ANALYSIS OF THE EXPRESSION PROFILES OF TWO ISOFORMS OF THE ANTIFUNGAL PROTEIN OSMOTIN FROM *Gossypium hirsutum*

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The expression of two cotton osmotin genes was evaluated in terms of the mRNA and protein expression patterns in response to chemical inducers such as ethylene, hydrogen peroxide, and sodium chloride. Reverse transcriptase-polymerase chain reactions (RT-PCR) indicated that osmotin mRNAs are expressed constitutively in root tissues of cotton plants, and that they are rapidly induced in leaf and stem tissues upon ethylene treatment. Real time RT-PCR indicated that osmotin transcript levels were induced 2 to 4 h after treatment with ethephon. The osmotin mRNA levels appear to increase 12 h after treatment, decrease, and then increase again. The osmotin protein expression patterns were analyzed in Western blot analyses using an anti-osmotin antibody preparation. A 24-KDa protein band was detected from cotton plants treated with the inducers. The 24-KDa osmotin proteins were induced 4 h after treatment with ethephon, while down-regulated 96 h after treatment. Multiple osmotin isoforms were observed to be induced in cotton plants upon treatment with ethephon by two-dimensional gel electrophoresis. One goal of this dissertation research was to genetically engineer two cotton osmotin genes to routinely overproduce their antifungal proteins in transgenic *Arabidopsis* and cotton plants as a natural defense against fungal infections, using co-cultivation with *Agrobacterium tumefaciens* cells harboring pCAMBIA 2301 vector constructs containing the osmotin genes. Many transgenic *Arabidopsis* and cotton plants were generated. However, genomic blotting analyses indicated the absence of the osmotin transgenes, but the presence of *GUS* genes from

the vector cassette. Alkaline blot analyses of the vector DNAs from transformed *Agrobacterium* cells confirmed that an anomalous DNA structural rearrangement or aberrant recombination event probably occurred in the A*grobacterium* cells, interdicting the integration of osmotin transgenes into the *Arabidopsis* and cotton plants. This research provides crucial baseline information on expression of cotton osmotin mRNAs and proteins.

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CHAPTER 1

INTRODUCTION

Cotton is the most valuable source of natural fiber, making it one of the largest crops in terms of economic value in the USA (Zapata et al., 1999; Sunilkumar and Rathore, 2001; Zhang et al., 2001; Ikram-Ul-Haq, 2004; Wu et al., 2005). Unfortunately, fungal pathogens destroy a large percentage of the cotton crop and consequently result in substantial economic losses every year (Bajaj, 1998; Lyon and Becerra-LopezLavalle, 2000; Dong et al., 2002; Rep et al., 2002; Cornelissen and Melchers, 1993; Emani et al., 2003; Roncero et al., 2003; Palmateer et al., 2004). There are over 30 different fungal pathogens capable of infecting cotton (Lyon and Becerra-LopezLavalle, 2000; Palmateer et al., 2004). Common fungal pathogens causing cotton seedling diseases include *Rhizoctonia solani*, *Pythium* species, *Fusarium* species, and *Thielaviopsis basicola* (Emani et al., 2003; Palmateer et al., 2004). These soil fungi may occur separately or in different combinations to cause seed decay before germination, seedling decay before emergence, girdling of the seedling after emergence at or near the soil surface, and rotting of root tips (Emani et al., 2003; http://plant pathology.tamu.edu/Texlab/Fiber/Cotton/csdc.html).

Other fungal pathogens that result in large economic loss every year are *Phymatotrichum omnivorum*, *Fusarium oxysporum* f. sp. *vasinfectum*, *Verticillium albo-atrum*, and *Verticillium dahliae* (Bajaj, 1998; Lyon and Becerra-LopezLavalle, 2000; Dong et al., 2003; Palmateer et al., 2004). *Phymatotrichum omnivorum* is the fungal pathogen responsible for root rot, in which the root system of the affected plant decays and results in rapid wilting and subsequent death of the plant within a matter of days.

This devastating disease kills plants in circular areas up to an acre or more in size (http://plantpathology.tamu.edu/Texlab/Fiber/Cotton/crr.html). *Verticillium* and *Fusarium* species are soilborne pathogens that can survive in the soil for extended periods of time. They penetrate the plant roots, enter into the vascular system, and then clog the xylem vessels to result in plant wilting (Rep et al., 2002; Roncero et al., 2003). *Verticillium albo-atrum* and *Verticillium dahliae* cause the fungal infection known as Verticillium wilt, in which chlorosis or necrosis of the leaf margins and between the major veins occurs, and a decrease in photosynthesis and increase in respiration cause plants to lose their leaves and most of their bolls (Dong et al., 2003; http://plant pathology.tamu.edu/Texlab/Fiber/Cotton/cvw.html). *Fusarium oxysporum* f. sp. *vasinfectum* is a fungal pathogen responsible for Fusarium wilt, which normally occurs in more mature plants. Like Verticillium wilt, chlorosis first appears on the margins of the lower leaves and then moves toward the top of the plant. Plants infected with *Fusarium oxysporum* f. sp. *vasinfectum* fruit earlier than normal and produce smaller bolls that open prematurely (http://plantpathology.tamu.edu/Texlab/Fiber/Cotton/cfw. html).

Plants have remarkable defense systems, considering they are constantly being challenged by pathogenic microorganisms and yet the development of disease is relatively rare (Glazebrook, 2001; Selitrennikoff, 2001; Brown et al., 2003). Three ways in which plants react to pathogen attack are 1) a non-host interaction, 2) compatible interaction, and 3) an incompatible interaction (Linthorst, 1991; Ausubel et al., 1995; Greenberg, 1997). With a non-host interaction, which is the most common, the pathogen is unable to replicate and the plant remains unharmed. With a compatible

interaction, the pathogen is able to infect the plant and spread systemically throughout to result in the accumulation of large amounts of the pathogen and disease of the plants that can range from very mild to very severe. With an incompatible interaction, the pathogen initially infects the plant at the site of entry, but is unable to systemically spread through the plant. So, the plant defense response is first activated by an initial pathogen infection, and a subsequent network of signal transduction processes, which are regulated by secondary signal molecules (e.g., salicylic acid, jasmonic acid, and ethylene), results in the rapid activation of defense gene expression (Bowles, 1990; Liu et al., 1995; Greenberg, 1997; Vidhyasekaran, 1997; Schenk et al., 2000; Dong, 2001; Brown et al., 2003).

With an incompatible host-pathogen interaction, the pathogen damage is most effectively restricted by the plants' hypersensitive response, in which there is a rapid necrosis of the cells just around the infection site. This hypersensitive response not only serves to prevent the further spread of the pathogen, but also limits subsequent infection by other pathogens (van Loon and van Strien, 1999; Glazebrook, 2001). During the hypersensitive response, ion fluxes across the plant cell membrane are altered, active oxygen species are generated, regulatory proteins and plant defense systems are activated, and various antimicrobial molecules (e.g., phytoalexins) accumulate at the infection site; an additional change in the cells surrounding the infection site is the thickening of the cell wall by the deposition of various macro-molecules such as callose, lignin, suberin, and hydroxyproline-rich proteins (Bol et al., 1990; Ward et al., 1991; Stintzi et al., 1993; Ausubel et al., 1995; Zhang et al., 1995;

Greenberg, 1997; Yang et al., 1997; Wei et al., 1998; van Loon and van Strien, 1999; Hancock et al., 2002).

Another occurrence following pathogen infection is the induction of pathogenesis-related proteins (PR proteins). PR proteins are encoded by the genomes of almost all plants, and are specifically induced in response to pathogenic infections by fungi, bacteria, and viruses. Some PR proteins are also induced by related situations and adverse environmental factors (Bol et al., 1990; Stintzi et al., 1993; Datta et al., 1999; Kitajima and Sato, 1999; van Loon and van Strien, 1999; Rep et al., 2002; Poupard et al., 2003). 'Related situations' refers to wounding of the plant tissue, which gives rise to proteins that are also induced during infections, and the application of chemicals (e.g., ethephon and salicylic acid) that imitate the effect of pathogen infection or induce some aspect of the host plant response (Boller et al., 1983; Bol et al., 1990; van Loon et al., 1994; Jia and Martin, 1999; Guo et al., 2000; Okushima et al., 2000; Park et al., 2001). The PR proteins react hypersensitively and contribute to the development of systemic acquired resistance against further pathogenic infection. This means that these novel proteins not only accumulate locally in the infected area, but are also induced in tissues distant from the initial infection site (Bol et al., 1990; Ward et al., 1991; van Loon and van Strien, 1999). It has been shown that the induction of PR proteins occurs relatively late in the hypersensitive response, so their contribution to resistance against the initial infection is thought to be limited. Therefore, it is likely that PR proteins are defense proteins that mainly serve in limiting pathogen multiplication and/or spread (Vidhyasekaran, 1997; Datta et al., 1999).

PR proteins were first independently discovered by two different groups in tobacco leaves following infection with tobacco mosaic virus (van Loon and van Kammen, 1970; van Loon et al., 1987; Bol et al., 1990). To be considered a PR protein, the protein has to meet two criteria (van Loon and van Strien, 1999). First, the protein has to be induced by a pathogen in tissues where the protein is not normally expressed. Second, this induced expression has to occur in at least two different plant-pathogen combinations, or expression can be demonstrated in only one plant-pathogen combination by two separate laboratories. PR proteins were originally divided into five major groups or families (PR-1 to -5) based on similarities in molecular weights, amino acid composition, and serological properties (van Loon et al., 1987; Linthorst, 1991). Since that time, 12 other classes of PR proteins have been recognized, for a total of 17 families of PR proteins (Zhang et al., 1995; Wei et al., 1998; van Loon and van Strien, 1999; Okushima et al., 2000). Within each of these PR families, there is a defined type member. The mRNA sequence of each representative member is used to search for homologues in other plants (van Loon and van Strien, 1999). The 17 PR protein families, along with their representative member, are given in Table 1. It should be noted that no matter what class they belong to, all PR proteins share a few characteristic properties that enable their detection and isolation. They are very stable at low pH; relatively resistant to the action of proteolytic enzymes; localized in compartments such as the vacuole, the cell wall, and/or the apoplast; and most are monomers of low molecular mass (8-50 kDa) (Linthorst, 1991; Stintzi et al., 1993; Cheong et al., 1997).

Family	Type Member	Properties
PR-1	Tobacco PR-1a	Unknown
PR-2	Tobacco PR-2	β-1,3-Glucanase
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco 'R'	Chitinase type I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato inhibitor I	Proteinase-inhibitor
PR-7	Tomato P_{69}	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco 'lignin-forming peroxidase'	Peroxidase
PR-10	Parsley 'PR1'	'Ribonuclease-like'
PR-11	Tobacco class V chitinase	Chitinase type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	*Arabidopsis* THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	'Oxalate oxidase-like'
PR-17	Tobacco PRp27	Unknown

Table 1. Recognized families of pathogenesis-related proteins, along with their representative member (modified from http://www.bio.uu.nl/~fytopath/PR-families.htm) (Zhang et al., 1995; Wei et al., 1998; van Loon and van Strien, 1999; Okushima et al., 2000).

The unifying classification that is currently used for PR proteins was proposed in 1994 to accommodate the induced and newly expressed proteins of both known and unknown function in the reaction of a plant to a pathogen (van Loon et al., 1994). This classification is based on their grouping into plant-wide families sharing amino acid sequences, serological relationship, and/or enzymatic or biological activity. The families are numbered using arabic numerals, and the different members within each family are named by lowercase letters in the order that they are described. Although incorrect, newly discovered mRNAs or cDNAs that are induced by pathogen attack are often considered to be progenitors of PR proteins. However, as van Loon and van Strien (1999) point out, these mRNAs or cDNAs cannot be classified as PR proteins until data on the cognate proteins are obtained. Until information at both the nucleic acid and the protein level are obtained and until both criteria are met, these sequences are termed PR-like proteins. Homologous proteins found in healthy tissues where no induction by pathogen infection has occurred are also referred to as PR-like proteins (van Loon et al., 1994).

To date, several *cis*-regulatory promoter elements have been identified that are involved in PR protein gene expression upon wounding or exposure to various elicitors (e.g., ethylene, jasmonic acid, and salicylic acid) (Guilfoyle, 1997; Xu et al., 1998; Datta et al., 1999). One of these promoter elements is the *cis* element 5'-AGCCGCC-3', which is called the GCC box, PR box, or ethylene response element (Guilfoyle, 1997). The PR box is found in the promoter region of numerous genes encoding PR proteins (Zhou et al., 1997; Jia and Martin, 1999; Kitajima and Sato, 1999; Koyama et al., 2001; Brown et al., 2003) and is required for ethylene induction of gene expression (Ohme-

Takagi and Shinshi, 1995; Sessa et al., 1995; Sato et al., 1996). Other *cis*-regulatory elements that mediate pathogen-induced PR gene expression include the W-box (consensus TTGACC or TGAC-$[N]_x$-GTCA), MRE-like sequence (consensus A[A/C]C [A/T]A[A/C]C), G-box (consensus CACGTG), and an SA-responsive element (SARE, consensus TTCGACCTCC) (Liu et al., 1995; Zhu et al., 1996; Yang et al., 1997; Rushton and Somssich, 1998).

PR protein isoforms are categorized as acidic, neutral, or basic based on their isoelectric points. Genes encoding the neutral/acidic PR proteins are induced in both local and systemic tissues after an accumulation of salicylic acid due to pathogenic infection. They are mostly extracellular and secreted into the plant apoplast. Basic proteins, on the other hand, are induced by ethylene instead of salicylic acid and are transported to the vacuole (Bol et al., 1990; Brederode et al., 1991; Kitajima and Sato, 1999; Santamaria et al., 2001). PR proteins are synthesized as preproteins that contain a hydrophobic N-terminal signal sequence to transport the polypeptide chain across the membrane of the endoplasmic reticulum (Melchers et al., 1993). Basic isoforms of PR proteins also have an additional positive C-terminal sorting signal, which is necessary and sufficient for vacuolar targeting, that is removed during or after transport to the target sites. Because the neutral/acidic proteins lack this C-terminal signal sequence for vacuolar targeting, they are secreted into the extracellular matrix (Linthorst, 1991; Neuhaus et al., 1991; Melchers et al., 1993; Sato et al., 1995; Liu et al. 1996; Cheong et al., 1997; Kim et al., 2002). It should be noted that acidic and basic isoforms of PR proteins have different degrees and specificities of antifungal activity (Bol et al., 1990; Liu et al., 1996; Kim et al., 2002). It has been proposed that the extracellular, acidic

isoforms may serve as a first line of defense against invading pathogens, while the intracellular, basic isoforms stored in the vacuole serve as a second line of defense (Mauch and Staehelin, 1989; Velazhahan et al., 1999).

Despite the elaborate defense response of cotton plants and the improvements made to the tolerance of cotton plants through conventional plant breeding, fungal pathogens are still important factors that limit crop yields and consequently result in financial losses every year (Ikram-Ul-Haq, 2004; Wu et al., 2005). Since the beginning of the molecular era of plant biology in the early 1980s, genetic engineering has been used to produce crops with enhanced resistance to fungal pathogens, as well as bacterial pathogens, pests, and various environmental stresses. Punja (2001) did an extensive review of the various approaches taken to genetically engineer plants for fungal disease resistance and grouped them into five general categories. Four of these categories include the expression of gene products that 1) either destroy or neutralize part of the pathogen (e.g., lipase, oxalic acid, and polygalacturonase); 2) enhance the structural defenses in the plant (e.g., elevated levels of lignin and peroxidase); 3) regulate plant defenses (e.g., ethylene, hydrogen peroxide, and salicylic acid); or 4) are involved in the hypersensitive response. The fifth category involves the expression of gene products that can either destroy the fungal pathogens directly or reduce their growth, such as PR proteins.

The amino acid sequences of the PR-5 proteins are highly similar to that of the sweet-tasting protein thaumatin isolated from the fruits of the West African monocotyledon rain forest shrub *Thaumatococcus daniellii* Benth (van der Wel and Loeve, 1972; de Vos et al., 1985). There is significant identity observed in their amino

acid sequences, and the proteins share several characteristics, such as similar molecular weights, the presence of several disulfide bonds and the lack of free sulfhydral groups, a high concentration of proline residues, and basic isoelectric points (Singh et al., 1987; Masuda et al., 2004). Osmotin, as well as the other PR-5 proteins, is therefore referred to as a thaumatin-like protein. PR-5 proteins are evolutionarily conserved in the plant kingdom and, like the other groups of PR proteins, consist of acidic, basic, and neutral isoforms (Stintzi et al., 1993; Liu et al., 1996; Capelli et al., 1997). So, the three subgroups of PR-5 proteins are acidic (PR-S), basic (osmotin), and neutral (osmotin-like-protein, OLP) (Kim et al., 2002). Like other PR proteins, thaumatin-like proteins are resistant to proteases and denaturation by pH or heat. The presence of 16 conserved cystienes that form eight disulfide bonds are most likely responsible for this resilience (Roberts and Selitrennikoff, 1990; Breiteneder, 2004).

Bressan and coworkers first isolated osmotin from salt-adapted tobacco cells (Singh et al., 1985 and 1987; Nelson et al., 1992). Osmotin-like proteins have also been found in many other plant species, such as potato (Pierpoint et al., 1990), maize (Roberts and Selitrennikoff, 1990; Vigers et al., 1991), tomato (Rep et al., 2002), *Arabidopsis* (Capelli et al., 1997; Hu and Reddy, 1997), rice (Reimman and Dudler, 1993), flax (Anžlovar et al., 1998), apple (Oh et al., 2000), black nightshade (Campos et al., 2002), and petunia (Kim et al., 2002). In addition to plants, thaumatin-like proteins have also been discovered in the nematode *Caenorhabditis elegans* (Kitajima and Sato, 1999) and the desert locust *Schistocerca gregaria* (Brandazza et al., 2004).

Several studies have shown that osmotin gene expression can be induced by several different hormonal and environmental signals. These include fungal infection,

abscisic acid, ethylene, high salt concentrations, wounding, tobacco mosaic virus, low temperature, and osmotic stress (Singh et al., 1987; La Rosa et al., 1992; Nelson et al., 1992; Raghothama et al., 1993; Liu et al., 1994; Zhu et al., 1995; Chang et al., 1997; Kitajima et al., 1998; Xu et al., 1998; Kitajima and Sato, 1999; Velazhahan et al., 1999; Helleboid et al., 2000; Newton and Duman, 2000; Koyama et al., 2001). Numerous experiments have shown that PR-5 proteins have antifungal activity *in vitro*, as they inhibit hyphal or spore growth of different fungi (Roberts and Selitrennikoff, 1990; Vigers et al., 1992; Melchers et al., 1993; Liu et al., 1994; Abad et al., 1996; Hu and Reddy, 1997; Koiwa et al., 1997; Ibeas et al., 2000 and 2001). Although the exact mechanistic action of PR-5 proteins has not been completely determined, it has been proposed that this antifungal activity is a result of the proteins specifically interacting with the fungal plasma membrane to form a transmembrane pore to cause water-influx and subsequent rupture of the fungal membrane (Bowles, 1990; Bohlmann and Apel, 1991; Cheong et al., 1997; Yun et al., 1997; Anžlovar et al., 1998; Kitajima and Sato, 1999). Like thionin, another type of plant defense protein, the PR-5 proteins are thought to form amphipathic domains resulting from a secondary structure mediated by disulfide bonds (Bohlmann and Apel, 1991; Cheong et al., 1997). Thionin functions by inserting its hydrophobic domain through the lipid bilayer of the fungal membrane to form a pore and cause subsequent membrane lysis.

An alternative theory for the action of PR-5 proteins is that they indirectly control the water permeability of fungal cell membranes by electrostatic interaction with membrane ion channels or osmotic receptors, which would result in the fungal cell's loss of osmotic balance and the subsequent lysis of its plasma membrane (Batalia et

al., 1996). With either scenario, the invariant number and position of the 16 cystiene residues observed in all PR-5 proteins is assumed to have a significant role in the formation of their amphiphilic secondary structures (Nelson et al., 1992; Raghothama et al., 1993; Stintzi et al., 1993; Cheong et al., 1997; Hu and Reddy, 1997; Raghothama et al., 1997; Kitajima and Sato, 1999; Velazhahan et al., 1999; Selitrennikoff, 2001).

Similar to other discovered PR multigene families (King et al., 1988; Zhu et al., 1995; Lin et al., 1996; Chlan and Bourgeois, 2001; Campos et al., 2002), our laboratory isolated a cluster of two presumptive cotton osmotin-like genes (*OSMI* and *OSMII*) and two pseudogenes (*OSMIII* and *OSMIV*), which contain internal stop codons within their coding regions (Wilkinson, 2003; Wilkinson et al., 2005). The two osmotin genes have an identity of 92% and, like other PR-5 proteins, lack introns (Nelson et al., 1992; Melchers et al., 1993; Zhu et al., 1995; Shih et al., 2001). The predicted isoelectric points of the mature proteins are 7.92 for OSMI and 7.56 for OSMII, and the calculated molecular weights are 23,885 kDa and 23,984 kDa for the OSMI and OSMII polypeptides, respectively (http://www.expasy.org). Another similarity to other neutral PR-5 proteins is that *OSMI* and *OSMII* have predicted N-terminal signal sequences of 24 amino acids and lack a C-terminal vacuolar targeting signal (Linthorst, 1991; Neuhaus et al., 1991; Sato et al., 1995; Liu et al., 1996; Cheong et al., 1997; Kim et al., 2002). Therefore, the mature proteins are predicted to be secreted into the extracellular matrix (Singh et al., 1987; Melchers et al., 1993). The predicted three-dimensional structure of the mature OSMI and OSMII proteins was generated by the software program 3D-JIGSAW (www.bmm.icnet.uk/servers/3djigsaw/). They also correspond to the other highly conserved PR-5 proteins, having 16 invariant cystiene residues that are

presumed to form eight disulfide bonds for the stability of three domains in a β-sheet/sandwich structure (de Vos et al., 1985; Ogata et al., 1992; Batalia et al., 1996; Koiwa et al., 1997 and 1999; Shih et al., 2001; Min et al., 2004).

There are several putative promoter elements located in the 5'-flanking region of the *OSMI* and *OSMII* genes that could bind to different transcription factors for expression activation (Raghothama et al., 1993; Thomas, 1993; Guilifoyle, 1997; Raghothama et al., 1997). These include three possible basic-region helix-loop-helix or E box motifs (CANNTG) (Kawagoe et al., 1994), an H-box or AC-element (5'-CCTAC $C(N_7)CT(N_4)A$-3') (Hatton et al., 1995), and four assumed GATA motifs (5'-GATAA-3') (Lam and Chua, 1989; Guilifoyle, 1997). Each of the osmotin genes also contain two supposed GCC boxes, or ethylene response elements, which, as stated above, are in the promoter motifs of many genes encoding PR proteins (Nelson et al., 1992; Raghothama et al., 1993 and 1997; Zhou et al., 1997; Kitajima et al., 1998; Jia and Martin, 1999; Kitajima and Sato, 1999; Koyama et al., 2001; Brown et al., 2003). Ethylene has been shown to be a potent inducer for a number of PR proteins (Bol et al., 1990; Linthorst, 1991). Ethylene is an endogenous plant hormone that influences many factors during plant growth and development, such as germination, senescence, epinasty, abscission, and fruit ripening (Ohme-Takagi and Shinshi, 1995). Yang and Hoffman (1984) demonstrated that the biosynthesis of ethylene increases rapidly during plant-pathogen interactions, and that ethylene subsequently induces the transcription of genes encoding PR proteins.

Several laboratories have shown that PR-5 proteins are constitutively expressed in plant roots and in cultured cells. They are not naturally present in healthy leaves, but

ethylene treatment causes a rapid induction in leaves (LaRosa et al., 1992; Sato et al., 1996; Kitajima and Sato, 1999). This constitutive expression of PR proteins in the roots is thought to be of significance for plant defense because the roots are typically surrounded by soil, which has numerous microorganisms; therefore, plants are likely defending themselves by having a preexisting defense mechanism in place, in addition to an induced one in other organs (Kitajima and Sato, 1999).

One of the goals of this dissertation research was to genetically engineer two cotton osmotin genes (the *OSMI* and *OSMII* genes) to routinely overproduce the osmotin proteins in transgenic cotton plants as a natural defense against fungal infections. In general, plant transformation is based on the introduction of foreign DNA into plant cells, followed by the regeneration of these transformed cells into whole plants. Plants are totipotent, meaning that each cell has the genetic potential to regenerate an entire fertile plant, and this remarkable characteristic is the genetic basis for plant tissue culture (Barz and Oksman-Caldentey, 2002). Plant transformation requires an efficient method for introducing DNA of interest into plant cells, plant tissues or cells that can regenerate whole plants, and a selection system that allows only the transformed cells to proliferate (Twyman et al., 2002).

A major breakthrough for plant transformation occurred in the late 1970s when van Larebeke et al. (1974) deduced the mechanism of crown gall formation by *Agrobacterium tumefaciens*. It was determined that virulent strains of *A. tumefaciens* carried a large plasmid that was able to induce crown galls and that part of the plasmid (the T-DNA plasmid) was transferred to the plant genome of crown gall cells (Chilton et al., 1977). This discovery therefore provided a natural gene transfer mechanism that

could effectively be used for plant transformation. The gene of interest is ligated within the T-DNA and transferred to the plant cells through infection. After the T-DNA has stably integrated into the plant genome, the transgene is inherited in a Mendelian fashion (Bhattacharyya et al., 1994). This technique was used in 1981 to transform tobacco, but the genes introduced into the transgenic plants were driven by their own promoters and were not expressed in plant cells (Otten et al., 1981). Since that time, *Agrobacterium*-mediated transformation has been refined and successfully used to transform a number of plants, such as cotton (Firoozabady et al., 1987; Umbeck et al., 1991; Gould and Magallanes-Cedeno, 1998; Zapata et al., 1999; Sawahel, 2001; Sunilkumar and Rathore, 2001; Zhang et al., 2001; Hussain et al., 2004; Ikram-Ul-Haq, 2004; Wu et al., 2005).

The only requirements of T-DNA transfer into the plant genome are the *vir* genes, which are responsible for the transfer of T-DNA, and the 24-bp direct repeat sequences that mark the left and right borders of the T-DNA (Zupan et al., 2000; Veena et al., 2003); therefore, disarmed Ti plasmids (Zambryski et al., 1983; Hajdukiewicz et al., 1994) were developed that lacked the oncogenes. This made it possible to transfer the T-DNA into plant cells without causing tumor formation. Because these large Ti plasmids were somewhat inefficient for transformation of plant cells, binary vectors with multiple restriction sites (Hoekema et al., 1983; Bevan, 1984) were developed in which the *vir* genes and the T-DNA were cloned on separate plasmids before being introduced into *A. tumefaciens* for plant transformation. This development alleviated the problem of inefficient transformation due to large Ti plasmids that often fragmented and also lacked unique restriction sites for subcloning (Twyman et al., 2002). Numerous techniques in

molecular biology and gene technology have improved the genetic engineering of plants, with the discovery of *Agrobacterium tumefaciens* and the development of an efficient T-DNA system for DNA transfer being some of the greatest. It is now the most commonly used method for plant transformation (Nain et al., 2005).

One goal of this dissertation research was to evaluate the expression of two cotton osmotin-like genes and their protein expression patterns potentially up-regulated by inducers such as ethylene and sodium chloride, using real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses with an anti-osmotin antibody preparation. A second goal, as mentioned above, was to genetically engineer these cotton osmotin-like genes to routinely overproduce the antifungal protein osmotin, a member of the PR-5 family, in transgenic *Arabidopsis* model plants and in transgenic cotton plants as a natural defense against fungal infections. This strategy is currently being used by many agribusiness companies engaged in the application of genetic engineering and biotechnology to develop transgenic plants with genes encoding PR proteins (Datta et al., 1999; Chrispeels and Sadava, 2003). Due to the wide-ranging fungal effect of the osmotin gene, it should be possible to use the osmotin gene as a novel defense that is effective against numerous pathogenic fungi. In fact, transgenic potato plants that routinely overproduce osmotin are highly resistant to infection by fungal pathogens (Liu et al., 1994; Zhu et al., 1996). Therefore, genetically engineering the cotton osmotin genes isolated in our laboratory to routinely overproduce the antifungal protein osmotin in transgenic cotton plants as a natural defense against fungal infections should increase the crop yield, as well as prevent the overusage of chemical fungicide treatments that could be harmful to humans and the environment.

CHAPTER 2

MATERIALS AND METHODS

Subcloning and Sequence Analysis of a 3.9-kb *Eco*RI Fragment from the Genomic Clone LCgOSM7B

Dr. Robert Pirtle, Dr. Irma Pirtle, and Dr. Jeffery Wilkinson of our laboratory isolated the genomic clone LCgOSM7B from a cotton (*Gossypium hirsutum* L., cv. Acala SJ3) genomic library (Wilkinson, 2003; Wilkinson et al., 2005) that was a gift of Dr. Thea Wilkins (University of California, Davis) (Leandro and Wilkins, 1998). The DNA from the genomic clone LCgOSM7B was digested with several different restriction endonucleases for physical mapping. The map was then compared to two other physical maps of the genomic clones LCgOSM16B and LCgOSM12A, generated by Drs. Robert Pirtle, David Yoder, and Jeffery Wilkinson of our laboratory (Wilkinson, 2003; Wilkinson et al., 2005). The positions of the *OSMI* and *OSMII* genes were deduced within the genomic clone LCgOSM7B, as well as the extent of overlap between the cotton genomic segments in the three separate lambda clones (LCgOSM16B, LCgOSM12A, and LCgOSM7B).

One microgram of the LCgOSM7B DNA was digested with the restriction endonuclease *Eco*RI (Promega) for a period of 3 h at 37˚C. This aspect of the research was done in collaboration with Dr. Jeffery Wilkinson of this laboratory. The digest mixture was mixed with 6X blue/orange 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 Na_2EDTA (pH 8.0)), and the restriction fragments were separated on a 0.8% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml in

TAE buffer (400 mM Tris-acetate and 2 mM Na_2EDTA (pH 8.5)). After analyzing the DNA fragments, a 3.9-kb fragment from the LCgOSM7B genomic clone was chosen for sequence analysis based on its location in the physical map.

To increase the yield of the 3.9-kb fragment, 80 µg of the LCgOSM7B DNA was digested with the restriction endonuclease *Eco*RI (Promega) and electrophoresed as before. Ten microliters of a pUC18 vector was also digested with *EcoR*I. The 3.9-kb LCgOSM7B DNA fragment and the 2.6-kb pUC18 fragment were excised from the gel, weighed, and purified using the QIAquick Gel Extraction Kit (QIAGEN). Three volumes of Buffer QG (QIAGEN proprietary composition) were added to each volume of gel slice. The mixtures were incubated in a 50˚C waterbath for 10 min, with vortexing every 2-3 min, until the gel pieces had completely dissolved. One gel volume of isopropanol was added, and the samples were vortexed. The mixtures were transferred to QIAquick columns and centrifuged for 1 min to bind the DNAs to the membranes. To remove any traces of residual agarose, 500 µl of Buffer QG was added, and the columns were centrifuged for 1 min. The columns were washed with the addition of 750 µl of Buffer PE (QIAGEN proprietary composition) and subsequent 1 min centrifugation. The columns were then dried with an additional 1 min centrifugation. The columns were placed into clean 1.5 ml microcentrifuge tubes, and the DNAs were each eluted with 50 µl of water, after a 1 min incubation and centrifugation. The purity and concentration of the purified products were assessed by gel electrophoresis on a 0.8% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml.

The 5'-end of linearized pUC18 vector DNA was dephosphorylated using calf intestine alkaline phosphatase (CIAP) (Gibco BRL) to reduce the possibility of self-

ligation. Six hundred nanograms of vector DNA were incubated with 10 µl of 10X CIAP Reaction Buffer (500 mM Tris-HCl (pH 8.5) and 1 mM Na_2EDTA) and 2 µl of CIAP in a total volume of 100 µl. The reactions were incubated for 15 min at 37˚C and then for 15 min at 56˚C. An additional 2 µl of CIAP was added to the reaction, and the two 15 min incubations were repeated. To terminate and deproteinize the dephosphorylation reaction, the sample was passed through a QIAquick PCR Purification Column (QIAGEN). Five volumes (500 µl) of Buffer PB (QIAGEN proprietary composition) were added to the reaction. The mixture was transferred to a QIAquick column and centrifuged for 1 min to bind the DNA to the membrane. After discarding the flow-through, the column was eluted by centrifugation with 750 µl of Buffer PE (QIAGEN proprietary composition). The column was then dried with an additional 1 min centrifugation. The column was placed into a clean 1.5 ml microcentrifuge tube, and the DNA was eluted with 30 µl of Buffer EB (10 mM Tris-HCl (pH 8.5)), after a 1 min incubation and centrifugation.

One hundred nanograms of the dephosphorylated 2.6-kb pUC18 fragment and 144 ng of the 3.9-kb LCgOSM7B DNA fragment were combined with 20 µg of carrier 5S rRNA in 30 µl. The DNA mixture was precipitated with a 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol overnight at -20˚C. The precipitated DNA was pelleted by a 30 min centrifugation at maximum speed (14,000xg) and 4˚C in an Eppendorf microfuge, washed three times with 70% ethanol, air-dried on ice, and resuspended in 7 µl of water. The DNAs were ligated together in a solution of 2 µl of 5X T4 DNA Ligase Buffer (250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM

DTT, and 25% (w/v) polyethylene glycol-8000) and 1 unit of T4 DNA ligase (Gibco BRL) at 22˚C overnight.

The ligation mixture was diluted 1:5 with water and transformed into electro-competent *E. coli* DH5α cells previously prepared in this laboratory by Dr. Jeffery Wilkinson. Ten microliters of the diluted ligation mixture was mixed with 40 µl of *E. coli* DH5α cells in a chilled 1 mm gap cuvette (BTX® P/N 610) and electroporated with an ECM® 395 Electroporator (BTX®, San Diego, CA) set on the HV mode/3 kV using a single 1.5 kV electrical pulse, resulting in a 15.0 kV/cm field strength with an exponential decay constant of 5 msec. Nine hundred sixty microliters of LB broth (Luria-Bertani; 10 g/l NaCl, 10 g/l bacto-tryptone, and 5 g/l yeast extract) was immediately added to the mixture, and the 1 ml mixture was transferred to a 14 ml Falcon™ tube. The mixture was incubated for 1 h at 37˚C in a New Brunswick incubator/shaker at 225 rpm. Aliquots (200 µl) of the mixture were spread on LB plates containing 50 µg/ml of ampicillin, 800 µg of IPTG (isopropyl thio-β-D-galactoside), and 800 µg of X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactoside). The plates were then incubated at 37˚C overnight for colony formation. Twelve colonies, which were assumed to contain the recombinant plasmid based on their white appearance, were selected and re-streaked on fresh LB plates containing 50 µg/ml of ampicillin, 800 µg of IPTG, and 800 µg of X-gal and re-grown to ensure pure colonies.

After a 24-h incubation at 37˚C, nine isolated colonies were inoculated into separate flasks with 25 ml LB broth containing 50 µg/ml ampicillin and incubated at 37˚C in an incubator/shaker at 220 rpm for 24 h. The plasmid DNAs were then isolated using the Wizard® *Plus* Minipreps DNA Purification System (Promega), following the

manufacturer instructions. Ten milliliters of each culture were transferred to 14 ml Falcon™ tubes and centrifuged for 15 min at maximum speed using a Sorvall GLC-4 tabletop centrifuge. The supernatants were discarded, and the cells were resuspended in 400 µl of Cell Resuspension Solution (50 mM Tris-HCl (pH 7.5), 10 mM Na_2EDTA, and 100 µg/ml RNase A) and subsequently transferred to 1.5 ml microcentrifuge tubes. The cells were lysed while inverting four times with 400 µl of Cell Lysis Solution (0.2 M NaOH and 1% SDS). After a 5 min incubation, the mixtures were neutralized with 400 µl of Neutralization Solution (1.32 M potassium acetate) by inversion. To pellet the bacterial DNA and cellular debris, the mixtures were centrifuged for 5 min at maximum speed using a Model 235C Fisher Scientific Microcentrifuge. Each of the supernatants were transferred to separate Wizard® Miniprep Columns through 1 ml of Wizard® Minipreps DNA Purification Resin within a 3 ml Syringe Barrel using a Vacuum Manifold (Promega). The columns were each washed with 2 ml of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCl (pH 7.5), 40 µM Na_2EDTA, and 55% ethanol), and subsequently dried for 30 sec by vacuum. The minicolumns were transferred to 1.5 ml microcentrifuge tubes and centrifuged at maximum speed for 2 min to remove any residual Column Wash Solution.

The minicolumns were then transferred to another set of 1.5 ml microcentrifuge tubes, and the DNAs were each eluted with 50 µl of water after a 1 min incubation via a 20 sec centrifugation at maximum speed. The concentration and purity of the plasmid DNAs were determined by taking spectrophotometric readings at 260 nm and 280 nm using a Varian DMS90 UV-Visible spectrophotometer. The plasmid DNAs were digested with the restriction endonuclease *Hind*III and mixed with 6X blue/orange

loading dye and electrophoresed on a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide. The sizes of the fragments produced from the *Hind*III digests were determined and compared with the physical maps. Transformed *E. coli* DH5α cells harboring two of the recombinant plasmid DNAs (p7B *Eco*RI #3 and p7B *Eco*RI #10) yielding fragments of the predicted size were stored in glycerol slants. Both strands of p7B *Eco*RI #10 plasmid (designated as pCgOSM7B-*Eco*RI) were sequenced using a primer-based approach on an Applied Biosystems Model 377XL DNA Sequencer by Lone Star Labs (Houston, TX) with the universal M13 phage sequencing primers and with primers designed specifically for the pCgOSM7B-*Eco*RI plasmid. The DNA sequences were subsequently analyzed using DNASIS® version 2.1 software (Hitachi Software Engineering Co., Yokohama, Japan).

Induction and Isolation of RNA from Cotton Plant Extracts

Three week-old cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5 or cv. Acala Maxxa) were treated with water, 100 mM hydrogen peroxide, or 1 mM ethephon and placed in separate one gallon plastic bags for a period of 0, 1, or 2 h. The water served as a control, while the hydrogen peroxide and ethephon both functioned as chemical inducers. As an additional control, another three week-old cotton plant (*Gossypium hirsutum* L., cv. Acala SJ5) that was neither treated nor placed in a plastic bag was also examined. After the appropriate amount of time, the plants were removed from the bags, and the soil was gently washed away from the roots using distilled water. Excess water was removed by blotting the plants on paper towels, and approximately 1 g of

leaf, stem, and root tissues each were weighed out. This aspect of the work was done in conjunction with Dr. Jeffery Wilkinson of our laboratory.

Total RNA was then isolated from the leaf, stem, and root tissues by a modified protocol (Chang et al., 1993; McKenzie et al., 1997) using the RNeasy Plant Mini Kit (QIAGEN), using the manufacturer instructions. The plant tissues were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The frozen tissue powders were transferred to 50 ml polypropylene centrifuge tubes containing 15 ml of extraction buffer (2% hexadecyltrimethyl-ammonium bromide (CTAB), 2% polyvinyl-pyrrolidone (PVP), 100 mM Tris-HCl (pH 8.0), 25 mM Na_2EDTA, 2 M NaCl, 0.5 g/l spermidine (N-[3-aminopropyl]-1,4-butane-diamine), and 2% β-mercaptoethanol (BME), added just before use) that was warmed to 65˚C in a waterbath. The tubes were vigorously shaken to resuspend the tissues, and they were kept at 65˚C until all of the samples were homogenized. Fifteen milliliters of chloroform was added to each of the extracts and mixed well. The samples were centrifuged at 9,000xg for 20 min at 4˚C in a microfuge, and the resulting top (aqueous) layers were transferred to fresh 50 ml tubes. The chloroform extractions were then repeated one time for stem and root extracts, and two times for leaf extracts. Five milliliters of 8 M LiCl was added to the final supernatants, the samples were thoroughly mixed by vortexing, and the RNA was precipitated at 4˚C overnight.

The precipitated RNAs were pelleted by centrifugation at 9,000xg for 30 min at 4˚C. The cells were lysed by resuspending the pellets in 500 µl of QIAGEN buffer RLT (proprietary composition), containing 5 µl BME, and then mixed with 250 µl of ethanol. The resulting 750 µl mixtures (including any precipitate) were transferred to RNeasy

mini-columns (QIAGEN), which were placed in 2 ml collection tubes, and centrifuged for 1 min at 14,000xg and 4˚C in a microfuge. The columns were washed with 700 µl of QIAGEN buffer RW1 (proprietary composition) and centrifuged for 15 sec at 8,000xg and 4˚C. The columns were then placed in new collection tubes and washed twice with 500 µl of QIAGEN buffer RPE (proprietary composition). The first wash was centrifuged for 15 sec at 8,000xg and 4˚C, and the second wash was centrifuged for 2 min under the same conditions to dry the columns. The columns were transferred to 1.5 ml centrifuge tubes, and the RNAs were eluted by adding 50 µl of RNase-free water into the columns and centrifuging 1 min at 14,000xg and 4˚C. The elution was carried out again with another 50 µl of RNase-free water to ensure all of the RNA was collected from each of the columns. The concentration and purity of each RNA sample was determined by taking spectrophotometric readings at 260 nm and 280 nm using a Varian DMS90 UV-Visible spectrophotometer.

Northern Blot Hybridization of RNA from Cotton Plant Extracts

Total RNA was extracted from three week-old cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5) that had been treated with water (as control), 100 mM hydrogen peroxide, or 1 mM ethephon for a period of 0, 1, or 2 h, as described above. Because the concentrations of two RNA extracts were low, 7.5 µg of all samples were lyophilized to a dry powder, and then dissolved in 5 µl of RNase-free water. Two volumes (10 µl) of 1.5X RNA sample/loading buffer (0.06% bromophenol blue, 0.06% xylene cyanol, 1.5X 3-[N-Morpholino]propanesulfonic acid (MOPS) buffer (see below), 9% formaldehyde, and 60% deionized formamide) were added to each sample. The samples were

subsequently heat-treated for 15 min at 65˚C. The samples were chilled on ice, and 1 µl of ethidium bromide (1 mg/ml) was added to each. The RNA samples were electrophoresed on 1% denaturing formaldehyde gels (1.5 g LE agarose (SeaKem®), 111 ml H_2O, 15 ml 10X MOPS (200 mM MOPS, 50 mM sodium acetate, 10 mM Na_2EDTA (pH 7.0)), and 37% formaldehyde) (Sambrook and Russell, 2001), with 3 µl of RNA Markers (Promega, Catalog # G3191). The RNAs were electrophoresed at 5 V/cm, using 1X MOPS as the running buffer.

A Polaroid photograph was taken of each gel with a ruler placed on top to calibrate the mobilities of the standard markers. The RNA samples immobilized in the gels were then transferred to Hybond-N^+ nylon membrane filter replicas (Amersham™, now part of General Electric Healthcare Life Sciences) with 20X sodium standard citrate buffer (SSC) (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) by the capillary action protocol described in Sambrook and Russell (2001). To do this, the gels were soaked twice in 5X SSC for 20 min each with gentle shaking. The nylon membrane, four Whatman 3MM® filter papers, and an 8 cm stack of paper towels were cut to the size of each gel. Two pieces of Whatman 3MM® filter papers that were wider and longer than each gel were also cut and used as wicks. The wicks were placed on a glass support plate inside a large baking dish containing 20X SSC, with the ends of the filter papers draping over the edges of the plate. Each gel was inverted and placed on top of the wicks, followed by the nylon membrane, which was floated on water and then immersed in transfer buffer for at least 5 min. Four pieces of Parafilm were cut and placed around the gel to prevent buffer from flowing directly to the paper towels. Two Whatman 3MM® filter papers were wetted in transfer buffer and placed on top of the

membrane, followed by two dry pieces of filter paper and the 8 cm stack of paper towels. Finally, a glass plate and a 400 g weight were placed on the stack of towels, and the RNAs were allowed to transfer via capillary action overnight. The next day, the membranes were dried for 30 min at 60˚C in a Blue M oven and then baked for 2 h at 80˚C in a vacuum oven. Once the RNAs were permanently fixed to the nylon filter membranes, they were wrapped in Whatman 3MM® filter paper and stored at -80˚C.

The membrane filter replicas were then used for Northern blot hybridization using a homologous cotton osmotin ^{32}P-labeled probe. Dr. Irma Pirtle of our laboratory used an 884-bp *Eco*RV fragment from the genomic plasmid subclone pCgOSM16B (Wilkinson, 2003; Wilkinson et al., 2005) as template to generate this probe by the random priming method (Feinberg and Vogelstein, 1983), using the Prime-a-Gene® Labeling System (Promega). The filters were wetted in 5X SSC, 0.1% sodium dodecyl sulfate (SDS) briefly at room temperature. The wetted filters were each placed in hybridization bottles with 40 ml of prehybridization/hybridization solution (5X SSC, 5X Denhardt's solution (50X Denhardt's solution is 1% (w/v) Ficoll® 400, 1% (w/v) PVP, and 1% (w/v) bovine serum albumin (BSA, fraction V)), 0.5% SDS, 50% deionized formamide, 20 mM Tris-HCl (pH 8.0), 2 mM Na_2EDTA (pH 7.5), 2.5 mM sodium pyrophosphate (pH 8.0), and 100 µg/ml denatured salmon sperm DNA), and prehybridized for 4 h at 42˚C in a Techne Hybridizer HB-1D oven. After the 4 h prehybridization, the solutions in the bottles were decanted, and 40 ml of fresh prehybridization/hybridization solution was added to each of the bottles, with 40 µl of a homologous cotton osmotin ^{32}P-labeled probe. Hybridization was carried out at 42˚C overnight.

After hybridization, the filters were rinsed with fresh prehybridization/hybridization solution for 30 min at room temperature. They were then washed twice with 2X SSC, 0.1% SDS for 30 min at room temperature, once with 1X SSC, 0.1% SDS for 30 min at room temperature, and once with 0.2X SSC, 0.1% SDS for 45 min at 55˚C. The blots were then dried for 30 min at 60˚C in a Blue M oven before they were exposed to Kodak X-OMAT® film with lanthanide intensifying screens at -80˚C for 10 weeks.

RT-PCR Analyses of RNA from Cotton Plant Extracts

Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were performed to determine which *OSM* transcripts (*I*, *II*, or *III*) were present in total RNA extracts from leaves, stems, and roots of cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5) that had been treated with water (as control), 100 mM hydrogen peroxide, or 1 mM ethephon for a period of 2 h (as described above). Leaf, stem, and root extracts from a control plant that was neither treated nor placed in a plastic bag were also analyzed by RT-PCR. Residual DNA was first removed from the RNA samples by incubating 1 µg of each total RNA extract 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. One microliter of 25 mM Na_2EDTA was added to the reactions to inactivate the DNase I, and the reaction mixtures were heated for 10 min in a 65˚C waterbath.

Unique oligonucleotide primers were specifically designed for the 5'- and 3'-untranslated regions (UTRs) of the osmotin gene sequence to amplify products from the *OSMI*, *OSMII*, and *OSMIII* genes (Wilkinson, 2003; Wilkinson et al., 2005). The primers

used for the *OSMI* mRNA were RT-PCR *OSMI* Forward (5'-ACAAATCCCAAAGTAAGA GCTAACC-3') and RT-PCR *OSMI* Reverse (5'-CAAGGCGCAGATTAACTAGACC-3'). The primers used for the *OSMII* mRNA were RT-PCR *OSMII* Forward (5'-CAAATCAC CAAGTAAAAACCAACC-3') and RT-PCR *OSMII* Reverse (5'-CCAAATGCAAATCAAC TACTCC-3'). The primers used for the *OSMIII* mRNA were RT-PCR *OSMIII* Forward (5'-CTCCATGCAATACAAAGC TAGC-3') and RT-PCR *OSMIII* Reverse (5'-CATAGCA ATGATTTGATTCTCTCG-3'). Primers for the cotton actin mRNA (a constitutively expressed housekeeping mRNA) were designed from the partial actin cDNA sequence (GenBank Accession D88414; Shimizu et al., 1997) and used as a positive control. The primers used for the cotton actin mRNA were ActinForA (5'-ATGTTCACCACCACTGC TGAA-3') and ActinRevC (5'-AGCTTCCATCC CGATGAAA-3').

