicillin (100 µg/ml) with biotin (2 µM) were grown for one hour at 37°C with shaking. Protein expression was induced by adding stock 1 M IPTG to a final concentration of 100 µM and incubating the cultures for 4 to 5 hours at 37°C with shaking. The cultures were centrifuged at 10,000 xg for 15 min. at 4°C to pellet the cells. The supernatants
were discarded and the cells were frozen at -20°C overnight. The pellets were resuspended in 10 ml Lysis Buffer (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 5% glycerol) per gram of cells at 4°C. After the cells were completely resuspended, the mixtures were brought to a final concentration of 1 mg lysozyme per ml (5 mg/ml in TEN (40 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 150 mM NaCl) and mixed for 20 min. at 4°C. Sodium deoxycholate (DOC) was added to a final concentration of 0.1% (w/v) and mixed for 5 min. at 4°C. After mixing with of 200 units of DNase I for 10 min. at 4°C, the samples were centrifuged for 15 min. at 10,000xg and 4°C. The supernatants were saved and the pellets discarded. The same procedure was carried out for 50 ml cultures of *E. coli* cells transformed with either the PinPoint™ Xa Control vector, the PinPoint™ Xa-3 vector, or untransformed cells (no vector). The tentative osmotin fusion proteins were then isolated using the biotinylated tag and SoftLink™ Soft Release Avidin Resin (Promega) according to the manufacturer’s protocols.

One ml of the SoftLink™ Soft Release Avidin Resin (Promega) was placed in a 15 ml Falcon polypropylene tube and equilibrated with Cell Lysis Buffer at 4°C. The resin was centrifuged at about 500 rpm (about 700 xg) for 5 min. and the supernatants were removed. One ml of the cell lysates from the 500 ml cultures transformed with the PinPoint-Xa-3-*OSMI* #4 and PinPoint-Xa-3-*OSMII* #86 constructs was added to the Falcon tubes containing the SoftLink™ Soft Release Avidin Resin (Promega) and incubated overnight with shaking at 4°C. The Falcon tubes containing the SoftLink™ Soft Release Avidin Resin (Promega) were centrifuged at about 500 rpm (about 700 xg) and transferred to clean tubes. The resins were washed with 10 volumes of 100 mM sodium citrate (pH 5.0) for 10 min. at 4°C and then
centrifuged for 5 min. to settle the resin in the tubes. The supernatants were removed and the resins washed twice with 10 volumes of Cell Lysis Buffer for 10 min. at 4°C. The bound fusion proteins were eluted by shaking the resin at 4°C with one volume of 50 mM Tris-HCl (pH 7.5) and 5 mM biotin for a minimum of 60 hours. The resin was collected by centrifuging for 5 min. at about 500 rpm (about 700 xg). The supernatants were collected and the amounts of protein were estimated using the Bradford protein determination assay. The fusion proteins were then stored at -80°C.

Using Factor Xa protease, the fusion proteins were digested to remove the N-terminal fusion tags. The reaction mixture of 1 µg of fusion polypeptide and 3-6 µg of Factor Xa protease (180 units/mg) was incubated overnight at 37°C. The resulting digest was lyophilized to partially reduce the volume. All samples of the osmotin fusion proteins, as well as the whole extracts from the control samples, were separated on a 15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting as described previously. Detection of proteins was performed using both the strepavidin-alkaline phosphatase and the anti-osmotin antibody as described above.

**Construction of Cassettes Containing the Osmotin Coding Regions and the 35S CaMV Promoter**

Unique PCR primers were designed to amplify the coding regions of the two presumptive osmotin genes, *OSMI* and *OSMII*, without the 5’- and 3’-untranslated regions (UTRs) and containing the sequences that code for the N-terminal targeting domains, in order to
construct an *Agrobacterium tumefaciens* binary plasmid vector for constitutive gene expression of the *OSMI* and *OSMII* genes in a eukaryotic system. For amplification of the *OSMI* gene, the forward amplimer was 5’-

CACGTATTCTAGAAAGTAAGAGCTAACCATGAGC-3’. This primer contains a *Xba*I restriction site (single underline), an in-frame stop codon (dotted underline), the optimal Kozak initiation sequence (double underline) (Kozak, 1991), and the methionine translation initiation codon (dashed underline). The reverse amplimer was 5’-

CCGAATTCCTAGACCAAGAGATGATCCC-3’, with an *EcoRI* restriction site (underlined). For amplification of the *OSMII* gene, the forward amplimer was 5’-

CACGTATTCTAGATAAAAACCAACCATGAGC-3’. This primer contains a *Xba*I restriction site (single underline), an in-frame translation termination codon (dotted underline), the Kozak sequence (double underline) (Kozak, 1991), and a methionine initiation codon (dashed underline). The reverse amplimer was 5’-

CAGAGAATTCCTACTCCAAGAGATCATCCC-3’, with an *EcoRI* restriction site (underlined).

The PCR amplification mixture contained 1 µg of DNA template (the plasmid pCgOSM16B-*Xhol/BamHI* for the *OSMI* gene and the plasmid pCgOSM12A for the *OSMII* gene), 10X High Fidelity Buffer (600 mM Tris-SO₄ (pH 8.9), 180 mM (NH₄)₂SO₄, Invitrogen), 10 mM dNTPs, 0.2 nmole of the forward and reverse amplimers, and 10 units of Platinum *Taq* DNA polymerase High Fidelity (Invitrogen). In a thermal cycler, the reaction mixtures were denatured for 5 min. at 94°C. Subsequently, the amplification reactions ran for 5
cycles, were denatured for 30 seconds at 94°C, annealed for 30 seconds at 56°C (the *OSMI* gene) or 55°C (the *OSMII* gene), and polymerized for 30 seconds at 72°C. The reaction mixtures were then subjected to 35 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 65°C, and polymerization for 30 seconds at 72°C. After the final cycle, the reactions were maintained at 72°C for seven min. and then cooled to 4°C (Innis and Gelfand, 1990).

The reaction mixtures were extracted using a QIAquick PCR extraction kit (QIAGEN). Briefly, five volumes of PB buffer (QIAGEN) was added to the PCR reaction mixtures. The mixtures were pipetted into QIAquick columns, and the columns microfuged for 30 to 60 seconds. The flow-through was discarded and the columns were washed with 750 µl of PE buffer (QIAGEN) by centrifugation for 30 to 60 seconds. The columns were dried for one min. in the microfuge, after the flow-through had been discarded. After the columns had been incubated with 30 µl of sterile water, the DNAs were eluted by centrifugation for one min. The 787-bp *OSMI* gene PCR product, the 728-bp *OSMII* gene PCR fragment, and a 2.4-kb pUC-based vector (from the John Innes Center, Norwich, UK, www.pgreen.ac.uk; Hellens et al., 2000) containing the 35S Cauliflower Mosaic Virus (CaMV) promoter-terminator regions (Figure 1) were digested with the restriction endonucleases *Xba*I and *EcoRI*.

The digested PCR products and the linearized vector were then purified using the QIAquick PCR Purification Kit. Briefly, three volumes of QG buffer (QIAGEN) were added to one volume of gel, determined from the weight of the gel slice. The gel slices were incubated at 50°C, vortexing every 2-3 min., for a total of 10 min. or until the gel slices were completely
dissolved. The mixtures were pipetted into QIAquick columns and the columns microfuged for one min. The flow-through was discarded and the columns were washed with 750 µl of PE buffer (QIAGEN) by centrifugation for 30 to 60 seconds. The columns were dried for one min. in the microfuge after the flow-thru had been discarded. The DNAs were eluted by centrifugation for one min. after the columns had been incubated for one minute with 30 µl of sterile water.

The purified PCR products and linearized vector were then analyzed on a 0.8% agarose gel to confirm their size and to estimate concentrations. The yields were estimated by comparison with standard marker bands with known sizes and amounts of DNA. The PCR products for the OSMI and OSMII genes were then subcloned into the XbaI and EcoRI sites of a 2.4-kb pUC-based vector (Hellens et al., 2000; www.pgreen.ac.uk) containing the 35S Cauliflower Mosaic Virus (CaMV) promoter-terminator regions. The osmotin gene I and II PCR fragments and the pUC-derived vector were mixed in a 3:1 ratio (insert:vector) for ligation.

Electrocompetent E. coli DH5a cells were electroporated with 1-2 ng of the osmotin gene I and II DNAs as described before. The transformed cells were plated in 200 µl aliquots on LB-ampicillin (50 µg/ml) plates and incubated overnight at 37°C. Several colonies were selected and re-streaked on fresh LB-ampicillin (50 µg/ml) plates to ensure that each colony represented a single recombinant plasmid clone and to serve as master plates. To screen for recombinant cells, selected colonies from the master plates were inoculated into 25 ml LB-ampicillin (50 µg/ml) media and grown overnight at 37°C with shaking. DNAs from the cultures
Figure 1. 35S Cauliflower mosaic virus (CaMV) cassette. The 672-bp 35S CaMV cassette is contained in a pUC-based plasmid vector of 2.4 kb from the John Innes Center, Norwich, UK (www.pgreen.ac.uk; Hellens et al., 2000) for E. coli-mediated transformation.
were isolated by the Wizard® Minipreps Kit (Promega) as described earlier. The isolated plasmid DNAs were doubly digested with the restriction endonucleases \textit{XbaI} and \textit{EcoRI} and electrophoresed on 0.8% LE agarose gels. The subclones yielding the appropriate sized fragments were designated p35S-\textit{OSMI} and p35S-\textit{OSMII}. Both strands of the coding regions and multiple cloning sites of the p35S-\textit{OSMI} and p35S-\textit{OSMII} constructs were sequenced, using a primer-based approach, with primers designed specifically for the coding regions of the \textit{OSMI} gene and the \textit{OSMII} gene.

**Construction of a \textit{Agrobacterium tumefaciens} Binary Vectors for Transformation of \textit{Arabidopsis} and Cotton Plants for Constitutive Expression of the Osmotin Genes**

To construct \textit{Agrobacterium tumefaciens} binary plasmid vectors for transformation of \textit{Arabidopsis} and cotton plants for constitutive gene expression of the osmotin genes, the 35S CaMV promoter-terminator cassettes were inserted into the pCAMBIA 2301 vector (Hajdukiewicz et al., 1994). To isolate the 1.4-kb 35S-\textit{OSMI} and 35S-\textit{OSMII} cassettes from the p35S-\textit{OSMI} and p35S-\textit{OSMII} constructs in the pUC based vector (Hellens et al., 2000; www.pgreen.ac.uk), the constructs were both digested to completion with the restriction endonuclease \textit{EcoRV}. The pCAMBIA 2301 vector (Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia, www.pcambia.org.au) was digested to completion with the restriction endonuclease \textit{SmaI} to prepare for a blunt-end ligation reaction.
The 1.4-kb *EcoRV* fragment from the p35S-OSMI and p35S-OSMII plasmids and the linearized pCAMBIA vector were electrophoresed on a 0.8% agarose gel and extracted using the QIAquick Gel Extraction Kit (QIAGEN) as previously described. The purity and concentrations of the eluted 1.4-kb *EcoRV* fragments containing the 35S-OSMI and 35S-OSMII cassettes and the linearized 11.6-kb pCAMBIA vector were determined by electrophoresis on a 0.8% agarose gel. The *EcoRV*-digested 35S-cassettes from the p35S-OSMI and p35S-OSMII constructs were then blunt-end ligated into the *SmaI* site of the pCAMBIA 2301 vector. The 35S-OSMI and 35S-OSMII cassettes and the linearized pCAMBIA 2301 vector were mixed in a 3:1 ratio (insert:vector). Two (2) µl of 5x Ligase Buffer (250 mM Tris-HCl (pH 7.6), 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000) and 1.0 µl (1 unit) of T4 DNA ligase were added to 7 µl of the vector/insert mixtures. The ligation mixtures were incubated at 18°C for 16 hours.

To transform electrocompetent *E. coli* DH5a cells, 1-2 ng of the recombinant plasmid DNAs resulting from the ligation of the 35S-OSMI and 35S-OSMII cassettes and the linearized pCAMBIA 2301 vector were mixed with 40 µl of the electrocompetent cells in disposable BTX® cuvettes with a 1 mm gap. After chilling on ice for one min., the plasmids were electroporated into the *E. coli* cells using a BTX® Electro Cell Manipulator® (ECM 395) set in the HV mode/3 kV with a 1.5 kV set charge having a 5-6 msec pulse length. Immediately following the electric pulse, one ml of LB media was added to the cuvettes and gently mixed by pipetting. The cell mixtures were transferred into a clean polypropylene tube and incubated for
60 min. at 37°C with shaking at 225 rpm. The transformed cells were plated in 200 µl aliquots on LB-kanamycin (50 µg/ml) plates and incubated overnight at 37°C.

Several colonies were selected and re-streaked on fresh LB-kanamycin (50 µg/ml) plates to ensure that each colony represented a single recombinant plasmid clone and to serve as master plates. The master plates were incubated at 37°C overnight and then stored at 4°C.

To screen for recombinant cells, selected colonies from the master plates were inoculated into 25 ml LB-kanamycin (50 µg/ml) and grown overnight at 37°C with shaking. DNAs from the cultures were isolated using the Wizard® Minipreps Kit (Promega) as described earlier. The isolated plasmid DNAs were doubly digested with the restriction endonucleases KpnI and PstI and electrophoresed on 0.8% LE agarose gels. The subclones yielding the appropriate sized fragments were designated pCambia-35S-OSMI and pCambia-35S-OSMII. Both strands of the coding regions and multiple cloning sites of pCambia-35S-OSMI and pCambia-35S-OSMII were sequenced, using a primer-based approach, with primers designed specifically for the coding regions of the OSMI and OSMII genes.

Preparation of Electrocompetent Agrobacterium tumefaciens LBA4404 Cells

Agrobacterium tumefaciens LBA4404 cells contain the disarmed Ti plasmid pAL4404, which has only the virulence and origin of replication regions of the Ti plasmid (Wenjun and Forde, 1989). Introduction of the recombinant T-DNA into A. tumefaciens cells can be performed by electroporation. The recombinant DNA is then transferred from the A.
tumefaciens cells into plant cells using components provided by the plasmid pAL4404 (Wen-jun and Forde, 1989).

For preparation of electrocompetent cells, 5 µl of a frozen stock of Electro MAX
Agrobacterium tumefaciens LBA4404 cells (Invitrogen, Inc.) was inoculated into five ml of
LB broth (Luria-Bertani media: 10 grams of Bactotryptone, 10 grams of NaCl, and 5 grams of
Bacto Yeast Extract per liter). The culture was grown overnight at 28°C with shaking in a New
Brunswick shaker/incubator at 100 rpm. Two 500 ml flasks containing LB media were
inoculated with the culture and incubated at 30°C with shaking at 100 rpm until an A_{600} of 1.5-
2.0 was achieved. The bacterial cells were then harvested by centrifugation in a Sorvall GS-3
rotor at 5,500 rpm (4,300xg) for 15 min. at 4°C. The supernatants were removed and the cells
resuspended in one volume of sterile ice-cold water and re-pelleted by centrifugation in a
Sorvall GS-3 rotor at 5,500 rpm (4,300xg) for 15 min. at 4°C (a total of seven times). Finally,
the cells were re-suspended in 1.5 ml of cold filter-sterilized 10% glycerol. The
electrocompetent cells were then aliquotted and frozen at -80°C.

