GENETIC AND MOLECULAR CHARACTERIZATION OF *OSMII* OVER-EXPRESSING TRANSGENIC UPLAND COTTON PLANTS

Shanmukh Salimath
Swati Tripathy
Kent Chapman
University of North Texas
Denton, TX
David Kerns
Texas AgriLife Research and Extension Center Lubbock
Lubbock, TX

Abstract

With the main objective of engineering cotton plants for improved tolerance to biotic and abiotic stress, upland cotton *(Gossypium hirsutum* L. cv Coker 312) was engineered to constitutively over-express one of its own inducible osmotin genes (*OSMII*). Osmotin or osmotin like proteins have been shown to provide protection against biotic and abiotic stresses in plants. A modified method utilizing the cotton embryogenic cell lines and *Agrobacterium*-mediated transformation was used to generate four independent transgenic events. The coding sequence of cotton *OSMII* (~800 bp) under the control of the CaMV 35S promoter was engineered into the Gateway binary vector, pMDC32 and pMDC43, for transformation. Transgenic cells and embryos were selected on hygromycin and regenerated plants were transferred to the greenhouse for maturation and seed production.

Transgenic T0 plants and T1 progeny derived from two independent fertile T0 transgenic lines were confirmed as transgenic by PCR amplification of the transgene promoter sequence. Genotyped transgenic T1 seedlings over-expressing *OSMII* were compared with wild type (non-transgenic) Coker 312 seedlings for their tolerance to cotton aphids (*Aphis gossypii*). Preliminary experiments suggest that *OSMII* over-expression confers some tolerance to aphids, slowing their rate of reproduction.

Introduction

Gossypium hirsutum L., commonly called the upland cotton, is an economically important crop that is widely cultivated across the Southern belt of the United States of America. In the United States, cotton is a multibillion dollar industry, and is highly valued for its durable natural fiber and seeds that contain high per cent (up to 20%) edible oil (www.usda.gov, Chapman et al 2001, Jones and King 1996). Destruction of cotton crop worldwide due to biological organisms such as fungi and insects (Davis 2009, Qaim and Zilberman 2003) and abiotic factors, e.g., drought, (Quisenberry et al 1985) can result in huge economic losses. Engineering varieties for the high yield of cotton fiber and modified seed oil have mostly been the driving forces of cotton crop improvement programs for decades (Liu et al 2009).

Plants naturally produce defense proteins called pathogenesis-related (PR) proteins directed against fungal pathogens (Abad et al 1996, Selitrennikoff 2001). Osmotin or osmotin like proteins belong to the class of PR proteins and are made in response to fungal stress or osmotic stress (water deprivation or salt exposure). It may be possible to use the osmotin gene as a defense gene effective against biotic and/or abiotic stresses (Rajam et al 2007). Transgenic potato and rice plants that overproduce osmotin are shown to provide resistance against fungal diseases (Liu et al 1994, Zhu et al 1996). Cotton plants engineered with tobacco osmotin were shown to confer resistance against drought (Parkhi et al 2009). Thus, the main objective of this research is to genetically engineer cottons with one of its own osmotin genes to overproduce the PR protein in transgenic cotton plants to provide natural resistance against biotic (fungal pathogens and/ or insects) and/or abiotic (drought resistance etc.) stresses. In this project we have concentrated on understanding and analyzing the interactions between *Gh. OSMII* engineered cotton plants and cotton aphid pest (*Aphis gossypii*).

Materials and Methods

Gh-OSMII gene construct for plant transformation

Cotton Osmotin coding sequence (*OSMII* - cDNA, 789 bp) was used as a preliminary template for TOPO cloning (Invitrogen, Cat # K2420-20). The PCR product, generated from gene specific primers, (i.e., *OSMII* - forward 5'-CACCTCACCAAGTAAAAACCAACC and *OSMII* - reverse 5'-AATGCAAATCAACTACTCCAAGAG), was used for directional cloning in pENTR TOPO cloning reaction with pENTR TOPO DNA within. The entry construct produced from this reaction was transformed into one shot chemically competent *E. coli* cells. To determine the directional insertion of *OSMII* gene product, the positive *OSMII*-pENTR TOPO clone selected on LB with kanamycin was analyzed by PCR with plasmid DNA purified from single isolated *OSMII*-pENTR TOPO colony and *OSMII* gene specific primers and/or M13 - forward 5'- GTAAAACGACGGCCAG and M13 - reverse (5'-GTCATAGCTGTTTCCTG) primers. The linearized gene product from *OSMII*pENTR TOPO DNA was further used in LR clonase recombination reaction with plasmid DNA from Gateway-compatible destination plant transformation vector, pMDC32 (with no GFP marker) driven by 35S CaMV promoter (Invitrogen, Cat # 12538-013, Curtis and Grossniklaus 2003) to produce the pMDC32-*OSMII* destination clone (selected on LB with kanamycin and analyzed with *OSMII* gene specific and pMDC32 vector specific PCR primers) for introduction into *A. tumefaciens* LB4404 (BIORAD PC module Gene Pulser X Cell Electroporation system). The *Agro-pMDC32-OSMII* gene construct (Fig. 1) was used for plant transformation and over production of osmotin protein in transgenics.