RT-PCR was done using the SuperScript™ One-Step RT-PCR with Platinum® *Taq* Kit (Invitrogen™), RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen™), the unique oligonucleotide primers, and the DNase I-treated RNA samples. Each RT-PCR reaction of 50 µl contained 1X Reaction Mix (0.2 mM of each dNTP and 1.2 mM $MgSO_4$), 2.5 units of RNaseOUT™, 200 ng of DNase I-treated RNA template, 0.2 µM of each mRNA-specific primer, and 1 µl of RT/Platinum® *Taq* Mix. The reactions were placed in a thermal cycler (Perkin Elmer GeneAmp PCR system 2400) and incubated for 30 min at 50˚C for ribonuclease inhibition, followed by a 2 min denaturation step at 94˚C. Once denatured, the reactions were subjected to 35 cycles, including a 15 sec step at 94˚C to denature the double-stranded template, a 30 sec step at 59˚C to allow the gene-specific primers to anneal to the templates, and a 1 min step at 72˚C to allow

the primers to extend with the available DNA polymerase. After the last cycle, the reactions were held at 72˚C for 10 min and then cooled to 4˚C.

The resulting RT-PCR products were mixed with 6X blue/orange loading dye and electrophoresed on a 1.0% (for *OSM* products) or a 2.0% (for actin products) agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM® DNA Markers (Promega, for *OSM* products) or 200-bp DNA Step Ladder (Promega, for actin products) to determine the product sizes and detect the presence of the *OSM* transcripts in each of the tissues examined. The gel fragments containing the 788-bp and 789-bp *OSMI* and *OSMII* products were excised from the gels, weighed, and the DNA products purified using the QIAquick Gel Extraction Kit (QIAGEN) as previously described. The purity and concentration of the purified products were determined by gel electrophoresis. Ten microliters of each purified product were mixed with 6X blue/orange loading dye and electrophoresed on a 1.0% agarose gel, with pGEM® DNA Markers (Promega) to confirm the RT-PCR product sizes and estimate relative yields. Both strands of the purified RT-PCR products were then sequenced on an Applied Biosystems Model 377XL DNA Sequencer by Lone Star Labs (Houston, Texas) using the gene-specific primers described above. The identities of the osmotin transcripts were subsequently confirmed by analyzing the DNA sequences using DNASIS® version 2.1 software (Hitachi Software Engineering Co., Yokohama, Japan).

Because the sequence for the *OSMII* product was somewhat garbled with extraneous background, and thus appeared to have more than one transcript sequence, the *OSMII* RT-PCR product was digested with either the restriction endonuclease *Sac*I (Promega) (designated *OSMII Sac*I) or *Ban*I (New England BioLabs) (designated *OSMII*

*Ban*I). The digested DNA fragments were electrophoresed on a 0.8% agarose gel, with pGEM® DNA Markers (Promega) for size determination. The gel slices containing the resulting 788-bp products from each digest were excised and purified using the QIAquick Gel Extraction Kit (QIAGEN) as described above, and the purity and concentration of the products were determined by gel electrophoresis. Both strands of the purified RT-PCR products were sent to Lone Star Labs (Houston, Texas) for DNA sequencing using the gene-specific primers RT-PCR *OSMII* Forward and RT-PCR *OSMII* Reverse primers mentioned above. Additional primers for the *OSMII Sac*I product were SacFor2 (5'-ACGACATTTCCTTAATTGACGG-3') and SacRev2 (5'-TAAGTGAATATTCAGCCAGGG-3'). Additional primers for the *OSMII Ban*I product were BanFor2 (5'-AAACACCCTGGCTGAATATGC-3'), BanRev2 (5'-CAGGAGCTCT TAACTCATTCG-3'), BanFor3 (5'-AAGCACCTTTACTTGCCCTGC-3'), and BanRev3 (5'-ACTTTGCCAAGGGTCTAGG-3'). The identities of the transcripts were subsequently confirmed by analyzing the DNA sequences using DNASIS® version 2.1 software (Hitachi Software Engineering Co., Yokohama, Japan).

Real Time RT-PCR Analyses of RNA from Cotton Plant Extracts

The induction curves of the *OSMI* and *OSMII* mRNAs was determined using real time RT-PCR (Giulietti et al., 2001; Livak and Schmittgen, 2001; Wilhem and Pingoud, 2003; Bustin and Nolan, 2004; Wong and Medrano, 2005). Total RNA was extracted from leaves, stems, and roots of one to two week-old cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5 and cv. Acala Maxxa) that had been treated with water (as control) or 1 mM ethephon for a period of 0, 1, 2, 3, 4, 8, 12, 24, 48, or 96 h, as

described above. Once the concentration and purity of the RNA extracts was determined by spectrophotometric readings at 260 nm and 280 nm, 5 µg of each RNA sample was electrophoresed on denaturing gels in 1% formaldehyde (Sambrook and Russell, 2001) as another confirmation of RNA quality.

Unique amplimer pairs were designed to amplify short DNA fragments (100-250 bp) of the *OSMI* and *OSMII* genes. The amplimers for *OSMI* were *OSMI*ForD (5'-CTCA TATAGAGATGGTCGGAAGT-3') and *OSMI*RevE (5'-CCAAGTAGAACCACGTGTTTG-3'); and the amplimers for *OSMII* were *OSMII*ForD (5'- AACCTGTGGTCCGACCTATT TC-3') and *OSMII*RevD (5'- ATTCCCAAATGCAAATCAACTACTC-3'). In order to standardize the results, the PCR products generated from the actin gene (Shimizu et al., 1997) and the *SadI* gene (the cotton stearoyl-acyl carrier protein desaturase gene) (Yang et al., 2005) were used as endogenous controls. The gene-specific primers used for the actin gene are given above (ActinForA and ActinRevC). The amplimer pair used for the *SadI* gene were *SadI*For (5'-CCAAAGGAGGTGCCTGTTCA-3') and *SadI*Rev (5'-TTGAGGTGAGTCAGAATGTTGTTC-3') (designed by Yang et al., 2005).

Before running the reactions, residual DNA was removed from the RNA samples using DNase I (Amplification Grade, Invitrogen™), as detailed above. Real time RT-PCR reactions were then carried out using the TaKaRa Real Time One Step RNA PCR Kit Version 2.0 (TAKARA BIO INC.), SYBR® Green I intercalating dye (CAMBREX Bio Science Rockland, Inc., Rockland, ME), the gene-specific primers, and the DNase I-treated RNA extracts. Each 25 µl RT-PCR reaction contained 1X One Step RNA PCR Buffer (containing a mixture of dNTPs and Mg^{2+}), 2.5 units of *TaKaRa Ex Taq*™ HS, 2.5 units of avian myeloblastosis virus (AMV) Reverse Transcriptase XL, 20 units of RNase

inhibitor, 2.5 µl of SYBR® Green I (10,000X concentrate diluted 2,000 times with water), 250 ng of DNase I-treated RNA template, and 0.2 µM of each gene-specific primer.

Duplicate real time RT-PCR reactions were done for all time points (0, 1, 2, 3, 4, 8, 12, 24, 48, and 96 h) for each of the RNA extracts from leaves, stems, and roots treated either with water (as control) or 1 mM ethephon. The reactions were run in three stages using the Smart Cycler® System (Cepheid). Stage 1 consisted of a 15 min hold at 42˚C and a 2 min hold at 95˚C for reverse transcription and inactivation of the reverse transcriptase. Stage 2 consisted of 35 PCR cycles, including a 10 sec step at 94˚C to denature the double-stranded template, a 25 sec step at the appropriate melting temperature (58˚C for actin and *OSMI* primers, 59˚C for *SadI* primers, and 61˚C for *OSMII* primers) to allow the gene-specific primers to anneal to the templates, and a 25 sec step at 72˚C to allow the primers to extend with the DNA polymerase. Stage 3 recorded the melt curves of the RT-PCR products from 60˚C to 95˚C at 0.2 degrees/sec. Some of the resulting RT-PCR products were then electrophoresed on 2.0% LE agarose gels, with a 200-bp DNA Step Ladder (Promega) to confirm product sizes.

The relative quantification of *OSMI* and *OSMII* transcript levels was done using the comparative threshold cycle (C_T) method ($\Delta\Delta C_T$) (Giulietti et al., 2001; Livak and Schmittgen, 2001; Wilhem and Pingoud, 2003; Bustin and Nolan, 2004; Wong and Medrano, 2005). The first calculation for the quantitation involved averaging the C_T values of the duplicate PCR reactions and getting the differences (ΔC_T's) between these averaged C_T values of the target (the *OSMI* or *OSMII* mRNAs) and the endogenous

control, or normalizer, (the *SadI* mRNA). The *SadI* mRNA appeared to be more stable than actin mRNA, so it was chosen as the endogenous control for all of the calculations.

$$\Delta C_T = C_T \text{ (target)} - C_T \text{ (normalizer)}$$

The control samples were then chosen as the reference (baseline) for each comparison to be made, and the comparative $\Delta\Delta C_T$ calculations were done to find the differences between the values for the control sample ΔC_T's and the treated sample ΔC_T's (or to find the cycle differences between the control samples and treated samples for the *OSMI* and *OSMII* mRNAs):

$$\Delta\Delta C_T = \Delta C_T \text{ (ethephon-treated)} - \Delta C_T \text{ (control)}$$

The resulting $\Delta\Delta C_T$ values were a mixture of negative and positive values, because the expression was higher in some samples and lower in others. So, the last step in quantitation was to transform all of these values (which are the fold differences) into absolute values and find the comparative gene expression level. This step indicates how much greater the expression level of *OSMI* or *OSMII* is in each tissue after treatment. The formula for this is $2^{-\Delta\Delta CT}$ (Giulietti et al., 2001; Livak and Schmittgen, 2001; Wilhem and Pingoud, 2003; Bustin and Nolan, 2004; Wong and Medrano, 2005). Using these values, Microsoft Excel® software was used to prepare scatter charts to demonstrate the relative induction of the *OSMI* and *OSMII* mRNAs.

Induction and Isolation of Protein from Cotton Plants for One-Dimensional SDS-PAGE and Western Blot Analyses

Working in conjunction with Dr. Jeffery Wilkinson of our laboratory, three to four week-old cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5) were treated with water,

100 mM hydrogen peroxide, 550 mM NaCl, or 1 mM ethephon and placed in separate gallon plastic bags for periods of 0 to 96 h. The water served as a control, while the hydrogen peroxide, NaCl, and ethephon functioned as chemical inducers. After appropriate amounts of time, the plants were removed from the bags, and the soil was gently washed away from the roots using distilled water. Excess water was removed by blotting the plants on paper towels.

Total protein was then extracted from the whole plants using a protocol modified from that of Chlan and Bourgeois (2001). The plants were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The frozen tissue powders were transferred to pre-weighed 50 ml polypropylene centrifuge tubes. After determining the tissue weights, 1 ml/g of 0.1 M sodium citrate buffer (pH 5.0) was added to each tube and vigorously shaken. The mixtures were centrifuged for 20 min at 11,000xg and 4˚C in a preparative RC-5C Sorvall centrifuge, and the resulting supernatants were filtered through a folded piece of Miracloth (Calbiochem) into clean 50 ml polypropylene tubes. The concentration of each protein extract was determined by Bradford analysis (using reagents from Sigma) in a Varian DMS90 UV-Visible spectrophotometer.

The total protein extracts were mixed with 6X sample buffer (350 mM Tris-HCl (pH 6.8), 30% (v/v) glycerol, 10% (w/v) sodium dodecyl sulfate (SDS), 600 mM dithiothreitol (DTT), and 0.012% (w/v) bromophenol blue) and 2 µl of β-mercaptoethanol (BME). They were heat-treated for 5 min in a 95˚C waterbath and cooled to room temperature. The protein extracts were resolved on a discontinuous buffer system, consisting of a stacking gel and a separating gel (Laemmli, 1970), with 10 µl of Full Range Rainbow™ recombinant protein molecular weight markers (Amersham™) for one-

dimensional (1-D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The stacking gel 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. The separating gel contained 15% acrylamide/bis-acrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.1% APS, and 0.04% TEMED. The proteins were electrophoresed at 30 mA per gel using 1X Tris-Glycine Electrophoresis Running Buffer (25 mM Tris base (pH 8.3), 192 mM glycine, and 0.1% (w/v) SDS) in a Mini-PROTEAN® 3 cell (Bio-Rad).

Following electrophoresis, the proteins were transferred to nitrocellulose membranes (PROTRAN™ Pure Nitrocellulose Transfer and Immobilization Membrane, Schleicher & Schuell, Keene, NH) using 1X Tris/Glycine Transfer Buffer (48 mM Tris base (pH 9.2), 39 mM glycine, and 20% (v/v) methanol) and a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). Each separation gel was equilibrated in the transfer buffer, with the nitrocellulose membrane, two pieces of Whatman 3MM® filter paper, and two fiber pads, for 45 min before being placed in a gel/membrane sandwich. The gel/membrane sandwiches, an ice block, and 1X Tris/Glycine Transfer Buffer were then placed in the transfer cell, and the proteins were transferred to the nitrocellulose membranes at 90 mA overnight using a Model 250/2.5 Bio-Rad Power Supply.

The following day, each nitrocellulose membrane was placed in a blocking solution of 5% milk:TBS (5% (w/v) dry milk, 20 mM Tris-HCl (pH 7.5), and 150 mM NaCl) for 2 h at room temperature with shaking. This was followed by two washes with TBS-T (TBS with 0.35% (v/v) Tween® 20 (polyoxyethylenesorbitan monolaurate)) for 10 min at room temperature with shaking. The membranes were incubated with a polyclonal anti-osmotin antibody (prepared commercially by Biosynthesis, Inc.,

Lewisville, TX). The antibody was prepared using an antigenic oligopeptide designed from the peptide sequence of the last 18 amino acids comprising the C-terminal end of the OSMI polypeptide (Wilkinson, 2003; Wilkinson et al., 2005). The antibody was used at a 1:1,000 dilution in 5% milk:TBS-T for 1 h at room temperature with gentle shaking. The membranes were then washed with TBS-T as before, and incubated with a 1:1,500 dilution of a secondary antibody (Anti-rabbit IgG, peroxidase-linked species-specific whole antibody from donkey from Amersham Pharmacia Biotech, now part of General Electric Healthcare Life Sciences) for 45 min at room temperature with gentle shaking. The membranes were washed one last time with TBS-T as before, and the bound secondary antibodies were visualized after incubating the membranes in a 1:1 mixture of ECL Western blotting detection reagents (Amersham Pharmacia Biotech) for 1 min at room temperature and subsequently exposing them to Kodak X-OMAT® film.

Purification of Polyclonal Anti-Osmotin Antibody

Due to nonspecific binding seen in the previous Western blots, portions of the polyclonal anti-osmotin antibody preparation were purified using the Melon™ Gel IgG Spin Purification Kit (Pierce), according to the instructions of the manufacturer. The Melon™ Gel IgG Purification Support and Purification Buffer were first equilibrated at room temperature, and 500 µl of the Purification Support solution (Pierce proprietary solution) was then transferred to a Handee™ Mini-Spin Column placed in a 1.5 ml microcentrifuge tube. The uncapped column/tube assembly was centrifuged for 1 min at 6,000xg and 4˚C. After the flowthrough was discarded, the column was washed twice with 300 µl of Purification Buffer (Pierce proprietary solution) with a 10 sec

centrifugation each time. The second flowthrough was discarded, and the bottom cap was placed on the column. Fifty microliters of the anti-osmotin antibody serum was diluted 1:10 with 450 µl of Melon™ Gel Purification Buffer (proprietary composition) and added to the column. The top cap was placed on the column, and the column was incubated for 5 min at room temperature with gentle end-over-end mixing. After the incubation period, the bottom cap was removed from the column, and the top cap was loosened so that the purified antibody could be collected in the 1.5 ml microcentrifuge tube with a 1 min centrifugation at 6,000xg and 4˚C. The purified antibody was stored at -80˚C until used in Western blot analyses.

To determine the best dilutions of primary and secondary antibody to use for the Western blot analyses, six sets of protein (5 µg each) isolated from three week-old cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) that had been treated with water as control or 1 mM ethephon for a period of 24 h were electrophoresed on a discontinuous buffer system as described above, with 10 µl of SeeBlue® Plus2 Pre-Stained Standards (Invitrogen™). The proteins were extracted from the cotton plants using the modified method from Ferguson et al. (1996) described below, and further purified using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad) described below. Following electrophoresis, the proteins were transferred to nitrocellulose membranes and used in Western blot analyses. Different dilutions of the purified primary antibody (anti-osmotin antibody partially purified using the Pierce Melon™ Gel IgG Spin Purification Kit) and secondary antibody (Anti-rabbit IgG, peroxidase-linked species-specific whole antibody from donkey from Amersham Pharmacia Biotech) were used in the Western blot analyses. The first set of protein extracts was incubated with a 1:1,000 dilution of

primary antibody and 1:2,500 dilution of secondary antibody. The second set of protein extracts was incubated with a 1:1,000 dilution of primary antibody and 1:5,000 dilution of secondary antibody. The third set of protein extracts was incubated with a 1:100 dilution of primary antibody and 1:5,000 dilution of secondary antibody. A fourth set of protein extracts was incubated with a 1:2,500 dilution of primary antibody and 1:5,000 dilution of secondary antibody. Finally, the fifth set of protein extracts was incubated with a 1:2,500 dilution of primary antibody and 1:10,000 dilution of secondary antibody.

Induction and Isolation of Protein from Cotton Plants for Two-Dimensional SDS-PAGE and Western Blot Analyses

As previously described, the polyclonal antibody preparation used in the previous Western blot analyses was prepared using an antigenic oligopeptide corresponding to the peptide sequence of the last 18 amino acids comprising the C-terminal end of the OSMI polypeptide (Wilkinson, 2003; Wilkinson et al., 2005). Because the mature isoforms of the putative osmotin proteins are practically the same size (estimated to be 23,885 KDa for OSMI and 23,984 KDa for OSMII) with nearly identical C-terminal regions (with only two amino acid variations), the presence of both osmotin isoforms is only presumed from Western blot analyses of 1-D SDS-PAGE. To determine that both osmotin proteins were indeed being induced, two-dimensional (2-D) gel electrophoresis was used to first separate the proteins by charge in the first dimension using their isoelectric points (estimated to be 7.92 for OSMI and 7.56 for OSMII) and by their size in the second dimension by SDS-PAGE.

Sample preparation is a major factor of successful 2-D gel electrophoresis of proteins (Saravanan and Rose, 2004; Carpentier et al., 2005). The modified protocol of Chlan and Bourgeois (2001) previously used to extract proteins from cotton plants did not yield protein of sufficient quality suitable for 2-D gel electrophoresis. Therefore, two other methods were used to extract total protein from the cotton plants that was free of any contaminating materials.

One protocol that was used involved a modified extraction procedure of Ferguson et al. (1996). With this method, 3 g of tissue from three week-old cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) treated with water as control or 1 mM ethephon for a period of 24 h was ground to a fine powder with a mortar and pestle under liquid nitrogen. The powdered tissues were then transferred to 50 ml polypropylene centrifuge tubes and further homogenized in 9 ml of extraction buffer (500 mM Tris-HCl (pH 8.65), 50 mM Na_2EDTA, 100 mM KCl, 2% (v/v) BME, and Complete, Mini Protease Inhibitor Cocktail Tablets (Roche)) and 9 ml of saturated phenol (phenol saturated with 1 M Tris-HCl (pH 7.9)) using a Polytron homogenizer for 1 min each on ice. The resulting homogenates were centrifuged for 15 min at 13,200xg and 20°C in the SS34 rotor of a preparative Sorvall RC-5C centrifuge to separate the aqueous and phenol phases. The aqueous phases and pelleted materials were discarded, and the interfaces and phenol phases, which contained the protein, of each sample were filtered through cheesecloth and re-extracted three more times by adding the volume of extraction buffer equal to the current volume of phenol, vortexing 3 min, centrifuging at 13,200xg and 20˚C to separate the phases, and discarding the aqueous phases. The final interfaces and phenol phases of each extract were then transferred to

clean 50 ml tubes, and the protein was precipitated by adding five volumes of 0.1 M ammonium acetate in methanol and storing at -20°C overnight. The precipitated proteins were collected by a 15 min centrifugation at 13,200xg and 20˚C. The protein pellets were then washed three times in 20 ml of 0.1 M ammonium acetate in methanol and once in 35 ml of cold (-20˚C) acetone. The protein pellets suspended in acetone were incubated at -20°C for 1 h before centrifuging 15 min at 13,200xg and -20°C. The protein pellets were then air-dried for 30 min at room temperature. Once dried, the pellets were resuspended in ReadyPrep Rehydration/Sample Buffer (8M urea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM DTT, and 0.2% (w/v) Bio-Lyte® 3/10 ampholytes) (Bio-Rad) with Mini Protease Inhibitor Cocktail Tablets (Roche) and centrifuged for 10 min at 12,000xg and 20°C to pellet any impurities. The resulting pellets were discarded, and the supernatants were transferred to clean tubes.

The concentration of each protein extract was determined using the *RC DC* Protein Assay kit (Bio-Rad), which is a colorimetric assay based on the Lowry method, with modification to be compatible with reducing agents and detergents. Once the protein concentrations had been determined for the control and ethephon-treated samples, 62.5 µg or 250 µg of each extract was diluted with water to a final volume of 100 µl and further purified using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad). Using this kit, several sets of control and ethephon-treated protein extracts were partially purified. First, 300 µl or 450 µl of precipitating agent 1 (Bio-Rad proprietary composition) was added to each sample and mixed well by vortexing. The mixtures were incubated on ice for 15 min, and then 300 µl or 450 µl of precipitating agent 2 (Bio-Rad proprietary

composition) was added to each mixture and vortexed. The samples were centrifuged at maximum speed using a Fisher Scientific Model 235C Microcentrifuge for 5 min, and the resulting supernatants were discarded. The tubes were centrifuged for another 30 sec, and the remaining supernatants were discarded. The pellets were washed with 40 µl or 60 µl of wash reagent 1 (Bio-Rad proprietary composition) and centrifuged at maximum speed for 5 min. Wash 1 was discarded, and the pellets were rinsed with 25 µl or 50 µl of water by vortexing for 20 sec. Then, 1 ml or 1.5 ml of wash reagent 2 (Bio-Rad proprietary composition, prechilled at -20˚C) and 5 µl of wash 2 additive (Bio-Rad proprietary composition) were then added to the tubes and vortexed for 1 min. The samples were incubated at -20˚C for 30 min, with a 30 sec vortex every 10 min. After the incubation period, the tubes were centrifuged at maximum speed for 5 min, and the supernatants were discarded. The tubes were centrifuged again for 30 sec, and the remaining supernatants were discarded. The pellets were then air-dried for 5 min at room temperature before 185 µl or 200 µl of ReadyPrep Rehydration/Sample Buffer (Bio-Rad) was added to each. To resuspend the pellets in this buffer, the tubes were vortexed for 30 sec, incubated at room temperature for 5 min, and vortexed again for 1 min. The tubes were then centrifuged at maximum speed for 5 min at room temperature to pellet any impurities, and the clarified protein samples were transferred to clean 1.5 ml microcentrifuge tubes.

The cleaned protein samples were applied to various isoelectric focusing (IEF) ReadyStrip™ IPG Strips (pH 3-10, pH 5-8, and pH 7-10) (Bio-Rad) in a focusing tray and allowed to rehydrate passively for 12 h at 20˚C. The protein samples were then focused (or separated by their isoelectric points) by a four-step protocol using a Bio-Rad

PROTEAN IEF cell. This four-step protocol included: 1) 250 V for 20 min with a linear ramp; 2) 8,000 V for 2 h with a linear ramp; 3) 8,000 V for 40,000 V-hours with a rapid ramp; and 4) 1,000 V for 25,000 V-hours with a rapid ramp.

After the first-dimension (1-D) separation, the IPG strips were transferred to rehydration/equilibration trays and prepared for the second-dimension (2-D) separation. Each of the strips was first equilibrated with 4 ml of equilibration buffer I (6 M urea, 2% (w/v) SDS, 0.375 M Tris-HCl (pH 8.8), 20% (v/v) glycerol, and 2% (w/v) DTT) for 15 min with gentle rocking. Buffer I was then decanted from the trays, and each strip was further equilibrated with equilibration buffer II (6 M urea, 2% (w/v) SDS, 0.375 M Tris-HCl (pH 8.8), 20% (v/v) glycerol, and 500 mg of iodoacetamide) for 15 min with gentle rocking. The equilibrated strips were then dipped in 1X Tris-Glycine Electrophoresis Running Buffer (pH 8.3) and placed on 8-16% Criterion Tris-HCl gels (Bio-Rad), along with 10 µl of Precision Plus Protein Dual Color Standards (for the pH 3-10 strips) (Bio-Rad) or 10 µl of SeeBlue® Plus2 Pre-Stained Standards (for the pH 5-8 and pH 7-10 strips) (Invitrogen™). Melted overlay agarose solution was then added on top of the IPG strips and protein standards before the proteins were separated by size at 200 V by SDS-PAGE.

The second-dimension gels containing the separated proteins were either stained with SYPRO® Ruby protein gel stain (Bio-Rad) and analyzed using The Discovery Series™ PDQuest™ 2-D Analysis Software (Bio-Rad), or they were transferred to Sequi-Blot PVDF (polyvinylidene fluoride) (for the pH 3-10 strips) (Bio-Rad) or nitrocellulose membranes (for the pH 5-8 and 7-10 strips) for Western blot analysis (as detailed above) to localize the positions of the presumptive osmotin

proteins using the purified anti-osmotin antibody. The proteins of interest were extracted from the gels using the EXQuest Spot Cutter (Bio-Rad) and sent to Dr. Bill Russell of the Chemistry Department Laboratory for Biological Mass Spectrometry at Texas A&M University, College Station, TX, for sequence analysis.

A second extraction procedure used to isolate high-quality protein from cotton plants used the Plant Total Protein Extraction Kit (Sigma). Following the instructions of the manufacturer, total protein was extracted from the leaves, stems, and roots of two week-old cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) that had been treated with water (as control) or 1 mM ethephon for a period of 24 h. Approximately 400 mg of each tissue was ground to a fine powder with a mortar and pestle under liquid nitrogen. The powdered tissues were then transferred to cold (-20˚C) pre-weighed 2 ml micro-centrifuge tubes, and 1.5 ml of cold (-20˚C) Methanol Solution (containing a 1:100 dilution of the Protease Inhibitor Cocktail) was added to each. The mixtures were vortexed and incubated at -20˚C for 5 min with periodic vortexing. The suspensions were centrifuged for 5 min at 16,000xg and 4˚C in a microfuge to pellet the proteins and plant debris. The supernatants were discarded, and the methanol wash was repeated three more times. After the final supernatants were discarded, the tubes were inverted over paper towels to allow any remaining Methanol Solution to drain. After the Methanol Solution had drained from the tubes, 1.5 ml of cold (-20˚C) acetone was added to each tube and vortexed for 30 sec before being incubated at -20˚C for 5 min. The mixtures were centrifuged for 5 min at 16,000xg and 4˚C to pellet the proteins and plant debris. After the supernatants were discarded, the acetone extractions were repeated one time. The resulting supernatants were discarded, and the pellets were

air-dried for 5 min at room temperature. After the pellets were dried, each tube was weighed and its predetermined mass was subtracted to determine the plant tissue mass. The tissue pellets were then suspended in 4 µl of Reagent Type 2 Working Solution (Sigma proprietary composition, a chaotropic reagent to dissolve hydrophobic proteins with a 1:100 dilution of the Protease Inhibitor Cocktail) per mg of plant tissue by vortexing. The mixtures were incubated for 15 min at room temperature with rocking and intermittent vortexing. The tubes were then centrifuged for 30 min at 16,000xg and room temperature to pellet the plant debris. The supernatants, which contained the total protein, were finally transferred to clean 1.5 ml microcentrifuge tubes.

The concentrations of the protein extracts were determined using Bradford assay solution (Sigma). Once the protein concentrations had been determined for the control and ethephon-treated samples, 20 µg of each extract and 10 µl of SeeBlue® Plus2 Pre-Stained Standards (Invitrogen™) were electrophoresed in duplicate on two denaturing SDS-PAGE gels at 130 V. One gel was stained with Bio-Safe™ Coomassie Stain (Bio-Rad), and the other was transferred to a nitrocellulose membrane for Western blot analysis. Once the presence of the putative osmotin proteins was determined in the ethephon-treated extracts, 200 µg from each extract from the leaf, stem, and root tissues were combined for a total of 600 µg protein, and cleaned using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad) as before. Using the cleanup kit, two sets of ethephon-treated protein extracts were cleaned and resuspended in 300 µl of ReadyPrep 2-D Rehydration/Sample Buffer 1 (7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 40 mM Tris-HCl; from Bio-Rad) containing 1X ReadyStrip pH 6.3-8.3 Buffer (Bio-Rad proprietary composition). The cleaned protein samples were then applied to pH 6.3-8.3

ReadyStrip™ IPG Strips and electrophoresed on the 2-D system as before. The four-step protocol used to focus the proteins included: 1) 250 V for 15 min with a rapid ramp; 2) 8,000 V for 1 h with a slow ramp; 3) 8,000 V for 45,000 V-hours with a rapid ramp; and 4) 1,000 V for 25,000 V-hours with a rapid ramp. This time, the second dimension gels containing the separated proteins were either stained with Bio-Safe™ Coomassie Stain (Bio-Rad), or they were transferred to a nitrocellulose membrane for Western blot analysis. To overlay the Western blot profile with the spots on the stained gel, the nitrocellulose membrane was stained with MemCode™ Reversible Protein Stain Kit- For Nitrocellulose Membrane (Pierce) before the blocking step of the Western analysis. After analyzing the Western blot, the proteins of interest were excised from the gel using a sterile scalpel blade and given to Mr. Prem Adhikari of Dr. Barney Venables' laboratory here at the University of North Texas for sequence analysis by mass spectrometry.

Transformation, Regeneration, and Screening of *Arabidopsis* Plants

Dr. Jeffery R. Wilkinson of our laboratory previously constructed the *Agrobacterium tumefaciens* binary vector constructs containing the cotton *OSMI* and *OSMII* genes in order to transform *Arabidopsis* and cotton plants for constitutive expression of the cotton osmotin polypeptides (Wilkinson, 2003). The coding regions, along with the N-terminal signal targeting domains, of the *OSMI* and *OSMII* genes were amplified (Innis and Gelfand, 1990; Kozak, 1991). The 787-bp *OSMI* and 728-bp *OSMII* products were each cloned into the *Xba*I and *EcoR*I sites of a 2.4-kb pUC-based pGreen vector containing a 677-bp 35S cauliflower mosaic virus (CaMV) promoter and

terminator (Figure 1; Hellens et al., 2000; www.pgreen.ac.uk; Wilkinson, 2003). These 1.4-kb cassettes were then removed from the pUC-based vector using *EcoR*V and subcloned into the *Sma*I site of an 11.6-kb pCAMBIA 2301 binary vector (GenBank Accession AF234316; Figure 2) from the Center for Application of Molecular Biology to International Agriculture (CAMBIA, Canberra, Australia, www.pcambia.org.au). The plasmid constructs were then electroporated into electrocompetent Ag*robacterium tumefaciens* LBA4404 cells (Invitrogen™) and the process confirmed by PCR analyses. The plasmid constructs in the transformants that generated the appropriate-sized PCR fragments were designated pCAMBIA-35S-*OSMI* #16 (H1, M3, and G3) and pCAMBIA-35S-*OSMII* #11 (A, F, G, and J). The transformed *Agrobacterium* cells were stored as glycerol stocks at -80˚C until used for plant transformation.

The vector constructs pCAMBIA-35S-*OSMI* #16-M, pCAMBIA-35S-*OSMII* #11-A, and pCAMBIA 2301-H (empty vector) harbored in *Agrobacterium tumefaciens* were used to transform *Arabidopsis thaliana* (ecotype Columbia) plants using the floral dip method of Clough and Bent (1998). The primary bolts of the *Arabidopsis* plants were clipped daily to encourage more flowering, which increased the efficiency of transformation and delayed the plants. The plants were then dipped into separate solutions of *Agrobacterium* cells that were prepared as described below.

The three *Agrobacterium tumefaciens* stocks were each inoculated into 2 ml of YEP broth (Yeast Extract Peptone: 10 g/l bactopeptone, 10 g/l yeast extract, and 5 g/l NaCl) containing 50 µg/ml kanamycin, and incubated for 24 h at 28^{o}C with shaking at 200 rpm in a New Brunswick shaker/incubator. After the 24 h incubation, the 2 ml cultures were added to 50 ml YEP containing 50 µg/ml kanamycin, and the cultures

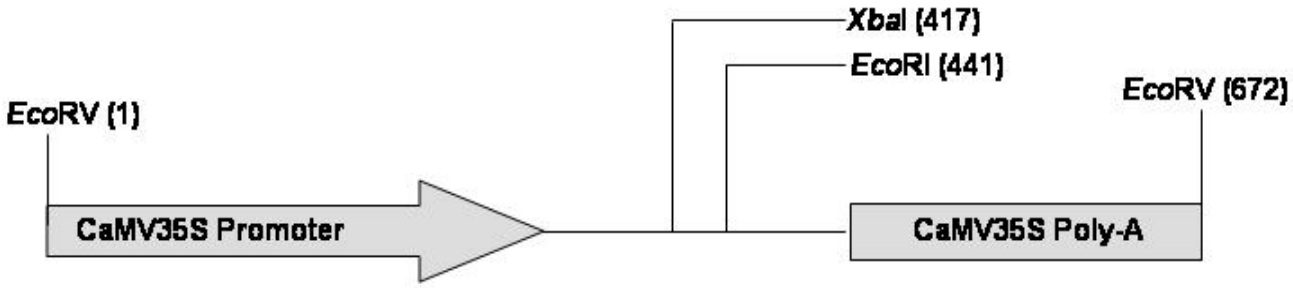

Figure 1. Diagram of the 35S cauliflower mosaic virus (CaMV) cassette in the 2.4-kb pUC-based pGreen plasmid vector from the John Innes Center, Norwich, UK (Hellens et al., 2000; modified and redrawn from www.pgreen.ac.uk). The 787-bp *OSMI* and 728-bp *OSMII* PCR products were each cloned into the *Xba*I and *EcoR*I sites of this 672-bp cassette to generate 1.4-kb cassettes in the vector constructs (Wilkinson, 2003).

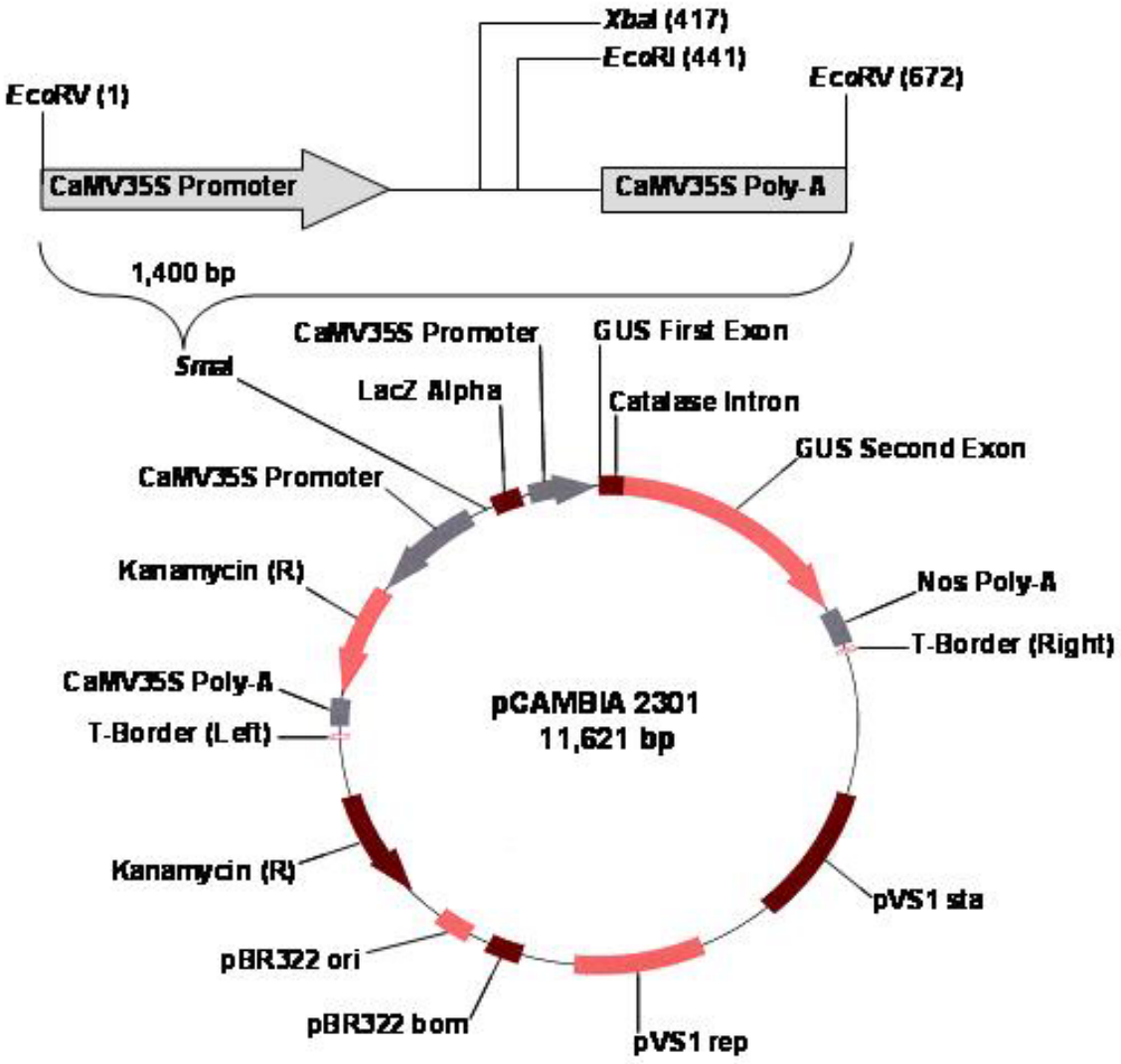

Figure 2. Diagram of the pCAMBIA 2301 binary vector from the Center for Application of Molecular Biology to International Agriculture, Canberra, Australia (modified and redrawn from www.cambia.org/daisy/bios/585.html using the BVTech Plasmid drawing software). The 1.4-kb 35S-*OSMI* and 35S-*OSMII* cassettes (www.pgreen.ac.uk) were ligated into the *Sma*I site by blunt-end ligation (Wilkinson, 2003).

were grown at 28°C with shaking at 200 rpm until an A_{600} of 1.8-2.0 (turbidity measurement) was reached. The cultures were centrifuged at 5,500xg for 20 min at room temperature, and the cells were resuspended in 5% sucrose to an A_{600} of about 0.8. Silwet L-77 (VAC-IN-STUFF, LEHLE seeds, Round Rock, TX), a surfactant that enables the *Agrobacterium* cells to penetrate the plant cell walls and membranes, was added to each of the diluted cultures to a concentration of 0.04%.

The parts of the *Arabidopsis* plants above ground were dipped in the diluted *Agrobacterium* solutions for 2-3 sec, with gentle agitation. The dipped plants were then sprayed with water to prevent an overgrowth of *Agrobacterium*, and the plants were immediately covered with a trashcan to maintain a high humidity. The trashcan was removed 24 h later, and the plants were transferred to a 22°C growth room with a 16 h photoperiod. Six days after the first dip, the plants were dipped again in fresh *Agrobacterium* solutions as before. Twenty-four hours after the second dip, the plants were transferred back to the growth room, where they were grown until the seeds became mature. The dried seeds were then harvested and stored in 20 ml glass scintillation vials.

The harvested seeds were sterilized using a vapor-phase sterilization method (Clough and Bent, 1998). Approximately 50 µl of seeds were transferred to 1.5 ml microcentrifuge tubes. The tubes were then placed into a bell jar, along with a beaker containing 100 ml of bleach. Just before sealing the jar, 3 ml of concentrated HCl was added into the bleach. The chlorine fumes then sterilized the seeds in the bell jar for 6 h. The sterilized seeds were subsequently plated on 100 x 20mm^2 kanamycin selection plates (0.5X MS (Murashige & Skoog) salts with micronutrients (iron, manganese, zinc,

boron, copper, molybdenum, cobalt; Sigma, Catalog# M0529; Murashige and Skoog, 1962), 0.5X MS salts with macronutrients (nitrogen, phosphorous, potassium, calcium, magnesium, sulfur; Sigma, Catalog# M0654; Murashige and Skoog, 1962), 0.25% Gelrite gellan gum, and 100 µg/ml kanamycin (pH 5.6)). Also plated on kanamycin selection plates were wild type *Arabidopsis thaliana* (ecotype Columbia) seeds (to serve as a negative control) and kanamycin-resistant seeds, a gift from Dr. Brian Ayre of Biological Sciences at the University of North Texas (to serve as a positive control). The plated seeds were cold-treated for 48 h before they were transferred to the growth room under a 16 h light/8 h dark cycle regimen. The plated seeds were left in the growth room until seedlings that produced green secondary leaves and established root systems were visible.

Once the putative *Arabidopsis* T_1 transformants were tentatively identified on the kanamycin selection plates, they were carefully transplanted to soil and covered with Saran wrap to keep the seedlings moist. After 48 h, the seedlings were gradually hardened over another 48-72 h time period by cutting small tears in the Saran wrap. These *Arabidopsis* T_1 plants were grown until the seeds became mature.

For further confirmation, the supposed transformed plants were also subjected to PCR analyses using the REDExtract-N-Amp™ Plant PCR Kit (Sigma) with an amplimer pair designed specifically for a 376-bp region of the *GUS* second exon within the pCAMBIA 2301 vector DNA. These primers were pCAMBIA For (5'-ACTGTAACCA CGCGTCTGTT-3') and pCAMBIA Rev (5'-AATCCAGTCCATTAATGCGT-3'). A unique amplimer pair for a 265-bp segment of the constitutively expressed actin 8 gene (An et al., 1996) was also used as a positive control. These primers, ACT8 FWRD (5'-GTTA

AGGCTGGATTCGCTGG-3') and ACT8 RVSE1 (5'-GTTAAGAGGAGCCT CGGTAAG-3'), were designed by Mr. Neal Teaster of Dr. Kent Chapman's laboratory of our department at UNT. A hole punch was used to cut a 0.7 cm disk from a leaf of each *Arabidopsis* T_1 plant examined. The leaf samples were each placed in 2 ml collection tubes containing 100 µl of Extraction Solution (Sigma proprietary mixture) and vortexed briefly. The tubes were placed in a 95˚C waterbath for 10 min. After the 10 min incubation, 100 µl of Dilution Solution (Sigma proprietary mixture) was added to each tube, and the solutions were mixed by vortexing briefly. The diluted leaf extracts were then subjected to PCR amplification using the Sigma REDExtract-N-Amp PCR Reaction Mix (containing a proprietary mixture of buffer, salts, dNTPs, *Taq* DNA polymerase, and TaqStart antibody for specific hot start amplification). Each 20 µl PCR reaction contained 1X REDExtract-N-Amp PCR Reaction Mix, 0.4 µM of each appropriate gene-specific primer, and 4 µl of diluted leaf disk extract. The reactions were placed in a thermal cycler (Perkin Elmer GeneAmp PCR system 2400) and denatured for 3 min at 94˚C. Once denatured, the reactions were subjected to 35 cycles, including a 30 sec step at 94˚C to denature the double-stranded template, a 30 sec step at 59˚C to allow the gene-specific primers to anneal to the template, and a 1 min step at 72˚C to allow the primers to extend with the supplied *Taq* polymerase. After the last cycle, the reactions were held at 72˚C for 10 min and then cooled to 4˚C. The PCR products were electrophoresed on a 2.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM® DNA Markers (Promega) as standards for size determination.

The *Arabidopsis* T_2 seeds were harvested from the *Arabidopsis* T_1 plants that gave rise to pCAMBIA 2301 PCR products and were stored in glass scintillation vials. These seeds were subjected to kanamycin selection and the DNAs analyzed by PCR as detailed above. The *Arabidopsis* T_2 plants that appeared to contain appropriate-sized inserts were further analyzed by Western blotting using the anti-osmotin antibody preparation. Total protein was extracted (Tripathy et al., 2003) from the leaves of a wild type *Arabidopsis thaliana* (ecotype Columbia) plant, and 44 of the putative transgenic *Arabidopsis* T_2 plants transformed with the pCAMBIA 2301 vector construct that generated the vector DNA PCR products. The *Arabidopsis* T_2 leaves were frozen in liquid nitrogen, and then homogenized in four volumes of 100 mM potassium-phosphate buffer (400 mM sucrose, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 mM ascorbate, and 5 mM DTT (pH 7.2)) using Kontes disposable PELLET PESTLES® and a PELLET PESTLE® Cordless Motor. The crude homogenates were centrifuged at 650xg for 10 min at 4˚C. The resulting supernatants were transferred to new tubes and centrifuged at 10,000xg for 20 min at 4˚C. The supernatants from the second centrifugation were then transferred to another set of tubes, and their concentrations were determined by Bradford assays (Sigma). Ten micrograms of each protein sample were mixed with 6X sample buffer, boiled for 5 min, and electrophoresed on a denaturing SDS-polyacrylamide gel with 10 µl of SeeBlue® Plus2 Pre-Stained Standards (Invitrogen™). A protein extract from an ethephon-treated cotton plant was also run as a positive control. Following electrophoresis, the proteins were transferred to nitrocellulose filter membranes for Western blot analyses using the polyclonal anti-osmotin antibody preparation as described previously. Once the *Arabidopsis* T_2 plants were grown and

dried, the *Arabidopsis* T_3 seeds were collected and stored in 20 ml glass scintillation vials.

Transformation, Regeneration, and Screening of Cotton Plants

The vector constructs pCAMBIA-35S-*OSMI* #16-M, pCAMBIA-35S-*OSMII* #11-A, and pCAMBIA 2301-H harbored in *Agrobacterium tumefaciens* cells were also used to transform cotton plants (*Gossypium hirsutum* L., cv. Coker 312) by modified methods of Sunilkumar and Rathore (2001) and Zhang et al. (2001).

The cv. Coker 312 seeds (a gift from Dr. Kent Chapman of the University of North Texas) were delinted using sulfuric acid (Zhang et al., 2001). The seeds were covered with sulfuric acid in a 250 ml beaker and immediately stirred until delinted (~30 seconds). The delinted seeds were transferred to a 500 ml beaker and washed twice with sterile water for 2 min with rotation. After the second wash, the seeds were placed in a 2 l beaker and washed thoroughly with sterile water for 10 min while stirring. They were then placed on a folded piece of Whatman 3MM® filter paper to dry overnight.

The next day, the seeds were surface-sterilized in a 600 ml beaker by first soaking them in 70% ethanol for 1 min with rotation, followed by two brief washes with sterile water. They were then treated for 20 min with 100 ml of 20% bleach and a drop of Tween 20 while stirring, followed by three brief washes with sterile water. The sterilized seeds were imbibed in a Petri dish by adding just enough sterile water to cover the seeds. The dish was wrapped in Parafilm, covered in aluminum foil, and placed in a 28˚C incubator for 48 h. After the 48 h incubation, the seed coats were removed using sterilized forceps. The seeds were then germinated on MSO medium

(0.5X MS salts with micronutrients, 0.5X MS salts with macronutrients, 2% glucose, pH 5.8, and solidified with 0.22% Phytagel) in Magenta boxes at 28˚C under a 16 h photoperiod (~85 µmol m^{-2} s^{-1}).