To transform the electrocompetent Agrobacterium tumefaciens LBA4404 cells,
approximately 3 ng of pCambia-35S-OSMI plasmid DNA and 10 ng of pCambia-35S-OSMII
plasmid DNA were mixed with 40 µl of the electrocompetent cells. Disposable cuvettes (BTX
P/N 610, 1 mm gap) were filled with the transformation mixtures. The cuvettes containing the
mixtures were then chilled on ice for one minute. The electroporator ECM apparatus was set
on the HV mode/3 kV and a single 1.44kV electrical pulse was applied to the chilled cuvettes,
resulting in a field strength of 14.4 kV/cm with an exponential decay constant of approximately
5-6 milliseconds. Immediately following the electroporation, one ml of YEP (Yeast Extract Peptone: 20 g Bactopeptone, and 10 g Yeast Extract per liter) were added to the cuvettes and mixed by pipetting. The resuspended cells were transferred to a 3 ml Falcon polypropylene tube and incubated without shaking at 28°C for one hour. Aliquots of 200 µl of the transformed cells were plated on YEP plates containing streptomycin (10 µg/ml) and kanamycin (10 µg/ml) and grown at 28°C. One to three days after incubation, numerous colonies were found. Isolated colonies were selected and re-plated on YEP plates containing streptomycin and kanamycin (10 µg/ml each) and grown at 28°C to ensure pure colonies. The resulting colonies were then inoculated into 25 ml of LB-broth containing streptomycin and kanamycin (10 µg/ml each) and grown at 28°C. The recombinant plasmid DNAs were isolated by a modified mini-scale plasmid DNA preparation described below.

The cells were pelleted by centrifugation of five ml of culture in a Sorvall GLC-4 tabletop centrifuge at 3,000 rpm (2,500 xg) for 15 min. The supernatants were discarded and the cells resuspended in 200 µl of Solution W (50 mM Tris-HCl (pH 8.0), 100 mM EDTA, and 100 µg RNaseA per ml) and transferred to 1.5 ml Eppendorf tubes. To the resuspended cells, 200 µl of Solution P (0.2 N NaOH and 1% SDS) was added and gently mixed. After 200 µl of Solution T (1.32 M KOAc (pH 4.8)) was added and mixed, the solutions were microcentrifuged for 5 min. in a Fisher Scientific Microcentrifuge (Model 235C). The resulting supernatants were transferred to clean 1.5 ml Eppendorf tubes and mixed with one volume of phenol/chloroform (1:1), followed by centrifugation for 5 min. The upper phases were transferred to clean tubes and extracted by mixing one volume of chloroform/isoamyl alcohol
(24:1) and centrifuging for 5 min. The upper phases were transferred, leaving the interfaces, to

clean tubes and the DNAs were precipitated by adding 0.1 volumes of 3 M sodium acetate (pH
5.2) and 2 volumes of 100% ethanol and incubating the samples at -20°C overnight. The
DNAs were collected by microcentrifuging the tubes at 4°C for 15 min. The resulting pellets
were washed three times with 70% ethanol, vacuum-dried, and resuspended in 50 µl of sterile
distilled water. The isolated DNAs were doubly-digested with the restriction endonucleases
KpnI and PstI and the fragments were resolved by electrophoresis on 0.8% agarose gels. The
samples of sufficient purity were selected for PCR amplification.

Selected DNA samples isolated from the Agrobacterium transformants were subjected
to amplification of the coding regions of the osmotin constructs. For DNA samples isolated
from Agrobacterium transformed with pCambia-35S-OSMI, the PCR primers 5’-
ATAACTGTATGAATCACGGC-3’ (p16BR1) and 5’-TTTCATTTTCCGCTCATGCAG-3’
(p16BF1) were used. The PCR amplification mixture contained 10 µl of DNA template, 10X
Buffer (100 mM Tris-HCl (pH 9.0), 500 mM KCl, 15 mM MgCl₂, and 1% Triton® X-100)
(Promega), 10 mM dNTPs, 2 nmole of each primer, and 10 units of Taq DNA polymerase
(Promega). The reaction mixtures were then placed in a thermal cycler and denatured for 5
min. at 94°C. The reactions then ran for 30 cycles, which denatured for 30 seconds at 95°C,
annealed for 30 seconds at 50°C, and polymerized for 30 seconds at 72°C. After the final
cycle, the reactions were maintained at 72°C for seven min. and then cooled to 4°C (Innis and
Gelfand, 1990).
For DNA samples isolated from *Agrobacterium* transformed with pCambia-35S-
*OSMII*, the PCR primers 5’-CACGTATTCTAGAGTAAAAACCAACCATGAGC-3’ and 5’-CAGAGAATTCCTACTCCAAGAGATCATCCC-3’ were used. The PCR amplification mixtures contained 5 µl of DNA template, 10X Assay Buffer A (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂, and 0.01% (w/v) gelatin) (Fisher Biotech), 10 mM dNTPs, 2 nmole of each primer, and 10 units of FisherBiotech Taq DNA polymerase. The reaction mixtures were then placed in a thermal cycler and denatured for 5 min. at 94°C. The reactions then ran for 5 cycles, denaturing for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and polymerizing for 30 seconds at 72°C. This regimen was followed by 30 cycles with denaturation for 30 seconds at 94°C, annealing for 30 seconds at 65°C, and polymerization for 30 seconds at 72°C. After the final cycle, the reactions were maintained at 72°C for seven min. and then cooled to 4°C (Innis and Gelfand, 1990). The PCR reactions were electrophoresed on 0.8% agarose gels to confirm the presence and size of the fragments derived from the osmotin genes. The transformants that had the appropriate-sized PCR fragments were designated pCambia-35S-OSMI (H1, M3 and G3) and pCambia-35S-OSMII (A, F, G, and J) and stored as glycerol stocks (33% autoclaved glycerol) at -80°C.

**Plant Materials**

Cotton plants (*Gossypium hirsutum* L., cv. Paymaster HS26, and cv. Acala SJ5) were grown in the greenhouse under conditions previously described (Chapman and Sprinkle, 1996). Whole plants or young leaves were harvested as needed for protein, RNA, and DNA
isolations. The Paymaster HS26 seeds were a gift from Dr. John J. Burke (USDA-ARS, Lubbock).

Induction, Isolation, and RT-PCR of RNA from Cotton Plant Extracts

To extract total RNA for use in Northern blot analysis and in reverse transcriptase-polymerase chain reactions (RT-PCR), 4 to 6 week-old cotton plants were sprayed with various chemical inducers and various tissues were collected using a modified protocol (Chang et al., 1993; McKenzie et al., 1997) using the QIAGEN RNeasy Mini Kit (QIAGEN). This work was done in conjunction with Ms. Kimberly Spradling of our laboratory. Three to four cotton plants from a single pot were sprayed with distilled water, 100 mM H$_2$O$_2$, or 1 mM ethephon, and then placed in a 1 gallon clear Ziplock bag. After an appropriate amount of time, the plants were removed from the bags and all soil was removed from the plants by washing gently with distilled water. The plants were washed briefly with distilled water and blotted dry. After breaking the plants into roots, stems, and leaves, the tissues were weighed. Less than one gram of each tissue sample was frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle. The ground frozen tissue samples were transferred to 50 ml centrifuge tubes containing 15 ml of hot (65°C) extraction buffer (2% CTAB (hexadecyltrimethyl-ammonium bromide), 2% PVP (polyvinylpyrrolidone), 100 mM Tris-HCl (pH 8.0), 25 mM disodium EDTA, 2 M NaCl, spermidine (N-[3-aminopropyl]-1,4-butane-diamine) (0.5 g/l), and 2% ß-mercaptoethanol (BME, added just before use)) and mixed until all the tissue samples were suspended. The mixtures were maintained at 65°C in a water bath and 15 ml of chloroform
mixed with the solutions. The plant debris was pelleted by centrifugation at 9,000xg (10,000 rpm with a Sorvall SS-24 rotor) for 20 min. at 4°C. The aqueous (upper) layers were transferred to clean 50 ml polypropylene centrifuge tubes and the chloroform extractions were repeated (for leaves, the extractions were repeated two times).

After the last chloroform extraction, the upper layers were transferred to clean 50 ml polypropylene centrifuge tubes mixed with 5 ml of 8 M lithium chloride, and stored at 4°C overnight. The LiCl-precipitated RNA samples were centrifuged for 30 min. at 9,000xg at 4°C. The supernatants were discarded and the pellets were resuspended in 500 µl of QIAGEN buffer RLT (proprietary composition) that had been combined with 5 µl of BME (β-mercaptoethanol), and mixed with 250 µl of 100% ethanol. The entire 750 µl sample (including any precipitates) were transferred to RNeasy mini-columns (QIAGEN) and centrifuged for one min. at 8,000 xg and 4°C. The columns were first washed with 700 µl of QIAGEN buffer RW1 (proprietary composition) and centrifuged for 15 seconds at 8,000 xg and 4°C. This step was followed by two washes with 500 µl of QIAGEN buffer RPE (proprietary composition), centrifuging the first wash for 15 seconds at 8,000 xg and 4°C and the second wash for 2 min. at 8,000 xg and 4°C (to dry the columns). The RNA samples were eluted by pipetting 50 µl of RNase-free sterile distilled water directly into the columns and centrifuging for 1 min. at 8,000 xg and 4°C. The procedure was repeated once, and the fractions were pooled. The RNA concentrations were determined by taking A_{260} readings before use in Northern blot and RT-PCR experiments, in collaboration with Ms. Kimberly Spradling of our laboratory.
A total of 1.5 µg of RNA for each sample was run in each lane on a 1% formaldehyde denaturing gel (1.5 g LE agarose, 111 ml H₂O, 15 ml 10X MOPS (200 mM MOPS (3-[N-Morpholino]propanesulfonic acid), 50 mM sodium acetate, 1 mM EDTA (pH 7.0)), and 37% formaldehyde. Before running, the samples were mixed with two volumes of 1.5X sample buffer/loading buffer (0.06% bromophenol blue, 0.06% xylene cyanol, 1.5X MOPS, 9% formaldehyde, and 60% deionized formamide) and heat treated at 65°C for 15 min. After heat treatment, 1 µl of ethidium bromide (1 mg/ml) was added to each sample. The samples were chilled on ice, loaded on the 1% formaldehyde gel, and run at 5 V/cm using 1X MOPS as the electrophoresis running buffer.

After making a photographic record of the gel, the RNA samples were transferred to a Hybond-N⁺ nylon membrane (Amersham) with 20XSSC. The gel was soaked 2X 20 minutes in 5X SSC, before being transferred. The pre-soaked gel was inverted and placed on a blotting station consisting of two filter paper “wicks” pre-wetted with 20X SSC and arranged to soak up excess 20X SSC from a reservoir by capillary action. The Hybond-N⁺ membrane was immersed in water and placed on the inverted gel. Two filter papers (cut to the size of the gel) and the membrane (that had been pre-soaked in 20X SSC) were placed on the gel. After all air bubbles had been removed from between the membranes and the gel, two additional dry filter papers were placed on the stack, followed by a generous layer of paper towels. A glass plate was then placed above the paper towels to ensure an even transfer and the filter paper “wicks” were layered with plastic wrap to prevent desiccation (Sambrook and Russell, 2002). After the RNA samples had been transferred overnight by capillary action, the membrane was
dried, first at 60°C and then baked for 2 hours at 80°C in a vacuum oven, before storing at -80°C. The Hybond N+ nylon membrane filter replicas were then used for Northern blot hybridization.

Prehybridization was done for 3 hours at 42°C in a solution containing 5X SSC (1X SSC is 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0), 5X Denhardt’s reagent, 0.5% SDS, 50% deionized formamide, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA (pH 7.5), 2.5 mM sodium pyrophosphate (pH 8.0), and denatured sheared salmon sperm DNA (100 µg/ml). Subsequently, hybridization was done overnight at 42°C using a solution containing 5X SSC (1X SSC is 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0), 5X Denhardt’s reagent, 0.5% SDS, 50% deionized formamide, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA (pH 7.5), 2.5 mM sodium pyrophosphate (pH 8.0), denatured sheared salmon sperm DNA (100 µg/ml), and the homologous cotton osmotin 32P-labeled probe prepared by Dr. Irma Pirtle as described previously. After hybridization, the membranes were rinsed 30 min. at room temperature with prehybridization solution. This was followed by two 30 min. washes with 2X SSC, 0.1% SDS at room temperature, one wash with 1X SSC, 0.1% SDS at room temperature for 30 min., and one 45 min. wash with 0.2X SSC, 0.1% SDS at 55°C. The nylon membrane replicas were dried at 37°C and placed in autoradiography using Kodak X-OMAT® film with an intensifying screen at -80°C.

The RNA samples were amplified with primers designed specifically for the 5’- and 3’-UTRs of the OSMI, OSMII, and OSMIII genes. Residual DNA was removed from the total RNA samples by incubating 1 µg of RNA with 1X DNase I Reaction Buffer (20 mM Tris-HCl
(pH 8.4), 2 mM MgCl$_2$, and 50 mM KCl) and 1 unit of DNase I (Amplification Grade, Invitrogen) for 15 min. at room temperature. The DNase I was inactivated by heating the samples for 10 min. at 65°C after bringing the samples to final concentrations of 2.5 mM EDTA. The DNase I-treated RNA samples were then used as template for RT-PCR analyses.

The RNA samples isolated from the treated cotton plants were used as templates for primers designed for the OSMI, OSMII, and OSMIII genes. The forward and reverse primers were: 5’-ACAAATCCCCAGTAAGAGCTAACC-3’ and 5’-CAAGGCGCAGATTAACTAGACC-3’ for the OSMI gene;

5’-CAAATCACCAAGTAAAAACCAACC-3’ and 5’-CCAAATGCAAATCAACTACTCC-3’, for the OSMII gene; and 5’-CTCCATGCAATACAAAGCTAGC-3’ and 5’-CATAGCAATGATTTGATTCTCTCG-3’ for the OSMIII gene. Using the Invitrogen SuperScript™ One-Step RT-PCR with Platinum® Taq Kit (Invitrogen) and the gene-specific primers, the RNA samples were amplified by RT-PCR. The RT-PCR amplification mixtures contained 200 ng of RNA template, 1X Reaction Mix (0.2 mM of each dNTP, and 1.2 mM MgSO$_4$), 50 units of RNaseOUT™ (Invitrogen), 0.2 µmole of each primer, and 1 µl of RT/Platinum® Taq mix in a final volume of 50 µl. The reaction mixtures were then placed in a thermal cycler and incubated at 50°C for 30 min. to allow the RNaseOUT to inhibit any ribonucleases that were present. The mixtures were denatured for 2 min. at 94°C. The reactions then ran for 35 cycles, denaturing for 15 seconds at 94°C, annealing for 30 seconds at 59°C, and polymerizing for one min. at 72°C. After the final cycle, the reactions were
maintained at 72°C for 10 min. and then cooled to 4°C. The PCR reactions were electrophoresed on a 1.0% agarose gel to confirm the presence of the appropriate PCR fragments derived from the osmotin genes. This work was done with Ms. Kimberly Spradling of our laboratory.

**Induction, Isolation and Western Blotting of Osmotin Proteins from Cotton Plants**

Using a modified protocol of Chlan and Bourgeois (2001), protein extracts were prepared from 4 to 6 week old cotton plants that had been treated with various chemical inducers working with Ms. Kimberly Spradling of our laboratory. Three to four cotton plants from a single pot were sprayed with distilled water, 100 mM H$_2$O$_2$, 550 mM NaCl, or 1 mM ethephon and placed in a one gallon clear Ziplock bag. After an appropriate amount of time, plants were removed from the bags and all soil was removed from the plant by washing gently with distilled water. The plant materials were washed briefly with distilled water and blotted dry. The plant tissues were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The frozen ground tissues were transferred to pre-weighed 50 ml polypropylene centrifuge tubes and placed on ice. The liquid nitrogen was allowed to evaporate and 100 mM sodium citrate (pH 5.0) was added at one ml per gram of tissue. The tissues and buffers were mixed thoroughly, and the slurries were centrifuged for 10 min. at 11,000 xg and 4°C. The resulting supernatants were filtered through Miracloth (Calbiochem) and collected in sterile 50 ml Falcon polypropylene tubes. Bradford assays were performed to determine protein
concentrations before use in SDS-PAGE and Western blot analysis, in collaboration with Ms. Kimberly Spradling.

The isolated protein samples were separated on a discontinuous buffer system (Laemmli, 1970). The system has two sections, the upper portion or stacking gel containing 5% acrylamide/bis-acrylamide (29:1), 0.15 M Tris-HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate (APS), and 0.12% TEMED and the separating portion, containing 15% acrylamide/bis-acrylamide (29:1), 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate (APS), and 0.04% TEMED. Before electrophoresis on the 5% stacking/15% separating SDS-polyacrylamide gel, 10 µl of 6X sample buffer (350 mM Tris-HCl (pH 6.8), 30% glycerol, 10% SDS, 9.3% DTT, and 0.012% bromophenol blue dye) was added to protein samples suspended in sodium citrate buffer. All samples were heat-treated at 100°C for 5 min. and cooled to room temperature before loading. A total of 40 µl of each sample was loaded per well and separated at 30 mA per gel using 1X Running Buffer (25 mM Trizma base, 250 mM glycine, 0.1% (w/v) SDS) in a Bio-Rad mini-protean II apparatus (Bio-Rad). After the proteins had been separated by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane by electroblotting using the Mini Trans-blot Electrophoretic Transfer Cell (Bio-Rad). Following the manufacturer’s protocol, the separating portion of the gel, a nitrocellulose membrane, and all other appropriate supplies were soaked for 45 min. in Transfer Buffer (48 mM Tris-HCl, 39 mM glycine, 20% (v/v) methanol, pH 9.2). The gel and membrane were arranged according to the manufacturer’s protocol and transferred overnight at 70-90 mA using a Model 250/2.5 Bio-Rad power supply. The resulting membrane containing
the separated proteins was tested for the presence of osmotin proteins using the polyclonal anti-osmotin antibody preparation.

The membranes were blocked with 5% milk-TBS (5% (w/v) dry milk, 20 mM Tris-HCl, 150 mM NaCl) for two hours at room temperature with shaking. The membranes were washed twice with TBS-T (TBS with 0.35% Tween 20 (polyoxyethylene-sorbitan monolaureate)) for 10 min. with gentle shaking and subsequently incubated with anti-osmotin antibody (Biosynthesis Inc.) at a 1:1,000 dilution in 5% milk-TBS-T for one hour at room temperature. The membranes were washed twice with TBS-T for 10 min. with gentle shaking and then incubated with a 1:1,500 dilution of the secondary antibody (Anti-rabbit Ig, Horseradish Peroxidase linked whole antibody from donkey; Amersham). The excess secondary antibodies were removed by washing the membranes two times for 10 min. with TBS-T. The bound antibodies were then visualized by incubating the membrane with ECL Western Blotting Detection Reagents (Amersham Pharmacia biotech), in a 1:1 mixture for one min. and then exposing the membranes to Kodak X-OMAT® film.

Genomic Blot Analysis

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 to confirm that the DNA fragments derived from the osmotin gene in pCOSM16B occur in
authentic genomic DNA and to determine the number of similar osmotin genes in the cotton genome. The prehybridization and hybridization conditions with the homologous 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.

Construction of Three-Dimensional Models of the OSMI and OSMII Proteins

To study the core structure of the osmotin proteins formed by the β-sheet network predicted by the DNASIS software version 2.1 (Hitachi) and Antheprot V5.0 (http://pbil.ibcp.fr/ANTHEPROT), three-dimensional models of the OSMI and OSMII proteins were generated. The predicted three-dimensional structures of cotton osmotin proteins I and II were generated by comparing the osmotins to the crystal structure of tobacco PR-5d protein (Koiwa et al., 1997; 1999) using the 3D-JIGSAW program (www.bmm.icnet.uk/servers/3djigsaw/; Bates and Sternberg, 1999; Bates et al., 2001; Contreras-Moreira and Bates, 2002). The resulting models were viewed and manipulated using RASMOL (RasWin Molecular Graphics 2.6) (http://www-fbsc.ncifcrf.gov).
CHAPTER 3

RESULTS

CHARACTERIZATION OF A COTTON OSMOTIN GENE CLUSTER

Isolation and DNA Sequence Analysis of Cotton Osmotin cDNA Clones

Approximately 200,000 plaques from a 48-hour cotton cotyledon cDNA library, cultivar Delta Pine 62 (Ni and Trelease, 1991) harbored in the UniZap (Stratagene) lambda vector were screened by the plaque-hybridization method of Benton and Davis (1977). A 884-bp \textit{HindIII/SalI} fragment from a tobacco osmotin gene (Nelson et al., 1992) was used as template to generate radioactive hybridization probe by random priming (Feinberg and Vogelstein, 1983). The probe was used for hybridization of DNA fragments from the cDNA library immobilized on positively-charged nylon membrane replicas. Several plaques that had intense hybridization signals were selected for plaque purification and phagemid rescue. The pBluescript SK (-) phagemid DNAs encoding putative osmotin-like transcripts were rescued \textit{in vivo}, isolated by a mini-plasmid preparation (Titus, 1991), doubly digested with the restriction endonucleases \textit{KpnI} and \textit{SalI}, and the DNA fragments resolved by electrophoresis on a 1.0% agarose gel. The agarose gel was then alkaline-blotted and hybridized with the radioactive probe derived from the tobacco osmotin-like gene (Nelson et al., 1992). Figure 2 is a representative alkaline-blot hybridization showing a number of hybridizing cDNA clones encoding putative osmotin-like PR5 proteins. The actual cDNA inserts of about 1 kb (kilobase) in size, not including the poly(A) tails, are larger than the apparent 700-bp size inserts.
Figure 2. Autoradiogram of representative alkaline-blot hybridization of isolated phagemid DNAs encompassing putative cotton osmotin-like cDNA clones. The cDNA inserts were cleaved from the phagemid DNAs by double digestion with the restriction endonucleases $KpnI$ and $SalI$. A 884-bp $HindIII/SalI$ fragment from the tobacco osmotin-like genomic subclone (Nelson et al., 1992) was used as template to generate the $^{32}P$-labeled hybridization probe by random priming (Feinberg and Vogelstein, 1983). Weakly hybridizing fragments of the linearized recombinant 2.6 kb Bluescript SK (+) vector (Stratagene) are seen, and the more intensely hybridizing fragments of the presumptive osmotin cDNA inserts (1.2 kb and 0.7 kb) are also observed. The relative mobilities of the standard pGEM DNA markers (Promega) are shown on the right side. The cDNA clones whose profiles are shown in lanes 47B2 and 52A2 (indicated by vertical arrows) were selected and the corresponding DNAs purified using a Wizard Miniprep Kit (Promega). The cDNA inserts were sequenced on both strands using the M13 -40 forward and the -50 reverse universal primers. The excised phagemid cotton cDNA clones were designated pCcOSM47B (as described in Yoder, 2002) and pCcOSM52A.
indicated by the relative mobilities on the electrophoresis gels. This difference in the estimated cDNA insert sizes is due to the numerous AT-rich regions found in the 3’-regions of the coding sequences, as well as the 3’-untranslated regions (UTRs), of the putative osmotin-like cDNA clones, causing the DNA fragments to migrate with apparent relative mobilities greater than DNA fragments of similar sizes and nucleotide compositions. This part of the work was done in conjunction with Dr. David Yoder of our laboratory.

The cotton cDNA phagemid clones designated pCcOSM47B (described in Yoder, 2002) and pCcOSM52A were among those selected for purification using the Wizard Miniprep Kit (Promega). The cDNA inserts were initially sequenced on both strands using the M13 -40 forward and M13 -50 reverse universal primers (Fan et al., 1996). The entire cDNA inserts of pCcOSM47B and pCcOSM52A were sequenced on both strands using a primer-based approach, and the cDNA inserts were determined to be 1,052 bp and 878 bp in size, respectively (Figures 3 and 4), excluding the poly(A) tails. Both sequences contain putative poly(A) polymerase near-upstream elements (Hunt, 1994) in the 3’-UTRs. For the OSM1 cDNA insert in pCcOSM47B, the near upstream element, with the canonical sequence of AATAAA, is located from nucleotides 1,028 to 1,033. For the OSMII cDNA insert in pCcOSM52A, the element is located from nucleotides 868 to 873. Based upon the genomic sequences (Appendices A and B), the cDNA insert of pCcOSM47B was inferred to lack 10 basepairs at the 5’-end of the open reading frame (ORF), and the cDNA insert of pCcOSM52A was deduced to lack 101 bp at the 5-end of its open reading frame.
The cotton osmotin preproteins (Figures 5 and 6) of 242 amino acids, deduced from the cDNA sequences of pCcOSM47B and pCcOSM52A and the gene sequences of pCcOSM16B and pCcOSM12A were analyzed using the software package PSORT (http://psort.ims.u-tokyo.ac.jp/) to predict putative targeting sequences and the preprotein cleavage sites, and by Antheprot V5.0 (http://pbil.ibcp.fr/ANTHEPROT) to predict isoelectric points (pI) and preprotein cleavage sites. The cotton osmotin preproteins were predicted to have N-terminal preprotein signal sequences (underlined in figures 5 and 6) that direct the preproteins into the extracellular secretory pathway (Neuhaus and Rogers, 1998), resulting in the extracellular secretion of the mature isoforms. The predicted preprotein C-terminal regions lack the necessary signal sequences for vacuolar targeting. Both preprotein signal sequences are predicted to be cleaved between the 24th and 25th amino acids, which in both preproteins consist of two alanine residues. The mature polypeptides are predicted to have pI’s of 7.5 for the OSMI polypeptide and 7.2 for the OSMII polypeptide. Both proteins have 16 invariant cysteine residues (highlighted in Figures 5 and 6), which are an important features in the formation of eight disulfide bonds of the two proteins (de Vos et al., 1985; Ogata et al., 1992; Koiwa et al., 1994; Batalia et al., 1996; Koiwa et al., 1997). These invariant cysteine residues are also found in all other PR5 proteins, such as those from Vitis raparia (AF178653), N. tabacum (S40046), T. danielli (J01209), and A. thaliana (U83490).
(M S Y) L T I S Q I S S L L F F S V L F I