During the germination period, the cultures of *Agrobacterium tumefaciens* harboring the vector constructs pCAMBIA-35S-*OSMI* #16-M, pCAMBIA-35S-*OSMII* #11-A, and pCAMBIA 2301-H were prepared for transformation. The transformed *Agrobacterium* cells were streaked on YEP plates (10 g/l bactopeptone, 10 g/l yeast extract, 5 g/l NaCl, and solidified with 15 g/l agar) containing 10 mg/l rifampicin and 50 mg/l kanamycin for single colony isolation. After a 48 h incubation period at 28˚C, five single colonies were chosen for each strain and individually inoculated in 14 ml tubes containing 2 ml of YEP broth with 10 mg/l rifampicin and 50 mg/l kanamycin. The cultures were grown for 36 h at 28˚C with 200 rpm shaking. Cells from the five tubes were then combined, centrifuged at 3,900xg for 15 min at 16˚C, and resuspended in 10 ml of pre-induction medium (1% glucose, 7.5 mM MES hydrate (2-(N-morpholino) ethanesulfonic acid hydrate) (pH 5.6), 2 mM sodium phosphate buffer (pH 5.6), AB salts (20 g/l NH_4Cl, 2.93 g/l $MgSO_4$, 3 g/l KCl, 0.2 g/l $CaCl_2$, 0.05 g/l $FeSO_4 \cdot 7H_2O$), and 100 µM acetosyringone). The cultures were grown in 125 ml flasks for 24 h at 28˚C with 200 rpm shaking. After the 24 h incubation (and just prior to co-cultivation), additional acetosyringone (Sigma) was added to each of the cultures at a final concentration of 100 µM.

For the transformation of cotton plant tissues, hypocotyl segments (3-10 mm in length) and cotyledon pieces (10-16 mm^2 surface area) were excised from seven day-old seedlings using a sterile scalpel and placed on Whatman 3MM® filter paper disks on

modified cotton callus initiation medium (G2) (Firoozabady et al., 1987; Sunilkumar and Rathore, 2001) with and without zeatin (cytokinin). The modified G2 media with zeatin contained 0.5X MS salts with micronutrients, 0.5X MS salts with macronutrients, 100 mg/l myo-inositol, 0.4 mg/l thiamine-HCl, 0.5 mg/l zeatin, 3% glucose, 1 g/l $MgCl_2 \cdot 6H_2O$, 100 µM acetosyringone, pH 5.5, and solidified with 0.22% Phytagel (Sigma). The media without zeatin contained 0.5X MS salts with micronutrients, 0.5X MS salts with macronutrients, 100 mg/l myo-inositol, 0.4 mg/l thiamine-HCl, 5 mg/L N^6-(2-isopentenyl) adenine, 0.1 mg/l naphthaleneacetic acid, 3% glucose, 1 g/l $MgCl_2 \cdot 6H_2O$, 100 µM acetosyringone, pH 5.5, and solidified with 0.22% Phytagel. After being trimmed/ wounded on all edges and punctured with a scalpel blade, the hypocotyl segments were laid horizontally and the cotyledon pieces were placed with their adaxial side facing down on the filter paper. The pre-induced *Agrobacterium* cultures were applied to the wound sites of each explant using a P-1,000 Pipetman pipette. Co-cultivation was carried out for 72 h at 25˚C under a 16 h photoperiod (~85 µmol m^{-2} s^{-1}).

The hypocotyl and cotyledon pieces were transferred to the same medium supplemented with antibiotics (P1-c4k50) to control bacterial growth (Firoozabady et al., 1987; Sunilkumar and Rathore, 2001) and for the induction and proliferation of callus tissue. Again, P1-c4k50 media with and without zeatin were prepared. The P1-c4k50 medium with zeatin contained 0.5X MS salts with micronutrients, 0.5X MS salts with macronutrients, 100 mg/l myo-inositol, 0.4 mg/l thiamine-HCl, 0.5 mg/l zeatin, 3% glucose, 1 g/l $MgCl_2 \cdot 6H_2O$, 50 mg/l kanamycin, 400 mg/l carbenicillin, pH 5.5, and solidified with 0.22% Phytagel. The P1-c4k50 medium without zeatin contained 0.5X MS salts with micronutrients, 0.5X MS salts with macronutrients, 100 mg/l myo-inositol,

0.4 mg/l thiamine-HCl, 5 mg/l N^6-(2-isopentenyl)adenine, 0.1 mg/l naphthaleneacetic acid, 3% glucose, 1 g/l $MgCl_2 \cdot 6H_2O$, 50 mg/l kanamycin, 400 mg/l carbenicillin, pH 5.5, and solidified with 0.22% Phytagel. The explants were incubated on this medium for 24 days at 26˚C under a 16 h photoperiod (~85 µmol m^{-2} s^{-1}).

The kanamycin-resistant calli growing on the hypocotyl and cotyledon pieces, along with some of the plant tissue, were excised using a sterile scalpel and transferred to modified embryogenic medium (G3) (Firoozabady et al., 1987; Sunilkumar and Rathore, 2001) with and without zeatin so that the embryonic calli could proliferate. The G3 medium with zeatin contained 0.5X MS salts with micronutrients, 0.5X MS salts with macronutrients, 100 mg/l myo-inositol, 0.4 mg/l thiamine-HCl, 0.5 mg/l zeatin, 3% glucose, 1 g/l $MgCl_2 \cdot 6H_2O$, 1 mg/l 2,4-dichlorophenoxyacetic acid, 0.5 mg/l kinetin, 50 mg/l kanamycin, 400 mg/l carbenicillin, pH 5.5, and solidified with 0.22% Phytagel. The G3 medium without zeatin contained 0.5X MS salts with micronutrients, 0.5X MS salts with macronutrients, 100 mg/l myo-inositol, 0.4 mg/l thiamine-HCl, 0.1 mg/l N^6-(2-isopentenyl)adenine, 5 mg/l naphthaleneacetic acid, 3% glucose, 1 g/l $MgCl_2 \cdot 6H_2O$, 50 mg/l kanamycin, 400 mg/l carbenicillin, pH 5.5, and solidified with 0.22% Phytagel. The calli were placed in a 26˚C growth chamber under a 16 h photoperiod (~10 µmol m^{-2} s^{-1}). The calli were maintained on G3 medium for 12 weeks, with regular subculture to fresh medium every four weeks.

After the calli were subcultured on the modified G3 medium three times, the calli were classified as either nonembryogenic or embryogenic. Embryogenic calli were fast-growing, light yellow, and loose (Sunilkumar and Rathore, 2001; Zhang et al., 2001). Nonembryogenic calli were slow-growing, compact, and light brown or light/dark green.

The embryogenic calli were transferred to MSBOK medium (Sunilkumar and Rathore, 2001) with and without zeatin. The MSBOK medium with zeatin contained 0.5X MS salts with micronutrients, 0.5X MS salts with macronutrients, 100 mg/l myo-inositol, 0.1 mg/l zeatin, 3% glucose, 1 g/l $MgCl_2 \cdot 6H_2O$, B-5 organics, additional 1.9 g/l KNO_3, 2 g/l activated charcoal, 25 mg/l kanamycin, 200 mg/l carbenicillin, pH 5.5, and solidified with 0.22% Phytagel. The MSBOK medium without zeatin contained 0.5X MS salts with micronutrients, 0.5X MS salts with macronutrients, 100 mg/l myo-inositol, 3% glucose, 1 g/l $MgCl_2 \cdot 6H_2O$, B-5 organics, additional 1.9 g/l KNO_3, 25 mg/l kanamycin, 200 mg/l carbenicillin, pH 5.5, and solidified with 0.22% Phytagel. The calli were maintained on this medium either in a 26˚C growth chamber or on a growth stand in the laboratory under a 16 h photoperiod (~10 µmol m^{-2} s^{-1}) with monthly subculture until somatic embryos developed. After the first two rounds of culture on the MSBOK medium, kanamycin was omitted from the medium.

Somatic embryos that were at least 5 mm in length were transferred onto Whatman 3MM® filter paper disks on EG3 medium (Sunilkumar and Rathore, 2001) (0.25X MS salts with micronutrients, 0.25X MS salts with macronutrients, 100 mg/l myo-inositol, 0.5% glucose, 0.4 mg/l thiamine-HCl, pH 5.5, and solidified with 0.22% Phytagel) for germination. The somatic embryos were maintained on the EG3 medium at 26˚C under a 16 h photoperiod (~70 µmol m^{-2} s^{-1}) until germination occurred.

After germination, plantlets that had 2 to 3 cm long shoots and true leaves were transferred to Magenta boxes or jars containing MS3 medium (Sunilkumar and Rathore, 2001). This medium contained 0.25X MS salts with micronutrients, 0.25X MS salts with macronutrients, 0.5% glucose, 0.14 mg/l thiamine-HCl, 0.1 mg/l pyridoxine, 0.1 mg/l

nicotinic acid, pH 5.5, and solidified with 0.08% Phytagel and 0.4% Bacto agar. The plantlets were incubated at 26˚C under a 16 h photoperiod (~70 µmol m^{-2} s^{-1}) until their shoots were 5 to 7 cm long and they had a well developed root system. At this point, the plantlets were transferred to soil and kept under high humidity in plastic bags for two weeks. After the two week period, the plants were gradually hardened for another two weeks by slowly opening the bags. After this, they were taken out of the bags and placed on a growth stand in the laboratory under a 16 h photoperiod (200 µmol m^{-2} s^{-1}) for three or four weeks. Once they had hardened, the plants were transferred to large pots and grown to maturity either in a 28˚C growth room or in a transgenic greenhouse.

Genomic DNAs were isolated from young leaves of a wild type cotton plant (*Gossypium hirsutum* L., cv. Coker 312) and the supposed transgenic cotton plants using the method of Paterson et al. (1993). With this method, 2 to 4 g of young cotton leaves were homogenized under liquid nitrogen using a mortar and pestle with a pinch of sand. The ground tissues were transferred to 50 ml centrifuge tubes, and 20 ml of ice-cold extraction buffer (0.35M glucose, 0.1M Tris-HCl (pH 8.0), 5 mM Na_2EDTA (pH 8.0), 2% (w/v) PVP, 0.1% (w/v) diethyldithiocarbamic acid (DIECA), 0.1% (w/v) ascorbic acid, and 0.2% (w/v) BME (pH 7.5)) was added. The samples were centrifuged at 2,700xg for 20 min at 4˚C. The resulting supernatants were discarded, the pellets were resuspended in 8 ml of nuclei lysis buffer (0.1M Tris-HCl (pH 8.0), 1.4M NaCl, 20 mM Na_2EDTA (pH 8.0), 2% (w/v) hexadecyl-triammonium bromide (CTAB), 2% (w/v) PVP, 0.1% (w/v) DIECA, 0.1% (w/v) ascorbic acid, and 0.2% (w/v) BME) by vortexing, and the samples were incubated at 65˚C for 30 min. After the 30 min incubation, 10 ml of chloroform:isoamyl alcohol (24:1) was added, and the samples were mixed by inverting

the tubes 50 times. The samples were centrifuged at 2,700xg for 5 min at 4˚C, and the upper (aqueous) phases were transferred to clean tubes. This chloroform:isoamyl alcohol extraction was repeated one more time. The aqueous phases were then transferred to new tubes, 0.6 volumes of ice-cold isopropanol were added to each, and the tubes were inverted until the DNA precipitates aggregated. The samples were centrifuged for 5 min at 10,000xg and 4˚C to pellet the DNAs. The pellets were washed with 1 ml of 70% ethanol and gentle vortexing. The extracts were centrifuged at 2,700xg for 10 min and 4˚C before the ethanol was removed, and the pellets were air-dried for 10 min. The dried pellets were then dissolved in 1 ml TE (10 mM Tris-HCl, 1 mM Na_2EDTA) (pH 8.0) at 65˚C for 30 min. After the DNAs were dissolved, the samples were centrifuged for 5 min at 10,000xg and 4˚C in order to pellet any remaining impurities. The resulting pellets were discarded, and the supernatants were finally transferred to clean 1.5 ml tubes. The concentration and purity of each DNA sample was assessed by absorption spectroscopy at A_{260} and at A_{280} using a Varian DMS90 UV-Visible spectrophotometer. The quality of the DNAs was also confirmed by running 200 ng of each sample on 0.8% agarose gels containing ethidium bromide at a final concentration of 0.5 µg/ml, with 1-kb DNA Extension Ladder standards (Gibco BRL).

Several PCR reactions were performed with the genomic DNA extracts from the prospective transgenic cotton plants using unique amplimer pairs to amplify the CaMV 35S cassette promoter and part of the coding regions of the *OSMI* and *OSMII* genes. These primers were OSMCheck For (5'-GAAGTTCATTTCATTTGGAGAGGACAGCCC-'3), complementary to a region of the 35S cassette, and OSMCheck Rev (5'-GGCAAAA CACGACCCTGTAATTGGAACC-3'), complementary to the 5'-ends of the *OSMI* and

OSMII genes. Primers designed specifically for the *GUS* second exon within the pCAMBIA 2301 vector (the pCAMBIA For and pCAMBIA Rev primers described above) were also used, as well as the gene-specific primers for the constitutive *SadI* gene (the *SadI*For and *SadI*Rev primers described above), which served as a positive control. In addition, the vector construct pCAMBIA-35S-*OSMI* #16 DNA from transformed *E. coli* DH5α cells was also used as a positive control for the OSMCheck primers. The construct pCAMBIA-35S-*OSMI* #16 DNA was isolated from the transformed *E. coli* DH5α cells using the QIAprep® Miniprep Kit (QIAGEN). Single colonies harboring each construct were inoculated into 30 ml of LB broth (Luria-Bertani; 10 g/l NaCl, 10 g/l bacto-tryptone, and 5 g/l yeast extract) with 50 µg/ml kanamycin and grown at 37˚C in a New Brunswick shaker/incubator at 200 rpm for 48 h. Ten milliliters of each culture was centrifuged at maximum speed using a Sorvall GLC-4 tabletop centrifuge at room temperature. The bacterial pellets were resuspended in 500 µl of Buffer P1 (QIAGEN proprietary composition) and transferred to 2 ml microcentrifuge tubes. Next, 500 µl of Buffer P2 (QIAGEN proprietary composition) was added, and the suspensions were gently mixed by inverting the tubes six times each. Seven hundred microliters of Buffer N3 (QIAGEN proprietary composition) was added, and the suspensions were mixed again by inverting 10 times. The mixtures were centrifuged at room temperature for 10 min at 17,900xg. The resulting supernatants were applied to QIAprep spin columns and centrifuged for 1 min. After discarding the flow-through, the spin columns were washed twice. The first wash was with 500 µl of Buffer PB (QIAGEN proprietary composition), and the second wash was with 750 µl of Buffer PE (QIAGEN proprietary composition). Each wash was followed by a 1 min centrifugation. After the second flowthrough was

discarded, the columns were centrifuged for an additional minute to dry. The columns were then placed in new 1.5 ml microcentrifuge tubes, and the DNAs were eluted from the columns after a 1 min incubation using 50 µl of water and a 1 min centrifugation.

The PCR reactions were done using SmartMix™ HM lyophilized beads (Cepheid), the appropriate gene-specific primers, and the genomic DNA extracts. Each 25 µl PCR reaction contained 200 µM dNTPs, 4 mM MgCl, 4.25 mM HEPES buffer (pH 7.2 ± 0.1), 3 units of hot start *Taq* polymerase, 100 ng of cotton genomic DNA, and 0.4 µM of each gene-specific primer. The reactions were placed in a thermal cycler (Perkin Elmer GeneAmp PCR system 2400), and the DNA templates were denatured at 95˚C for 5 min. Once the templates were denatured, the reactions were subjected to 30 cycles, including a 15 sec step at 95˚C to denature the double-stranded template, a 30 sec step at the appropriate melting temperature (59˚C for *Sad*I primers and a range from 50˚C to 72˚C for OSMCheck primers) to allow the gene-specific primers to anneal to the template DNAs, and a 30 sec step at 72˚C to allow the primers to extend with the supplied DNA polymerase. After the last cycle, the reactions were held at 72˚C for 10 min and then cooled to 4˚C. The resulting PCR products were mixed with 6X blue/ orange loading dye and electrophoresed on a 2.0% agarose gel (for the *SadI* gene PCR products) or a 1.0% agarose gel (for OSMCheck PCR products) containing ethidium bromide at a final concentration of 0.5 µg/ml, with 200-bp DNA Step Ladder standards (Promega) to confirm PCR product sizes.

Leaf samples of the putative transgenic cotton plants were also sent to BioDiagnostics, Inc. (http://www.biodiagnostics.net/; River Falls, WI) to verify the presence of the supposed transgenes in the cotton plants by PCR analyses. The

amplimer pairs used in these analyses were the OSM Check primers (OSMCheck For and OSMCheck Rev) mentioned above, as well as OSMCheck FOR3 (5'-ATTGTTGCA CTCAAGGGTACG-3') and OSMCheck REV3 (5'-AGCGAAACCCTATAAGAACCC-3'). One or two of the youngest leaves from 35 different tentative transgenic cotton plants (one cv. Coker 312 wild type, one plant supposedly transformed with the empty pCAMBIA 2301 vector, 31 putative *OSMI* transgenic plants, and two prospective *OSMII* transgenic plants) were sent, along with purified pCAMBIA-35S-*OSMI* #16 plasmid DNA for use as a positive control template in the PCR analyses.

In another method to verify that the osmotin vector constructs were present in the putative transgenic cotton plants, kanamycin resistance tests were performed to detect the expression of the *npt*II gene, as was done by Wu et al. (2005) and Zapata et al. (1999) from the integrated pCAMBIA 2301 vector DNA. The lowest concentration of kanamycin that would affect untransformed cv. Coker 312 plants was first established. A sterile cotton swab was used to apply 0, 0.1, 1.0, 2.0, and 3.0% (w/v) kanamycin solutions to separate leaves of a non-transgenic control plant. Ten days after application, the leaves were cut from the plant and analyzed. From this, it was decided to apply 1.0 and 2.0% kanamycin solutions to the leaves of nine putative transgenic cotton plants. Kanamycin solutions (0, 0.1, 1.0, and 2.0%) were again applied to a cv. Coker 312 wild type plant as a control. Then, the 1.0 and 2.0% kanamycin solutions were applied to separate leaves of each of the putative transgenic plants. Ten days after application, the leaves were cut from the plants and analyzed.

To determine how many times the osmotin transgenes might have been inserted, genomic blot analyses were performed on DNAs from each of the supposed transgenic

cotton plants, as well on the DNA from a cv. Coker 312 wild type plant as a control. In hopes that the use of ^{32}P radiation could be precluded, the Pierce North2South® Biotin Random Prime Labeling Kit, along with the North2South® Chemiluminescent Hybridization and Detection Kit, was initially used. With the North2South® Biotin Random Prime Labeling Kit, two different HPLC-purified oligonucleotides (each 50 residues in length) and control DNA (supplied with the kit) were labeled with biotin by the random-priming procedure of Feinberg and Vogelstein (1983). One oligonucleotide (osmotin probe) was for hybridization to the osmotin genes, and the other oligonucleotide (pCAMBIA probe) was for hybridization to the pCAMBIA vector DNA. The sequence of the osmotin gene probe was 5'-ATGAGCTACTTAACCATTTCCCAA ATCTCCTCCCTCCTCTTCTTTAGCGT-3'. The sequence of the pCAMBIA vector DNA probe was 5'-AACAGTTCCTGATTAACCACAAACCGTTCTACTTTACTGGCTTTGG TCGT-3'. This labeling method involves using random heptanucleotides annealing to the denatured oligonucleotides to act as primers for complementary strand synthesis by the Klenow fragment of *E. coli* DNA Polymerase I (lacking the 3'-exonuclease activity). Biotinylated nucleotides were added to each of the reactions to ensure that the newly synthesized DNA strands were labeled with biotin. After the labeling reactions were completed, ethanol precipitation was used to remove any unincorporated nucleotides and absorption spectroscopy at A_{260} was used to quantify each of the labeled probes. Several dilutions of the biotin-labeled control probe were spotted onto nitrocellulose and detected using the Pierce Streptavidin-HRP from the North2South® Hybridization and Detection Kit, in order to confirm that the control labeling reactions were successful.

The biotinylated oligonucleotides were then used for genomic blot analysis. To do this, 10 µg of genomic DNA isolated from a wild type cv. Coker 312 plant and five different putative transgenic cotton plants (described above) were digested with the restriction enzymes *Stu*I and *Xho*I (5 units of each enzyme per µg DNA) overnight at 37˚C. The digests were electrophoresed on a 0.8% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, along with 1-kb DNA Extension Ladder standards (Gibco BRL).

The DNA fragments were transferred to a Hybond™-N^+ filter membrane (Amersham™) replica by the alkaline transfer method described in Sambrook and Russell (2001). The gel was soaked in 0.2 N HCl for 10 min to nick the DNA in preparation for transfer. This was followed by three rinses with water. The gel was soaked in alkaline transfer buffer (0.4 N NaOH, 1 M NaCl) for 15 min, followed by another 20 min soak in fresh buffer. The DNA fragments were then transferred to the membrane via capillary action overnight using the alkaline transfer buffer. The following morning, the membrane was soaked for 15 min in neutralization solution (0.5 M Tris-HCl (pH 7.2), 1 M NaCl), and the DNA fragments were immobilized on the membranes by baking in a vacuum oven for 2 h at 80˚C. Using the North2South® Chemiluminescent Hybridization and Detection Kit, the membranes and labeled probes were used for genomic blot analysis. Using the solutions in this kit, the membrane was pre-hybridized for 2 h at 55˚C and hybridized with each of the denatured biotinylated probes overnight at 55˚C. The following day, the membrane was washed three times for 15 min each with 2X SSC/0.1% SDS at 55˚C. To detect the probe, the nonspecific sites on the membrane were blocked and Streptavidin-HRP was added. The excess Streptavidin-

HRP was subsequently washed off, and equal volumes of luminol/enhancer solution and stable peroxide solution were mixed together and poured over the membrane. The membranes were then wrapped in Saran wrap and exposed to film for a period of 1 min to overnight.

Because a strong hybridization signal was never detected with the Pierce North2South® Chemiluminescent Hybridization and Detection Kit, genomic blot analyses were done instead using classical radioactive ^{32}P-labeled probes. Five different primer sets were synthesized and used to amplify different regions of the pCAMBIA 2301 vector DNA (the *npt*II gene, *Lac*Z gene, two different regions of the *GUS* second exon, and the *nos* terminator). A new amplimer pair was also synthesized for the *OSMI* gene. The primers designed for the *npt*II gene were Kan For (5'-ATCGCCATGTGTCACGA CG-3') and Kan Rev (5'-AATGAACTCCAGGACGAGGC-3'). The primers designed for the *Lac*Z gene were LacZ For (5'-AGGCATGCAAGCTTGGCAC-3') and LacZ Rev (5'-AGGCTGCGCAACTGTTGG-3'). The primers designed for the two different regions of the *GUS* second exon were Gus2Ex ForA (5'-AATTGATCAGCGTTGGTGGG-3'), Gus2Ex RevA (5'-GTCGGTAATCACCATTCCCG-3'), Gus2Ex ForB (5'-AGCAAGCG CACTTACAGGC-3'), and Gus2Ex RevB (5'-AAATTCCATACCTGTTCACCGAC-3'). The primers designed for the *nos* terminator were NosTerm For (5'-CTTAAGATTGAAT CCTGTTGCCG-3') and NosTerm Rev (5'-CGATCTAGTAACATAGATGACACCGC-3'). These new primers were used to generate their respective PCR products from pCAMBIA-35S-*OSMI* #16 plasmid DNA.

The PCR reactions were carried out using AccuPrime™ *Pfx* DNA Polymerase (Invitrogen™), the appropriate amplimer pairs, and the pCAMBIA-35S-*OSMI* #16

plasmid DNA. Each 50 µl PCR reaction contained 1X AccuPrime™ *Pfx* Reaction mix (containing dNTPs, $MgSO_4$, and thermostable AccuPrime™ proteins (that improve specific primer-template hybridization)), 200 ng of pCAMBIA-35S-*OSMI* #16 plasmid DNA, 0.3 µM of each gene-specific primer, and 1.25 units of AccuPrime™ *Pfx* DNA Polymerase. The reactions were placed in a thermal cycler (Perkin Elmer GeneAmp PCR system 2400), and the DNA templates were denatured at 94˚C for 2 min. Once the templates were denatured, the reactions were subjected to 30 cycles, including a 15 sec step at 95˚C to denature the double-stranded template, a 30 sec step at 62˚C to allow the gene-specific primers to anneal to the template, and a 30 sec step at 68˚C to allow the primers to extend with the supplied DNA polymerase. After the last cycle, the reactions were held at 68˚C for 10 min and then cooled to 4˚C.

The resulting PCR products were mixed with 6X blue/orange loading dye and electrophoresed at 20 V overnight on a 1.0% LE agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with 200-bp DNA Step Ladder standards (Promega) to confirm PCR product sizes. All PCR fragments, except the *Lac*Z PCR product, were excised from the gel, weighed, and purified using the QIAquick Gel Extraction Kit (QIAGEN) as described previously. The purity and concentration of the purified products were determined by gel electrophoresis. Five microliters of each purified product were mixed with 6X blue/orange loading dye and electrophoresed on a 1.0% agarose gel, with the pGEM® DNA Marker standards (Promega) to estimate relative yields. Both strands of the purified PCR products were sequenced on an Applied Biosystems Model 377XL DNA Sequencer by Lone Star Labs (Houston, Texas) using the gene-specific primers described above, and the sequences were analyzed

using DNASIS® version 2.1 software (Hitachi Software Engineering Co., Yokohama, Japan).

Of the five purified PCR fragments, the *GUS* second exon A and *OSMI* PCR products were selected as templates to generate probes for the genomic blot analyses. The two PCR products were used as templates to generate ^{32}P-labeled probes by the random priming procedure of Feinberg and Vogelstein (1983). The fragments were labeled by Drs. Robert and Irma Pirtle in our laboratory using [α-^{32}P] dCTP (Perkin Elmer) and purified using Sephadex® G-50 NICK Columns (Amersham Biosciences).

To try to confirm that the *GUS* second exon A and *OSMI* PCR products were integrated into the DNAs of the putative transgenic plants before doing the genomic blot analyses, their corresponding sequence specific amplimer pairs were used to amplify their respective PCR products from the cotton genomic DNAs. The PCR reactions were carried out using SmartMix™ HM lyophilized beads (Cepheid) as described above. The annealing temperature used for all reactions was 66˚C. The resulting PCR products were mixed with 6X blue/orange loading dye and electrophoresed on a 1.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml.

Once the *OSMI* gene and *GUS* second exon A gene hybridization targets were shown to be present in the genomic DNA samples, 10 µg of each genomic DNA was digested with 50 units of the restriction endonuclease *Nde*I (New England BioLabs). The pCAMBIA-35S-*OSMI* #16 plasmid DNA, as control, was also digested with *Nde*I (New England BioLabs, 10 units per microgram). The DNA, water, and 10X NEBuffer 4 were mixed together and incubated for 3 h at 4˚C, with gentle mixing every 30 min. The

*Nde*I was then added to each reaction, and the mixtures were incubated in a 37˚C waterbath for 16 h. The digests were terminated by placing the reaction tubes in a 65˚C waterbath for 20 min. The digests were then mixed with 6X blue/orange loading dye and electrophoresed on 0.7% agarose gels. Serial dilutions (0.5 µg, 0.1 µg, 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg) of the digested control plasmid DNA were also electrophoresed and stained with 0.5 µg/ml ethidium bromide.

After photographing the gels, the DNA was then transferred to Hybond™-N^+ nylon membrane filters (Amersham™) according to the alkaline blot transfer method described in Sambrook and Russell (2001). The DNA in the gel was depurinated by placing the gels in 0.125 M HCl for 10 min with gentle agitation, and the HCl was removed by rinsing the gels with water five times for 1 min each and then once for 5 min. The gels were then soaked in alkaline transfer buffer (0.4 N NaOH, 1 M NaCl) for 15 min with gentle agitation. This wash was removed, and the gels were soaked for another 20 min with fresh alkaline transfer buffer. The nylon membrane, two Whatman 3MM® filter papers, and an 8 cm stack of paper towels were cut to the size of each gel. Two larger Whatman 3MM® filter papers were placed on a glass support plate inside a large baking dish containing alkaline transfer buffer. After the 20 min denaturation step, each gel was inverted and placed on top of the wicks, followed by the nylon membrane, which had been floated on water and immersed in alkaline transfer buffer for 5 min. Four pieces of Parafilm were cut and placed around the gel to prevent the buffer from flowing directly to the paper towels. The two pieces of filter paper were wet in transfer buffer and placed on top of the membrane. This was followed by the stack of paper towels, and weighted with a glass plate and 400 g weight. The DNAs were transferred

from the gels to the nylon membranes overnight by capillary action using the alkaline transfer buffer. The following day, the blots were soaked in neutralization buffer (0.5 M Tris-HCl (pH 7.2), 1 M NaCl) for 15 min and then air-dried at room temperature. Once dried, the DNAs were permanently immobilized on the nylon membranes by baking them for 2 h at 80˚C in a vacuum oven. The membranes were wrapped in Whatman 3 MM® filter paper and aluminum foil before they were stored at room temperature under vacuum until prehybridization. All gels were run in duplicate. There were eight blots containing digested genomic DNAs and two blots containing digested plasmid DNA as a control.

The membranes containing the immobilized DNAs and the ^{32}P-labeled *OSMI* and *GUS* second exon A probes were used for genomic blot analyses. The nylon membranes were wetted underneath with 6X SSC, 0.1% SDS and then submerged for 2 min. Once completely soaked, the immobilized DNAs on the membranes were prehybridized in a solution of 6X SSC, 5X Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA for 4 h at 60˚C. After the 4 h, the blots were hybridized in a solution of 6X SSC, 5X Denhardt's solution, 0.5% SDS, 20 mM Tris-HCl (pH 8.0), 2 mM Na_2EDTA (pH 7.5), 2.5 mM sodium pyrophosphate (pH 8.0), 100 µg/ml denatured salmon sperm DNA, and ^{32}P-labeled probe overnight at 60˚C. The next day, the hybridized blots were washed with 2X SSC, 0.1% SDS for 5 min at room temperature. This was followed by a 30 min wash with 2X SSC, 0.1% SDS at 60˚C. Then the blots were washed twice at 60˚C with 1X SSC, 0.1% SDS. The first wash was 30 min and the second wash was 60 min. After the blots were thoroughly dried at room temperature, they were autoradiographed using Kodak X-OMAT® film with lanthanide

intensifying screens at -80˚C. The blots containing the digested plasmid DNA were exposed to film for 5 min up to 48 h. The blots containing the digested genomic DNAs were exposed to film for one month. Following the appropriate exposure time, the films were developed and analyzed.

Because the results of the *Nde*I digests were somewhat ambiguous, 11 of the putative transgenic cotton plants were chosen for further analysis. Genomic DNAs extracted from the cv. Coker 312 wild type plant and the 11 supposed transgenic cotton plants were each digested with the restriction enzymes *Nde*I + *Afl*II, *Nsi*I, and *Ase*I. The pCAMBIA-35S-*OSMI* #16 plasmid DNA was also digested with *Nsi*I (New England BioLabs, 10 units per microgram). This series of genomic blots (digestion, electrophoresis, blotting, and hybridization) was performed as before, and the blots were exposed to Kodak X-OMAT® film for a period of 49 days instead of one month.

Confirmation of *Agrobacterium tumefaciens* Constructs

All attempts made to verify the presence of the osmotin transgenes in the putative *Arabidopsis thaliana* and cotton transgenic plants failed. Thus, the plasmid construct DNAs were re-isolated from the *Agrobacterium tumefaciens* stocks originally used to transform the plants. The purified plasmid construct DNAs were analyzed by restriction endonuclease digestion and PCR analyses. The plasmid DNAs used in the analyses were isolated from the *Agrobacterium* cells using a modified protocol of Li et al. (1995). The *Agrobacterium* cultures (pCAMBIA 2301 in Agro-H, pCAMBIA-35S-*OSMI* #16-M3, and pCAMBIA-35S-*OSMII* #11-A) were inoculated into 50 ml of LB broth containing 10 µg/ml streptomycin and 50 µg/ml kanamycin and grown for 48 h at 28˚C

in an incubator/shaker at 200 rpm. One milliliter of each culture was transferred to four 2 ml microcentrifuge tubes and centrifuged for 2 min at maximum speed using a Model 235C Fisher Scientific Microcentrifuge at room temperature. The supernatants were discarded, and an additional 1 ml of cells was added to each tube, and the cells were pelleted with another 2 min centrifugation. After the supernatants were discarded, the cells were resuspended in 100 µl of Solution I (50 mM glucose, 10 mM Na_2EDTA, and 25 mM Tris-HCl (pH 8.0)) and incubated for 5 min at room temperature. Twenty microliters of 20 mg/ml lysozyme solution was added to each suspension, mixed by inversion, and incubated for 15 min at 37˚C. The bacterial cells were lysed with 200 µl of Solution II (0.2 N NaOH and 1% (w/v) SDS) while inverting the tubes four times and incubated on ice for 5 min. The suspensions were neutralized with 150 µl of cold (-20˚C) Solution III (5 M potassium acetate and 11.5% (v/v) glacial acetic acid (pH 4.8)), inverted several times, and incubated on ice for another 5 min. The cell debris was removed from each tube by centrifuging for 5 min, and the supernatants were transferred to clean microcentrifuge tubes.

RNase A (10 µg/µl) was added to each supernatant at a final concentration of 20 µg/ml and incubated for 20 min at 37˚C. One volume of phenol:chloroform (1:1) was added to each sample, vortexed briefly, and centrifuged for 5 min. Each aqueous phase was transferred to a new tube, and one volume of chloroform:isoamyl alcohol (24:1) was added. After the suspensions were vortexed briefly and centrifuged for 5 min, each aqueous phase was transferred to another fresh microcentrifuge tube. Two volumes of 95% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) were added to each and inverted. The DNAs were precipitated for 30 min in a dry ice/alcohol bath and

then collected with a 15 min centrifugation at 4˚C. The pellets were washed with 100 µl of 70% ethanol, air-dried for 10 min at room temperature, and each dissolved in 20 µl of water. The DNAs of each construct were combined into one tube, and their concentrations were determined at 260 nm and 280 nm.

The isolated plasmid DNAs were then subjected to PCR analyses. The plasmid DNA isolated from pCAMBIA 2301 in Agro-H was analyzed using the pCAMBIA For and pCAMBIA Rev primers, and the plasmid DNAs isolated from pCAMBIA-35S-*OSMI* #16-M3 and pCAMBIA-35S-*OSMII* #11-A were analyzed using the OSMCheck For and OSMCheck Rev primers described earlier. The PCR reactions were carried out using SmartMix™ HM lyophilized beads (Cepheid). The PCR products were mixed with 6X blue/orange loading dye and electrophoresed on a 1.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml. The gel slices containing the resulting PCR fragments were excised from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN). The purity and concentration of the purified products were determined by gel electrophoresis with pGEM® DNA Marker standards (Promega) to estimate yields. Both strands of the purified PCR products were then sequenced on an Applied Biosystems Model 377XL DNA Sequencer by Lone Star Labs (Houston, Texas) using the pCAMBIA and OSMCheck primers and subsequently analyzed using DNASIS® version 2.1 software (Hitachi Software Engineering Co., Yokohama, Japan).

As another confirmation, 12 µl of the plasmid DNAs isolated from the *Agrobacterium* cells were digested with the restriction endonuclease *Sal*I (Gibco BRL). Also, 1 µg of plasmid DNAs isolated from transformed *E. coli* DH5α cells (pCAMBIA 2301 in DH5α, pCAMBIA-35S-*OSMI* #16, and pCAMBIA-35S-*OSMII* #11) were also

digested. The digested fragments were separated on a 0.6% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml.

Second Transformation of *Agrobacterium tumefaciens* with the Binary Plasmid Vector Constructs

The plasmid DNAs (pCAMBIA 2301 in DH5α, pCAMBIA-35S-*OSMI* #16, and pCAMBIA-35S-*OSMII* #11) were isolated from transformed *E. coli* DH5α cells using the QIAprep® Miniprep Kit (QIAGEN). The DNAs were then precipitated with two volumes of cold (-20˚C) 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2), on ice for 1 h. The DNAs were collected by a 15 min centrifugation at maximum speed and 4˚C. The DNA pellets were washed with 500 µl of 70% ethanol and subsequently air-dried for 10 min at room temperature before being dissolved in 25 µl of TE buffer (pH 7.5). The concentration and purity of each plasmid DNA sample was determined at 260 nm and 280 nm, and then 50 ng/µl stocks were made of each. One microliter of each stock was then mixed with 20 µl of ElectroMAX™ *Agrobacterium tumefaciens* LBA4404 Cells (Invitrogen™) in a 1.5 ml microcentrifuge tube on ice. The mixtures were transferred to chilled 1 mm gap cuvettes (BTX® P/N 610) and electroporated with an ECM® Electroporation System (BTX®, San Diego, CA) set on the HV mode/3 kV using a single 1.44 kV electrical pulse that resulted in a 14.4 kV/cm field strength with an exponential decay constant of 5 msec. One milliliter of YM medium (0.04% (w/v) yeast extract, 1.0% (w/v) mannitol, 1.7 mM NaCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, and 2.2 mM $K_2HPO_4 \cdot 3H_2O$, pH 7.0) was immediately added to each mixture and transferred to 14 ml Falcon™ tubes. The mixtures were incubated for 3 h in an incubator/shaker at 30˚C at 225 rpm, and

then diluted 1:10 with YM broth prewarmed to 30˚C. Fifty microliters, 100 µl, and 200 µl of each diluted mixture were spread on prewarmed YM plates containing 100 µg/ml streptomycin and 50 µg/ml kanamycin. The plates were then incubated at 30˚C for 72 h. Isolated colonies were selected and re-streaked on YM plates containing 100 µg/ml streptomycin and 50 µg/ml kanamycin to ensure pure colonies.

After a 48 h incubation at 30˚C, isolated colonies were selected for colony PCR using the Gus2Ex ForA and Gus2Ex RevA primers previously described. The PCR reactions were carried out using SmartMix™ HM lyophilized beads (Cepheid), and the pCAMBIA-35S-*OSMI* #16 plasmid DNA was used as a positive control. The PCR products were electrophoresed on a 1.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml. Two colonies that produced a *GUS*2ExA PCR product were chosen for each plasmid construct (designated as pCAMBIA 2301 in Agro DD, pCAMBIA 2301 in Agro EE, pCAMBIA-35S-*OSMI* #16 DD, pCAMBIA-35S-*OSMI* #16 EE, pCAMBIA-35S-*OSMII* #11 DD, and pCAMBIA-35S-*OSMII* #11 EE) and inoculated into 50 ml YEP broth containing 100 µg/ml streptomycin and 50 µg/ml kanamycin and incubated at 30˚C with 220 rpm shaking for 48 h. The plasmid DNAs were then isolated using the modified protocol of Li et al. (1995).

Fifteen microliters of each plasmid DNA isolated from the cells of this second *Agrobacterium* transformation, 15 µl of each plasmid DNA isolated from the original *Agrobacterium* cells, and 1 µg of pCAMBIA-35S-*OSMI* #16 plasmid DNA were digested in duplicate with the restriction enzymes *Bam*HI and *Kpn*I at 37˚C for 5 h. The digests of the plasmid DNAs isolated from *Agrobacterium* cells and 0.01 µg of the pCAMBIA-35S-*OSMI* #16 plasmid DNA digest were then electrophoresed on 0.7% agarose gels

containing ethidium bromide at a final concentration of 0.5 µg/ml, along with a 1-kb DNA Extension Ladder (Gibco BRL) to confirm product sizes. The DNAs were then transferred to Hybond™-N^+ nylon membranes (Amersham™) according to the alkaline transfer method in Sambrook and Russell (2001). The membranes containing the immobilized plasmid DNAs and the ^{32}P-labeled *OSMI* and *GUS* second exon A probes were used for alkaline blot analyses and subsequently exposed to Kodak X-OMAT® film without screens at room temperature for a period of 1.5 h to 24 h.

CHAPTER 3

RESULTS

Subcloning and Sequence Analysis of a 3.9-kb *Eco*RI Fragment from the Genomic Clone LCgOSM7B

Drs. Robert Pirtle and Jeffery Wilkinson of our laboratory (Wilkinson, 2003; Wilkinson et al., 2005) isolated the genomic clone LCgOSM7B from a cotton (*Gossypium hirsutum* L., cv. Acala SJ3) genomic library (Leandro and Wilkins, 1998) provided by Dr. Thea Wilkins (University of California, Davis). The LCgOSM7B genomic DNA was digested with several different restriction endonucleases for physical mapping. The map was then compared to two other physical maps of the genomic clones LCgOSM16B and LCgOSM12A, previously constructed by Drs. Robert Pirtle, David Yoder, and Jeffery Wilkinson of our laboratory (Wilkinson, 2003; Wilkinson et al., 2005). From this comparison, the positions of the *OSMI* and *OSMII* genes were deduced within the LCgOSM7B genomic clone, as well as the extent of overlap between the cotton genomic segments in the three separate lambda clones (LCgOSM16B, LCgOSM12A, and LCgOSM7B). The physical maps of these three overlapping cotton genomic clones are shown in Appendix A (Wilkinson, 2003; Wilkinson et al., 2005).

While working with Dr. Jeffery Wilkinson, the LCgOSM7B DNA was digested with the restriction endonuclease *Eco*RI (Promega), and the restriction fragments were separated on a 0.8% agarose gel. Using the mobilities of the pGEM® DNA Markers (Promega) and Lambda DNA/*Hind*III Markers (Promega), the *Eco*RI DNA fragments were determined to be 3.9 kb, 4.5 kb, 13.5 kb, and 23 kb in size. Based on the physical

map of the LCgOSM7B, the 3.9-kb fragment was chosen for sequence analysis. After analyzing the sequences of this 3.9-kb *Eco*RI fragment and an overlapping 3.4-kb *Hind*III fragment from the genomic clone LCgOSM7B (Wilkinson, 2003), the segment of cotton DNA was discerned to have two osmotin pseudogenes (*OSMIII* and *OSMIV*) that have internal termination codons, which would prevent the translation of the coding regions (Wilkinson et al., 2005). This work completed the 16,007 bp of sequenced cotton genomic DNA represented in the physical maps shown in Appendix A (Wilkinson, 2003; Wilkinson et al., 2005). The GenBank Accession numbers for the sequences are AF304007 and AY303690 (Wilkinson et al., 2005).

Induction and Isolation of RNA from Cotton Plant Extracts

Total RNA was extracted from leaf, stem, and root tissues of cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5 or cv. Acala Maxxa) treated with water, 100 mM hydrogen peroxide, or 1 mM ethephon and placed in separate gallon plastic bags for periods from 0 h up to 96 h. The water was the control, and the hydrogen peroxide and ethephon were chemical inducers. As another control, total RNA was also extracted from other three week-old cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5) that had neither been treated nor placed in plastic bags. After the concentration and purity of each RNA sample was determined by spectrophotometric readings at 260 nm and 280 nm, 5 µg of each extract was electrophoresed at 5 V/cm on a denaturing gel in 1% formaldehyde (Sambrook and Russell, 2001), with 3 µl of RNA Marker standards (Promega), to examine the quality (shown in Figure 3).

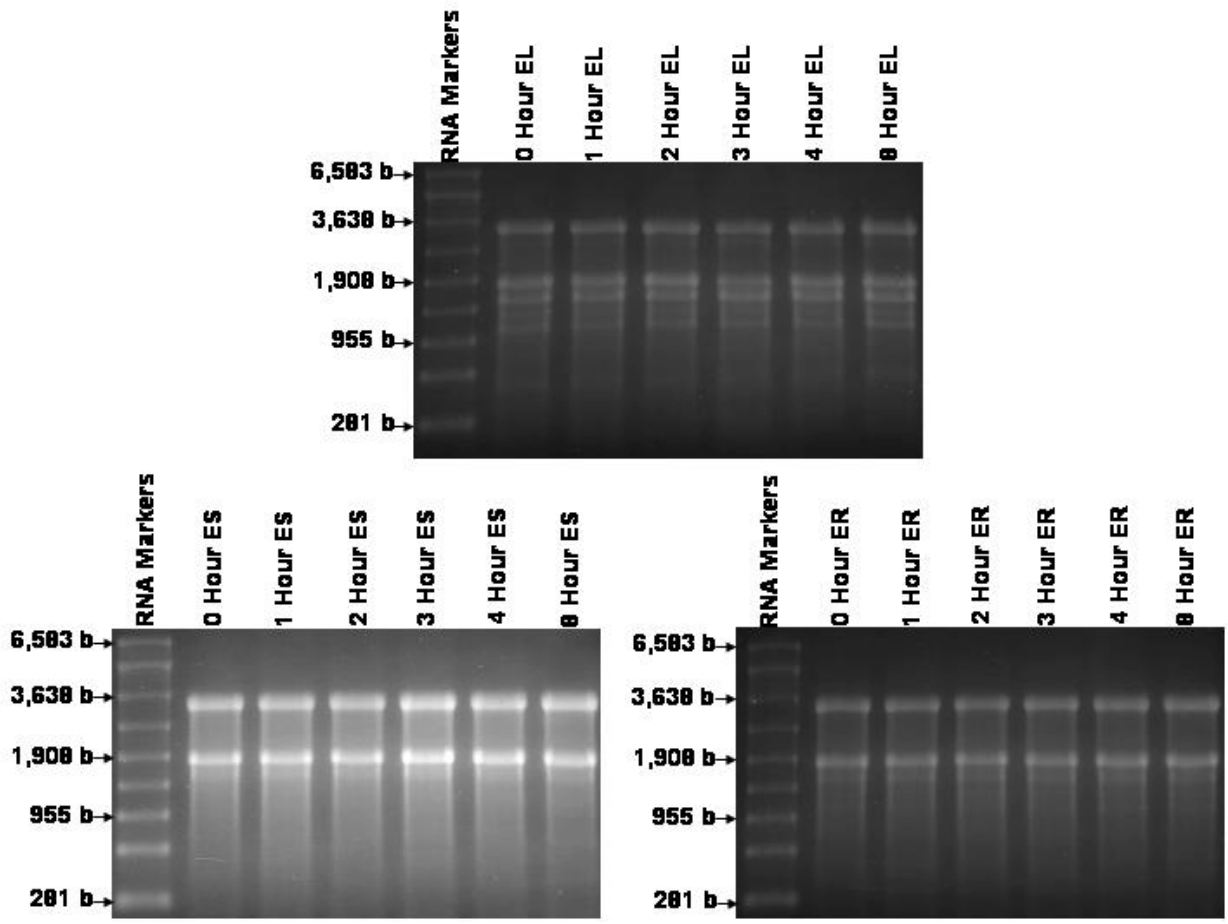

Figure 3. Total RNA extracted from leaf, stem, and root tissues of cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5 or cv. Acala Maxxa) treated with water or 1 mM ethephon for periods from 0 to 8 h. After the concentration and purity of each RNA sample was determined at 260 nm and 280 nm, 5 µg of each extract was electrophoresed at 5 V/cm on a denaturing gel in 1% formaldehyde (Sambrook and Russell, 2001), with 3 µl of RNA Marker standards (Promega), to examine the quality.

Northern Blot Hybridization of RNA from Cotton Plant Extracts

Total RNA extracts from the leaves, stems, and roots of three week-old cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5) that had been treated with water (as control), 100 mM hydrogen peroxide, or 1 mM ethephon for a period of 0, 1, or 2 h were electrophoresed on denaturing gels in 1% formaldehyde and transferred to positively-charged nylon membranes for Northern blot hybridization using a homologous radioactive cotton osmotin probe. After ten weeks of autoradiography, there was a high background on the films, making it difficult for presentation in this dissertation. However, the autoradiograms did clearly indicate the presence of osmotin transcripts in all control and induced samples, with the signal most intense after 2 h of treatment (data not shown). Therefore, it appeared that the osmotin mRNAs were generated in all three tissues (leaf, stem, and root) using all three treatments (water, hydrogen peroxide, and ethephon).

RT-PCR Analysis of RNA from Cotton Plant Extracts

Reverse transcriptase-polymerase chain reactions (RT-PCR) were performed to assess the nature of the osmotin transcripts in total RNA extracts from leaf, stem, and root tissues of cotton plants that had been treated with water (as control), 100 mM hydrogen peroxide, or 1 mM ethephon for a period of 2 h. Leaf, stem, and root tissue extracts from a control plant that was neither treated nor placed in a plastic bag were also analyzed by RT-PCR. The unique amplimer pairs used in these analyses were designed specifically for the 5'- and 3'-untranslated regions (UTRs) of the osmotin gene sequences to amplify products from the *OSMI*, *OSMII*, and *OSMIII* genes. An amplimer

pair for the actin mRNA (An et al., 1996) was also designed and used to generate a positive control product.

After electrophoresing the RT-PCR products on agarose gels, the expected 788-bp *OSMI* and 789-bp *OSMII* products were observed in each of the 2 h RNA extracts examined (Figure 4). The predicted 218-bp actin control products were also generated from each of the samples examined (Figure 5). However, no product was produced with the *OSMIII* amplimer pair (Figure 6).

Gel slices containing the *OSMI* and *OSMII* RT-PCR products produced from the 2 h RNA extracts were excised from the gels, and the products were purified and sequenced. The RT-PCR product made with the *OSMI* gene-specific primers was confirmed to be identical to the *OSMI* open reading frame by sequence analysis. Upon analyzing the sequence of the supposed *OSMII* product, it was suspected to contain more than one transcript because the sequence had a high degree of variability. Therefore, the *OSMII* RT-PCR product was digested with either the restriction endonuclease *Sac*I (Promega), and called *OSMII Sac*I, or *Ban*I (New England BioLabs), and designated *OSMII Ban*I. These enzymes were chosen because there are no *Sac*I restriction sites within the *OSMII* coding region, but there is a single predicted *Sac*I cleavage site in the presumptive new osmotin gene (called the *OSMIIB* gene). On the other hand, there are no predicted *Ban*I restriction sites in the *OSMIIB* gene, but there is one known *Sac*I cutting site within the *OSMII* coding region.

Three bands of the predicted sizes were observed when the digested DNA fragments were electrophoresed on a 0.8% agarose gel. The *Sac*I digest generated 788-bp, 473-bp, and 315-bp fragments. The *Ban*I digest generated 788-bp, 587-bp,

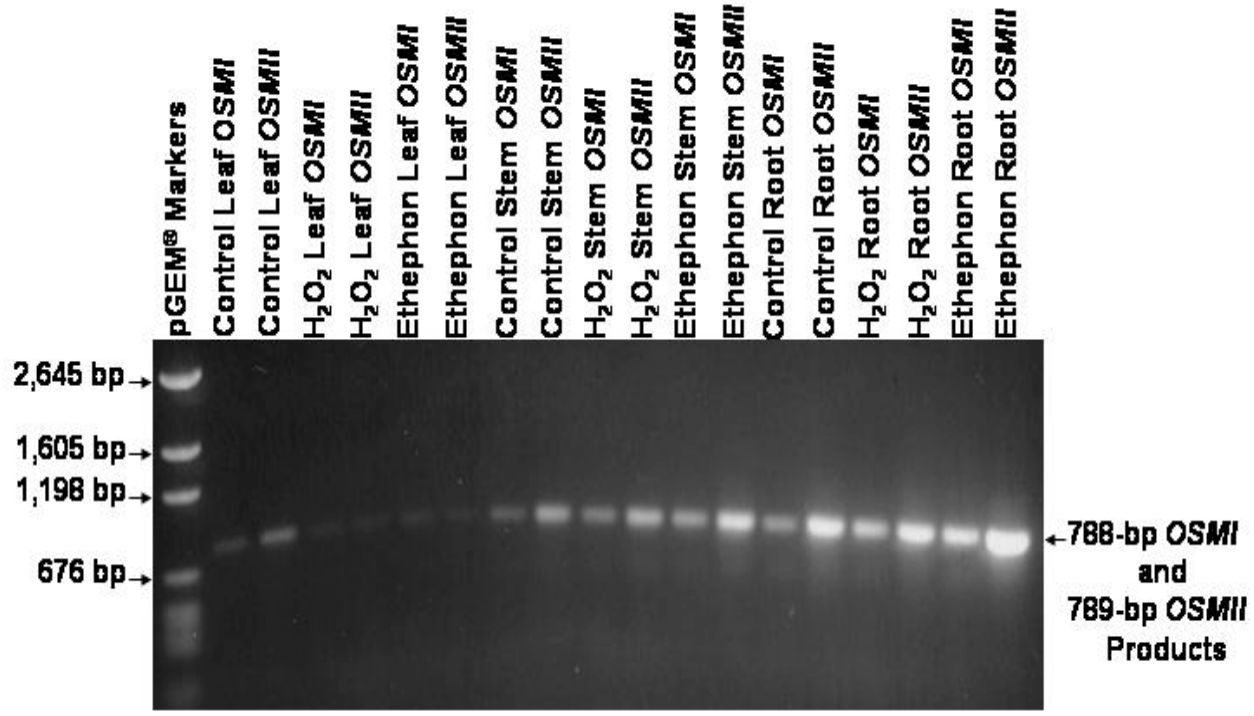

Figure 4. Reverse transcriptase-polymerase chain reaction (RT-PCR) products produced from total RNA extracts from leaves, stems, and roots of cotton plants that had been treated with water (as control), 100 mM hydrogen peroxide, or 1 mM ethephon for a period of 2 h, using unique oligonucleotide primers designed specifically for the 5'- and 3'-untranslated regions (UTRs) of the *OSMI* and *OSMII* genes. The RT-PCR products were electrophoresed on a 1.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, along with pGEM® DNA Marker standards (Promega) to determine product sizes. The resulting 788-bp and 789-bp *OSMI* and *OSMII* PCR products seen in all sample lanes correspond to their predicted sizes.

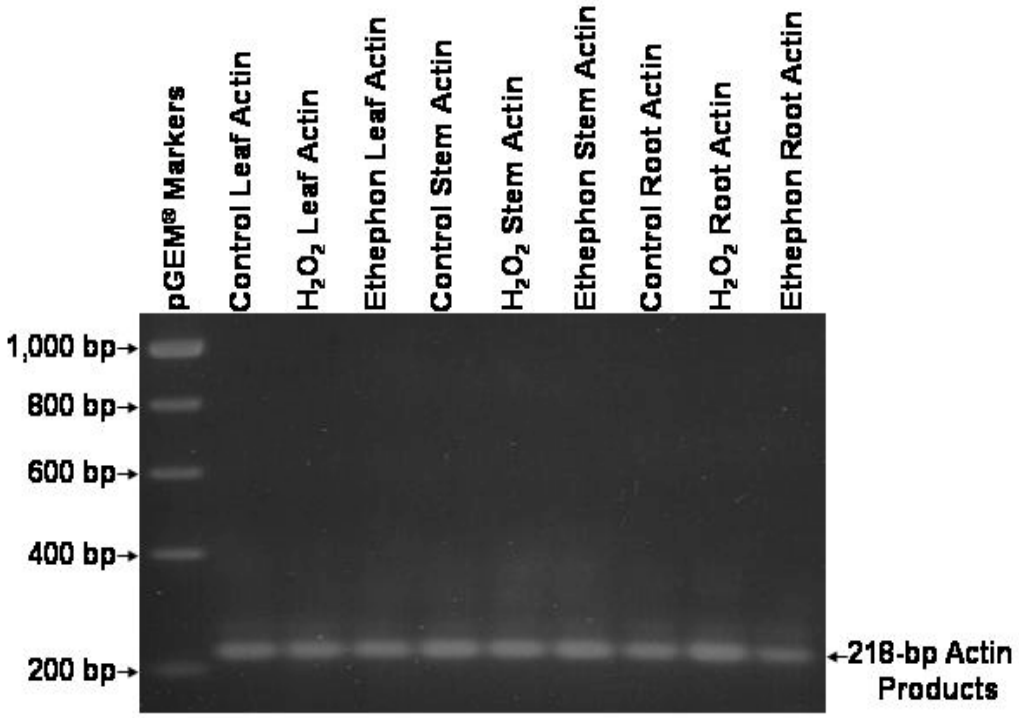

Figure 5. Reverse transcriptase-polymerase chain reaction (RT-PCR) products formed from total RNA extracts from leaves, stems, and roots of cotton plants treated with water (as control), 100 mM hydrogen peroxide, or 1 mM ethephon for a period of 2 h, using gene-specific primers for the constitutive actin mRNA (Shimizu et al., 1997) as a positive control. The RT-PCR products were electrophoresed on a 2.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, along with a 200-bp DNA Step Ladder (Promega) to determine product sizes. The expected 218-bp actin control DNA product was generated from each of the samples.

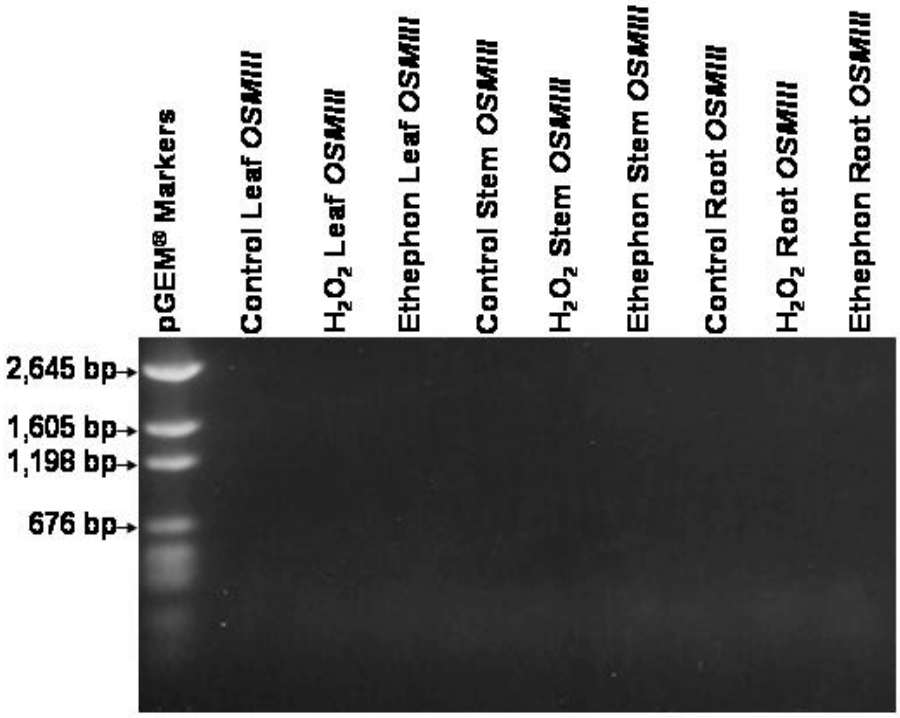

Figure 6. Reverse transcriptase-polymerase chain reaction (RT-PCR) products from total RNA extracts from leaves, stems, and roots of cotton plants treated with water (as control), 100 mM hydrogen peroxide, or 1 mM ethephon for a period of 2 h, using unique oligonucleotide primers designed specifically for the 5'- and 3'-untranslated regions (UTRs) of the *OSMIII* gene. The RT-PCR reactions were electrophoresed on a 1.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, along with pGEM® DNA Marker standards (Promega). No products were detected when using the *OSMIII* primers.

and 201-bp fragments (Figure 7). The 473-bp and 315-bp fragments from the *Sac*I digest correspond to the fragments of *OSMIIB* product, while the 587-bp and 201-bp fragments of the *Ban*I digest correspond to fragments of the *OSMII* product. The 788-bp fragments from the digests were assumed to correspond to a single transcript, the *OSMII* product from the *Sac*I digest and the *OSMIIB* product from the *Ban*I digest. These 788-bp fragments were excised from the gel, purified, and sent for sequence analysis to Lone Star Labs, Houston, TX, to confirm their identities.

After analyzing the sequence of both strands of the *OSMII* and *OSMIIB* products, the 788-bp fragment generated from the *Sac*I digestion was confirmed to correspond to the *OSMII* open reading frame, and the 788-bp fragment generated from the *Ban*I digestion was found to be a presumptive new osmotin gene, now designated the *OSMV* gene. Therefore, the RT-PCR results indicated that the *OSMI*, *OSMII*, and *OSMV* mRNAs are present in all examined tissues (leaves, stems, and roots), after a 2 h treatment with water, ethephon, or hydrogen peroxide in plastic bags.

To determine that the presence of the osmotin mRNAs is constitutive, and not induced by experimental treatments, the RNAs isolated from the control plants (neither treated nor placed in plastic bags) were also subjected to RT-PCR analyses and the products were electrophoresed on a 0.8% agarose gel. No osmotin mRNA was observed from leaf or stem tissues. However, RT-PCR products for both the *OSMI* gene and *OSMII* gene were detected in root tissues (Figure 8). This data may indicate that previous growth or experimental conditions may have been affecting the production of osmotin mRNAs. A more thorough examination of the *OSMI* and *OSMII* mRNA expression levels was done as described in the next section.

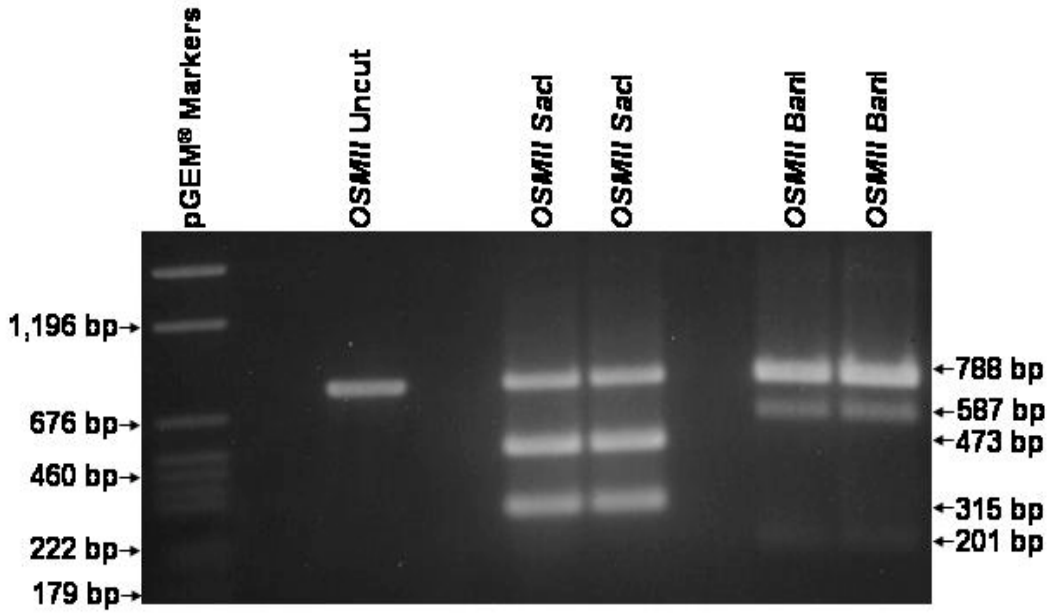

Figure 7. Digestion products from the *OSMII* RT-PCR products after digestion with either *Sac*I (designated *OSMII Sac*I) or *Ban*I (designated *OSMII Ban*I). The digested DNA fragments were electrophoresed on a 0.8% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM® DNA Marker standards (Promega). The *Sac*I digest generated 788-bp, 473-bp, and 315-bp fragments. The *Ban*I digest generated 788-bp, 587-bp, and 201-bp fragments. The 473-bp and 315-bp fragments from the *Sac*I digest were derived from the *OSMIIB* RT-PCR product, while the 587-bp and 201-bp fragments of the *Ban*I digest were from the *OSMII* RT-PCR product. The 788-bp fragments resulting from the digests were assumed to each contain a single product, the *OSMII* product from the *Sac*I digest and the *OSMIIB* product from the *Ban*I digest.

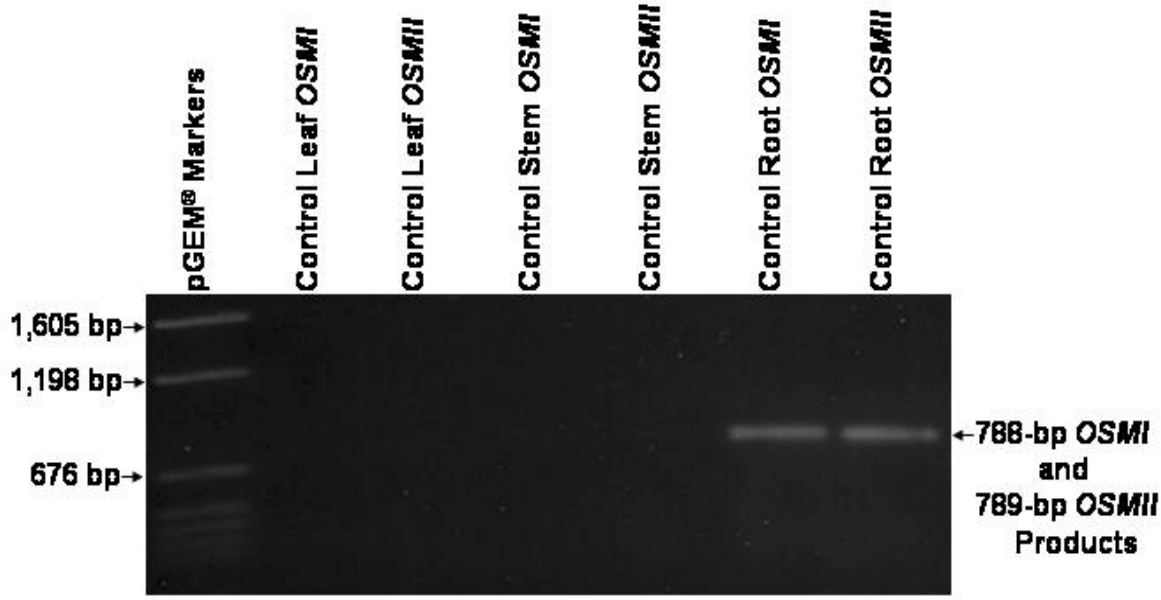

Figure 8. RT-PCR products from total RNA extracts isolated from control plants that were neither treated nor placed in plastic bags. The RT-PCR products were electrophoresed on a 0.8% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM® DNA Marker standards (Promega). No apparent osmotin mRNA product was detected from leaf or stem tissues. However, the expected 788-bp *OSMI* and 789-bp *OSMII* RT-PCR products were detected from root tissues.

Real Time RT-PCR Analyses of RNA from Cotton Plant Extracts

The next analysis of the *OSMI* and *OSMII* mRNA expression levels was to determine their induction curves by real time RT-PCR. Although Northern blot hybridization and RT-PCR are commonly used for the quantitation of mRNA, real time RT-PCR is much more sensitive (Giulietti et al., 2001; Wong and Medrano, 2005). RNA extracts from leaf, stem, and root tissues of one to two week-old cotton plants treated with water (as control) or 1 mM ethephon for periods of 0, 1, 2, 3, 4, 8, 12, 24, 48, or 96 h were examined to determine the times and levels that the osmotin mRNAs might be induced by ethephon.

The relative quantification of the *OSMI* and *OSMII* transcript levels was determined using the comparative threshold cycle method (Giulietti et al., 2001; Livak and Schmittgen, 2001; Wilhem and Pingoud, 2003; Bustin and Nolan, 2004; Wong and Medrano, 2005) and the *SadI* transcript (Yang et al., 2005) as the endogenous control because it appeared slightly more stable than the actin transcript (An et al., 1996). A C_T value represents a cycle number at which the fluorescence is first significantly detected above background. In the real time reactions, the SYBR® Green I intercalating dye was used as an indicator of PCR product formation, as it only fluoresces when bound to double stranded DNA (Wong and Medrano, 2005). The comparative *OSMI* and *OSMII* gene expression levels determined for all RNA extracts (which indicates how much greater the abundance of the *OSMI* and *OSMII* transcripts is in each tissue after treatment with 1 mM ethephon) are shown in Tables 2 and 3. It appears that both the *OSMI* and *OSMII* transcripts are induced upon treatment with 1 mM ethephon, with the *OSMII* transcript apparently being induced the greatest, in all tissue types examined.

RNA Sample	Abundance of *OSMI*	RNA Sample	Abundance of *OSMI*	RNA Sample	Abundance of *OSMI*
0 h Leaf	1.66x↑	0 h Stem	7.31x↑	0 h Root	7.62x↓
1 h Leaf	3.05x↑	1 h Stem	2.57x↑	1 h Root	7.21x↑
2 h Leaf	20.82x↑	2 h Stem	26.91x↑	2 h Root	7.01x↑
3 h Leaf	18.90x↑	3 h Stem	28.44x↑	3 h Root	29.24x↑
4 h Leaf	35.51x↑	4 h Stem	17.15x↑	4 h Root	46.85x↑
8 h Leaf	108.38x↑	8 h Stem	103.25x↑	8 h Root	50.91x↑
12 h Leaf	781.44x↑	12 h Stem	86.22x↑	12 h Root	186.11x↑
24 h Leaf	38.59x↑	24 h Stem	630.35x↑	24 h Root	96.34x↑
48 h Leaf	6,038.61x↑	48 h Stem	212.31x↑	48 h Root	8.57x↑
96 h Leaf	292.04x↑	96 h Stem	140.07x↑	96 h Root	42.81x↑

Table 2. Relative quantification of *OSMI* transcript levels in RNA extracts from leaf, stem, and root tissues of one to two week-old cotton plants treated with water (as control) or 1 mM ethephon for periods of 0, 1, 2, 3, 4, 8, 12, 24, 48, or 96 h. The RNA extracts were examined using real time RT-PCR to determine the time frames and the extent to which osmotin mRNAs were induced by ethephon. The relative quantification of *OSMI* transcript levels, which indicates how many times greater the abundance of the *OSMI* transcript is in each tissue after treatment with 1 mM ethephon, was done using the comparative threshold cycle method (Giulietti et al., 2001; Livak and Schmittgen, 2001; Wilhem and Pingoud, 2003; Bustin and Nolan, 2004; Wong and Medrano, 2005) and the *SadI* transcript as the endogenous control (Yang et al., 2005).

RNA Sample	Abundance of *OSMII*	RNA Sample	Abundance of *OSMII*	RNA Sample	Abundance of *OSMII*
0 h Leaf	1.74x↓	0 h Stem	1.06x↓	0 h Root	1.21x↓
1 h Leaf	4.69x↑	1 h Stem	29.04x↑	1 h Root	5.66x↑
2 h Leaf	59.71x↑	2 h Stem	25.46x↑	2 h Root	3.43x↑
3 h Leaf	152.22x↑	3 h Stem	1,074.91x↑	3 h Root	12.47x↑
4 h Leaf	184.82x↑	4 h Stem	617.37x↑	4 h Root	53.45x↑
8 h Leaf	1,488.87x↑	8 h Stem	32,093.64x↑	8 h Root	77.71x↑
12 h Leaf	14,972.21x↑	12 h Stem	241,221.67x↑	12 h Root	1,509.65x↑
24 h Leaf	317.37x↑	24 h Stem	52,136.28x↑	24 h Root	354.59x↑
48 h Leaf	16,046.82x↑	48 h Stem	45,073.75x↑	48 h Root	6.36x↑
96 h Leaf	3,258.52x↑	96 h Stem	225.97x↑	96 h Root	5,996.90x↑

Table 3. Relative quantification of *OSMII* transcript levels in RNA extracts from leaf, stem, and root tissues of one to two week-old cotton plants treated with water (as control) or 1 mM ethephon for periods of 0, 1, 2, 3, 4, 8, 12, 24, 48, or 96 h. The RNA extracts were examined using real time RT-PCR to determine the time frames and the extent to which the osmotin mRNAs were being induced by ethephon. The relative quantification of *OSMII* transcript levels, which indicates how many times greater the abundance of the *OSMII* transcript is in each tissue after treatment with 1 mM ethephon, was done using the comparative threshold cycle method (Giulietti et al., 2001; Livak and Schmittgen, 2001; Wilhem and Pingoud, 2003; Bustin and Nolan, 2004; Wong and Medrano, 2005) and the *SadI* transcript as the endogenous control (Yang et al., 2005).

The greatest induction of the *OSMI* transcript was seen in leaves. The expression level was determined to be 781.4 times greater in the ethephon-treated sample than the control sample after 12 h. The level dropped to 38.6 times greater at 24 h, and then elevated up to 6,038.6 times greater expression after 48 h of treatment. Although the increase in the *OSMI* mRNA abundance was not as great as that seen in leaves, there was still a considerable boost of the transcript abundance in stem and root tissues following ethephon treatment. For stem tissues, the expression level was 103.3 times greater in the ethephon-treated extract than the control extract after 8 h. The level dipped down slightly to 86.2 times greater at 12 h, and then increased up to 630.4 times greater after 24 h of ethephon treatment. For root tissues, the expression level peaked at 12 h with 186.1 times greater expression in the ethephon-treated sample than the control sample. The level then dropped down to 8.6 times at 48 h, and then went back up slightly to 42.8 times greater after 96 h of treatment.

As mentioned above, the increase in the abundance of the *OSMII* mRNA was even more significant than that for the *OSMI* mRNA following treatment with 1 mM ethephon. However, the greatest induction of the *OSMII* transcript was seen in stem tissues, rather than leaf tissues. The expression level was 1,074.9 times greater in the ethephon-treated sample than in the control sample after only 3 h. The level dropped to 617.4 times greater at 4 h, but then increased greatly to 241,221.7 times greater expression after 12 h of treatment, before steadily dropping back down. Looking at the *OSMII* mRNA abundance in leaves, the expression level increased to 14,972.2 times greater in the ethephon-treated extract than the control extract after 12 h. The level dipped down to 317.4 times greater at 24 h, and then went back up to 16,046.8 times

greater after 48 h of ethephon treatment, before dropping back down. For root tissues, the expression level in the ethephon-treated sample was 1,509.7 times greater than the control sample after 12 h. The level then dropped down to 6.4 times at 48 h, and then dramatically increased to 5,996.9 times greater after 96 h of treatment.

Microsoft Excel® was used to prepare scatter charts of the results shown in Tables 2 and 3, to demonstrate the induction of the *OSMI* and *OSMII* mRNAs (Figures 9-11). These graphical representations indicate the apparent induction in the *OSM* transcript levels in leaves, stems, and roots after treatment with 1 mM ethephon. The levels of osmotin mRNAs tend to go up 12 h after treatment, decrease, and then increase again.

Induction and Isolation of Protein from Cotton Plants for One-Dimensional SDS-PAGE and Western Blot Analyses

While working with Dr. Jeffery Wilkinson of our laboratory, total protein was isolated (Chlan and Bourgeois, 2001) from three to four week-old cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5) that had been treated with water (as a control), 100 mM hydrogen peroxide, 550 mM NaCl, or 1 mM ethephon and placed in separate one gallon plastic bags for a period of 0 to 96 h. After determining the protein concentrations of the extracts, the proteins were separated on a discontinuous buffer system (Laemmli, 1970), along with Full Range Rainbow™ recombinant protein molecular weight markers (Amersham™). Following electrophoresis, the proteins were transferred to a nitrocellulose membrane filter for Western blot analyses using a polyclonal anti-osmotin antibody preparation (Biosynthesis Inc., Lewisville, TX). The

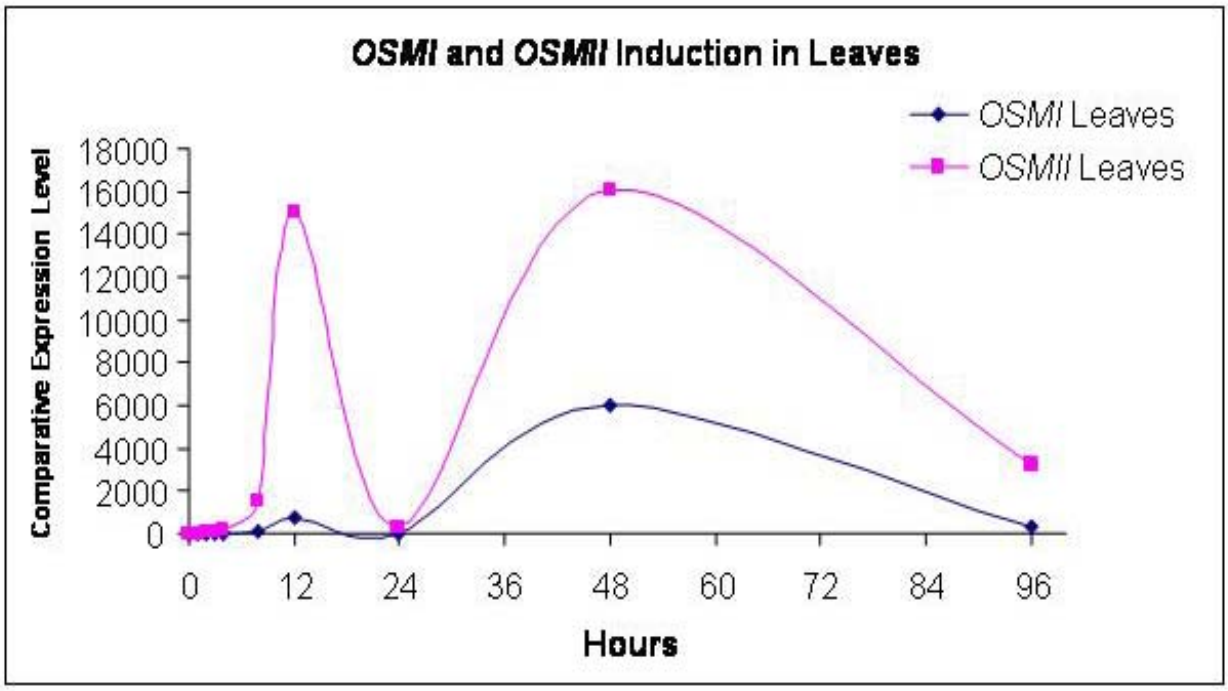

Figure 9. Scatter chart of the real time RT-PCR data presented in Tables 2 and 3, in order to demonstrate the average relative induction profiles of the *OSMI* and *OSMII* mRNAs in leaf tissues. The values on the Y-axis indicate the comparative expression levels of the *OSMI* and *OSMII* transcripts between the ethephon-treated sample and the control sample for that particular time point. An apparent induction in *OSM* transcript levels occurs in leaves after treatment with 1 mM ethephon over a 96-h time period. The levels of osmotin mRNAs went up 12 h after treatment, back down at 24 h, and then up again after 48 h of treatment before tapering off at 96 h.

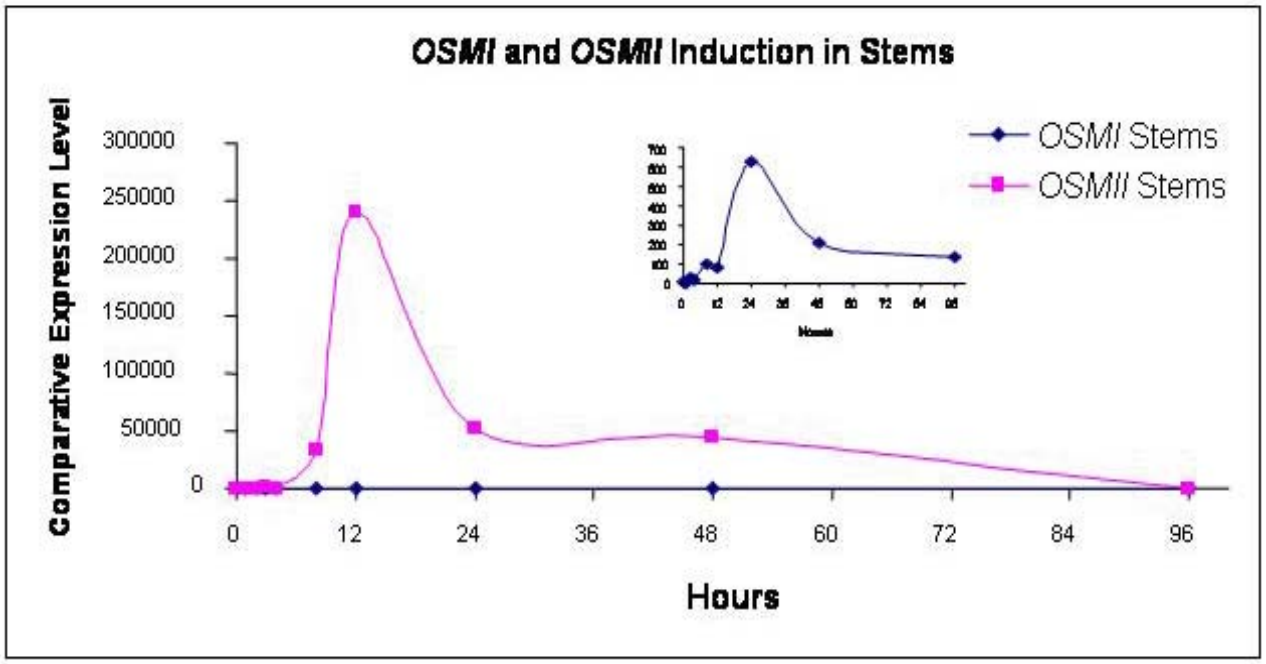

Figure 10. Scatter chart of the real time RT-PCR data presented in Tables 2 and 3 in order to demonstrate the average relative induction of the *OSMI* and *OSMII* mRNAs in stem tissues. The values on the Y-axis indicate the comparative expression levels of the *OSMI* and *OSMII* transcripts between the ethephon-treated sample and the control sample for that particular time point. An apparent induction in *OSM* transcript levels occurs in stems after treatment with 1 mM ethephon over a 96-h time period. The abundance of *OSMII* mRNA was considerably higher than the *OSMI* mRNA. Therefore, an inset graph of the *OSMI* profile is also shown. While the accumulation of *OSMII* mRNA peaked at 12 h after treatment and then tapered off by 96 h, the accumulation of the *OSMI* mRNA peaked later at 24 h post-treatment and then tapered off.

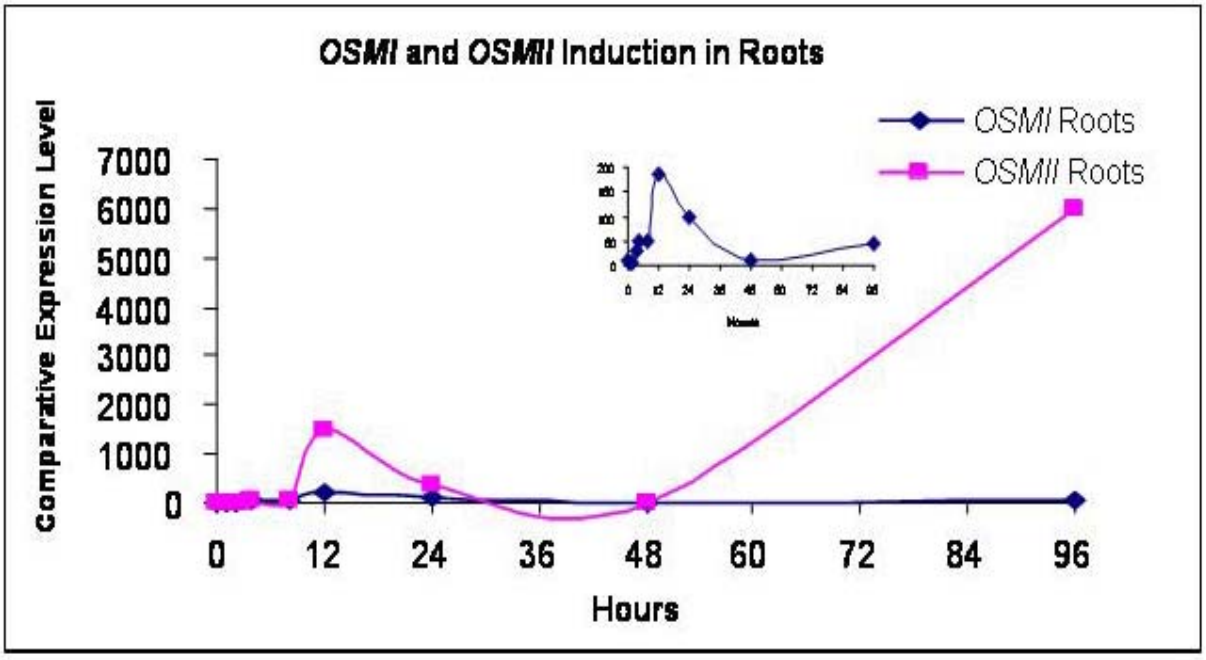

Figure 11. Scatter chart of the real time RT-PCR data presented in Tables 2 and 3 in order to demonstrate the average relative induction of the *OSMI* and *OSMII* mRNAs in root tissues. The values on the Y-axis indicate the comparative expression levels of the *OSMI* and *OSMII* transcripts between the ethephon-treated sample and the control sample for that particular time point. An apparent induction in the *OSM* transcript levels occurs in roots after treatment with 1 mM ethephon over a 96-h time period. The abundance of the *OSMII* mRNA was considerably higher at 96 h post-treatment than the *OSMI* mRNA. Therefore, an inset graph of the *OSMI* profile is also shown. The pattern of accumulation for both the *OSMI* and *OSMII* mRNAs were similar, because they peaked at 12 h after treatment, dropped back down, and then went back up 96 h after ethephon treatment.

bound secondary antibodies were then visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and exposure to Kodak X-OMAT® film.

The autoradiogram of the Western blot analysis of total protein extracts from cotton plants treated with water, 100 mM hydrogen peroxide, 550 mM NaCl, or 1 mM ethephon for a period of 24 h indicated the presence of a 24-KDa protein band in all sample lanes except the water control lane (Figure 12). The presumed mature 24-KDa osmotin isoforms were the most intense after treatment with 1 mM ethephon, followed by 100 mM hydrogen peroxide, and the least intense after treatment with 550 mM NaCl.

Because the induction of the putative osmotin protein(s) seemed to be the greatest after treatment with ethephon, a time course was done comparing protein extracts from cotton plants that had been treated with water (as control) or 1 mM ethephon. Total protein was isolated from three to four week-old cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5) that had been treated with water or 1 mM ethephon and placed in separate one gallon plastic bags for periods from 0 to 96 h. The whole plant tissues were collected every 4 h from 0 to 24 h, with additional collections made at 48 and 96 h. After determining the protein concentrations, the proteins were resolved on two separate discontinuous buffer systems (Laemmli, 1970), with Full Range Rainbow™ recombinant protein molecular weight markers (Amersham™). Following electrophoresis, the proteins were transferred to nitrocellulose membrane filters for Western blot analyses using a 1:1,000 dilution of the anti-osmotin antibody. A 1:1,500 dilution of the bound secondary antibody was used to detect the osmotin proteins with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and autoradiography.

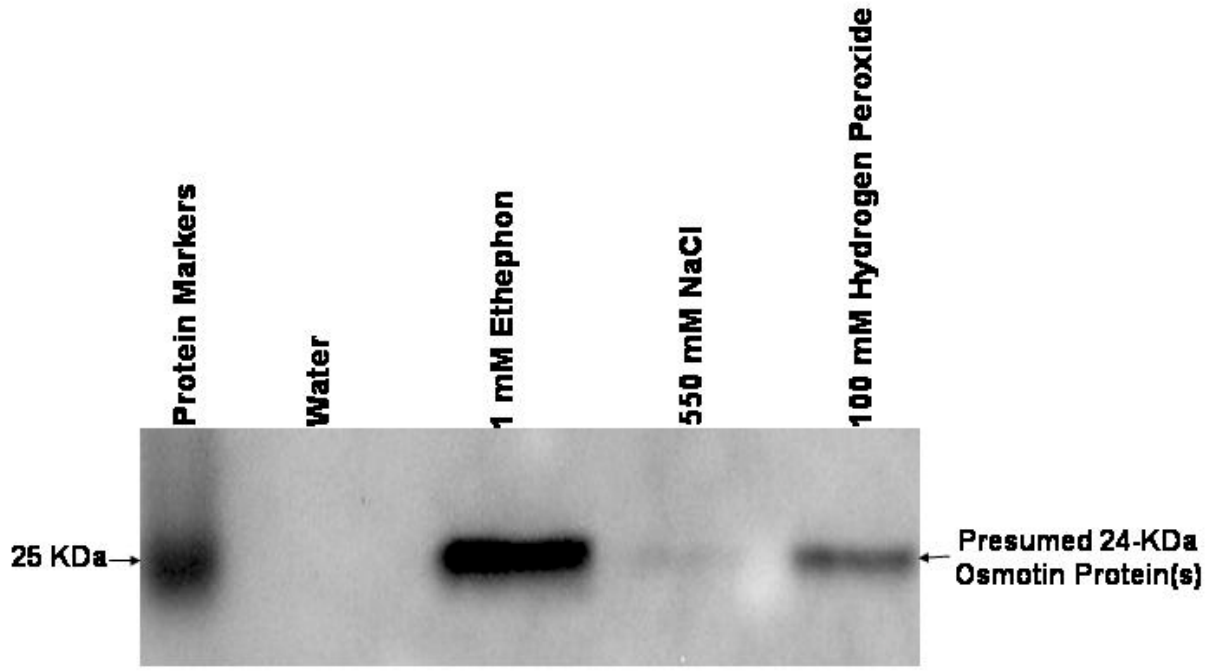

Figure 12. Western blot of total proteins isolated from cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5) treated with water (as control), 100 mM hydrogen peroxide, 550 mM NaCl, or 1 mM ethephon for a period of 24 h. After determining the concentration of each extract, the proteins were electrophoresed in a discontinuous buffer system, consisting of a stacking gel and a separating gel (Laemmli, 1970), with Full Range Rainbow™ recombinant protein molecular weight markers (Amersham™). The proteins embedded in the gels were transferred to a nitrocellulose membrane for Western blot analysis with a 1:1,000 dilution of a polyclonal anti-osmotin antibody preparation. A 1:1,500 dilution of the secondary antibody was then visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and exposure to Kodak X-OMAT® film for 2 min. A 24-KDa band was visualized in all sample lanes except the control. The bands corresponding to the presumed 24-KDa osmotin protein(s) was (were) from the most abundant to the least abundant: after treatment with 1 mM ethephon; after treatment with 100 mM hydrogen peroxide; and after treatment with 550 mM NaCl.

After a 45 sec autoradiography, the Western blot analyses indicated the presence of a 24-KDa protein band in the ethephon-treated extracts, but this band was not observed in the control extracts (Figure 13). The presumed 24-KDa osmotin protein(s) seemed to be induced 4 h after treatment with ethephon, and the band intensities dropped off 96 h after treatment. There were also two smaller protein bands, estimated to contain proteins 15 and 20 KDa in size, in the total protein extracts from both the control and ethephon-treated plants. These two bands are thought to be two predicted polypeptides of 14.0 and 19.5 KDa that would result from internal translation start codons in both the *OSMI* and *OSMII* genes (Wilkinson et al., 2005).

Purification of Anti-Osmotin Antibody

Due to nonspecific binding seen in the previous Western blots, the polyclonal anti-osmotin antibody preparation was purified using the Melon™ Gel IgG Spin Purification Kit (Pierce). To determine the best dilutions of primary and secondary antibodies to use for the Western blot analyses, six sets of protein extracts (5 µg each) isolated from three week-old cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) that had been treated with water (as control) or 1 mM ethephon for a period of 24 h were electrophoresed on a discontinuous buffer system (Laemmli, 1970), with 10 µl of SeeBlue® Plus2 Pre-Stained Standards (Invitrogen™). Following electrophoresis, the proteins were transferred to nitrocellulose membranes and used in Western blot analyses using different dilutions of the purified primary antibody (anti-osmotin antibody cleaned using the Pierce Melon™ Gel IgG Spin Purification Kit) and secondary antibody (Anti-rabbit IgG, peroxidase-linked species-specific whole antibody from donkey;

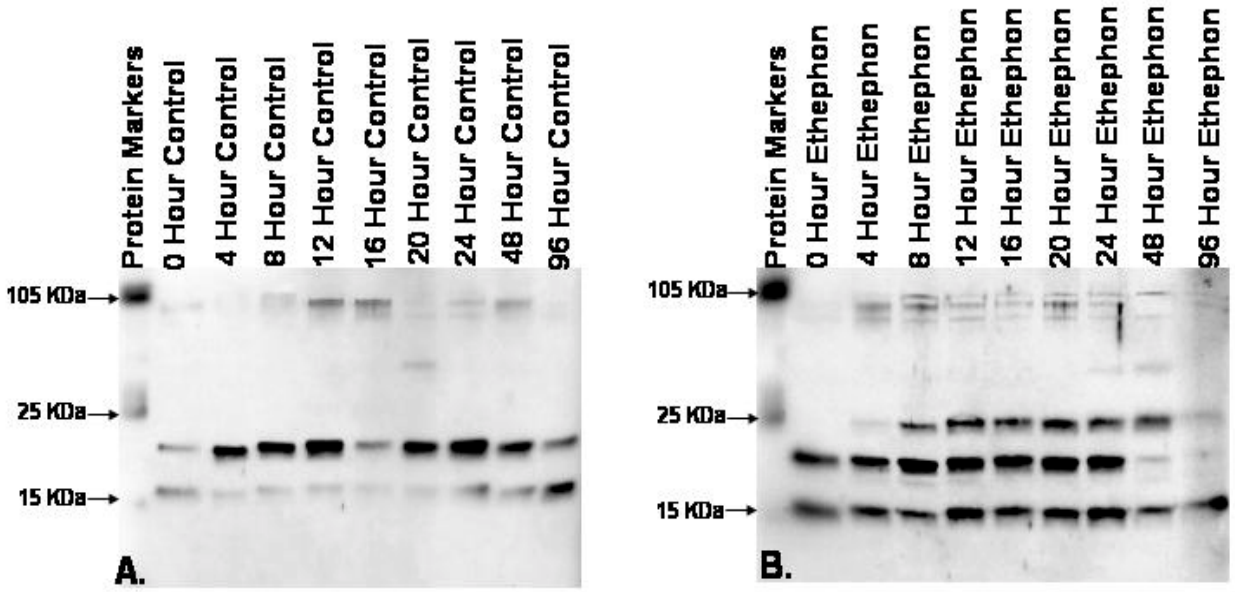

Figure 13. Western blot analyses of protein extracts from cotton plants (*Gossypium hirsutum* L., cv. Acala SJ5) treated with water or 1 mM ethephon for a period of 0 to 96 h. Whole plant tissues were collected every 4 h from 0 to 24 h, with additional collections made at 48 and 96 h. After determining the concentrations of the extracts, the proteins were separated on two separate discontinuous buffer systems, consisting of a stacking gel and a separating gel (Laemmli, 1970), along with Full Range Rainbow™ recombinant protein molecular weight markers (Amersham™). The proteins embedded in the gels were transferred to nitrocellulose membranes for Western blot analyses using a 1:1,000 dilution of an anti-osmotin antibody. A 1:1,500 dilution of the secondary antibody was then used to visualize the protein bands with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and exposure to Kodak X-OMAT® film for 45 sec. A band corresponding to the mature 24-KDa osmotin proteins was not visualized in the control extracts (A), but in the ethephon-treated extracts (B). There were also two smaller bands containing proteins, estimated to be 15 and 20 KDa in size, detected in the total protein extracts from both the control and ethephon-treated plants.