ATGGACGCTAC TAA CAT TTT CCA AAT TCT TCC TCC CTC TCT TTT TAC GT TCC TTT TTC

SAH AFE I R E N C S Y T V W A A

+61
TTCCGTCATCAGCCGGCGCTTGAATCTCCCAATAGAAGTGCTCTCAAGGGTCTGACGCA

ASPGGGRDRPRQSWSWTIDV

+121
GGCTTCTCGTTGCTGGCCTGCCTTCTAACCTTGCCGAAGTTGGACCAGTGATGCTGCT

AGTAMARIGRTNCNFASG

+181
GCTGACACTGCTATGGCTGTATTTGGGTCGAAACCACATGCAATCCGAGCTGGT

RGCQHTGDCCGGLLQCKGKGVD

+241
AGGGTCATTCGCAACCGGTAATTGGTGGGTGACTCCCTCAAATGCAAGTTGGGTTGTA

PPNTLAEYNQFNGMDFYD

+301
CCCTCAACACCCCTGGCCTGAATAATTGACCTTAACCAATTTGAAACATGGATTTCTACGAC

ISLVDGFNNIPMVGPNTNGGC

+361
ATTTTCTTAGTTGATGGGTTTTACATCCCCCCATGGTGTGTGGTCTACAACTAGTGCGGTGGTA

HNIRCTADINGQCPNELRAP

+421
CACACATCTCGGTGACACTGAGCACAATCAACCGGAATCGCGATGTTAAGACTCTCT

GGCNNPNCTVFKTNERYCCTQG

+481
GGTGGGCATATAACCCACGCGGTGTTTAAAGCAATAATTGGTGCACCTCAAGGG

YGTCGPGTTRYSRFFKDKCRDS

+541
TACGGACCTGTCGTCGCCGCCCCGTATTTCAAGGACGGGTCGCTGGTATTTCA

YSYPQDDDSPSTTFCAPASN

+601
TACAGTTATATCCTCAAGATTGTATCTTGCGACCTTTAATTTGCTCTGTGGTTCCAAATAC

RVPFCPRGSPHIEMVGSKSQ

+661
AGGGTGCTGTTTTCCCCGACGCCATCCTCCTATAGAGATGTCGGAGATAGCGCCAAC

EKTER

+721
GAAAAATAAGGATCACTCTTCATCCATATCTGCGGCTGGGACCTAAAATAAAA

+781
AAAGTCATTGGGATTCCAAATGATATAAACCACGCGGTCTACTTGGTTTTATTTTACT

+841
ATGTTTTGTGTTAATATGTCGCGTTTCAAACAGCCACAGACAGCTCAACAGGTCTAT

+901
TCGCTTCATCAAAAAACTCATAAAATACCCATATATAAAAAATTTGATAGAATATTGAT

+961
TATTTTTTTATGGAATATAATTGATGATATTTATATATATATATATATATTATATTATCATATTG

Poly A Poly A Site

+1021
ATACGTAATAAAAATTATTTTTAAATTTATATTTATCATTATTPA

+1061
Figure 3. DNA sequence of the coding region and 3’-untranslated region (3’-UTR) for the presumptive cotton osmotin cDNA clone designated pCcOSM47B. The ten 5’-terminal basepairs (bp) were determined from the DNA sequence of the cognate cotton osmotin gene encoded by the genomic clone designated pCgOSM16B (Appendix A), indicated by parentheses. The presumptive poly(A) signal sequence (AATAAA) and poly(A) site are underlined. The deduced preprotein amino acid sequence is shown above the DNA sequence using the single-letter amino acid code. This putative cotton osmotin cDNA sequence has been assigned the GenBank Accession number AF192271.
(M S Y L T I S Q I S S I L F F S V L F I
+1
ATGAGCTCTTACATACATCTTCCAGATCTTCCACATCTTCTTCCAGCTTCTTCTTTTCAT
S A H A R F E I R N E C P) Y T V W A A
+61
TCGGGCCCATGCAGCGCGCTGGATAGGCCATCAATATGAGGCACTTCCAGCTTTTCCAT
A S P G G G R R L D P R Q S W T I N V P
+121
GGCGCTCCTGGTAGTAGACCCAGGGCAAGTGAGGACCATCAATATGCCT
A G T A M A R I W G R T N C N F D A N G
+181
GCTGGCACAGCCATGGGCTGATCTCTGGGGTCGGCACAAATGCAAATTCGATGCCAATGTG
R G H C Q T G D C G G L L E C Q G W G V
+241
AGGGGTCATTTGCCAAACCGGTTACTGTTGGACTCATTGAGTCCAAAGGGTTGGGGTGTC
P P N T L A E Y S L N Q F G N M D F Y D
+301
CCTCCAAACACCTTTGGCTGAATACCTTAAATCTAAATTTGGAACATGGGTGTTCTACGAC
I S L D G F N I P M V F G P T N G G C
+361
ATTTCCTTAATTGACGGGTACATCAACCTCCTCCATGTTGGTTCTCCACCTATGCGGTTGT
H N I R C T A D L K G Q C P N E L R A P
+421
CACACATTCGCTGACACAGCAGCTTCACAGGACATGCCGAATGTTTAAAGGCCTCCT
G G C N N P C T V F K T N E Y C T Q G
+481
GGTGGGTCGCAAAACGAGCAGGCTCAAAACCAATGAGGTATATTGCAACTCAGGAGCC
Y G T C G P T Y F S R F F K D R C H D S
+541
TACGGAACTGTTGGTCCGGACCCTACTCTCAAGTTTCAAGGACGCGGTCCTAGATTCTCA
Y S Y P Q D D P S S T F T C P A G S N Y
+601
TACAGTATCTCAGGATCTGCTTTAAGCTCCCTAGGTCGTCCGGATCATTAC
R V V F C P R G S P R I E M V G S K N Q
+661
AGGGGGTCATTTGCCAAGGCTACGGCGGAGAGAGATGTTGGAGCGGAAGAACCAAA
E K *
+721
GAAAAGTCTAAAGGGATGATCCTTGGGATGGTTGATTTGCAATTGGGATTTGGAATTAAAAAAATAAG
+781
A2CATTTGGCTTTTGGCAAAATGATATTGACATTTGCTATTGCTTTTACTCAATAT
Poly A Signal Poly A SiteA
+841
GTTTTGTTTAATGGGAGAGGAAGAAAAAACAATATAATACTA
+878
Figure 4. DNA sequence of the coding region and 3’-untranslated region (3’-UTR) for the presumptive cotton osmotin-like cDNA clone designated pCcOSM52A. The 101 5’-terminal basepairs (bp) were determined from the DNA sequence of the cognate cotton osmotin gene II encoded by the genomic clone designated pCgOSM12A (Appendix B), and are indicated by parentheses. The presumptive poly(A) signal sequence (AATAAA) and poly(A) site are underlined. The deduced preprotein amino acid sequence is shown above the DNA sequence using the single-letter amino acid code. This putative cotton osmotin cDNA sequence has been assigned the GenBank Accession number AY301283.
Figure 5. The deduced amino acid sequence of the cotton osmotin preprotein I, based upon the DNA sequence of the *OSMI* open reading frame in the plasmid pCcOSM47B in Figure 3 and the cotton osmotin gene (*OSMI*) in the plasmid pCgOSM16B in Figure 12. The N-terminal sequence, predicted by the software program PSORT (http://psort.ims.u-tokyo.ac.jp/), for extracellular targeting (Neuhaus and Rogers, 1998) is underlined, and the presumptive cleavage site is denoted by the pound (#) symbol. This putative neutral osmotin protein I is likely secreted to the extracellular matrix, due to the absence of a necessary C-terminal vacuole targeting sequence (Neuhaus et al., 1991; Liu et al., 1996). The 16 conserved cysteine residues are numbered and highlighted.
Figure 6. The amino acid sequence of the cotton osmotin-like preprotein, deduced from the DNA sequence of the OSMII open reading frame in the plasmid pCcOSM52A shown in Figure 4 and the cotton osmotin (OSMII) gene in the pCgOSM12A plasmid in Figure 13. The N-terminal sequence, predicted by the software program PSORT (http://psort.ims.u-tokyo.ac.jp/), for extracellular targeting (Neuhaus and Rogers, 1998) is underlined, and the presumptive cleavage site is denoted by the pound (#) symbol. This putative neutral osmotin protein II is likely secreted to the extracellular matrix, due to the absence of a necessary C-terminal vacuole targeting sequence (Neuhaus et al., 1991; Liu et al., 1996). The 16 conserved cysteine residues are numbered and highlighted.
As shown in Figure 7, the coding regions of a number of osmotin-like protein cDNAs/genes were compared at the nucleotide sequence level. The highest degree of identity (75%) with the cotton osmotin coding region is with the *Fagus sylvatica* (beech tree) osmotin coding region (AJ298303). As would be expected, tobacco osmotin coding sequences have the highest identities with each other (greater than 90%), and the grape osmotin sequences also have high identities of more than 60% with each other. In contrast, the osmotin coding regions of several species have low sequence identities, such as between *Arabidopsis* and *Atriplex*, with identities of 32% and 37%. From comparison of the alignments of the deduced amino acid sequences for the putative cotton osmotin preproteins and other putative plant osmotin preproteins in Figure 8, a 59% identity occurs between the cotton and grape osmotin proteins, a 48% identity occurs between the cotton and tobacco preproteins, about 35% identities occur between the cotton and the thaumatin and maize preproteins, and only a 17% identity occur between the cotton and *Arabidopsis* preproteins.

The three-dimensional crystal 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 by X-ray crystallography. All three protein structures are highly similar (Kitajima and Sato, 1999). The three proteins have an extensive β-strand network that forms a β-sheet motif called a flattened β-barrel (or β-sandwich), and also have a second structural motif composed of small turns and loops stabilized by eight disulfide bonds. As seen from inspection of the amino acid sequence alignment (Figure 9), there are 16 invariant
Figure 7. Comparison of a number of the DNA sequences of plant osmotin gene and cDNA coding regions. The DNA sequences obtained from the GenBank Database were aligned using DNASIS software (Hitachi). The sequence identities are shown above as percentages.
Figure 8. Selected plant osmotin amino acid sequences from the GenBank Database were aligned using DNASIS software (Hitachi). The sequence identities are shown above as percentages. The osmotin sequences compared are: *G. hirsutum* OSMI (AY303690), *G. hirsutum* OSMII (AF304007), *Vitis raparia* (AF178653), *N. tabacum* (S40046), *T. danielli* (J01209), and *A. thaliana* (U83490).
cysteine residues in the deduced primary structures of all six PR5 proteins. de Vos et al. (1985) observed that the turn and loop motif 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 structural feature could be important for binding to fungal membrane receptors. The putative cotton osmotins also have 16 invariant cysteine residues and have similar \( \beta \)-strand structures that occur in thaumatin, zeamatin, and tobacco protein Pr-5d, and hence could potentially adopt the \( \beta \)-sandwich three-dimensional structure for interacting with fungal membrane receptor proteins.

The three-dimensional structure of tobacco protein Pr-5d (Kitajima and Sato, 1999; GenBank Locus 2981950) was used as a model for target-template alignment to generate three-dimensional models of the mature cotton osmotin proteins. These models are shown in Figure 10 and the major structural domains of the flattened \( \beta \)-barrels and turns/loop regions that are found in PR-5d are evident in both mature cotton osmotin isoforms.

### Isolation and DNA Sequence Analysis of a Cotton Osmotin Gene Cluster with Two Presumptive Genes and Two Pseudogenes Harbored in Three Overlapping Genomic Clones

Prospective genomic clones encompassing osmotin genes were isolated from two cotton genomic libraries harbored in the EMBL3 (Acala SJ-3) and FIXII (Acala SJ-5) lambda vectors. The DNAs from the genomic clones encompassing presumptive osmotin genes were isolated by mini-lysate preparations. The DNAs were digested with various restriction endonucleases and the fragments resolved by agarose gel electrophoresis. The cotton genomic
<table>
<thead>
<tr>
<th>Species</th>
<th>Cigotoplasma genus</th>
<th>Database Accession</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gossypium hirsutum OSMI</td>
<td>1</td>
<td>PSEK</td>
<td>43</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMII</td>
<td>1</td>
<td>PSEK</td>
<td>43</td>
</tr>
<tr>
<td>Vitis raparia</td>
<td>1</td>
<td>PSEK</td>
<td>47</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>1</td>
<td>PSEK</td>
<td>40</td>
</tr>
<tr>
<td>Thaumatococcus danielli</td>
<td>1</td>
<td>PSEK</td>
<td>43</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>1</td>
<td>PSEK</td>
<td>44</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMI</td>
<td>44</td>
<td>PSEK</td>
<td>88</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMII</td>
<td>44</td>
<td>PSEK</td>
<td>88</td>
</tr>
<tr>
<td>Vitis raparia</td>
<td>48</td>
<td>PSEK</td>
<td>92</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>41</td>
<td>PSEK</td>
<td>86</td>
</tr>
<tr>
<td>Thaumatococcus danielli</td>
<td>44</td>
<td>PSEK</td>
<td>92</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>45</td>
<td>PSEK</td>
<td>93</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMI</td>
<td>89</td>
<td>PSEK</td>
<td>137</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMII</td>
<td>89</td>
<td>PSEK</td>
<td>137</td>
</tr>
<tr>
<td>Vitis raparia</td>
<td>93</td>
<td>PSEK</td>
<td>141</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>87</td>
<td>PSEK</td>
<td>135</td>
</tr>
<tr>
<td>Thaumatococcus danielli</td>
<td>93</td>
<td>PSEK</td>
<td>140</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>94</td>
<td>PSEK</td>
<td>143</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMI</td>
<td>138</td>
<td>PSEK</td>
<td>177</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMII</td>
<td>138</td>
<td>PSEK</td>
<td>177</td>
</tr>
<tr>
<td>Vitis raparia</td>
<td>142</td>
<td>PSEK</td>
<td>181</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>136</td>
<td>PSEK</td>
<td>178</td>
</tr>
<tr>
<td>Thaumatococcus danielli</td>
<td>141</td>
<td>PSEK</td>
<td>181</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>144</td>
<td>PSEK</td>
<td>191</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMI</td>
<td>178</td>
<td>PSEK</td>
<td>223</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMII</td>
<td>178</td>
<td>PSEK</td>
<td>223</td>
</tr>
<tr>
<td>Vitis raparia</td>
<td>182</td>
<td>PSEK</td>
<td>225</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>179</td>
<td>PSEK</td>
<td>223</td>
</tr>
<tr>
<td>Thaumatococcus danielli</td>
<td>182</td>
<td>PSEK</td>
<td>224</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>192</td>
<td>PSEK</td>
<td>239</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMI</td>
<td>224</td>
<td>PSEK</td>
<td>242</td>
</tr>
<tr>
<td>Gossypium hirsutum OSMII</td>
<td>224</td>
<td>PSEK</td>
<td>242</td>
</tr>
<tr>
<td>Vitis raparia</td>
<td>225</td>
<td>PSEK</td>
<td>246</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>224</td>
<td>PSEK</td>
<td>236</td>
</tr>
<tr>
<td>Thaumatococcus danielli</td>
<td>223</td>
<td>PSEK</td>
<td>233</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>240</td>
<td>PSEK</td>
<td>245</td>
</tr>
</tbody>
</table>
Figure 9. Alignments of predicted amino acid sequences of several plant osmotin preproteins.

The osmotin sequences compared are: *G. hirsutum* OSMI (AY303690), *G. hirsutum* OSMII (AF304007), *Vitis raparia* (AF178653), *N. tabacum* (S40046), *T. danielli* (J01209), and *A. thaliana* (U83490). Completely conserved amino acid residues are indicated in reverse contrast. Inspection of the sequences indicates the presence of 16 invariant cysteine residues (shown by vertical gray bars), possibly involved in disulfide bond formation in the three-dimensional structures of the proteins. The amino acid sequences were aligned using DNASIS software from Hitachi.
Figure 10. Predicted three-dimensional structures of cotton osmotin proteins I and II compared to the crystal structure of tobacco PR-5d protein (Koiwa et al., 1997; 1999), generated by the software program 3D-JIGSAW (www.bmm.icnet.uk/servers/3djigsaw/; Bates and Sternberg, 1999; Bates et al., 2001; Contreras-Moreira and Bates, 2002). Predicted tertiary structures of (A) tobacco protein PR-5d, (B) cotton osmotin protein I (OSMI); and (C) cotton osmotin protein II (OSMII). The core structure of all three proteins is formed by a β-sheet network which forms a β-sandwich (upper right of diagrams). The β-sandwich structure could be important for interaction with fungal membrane receptor proteins (Koiwa et al., 1997; 1999). A three-dimensional domain of turns and loops (left of diagrams) is stabilized by eight disulfide bonds formed by 16 invariant cysteine residues. The turn and loop domains occur in other proteins such as toxins and agglutinins, and may be crucial for binding to membrane receptors in fungal membranes (de Vos et al., 1985).
DNA inserts in the cotton genomic clones designated LCgOSM16B (in the EMBL3 vector), LCgOSM12A (in the FIXII vector), and LCgOSM7B (in the FIXII vector) were selected for a large-scale lambda DNA preparations. Physical mapping was accomplished by digestion of the LCgOSM16B, LCgOSM12A, and LCgOSM7B DNAs with a number of restriction endonucleases. The DNA fragments were separated by electrophoresis on 0.8%, 1.0%, 2.0%, and 4.0% agarose gels. Using either a heterologous tobacco osmotin probe or a homologous cotton osmotin probe, alkaline blot hybridization of the immobilized DNAs was done (Reed and Mann, 1985).

The cotton genomic segments contained within the LCgOSM16B, LCgOSM12A, and LCgOSM7B clones were found to encompass two presumptive osmotin PR5 genes and two osmotin pseudogenes. The cotton genomic inserts in the LCgOSM16B, LCgOSM12A, and LCgOSM7B clones were determined to be 16.0 kb, 15.5 kb, and 16.5 kb, respectively, by physical mapping. The three overlapping lambda clones encompass a total of 29.0 kb of genomic DNA, shown with the positions of the intronless osmotin genes or transcription units in Figure 11. Four subclones were generated from the three lambda clones to contain the two potential genes and two pseudogenes. A 7.2-kb SalI/BamHI subclone from LCgOSM16B was shown to encompass the complete osmotin gene I corresponding to the cDNA pCcOSM47B (described by Yoder, 2002, and designated OSMI, Figure 12) and an almost full-length open reading frame corresponding to the cDNA pCcOSM52A (designated OSMII, Figure 13). A 4.2-kb EcoRI subclone from the clone LCgOSM12A that encompasses the complete osmotin gene OSMII was also generated. A 3.6-kb EcoRI fragment and a 3.5-kb
HindIII fragment derived from the clone LCgOSM7B, which contained two osmotin pseudogenes, were subcloned in pUC18 and the pseudogenes designated OSMIII and OSMIV (See Appendix B). Both have internal termination codons which would render the coding regions nonfunctional for translation.

Both strands of the subcloned cotton genomic DNA segments were sequenced using an oligonucleotide primer-based approach, including semi-automated DNA sequencing and terminator cycle sequencing. This work was done in collaboration with Dr. David Yoder and Ms. Kimberly Spradling of our laboratory. A total of 16,007-bp of cotton genomic DNA sequence was determined for the four overlapping subclones. The LCgOSM16B clone in the EMBL3 vector had a total of 7,127 bp sequenced (GenBank AY303690), and the clones LCgOSM12A and LCgOSM7B in the FIXII vector had a total of 8,914 bp determined (GenBank AF304007) as shown in Appendices A and B. The cotton genomic segments contain two complete osmotin genes which lack introns, and are the cognate genomic clones corresponding to the presumptive osmotin cDNA clones designated pCcOSM47B and pCcOSM52A, respectively. The two putative cotton osmotin genes have a wide variety of potential promoter elements in their 5’-flanking regions which could bind various transcription factors for activation of their expression in different environmental situations, perhaps even through synergistic effects (Mitchell and Tjian, 1989; Young, 1991; Thomas, 1993). A prospective cap site for the 5’-end of the mature osmotin mRNAs is located 37 nucleotides (nt) before the ATG initiation codon for the OSMI gene and 36 nt for the OSMII gene. Putative
Figure 11. Physical maps of three overlapping cotton genomic clones encompassing a cluster of two osmotin genes (*OSMI* and *OSMII*) and two osmotin pseudogenes (*OSMIII* and *OSMIV*). The clone designated LCgOSM16B (shown in A) in the lambda EMBL3 vector contains a 16.0-kb cotton DNA segment encompassing the *OSMI* gene and the 3’-half of the *OSMII* gene. The overlapping clones designated LCgOSM12A (shown in B) and LCgOSM7B (shown in C), in the lambda FIXII vector, contain cotton DNA inserts of 15.5 kb and 16.5 kb, respectively, and both clones encompass two genes (*OSMI* and *OSMII*) and two pseudogenes (*OSMIII* and *OSMIV*). The 3’-flanking region of the *OSMI* gene is not contained in the clone LCgOSM7B. An overall 29.0-kb region of cotton genomic DNA is harbored in the three clones. A 16.0-kb segment of cotton DNA overlapping the three clones was sequenced from the proximal *Eco*RI site in the 5’-flanking region of the *OSMIV* pseudogene in clone LCgOSM7B (C) to the proximal *Bam*HI site in the 3’-flanking region of the *OSMI* gene in clone LCgOSM16B (A). The arrows denote the relative polarities of the genes from 5’ to 3’. From right to left, the coding regions of the *OSMI* and *OSMII* genes are 2.2 kb apart, the coding regions of *OSMII* and *OSMIII* are 2.7 kb apart, and the coding regions of the *OSMIII* and *OSMIV* pseudogenes are 0.3 kb apart. The left and right arms of the lambda vectors are indicated by \( \lambda_L \) and \( \lambda_R \), respectively.
Figure 12. The nucleotide sequence of a DNA segment encompassing only the open reading frame and the 5’- and 3’-untranslated regions of the cotton osmotin gene I (OSMI) is shown. The numbering indicates number of nucleotides relative to the beginning of the coding region. The OSMI gene has an open reading frame of 729 bp, including the termination codon, and has no introns. The deduced amino acid sequence of 242 amino acids of the osmotin polypeptide is indicated above the coding region. The putative cap site (underlined) for the 5’-end of the mature OSMI mRNA occurs 37 bp upstream from the initiation codon. The DNA genomic sequence corresponds to nt 2628 to 4067 from the LCgOSM16B genomic clone (GenBank Accession Number AY303690).