Amersham Pharmacia Biotech). The first set of protein extracts was incubated with a 1:1,000 dilution of primary antibody and 1:2,500 dilution of secondary antibody; the second set of protein extracts was incubated with a 1:1,000 dilution of primary antibody and 1:5,000 dilution of secondary antibody; the third set of protein extracts was incubated with a 1:100 dilution of primary antibody and 1:5,000 dilution of secondary antibody; the fourth set of protein extracts was incubated with a 1:2,500 dilution of primary antibody and 1:5,000 dilution of secondary antibody, and the fifth set of protein extracts was incubated with a 1:2,500 dilution of primary antibody and 1:10,000 dilution of secondary antibody. It was determined that a 1:100 dilution of purified primary antibody and a 1:5,000 dilution of secondary antibody provided the best result, providing an intense 24-KDa protein band only in the ethephon-treated sample and two larger nonspecific protein bands in both the control and treated samples (Figure 14).

Induction and Isolation of Protein from Cotton Plants for Two-Dimensional SDS-PAGE and Western Blot Analyses

As mentioned above, the polyclonal antibody preparation used in the previous Western blot analyses was generated using an antigenic oligopeptide corresponding to the last 18 amino acids of the C-terminal end of the OSMI polypeptide (Wilkinson, 2003; Wilkinson et al., 2005). Because the putative mature isoforms of the osmotin protein are practically the same size (23,885 KDa for OSMI and 23,984 KDa for OSMII) with essentially identical C-terminal regions (with only two amino acid variations), the presence of both osmotin isoforms can be only presumed from Western blot analyses of one dimensional (1-D) SDS-PAGE. To determine that both osmotin proteins are indeed

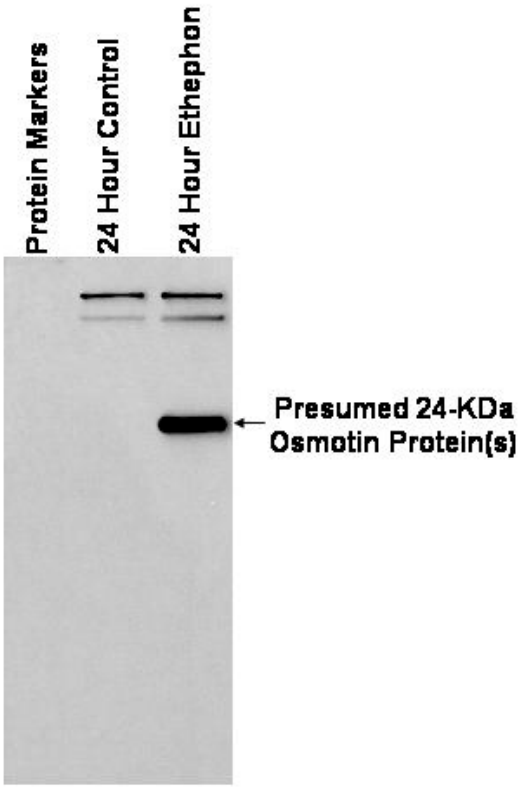

Figure 14. Western blot analysis of protein extracts from cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) treated with water or 1 mM ethephon for a period of 24 h. Different dilutions of the purified primary antibody (anti-osmotin antibody cleaned using the Pierce Melon™ Gel IgG Spin Purification Kit) and secondary antibody (Anti-rabbit IgG, peroxidase-linked species-specific whole antibody from donkey; Amersham Pharmacia Biotech) were used to optimize the best dilution of antibody. A 1:100 dilution of purified primary antibody and a 1:5,000 dilution of secondary antibody provided the best signal, revealing a band corresponding to the mature 24-KDa osmotin isoforms only in the ethephon-treated sample and two larger bands containing proteins of unknown function in both the control and treated samples.

being induced, two-dimensional (2-D) gel electrophoresis was used to resolve the proteins on the basis of charge by their isoelectric points (7.92 for OSMI and 7.56 for OSMII) in the first dimension and then by their size on SDS-PAGE in the second dimension.

Sample preparation is a major factor of successful 2-D gel electrophoresis of proteins. Because the modified protocol of Chlan and Bourgeois (2001) previously used to extract protein from cotton plants did not yield protein of high enough quality to be used for 2-D gel electrophoresis, two other protocols were used to try to extract total protein from the cotton plants that was free of any contaminating materials.

One protocol that was used involved the modified extraction procedure of Ferguson et al. (1996). With this procedure, total protein was isolated from whole cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) treated with water (as control) or 1 mM ethephon for a period of 24 h. After the concentrations of the protein extracts were determined using the *RC DC* Protein Assay (Bio-Rad), 62.5 µg or 250 µg of each were further purified using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad). The cleaned protein samples were focused (or separated by their isoelectric points) on various ReadyStrip™ IPG Strips (pH 3-10, pH 5-8, and pH 7-10). There were 22 different IPG strips for (three control extracts, pH 3-10; three ethephon-treated extracts, pH 3-10; four control extracts, pH 5-8; four ethephon-treated extracts, pH 5-8; four control extracts, pH 7-10; and four ethephon-treated extracts, pH 7-10). Once separated by their isoelectric points, the protein extracts were then resolved by size on 8-16% Criterion Tris-HCl gels (Bio-Rad), with 10 µl of protein standards.

Sixteen of the gels (eight control and eight treated) containing the separated proteins were stained with SYPRO® Ruby protein gel stain (Bio-Rad) and analyzed using The Discovery Series™ PDQuest™ 2-D Analysis Software (Bio-Rad). The proteins on the other six gels (three control and three treated) were transferred to Sequi-Blot PVDF membranes (for the pH 3-10 gels; Bio-Rad) or nitrocellulose membranes (for the pH 5-8 and 7-10 gels) for Western blot analyses to help localize the presumptive osmotin proteins with the purified anti-osmotin antibody.

After analyzing the Western blots of the pH 3-10 gels, a row of protein spots at approximately 24 KDa was detected on the blot of the ethephon-treated sample, but these 24-KDa protein spots were absent from the control blot (Figure 15). A 14-KDa protein spot and a 19-KDa protein spot were detected from both the control and ethephon-treated samples, which again, are thought to be two predicted polypeptides of 14.0 and 19.5 KDa that would result from initiation at internal AUG translation start codons in both the *OSMI* and *OSMII* genes (Wilkinson et al., 2005). Also seen on both of the pH 3-10 control and ethephon blots were a 40-KDa protein spot and a row of protein spots at 80 KDa. After analyzing the Western blots of the pH 5-8 and pH 7-10 gels, a row of protein spots at approximately 24 KDa was again observed on the blots of the ethephon-treated samples, but not detected on the control blots (Figures 16 and 17). The 14-KDa, 19-KDa, 40-KDa, and 80-KDa protein spots were again seen on both the control and ethephon blots.

After completing the analysis of the gels and Western blots of the proteins separated on pH 3-10, pH 5-8, and pH 7-10 gels, 15 protein spots of interest (13 from pH 3-10 gels and two from pH 5-8 gels) were extracted from the gels using The

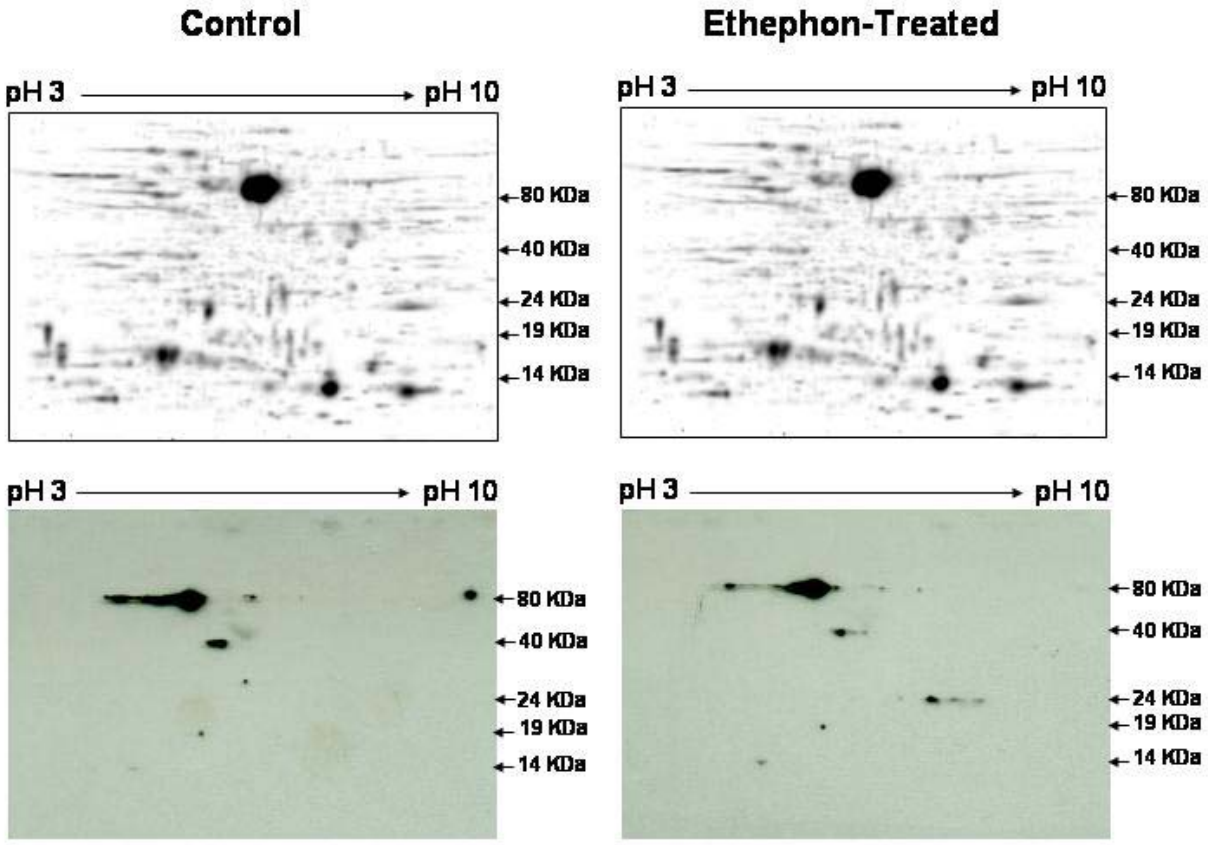

Figure 15. Two-dimensional (2-D) separation of protein extracts from cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) treated with water or 1 mM ethephon for a period of 24 h. After the concentration of each protein extract was determined using the Bio-Rad *RC DC* Protein Assay, 250 µg of each were further purified using the Bio-Rad ReadyPrep™ 2-D Cleanup Kit, removing ionic detergents, salts, and phenolic compounds that are known to interfere with isoelectric focusing (IEF). The cleaned protein samples were first separated by their isoelectric points on pH 3-10 ReadyStrip™ IPG Strips and then separated by size on 8-16% Criterion Tris-HCl gels, with 10 µl of Precision Plus Protein Dual Color Standards. The separated proteins were either stained with SYPRO® Ruby protein gel stain (Bio-Rad) (top) or transferred to Sequi-Blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad) and analyzed by Western blot analyses using the purified anti-osmotin antibody to help localize the presumptive osmotin proteins (bottom). A row of protein spots at approximately 24 KDa was detected on the blot of the ethephon-treated sample, but these 24-KDa protein spots were absent from the control blot. A 14-KDa protein spot and a 19-KDa protein spot were detected in both the control and ethephon-treated samples. Also seen on both of the pH 3-10 control and ethephon Western blots were a row of nonspecific protein spots at 40 KDa and 80 KDa.

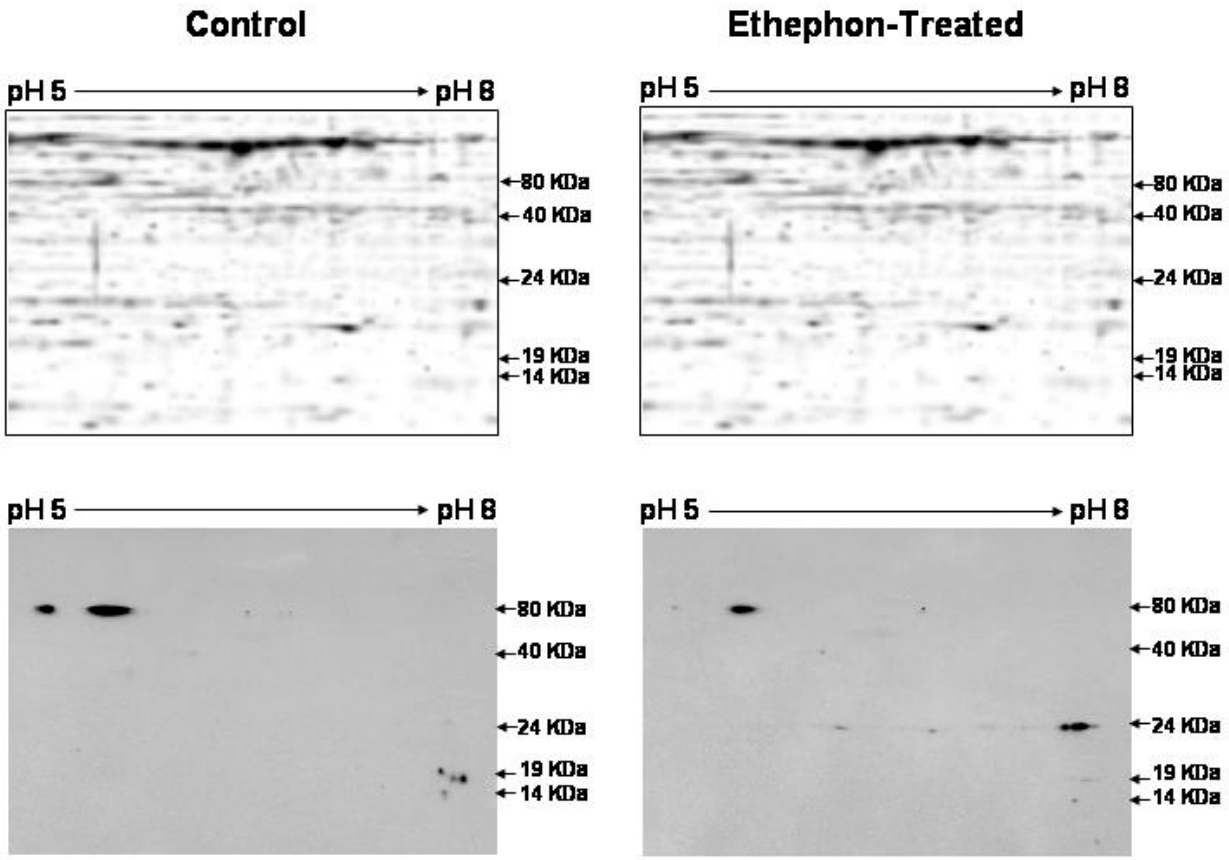

Figure 16. Two-dimensional (2-D) separation of protein extracts from cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) treated with water or 1 mM ethephon for a period of 24 h. After the concentration of each protein extract was determined using the *RC DC* Protein Assay, 62.5 µg of each was further purified using the ReadyPrep™ 2-D Cleanup Kit. The cleaned protein samples were first separated by charge by their isoelectric points on pH 5-8 ReadyStrip™ IPG Strips, and then separated by size on 8-16% Criterion Tris-HCl gels, with 10 µl of SeeBlue® Plus2 Pre-Stained Standards. The resolved proteins were either stained with SYPRO® Ruby protein gel stain (Bio-Rad) (top) or transferred to nitrocellulose membranes and analyzed by Western blot analyses with the purified anti-osmotin antibody to localize the presumptive osmotin protein spots (bottom). A row of protein spots at about 24 KDa was detected on the blot of the ethephon-treated sample, but these 24-KDa protein spots were lacking from the control blot. A 14-KDa protein spot and a 19-KDa protein spot were observed in both the control and ethephon-treated extracts. Also seen on both of the pH 5-8 control and ethephon blots were a row of nonspecific protein spots at about 40 KDa and 80 KDa.

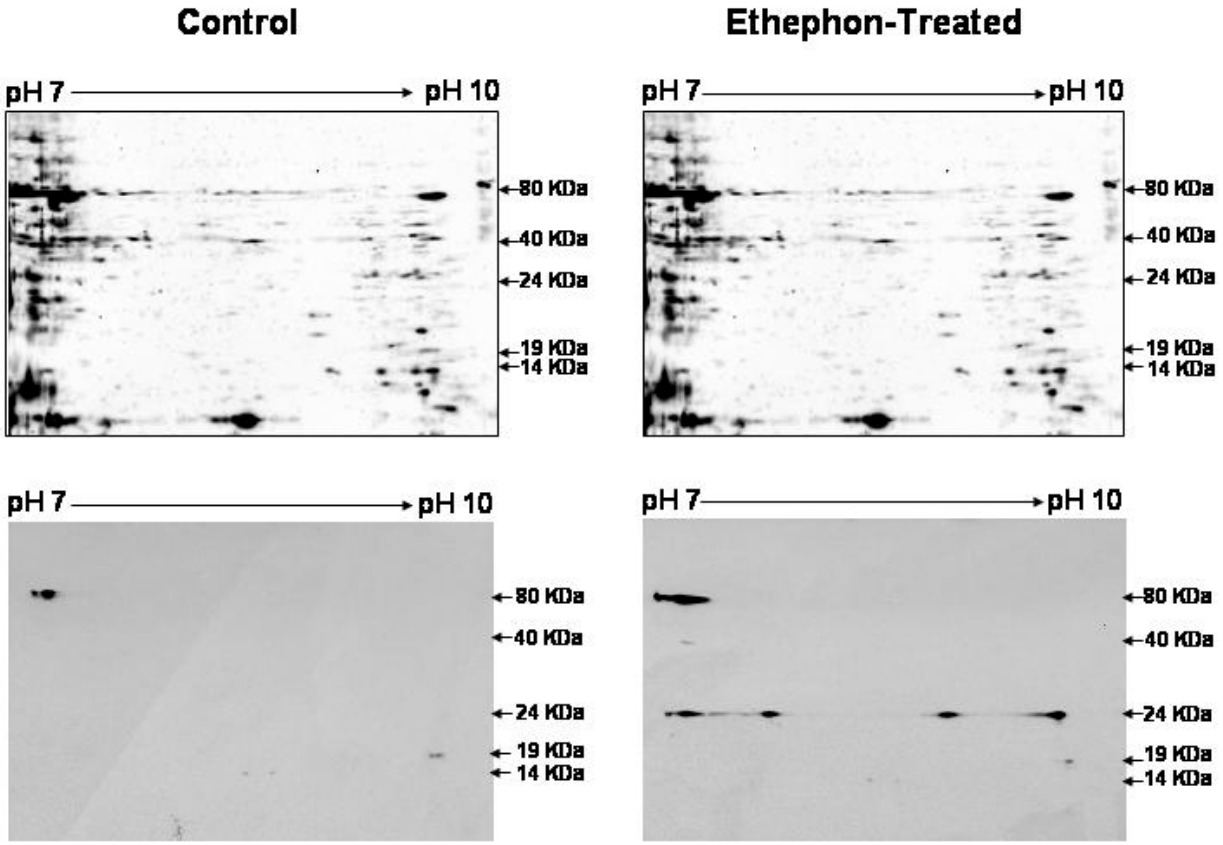

Figure 17. Two-dimensional (2-D) separation of protein extracts from cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) treated with water or 1 mM ethephon for a period of 24 h. After the concentration of each protein extract was determined using the *RC DC* Protein Assay, 62.5 µg of each was further purified using the ReadyPrep™ 2-D Cleanup Kit. The cleaned protein samples were first separated by their isoelectric points on pH 7-10 ReadyStrip™ IPG Strips, and then separated by size on 8-16% Criterion Tris-HCl gels, with 10 µl of SeeBlue® Plus2 Pre-Stained Standards. The separated proteins were either stained with SYPRO® Ruby protein gel stain (Bio-Rad) (top) or transferred to nitrocellulose membranes and analyzed by Western blot analyses with the purified anti-osmotin antibody to localize the presumptive osmotin protein spots (bottom). A row of protein spots at approximately 24 KDa was seen on the blot of the ethephon-treated sample, but these 24-KDa protein spots were lacking from the control blot. A 14-KDa protein spot and a 19-KDa protein spot were detected in both the control and ethephon-treated samples. Also seen on both of the pH 7-10 control and ethephon blots were a row of nonspecific protein spots at about 40 KDa and 80 KDa.

EXQuest Spot Cutter (Bio-Rad). Twelve of the protein spots cut from the pH 3-10 gels corresponded to proteins of approximately 24 KDa; these protein spots were either present only in the ethephon-treated sample, or they were more intense after ethephon treatment. The other protein spot excised from the pH 3-10 gels was the 40-KDa protein spot present in both the control and treated extracts. The two protein spots cut from the pH 5-8 gels were among the row of 80-KDa protein spots observed in both the control and ethephon-treated samples (Figure 18). These 15 selected protein spots were sent to Dr. Bill Russell of the Chemistry Department Laboratory for Biological Mass Spectrometry at Texas A&M University, College Station, TX, for sequence analysis by mass spectrometry. Unfortunately, there were no strong identifications made from any of the protein samples analyzed.

The other extraction procedure used to isolate high-quality protein from cotton plants involved the Plant Total Protein Extraction Kit from Sigma. Using this kit, total protein was extracted from the leaf, stem, and root tissues of two week-old cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) treated with water (as control) or 1 mM ethephon for a period of 24 h. Once the protein concentrations had been determined for the control and ethephon-treated samples, 20 µg of each extract and 10 µl of SeeBlue® Plus2 Pre-Stained Standards were electrophoresed in duplicate on two denaturing SDS-PAGE gels at 130 V. One gel was stained with Bio-Safe™ Coomassie Stain, and the other was transferred to a nitrocellulose membrane for Western blot analysis. After only a 30 sec exposure, an intense 24-KDa protein band appeared in all of the ethephon-treated extract lanes (for leaf, stem, and root tissues), but not in the control sample lanes (Figure 19).

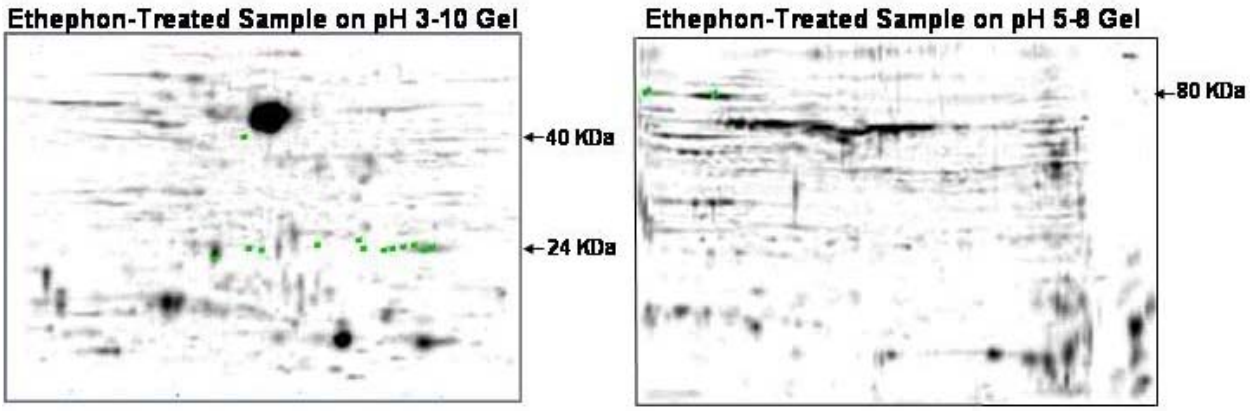

Figure 18. Proteins selected for mass spectrophotometric sequence analysis. After analyzing the 2-D gels and Western blots of the proteins resolved on pH 3-10, pH 5-8, and pH 7-10 2-D gels, 15 protein spots (13 from pH 3-10 gels and 2 from pH 5-8 gels; shown in green) were chosen for sequence analysis. These proteins were excised from the gels using The EXQuest Spot Cutter. Twelve of the protein spots cut from the pH 3-10 gels were approximately 24 KDa; these proteins were either present only in the ethephon-treated sample, or they were induced after ethephon treatment. The other protein spot excised from the pH 3-10 gels was the 40-KDa protein spot present in both the control and treated extracts. The two protein spots cut from the pH 5-8 gels were among the row of 80-KDa protein spots observed in both the control and ethephon-treated samples.

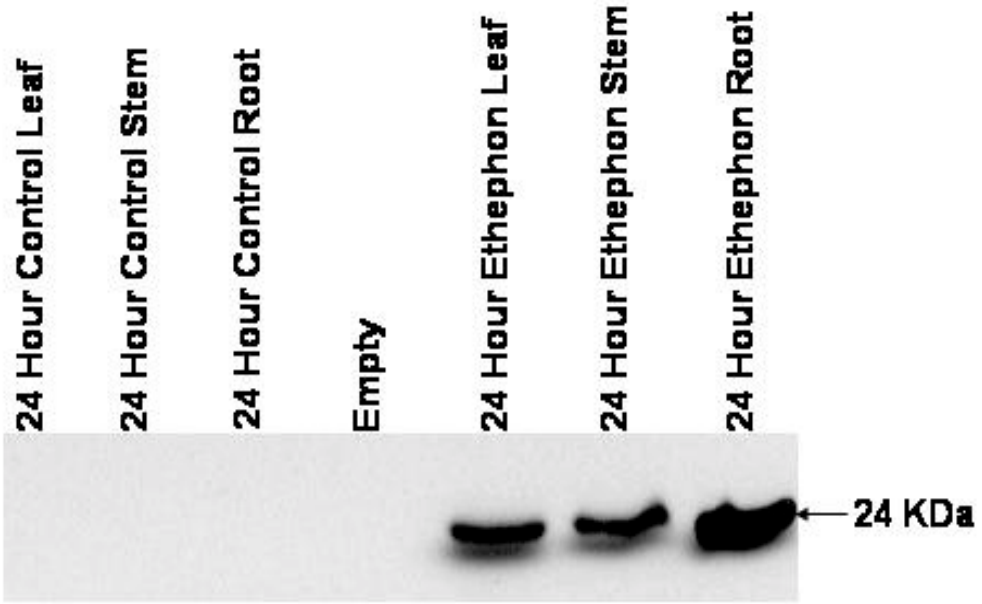

Figure 19. Western blot analysis of proteins extracted from the leaves, stems, and roots of two week-old cotton plants (*Gossypium hirsutum* L., cv. Acala Maxxa) treated with water or 1 mM ethephon for a period of 24 h. The plant proteins were extracted with the Plant Total Protein Extraction Kit (Sigma). Once the protein concentrations had been determined for the control and ethephon-treated samples, 20 µg of each were electrophoresed on a denaturing SDS-PAGE gel and transferred to a nitrocellulose membrane for Western blot analysis. After a 30 sec exposure to film, an intense 24-KDa band corresponding to the osmotin isoform band was observed in all of the ethephon-treated extracts (leaf, stem, and root), but was not observed in the control samples.

Once the presence of the putative osmotin proteins was confirmed in the ethephon-treated extracts, 200 µg of each extract (from leaf, stem, and root tissues) were combined for a total of 600 µg, and cleaned using the ReadyPrep™ 2-D Cleanup Kit. The samples were then applied to pH 6.3-8.3 ReadyStrip™ IPG Strips and resolved by 2-D electrophoresis. The second dimension gels containing the separated proteins were either stained with Bio-Safe™ Coomassie Stain, or they were transferred to a nitrocellulose membrane for Western blot analysis. To align the blot with the stained gel, the nitrocellulose membrane was stained with MemCode™ Reversible Protein Stain Kit- For Nitrocellulose Membrane (Pierce) before the blocking step of the Western analysis.

After analyzing the Western blots of the pH 6.3-8.3 gels, a row of protein spots at approximately 24 KDa was observed on the blot of the ethephon-treated samples (Figure 20). Protein spots corresponding to proteins of 14 KDa, 19 KDa, 40 KDa, and 80 KDa were also seen on the Western blot. The gel stained with Bio-Safe™ Coomassie Stain was aligned with the Western blot, and seven 24-KDa protein spots were excised from the gel using a sterile scalpel blade and given to Mr. Prem Adhikari of Dr. Barney Venables' laboratory here at the University of North Texas for sequence analysis by mass spectrometry. Unfortunately, there were still no strong identifications made from any of the protein samples analyzed.

Transformation, Regeneration, and Screening of *Arabidopsis* Plants

The binary vector constructs designated pCAMBIA-35S-*OSMI* #16-M, pCAMBIA-35S-*OSMII* #11-A, and pCAMBIA 2301-H (empty vector) harbored in *Agrobacterium*

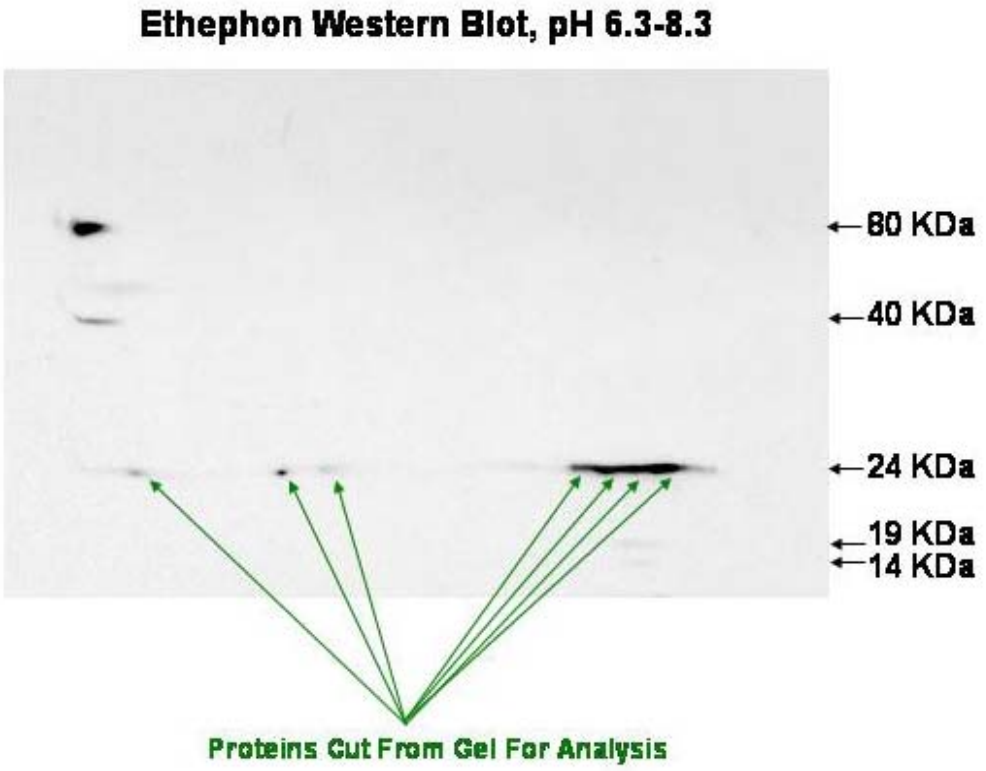

Figure 20. Western blot analysis of protein extract from cotton plant (*Gossypium hirsutum* L., cv. Acala Maxxa) treated with 1 mM ethephon for a period of 24 h and resolved by 2-D gel electrophoresis. After the concentration of the protein extract was determined using the Bio-Rad *RC DC* Protein Assay, 600 µg was further purified using the Bio-Rad ReadyPrep™ 2-D Cleanup Kit. The cleaned protein sample was first separated according to the protein isoelectric points on ReadyStrip™ IPG Strips, pH 6.3-8.3, and then separated by size on 10.5-14% Criterion Tris-HCl gels, with SeeBlue® Plus2 Pre-Stained Standards. The resolved proteins were transferred to a nitrocellulose membrane and analyzed by Western blot analysis using the purified anti-osmotin antibody to localize the presumptive osmotin proteins. After aligning the blot with the corresponding gel (stained with Bio-Rad Bio-Safe™ Coomassie Stain), a row of 24-KDa protein spots (marked with green arrows) was excised from the 2-D gel for sequence analysis.

tumefaciens were made by Dr. Jeffery R. Wilkinson of our laboratory (diagram shown in Figure 2; Wilkinson, 2003). The pCAMBIA-35S-*OSMI* #16-M and pCAMBIA-35S-*OSMII* #11-A constructs contained the 787-bp *OSMI* coding region with an N-terminal signal targeting domain (Neuhaus et al., 1991) and the 728-bp *OSMII* coding region with an N-terminal targeting domain (Neuhaus et al., 1991), respectively. For this dissertation work, these constructs were used to transform *Arabidopsis thaliana* (ecotype Columbia) plants using the floral dip method of Clough and Bent (1998).

Once the dipped *Arabidopsis* T_0 plants were grown to maturity and allowed to dry, the *Arabidopsis* T_1 seeds were harvested and subsequently screened on kanamycin selection plates. Wild type *Arabidopsis thaliana* (ecotype Columbia) seeds were also plated on kanamycin selection plates to serve as a negative control, and kanamycin-resistant seeds were plated on kanamycin selection plates to serve as a positive control. Approximately one month after plating, the seedlings that contained the *nptII* (kanamycin resistance) gene from the pCAMBIA 2301 vector produced green secondary leaves and established good root systems, whereas the other plantlets without the vector constructs were yellow and dying (Figure 21). Once the *Arabidopsis* T_1 transformants were selected on the kanamycin plates, they were transplanted to soil and grown in a 22˚C growth room.

In another analysis, the plants that grew on the kanamycin plates were also subjected to PCR analyses using the REDExtract-N-Amp™ Plant PCR Kit (Sigma) and an amplimer pair designed specifically for a 376-bp region of the *GUS* second exon within the pCAMBIA 2301 vector sequence. A unique amplimer pair for a 265-bp segment of the constitutively expressed actin 8 gene (An et al., 1996) was also used as

Figure 21. Putative transgenic *Arabidopsis thaliana* (ecotype Columbia) plants on a kanamycin selection plate. The seeds harvested from each generation of prospective transgenic *Arabidopsis* plants were screened on kanamycin selection plates. Approximately one month after plating, the seedlings that appeared to contain the *neomycin phosphotransferase* II (*npt*II, kanamycin resistance) gene from the pCAMBIA 2301 vector produced green secondary leaves and established good root systems, whereas the other plantlets without the vector constructs were yellow and dying.

a positive control. The PCR products were electrophoresed on 2.0% agarose gels (example shown in Figure 22). There were 18 supposed *Arabidopsis* T_1 plants transformed with the empty vector construct (pCAMBIA 2301-H) that grew on kanamycin selection plates, and their genomic DNAs generated a 376-bp pCAMBIA 2301 PCR product. There were 11 *Arabidopsis* T_1 plants supposedly transformed with the pCAMBIA-35S-*OSMI* #16-M construct and 22 *Arabidopsis* T_1 plants putatively transformed with the pCAMBIA-35S-*OSMII* #11-A construct. The plants also grew on kanamycin selection plates, and their genomic DNAs generated a 376-bp pCAMBIA PCR product.

The *Arabidopsis* T_2 seeds were harvested from these *Arabidopsis* T_1 plants and subjected to kanamycin selection and PCR analysis confirmation as above. There were 12 *Arabidopsis* T_2 plants thought to be transformed with the empty vector construct (pCAMBIA 2301-H), 11 *Arabidopsis* T_2 plants tentatively transformed with the pCAMBIA-35S-*OSMI* #16-M construct, and 22 *Arabidopsis* T_2 plants supposedly transformed with the pCAMBIA-35S-*OSMII* #11-A construct that grew on kanamycin selection plates, and their genomic DNAs generated a 376-bp pCAMBIA PCR product.

The *Arabidopsis* T_2 plants that appeared to contain the osmotin construct cassette insert were then further analyzed by Western blotting using the anti-osmotin antibody preparation, in order to confirm osmotin expression. Total protein was extracted (Tripathy et al., 2003) from the leaves of a wild type *Arabidopsis thaliana* (ecotype Columbia) plant, and 44 of the *Arabidopsis* T_2 plants that generated pCAMBIA PCR products. Ten micrograms of each protein sample were electrophoresed on a denaturing SDS-polyacrylamide gel. Protein previously isolated from an ethephon-

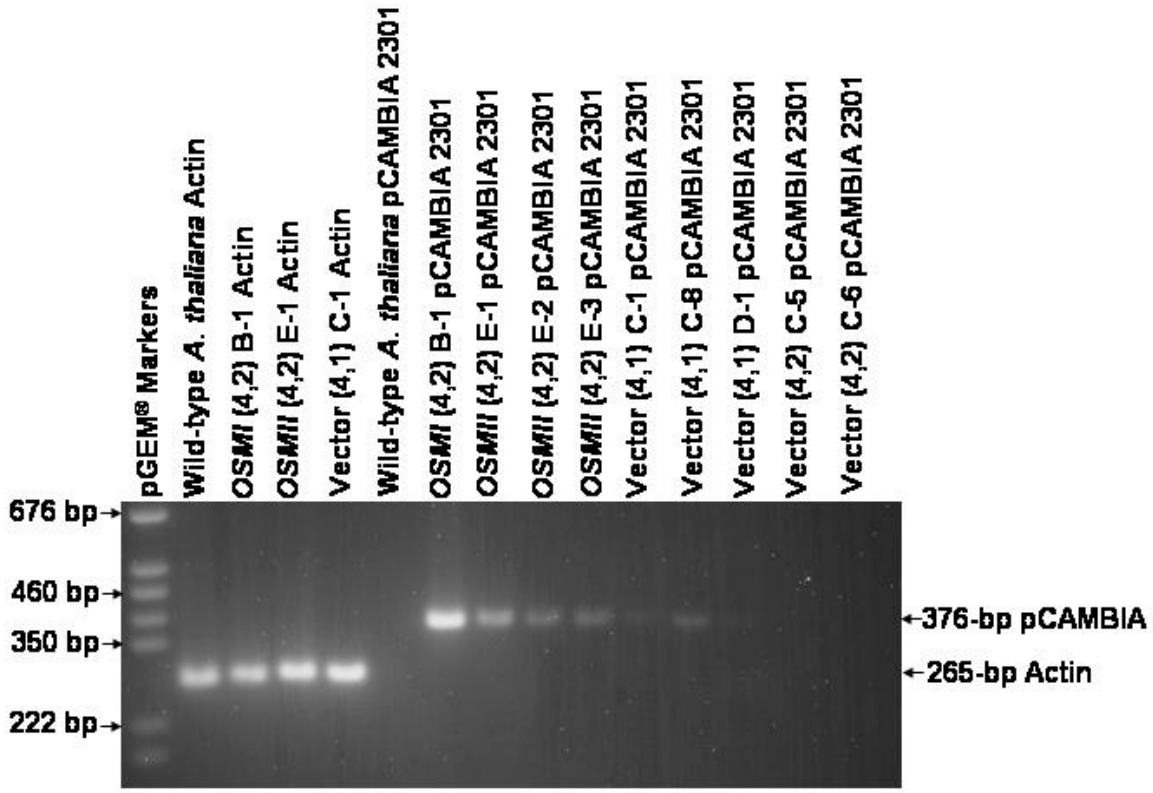

Figure 22. PCR analysis of putative transformed *Arabidopsis thaliana* plants (ecotype Columbia) grown on kanamycin selection media. DNA was extracted from the putative transformants and subjected to PCR analyses using the REDExtract-N-Amp™ Plant PCR Kit (Sigma) and an amplimer pair designed specifically for a 376-bp region of the *GUS* second exon within the pCAMBIA 2301 vector and an amplimer pair specific for a 265-bp segment of the constitutively expressed actin 8 gene (An et al., 1996) as a positive control. The PCR products were electrophoresed on 2.0% agarose gels containing ethidium bromide at a final concentration of 0.5 µg/ml, along with pGEM® DNA Marker standards (Promega). The actin PCR product was present for all *Arabidopsis* plants tested, including wild type plants. The product for the *GUS* second exon of the pCAMBIA 2301 vector can be seen in seven of the plants tested. As expected, no *GUS* second exon A product was generated from the wild type plant.

treated cotton plant was also run as a positive control. Following electrophoresis, the proteins were transferred to a nitrocellulose membrane for Western blot analysis using the anti-osmotin antibody. The antibody bound to the ethephon-treated cotton extract but not to the *Arabidopsis* protein extracts, except for two large, nonspecific bands that were seen in every lane (wild type plant and transformants) (data not shown). Once the Arabidopsis T_2 plants were grown to maturity and dried, the T_3 seeds were collected and stored in glass scintillation vials.

Transformation, Regeneration, and Screening of Cotton Plants

The vector constructs pCAMBIA-35S-*OSMI* #16-M, pCAMBIA-35S-*OSMII* #11-A, and pCAMBIA 2301-H harbored in *Agrobacterium tumefaciens* cells (diagram shown in Figure 2) were also used to transform cotton plants (*Gossypium hirsutum* L., cv. Coker 312) by modified methods of Sunilkumar and Rathore (2001) and Zhang et al. (2001).

Hypocotyl segments and cotyledon pieces were excised from seven day-old cv. Coker 312 seedlings (Figure 23A) and wounded using a sterile scalpel. The *Agrobacterium* cultures were applied to the wound sites of each explant, and co-cultivation was carried out for 72 h on modified G2 medium. The hypocotyl and cotyledon pieces were then transferred to P1-c4k50 medium containing 50 mg/l kanamycin and 400 mg/l carbenicillin for a period of 24 days to allow the induction and proliferation of callus tissue (Figure 23B). The kanamycin-resistant calli growing on the hypocotyl and cotyledon pieces (Figures 23C and 23D) represented an individual stably transformed callus line. These calli were excised, along with some of the plant tissue, using a sterile scalpel and transferred to modified G3 medium containing 50 mg/l

kanamycin and 400 mg/l carbenicillin. The calli were maintained on this medium for 12 weeks, with regular subculturing to fresh medium every four weeks, so that the embryonic calli could proliferate.

After the calli were subcultured on the modified G3 medium three times, the calli were classified as either nonembryogenic or embryogenic. Embryogenic calli were fast-growing, light yellow, and loose (Sunilkumar and Rathore, 2001; Zhang et al., 2001) (Figure 23E). Nonembryogenic calli were slow-growing, compact, and light brown or light/dark green. These calli eventually turned brown or black and died. The embryogenic calli were maintained on MSBOK medium containing 25 mg/l kanamycin, only for the first two rounds of culture, and 200 mg/l carbenicillin with monthly subculture until somatic embryos developed (Figure 23F). At this stage, all of the calli being maintained on the MSBOK plates with zeatin turned brown and were discarded.

Somatic embryos that were at least 5 mm in length (Figure 23G) were transferred to EG3 medium for germination. The somatic embryos were maintained on the EG3 medium until germination occurred (Figure 23H). After germination, plantlets that had 2-3 cm long shoots and true leaves were transferred to Magenta boxes or jars containing MS3 medium (Figure 23I). The plantlets were left on this media until their shoots were 5-7 cm long and they had a well developed root system. At this point, the plantlets were transferred to soil and kept under high humidity in plastic bags for two weeks (Figures 23J and 24). After the two-week period, the plants were gradually hardened for another two weeks by slowly opening the bags. After this, they were taken out of the bags and placed on a growth cart in the lab for three or four weeks (Figures 23K and 24). Once they had hardened, the plants were transferred to large pots and

grown to maturity either in a 28˚C growth room or in the greenhouse (Figure 23L). A total of 17 cotton plants were thought to be transformed with the empty vector construct (pCAMBIA 2301-H), 32 cotton plants were thought to be transformed with the pCAMBIA-35S-*OSMI* #16-M construct, and three cotton plants were thought to be transformed with the pCAMBIA-35S-*OSMII* #11-A construct.

Genomic DNAs were isolated from young leaves of a wild-type cotton plant (*Gossypium hirsutum* L., cv. Coker 312) and the supposed transgenic cotton plants by the method of Paterson et al. (1993). The quality of the DNAs was analyzed by electrophoresis on agarose gels, as shown in Figure 25. Several PCR reactions were performed with the genomic DNA extracts using an amplimer pair specifically designed for a 376-bp region of the *GUS* second exon within the pCAMBIA 2301 vector, the gene-specific primers for the constitutive *SadI* gene (Yang et al., 2005), and an amplimer pair (OSMCheck For and OSMCheck Rev) that was designed to generate an overlap fragment for the vector-insert junction between the 35S cassette promoter and the 5' end of the *OSMI* and *OSMII* genes. The pCAMBIA-35S-*OSMI* #16 plasmid DNA isolated from transformed *E. coli* DH5α cells was used as a positive control. The PCR reactions were carried out using SmartMix™ HM lyophilized beads (Cepheid), and the resulting PCR products were electrophoresed on agarose gels. All of the DNAs, except for the pCAMBIA-35S-*OSMI* #16 plasmid DNA, generated PCR products when using primers for the constitutive *SadI* gene. Except for the cv. Coker 312 wild type extract, all of the DNAs generated PCR products using the *GUS* second exon primers. However, the only PCR product produced with the OSMCheck amplimer pair was amplified from the positive control (the pCAMBIA-35S-*OSMI* #16 plasmid DNA). None

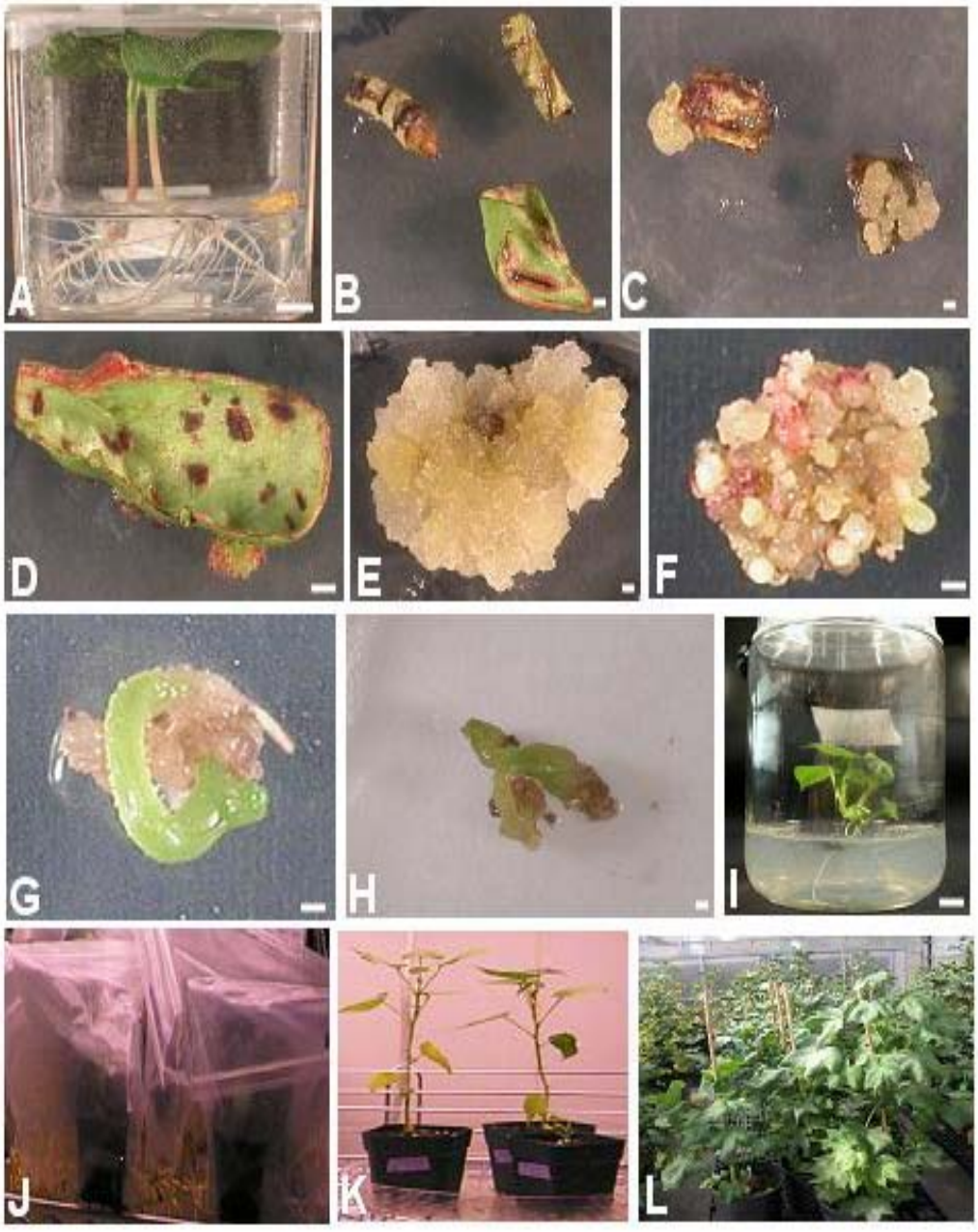
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Figure 23. Different stages of transformation and regeneration of cotton plants. A. Seven day-old cotton plant (*Gossypium hirsutum* L., cv. Coker 312) used for the transformation of plant tissues. B. Hypocotyl and cotyledon segments excised from cv. Coker 312 seedlings and wounded using a sterile scalpel on P1-c4k50 medium 72 h after co-cultivation with the *Agrobacterium tumefaciens* vector constructs. This medium provided the nutrients for the induction and proliferation of callus tissue. C. and D. Kanamycin-resistant calli growing on the hypocotyl (C) and cotyledon (D) segments, each representing an individual stable transformed callus line. These calli were excised, along with some of the plant tissue, and transferred to modified G3 medium for the proliferation of embryonic calli. E. Embryogenic callus, which was fast-growing, light yellow, and loose, on modified G3 medium. F. Somatic embryos developing from the callus on MSBOK medium. G. Somatic embryo developed from callus on MSBOK medium just prior to being transferred to EG3 medium. H. Somatic embryo after transfer to EG3 medium. I. Germinated embryo developed into a putative transgenic plantlet with true leaves and a good root system in a Magenta jar containing MS3 medium. J. Putative transgenic plants in soil being kept under high humidity in plastic bags for two weeks. After the two-week period, the plants were gradually hardened for another two weeks by slowly opening the bags. K. Supposed transgenic plants on a growth stand in the lab after being acclimated. The plants were kept in the laboratory for three or four weeks before being transferred to large pots. L. Putative transgenic plants in the greenhouse. The white bars are scaled for 1 mm.

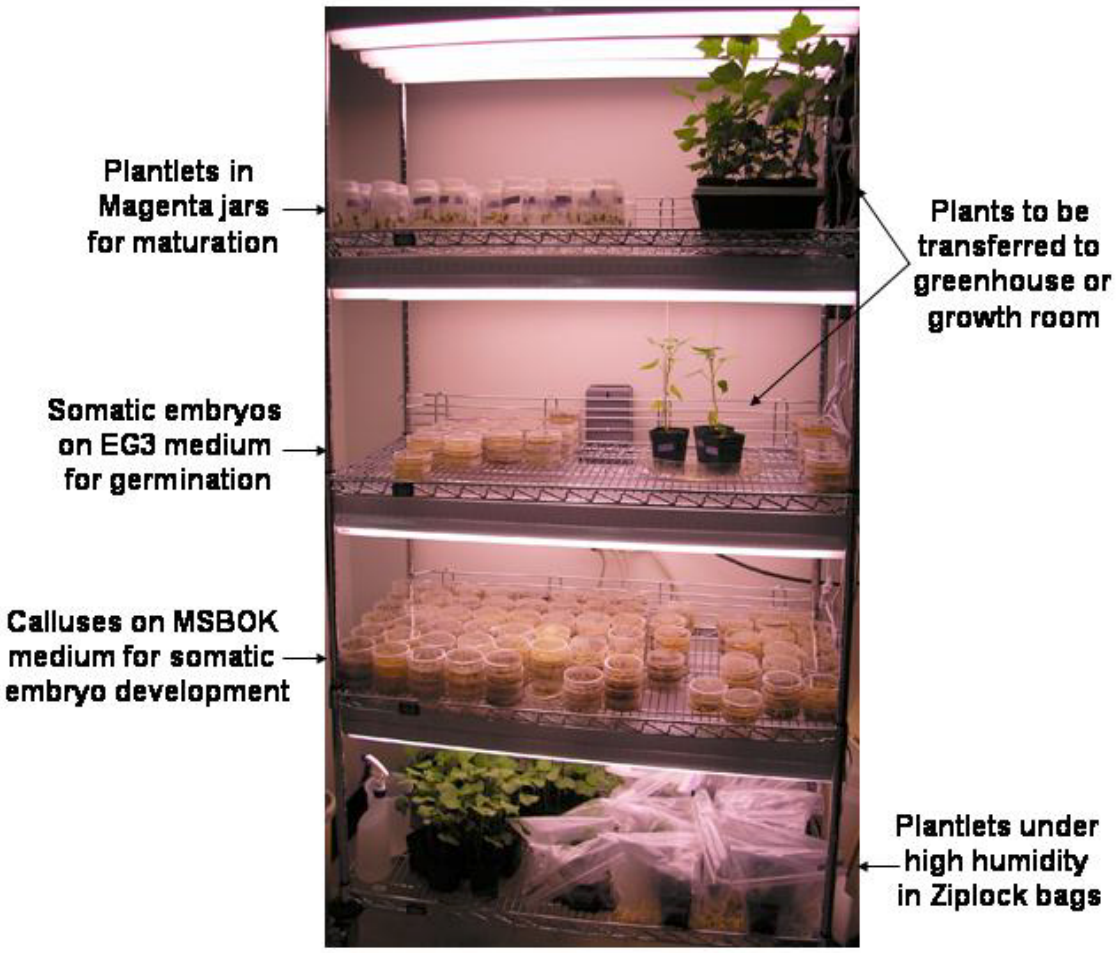


Figure 24. Growth stand in the laboratory in which calli and prospective transgenic plantlets coming out of culture were maintained.

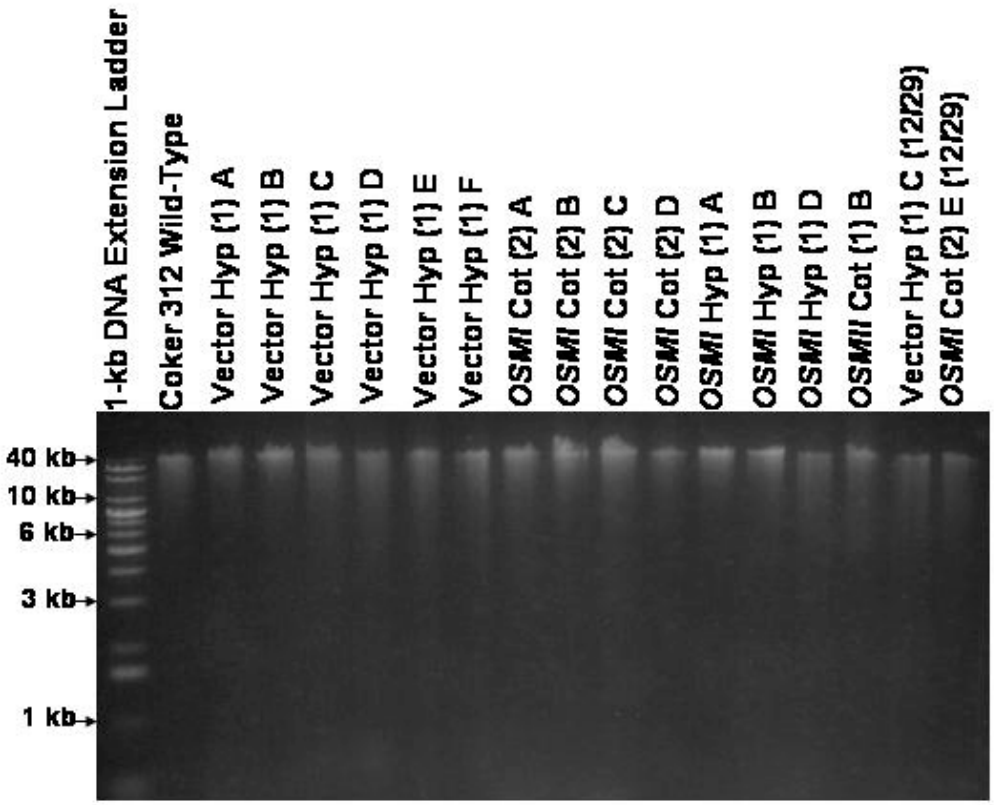


Figure 25. Genomic DNAs isolated from young leaves of a wild type cotton plant (*Gossypium hirsutum* L., cv. Coker 312) and the supposed transgenic cotton plants by the method of Paterson et al. (1993). After the concentration and purity of each DNA sample was assessed at $A_{260\ nm}$ and $A_{280\ nm}$, the quality of the DNAs was also confirmed by electrophoresing 200 ng of each sample on 0.8% agarose gels containing ethidium bromide at a final concentration of 0.5 µg/ml, with a 1-kb DNA Extension Ladder (Gibco BRL).

of the putative transgenic cotton plant DNAs generated the transgene PCR products (data not shown).

To verify the presence of the osmotin transgenes by PCR analyses using the OSMCheck For and OSMCheck Rev primers, leaf samples of the putative transgenic cotton plants were sent to BioDiagnostics, Inc. (http://www.biodiagnostics.net/; River Falls, WI), a company that routinely uses PCR to screen individual cotton seeds or plants for the presence of transgenes. One or two of the youngest leaves from 35 different cotton plants (one cv. Coker 312 wild type plant, one plant supposedly transformed with the empty vector, 31 putative transgenic plants with the *OSMI* gene, and two tentative transgenic plants with the *OSMII* gene) and purified pCAMBIA-35S-*OSMI* #16 plasmid DNA for use as a positive control, were sent to the company for PCR analyses. Unfortunately, all of the putative transformed cotton DNA samples tested negative for the OSMCheck PCR product, while the plasmid control DNA produced a very strong positive result. An internal control was run on all of the samples in order to confirm the quality of the DNA. Of the 35 samples that were sent, 24 generated a strong internal control band indicating quality DNA, while 11 samples did not. Based on these results, and the fact that part of the pCAMBIA vector could be amplified from the putative transgenic plant DNAs, it was suggested that there might be some degradation or recombination events occurring within the vector or in the transformed plants.

In an attempt to prove that there was a potential degradation or recombination problem, an amplimer pair designed to amplify the overlap region in the vector-insert junction between the 3' end of the osmotin gene (OSMCheck FOR3) and the CaMV polyA terminator region (OSMCheck REV3) was designed and used in PCR analyses of

the cotton DNA extracts. Unfortunately, these PCR assays turned out negative, while the plasmid control DNA generated an intense positive PCR product.

Using a different approach to verify that the constructs were present in the assumed transgenic cotton plants, kanamycin resistance assays were done to detect the expression of the *npt*II gene (as was done in Wu et al. (2005) and Zapata et al. (1999)) from the pCAMBIA 2301 vector cassette. The lowest concentration of kanamycin that would affect untransformed cv. Coker 312 plants was initially established by applying 0, 0.1, 1.0, 2.0, and 3.0% (w/v) kanamycin solutions to separate leaves of a non-transgenic control plant. Ten days after application, the leaves were cut from the plant and analyzed. It was found that 0% kanamycin solution had no effect, the 0.1% solution had a slight chlorotic effect where applied, the 1.0% and 2.0% solutions had a greater chlorotic effect, and the whole leaf was bleached with the 3.0% kanamycin solution (Figure 26).

It was then decided to apply 1.0 and 2.0% kanamycin solutions to the leaves of a cv. Coker 312 wild type plant and nine putative transgenic cotton plants. Ten days after application, the leaves were cut from the plants and analyzed. Kanamycin had the same chlorotic effects on the wild type plant as before, and all but two of the putative transgenics were unchanged, indicating the presence of the *npt*II gene. It should be noted that the two plants having the slight discolorations had several discolored leaves and appeared to be stressed from other non-experimental conditions. Examples of pictures from this experiment are shown in Figure 27.

Genomic blot analyses were done to verify that the putative transgenic cotton plants do indeed contain the osmotin gene inserts. Although PCR analyses should

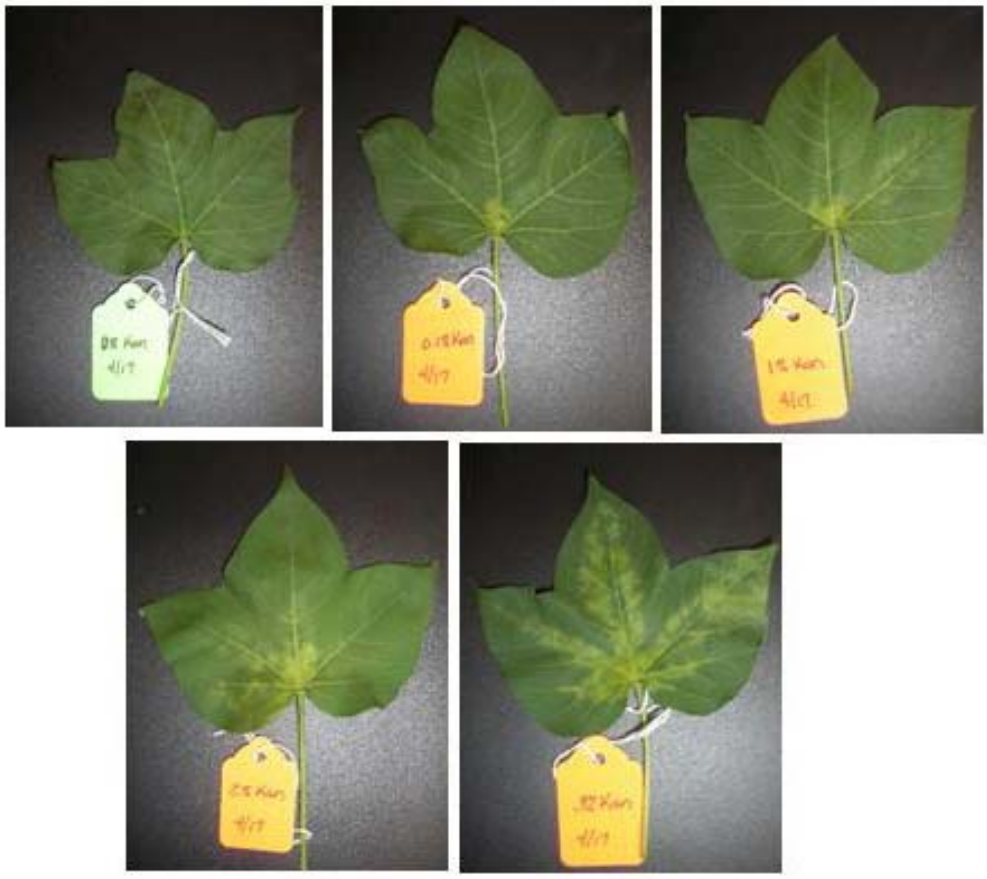


Figure 26. Kanamycin resistance assays performed on wild type cotton (Gossypium hirsutum L., cv. Coker 312) to determine the lowest concentration of kanamycin that would affect untransformed wild type cv. Coker 312 plants. Kanamycin solutions (0, 0.1, 1.0, 2.0, and 3.0% (w/v)) were applied to separate leaves of a wild type control plant. Ten days after application, the leaves were cut from the plant and analyzed. It was found that 0% kanamycin had no effect, the 0.1% solution had a slight chlorotic effect where applied, the 1.0% and 2.0% solutions had a greater chlorotic effect, and the whole leaf was bleached with the 3.0% kanamycin solution.

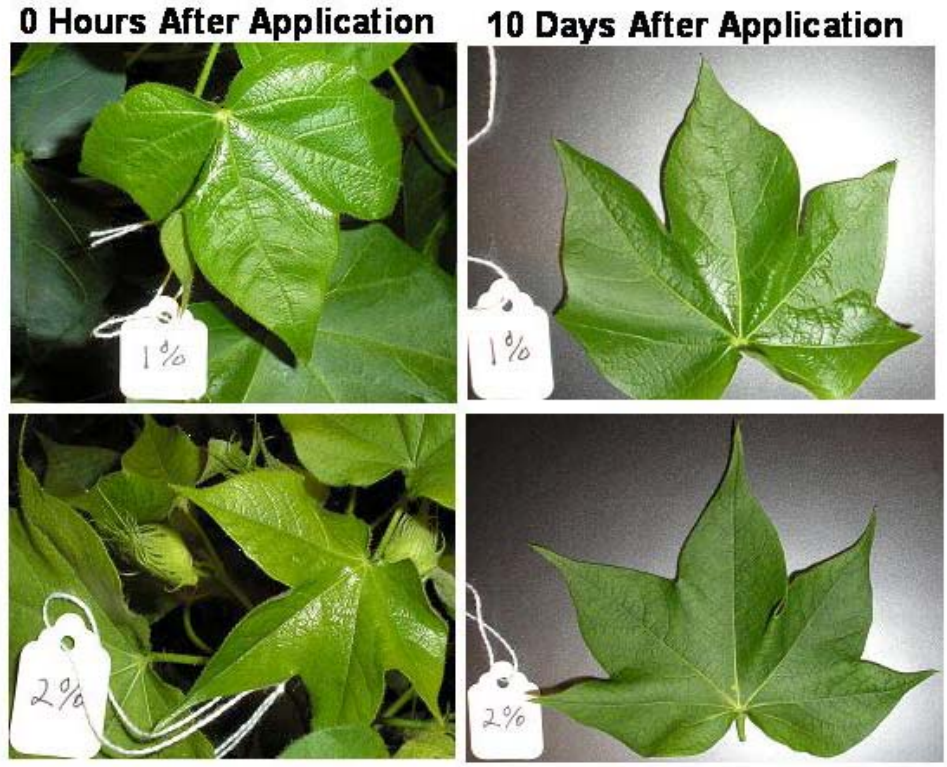


Figure 27. Results of kanamycin assay on two putative cotton transgenic plants containing the osmotin vector constructs. One and two percent kanamycin solutions were applied to the leaves of a cv. Coker 312 wild type plant and nine putative transgenic cotton plants. Ten days after application, the leaves were cut from the plants and analyzed. Kanamycin was found to have no effect on the treated leaves ten days after application, suggesting that the *npt*II gene is present in the plants.

indicate the presence or absence of the transgenes in the plants, genomic blot analyses are necessary to determine the reiteration frequency and arrangement (clustering or dispersion in the genome) of the putative transgenes in each tentative transgenic plant. To preclude the use of radiation, the Pierce North2South® Biotin Random Prime Labeling Kit, with the North2South® Chemiluminescent Hybridization and Detection Kit, was initially tried. With the North2South® Biotin Random Prime Labeling Kit, two different HPLC-purified oligonucleotides (each 50 nucleotides in length) and control DNA (supplied with the kit) were labeled with a fluorescent biotin probe based on the random-priming procedure of Feinberg and Vogelstein (1983). One oligonucleotide (osmotin probe) was for hybridization to the osmotin genes, and the other (pCAMBIA probe) was for hybridization to the pCAMBIA vector DNA. Several dilutions of the biotin-labeled control probe were spotted onto nitrocellulose and detected using the Pierce Streptavidin-HRP from the North2South® Hybridization and Detection Kit to confirm that the control labeling reaction was successful.

The labeled oligonucleotides were then used in genomic blot analyses. To do this, 10 µg of genomic DNA isolated from a cv. Coker 312 wild type plant and five different putative transgenic cotton plants were digested with the restriction enzymes *StuI* and *XhoI* and electrophoresed on a 0.8% agarose gel. The DNAs were transferred to a Hybond™-N^+ nylon membrane according to the alkaline transfer method in Sambrook and Russell (2001). Using the North2South® Chemiluminescent Hybridization and Detection Kit, the membrane and labeled probes were used for genomic blot analyses. Unfortunately, a strong signal was never generated using this nonradioactive approach (data not shown).

It became necessary to do the genomic blot analyses with classical ^{32}P-labeled oligonucleotide probes prepared by random priming (Feinberg and Vogelstein, 1983), as this laboratory has done previously. PCR fragments were generated for random-priming template DNAs from the pCAMBIA 2301 plasmid vector with specific amplimer pairs, representing different segments of the pCAMBIA 2301 vector construct containing the osmotin gene I insert: a PCR fragment (449 bp) generated from the *OSMI* gene, a PCR fragment (398 bp) generated from the *npt*II gene for kanamycin resistance, a PCR fragment (160 bp) generated from the *lac*Z alpha gene, a PCR fragment (366 bp) generated from the *GUS* reporter gene (second exon A), another PCR fragment (498 bp) generated from the *GUS* reporter gene (second exon B), and a PCR fragment (224 bp) generated from the polyA termination region (*nos* gene polyA region). The PCR fragments were purified by agarose gel electrophoresis (Figure 28A), and all the fragments except the *lac*Z gene PCR product were confirmed to be of high quality (Figure 28B). The sequences of both strands of each PCR product were confirmed using the appropriate primer pairs by Lone Star Labs (Houston, TX), and the sequences were analyzed by using DNASIS® version 2.1 software (Hitachi Software Engineering Co., Yokohama, Japan). Two PCR fragments, the osmotin gene template and the *GUS* second exon A template, were chosen to be used as probes for genomic blot analyses of the DNAs from the putative transgenic cotton plants. They were used as templates for generating ^{32}P-labeled DNA fragments by the random priming procedure of Feinberg and Vogelstein (1983) by Drs. Robert and Irma Pirtle in our laboratory.

Genomic DNAs from 43 putative transgenic cotton plants were prepared by the method of Paterson et al. (1993). The quantity and quality of all 43 genomic DNA

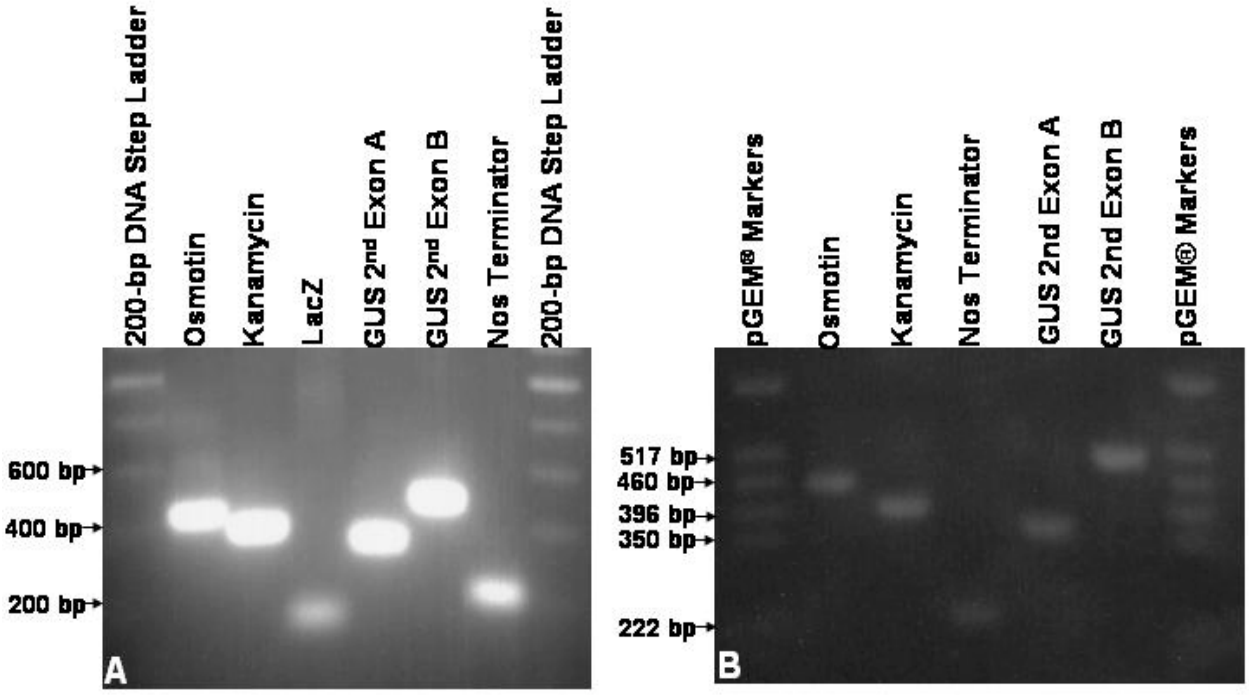


Figure 28. A. PCR products generated from five different regions of the pCAMBIA 2301 vector construct (*npt*II gene, *LacZ* gene, two different regions of the *GUS* second exon, and the *nos* terminator) and the *OSMI* gene. The PCR products were electrophoresed at 20 V overnight on a 1.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with 200-bp DNA Step Ladder standards (Promega). Except for the *LacZ* fragment, all gel slices containing the PCR fragments were excised from the gel, weighed, and purified using the QIAquick Gel Extraction Kit (QIAGEN). B. Gel electrophoresis showing the purity and concentration of the purified products. Five microliters of each purified product were electrophoresed on a 1.0% agarose gel, with pGEM® DNA Markers (Promega) to visually estimate yields.

samples was assessed by absorption spectroscopy and agarose gel electrophoresis. To ensure that the *GUS* second exon A and *OSMI* PCR products were generated from each of the putative transgenic plants, the specific amplimer pairs were used to amplify both products from the genomic DNA templates. PCR products were generated from each of the putative transgenic *OSMI* DNA samples, but two putative transgenic *OSMII* DNA samples did not yield a *GUS* second exon A product (Figure 29). As expected, the cv. Coker 312 wild type DNA sample did not yield a *GUS* second exon A product.

The 41 prospective transgenic genomic DNA samples were digested with the restriction enzyme *Nde*I, and the *Nde*I fragments were electrophoresed on 0.7% agarose gels (Figure 30). Serial dilutions (0.5 µg, 0.1 µg, 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg) of digested pCAMBIA-35S-*OSMI* #16 plasmid DNA were also electrophoresed (Figure 31A). After photographing the gels, the DNA was then transferred to Hybond™-N^+ membrane (Amersham™) by the alkaline transfer method (Sambrook and Russell, 2001). All gels were run in duplicate. There were eight blots containing digested genomic DNA and two blots containing digested plasmid DNA as a control.

The nylon membranes containing the immobilized DNAs were prehybridized for 4 h at 60˚C and subsequently hybridized with the ^{32}P-labeled *OSMI* and *GUS* second exon A probes overnight at 60˚C. The next day, the hybridized blots were washed to remove the extraneous probes and autoradiographed. The blots containing the digested plasmid DNA were exposed for a time period from 5 min up to 48 h to estimate the length of time needed for the genomic blot exposures. After only 5 min on film (without an intensifying screen), the expected 10.6-kb fragment could be readily seen in

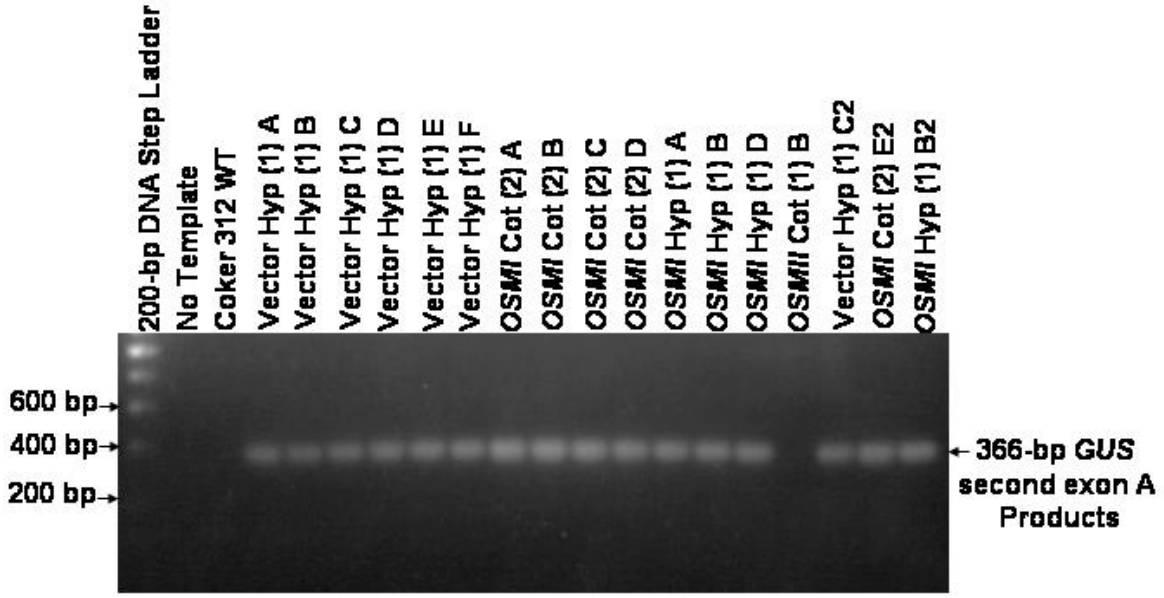


Figure 29. *GUS* second exon A PCR products generated from a number of the putative transgenic plants before doing the genomic blot analyses. The PCR products were mixed with 6X blue/orange loading dye and electrophoresed on a 1.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with a 200-bp DNA Step Ladder (Promega). All of the DNAs, except for the cv. Coker 312 wild type extract and two extracts from putative *OSMII* transformants (*OSMII* cotyledon (1) A2 and *OSMII* cotyledon (1) B), generated the predicted 366-bp PCR products when using the *GUS* second exon A amplimer pair.

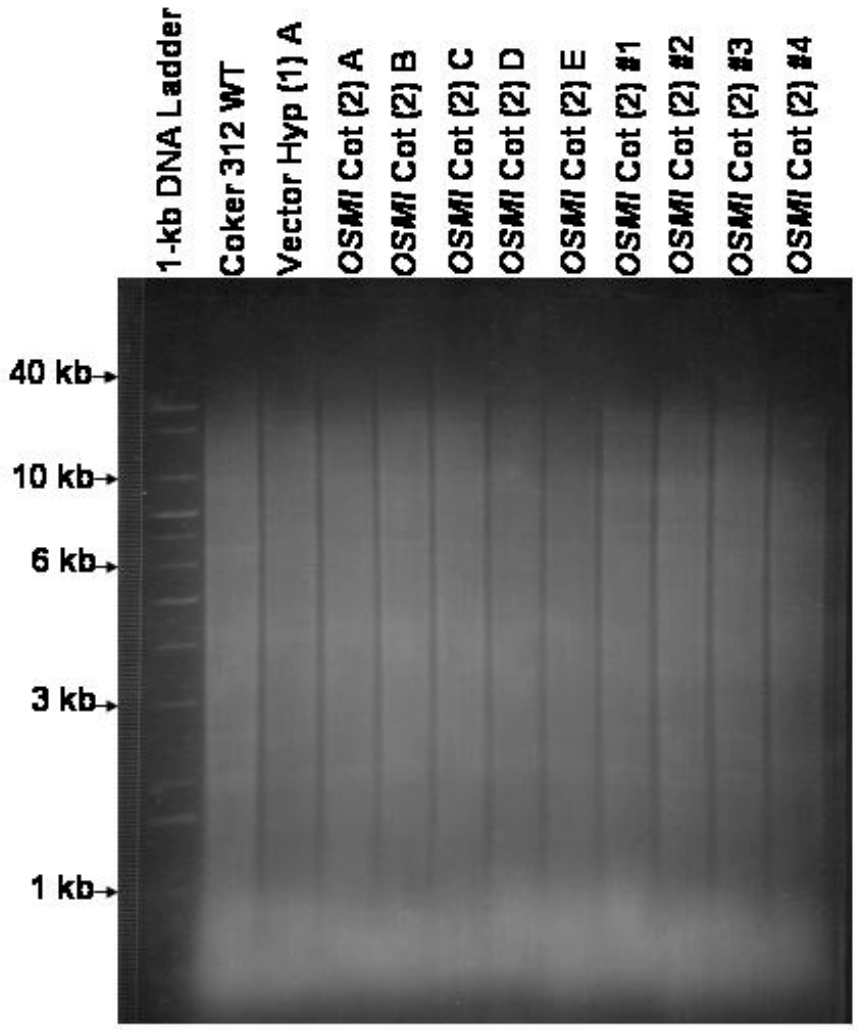


Figure 30. Ten micrograms of the cotton genomic DNA samples from the tentative transgenic cotton plants digested with 50 units of the restriction enzyme *Nde*I. The digests were mixed with 6X blue/orange loading dye and electrophoresed on 0.7% agarose gels. The gels were then stained with 0.5 µg/ml ethidium bromide and destained with water prior to photography.

the lanes containing 0.5 µg, 0.1 µg, and 10 ng of plasmid DNA (Figure 31B). After 48 h on film (with an intensifying screen), the 10.6-kb fragment could be seen in all of the lanes, except the last one containing only 1 pg of plasmid DNA (Figure 31C). From this, it was determined that the blots containing the digested genomic DNA should be exposed to film for about one month.

The autoradiograms shown in Figures 32 through 35 are ambiguous and somewhat difficult to interpret. The ^{32}P-labeled *OSMI* probe hybridized to four cotton genomic *NdeI* DNA fragments of about 3 kb, 7 kb, 8 kb, and 10 kb, for all of the cotton genomic DNA samples. After analysis of the 16-kb DNA sequence encompassing the osmotin gene cluster from our laboratory (Wilkinson, 2003; Wilkinson et al., 2005), it was deduced that the 3-kb *Nde*I genomic fragment contains the *OSMI* gene, that the 7-kb *Nde*I genomic fragment encompasses the *OSMII* gene, and that the 8-kb and 10-kb *Nde*I genomic fragments encompass two other cotton osmotin genes. The observation of four *Nde*I genomic fragments representing four similar osmotin genes in the cotton genome is consistent with our previous genomic blotting results of four similar osmotin genes (either paralogs or orthologs) in the cotton genome (Wilkinson, 2003; Wilkinson et al., 2005). However, no obvious *Nde*I genomic fragments were detected from any of the 41 presumptive transgenic cotton plants that would correspond to any possible osmotin transgenes, the worst results that could have been obtained.

The cotton plants supposedly containing the *OSMI* transgene yielded four different profiles when probed with the ^{32}P-labeled *GUS* second exon A probe. All the tentative transgenic *OSMI* cotyledon plants appear to have originated from the same cotyledon callus, as they all had a hybridizing *GUS Nde*I genomic fragment of 10 kb

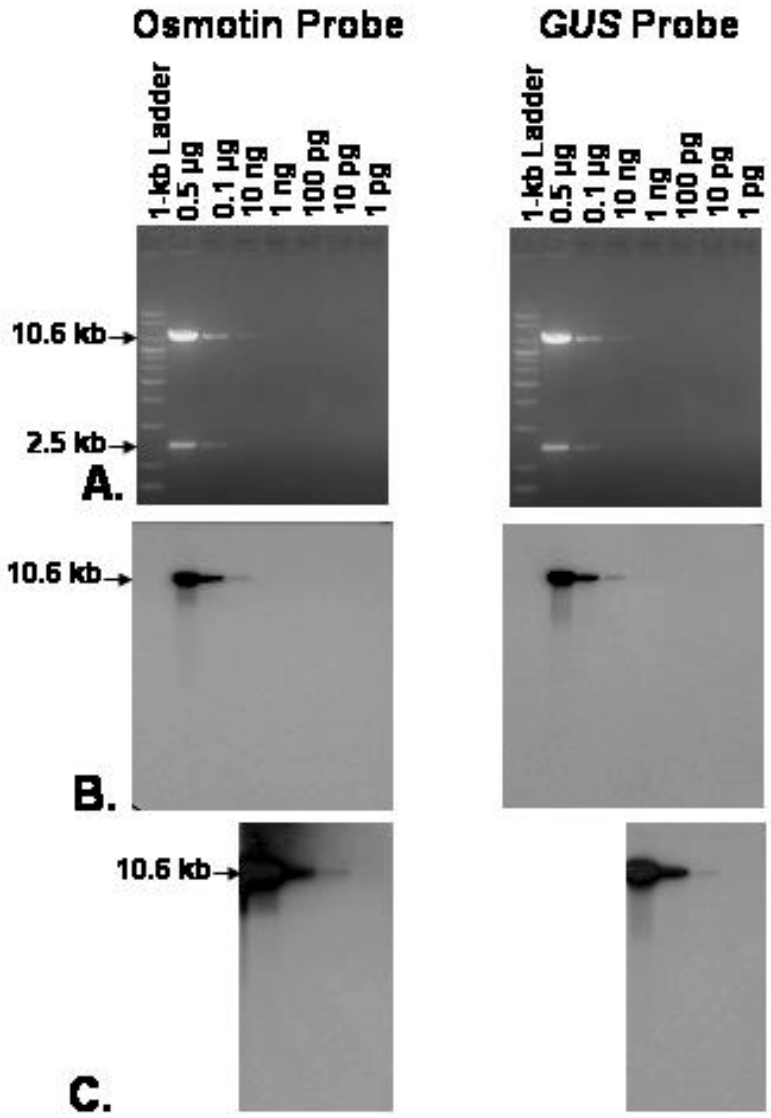


Figure 31. Serial dilutions (0.5 µg, 0.1 µg, 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg) of vector construct pCAMBIA-35S-*OSMI* #16 DNA digested with the restriction endonuclease *Nde*I. A) The *Nde*I DNA fragments were electrophoresed on duplicate 0.7% agarose gels. B) Autoradiograms showing the anticipated 10.6-kb fragment in the lanes containing 0.5 µg, 0.1 µg, and 10 ng of plasmid DNA after a 5 min autoradiography (without an intensifying screen). The DNAs hybridized with the ^{32}P-labeled *OSMI* probe are shown in the left panel, and the DNAs hybridized with the ^{32}P-labeled *GUS* second exon A probe are shown in the right panel. C) Autoradiograms showing the expected 10.6-kb fragment in the lanes containing 1 ng, 100 pg, and 10 pg of plasmid DNA after a 48 h autoradiography (with an intensifying screen). The DNAs hybridized with the ^{32}P-labeled *OSMI* probe are shown in the left panel, and the DNAs hybridized with the ^{32}P-labeled *GUS* second exon A probe are shown in the right panel.

(corresponding to the 10-kb *OSMI* fragment), as well as two other larger fragments that did not correspond to any band on the blot with the *OSMI* probe. Fourteen of the putative *OSMI* transgenic plants from hypocotyl callus appear to belong to the same line, because they all had a hybridizing *GUS Nde*I genomic fragment of 7 kb (corresponding to the 7-kb *OSMI* fragment). Two putative *OSMI* transgenic plants from hypocotyl callus, representing a third line, had hybridizing *GUS Nde*I genomic fragments of 7 kb and 10 kb (corresponding to the 7-kb and 10-kb *OSMI* fragments), as well as other hybridizing *GUS Nde*I fragments that did not match up to any *OSM* fragments. One tentative *OSMI* transgenic plant from hypocotyl callus, representing a fourth line, had a hybridizing *GUS Nde*I fragment that did not correspond to any hybridizing *OSMI Nde*I fragment. There was only one tentative transgenic plant containing the *OSMII* transgene construct with an 8-kb *NdeI* fragment that hybridized with the *GUS* probe (corresponding to the 8-kb *OSMI* fragment). The transgenic cotton plants presumably harboring the empty vector controls had three different hybridizing patterns. Three empty vector plants had a hybridizing *GUS Nde*I fragment that did not correspond to any hybridizing *OSMI* fragment; one empty vector plant had two hybridizing *GUS* fragments that did not correspond to any hybridizing *OSMI* fragment; and another empty vector plant had one hybridizing *GUS Nde*I fragment of 8 kb (corresponding to the 8-kb *OSMI* fragment).

Because no definitive hybridizing osmotin transgene fragments were detected and because several hybridizing *GUS* fragments seemed to actually overlap and coincidentally correspond with some of the hybridizing *OSMI Nde*I fragments, apparently none of the transgenic cotton plants were transformed with the osmotin gene

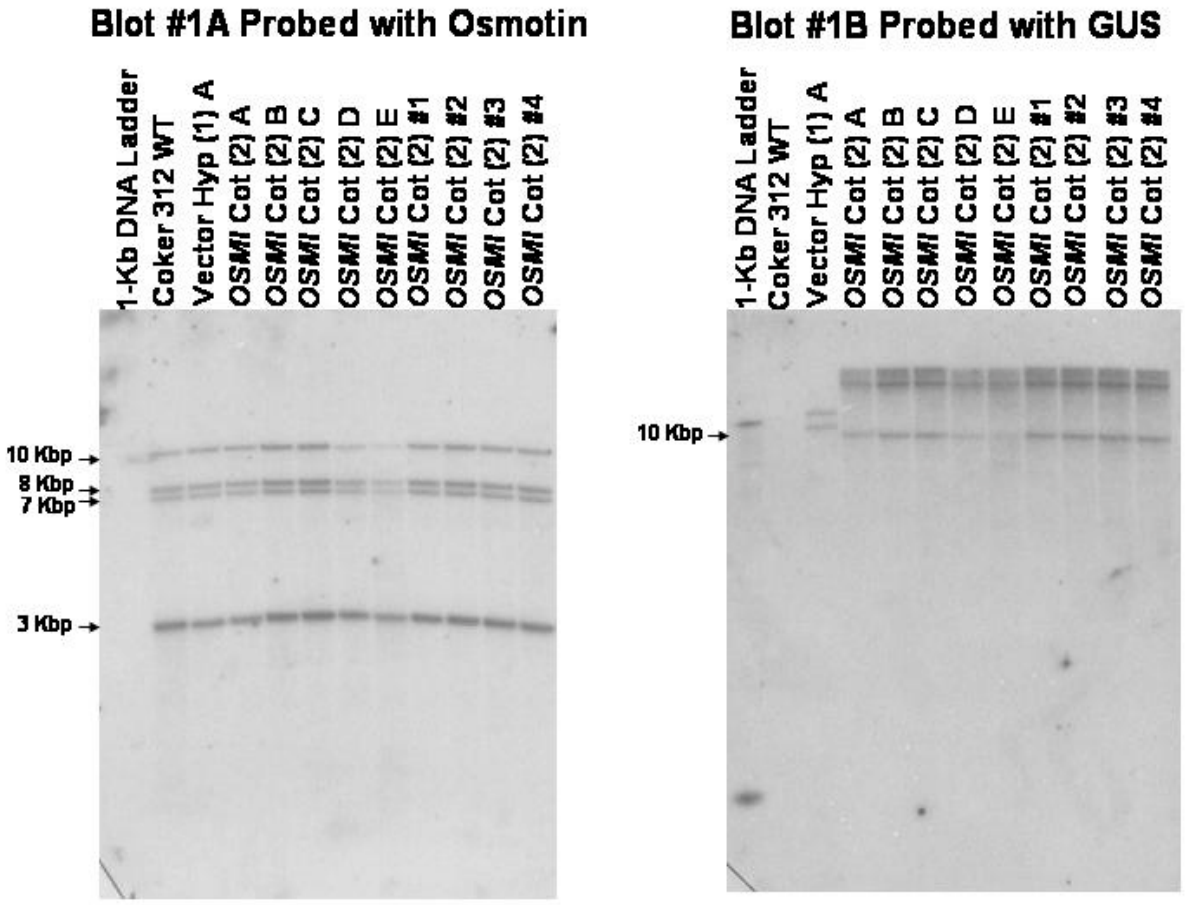


Figure 32. Autoradiograms of duplicate genomic blots of a wild type cotton plant (*Gossypium hirsutum* L., cv. Coker 312) and putative transgenic cotton plants with the osmotin vector constructs digested with *Nde*I. Left Panel) The ^{32}P-labeled *OSMI* probe hybridized to four fragments from all of the cotton genomic DNA samples. The fragments were about 3 kb, 7 kb, 8 kb, and 10 kb. Right Panel) All of the *OSMI* cotyledon transgenic plants had a *GUS* fragment hybridize at 10 kb (corresponding to the largest *OSM* fragment), as well as two other larger fragments that did not correspond to any fragment on the *OSM* blot. The digest of the genomic DNA isolated from the vector hypocotyl (1) A plant produced two *GUS* fragments that did not correspond to any *OSM* fragment. The ^{32}P-labeled *GUS* second exon A probe did not hybridize to any fragment in the wild type genomic DNA sample.

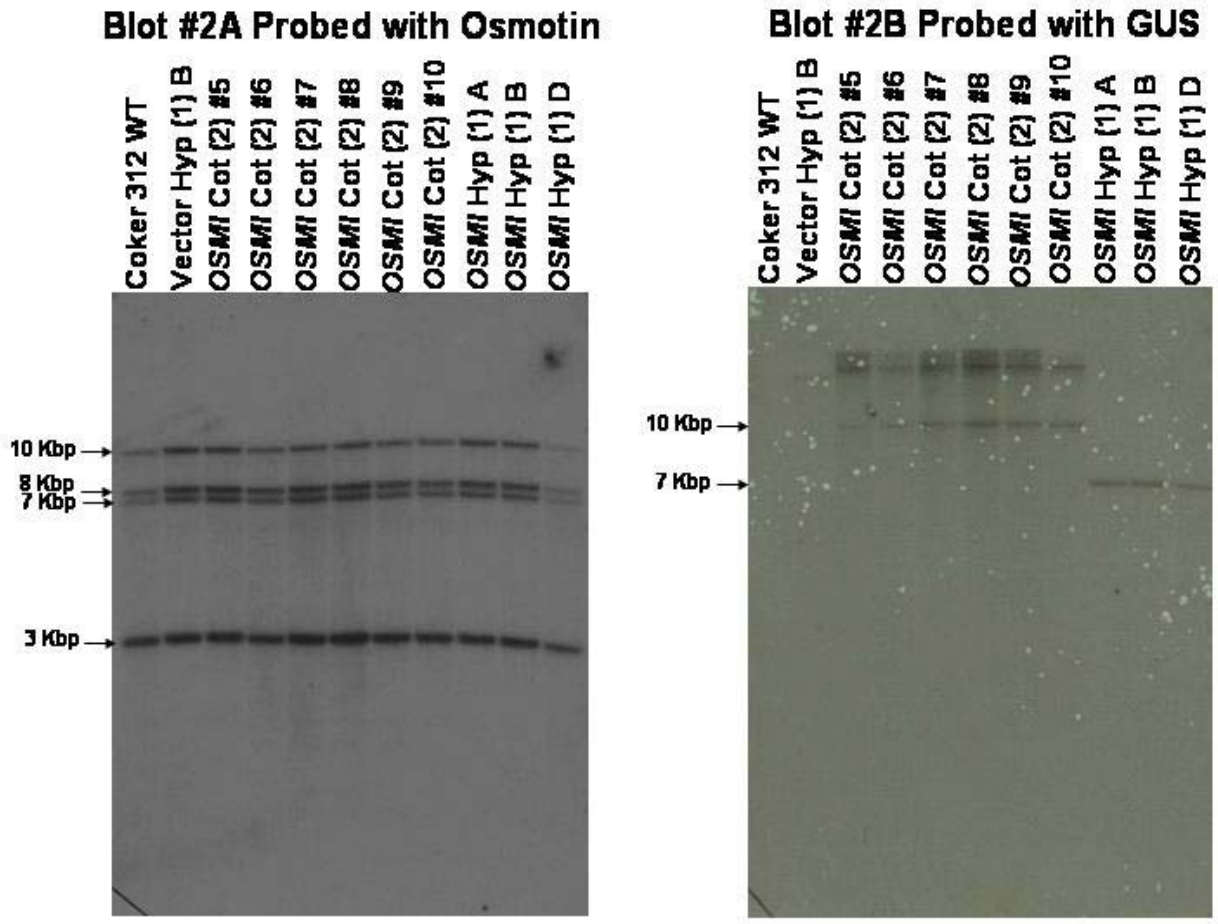


Figure 33. Autoradiograms of duplicate genomic blots of a wild type cotton plant (*Gossypium hirsutum* L., cv. Coker 312) and putative transgenic cotton plants with the osmotin vector constructs digested with *Nde*I. Left Panel) The ^{32}P-labeled *OSMI* probe hybridized to four fragments from all of the cotton genomic DNA samples. The fragments were about 3 kb, 7 kb, 8 kb, and 10 kb. Right Panel) All of the *OSMI* cotyledon transgenic plants had a fragment hybridize to the ^{32}P-labeled *GUS* probe at 10 kb (corresponding to the largest *OSM* fragment), as well as two other larger fragments that did not correspond to any fragment on the *OSM* blot. The digests of the genomic DNAs isolated from the three *OSMI* hypocotyl plants produced a *GUS* fragment at 7 kb (corresponding to the third largest *OSM* fragment). The digest of the genomic DNA isolated from the vector hypocotyl (1) B plant produced a *GUS* fragment that did not correspond to any *OSM* fragment. The ^{32}P-labeled *GUS* second exon A probe did not hybridize to any fragment in the wild type genomic DNA sample.