Potential regulatory promoter elements (Guilfoyle, 1997) occur in the 5’-flanking region of the osmotin I gene. A TATA basal promoter element (underlined) occurs 12 nucleotides (nt) upstream from the putative cap site. Two basic region helix-loop-helix (bHLH) or E box motifs (underlined) with the consensus sequence CANNTG occur 56 nt and 186 nt upstream from the CAP site in the OSMI gene. The E box motif has been shown to be a seed-specific regulatory element in the French bean β-phaseolin gene (Kawagoe et al., 1994). Two AGC boxes (underlined) are found at 63 nt and 127 nt upstream in the OSMI gene. The OSMI gene has a 3’-untranslated region (3’-UTR) of 337 bp, deduced by comparison with the 3’-flanking region of the corresponding cotton osmotin cDNA clone pCcOSM47B (GenBank Accession Number AF192271) sequenced in our laboratory by Dr. David Yoder (Yoder, 2001). The cDNA clone pCcOSM47B is almost full-length, from nt 10 to 1066 (nt shown by highlighting). The
near-upstream polyadenylation signal (AATAAA, nt 1026-1031, underlined) occurs 40 nt upstream from the 3’-polyadenylation site in the putative mRNA transcript.
Figure 13. The nucleotide sequence of a DNA segment encompassing only the open reading frame and the 5'- and 3'-untranslated flanking regions of the cotton osmotin gene II (OSMII). The numbering indicates number of nucleotides relative to the beginning of the coding region. The OSMII gene has an open reading frame of 729 bp, including the termination codon, and has no introns. The deduced amino acid sequence of 242 amino acids of the osmotin polypeptide is shown above the coding region. The putative cap site for the 5'-end of the mature OSMII mRNA occurs 36 bp upstream from the initiation codon. The DNA sequence in this figure corresponds to nucleotides 7573 to 8892 found in GenBank Submission AF304007, encompassing this gene.

Potential regulatory promoter elements (Guilfoyle, 1997) occur in the 5'-flanking region of the osmotin II gene. A TATA basal promoter element occurs 13 nucleotides (nt) upstream from the putative cap site. Two basic region helix-loop-helix (bHLH) or E box motifs with the consensus sequence CANNTG occur 49 nt and 146 nt upstream from the CAP site in the OSMII gene. The E box motif has been shown to be a seed-specific regulatory element in the French bean β-phaseolin gene (Kawagoe et al., 1994). Two AGC boxes are found at 122 nt and 63 nt upstream in the OSMII gene. The OSMII gene has a 3’-untranslated region (3’-UTR) of 148 bp, deduced by comparison with the 3’-flanking region of the corresponding cotton osmotin cDNA clone pCcOSM52A (GenBank Accession Number AY301283) sequenced in our laboratory. The cDNA clone pCcOSM52A is almost full-length, nt 102-878 (nt shown by highlighting). The near-upstream polyadenylation signal (AATAAA, nt 868-873) occurs 10 nt upstream from the 3’-polyadenylation site in the putative mRNA transcript.
TATA and CAAT basal promoter elements (Mitchell and Tjian, 1989; Young, 1991) are located at -49 and -103 nt for the OSMI gene, and -49 nt and -96 nt from the OSMII gene. Two presumptive ethylene response elements (GCC boxes) with the sequence 5’-AGCCGCCC-3’ occur -127 and -63 nt from the initiation codon for the OSMI gene and -63 and -122 nt for the OSMII gene. 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 at 486, 223, and 92 nt upstream for the OSMI gene and 445, 182, and 85 nt for the OSMII 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 (5’-CCTACC(N7)CT(N4)A-3’) in reverse orientation 1588 nt corresponding to the presumptive osmotin cDNA clones designated pCcOSM47B and pCcOSM52A.
The two putative cotton osmotin genes have a wide variety of potential promoter elements in their 5’-flanking regions which could bind various transcription factors for activation of their expression in upstream from the ATG initiation codon of the \textit{OSMI} gene (nt 1341-1360 in Appendix A). H-box motifs occur in the promoter region of the bean phenylalanine ammonia-lyase 2 (\textit{PAL2}) gene (Hatton et al., 1995) and may be important in developmental-specific gene activation. There are four prospective GATA motifs with the sequence 5’-GATAA-3’ at nt 737, 607, 411, and 307 upstream from the \textit{OSMI} gene and 856, 1100, 1252, 2136 nt upstream from the \textit{OSMII} gene. GATA elements occur in the promoter regions of many light-regulated genes (reviewed in Guilfoyle, 1997) as well as some promoters, like the CaMV35S RNA promoter, that are light-unresponsive (Lam and Chua, 1989). As described earlier, 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, an H-box element, and other promoter elements that are activated by various environmental and hormonal cues.

The 3’-flanking region of the cotton osmotin genes have a canonical near-upstream poly(A) polymerase element (Hunt and Messing, 1998) of 5’-AATAAA-3’ at 33 nt upstream from the poly(A) cleavage/polyadenylation site which is 296 nt downstream from the stop codon, inferred from comparison with the cDNA sequence. Interestingly, a 36-bp AT-rich perfect direct repeat sequence occurs at nt 6659-6694 and nt 6700-6735 in the 3’-flanking region of the \textit{OSMI} gene (Appendix A).
Genomic Blot Hybridization

Alkaline blot hybridization of cotton genomic DNA, as seen in Figure 14, was done with the homologous 884-bp EcoRV fragment purified from the OSMI gene in pCgOSM16B to confirm that DNA fragments derived from the cloned osmotin genes truly occur in genomic DNA, and also to estimate the number of similar osmotin genes in the cotton genome. Double digestion of cotton genomic DNA with XhoI and BamHI produces three hybridizing fragments of 16.9 kb (only barely detectable in this photograph), 7.6 kb, and 5.5 kb. Digestion of cotton genomic DNA with EcoRI generates four hybridizing fragments of 10.5 kb, 4.2 kb, 4.0 kb, and 3.2 kb. Cleavage of genomic DNA with EcoRV yields three hybridizing fragments of 5.2 kb, 3.3 kb, and 0.9 kb. The 5.5-kb XhoI/BamHI and 0.9-kb EcoRV genomic fragments directly correspond to the 5.5-kb XhoI/BamHI and 0.9-kb EcoRV DNA fragments generated from the cloned cotton DNA segment in pCgOSM16B, based on the DNA sequence and physical mapping. The 3.2-kb EcoRI genomic fragment directly corresponds to a 3.2-kb EcoRI restriction sites found near the OSMI gene, while the 4.2-kb genomic fragment corresponds to the OSMII gene. The other hybridizing fragments in the XhoI/BamHI, EcoRI, and EcoRV 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.
Figure 14. Genomic alkaline blot hybridization for cotton osmotin genes. Comparison of the cloned cotton osmotin genes/pseudogenes with cotton genomic DNA from cotton cultivar Paymaster HS26 leaf tissue by alkaline blot hybridization. (A) LCgOSM16b and LCgOSM12A DNAs (2 µg per reaction) were digested with the restriction endonucleases EcoRI, EcoRV, and XhoI/BamHI, and resolved on a 0.8% agarose gel. The DNA fragments were stained with ethidium bromide prior to photography. (B) Alkaline blot hybridization of (A) was with a radioactive probe derived from an 884-bp EcoRV fragment from pCOSMg16B. (C) Genomic DNAs from cotton cultivar Paymaster HS26 (10 µg per reaction) were digested with the restriction endonucleases EcoRI, EcoRV, and XhoI/BamHI, and resolved on a 0.8% agarose gel. Alkaline blot hybridization was done under the same conditions as panel (B). The sizes (in kb) of standard DNA fragments obtained from ? DNA digested with HindIII and pGEM DNA standard markers (Promega) are shown on the left side.
Western Blot Analysis of Osmotin Proteins in Cotton Tissue Extracts

A polyclonal anti-osmotin antibody preparation, antigenic to a polypeptide consisting of the last 18 amino acids on the C-terminal end of the OSMI polypeptide (i.e. CPRGSPHIEMVGSKSQEK), was used to detect osmotin proteins in crude cotton tissue extracts. In Western blots of crude protein extracts from 4 to 6 week-old cotton plants (cv. Acala SJ-5) that had been treated with various chemical inducers (1 mM ethephon, 550 mM NaCl, or 100 mM hydrogen peroxide), a 24 kilodaltons (kDa) protein was detected using the anti-osmotin antibody preparation. This protein was not observed in Western blots of crude cotton extracts treated with water as control (Figure 15). The molecular weights were estimated by comparison with known protein molecular weight standards (Amersham). The smaller molecular protein bands seen in the crude homogenate lanes was determined to be 14 kDa in size, and may be due to an alternative initiation start codon in the coding regions.

Since both osmotin genes I and II have prospective ethylene-response elements in their 5’-flanking promoter regions, ethephon induction should lead to over-expression of the osmotin genes. Similarly, hydrogen peroxide, as a reactive oxygen species, is well-known to serve as a general stress elicitor. Cotton is known to be a salt-tolerant species (Flowers et al., 1977). Therefore, although osmotin in tobacco is induced by salt stress, the same level of response is not seen in cotton. As the C-terminal regions of both osmotin proteins are nearly identical (with only two amino acid variations), the presence of both osmotin proteins is presumed. Working with Ms. Kim Spradling of our laboratory, the presence of both osmotin I and II mRNAs in
various cotton tissues and with various treatments by RT-PCR analysis using gene-specific amplimer pairs has been confirmed (Figure 16).

**Total RNA Extraction for Northern Blot Hybridization and RT-PCR Analysis**

The total RNA samples extracted from roots, stems and leaves of cotton plants that had been treated with 1 mM ethephon, 100 mM hydrogen peroxide, or water (control) were examined by electrophoresis on denaturing formaldehyde agarose gels. Northern blot hybridization with the homologous cotton osmotin probe revealed the presence of osmotin transcripts in both induced and un-induced root, leave, and stem tissues (data not shown). From the Northern blot analysis, it appears that the osmotin mRNAs are produced in all three tissues (leaf, stem, and root) under all three treatments (water, ethephon, and hydrogen peroxide). To ascertain if both *OSMI* and *OSMII* transcripts were present in the RNA extracts from the plant tissue, reverse transcriptase polymerase chain reactions (RT-PCR) were done.

To detect the presence of the two cotton osmotin mRNAs, unique oligonucleotide primers (See Materials and Methods) were designed for the 5’ and 3’- untranslated regions (UTRs) of the osmotin gene sequences (Figures 12 and 13). A 788-bp RT-PCR product was amplified for the *OSMI* gene and a 789-bp RT-PCR product was amplified for the *OSMII* gene. The total RNA extracted from leaf, stem and root tissues from 4 to 6 week-old cotton plants treated with distilled water as control, and induced with 100 mM H$_2$O$_2$, or 1 mM ethephon was amplified with unique primers. Both strands of the 788-bp and 789-bp *OSMI*
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (Water)</th>
<th>1 mM Ethephon</th>
<th>550 mM NaCl</th>
<th>100 mM H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>105 kDa</td>
</tr>
<tr>
<td>25 kDa</td>
</tr>
<tr>
<td>15 kDa</td>
</tr>
</tbody>
</table>

Osmotin (24 kDa)
Figure 15. Western blot analysis of tissue extracts from cotton plants (cv. Acala SJ-5) treated with either water (as control), or induced with 1 mM ethephon, 550 mM NaCl, or 100 mM hydrogen peroxide. Protein samples were prepared from whole cotton plants 24 hours after induction and homogenized in 0.1 M sodium citrate buffer (pH 5.0). A total of 1.5 µg of protein per lane (determined by Bradford analysis) were electrophoresed on a 15% SDS-polyacrylamide gel (Laemmli, 1970) and transferred to a nitrocellulose membrane for Western blot analysis. The membranes were incubated with a 1:1000 dilution of anti-osmotin antibody serum and visualized using horseradish peroxidase-linked anti-rabbit secondary antibody, followed by detection using the Amersham ECL® Detection Kit. Immunoreactive bands corresponding to the predicted mobilities of the osmotin proteins I and II (about 24 kDa) were detected in the ethephon-treated, peroxide-treated, and NaCl-treated samples.
and OSMII RT-PCR fragments generated were sequenced to confirm their identities. The expression of osmotin mRNAs, as determined by RT-PCR, indicated that OSMI and OSMII mRNAs are present in all tissues (including roots, stems, and leaves), when treated with water, ethephon, or hydrogen peroxide (Figure 16). At this point, these results are ambivalent and are being further investigated further by Ms. Kimberly Spradling of our laboratory. Despite the presence of the RT-PCR products under all treatment conditions, varying degrees of expression were seen, depending on the tissue type. The greatest level of expression is apparently in roots and stems, with a much lower level seen in leaves.

**Osmotin Expression Induced by Ethephon Treatment.**

La Rosa et al., (1992) reported that the presence or induction of osmotin mRNA production does not parallel increases in protein production. Thus, the induction of osmotin protein was investigated by comparing crude cotton tissue protein extracts from 4 to 6 week-old cotton plants (cv. Acala SJ-5) that had been treated with either 1 mM ethephon or water, and the tissues collected every 4 hours from 0 to 24 hours, with additional collections at 48 and 96 hours. Using the anti-osmotin antibody, a 23.8 kDa protein was found to be present in the ethephon-treated samples (Figure 17), but it was not present in the control samples (Figure 18). Its molecular weight was estimated by comparison with known protein molecular weight standards. Two smaller molecular protein bands can also be seen in the crude homogenates from both the ethephon-treated and the control samples, and were determined to be 19.8 kDa and 15.0 kDa in size. These two bands may correspond to two predicted polypeptides of 19.5
Figure 16. Total RNA was extracted from cotton plant tissues, treated with water (as control), or induced with 1 mM ethephon or 100 mM hydrogen peroxide. The RNA was amplified by RT-PCR using unique OSMI gene-specific and OSMII gene-specific amplifier pairs. The RT-PCR products and pGEM standard DNA markers (Promega) were electrophoresed and stained with ethidium bromide. Bands corresponding to the predicted mobilities of the osmotin mRNAs I and II (788 bp and 789 bp, respectively) are seen in all sample lanes.
Figure 17. Western blot analysis of cotton plants (cv. Acala SJ-5) treated with water as a control. Protein samples were collected every four hours from 0 to 24 hours, with additional collections at 48 and 96 hours after induction. Whole cotton plants were homogenized in 0.1 M sodium citrate buffer (pH 5.0), and a total of 3.0 µg of protein (per lane) were electrophoresed on a 15% SDS-polyacrylamide gel (Laemmli, 1970) and then transferred to a nitrocellulose membrane. The membranes were incubated with a 1:1000 dilution of anti-osmotin antibody serum and visualized using horseradish peroxidase-linked anti-rabbit secondary antibody followed by detection using the Amersham ECL® Detection Kit. Immunoreactive bands corresponding to the predicted mobilities of polypeptides that would result from alternative translation start codons were detected at 19.8 and 15.0 kDa.
and 14.0 kDa that would result from possible internal translation start codons located at nt 193-195 and 391-393 in both the OSMI and OSMII genes (Figures 12 and 13).

Construction of Plasmid Vectors for Expression of Osmotin Fusion Polypeptides in a Bacterial Expression System

Using specific DNA primers (See Materials and Methods) and either the XhoI/BamHI fragment from the plasmid subclone pCgOSM16B or the plasmid subclone pCgOSM12A (pOSMII) as template, a 700-bp PCR product was generated that contained the coding regions, minus the N-terminal targeting domains, for the OSMI and OSMII open reading frames. These PCR fragments were directionally subcloned into the HindIII/BglII site of the previously prepared PinPoint™ Xa-3 vector (Promega). The resulting 4-kb recombinant plasmids (Figure 19) were used to transform E. coli DH5a cells by electroporation. Plasmid DNAs were isolated from the potential transformants and subclones identified through physical mapping. Presumptive clones were sequenced to confirm the presence of the osmotin I and II coding regions. The constructs were then used to express fusion proteins once they were judged to be intact (i.e. having no deletions, additions, or mutations), as well as having the open reading frames in-frame with the bacterial lacZ N-terminal fusion polypeptide.
23.8 kDa (Osmotin)
19.8 kDa
15.0 kDa
Figure 18. Western blot analysis of cotton plants (cv. Acala SJ-5) treated with ethephon to induce osmotin protein expression. Protein samples were collected every four hours from 0 to 24 hours, with additional collections at 48 and 96 hours after induction. Whole cotton plants were homogenized in 0.1M sodium citrate buffer (pH 5.0), and a total of 3.0 µg of protein (per lane) were electrophoresed on a 15% SDS-polyacrylamide gel (Laemmli, 1970) and transferred to a nitrocellulose membrane. The membranes were incubated with a 1:1000 dilution of anti-osmotin antibody serum and visualized using horseradish peroxidase-linked anti-rabbit secondary antibody followed by detection using the Amersham ECL® Detection Kit. Immunoreactive bands corresponding to the predicted mobilities of polypeptides that would result from the mature polypeptide of 24 kDa and those generated from alternative translation start codons were detected at 19.8 and 15.0 kDa.
Figure 19. Diagram of the Pinpoint™ Xa-3 vector (redrawn from Promega catalog) showing the insertion site for the osmotin I an II PCR products. The recombinant plasmids were generated by inserting the 700-bp HindIII/BgII-PCR products for the OSMI and OSMII genes into the HindIII/BgII site of the 3.3-kb PinPoint™ Xa-3 vector (Promega).
Expression of Cotton Fusion Osmotin Polypeptides in an *E. coli* Expression System

The osmotin proteins were expressed as N-terminal biotinylated fusion polypeptides in *E. coli*. The proteins were induced using IPTG in *E. coli* transformants containing either no plasmid, the PinPoint™ Xa-3 vector (Promega) only, or one of the two osmotin plasmid constructs (See Materials and Methods). The protein samples were prepared by pelleting two 100 µl aliquots from each culture four hours after induction with IPTG. The crude protein extracts were resuspended in either 50 µl of 0.1 M sodium citrate (pH 5.0) or 50 µl of 1X Sample Buffer (See Materials and Methods). Before electrophoresis, 10 µl of 6X Sample Buffer was added to protein samples suspended in the sodium citrate buffer. A total of 40 µl of each sample was loaded per well and separated on a discontinuous buffer system after heat treatment (Laemmli, 1970). After the proteins had been separated by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane and assayed for the presence of biotinylated proteins and osmotin fusion polypeptides. A single biotinylated protein (22.5 kDa) is normally present in wild type *E. coli*. Therefore, cells that express the full-length fusion products would generate a polypeptide of 37 kDa as well as the native 22.5 kDa protein. Protein extracts isolated from the transformed cells containing the recombinant plasmids had the 37 kDa OSMI and OSMII polypeptides (Figure 20), as well as the native 22.5 kDa biotinylated protein. Western blot analyses were also done using the polyclonal anti-osmotin antibody to confirm the identity of the fusion products (data not shown due to picture intensity). The transformants that were found to contain polypeptides corresponding to the predicted mobilities of the fusion
proteins were expressed in large cultures to attain sufficient quantity of products, for isolation using the SoftLink™ Soft Release Avidin Resin (Promega).

Polypeptide expression was induced in the bacterial transformants by adding 1M IPTG to a final concentration of 100 μM and incubating the cultures for 4 to 5 hours. Protein extracts from cultures containing the vector constructs or the control were prepared by cell lysis (See Materials and Methods). The osmotin fusion proteins were then purified using the biotinylated tag and SoftLink™ Soft Release Avidin Resin (Promega). A total of 5.0 µg of protein (per lane) of the isolated fusion proteins and the crude extracts were electrophoresed on a 15% SDS-polyacrylamide gel (Laemmli, 1970), and transferred to a nitrocellulose membrane. The membranes were incubated with a 1:1000 dilution of anti-osmotin antibody serum and visualized using horseradish peroxidase-linked anti-rabbit secondary antibody, followed by detection using the Amersham ECL® Detection Kit. Immunoreactive bands corresponding to the predicted mobilities of osmotin proteins I and II were detected at 37 kDa (Figure 21). The molecular weight was estimated by comparison with known protein molecular weight standards (Amersham). The biotinylated fusion tags were removed from the osmotin fusion proteins by digestion with the Factor Xa protease (Promega).

Detection of proteins was performed using both the strepavidin-alkaline phosphatase and the anti-osmotin antibody. A total of 1.0 µg of protein of the digested isolated fusion proteins were electrophoresed by SDS-PAGE (15%) (Laemmli, 1970) and transferred to a nitrocellulose membrane. The membranes were incubated with a 1:1000 dilution of anti-osmotin antibody serum and visualized using horseradish peroxidase-linked anti-rabbit
secondary antibody followed by detection using the Amersham ECL® Detection Kit.

Immunoreactive bands corresponding to the predicted mobilities of osmotin fusion proteins (37 kDa) and multiple bands of the digested osmotin I protein were detected. The molecular weight was estimated by comparison with known protein molecular weight standards.

Western blot analyses were also done using Streptavidin Alkaline Phosphatase (Promega) and Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega). The anti-osmotin immunoreactive bands of 35 kDa, 23 kDa, 17 kDa, 16 kDa, and 11kDa were identified in the digest of the OSMI fusion protein samples (Figure 22). The 35-kDa band corresponds to fusion protein (37 kDa), and is seen on both Western analyses. The resulting mature OSMI polypeptide is seen as the 23 kDa immunoreactive band on the anti-osmotin Western blots and the biotinylated fusion tag is seen as a 13-kDa band in the strepavidin alkaline phosphatase Western blot analysis. The remaining immunoreactive bands should correspond to the osmotin I fusion protein after the 13-kDa biotinylated tag was removed.

Construction of Agrobacterium tumefaciens Binary Plasmid Vector Constructs for Transformation of Arabidopsis and Cotton Plants for Constitutive Expression of Cotton Osmotin Genes

To generate an Agrobacterium tumefaciens binary plasmid constructs for transformation of Arabidopsis and cotton plants for constitutive gene expression of the OSMI and OSMII polypeptides, the two presumptive osmotin genes, OSMI and OSMII, were amplified by PCR for expression in a eukaryotic system. Unique primers were designed to
<table>
<thead>
<tr>
<th>Size (kDa)</th>
<th>1X Sample Buffer</th>
<th>0.1 M Sodium Citrate (pH 5.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli with no vector</td>
<td>E. coli with no vector</td>
</tr>
<tr>
<td></td>
<td>PinPoint Xa-3 Vector</td>
<td>PinPoint-OSMII</td>
</tr>
<tr>
<td></td>
<td>PinPoint-OSMII</td>
<td>PinPoint-OSMII</td>
</tr>
<tr>
<td>50 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 kDa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Osmotin fusion polypeptides (37 kDa).

Native *E. coli* protein (22.5 kDa).
Fusion polypeptide with no insert (13 kDa).
Figure 20. Western blot analysis of osmotin fusion proteins from bacterial extracts from *E. coli* cells transformed with the PinPoint™ Xa-3 vector (Promega), the PinPoint Xa-3-OSMI gene construct, the PinPoint Xa-3-OSMII gene constructs, or no vector (as control). The protein extracts were resuspended in either 50 µl of 0.1 M sodium citrate (pH 5.0) or 50 µl of 1X Sample Buffer (25% Stacking Gel 4X buffer (500 mM Tris base and 0.4% SDS), 2% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.0025% bromophenol blue). A total of 40 µl of each sample was loaded per well and separated on a discontinuous buffer system after heat treatment (Laemmli, 1970). The biotinylated proteins were detected by incubating with a 1:5000 dilution of Streptavidin-Alkaline Phosphatase (Promega) and visualized using Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega). Immunoreactive bands corresponding to the predicted mobilities of the fusion proteins of 37 kDa were detected in the protein samples recovered from the transformed cells containing the PinPoint-Xa-3 OSMI gene and OSMII gene constructs.
Figure 21. Western blot analysis of osmotin fusion proteins from bacterial extracts from *E. coli* cells transformed with the PinPoint™ Xa-3 vector (Promega), the PinPoint Xa-3-OSMI gene construct, the PinPoint Xa-3-OSMII gene constructs, or no vector (as control). The biotinylated fusion polypeptides were purified using the SoftLink™ Soft Release Avidin Resin (Promega). A total of 5 µg of each sample was loaded per lane (after heat treatment) and the proteins separated on a discontinuous buffer system (Laemmli, 1970). After transfer, the protein samples immobilized on the nitrocellulose membranes were incubated with a 1:1000 dilution of anti-osmotin antibody serum and visualized using horseradish peroxidase-linked anti-rabbit secondary antibody (Amersham) followed by detection using the Amersham ECL® Detection Kit. Immunoreactive bands corresponding to the predicted mobilities of the fusion polypeptides of 37 kDa were detected in the protein samples recovered from the transformed cells containing the PinPoint-Xa-3 OSMI and OSMII constructs.
Figure 22. Western blot analysis of purified osmotin proteins from large-scale induction of *E. coli* cells transformed with either the PinPoint Xa-3-*OSMI* construct or the PinPoint Xa-3-*OSMII* construct. The biotinylated fusion proteins were isolated using the SoftLink™ Soft Release Avidin Resin (Promega). A total of 1µg of each sample was digested with the Factor Xa protease (Promega) and separated on a discontinuous buffer system after heat treatment (Laemmli, 1970). After Western blotting, the proteins immobilized on the nitrocellulose membranes were incubated with a 1:1000 dilution of anti-osmotin antibody serum and visualized using horseradish peroxidase-linked anti-rabbit secondary antibody, followed by detection using the Amersham ECL® Detection Kit. Immunoreactive bands corresponding to the predicted mobilities of the fusion polypeptides of 37 kDa were detected in the protein samples recovered from the transformed cells containing the PinPoint-Xa-3 *OSMI* and *OSMII* constructs. An immunoreactive band corresponding to the mature osmotin protein was detected only in the fractionated digest of the OSMI polypeptide. The biotinylated proteins were detected by incubating with a 1:5000 dilution of Streptavidin Alkaline Phosphatase (Promega) and visualized using Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega). The reactive protein band of 13 kDa, corresponding to the fusion tag, was detected in both OSMI and OSMII polypeptide sample lanes.
amplify a 787-bp *OSMI* gene PCR product and a 728-bp *OSMII* gene PCR fragment such that only the coding regions, without the 5’- and 3’-untranslated regions (UTRs) but containing the sequences that code for the N-terminal signal targeting domains, were amplified. These two PCR fragments were directionally cloned into the *XbaI/EcoRI* sites of a 2.4-kb pUC-based vector containing the 35S cauliflower mosaic virus (CaMV) promoter terminator (Figure 1; Hellens et al., 2000, www.pgreen.ac.uk). The resulting plasmids (designated p35S-*OSMI* and p35S-*OSMII*) contained the osmotin *I* and *II* genes under the control of the constitutive promoter from the tobacco cauliflower mosaic virus. This 1.4-kb cassette was isolated and subcloned into the 11.6-kb binary vector pCambia 2301 from the Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia (Figure 23, www.pcambia.org.au). The coding regions and vector-insert junctions of the 13-kb constructs designated pCambia-35S-*OSMI* and pCambia-35S-*OSMII* were confirmed by sequencing both strands.

The plasmid DNAs were isolated and used to transform electrocompetent *Agrobacterium tumefaciens* LBA4404 cells by electroporation. DNA samples isolated from selected *Agrobacterium* colonies were subjected to PCR amplification of the coding regions of the subcloned osmotin genes. The PCR primers 5’-ATAACTGTATGAATCACGGC-3’ (called p16BR1) and 5’-TTTCATTTCCGCTCATGCAG-3’ (called p16BF1) were used for amplification of DNA samples isolated from *Agrobacterium* transformed with the plasmid construct pCambia-35S-*OSMI*. The PCR primers 5’-

CACGTATTCTAGAGTTAAAAACCCAACCATGAGC-3’ and 5’-


CAGAGAATTCTACTCCAAGAGATCATCCC-3’ were used for amplification of DNA samples isolated from *Agrobacterium* transformed with the plasmid construct pCambia-35S-OSMII. The PCR products derived from the plasmid constructs in *Agrobacterium* were electrophoresed on 0.8% agarose gels to confirm the presence of the appropriate-sized fragments amplified from the two osmotin genes. The clones from transformed cells that showed the presence of the appropriate-sized PCR fragments were designated pCambia-35S-OSMI (clones H1, M3 and G3) (Figure 24) and pCambia-35S-OSMII (clones A, F, G, and J) (Figure 25). The cells were stored as glycerol stocks (33%) at -80°C.
Figure 23. Diagram of the pCAMBIA 2301 vector (Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia, www.pcambia.org.au) showing the insertion site for the 1.4-kb 35S-\textit{OSMI} and 35S-\textit{OSMII} cassettes, containing the osmotin genes under the control of the constitutive 35S CaMV promoter from the tobacco cauliflower mosaic virus (Hellens et al., 2000; www.pgreen.ac.uk). The 1.4-kb inserts were ligated into the \textit{SmaI} site of the pCAMBIA 2301 vector. The resulting 13.0–kb plasmid constructs were designated pCambia-35S-\textit{OSMI} and pCambia-35S-\textit{OSMII}, and were used to transform electrocompetent \textit{Agrobacterium tumefaciens} LBA4404 cells by electroporation.
Figure 24. Plasmid DNA isolated from *Agrobacterium tumefaciens* transformed with the plasmid construct pCambia-35S-*OSMI* and a 555-bp region amplified using the PCR primers 5’-ATAACTGTATGAATCACGGC-3’ (called p16BR1) and 5’-TTTCATTTCCGCTCATGCAG-3’ (called p16BF1). The PCR reaction products were electrophoresed with standard DNA markers and stained with ethidium bromide. Bands of 560-bp, 580-bp, and 580-bp corresponding to the predicted mobilities of the osmotin gene *I* PCR products are seen in sample lanes containing the transformants designated pCambia-35S-*OSMI* H1, M3 and G3.
Figure 25. Plasmid DNA isolated from *Agrobacterium tumefaciens* transformed with the plasmid construct pCambia-35S-*OSMII*. A 728-bp fragment was amplified using the PCR primers 5’-CACGTATTCTAGATTTAAAACCATGAGC-3’ and 5’-CAGAGAATTCTACTCCAGAGATCATCCC-3’. The PCR reaction products were electrophoresed with standard DNA markers and stained with ethidium bromide. Bands of 770-bp corresponding to the predicted mobilities of the osmotin II gene PCR products are seen in sample lanes from the transformants designated pCambia-35S-*OSMII* A, F, G, and J.
CHAPTER 4

DISCUSSION

Fungal or bacterial pathogen infection of crop plants can result in a large reduction of plant biomass and economic output. For disease control and to reduce chemical treatments, genetic engineering of plant defense genes has become an area of intense effort to improve plant defense mechanisms (Hamptom et al., 1990; Paplomatas et al., 1992; Dong et al., 2003). To develop such an approach for cotton, it is first necessary to gain an extensive knowledge of the defense mechanisms elicited in cotton-pathogen interactions. Cotton, as all plants, activates a number of pathways as part of a generalized defense response with different components such as recognition, signal transduction, and response rates for specific pathways (Cui et al., 2000; McFadden et al., 2001; Zhou et al., 2002). Knowledge of the various components of disease resistance should allow a cotton variety with enhanced resistance against multiple pathogens to be developed through genetic engineering. Any establishment of a direct causal relationship between biochemical defense mechanisms and disease resistance as well as the localization and contribution of different defense responses to total defense are important first steps in development of resistant cotton cultivars.