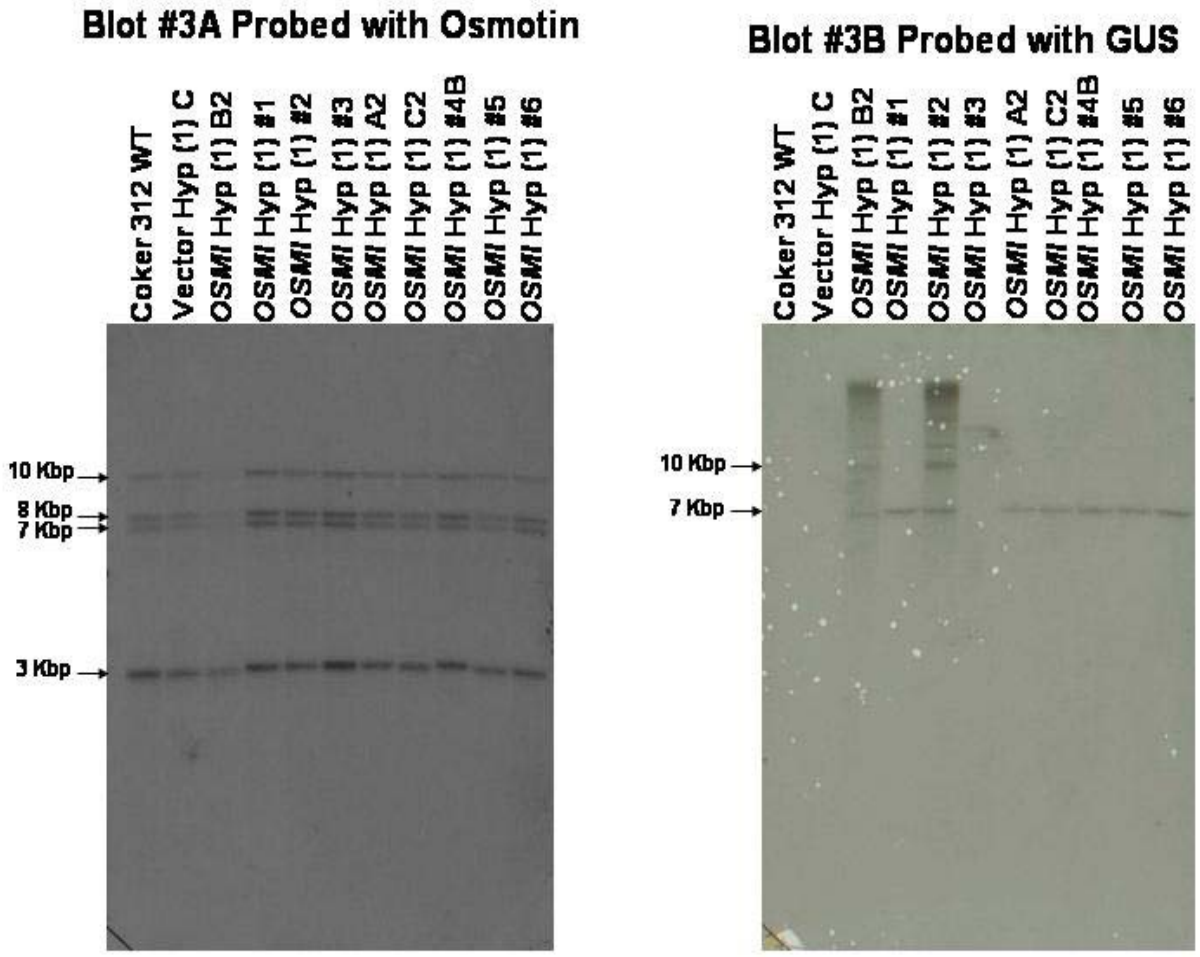


Figure 34. Autoradiograms of duplicate genomic blots of a wild type cotton plant (*Gossypium hirsutum* L., cv. Coker 312) and putative transgenic cotton plants with the osmotin vector constructs digested with *Nde*I. Left Panel) The ^{32}P-labeled *OSMI* probe hybridized to four fragments from all of the cotton genomic DNA samples. The fragments were about 3 kb, 7 kb, 8 kb, and 10 kb. Right Panel) Six of the *OSMI* hypocotyl plants (#1, A2, C2, #4B, #5, and #6) had a fragment hybridize at 7 kb to the ^{32}P-labeled *GUS* probe (corresponding to the third largest *OSM* fragment). Two of the *OSMI* hypocotyl plants (B2 and #2) had a *GUS* fragment hybridize at 7 kb and 10 kb (corresponding to the third largest and the largest *OSM* fragment), as well as other *GUS* fragments that did not correspond to any fragments on the *OSM* blot. The digests of the genomic DNAs isolated from the *OSMI* hypocotyl (1) #3 plant and the vector hypocotyl (1) C plant had one *GUS* fragment that did not correspond to any *OSM* fragment. The ^{32}P-labeled *GUS* second exon A probe did not hybridize to any fragment in the wild type genomic DNA sample.

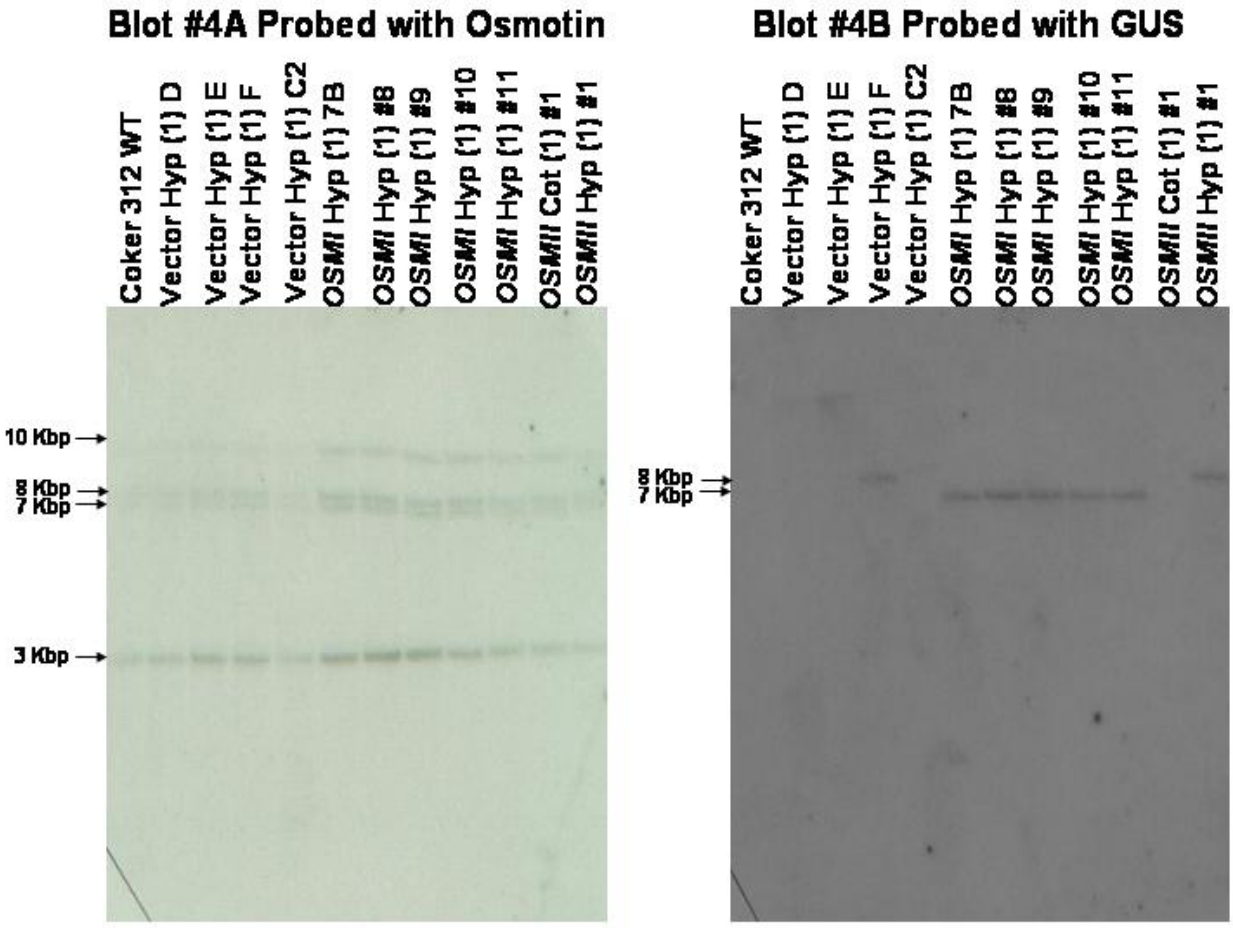


Figure 35. Autoradiograms of duplicate genomic blots of a wild type cotton plant (*Gossypium hirsutum* L., cv. Coker 312) and putative transgenic cotton plants with the osmotin vector constructs digested with *Nde*I. Left Panel) The ^{32}P-labeled *OSMI* probe hybridized to four fragments from all of the cotton genomic DNA samples. The fragments were about 3 kb, 7 kb, 8 kb, and 10 kb. Right Panel) The *OSMI* hypocotyl plant DNAs generated a fragment at 7 kb when hybridized with the ^{32}P-labeled *GUS* probe (corresponding to the third largest *OSM* fragment). The *OSMII* hypocotyl (1) #1 plant had a *GUS* fragment hybridize at 8 kb (corresponding to the second largest *OSM* fragment). The digest of the genomic DNA isolated from the vector hypocotyl (1) E plant produced one hybridizing *GUS* fragment that did not correspond to any fragment on the *OSM* blot. The vector hypocotyl (1) F plant had a hybridizing *GUS* fragment at 8 kb (corresponding to the second largest *OSM* band). The ^{32}P-labeled *GUS* second exon A probe did not hybridize to any fragment in the wild type, the vector hypocotyl (1) D, the vector hypocotyl C2, or the *OSMII* cotyledon (1) #1 genomic DNA samples.

constructs. Thus, a second series of genomic blot analyses was done (shown in Figures 36 through 38) with the two ^{32}P-labeled *GUS* second exon A and *OSMI* probes to assess whether the supposed transgenic cotton plants apparently did not have the osmotin gene inserts, and if the hybridizing *GUS* fragments from the first set of genomic blots were simply coincidental, overlapping DNA fragments. The previous negative results were confirmed by digesting cotton genomic DNA samples from each putative transgenic line with several additional restriction enzymes (*Nde*I/*Afl*II double digest, *Nsi*I, and *Ase*I), in order to obtain different restriction cleavage patterns. Based on autoradiograms of digested plasmid DNA, it was determined that the blots containing the digested genomic DNA should be exposed for one to two months.

When the cotton genomic DNAs were doubly digested with *Nde*I and *Afl*II, the genomic DNAs from the wild type and empty vector cotton plants yielded a pattern of four DNA fragments hybridizing to the *OSMI* probe: 3.2 kb (probably encompassing the *OSMI* gene), 4.1 kb (probably containing the O*SMII* gene), 4.4 kb , and 7.1 kb. When the tentative *OSMI* transgenic plants from the hypocotyl calli were digested with these same two enzymes, they yielded five prominent hybridizing fragments (the same four as above and an additional 8.9-kb fragment). Also, two of the tentative *OSMI* transgenic plants from the cotyledon calli appeared to have the 8.9 kb fragment as well, but only very faintly. There also appeared to be very faint bands corresponding to this extra 8.9-kb fragment for three of the supposed transgenic plants when hybridized with the *GUS* probe. The 8.9-kb fragments most likely are partial fragments from incomplete digestion of the genomic DNAs with one of the two enzymes in the double digestions. All of the putative *OSMI* transgenic plants from the hypocotyl and cotyledon calli had a hybridizing

GUS fragment of 3.2 kb (corresponding to the *OSMI* fragment thought to contain the *OSMI* gene). The three empty vector plants examined all displayed a single fragment of 4.0 kb when hybridized with the *GUS* probe.

When the cotton genomic DNA samples were digested with *Ase*I, all of the wild type and putative transgenic plants yielded the same pattern of three hybridizing fragments with the osmotin probe: 1.6 kb, 1.9 kb (probably encompassing the *OSMI* gene), and 2.9 kb (probably encompassing the *OSMII* gene). All of the putative *OSMI* transgenic plants from the hypocotyl and cotyledon calli examined had a faint hybridizing *GUS* fragment of 1.9 kb (corresponding to the *OSMI* fragment thought to contain the *OSMI* gene). This probably has no real significance. Two of the transgenic plants from the hypocotyl calli with the empty vector yielded a single 2.4-kb fragment when hybridized with the G*US* probe, while the other transgenic plant from the hypocotyl calli with the empty vector yielded a 1.5-kb fragment.

When the cotton genomic DNA samples were digested with *Nsi*I, all of the wild type and putative transgenic plants yielded the same two hybridizing fragments with the osmotin probe: 2.4 kb (probably encompassing the *OSMII* gene) and 6.7 kb (probably encompassing the *OSMI* gene). Unfortunately, none of the *GUS* fragments observed on the autoradiograms matched up with any of the hybridizing *OSM* fragments. Again, it should be noted that two of the empty vector transgenic plants yielded a single 4.0-kb fragment when hybridized with the *GUS* probe, while the other transgenic plant yielded a 6.9 kb-fragment. Clearly, the genomic blotting experiments indicate the complete absence of osmotin transgenes in the supposed transgenic cotton plants, but do indicate the presence of the *GUS* fragment from the vector cassette in the prospective

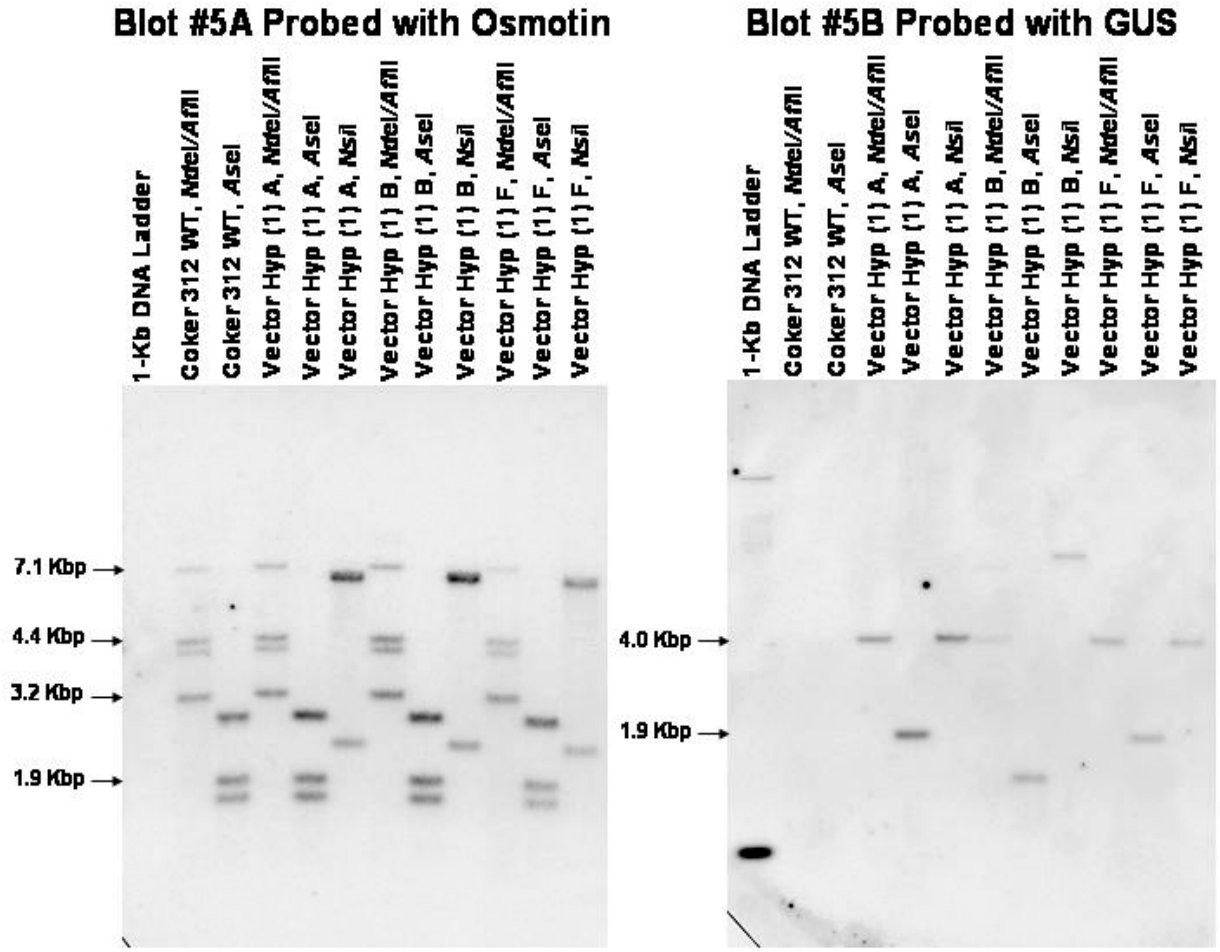


Figure 36. Autoradiograms of duplicate genomic blots of a wild type cotton plant (*Gossypium hirsutum* L., cv. Coker 312) and putative transgenic cotton plants with the osmotin vector constructs digested with *Nde*I + *Afl*II, *Nsi*I, and *Ase*I. Left Panel) The ^{32}P-labeled *OSMI* probe hybridized to four fragments from all of the cotton genomic DNA samples when digested with *Nde*I + *Afl*II. The fragments were about 3.2 kb, 4.1 kb, 4.4 kb, and 7.1 kb. The ^{32}P-labeled *OSMI* probe hybridized to three fragments from all of the cotton genomic DNA samples when digested with *Ase*I. The fragments were about 1.6 kb, 1.9 kb, and 2.9 kb. The ^{32}P-labeled *OSMI* probe hybridized to two fragments from all of the cotton genomic DNA samples when digested with *Nsi*I. The fragments were about 2.4 kb and 6.7 kb. Right Panel) The digests of the genomic DNAs isolated from the empty vector plants produced a 4.0-kb fragment when hybridized with the ^{32}P-labeled *GUS* probe. Two of the empty vector plants (vector hypocotyl (1) A and vector hypocotyl (1) F) generated a hybridizing 2.4-kb *GUS* fragment, while the other empty vector plant (vector hypocotyl (1) B) generated a 1.5-kb fragment that hybridized to the *GUS* probe. The genomic DNAs from two of the empty vector plants (vector hypocotyl (1) A and vector hypocotyl (1) F) generated a 4.0-kb fragment that hybridized to the *GUS* probe, while the other empty vector plant (vector hypocotyl (1) B) produced a 6.9-kb fragment. The ^{32}P-labeled *GUS* second exon A probe did not hybridize to any fragment in the wild type genomic DNA sample.

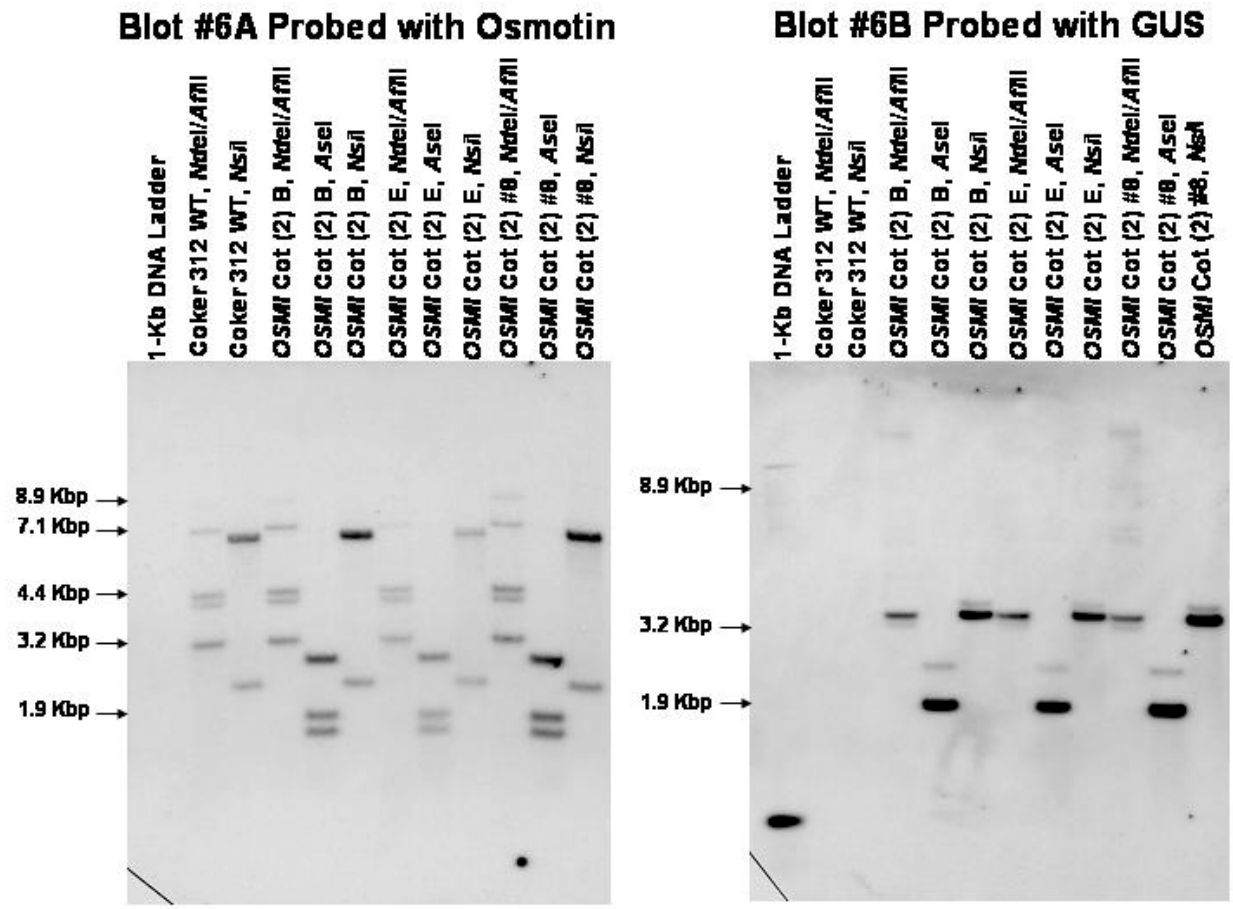


Figure 37. Autoradiograms of duplicate genomic blots of a wild type cotton plant (*Gossypium hirsutum* L., cv. Coker 312) and putative transgenic cotton plants with the osmotin vector constructs digested with *Nde*I + *Afl*II, *Nsi*I, and *Ase*I. Left Panel) The ^{32}P-labeled *OSMI* probe hybridized to four fragments from all of the cotton genomic DNA samples when digested with *Nde*I + *Afl*II. The fragments were about 3.2 kb, 4.1 kb, 4.4 kb, and 7.1 kb. Both ^{32}P-labeled *OSMI* and *GUS* probes hybridized to an extra 8.9-kb fragment from the genomic DNAs of two of the *OSMI* cotyledon plants (*OSMI* cotyledon (2) B and *OSMI* cotyledon (2) 8). The ^{32}P-labeled *OSMI* probe hybridized to three fragments from all of the cotton genomic DNA samples when digested with *Ase*I. The fragments were about 1.6 kb, 1.9 kb, and 2.9 kb. The ^{32}P-labeled *OSMI* probe hybridized to two fragments from all of the cotton genomic DNA samples when digested with *Nsi*I. The fragments were about 2.4 kb and 6.7 kb. Right Panel) All of the *OSMI* cotyledon plants generated a 3.2-kb *GUS* fragment (corresponding to the smallest *OSM* fragment) that hybridized to the ^{32}P-labeled *GUS* probe. All of the *OSMI* cotyledon plants produced a 1.9-kb *GUS* fragment (corresponding to the second largest *OSM* fragment) that hybridized to the ^{32}P-labeled *GUS* probe, as well as a larger fragment that did not correspond to any *OSM* fragments. All of the *OSMI* cotyledon plants produced two hybridizing *GUS* fragments that did not correspond to any *OSM* fragments. The ^{32}P-labeled *GUS* second exon A probe did not hybridize to any fragment in the wild type genomic DNA sample.

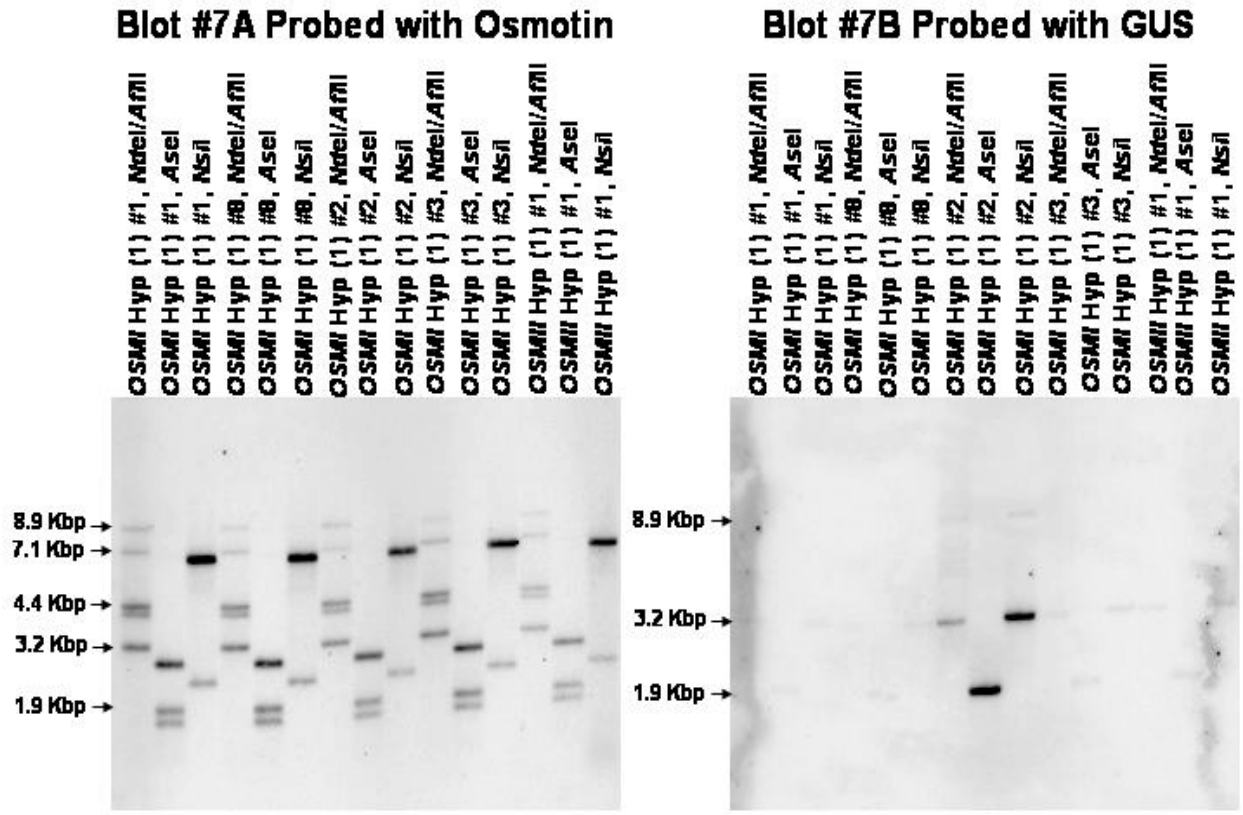


Figure 38. Autoradiograms of duplicate genomic blots of a wild type cotton plant (*Gossypium hirsutum* L., cv. Coker 312) and putative transgenic cotton plants with the osmotin vector constructs digested with *Nde*I + *Afl*II, *Nsi*I, and *Ase*I. Left Panel) The ^{32}P-labeled *OSMI* probe hybridized to five fragments from all of the *OSM* hypocotyl genomic DNA samples when digested with *Nde*I + *Afl*II. The fragments were about 3.2 kb, 4.1 kb, 4.4 kb, 7.1 kb, and 8.9 kb. The ^{32}P-labeled *OSMI* probe hybridized to three fragments from all of the cotton genomic DNA samples when digested with *Ase*I. The fragments were about 1.6 kb, 1.9 kb, and 2.9 kb. The ^{32}P-labeled *OSMI* probe hybridized to two fragments from all of the cotton genomic DNA samples when digested with *Nsi*I. The fragments were about 2.4 kb and 6.7 kb. Right Panel) All of the *OSM* hypocotyl plants generated a 3.2-kb *GUS* fragment (corresponding to the smallest *OSM* fragment) that hybridized to the ^{32}P-labeled *GUS* fragment. A hybridizing 8.9-kb *GUS* fragment (corresponding to the largest *OSM* fragment) is produced from the genomic DNA of the *OSMI* hypocotyl (1) 2 plant. All of the *OSM* hypocotyl plants produced a faintly hybridizing *GUS* fragment at 1.9 kb (corresponding to the second largest *OSM* fragment). The ^{32}P-labeled *GUS* second exon A probe did not hybridize to any fragment in the wild type genomic DNA sample.

transgenic cotton plants. It appears as though an anomalous DNA structural rearrangement or recombination artifacts occurred in vector constructs during the cloning or transformation processes.

Confirmation of *Agrobacterium tumefaciens* Constructs

Because all attempts made to verify the presence of the transgene in the putative *Arabidopsis thaliana* and cotton transgenic plants had failed, the original osmotin vector constructs in the transformed *E. coli* DH5α cells (the pCAMBIA 2301 empty vector in strain DH5α, the *OSMI* gene in pCAMBIA 2301 in strain DH5α, and the *OSMII* gene in pCAMBIA 2301 in strain DH5α) and in the transformed *Agrobacterium tumefaciens* LBA4404 cells (the pCAMBIA2301 empty vector, the *OSMI* gene in pCAMBIA2301, and the *OSMII* gene in pCAMBIA2301) were re-examined. After isolating the plasmid DNA constructs from *E. coli* and *Agrobacterium*, they were analyzed by PCR analysis and restriction digestion. Sequence analysis of the PCR products confirmed that the *Agrobacterium tumefaciens* cells contained the osmotin transgenes. Several restriction digestions on the DNAs were done with the restriction enzyme *Sal*I (Figure 39), which is a single cutter of only the pCAMBIA vector. The plasmid DNA isolated from *E. coli* appeared to be intact, yielding the single expected band of about 13 kb. However, the plasmid construct DNAs isolated from the *Agrobacterium* strains resulted in a 50-kb restriction fragment for the empty vector construct and for the *OSMII* construct, and there were multiple restriction fragments produced from the *OSMI* construct. These aberrant restriction patterns indicate that some aberrant DNA structural rearrangement or recombination events could have happened to the constructs at some point after they

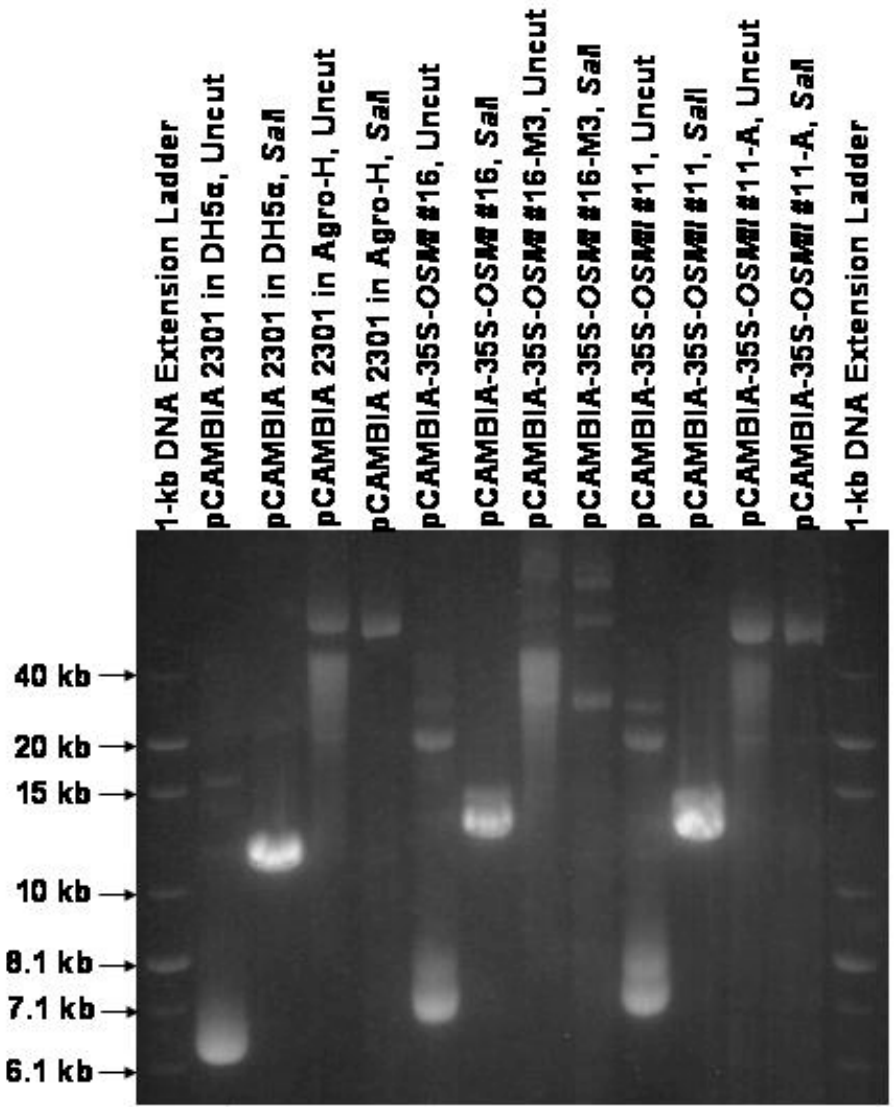


Figure 39. *Sal*I restriction digests of plasmid vector DNAs isolated from transformed *E. coli* and *Agrobacterium* cells. The digested fragments were separated on a 0.6% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with 0.5 µg of a 1-kb DNA Extension Ladder. The plasmid DNAs isolated from the *E. coli* cells gave rise to an 11.6-kb restriction fragment for the empty vector construct (pCAMBIA 2301 in DH5α), and a 13.0-kb restriction fragment for both the *OSMI* and *OSMII* vector constructs (called pCAMBIA-35S-*OSMI* #16 and pCAMBIA-35S-*OSMII* #11, respectively). The plasmid DNAs isolated from the *Agrobacterium* cells generated a 50-kb restriction fragment for the empty vector construct (pCAMBIA 2301 in Agro-H) and for the *OSMII* construct (pCAMBIA-35S-*OSMII* #11-A), and multiple restriction fragments for the *OSMI* construct (pCAMBIA-35S-*OSMI* #16-M3).

were transformed into the *Agrobacterium* cells, as described above in the PCR assay results from BioDiagnostics, Inc.

Second Transformation of *Agrobacterium tumefaciens* with the Binary Plasmid Vector Constructs

To address this issue, the osmotin vector constructs isolated from the transformed *E. coli* cells were re-transformed into fresh ElectroM*AX*™ *Agrobacterium* tumefaciens LBA4404 cells (Invitrogen™) by electroporation. Colony selection was carried out on YM medium containing 100 µg/ml streptomycin and 50 µg/ml kanamycin. Colony PCR amplification with the *GUS* second exon A amplimer pair confirmed that each of the transformed *Agrobacterium* cultures contained the osmotin vector construct, as all of the cultures generated *GUS* PCR products (Figure 40), indicative of the pCAMBIA construct. Plasmid DNAs were then isolated (Li et al., 1995) from each *Agrobacterium* culture, and doubly digested with the restriction enzymes *Bam*HI and *Kpn*I, which should cleave the 1.4-kb osmotin inserts from the pCAMBIA vector constructs. Plasmid DNAs from the original transformed *Agrobacterium* cells that were used to transform the cotton and *Arabidopsis* plants were also doubly digested with *Bam*HI/*Kpn*I. Duplicate *Bam*HI/*Kpn*I digestion reactions were done for each set of plasmid DNAs, and the DNA fragments electrophoresed on 0.7% agarose gels. The *Bam*HI/*Kpn*I fragments were transferred to nylon membrane replicas by alkaline blotting. The *Bam*HI/*Kpn*I fragments immobilized on the filters were probed with either the ^{32}P-labeled *OSMI* gene probe or the ^{32}P-labeled *GUS* second exon A probe, as done with the genomic blots.

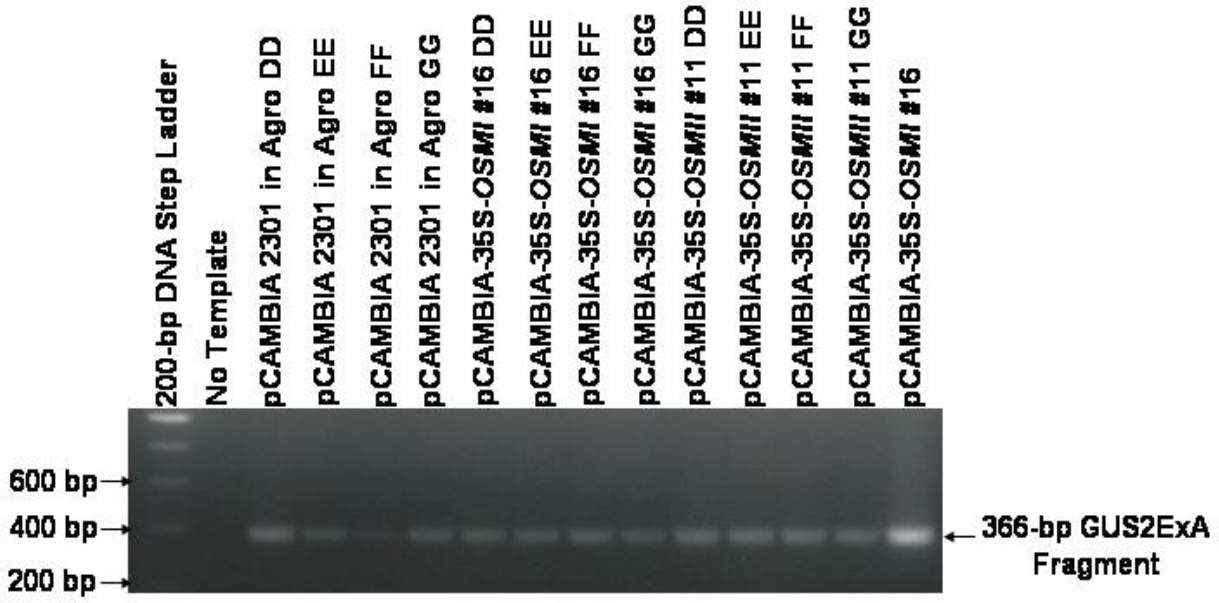


Figure 40. *GUS* second exon A (GUS2ExA) PCR products generated from colonies of transformed *Agrobacterium tumefaciens* cells harboring the osmotin vector constructs, selected from YM plates containing 100 µg/ml streptomycin and 50 µg/ml kanamycin. The plasmid vector DNAs (pCAMBIA 2301 vector, pCAMBIA-35S-*OSMI* #16, and pCAMBIA-35S-*OSMII* #11) were isolated from transformed *E. coli* DH5α cells and electroporated into fresh ElectroMAX™ *Agrobacterium tumefaciens* LBA4404 Cells (Invitrogen™). Isolated *Agrobacterium* colonies were selected for colony PCR using the Gus2Ex ForA and Gus2Ex RevA amplimer pair to confirm the presence of the transgenes. The pCAMBIA-35S-*OSMI* #16 plasmid DNA was also used as a positive control. The PCR products were electrophoresed on a 1.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with a 200-bp DNA Step Ladder (Promega). All of the selected colonies, as well as the positive control, generated 366-bp GUS2ExA PCR products. There was no product generated from the PCR reaction lacking a template.

The results for the new *Agrobacterium* constructs (Figure 41) were almost as expected, with the *Bam*HI/*Kpn*I double digests excising the 1.4-kb osmotin gene inserts out of the *OSMI* and the *OSMII* pCAMBIA constructs. The 1.4-kb *Bam*HI/*Kpn*I fragments encompassing the *OSMI* gene and *OSMII* gene clearly hybridize to the *OSMI* probe, and the 11.6-kb *Bam*HI/*Kpn*I fragment encompassing the *GUS* gene does not hybridize to the *OSMI* probe. Also, the 11.6-kb empty pCAMBIA vector fragment containing the *GUS* gene does not hybridize to the *OSMI* probe. The ^{32}P-labeled GUS probe hybridizes to the 11.6-kb *Bam*HI/*Kpn*I fragment but does not hybridize to the 1.4-kb *Bam*HI/*Kpn*I fragments encompassing the *OSMI* and *OSMII* genes. Also, the 11.6-kb empty pCAMBIA vector fragment containing the *GUS* gene hybridizes to the *GUS* probe. The sizes of the osmotin vector constructs are 13 kb, as expected. Surprisingly, an aberrant, minor hybridizing 19-kb *Bam*HI/*Kpn*I fragment was observed with the *GUS* probe, which is much larger than the 13-kb size of the osmotin vector constructs. The weakly hybridizing 19-kb *Bam*HI/*Kpn*I fragment is also observed in the genomic blot of the original *Agrobacterium OSMI* and the *OSMII* pCAMBIA constructs.

The results for the original *Agrobacterium OSMI* and the *OSMII* pCAMBIA constructs (Figure 42) were very disappointing. Aberrantly-sized 19-kb and 25-kb *Bam*HI/*Kpn*I hybridizing fragments were detected for the *OSMI* and the *OSMII* vector constructs when probed with the *GUS* probe, and the *OSMII* constructs looked identical to the empty vector constructs. More alarming was the failure to detect any hybridizing fragments with the *OSM* probe for either of the *OSMI* or *OSMII* vector constructs. Because the linearized plasmid vectors should only be 13 kb in size, it seems as though an anomalous DNA structural rearrangement or recombination occurred in the

Agrobacterium cells. In particular, the 25-kb *Bam*HI/*Kpn*I fragment would appear to have arisen by an anomalous dimerization event, with concomitant excision of the osmotin gene cassettes that were inserted into the empty vectors. However, these results are very odd, considering that we have previously been able to PCR amplify and sequence the osmotin gene cassettes from the *OSMI* and *OSMII* vector constructs transformed into the original *Agrobacterium* cells.

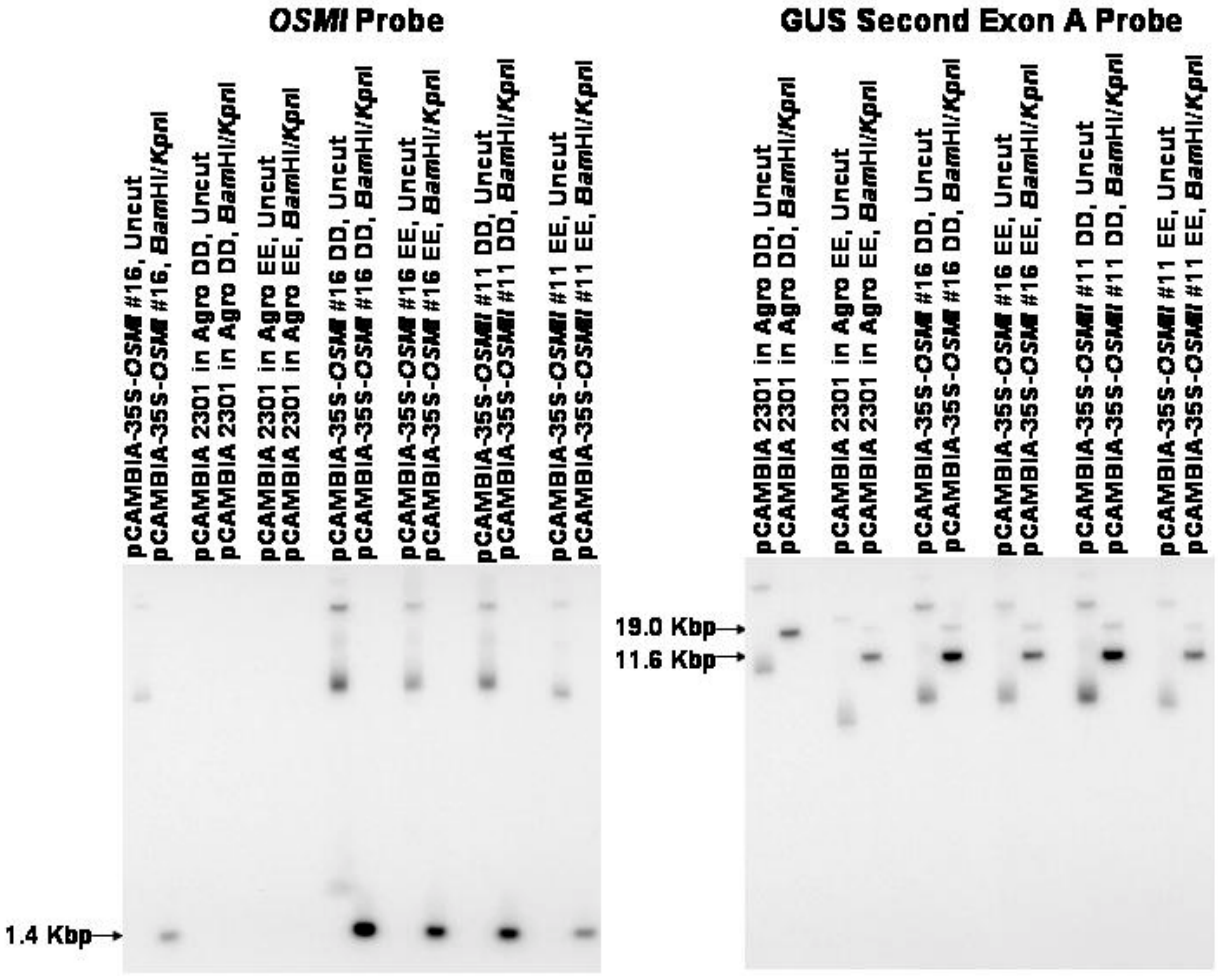


Figure 41. Alkaline blot analyses of *Bam*HI/*Kpn*I digests of plasmid DNAs isolated from the second set of transformed *Agrobacterium* cells. After a 4 h autoradiography, a 1.4-kb fragment hybridized to the *OSMI* probe in the digests of the pCAMBIA-35S-*OSMI* #16 DD, pCAMBIA-35S-*OSMI* #16 EE, pCAMBIA-35S-*OSMII* #11 DD, and pCAMBIA-35S-*OSMII* #11 EE plasmid DNAs, as well as to a 1.4-kb fragment in the digest of the positive control from *E. coli*. After a 3 h exposure, it was observed that the *GUS* second exon A probe hybridized to an 11.6-kb fragment in the digests of the pCAMBIA 2301 in Agro EE, pCAMBIA-35S-*OSMI* #16 DD, pCAMBIA-35S-*OSMI* #16 EE, pCAMBIA-35S-*OSMII* #11 DD, and pCAMBIA-35S-*OSMII* #11 EE plasmid DNAs. The *GUS* second exon A probe hybridized to a 19.0-kb fragment in the pCAMBIA 2301 in Agro DD digest. This 19.0-kb *Bam*HI/*Kpn*I fragment also weakly hybridized to the probe in all of the other digests.