Cotton responses to phytopathogens include the synthesis of antimicrobial phytoalexins or sesquiterpenoids (Brooks and Watson, 1991); cell wall reinforcement due to synthesis of hydroxyproline-rich glycoproteins and the deposition of lignins (Vance et al., 1980); and a major
quantitative change in soluble proteins, which includes several PR proteins. Research on the
PR-4 proteins (chitinases) and PR-2 proteins (1,3-ß-glucanases) have been an active area of
study in cotton for some time (Hudspeth et al., 1996; Levorsan and Chlan, 1996; 1997; Chlan
and Bourgeois, 2001). In contrast, no research has yet been reported on PR5 proteins in
cotton. This dissertation describes the first PR5 protein genes characterized in cotton, the first
osmotin gene cluster analyzed, and the first osmotin pseudogenes reported.

A total of 16,007-bp of cotton genomic DNA was determined from three overlapping
genomic clones designated LCgOSM16B, LCgOSM12A, and LCgOSM7B. The three clones
encompass a total of 29.0 kb of cotton genomic DNA. This work was done in collaboration
with Dr. David Yoder and Ms. Kimberly Spradling of this laboratory. A total of 7,127
basepairs was determined for the cotton genomic insert in the clone designated LCgOSM16B
(GenBank AY303690), and a total of 8,914 basepairs was sequenced for the genomic clones
designated LCgOSM12A and LCgOSM7B (GenBank AF304007), as shown in Appendices 1
and 2. The cotton genomic segments contain two complete osmotin genes or transcription units
(designated OSMI and OSMII), which lack introns and correspond identically with the
presumptive osmotin sequences from the cDNA clones designated pCcOSM47B and
pCcOSM52A, respectively. These osmotin genes would generate putative preproteins of 242
amino acids in length, and the proteins would have characteristic N-terminal signal sequences of
24 amino acids. The mature forms of the cotton osmotins would have 218 amino acids (24
kDa) and would likely be targeted for extracellular secretion as neutral isoforms, with isoelectric
points of 7.5 and 7.2 for the OSMI and OSMII polypeptides, respectively.
Osmotins are known to interact with receptor proteins in fungal membranes by a receptor-mediated signal cascade (Abad et al., 1996). Changes that diminish the receptor-osmotin interactions (either in the fungal receptors or in the ability of the osmotins to bind) ought to decrease the anti-pathogen activities of the osmotins. Increased resistance to osmotins, as seen in yeast cells, results in impairment in sexual reproduction due to mutated pheromone receptors (Abad et al., 1996). A reduction in efficacy of osmotin activity can also be caused by changes in the N-linked phosphomannans of the yeast cell-wall proteins (Abad et al., 1996; Liu et al., 1994; Ibeas et al., 2000). The importance of PR5 protein three-dimensional structure in protein-receptor interactions has been demonstrated by the loss of activity of thaumatin when cross-reacted with anti-thaumatin antibodies (de Vos et al., 1985).

The three-dimensional structures of several PR5 proteins, including thaumatin (de Vos et al., 1985), zeamatin (Batalia et al., 1996), and osmotin (Koiwa et al., 1997), have been determined to be very homologous (Kitajima and Sato, 1999). These three proteins have an extensive β-strand network (a flattened β-barrel) and also a domain of turns and loops stabilized by eight disulfide bonds formed from 16 cysteine residues. These 16 cysteine residues are invariant across all PR5 proteins. Alignment of the deduced amino acid sequences for the cotton osmotin preproteins and other plant osmotin preproteins in Figure 8 indicates that there is a 59% identity between the cotton and grape proteins, a 48% identity between the cotton and tobacco preproteins, about 35% identity with both the thaumatin and maize preproteins, and only a 17% identity with the Arabidopsis osmotin preprotein.
Using these similar proteins for comparison, the same conserved regions of β-sheets and turns and loops stabilized by the 16 cysteines are found. These domains form a highly conserved tertiary structure that has a cleft with two structurally important regions, one with electrostatic potential and one with a hydrophobic patch. In studies of tobacco osmotin, the domain of negative electrostatic potential has four acidic residues (E84, D97, D102 and D184) and the hydrophobic patch consists of two phenylalanines located at residues 90 and 95 (Koiwa et al., 1999; Campos et al., 2002). In cotton, the same two domains can be found in the mature proteins, with the acidic residues occurring at E83, D96, D101, D182 and D183, and the two phenylalanines occurring at residues 89 and 94. The presence of these residues in the active cleft, when considered with the structural modeling, may indicate that the cotton osmotins have antifungal activity.

The cotton osmotin genes have structural similarities to the extensively studied tobacco osmotin gene (Nelson et al., 1992; Raghothama et al., 1993; 1997), with similar sets of prospective promoter elements in the 5'-flanking regions, perhaps leading to the enhanced expression of the genes when confronted with various environmental and hormonal stimuli, similar to the expression of other PR-protein genes. As in the GCC elements (GCC boxes) of tobacco osmotin-like PR5 proteins (Kitajima et al., 1998), the expression of the presumptive osmotin genes appears to be constitutive in roots, stems, and leaves, since both OSMI and OSMII transcripts are detected by RT-PCR analysis in total RNA samples extracted from cotton plant tissues treated with water. Ms. Kimberly Spradling of our laboratory is further investigating the expression of the presumptive osmotin genes in roots, stems, and leaves to
determine if *OSMI* and *OSMII* gene expression is truly constitutive or if the presence of *OSMI* and *OSMII* transcripts in total RNA extracts of cotton plant tissues treated with water is due to environmental stimuli. However, the presence of the full-length osmotin mRNAs did not correlate with the production of the mature osmotin proteins under control conditions. Only upon induction with chemical inducers such as ethephon were the full-length cotton osmotin preproteins detected. This was indicated by the 24 kDa immunoreactive bands detected in crude extracts of 4 to 6 week-old cotton plants (cv. Acala SJ-5) that had been treated with various chemical inducers (1 mM ethephon, 550 mM NaCl, or 100 mM hydrogen peroxide). These proteins were not observed in Western blot analysis of crude cotton tissue extracts treated with water as control (Figure 15).

In tobacco plants, it has been demonstrated that the accumulation of osmotin mRNA and osmotin protein is under complex developmental control (LaRosa et al., 1992). When tobacco plants were treated with tobacco mosaic virus, ethylene, NaCl, abscisic acid, norobornadine, wounding, or auxin deprivation, the osmotin mRNA levels increased. However, only NaCl, ethylene, and auxin deprivation caused any concomitant increase in osmotin protein levels, with ethylene showing only a minor increase (LaRosa et al., 1992). Further investigations of the effects of abscisic acid indicate that the synthesis of osmotin proteins did indeed take place, but no net accumulation occurred, indicating that osmotins may be post-transcriptionally regulated (LaRosa et al., 1992). Cotton plants appear to constitutively produce osmotin mRNAs, but expression of the proteins could be by post-transcriptional regulation by chemical inducers. Unlike tobacco, the largest increase in osmotin expression in cotton plants is induced
by ethylene (ethephon). Only a slight increase in osmotin expression is seen with NaCl treatment, by the ratio of osmotin to total protein shown in Figure 15.

Like ethylene, hydrogen peroxide (H$_2$O$_2$) is a key signaling component in pathogen infection (Hancock et al., 2002). As one of the first reactive oxygen species produced in response to cellular damage from pathogens, H$_2$O$_2$ may function to coordinate and control cellular responses to pathogens (H Hancock et al., 2002). H$_2$O$_2$ is known as a signaling molecule for the hypersensitive response, as well as for cell wall thickening, and is produced in response to many environmental signals. It would be advantageous to cotton plants to activate osmotins as part of the generalized defense responses to the general symptoms of infection, instead of limiting responses to specific chemical signals provided by pathogens. In the Western blot analyses of cotton plant extracts, the 24 kDa osmotin proteins accumulate in response to NaCl, ethephon, and H$_2$O$_2$. The cotton osmotins appear to be induced to a greater degree by ethephon (ethylene), which is a specific signaling molecule for pathogen stress, than by H$_2$O$_2$, a general response signal to pathogen and environmental stress. Thus, it appears that osmotin proteins in cotton, although apparently induced by generalized cell stress, seem to be more dependent upon the specific pathway of ethylene signaling in pathogen stress.

Alkaline blot hybridization analysis of genomic cotton DNA demonstrates that the presumptive cotton osmotin genes are found in the cotton allotetraploid genome, and likely contains multiple orthologs. Multiple hybridizing fragments in the digested genomic DNAs indicate that there are at least three cotton osmotin-like PR5 genes in the cotton genome. Genomic blot hybridization probes can often fail to detect all the genes of a family of PR5
proteins (including osmotins), especially under stringent hybridization conditions, so that an under-representation of the number of potential osmotin genes can result (Velazhahan et al., 1998). Thus, the stringent hybridization conditions employed in the genomic blot hybridization may be an under-representation of the actual number of cotton osmotin genes in the cotton genome. The genomic organization of cotton osmotin genes appears to be unique from other plant osmotin genes, both in number and arrangement. Of the osmotin-like protein genes reported in the literature, only *Brassica campestris* (Cheong et al., 1997) seems to have a multigene family. Other plant genomes, such as petunia (Kim et al., 2002), *Arabidopsis* (Cappelli et al., 1997; Sato et al., 1995; Hu and Reddy, 1997), and *Nicotiana sylvestris* (Sato et al., 1990) seem to have single-copy osmotin genes, or at most, two copies in the case of *Nicotiana tabacum* (Sato et al., 1990). The increased copy number in the cotton genome may be due merely to the allotetraploid nature of the cotton genome. A unique arrangement of osmotin genes also occurs in the cotton genome, since two functional osmotin genes are organized in a cluster containing two osmotin pseudogenes. Organization of genes into multigenic families is a common feature in plant genomes, and some thioredoxin genes induced by pathogen attack have been shown to exist as a gene family which is differentially expressed (Reichheld et al., 2002).

Studies of *Gossypium* species have indicated that rapid genomic changes are not associated with allopolyploid formation in cotton. This is because formation of the allopolyploid cotton only occurred 1-2 million years ago, and also because most low-copy genomic loci have not interacted through gene conversion or other mechanisms (Liu et al., 2001). Thus, most low-
copy sequences should evolve independently in natural allopolyploid cotton, and are probably not subject to rapid genome changes, at least at the sequence level (Liu et al., 2001). In the cotton osmotin gene family, an example of gene duplication may be seen by the apparent duplication of the \textit{OSMI} and \textit{OSMII} genes. There is also evidence of a recombination event, since the osmotin pseudogenes \textit{III} and \textit{IV} lack fully functional promoter regions. For example, the \textit{OSMIV} pseudogene may have formed from an unequal cross-over event that placed it in the promoter region of the \textit{OSMIII} pseudogene, resulting in two non-functional pseudogenes. A more complete investigation of the osmotin gene family in the cotton genome must be done before any firm conclusions can be drawn. Genes in clusters may be differentially expressed, either in different tissues, under different stresses, or have completely different functions (Reichheld et al., 2002). It would appear that the \textit{OSMI} and \textit{OSMII} genes are expressed under the same conditions and in the same tissues. It remains unclear if they have the same function and would be effective against the same phytopathogens.

The cotton osmotin proteins were expressed as N-terminal biotinylated fusion proteins in an \textit{E. coli} expression system. The osmotin fusion polypeptides were purified using the biotinylated tag and SoftLink\textsuperscript{TM} Soft Release Avidin Resin (Promega). After the proteins had been separated by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane and tested for the presence of biotinylated proteins and osmotin proteins. Proteins isolated from the transformed cell extracts containing the recombinant osmotin gene plasmids demonstrated the presence of 37 kDa forms of the OSMI and OSMII polypeptides (Figures 19 and 21), as well as the native bacterial 22.5 kDa biotinylated protein. Western blot analyses were also done
with the polyclonal anti-osmotin antibody to confirm the identity of the fusion products. Immunoreactive bands corresponding to the predicted mobilities of the osmotin fusion polypeptide forms were detected at 37 kDa (Figure 20). The molecular weights were estimated by comparison with known protein molecular weight standards. The purified proteins were digested with Factor Xa protease (Promega). For the osmotin I fusion protein, a 24 kDa immunoreactive band, as well as a 13 kDa biotinylated band, were detected after digestion. Additional immunoreactive bands of 18 and 16 kDa were also detected after digestion with Factor Xa protease. The 24 kDa band corresponds to the mobility of the predicted mature osmotin protein, after removal of the 13 kDa biotinylated fusion tag. The two additional immunoreactive bands may possibly be due to the degraded or cleaved forms of the fusion proteins.

In order to construct an *Agrobacterium tumefaciens* binary plasmid vector for transformation of *Arabidopsis* and cotton plants for constitutive gene expression, the two presumptive *OSMI* and *OSMII* genes were amplified by PCR. The sequence and vector-insert junctions of the plasmid constructs designated p35S-OSMI and p35S-OSMII were confirmed by sequencing. The plasmid DNAs were isolated and used to transform electrocompetent *Agrobacterium tumefaciens* LBA4404 cells. Selected plasmid DNA samples isolated from *Agrobacterium* were subjected to amplification of the osmotin coding regions. The transformants that showed the presence of the appropriate-sized PCR fragments were designated pCambia-35S-OSMI and pCambia-35S-OSMII and are being used in transforming *Arabidopsis* and cotton plants to produce transgenic plants that constitutively overproduce
osmotin. These experiments are currently in progress in our laboratory by Ms. Kimberly Spradling.

The osmotin PR5 cDNA inserts in the clones pCcOSM47B and pCcOSM52A are identical to the coding regions of the cotton osmotin genes I and II, respectively, encompassed in the overlapping genomic clones LCgOSM16B, LCgOSM12A and LCgOSM7B. The two osmotin cDNA inserts and the two cognate genes have identical deduced amino acid sequences and positioning of polyadenylation signals. The deduced amino acid sequences show strong conservation of the N-terminal amino acid signal sequences, signal cleavage sites at two alanine residues, and the 16 invariant cysteine residues, when compared to each other and to other PR5 amino acid preprotein sequences. Both OSMI and OSMII mRNAs seem to be constitutively expressed in cotton plant leaves, stems, and roots. The mature 24 kDa osmotin proteins accumulate in plants treated with ethephon or H$_2$O$_2$. Thus, the cotton osmotin genes I and II encompassed in LCgOSM16B, LCgOSM12A and LCgOSM7B encode osmotin PR5 proteins. The coding regions of the OSMI and OSMII genes have been expressed and isolated as fusion polypeptides in a bacterial system. Binary vectors containing the open reading frames of the OSMI and OSMII genes under the control of the 35S CaMV promoter have also been constructed and are being transformed into Agrobacterium tumefaciens for transformation of Arabidopsis and cotton plants, in order to generate transgenic plants highly resistant to fungal invasion.
APPENDIX A

DNA SEQUENCE OF GENBANK ACCESSION AY303690 ENCOMPASSING THE COTTON OSMOTIN I GENE AND A PORTION OF THE OSMOTIN II GENE
Appendix A: DNA Sequence of GenBank Accession AY303690 Encompassing the cotton osmotin I gene and a portion of the osmotin II gene

OSMII

| 1  | GATCTCTTCCATCCTCTTCTTTTCATTTCGCCACATGCGGCAGGCTTTTG   |
| 2  | IRNECPYTVWAASPGGGGR   |
| 61 | AATCCGCAATGAGTGCCCCCTACACTGTTTGGCAGCCTCTTCTGCTGTGCTGCTGL   |
| 62 | LDPRQSWTINVPAGTAMARI   |
| 121 | CTTAGACCCACCGGAAGTTTGACATCAATGCTGCTGACAGCTGGCCTCTCTAT   |
| 122 | WGRTNCNFANDGRGHCQTGDF   |
| 181 | CTGGGTTGCAACATTTGCAATGCTAGGAGGTCATTGCGCAAACCGGTTG   |
| 182 | CGGLLECCQGWGVPPTLAEY   |
| 241 | CTGTGTTGAGACCTTCCTTGAGTCGAAAAGTTGGGGTCTGCTCTCCAAACACCTTGGCTGAATA   |
| 242 | SLNQFGNMDFYDSTRIDGFN   |
| 301 | TTCACTTTAATCAATTTGGAAAACATGGATTTCTACGACATTTTTCTTAATTGACGTTTAA   |
| 302 | IMPMFPGTPNGCHNIRCTAD   |
| 361 | CATCCCCATGTGGTTTGGTTCACACTAATGGCCGTTGCTCAACACATCTGTGCACACAGAG   |
| 362 | LKGQCPNELRAGPGCCNNPCT   |
| 421 | CCTCAAGGGACATGCCCCGAATGTTAGGAGCCTGTGTTGCTGCTAATACCTGACAC   |
| 422 | VFKTNEYCCTQGYGTCPGTY   |
| 481 | AGTTGTCCACAAACCATATGATAGATTGTCGACTCAAGGACTGTTGGTCGGGACTT   |
| 482 | FSRFKKDRCDHSYSYPQDDP   |
| 541 | TTTCCTAGGTTTTTTCAAGGGACCGGCTGCAATGCTACAGTCTATCGGATGTACC   |
| 542 | SSTFTCPAGSNYRVFCPRG   |
| 601 | TTCAAGCACCTTTACTTGGCCGATTTTACAGAGGGTTGTTTTTGCCCCTAGAG   |
| 602 | SPRIMEVGSNKQEK   |
| 661 | CTCAACCCCGCATAGAGATGGTCAAGGAACACAAAGGAAGATAAAAAGGATGATCTCCTT   |
| 721 | GGAGTAGGTGATTTGGCATTTGGGAAATAAAAAATAAAGATCATTTTGCTTTGGCAAATTGAT   |
| 781 | ACTGAACCTATGGATGTATTGTTTTTGGTACATATGGTTTTTTAATGGAGGCAAA   |

Poly A Signal Poly A Site

| 841 | AAACAAATATACATTTTTTTTTGGTGTATTTATAATAATAAAAGAACAATCTGAAAATAA   |
| 901 | ACTATTATTCAGATTTCCAATTTTGGATGATGATATTGGCCCACATCAGTTG   |
| 961 | TCAAACCTGCTTGGTGAATTGAATGAGACATCTCTGTGCTGGAAGAACAACACACTAACACAA   |
| 1021 | CGAATTTAAGGTAATGCTATTTTTTTAGCTTTCTTCTGCTCAACCTGATTCC   |
| 1081 | TGCTGAACTTATATATACATTTTTTTGGTTTTTTTTATGACCTTTTTCTGCTCAGTATTTCC   |
| 1141 | GTTCATGTCCTTTCTTTGGGTGTGCTGATGCTGCTGGCAAACCAGGGTCAAACCTCACCCTGTGAT   |
| 1201 | AAACCAATACACAATACATTTGGACACCCCTTCGCCAAGCAACACCGCTGGCAGCCCGAGTTATCT   |
| 1261 | CATCACTTCTGGTTTCGCTCCCTCGATCGAAGAACAACCGCCTCCAACTACACTCTATCACAC   |
| 1321 | TCTCTCGTTTTTTCAAGGGACACCACTATCTTCACCACCTCTTTGGCAAAACACACCATCTC   |

149
AGC Box

CAAT Box

DHLH

AGC Box

OSMI
APPENDIX B

DNA SEQUENCE OF GENBANK ACCESSION AY304007 ENCOMPASSING THE
COTTON OSMOTIN II GENE AND THE OSMOTIN III AND IV PSEUDOGENES
Appendix B: DNA Sequence of GenBank Accession AY304007 Encompassing the cotton osmotin II gene and the osmotin III and IV pseudogenes

1     GAATTCAGAGAGAGGAAGATTGGGTTTATAAAGAAACCTTTTGTTTTTTATGACAA
60
61    GATAGTGGGAAGAAGAAGACGTTTTTGGAAGTATGGAAGATGTGTTAAGAAAT
120
121   AACTATTTTTAGCAATAATAAGGGGATGAGAATGCTAGAATGTAATAGGCAATAA
180
181   AAACTATCTTTTAGCCAAATAAATGGGATGAGAATGCATAGAAGATGTAATAGGCA
240
241   TAATCACAGAGGCAGCTTTATCGGAAAAAGAGATGGCTGAGACAGCCATAACTCAATTTCA
300
301   CTCGTTAGTCATCTGTCTTTACTCTTTTGCGCTTTTCGATTTTCGAGCAAAAA
360
361   AAAGTTGGCCTCATATTATGAAGATTATATTATATTTATGGGATGATGGATGGAATCGAGTCAATT
420
421   TCAACATCAAATAATCCCAACTATGCCTATTTATGATTCCATATGTTATGGATTATTTTTC
480
481   TAAAGATCCCTTATCTCTAATTATTTATAGGATGATGATTGATTGAATCGATCAATT
540
541   GATCTTCTGTGGTTCTGTATTACAAACATCAAATCCCGAATTTCCCTCATTCTACGAGAT
600
601   TAGCATCACATGTTGTAATTGCTAAGTTTTAAGAGAAAATATTGGTATATCATCTGTCA
660
661   AGATCTTAGACTCTTCAAAATAATGCTCAAGATCATAATAAAATTTTATATAAAATATA
720
721   AAATATCAAAAAAAAATAAATATATAAAAAAAAAATATATGATATAATTATTTAGATGTTTGTAA
780
781   AATAAAAAATAACACTGTTAATGTATAAATTTACTTTAATTAAAATAAAATATAAC
840
841   AGATATTCACGTTCCTCTTCGAGCAGATCTTTTCAAATAAGTAAAAATAAATCACATT
900
901   TTGTTTCCCAGATTGTAGAGATATTTACCCCTACCCATATATTAAGATCTCTTA
960
961   GATTTTCAAGTAAAAATTATTAAAATAACACAACAAATATTATAATTAGAACAAAAAAAATTATA
1020
1021  TGAAAAAGAGTCACTGTTATAATTGGTTATTTTATGTTATAATTTTGGAGTTTTAAAAATAGTATAA
1080
1081  TAGTTCAACCTTATCCATATATGATTATGTATTATTACCTGATACTATTAAAATCA
1140
1141  ATTATTGGCATAATAGCAATTATATAGATTGTATTGTATTGTATGTTATGCATTGATAT
1200
1201  CGACATTATATCAAAACGAGGAGCATAAGTCTCAAAGTCTTTAAACCTTTATCCTC
1260
1261  CTAATATATGATAATTCTTAGGCAATTGTATAAAAAATAATAAAATTTTATTTCAAACACTA
1320
1321  AACCTAACTTTTCCAGACCTCAAGCAATTGTGAGTGACATTGTGCTCCTATTTATTTAT
1380
1381 ATAAATAATTTTTTTTACCATACCTATAAAACACTTGCAAAACCTCCATTTT
1440 1500
1441 GCTTGTTCAGTTTCAAAATATTATTTCCATAAATCCAAACTAAAACCAGAAAACACTCAAAAA
1500
1501 ATTTATGCATTGCTATGCAAGTGGCTTTGAAGAACAAATTCACGAACGTTTAACAGTGG
1560
1561 CATGGATTGAAGCTGTCATCCATTATATGGAAGGCTTTCTTTTATTACAAGCAAAAGCAAGATGC
1620
1621 TGAATATATAGTTGAAATTTTGGTTTATCAAGCATGATGGATAGGTACGAGATTGACT
1680
1681 CCCAAAGGTAAAAGCTGAGATCTGAGAAAAAGGGTGAGCTTTCTTTGGGAAGTGGCTAC
1740
1741 CATTTTTTCATTTCTCTTGTGCTTTAGTGCATCCAAAGCATCATCATGCAATATCAATT
1800
1801 CTTACAAACCATCCCCATGCCGTGATTACTCTAATGCTCTTCAAAGCAGAAGCAAAAGAATA
1860
1861 AGGATACATTCATATTGATATCCACCATTTCTGCTCAACAGAAAACAGAGATTGACT
1920
1921 TCCACTAAAGCTGAGCCTTCTCTCTCATATTGCTATTCTAATGCGTGGGTCCACAATC
1980
1981 AGTATAAAAATTCTAGGACTTTTACATATAAAAGTGCAATTGTATTTTATTTTTTCTACAT
2040
2041 AAAATAAAAACAAATATTTTCTCTGATATGACCATCAAAAAAGCAGAAGCATATTCTTTCTT
2100
2101 GTCAACAACAGATATACCTAAACATGTGCATGTGAACAGGCTAGTTATATTATATCAACATA
2160
2161 CCATTAGTTACCCCTTAAAAATAAGTTAAAAAATTTAATTTAAAAATAAAAAACTAATATACCTTTA
2220
2221 ATCTAAAAATCTAAAAATAATTTTTTTAAAAATAAAATGAGATGGTAAATGGATGAAAAAGA
2280
2281 ATGATGGCTTCTATTAAAACCGCCTTTAAACTCATCAGGTGCTTACGCTGGCTGTGGGTGA
2340
2341 CGGGTGTCAGTCTATCTGCATTTAAGTAGTGCTATTTGTCTGTCCTACAGTTTTTATAATTAA
2400
2401 TGAATATATCCTGTCAGCTGGAGCTTTCGCTGCTGGCTGCTGGATTGATGGAAT
2460
2461 AAAGATTTTCTTATTTTCCGGACCAATTTAATTTTAATTAATTTTTAATATTTTATTTTTAT
2520
2521 TTTAAATTTTCTTATTTAATTTTAAATTTTTAATATTTATTTTTTAAATATTTTTATTTTAT
2580
2581 TTTTTTTAGTCACTTAAATATTTATGCTCTTTTTATAATATTTAAAAATATTTTTAAAAT
2640
2641 TATGATTATCTTTTCAACAAATATTTAAATTAAAGTTTTTCCCTTTTGAGACGTTGGTAGC
2700
2701 CATTTCGGCTGGTTTTTTTCTTGTATTAAATATGCGATGTGGTGTGGTGGGTGTATATCC
2760
2761 ATGTAACATTTTGGGATTTTTTTTTTACATTTTTATTATTAAGTTTTTCTTCTCAATTAT
2820
2821 ATGGTTTAAAGTTTTTTTTTAAATTTTCTACCCCTCAATTAATAGGCGATTTTGCTCTAAT
2880
2881 AAATCAGGTTTAAATTTTCTTTATTTTAATTTAAGGACAATCTACCTGCAGTAAAGTT
2940
CGTATTTTTGGGTTGAGTCTTAGTTCAATTAACAAAAATATTGTACTAATGTAGG

AGGACGTAGGTTTAAATGCATTGAGTGGTGTTCTATTATTTATGGGTTTCTAGACATT

GTGTCAAAATATATAATACGAAACTTTAATAATTTAGATGTTTAACAAAAAATAATTACAT

TTTTAATGTTGAGACATATGAAATTTTACTATATAAAATGGTTTTTTAAAAATAATTTTCCTTAT

AAAATGTTTACAAAAATTTTCAATATATGAAATTTTTACAGAATTTTTTAATAAAAAAAAAATGAAAA

TGATTTCAAGCTATTACTTGAGATGTGGAGAAGTTAATGTATCTGCAGTATTATAATTAA

ATGAATTTTTATGCACATGACTAATATTATTTACAGAATTTTTAATAAAAAATGGAAAA

TGATTTCAAGCTATTACTTGAGATGTGGAGAAGTTAATGTATCTGCAGTATTATAATTAA

ATGAATTTTTATGCACATGACTAATATTATTTACAGAATTTTTAATAAAAAATGGAAAA

AAATTTATGTTGAACGAATATGAATTTTACTATAAAATGTTTTTAAAATAATTTTCTTAT

AAAATGTTACAAATTTTCAATATATGAA TTTTACAGAATTTTTAATAAAAAATGAAAA

ATGTCATGAAATTTGCTGTTAGTATGTGTAATGCATAAAAGCTCATGCGATCGATAATAC

ATCAAATATAATCTCTTATATAATTTATTCATGAATTAAAAAAGAAAAAATT

ATTTTAGATCATAATAACCCGTATGCTAGTTTCTTCATATAAATGCTTTCATATTTAAAAA

TTAATTATATGTCACAAATATTAATATTGTCAGATCAGATCAATTGAGACGATGAAACCCCTAA

ATATTAGACCAACATATAGGGTGCTGGCTGGTTATTTCTTTCTCATATAATGCGTTCATATTTAAA

AAAGTCTAATTTXTATTATGAAGATATATAATACGAGAAATTATAATTTTCTTAT

TTAATTATATGTCACAAATATTAATATTGTCAGATCAGATCAATTGAGACGATGAAACCCCTAA

ATTTTAGACCAACATATAGGGTGCTGGCTGGTTATTTCTTTCTCATATAATGCGTTCATATTTAAA

TTAATTATATGTCACAAATATTAATATTGTCAGATCAGATCAATTGAGACGATGAAACCCCTAA

AAATTTATGTTGAACGAATATGAATTTTACTATAAAATGTTTTTAAAATAATTTTCTTAT

AAAATGTTACAAATTTTCAATATATGAA TTTTACAGAATTTTTAATAAAAAATGAAAA

ATTTTATGTTGAACGAATATGAATTTTACTATAAAATGTTTTTAAAATAATTTTCTTAT

AAAATGTTACAAATTTTCAATATATGAA TTTTACAGAATTTTTAATAAAAAATGAAAA

ATTTTATGTTGAACGAATATGAATTTTACTATAAAATGTTTTTAAAATAATTTTCTTAT

AAAATGTTACAAATTTTCAATATATGAA TTTTACAGAATTTTTAATAAAAAATGAAAA