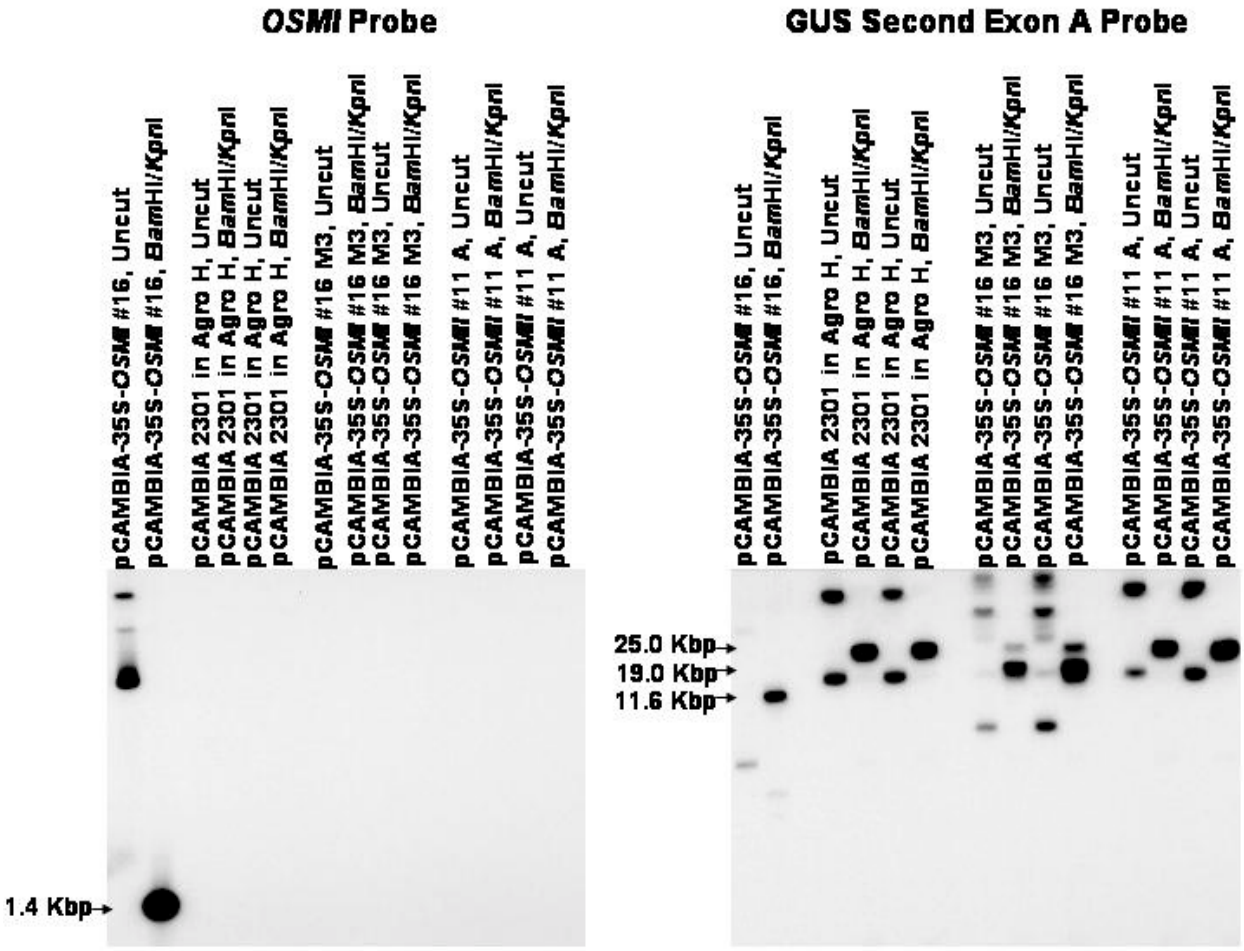


Figure 42. Alkaline blot analyses of *Bam*HI/*Kpn*I digests of plasmid DNAs isolated from the original set of transformed *Agrobacterium* cells harboring the osmotin vector constructs. After a 24 h autoradiography, it was observed that the *OSMI* probe only hybridized to a 1.4-kb fragment in the digest of the positive control from the *E. coli* cells. There were no fragments visible for any of the plasmid DNAs isolated from the original transformed *Agrobacterium* cells. After a 2.5 h autoradiography, a 25-kb hybridizing fragment was detected for the pCAMBIA 2301 in Agro-H, pCAMBIA-35S-*OSMI* #16-M3, and pCAMBIA-35S-*OSMII* #11-A digests when hybridized to the *GUS* second exon A probe. A 19.0-kb fragment was also detected from the *OSMI* construct. In addition, the *GUS* probe hybridized to the expected 11.6-kb fragment in the digest of the positive control from the transformed *E. coli* cells.

CHAPTER 4

DISCUSSION

Cotton is a valuable source of natural fiber, feed, and edible oil (Zapata et al., 1999; Sunilkumar and Rathore, 2001; Zhang et al., 2001; Ikram-Ul-Haq, 2004; Wu et al., 2005). Fungal diseases in cotton, such as various types of root rot and wilt, destroy much of the cotton crop and subsequently result in large economic losses every year (Bajaj, 1998; Lyon and Becerra-LopezLavalle, 2000; Dong et al., 2002; Rep et al., 2002; Emani et al., 2003; Roncero et al., 2003; Palmateer et al., 2004). Traditional breeding methods have produced varieties of cotton with improved agronomic traits, such as fungal resistance, but the lack of useful economic characters in commercial cotton cultivars has been a major challenge (Sawahel, 2001; Ikram-Ul-Haq, 2004; Wu et al., 2005). Although fungicides have helped control plant fungal infections, this chemical control is economically costly as well as environmentally undesirable. Therefore, new strategies are being used based on plant defense mechanisms to control fungal diseases to improve agricultural production and prevent the overusage of chemical fungicide treatments that could be harmful to humans and the environment.

Plants naturally produce their own defense proteins called pathogenesis-related (PR) proteins directed against bacterial and fungal pathogens. These proteins are routinely made by plants in low amounts, but accumulate in large amounts after pathogen infection (Bol et al., 1990; Datta et al., 1999; Kitajima and Sato, 1999; van Loon and van Strien, 1999; Rep et al., 2002). PR-5 proteins called osmotins have been isolated from a variety of crop plants (Singh et al., 1985 and 1987; Pierpoint et al., 1990; Roberts and Selitrennikoff, 1990; Vigers et al., 1991; Nelson et al., 1992; Reimman and

Dudler, 1993; Capelli et al., 1997; Hu and Reddy, 1997; Anžlovar et al., 1998; Oh et al., 2000; Campos et al., 2002; Kim et al., 2002; Rep et al., 2002). Osmotin genes have previously been shown to contain biological control regions or promoter elements for the overproduction of osmotin when the plants are exposed to a number of hormonal and environmental signals, including osmotic stress due to water deprivation or salt exposure, fungal infection, abscisic acid, wounding, ethylene, and low temperature (Singh et al., 1987; La Rosa et al., 1992; Nelson et al., 1992; Raghothama et al., 1993; Liu et al., 1994; Zhu et al., 1995; Chang et al., 1997; Guilfoyle, 1997; Kitajima et al., 1998; Xu et al., 1998; Kitajima and Sato, 1999; Velazhahan et al., 1999; Helleboid et al., 2000; Newton and Duman, 2000; Koyama et al., 2001).

Osmotins are thought to specifically interact with fungal membrane receptors to perhaps form transmembrane pores that lead to osmotic rupture and destruction of the fungal cells (Bowles, 1990; Roberts and Selitrennikoff, 1990; Bohlmann and Apel, 1991; Vigers et al., 1992; Melchers et al., 1993; Liu et al., 1994; Abad et al., 1996; Hu and Reddy, 1997; Koiwa et al., 1997; Yun et al., 1997; Anžlovar et al., 1998; Kitajima and Sato, 1999; Coca et al., 2000; Ibeas et al., 2000 and 2001). Due to the wide-ranging fungal effect of the osmotin gene, it may be possible to use the osmotin gene as a novel defense that is effective against numerous pathogenic fungi. In fact, transgenic potato plants that routinely overproduce osmotin are highly resistant to infection by fungal pathogens (Liu et al., 1994; Zhu et al., 1996). Therefore, the ultimate goal of this dissertation research was to try to genetically engineer cotton osmotin genes to routinely overproduce the antifungal protein osmotin in transgenic cotton plants as a natural defense against fungal infections.

The initial research presented in this dissertation was done in collaboration with Dr. Jeffery Wilkinson of our laboratory. It involved the final sequencing and analysis of the 16,007 bp of cotton genomic DNA depicted in the physical maps shown in Appendix A. A major component of the doctoral research of Dr. Wilkinson was the isolation of a cotton osmotin gene cluster containing two actual genes (*OSMI* and *OSMII*) and two pseudogenes (*OSMIII* and *OSMIV*) (Wilkinson, 2003; Wilkinson et al., 2005). The osmotin genes have a wide variety of potential promoter elements in their 5'-flanking regions that could bind various transcription factors for activation of their expression in different environmental situations (Raghothama et al., 1993; Thomas, 1993; Guilifoyle, 1997; Raghothama et al., 1997). Included in the putative promoter motifs are two presumptive ethylene response elements, which occur in the promoters of numerous PR-protein genes (Nelson et al., 1992; Raghothama et al., 1993 and 1997; Zhou et al., 1997; Kitajima et al., 1998; Jia and Martin, 1999; Kitajima and Sato, 1999; Koyama et al., 2001; Brown et al., 2003). Ethylene has been shown to be a potent inducer for a number of PR proteins (Bol et al., 1990; Linthorst, 1991).

One objective of this dissertation research was to evaluate the expression of the *OSMI* and *OSMII* genes in terms of the osmotin mRNA and protein expression patterns potentially up-regulated by inducers such as ethephon (which decomposes upon air oxidation to ethylene), hydrogen peroxide, and sodium chloride. These elicitors were chosen due to the presence of prospective ethylene-response elements in the 5'-flanking promoter regions of the *OSMI* and *OSMII* genes (Wilkinson, 2003; Wilkinson et al., 2005); because hydrogen peroxide is one of the first reactive oxygen species produced during the hypersensitive response (Hancock et al., 2002); and because the

tobacco osmotin gene has been shown to be induced by salt (Singh et al., 1987; LaRosa et al., 1992; Nelson et al., 1992; Raghothama et al., 1993 and 1997).

While working with Dr. Wilkinson, total RNA extracted from leaf, stem, and root tissues of cotton plants, treated with water as control, 100 mM hydrogen peroxide, or 1 mM ethephon for a period of 0, 1, or 2 h, were examined by Northern blot hybridization using a homologous cotton osmotin probe. After analyzing the autoradiograms, it appeared that the osmotin mRNAs were produced in all three tissues (leaf, stem, and root) under all three treatments (water, hydrogen peroxide, and ethephon), with the signal becoming the most intense after two hours of treatment.

Because the *OSMI* and *OSMII* genes have an identity of 92% (Wilkinson, 2003; Wilkinson et al., 2005), Northern blot analysis was not specific enough to determine if one or both osmotin mRNAs were present in the total RNA extracts. Therefore, reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was performed to determine which osmotin transcripts were present in the 2 h total RNA extracts using unique oligonucleotide primers designed specifically for the 5'- and 3'-untranslated regions (UTRs) of the osmotin gene sequences. The quality of the RNA samples was assessed by amplifying a cotton housekeeping actin mRNA transcript as a control, which was about the same intensity in all samples. Both the 788-bp *OSMI* and 789-bp *OSMII* RT-PCR products were generated from all of the 2 h RNA extracts, as shown in Figure 4. From sequence analysis of both strands of both the *OSMI* and *OSMII* products, the RT-PCR product generated with the *OSMI* gene-specific primers was confirmed to be identical with the *OSMI* open reading frame. The RT-PCR product generated with the *OSMII* primers was determined to contain two products, a product

from the *OSMII* transcript and a presumptive new product derived from a different osmotin transcript, now designated *OSMV*. Therefore, initial RT-PCR analyses revealed that the *OSMI*, *OSMII*, and *OSMV* mRNAs appear to be expressed under similar conditions in cotton plants, which was expected because they may have similar potential promoter elements, meaning they should be up-regulated by similar inducing agents. However, it was surprising that the osmotin transcripts appeared in the control leaf and stem extracts treated with water.

To determine if the presence of the osmotin mRNAs is constitutive, and not induced by growth or experimental conditions, total RNA isolated from control plants grown under more stringent conditions, neither treated nor placed in plastic bags, were also subjected to RT-PCR analyses. From this set of experiments, it was determined that apparently no osmotin mRNAs are present in leaf or stem tissues. However, RT-PCR products for both the *OSMI* gene and *OSMII* gene transcripts were detected in roots (Figure 8). This observation revealed an expression pattern of the osmotin transcripts that was more in agreement with present literature (Linthorst, 1991; LaRosa et al., 1992; Vigers et al., 1992; Sato et al., 1996; Kitajima et al., 1998; Kitajima and Sato, 1999; Okushima et al., 2000), and would indicate that previous growth conditions might be affecting the production of osmotin mRNAs. Several laboratories have shown that PR proteins are constitutively expressed only in the roots of healthy plants, but that ethylene treatment causes a rapid induction in leaves (Linthorst, 1991; LaRosa et al., 1992; Vigers et al., 1992; Sato et al., 1996; Kitajima et al., 1998; Kitajima and Sato, 1999; Okushima et al., 2000). The constitutive expression of PR proteins in roots may be of significance for plant defense, because roots are typically surrounded by soil

containing numerous microorganisms. Thus, plants are likely defending themselves by having a preexisting defense mechanism in place, in addition to an induced defense process in other tissues (Kitajima and Sato, 1999).

For a more thorough examination of the *OSMI* and *OSMII* mRNA expression levels, real time RT-PCR was used to determine their induction curves (Giulietti et al., 2001; Livak and Schmittgen, 2001; Wilhem and Pingoud, 2003; Bustin and Nolan, 2004; Wong and Medrano, 2005). Although Northern blot hybridization and RT-PCR are commonly used for the quantitation of mRNA, real time RT-PCR is much more accurate and sensitive; it is normally used for the relative estimation of mRNA levels. Total RNA extracts from leaf, stem, and root tissues of cotton plants treated with water (as control) or 1 mM ethephon for a period of 0, 1, 2, 3, 4, 8, 12, 24, 48, or 96 h were examined to determine the time periods and levels of osmotin mRNA induction by ethephon. It seems that both *OSMI* and *OSMII* mRNAs are induced upon treatment with 1 mM ethephon, with the *OSMII* mRNA being affected the most in all tissue types examined. There seems to be a considerable induction in *OSM* transcript levels 2 to 4 h after treatment with ethephon. As seen in Figures 9 through 11, the levels of osmotin mRNAs appear to go up 12 h after treatment, go back down, and then back up again. The greatest increase in expression is seen in the leaf and stem tissues. This may be due to the constitutive presence of the osmotin transcripts in the root tissues.

LaRosa et al. (1992) demonstrated that osmotin gene expression was post-transcriptionally regulated, showing that some strong inducers of mRNA do not lead to comparable protein accumulation. Therefore, one aspect of this dissertation work involved analyzing the OSMI and OSMII protein expression patterns upon treatment

with various inducers. As shown in Figure 12, a band corresponding to a 24-KDa protein was detected using the anti-osmotin antibody preparation in Western blot analyses of crude protein extracts from cotton plants treated with various chemical inducers (1 mM ethephon, 550 mM NaCl, or 100 mM hydrogen peroxide). The corresponding protein was not detected in Western blots of crude cotton extracts treated with water as control. It should be noted that the 24-KDa protein band was the most intense after treatment with ethephon, followed by the corresponding band after treatment with hydrogen peroxide. This is likely due to ethylene being a specific signaling molecule for pathogen stress, whereas hydrogen peroxide is a general response signal involved in the hypersensitive response to pathogen infection (Hancock et al., 2002). The 24-KDa presumptive osmotin protein band was the least intense after being induced with sodium chloride treatment, which would be likely, because cotton is known to be a salt-tolerant species (Flowers et al., 1977).

The induction of the cotton osmotin proteins was then studied by comparing total protein extracts from cotton plants treated with either water (as control) or 1 mM ethephon for a period of 0, 4, 8, 12, 16, 20, 24, 48, and 96 h. Using the anti-osmotin antibody, Western blot analyses detected the presence of a band corresponding to the 24-KDa mature osmotin isoforms in the ethephon-treated extracts, but the band did not occur in the control extracts, shown in Figure 13. The presumed 24-KDa osmotin protein(s) seemed to be up-regulated 4 h after treatment with ethephon and down-regulated 96 h after treatment. There were also two smaller protein bands, estimated to contain proteins about 15 and 20 KDa in size, in the total protein extracts from both the control and ethephon-treated plants. These two bands are thought to be two predicted

polypeptides of 14.0 and 19.5 KDa that could result from initiation at internal AUG translation start codons in both the *OSMI* and *OSMII* mRNA open reading frames (Wilkinson et al., 2005).

The polyclonal antibody preparation used in the Western blot analyses was prepared from an antigenic oligopeptide identical in sequence to the last 18 amino acids of the C-terminal end of the OSMI polypeptide (Wilkinson, 2003; Wilkinson et al., 2005). Because the putative mature isoforms of the osmotin protein are practically the same size (23,885 KDa for OSMI versus 23,984 KDa for OSMII) and have nearly identical C-terminal regions (with only two amino acid variations), the presence of both osmotin isoforms can only be presumed from Western blot analyses of one-dimensional (1-D) SDS-PAGE, which separates proteins on the basis of size. To determine that both osmotin proteins are indeed being induced, two-dimensional (2-D) gel electrophoresis was used to first separate the proteins by charge by their isoelectric points (7.92 for OSMI and 7.56 for OSMII), and then by their size.

After analyzing the first set of separated proteins with The Discovery Series™ PDQuest™ 2-D Analysis Software (Bio-Rad) and Western blot analysis, a row of protein spots at approximately 24 KDa was detected in the ethephon-treated samples, but these 24-KDa protein spots were lacking in the control samples, shown in Figures 15 through 17. A 14-KDa protein spot and a 19-KDa protein spot were detected in both the control and ethephon-treated samples, which are thought to be two predicted polypeptides of 14.0 and 19.5 KDa that would result from internal translation AUG start codons in both the *OSMI* and *OSMII* mRNA coding regions. Also, seen in both the

control and ethephon-treated extracts were a 40-KDa protein spot and a row of protein spots at 80 KDa.

A total of 15 protein spots, marked in Figure 18, were extracted from this first set of 2-D gels. Twelve of the protein spots were approximately 24 KDa. These protein spots were either present only in the ethephon-treated sample, or they were induced after ethephon-treatment. The other protein spots excised from the gels corresponded to the 40-KDa protein and two proteins from the row of 80-KDa proteins present in both the control and treated extracts. These 15 selected protein spots were sent to Dr. Bill Russell of the Chemistry Department Laboratory for Biological Mass Spectrometry at Texas A&M University, College Station, TX, for mass spectrophotometric sequence analysis. Unfortunately, there were no identifications made from any of the protein samples analyzed due to a lack of protein in the protein spots. Dr. Russell's mass spectrometry facility has had a high success rate with protein spots in Coomassie R-250-stained gel slices, but protein spots in silver stained-gel slices (and therefore SYPRO stained-gel slices) are more difficult to analyze, because they are much more sensitive than Coomassie stains (Patton, 2002; Rose et al., 2004).

The next set of protein separations was done using a much larger amount of total protein, and the separated proteins were stained with Bio-Safe™ Coomassie G-250 Stain, which is more sensitive than Coomassie Brilliant Blue R-250 (http://www.bio-rad.com/LifeScience/pdf/Bulletin_2423.pdf), but it does not require the use of methanol and acetic acid fixatives for destaining, which causes gel shrinkage and makes subsequent alignment difficult. As shown in Figure 20, Western blot analyses of the second set of 2-D gels revealed a row of protein spots at approximately 24 KDa in the

ethephon-treated samples but not in the control samples. Protein spots at 14 KDa, 19 KDa, 40 KDa, and 80 KDa were again visualized on the Western blots of both the control and treated extracts. From this second set of 2-D gels, seven 24-KDa protein spots were excised and given to Mr. Prem Adhikari of Dr. Barney Venables' laboratory of the Biological Sciences Department at the University of North Texas for sequence analysis. Unfortunately, no identifications could be made from any of the protein samples analyzed.

Two-dimensional gel electrophoresis is a valuable tool in proteomics research, as it is able to simultaneously resolve thousands of proteins in a complex mixture (Choe and Lee, 2003). Sample preparation is one of the most important factors in 2-D gel electrophoresis, which presents a problem with plants due to their relatively low protein concentrations as compared to bacterial or animal tissues, and also due to the presence of proteases and materials that interfere with protein separations and subsequent analyses (i.e. cell wall and vacuole components, storage polysaccharides, lipids, phenolic compounds, carbohydrates, and numerous secondary metabolites) (Saravanan and Rose, 2004; Carpentier et al., 2005; Isaacson et al., 2006). Although there have been many advances in the field of plant proteomics, there are still several classes of proteins that are not well represented on 2-D gels, including those that are expressed at low levels, hydrophobic or very basic proteins, and very small or very large proteins (Gygi et al., 1999; Gőrg et al., 2000; Harry et al., 2000; Rossignol, 2001; Rose et al., 2004). Therefore, the failure to obtain sufficient protein for sequence analysis is most likely due to the somewhat hydrophobic nature and the presence of eight disulfide bonds of the osmotin proteins, and the fact that they are likely masked by the more

abundant soluble proteins, such as Rubisco (ribulose bisphosphate decarboxylase/ oxygenase), which is the most abundant protein in the world and can comprise up to half of the total leaf protein in some plant species (Robertson et al., 1997; Rose et al., 2004; Saravanan and Rose, 2004).

To overcome this problem, a sequential extraction of proteins using a series of different solvents can be used to decrease protein complexity and enhance the detection of low abundance proteins without being contaminated by the more abundant soluble proteins (Robertson et al., 1997; Rose et al., 2004; Saravanan and Rose, 2004). A disadvantage of this type of multi-step extraction procedure involves reproducibility and sample loss. Another method to isolate low abundance proteins is to separate the protein mixture on narrow pH range gels, such as the pH 6.3-8.3 IPG strips used in the last 2-D separations. Separating total protein mixtures on a narrow-range isoelectric focusing pH gradient helps relieve the problems of only being able to see a small percentage of the proteome due to insufficient spatial resolution and also the difficulty of detecting low copy number proteins in the presence of the most abundant proteins (Gőrg et al., 2000; Harry et al., 2000). Although the identity of the osmotin isoforms was not confirmed by mass spectrophotometric sequence analysis, the Western blot analyses with the anti-osmotin antibody clearly indicate that there are several osmotin proteins in cotton, as shown in Figures 15 through 17 and 20.

The major goal of this dissertation research was to genetically engineer the *OSMI* and *OSMII* genes to routinely overproduce their antifungal proteins in transgenic cotton plants as a natural defense against fungal infections. Because cotton transformation and regeneration is quite labor-intensive and time-consuming, requiring

a year or more for completion, *Arabidopsis thaliana* was used as a model plant system because it is easy to grow, and relatively simple techniques for transformation of the plant, such as the floral dip method of Clough and Bent (1998), are routinely used in many laboratories. The initial plan was to evaluate the model *Arabidopsis* plants constitutively overexpressing the cotton osmotin genes for their resistance against various fungal pathogens before transforming cotton, but the transformation of cotton was started prior to analyzing the putative *Arabidopsis* transformants, due to the length of time needed for cotton transformation.

Dr. Jeffery Wilkinson of this laboratory originally engineered the pCAMBIA 2301 binary vector constructs (from the Center for Application of Molecular Biology to International Agriculture (CAMBIA), Canberra, Australia) containing the *OSMI* and *OSMII* coding regions that were used in the transformation of *Arabidopsis* and cotton plants using co-cultivation with *Agrobacterium tumefaciens* (Wilkinson, 2003). Unfortunately, it appears that the outcome of both transformation events was quite dismal. The prospective transgenic *Arabidopsis* plants produced green secondary leaves and a good root system on kanamycin selection media, whereas the other plantlets were yellow and dying (Figure 21). The putative transgenic plants contained the *GUS* second exon within the pCAMBIA 2301 vector as determined by PCR analyses (Figure 22), but they failed to produce PCR products when using amplimer pairs specifically for the osmotin transgenes or to produce any detectable protein bands on the autoradiograms when analyzed by Western blotting using the anti-osmotin antibody preparation.

The outcome of the cotton transformation with the osmotin vector constructs was also negative. As with the putative *Arabidopsis* transformants, the supposed transgenic cotton plants produced expected PCR products using amplimer pairs for the *GUS* second exon from the pCAMBIA 2301 vector (Figure 29), the constitutive *SadI* gene, and the endogenous cotton *OSMI* gene. However, the plants failed to generate any PCR products at all when using an amplimer pair designed specifically for the osmotin transgenes. After talking with Dr. Kent Chapman, our colleague in Biological Sciences at the University of North Texas, leaf samples from the putative transgenic cotton plants and a cv. Coker 312 wild type plant, as well as purified pCAMBIA-35S-*OSMI* #16 plasmid construct DNA for use as a positive control, were sent to BioDiagnostics, Inc. (River Falls, WI; http://www.biodiagnostics.net/). This is a company that specializes in the screening of individual seeds or plants for the presence of prospective transgenes. Unfortunately, all of the putative transformed cotton samples tested negative for the osmotin transgenes, while the plasmid control generated a very strong positive signal. An internal control was run on all of the samples in order to verify the presence of high-quality DNA. Of the 35 samples that were sent, 24 produced strong internal control bands indicating high-quality DNAs, while 11 samples did not.

Based on these results and the fact that part of the pCAMBIA 2301 vector DNA could be amplified in the putative transgenic plants, it would seem that some aberrant recombination or DNA structural rearrangement events could have occurred within the vector construct, either upon transformation into the *Agrobacterium* cells or upon transformation and integration of the construct DNAs into the cotton plant chromosomal DNA. In an attempt to prove that an anomalous DNA recombination or rearrangement

event may have occurred, amplimer pairs designed to amplify the 3'-half of the osmotin gene and the insert-vector DNA junction corresponding to the 3'-untranslated region (3'-UTR) of the CaMV polyA terminator region. Sadly, these PCR assays also turned out negative, while the plasmid control produced a strong positive signal.

Kanamycin resistance tests were performed to detect the expression of the *neomycin phosphotransferase* II (*npt*II) gene, as done by Wu et al. (2005) and Zapata et al. (1999), from the pCAMBIA 2301 vector, using a different approach to verify that the vector constructs with the osmotin genes were possibly integrated into the chromosomal DNAs of the assumed transgenic cotton plants. After establishing the lowest concentration of kanamycin that would affect wild type cv. Coker 312 plants (results shown in Figure 26), 1.0 and 2.0% kanamycin solutions were applied to the leaves of a cv. Coker 312 wild type plant and nine putative transgenic cotton plants. Ten days after application, the leaves were cut from the plants and analyzed. The kanamycin bleached almost the entire leaf of the wild type plant, and all but two of the putative transgenics were unchanged, indicating the expression of the *npt*II gene and thus cotton plants potentially transformed with the pCAMBIA 2301 vector (shown in Figure 27). It should be noted that the two potential transgenic plants having slight discolorations had several discolored leaves and appeared to be stressed from other conditions before the experiment. Therefore, the results obtained from the PCR analyses and the kanamycin resistance tests indicated that the pCAMBIA 2301 vector was integrated into the genomic DNA of the putative cotton plants. However, there was still concern due to the inability to amplify the osmotin transgenes from the plants by PCR analyses.

To resolve this issue, and to determine the reiteration frequency and arrangement (clustering or dispersion in the genome) of the putative osmotin transgenes, genomic blot analyses were performed in duplicate on each of the supposed transgenic cotton plants, as well as a cv. Coker 312 wild type plant and pCAMBIA-35S-*OSMI* #16 plasmid vector DNA as controls. Two separate ^{32}P-labeled oligonucleotide probes were used: one specific for hybridization to a region of the *GUS* second exon and the other specific for hybridization to the endogenous cotton *OSMI* gene. The plasmid and genomic DNAs were digested with the restriction enzyme *Nde*I for the first set of genomic blot analyses.

As shown in representative autoradiograms in Figures 32 through 35, the results were ambiguous and somewhat difficult to interpret. The ^{32}P-labeled *OSMI* probe hybridized to four cotton genomic *NdeI* DNA fragments of about 3 kb, 7 kb, 8, kb, and 10 kb for all of the cotton genomic DNA samples. After analysis of the 16-kb DNA sequence encompassing the osmotin gene cluster from this laboratory (Wilkinson, 2003; Wilkinson et al., 2005), it was deduced that the 3-kb *Nde*I genomic fragment contained the *OSMI* gene, that the 7-kb *Nde*I genomic fragment encompassed the *OSMII* gene, and that the 8-kb and 10-kb *Nde*I genomic fragments encompassed two other cotton osmotin genes. This observation of four *Nde*I genomic fragments representing four similar osmotin genes in the cotton genome is consistent with the previous genomic blotting results of four similar osmotin genes in the cotton genome (Wilkinson, 2003; Wilkinson et al., 2005). However, no obvious *Nde*I genomic fragments were detected from any of the presumptive transgenic cotton plants that would correspond to any possible osmotin transgenes.

The cotton plants supposedly containing the *OSMI* transgene yielded four different profiles when probed with the ^{32}P-labeled *GUS* second exon A probe. All of the tentative *OSMI* transgenic plants depicted in Figures 32 and 33 appeared to have originated from the same cotyledon callus, as they all had a hybridizing *GUS Nde*I genomic fragment of 10 kb (corresponding to the 10-kb *OSMI* fragment), as well as two other larger fragments that did not correspond to any band on the blot with the *OSMI* probe. The putative *OSMI* transgenic plants originating from hypocotyl calli, as shown in Figures 33 through 35, appear to belong to three separate lines. Fourteen of the tentative *OSMI* transgenic plants from hypocotyl callus had a hybridizing *GUS Nde*I genomic fragment of 7 kb (corresponding to the 7-kb *OSMI* fragment). Two of the supposed *OSMI* transgenic plants from hypocotyl callus had hybridizing *GUS Nde*I genomic fragments of 7 kb and 10 kb (corresponding to the 7-kb and 10-kb *OSMI* fragments), as well as other hybridizing *GUS Nde*I fragments that did not correspond to any *OSM* fragments. One tentative *OSMI* transgenic plant from a hypocotyl callus had a hybridizing *GUS Nde*I fragment that did not correspond to any hybridizing *OSMI Nde*I fragment.

There was only one supposed transgenic plant containing the *OSMII* transgene, with an 8-kb *Nde*I fragment that hybridized with the *GUS* probe (corresponding to the 8-kb *OSMI* fragment). The cotton plants presumably containing the empty vector yielded three different hybridizing patterns. Three empty vector plants, shown in Figures 33 through 35, had a hybridizing *GUS Nde*I fragment that did not correspond to any hybridizing *OSM* fragment. One empty vector plant, shown in Figure 32, had two hybridizing *GUS* fragments that did not correspond to any hybridizing *OSMI* fragment.

The other empty vector plant, shown in Figure 35, had one hybridizing *GUS Nde*I fragment of 8 kb (corresponding to the 8-kb *OSMI* fragment).

Because no definitive hybridizing osmotin transgene fragments were detected and because several hybridizing *GUS* fragments seemed to actually overlap and might have coincidentally corresponded with some of the hybridizing *OSMI Nde*I fragments, it appeared that none of the putative transgenic cotton plants contained the osmotin transgenes. To confirm these negative results, cotton genomic DNA samples from each putative transgenic line and a cv. Coker 312 wild type plant were chosen for a second series of genomic blot analyses using the two ^{32}P-labeled *GUS* second exon A and *OSMI* probes. Each of the DNA samples were digested with the restriction enzymes *Nde*I + *Afl*II, *Ase*I, and *Nsi*I to obtain different restriction cleavage patterns.

The representative autoradiograms from this second series of genomic blot analyses are shown in Figures 36 through 38. When the cotton DNA extracts were digested with *Nde*I and *Afl*II together, the ^{32}P-labeled *OSMI* probe hybridized to four cotton genomic DNA fragments of about 3.2 kb, 4.1 kb, 4.4 kb, and 7.1 kb for the wild type and empty vector cotton genomic DNA samples, as shown in Figure 36. From the 16-kb DNA sequence encompassing the osmotin gene cluster from this laboratory (Wilkinson, 2003; Wilkinson et al., 2005), it was deduced that the 3.2-kb *Nde*I/*Afl*II genomic fragment contained the *OSMI* gene, that the 4.1-kb *Nde*I/*Afl*II genomic fragment encompassed the *OSMII* gene, and that the 4.4-kb and 7.1-kb *Nde*I/*Afl*II genomic fragments encompassed two other cotton osmotin genes. Again, this observation of four *Nde*I/*Afl*II genomic fragments representing four similar osmotin genes in the cotton genome is consistent with the previous genomic blotting results of

four similar osmotin genes in the cotton genome (Wilkinson, 2003; Wilkinson et al., 2005).

When the tentative *OSMI* transgenic plants from the hypocotyl calli were digested with these same two enzymes, they yielded five prominent hybridizing fragments (the same four as above and an additional 8.9-kb fragment). Two of the putative *OSMI* transgenic plants from cotyledon callus, shown in Figure 37, also produced this same hybridizing pattern of five *Nde*I/*Afl*II hybridizing fragments, but they were very faint. There also appeared to be very faint bands corresponding to this extra 8.9-kb fragment for three of the supposed transgenic plants when hybridized with the *GUS* probe. All of the tentative *OSM* transgenic plants from the cotyledon and hypocotyl calli also produced a 3.2-kb *Nde*I/*Afl*II *GUS* fragment (corresponding to the 3.2-kb *OSMI* fragment). The three empty vector plants, shown in Figure 36, all displayed a single fragment of 4.0 kb when hybridized with the *GUS* probe.

When the cotton genomic DNA samples were digested with *Ase*I, all of the wild type and putative transgenic plants yielded the same pattern of three hybridizing fragments with the ^{32}P-labeled *OSMI* probe: 1.6 kb, 1.9 kb (probably encompassing the *OSMI* gene), and 2.9 kb (probably encompassing the *OSMII* gene). The ^{32}P-labeled *GUS* probe hybridized to a 1.9 kb *Ase*I fragment (corresponding to the 1.9-kb *OSMI* fragment thought to contain the *OSMI* gene) for all of the cotton plants supposedly transformed with the osmotin transgenes. This probably has no real significance. The cotton plants presumably containing the empty vector yielded two different hybridizing patterns. Two of the transgenic plants from the hypocotyl callus with the empty vector, shown in Figure 36, had a single hybridizing *GUS Ase*I fragment of 2.4 kb. The other

transgenic plant from the hypocotyl callus with the empty vector, also shown in Figure 36, had a hybridizing *GUS Ase*I fragment of 1.5 kb.

When the cotton genomic DNA samples were digested with *Nsi*I, all of the wild type and putative transgenic plants yielded the same two hybridizing fragments with the ^{32}P-labeled *OSMI* probe: 2.4 kb (probably encompassing the *OSMII* gene) and 6.7 kb (probably encompassing the *OSMI* gene). Unfortunately, none of the hybridizing *GUS* fragments observed on the autoradiograms matched up with any of the hybridizing *OSM* fragments. Two empty vector plants, shown in Figure 36, had a hybridizing GUS *Nsi*I fragment of 4.0 kb, while the other empty vector plant yielded a 6.9-kb fragment.

Although the extra 8.9-kb hybridizing fragment from the *Nde*I/*Afl*II digests seemed promising, these were most likely partial fragments due to incomplete digestion of the genomic DNAs with one of the two enzymes in the double digestions, because no extra corresponding bands were seen for the *Ase*I or *Nsi*I digests. Therefore, the genomic blotting experiments indicated the complete absence of the intact osmotin transgenes in the supposed transgenic cotton plants, but they did indicate the presence of the *GUS* fragment from the vector cassette in the prospective transgenic cotton plants. Thus, it appears as though an anomalous DNA structural rearrangement or recombination artifact occurred in the vector constructs during the cloning or transformation process.

Because all attempts made to verify the presence of the transgenes in the putative *Arabidopsis thaliana* and cotton transgenic plants failed, the plasmid vector DNAs were isolated from the original transformed *E. coli* DH5α cells and *Agrobacterium tumefaciens* LBA4404 cells and analyzed by PCR and restriction digestion analyses.

PCR and subsequent sequence analysis confirmed that the *Agrobacterium* stocks contained the cloned osmotin transgenes. As another confirmation, the plasmid DNAs were digested with the restriction endonuclease *Sal*I, which cleaves the pCAMBIA vector only once. As shown in Figure 39, the plasmid construct DNAs isolated from the transformed *E. coli* cells were clearly intact, yielding the single expected 11.6-kb restriction fragment for the empty vector construct and the single expected 13.0-kb restriction fragment for both the *OSMI* and *OSMII* vector constructs. However, the plasmid construct DNAs isolated from the transformed *Agrobacterium* cells generated a 50-kb restriction fragment for the empty vector and *OSMII* constructs, and multiple restriction fragments for the *OSMI* construct. These strange restriction patterns indicated that some aberrant DNA structural rearrangement or recombination event likely occurred to the construct DNAs at some point after they were transformed into the *Agrobacterium* cells, as discussed above in the PCR assay results from BioDiagnostics, Inc.

To address this issue, the original plasmid DNA constructs isolated from *E. coli* DH5α cells were re-transformed into fresh ElectroMAX™ *Agrobacterium tumefaciens* LBA4404 Cells. Isolated colonies were selected from streptomycin/kanamycin selection plates and subjected to colony PCR using amplimer pairs for the *GUS* second exon to confirm the presence of the transgenes. The colonies producing PCR products (Figure 40) were then inoculated and grown in streptomycin/kanamycin selection media, and the plasmid DNAs were isolated using the modified protocol of Li et al. (1995). The plasmid vector DNAs isolated from the cells of this second *Agrobacterium* transformation, the *OSMI* plasmid DNA from the transformed *E. coli* DH5α cells, and the plasmid

DNAs isolated from the original transformed *Agrobacterium* cells, were doubly digested in duplicate with the restriction enzymes *Bam*HI and *Kpn*I, which should cleave the 1.4-kb osmotin inserts from the pCAMBIA vector constructs. The restriction digests of the plasmid DNAs were analyzed by alkaline blot analysis using the ^{32}P-labeled *OSMI* and *GUS* second exon A probes.

The results for the newly transformed *Agrobacterium* cells harboring the vector constructs were almost as expected, with the *Bam*HI/*Kpn*I double digests excising the 1.4-kb osmotin gene inserts from the *OSMI* and *OSMII* pCAMBIA 2301 constructs. The autoradiograms of the alkaline blots with the ^{32}P-labeled *OSMI* gene and *GUS* second exon A probes are shown in Figure 41. In the left panel of Figure 41, the 1.4-kb *Bam*HI/*Kpn*I fragments encompassing the *OSMI* gene and *OSMII* gene clearly hybridize to the *OSMI* probe, and the 11.6-kb *Bam*HI/*Kpn*I fragment encompassing the *GUS* gene does not hybridize to the *OSMI* probe. Also, the 11.6-kb empty pCAMBIA vector fragment containing the *GUS* gene does not hybridize to the *OSMI* probe. In the right panel of Figure 41, the 11.6-kb *Bam*HI/*Kpn*I fragment hybridizes to the *GUS* probe but does not hybridize to the 1.4-kb *Bam*HI/*Kpn*I fragments encompassing the *OSMI* and *OSMII* genes. Also, the 11.6-kb empty pCAMBIA vector fragment containing the *GUS* gene does hybridize to the *GUS* probe. The sizes of the osmotin vector constructs are 13 kb, as expected. Surprisingly, an aberrant, minor hybridizing 19-kb *Bam*HI/*Kpn*I fragment was observed with the *GUS* probe, which is much larger than the 13-kb size of the osmotin vector constructs. The weakly hybridizing 19-kb *Bam*HI/*Kpn*I fragment is also observed in the alkaline blot of the original transformed *Agrobacterium* cells with the *OSMI* and *OSMII* pCAMBIA constructs.

The results for the original transformed *Agrobacterium* cells with the *OSMI* and *OSMII* pCAMBIA constructs were quite disappointing. As shown in Figure 42, aberrantly-sized 19-kb and 25-kb *Bam*HI/*Kpn*I hybridizing fragments were detected for the *OSMI* and the *OSMII* vector constructs when probed with the *GUS* probe, and the *OSMII* construct DNAs looked identical to the empty vector construct DNAs. More alarming was the failure to detect any hybridizing fragments using the *OSM* probe for either of the *OSMI* or *OSMII* vector constructs. Because the linearized plasmid vectors should only be 13 kb in size, it seems as though an anomalous DNA structural rearrangement or recombination occurred in the *Agrobacterium* cells. In particular, the 25-kb *Bam*HI/*Kpn*I fragment would appear to have arisen by an anomalous dimerization event, with concomitant excision of the osmotin gene cassettes that were inserted into the empty vectors. However, these results are very odd, considering that we have previously been able to PCR amplify and sequence the osmotin gene cassettes from the *OSMI* and *OSMII* vector constructs transformed into the original *Agrobacterium* cells. Even though the *Arabidopsis* and cotton plants were apparently transformed with the pCAMBIA 2301 vector constructs, the overall outcome was unsuccessful because the osmotin proteins were not expressed in the putative transgenic plants.

Although this lack of success in transforming the *Arabidopsis* and cotton plants with the osmotin vector constructs appears to have been attributed to a DNA structural rearrangement or recombination event that occurred in the transformed *Agrobacterium* cells, many researchers have reported that the transformation of cotton is quite difficult overall, partly due to the amount of time and labor involved (Zapata et al., 1999; Sawahel, 2001; Sunilkumar and Rathore, 2001; Zhang et al., 2001; Wu et al., 2005).

However, the challenges presented for cotton transformation are being met more and more every year. In fact, the control of diseases in modern agriculture is often achieved by genetically engineering plants for a multigene resistance strategy where multiple defense proteins are simultaneously overexpressed for a synergistic enhancement of resistance to pathogens (Ward et al., 1991; Cornelissen and Melchers, 1993; Liu et al., 1994; Jach et al., 1995; Abad et al., 1996; Punja, 2001). This multigene strategy allows the plants to have a broad range resistance to fungal pathogens. Also, fungal pathogens have displayed the ability to rapidly adapt their genetic structure in the face of selection forces (Liu et al., 1994; Punja, 2001). Therefore, the overexpression of multiple PR proteins, instead of one, could lessen the likelihood that the pathogens will evolve an adaptive strategy to defend against these defensive proteins (Jach et al., 1995; Punja, 2001).

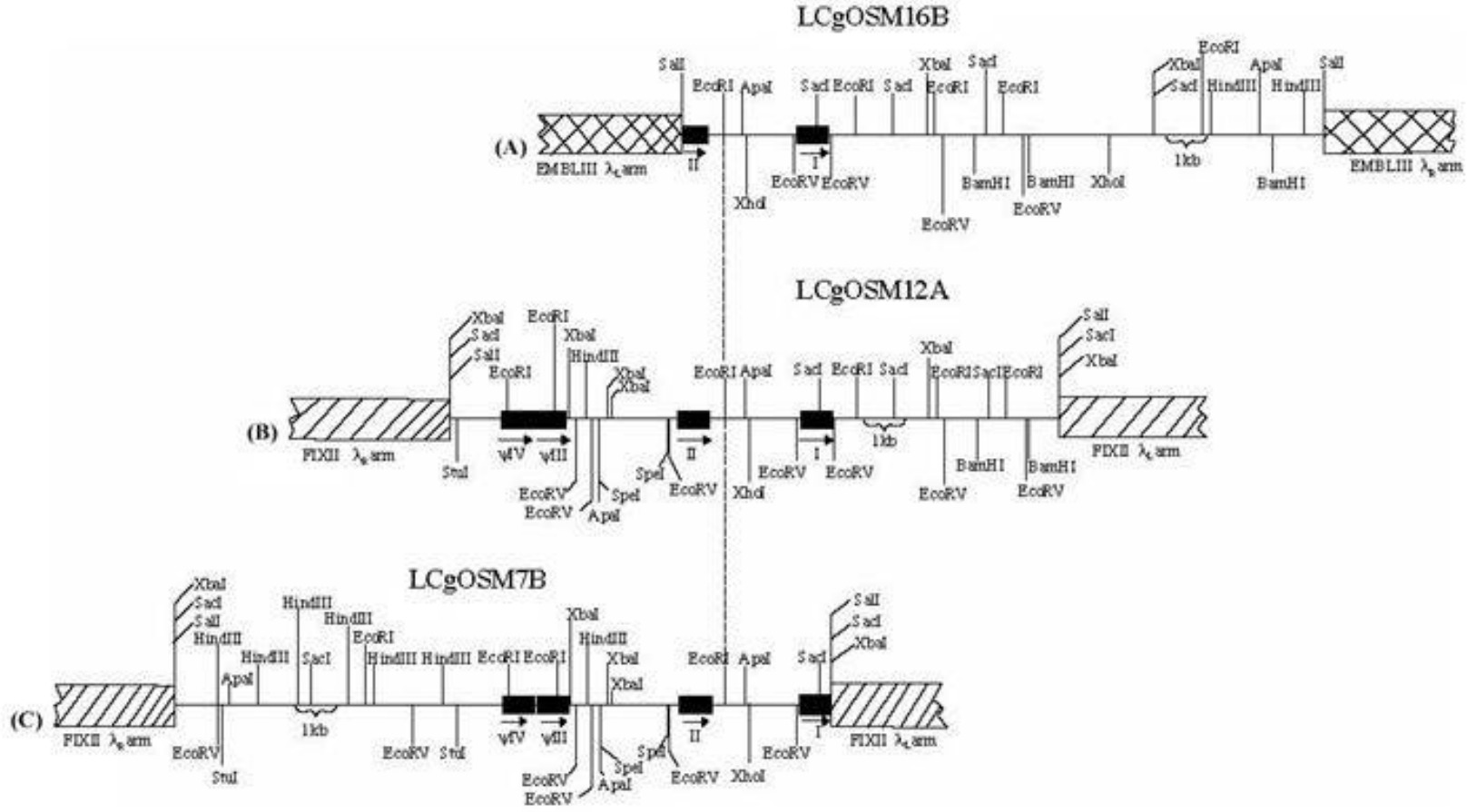
Plants are of great importance for medicine, pharmaceutical sciences, agriculture, and food production. So an important goal in breeding improved crop plants is to produce crops that are resistant to fungal pathogens. Although genetically engineering plants to be more resistant to fungal attack is a major advantage for agriculture, it is a subject of extensive and often very critical public debate (Stewart et al., 2000; Barz and Oksman-Caldentey, 2002). Some of the major challenges to the acceptance of genetically modified crops include the potential spread of the transgenes to closely related weedy species and the impact on their ecology ; the possibility of effecting beneficial microorganisms; the potential health risks of the overexpression of novel proteins in foods; and, as mentioned above, the possibility of promoting the evolution of pathogen strains to become resistant to the novel engineered trait (Stewart

et al., 2000; Punja, 2001; Breiteneder, 2004). Despite the risks that genetically modified plants present, plant transformation should be considered an indispensable tool for the improvement of valuable crops, such as cotton.

APPENDIX

PHYSICAL MAPS OF THREE OVERLAPPING COTTON GENOMIC CLONES CONTAINING A CLUSTER OF TWO OSMOTIN GENES (*OSMI* AND *OSMII*) AND TWO OSMOTIN PSEUDOGENES (*OSMIII* AND *OSMIV*)

(Wilkinson, 2003; Wilkinson et al., 2005)


LCgOSM16B
(A)
EMBLIII λ arm
II
I
1kb
EMBLIII λ arm
LCgOSM12A
(B)
FIXII λ arm
ψIV
ψIII
1kb
FIXII λ arm
LCgOSM7B
(C)
FIXII λ arm
1kb
ψIV
ψIII
FIXII λ arm

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