Invitrogen pENTR/SD/D -TOPO entry cloning vector showing directional insertional site for Osmotin gene

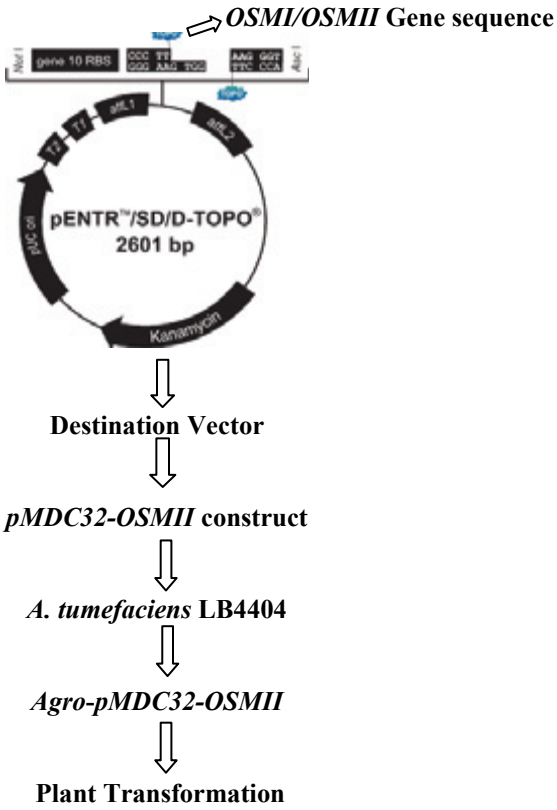

Figure 1. pMDC32-OSMII gene construct for cotton transformation.

Cotton Tissue Culture and Genetic Transformation

Cotton tissue culture and genetic transformation methods, modified from Leelavathi et. al. (2004) and Rathore et. al. (2006), were carried out under two main headings 1. Generation of Embryogenic Cell Lines (ECLs) and 2. Co - culture and selection of transgenics on MSM media with hygromycin B.

1. Embryogenic Cell Lines of Cotton

•Initiated cotton seed germination on MSM (Murashige and Skoog basal salts+D-Maltose) solid media in petriplate(s).
•Transferred germinated cotton seeds to MSM liquid media to aid in cotyledon expansion.
•Harvested expanded leaves and continued culture of cotyledon pieces on MSMK (MSM + KNO3) plates.
•Sub-cultured to fresh medium until callus production.
•Collected Somatic Embryogenic Cells (SECs) from callus cultures (Fig. 2A) and initiated generation of SECs in MSM liquid medium (3 mL per well) in 12 well tissue culture plates on a gyratory shaker (110rpm, 16hr light/8hr dark).
•Multiplied SECs in MSM liquid medium (3mL) in 12 well tissue culture plate for use in transformation experiments.

2. *Agrobacterium*-Mediated Cotton Transformation

•Actively growing cotton Somatic Embryogenic Cell (SEC) lines, maintained at 30ºC, were used for *Agrobacterium* mediated transformation.
•*Agrobacterium tumefactions* strain LBA4404 cells (containing *Gh.-OSMII* gene of interest in binary plant transformation vector pMDC32 and pMDC43) were cultured at 28ºC for 36-40 hours in LB medium with anamycin (50 µg/mL) and streptomycin (100µg/mL).
•SECs and *Agrobacterium* cells were co-cultured in the dark for 48 hours on MSM solid plates layered with sterile filter paper disc and MSM liquid medium containing 100µM acetosyringone.
•SECs, in small (2-5mm) tissue mounds, were transferred to MSMK (MSM + KNO3) solidified media containing carbenicillin (400µg/mL) and hygromycin B (15 and/or 25µg/mL) to select for developing transgenic embryos (Fig. 2B).
•Collected differentiating embryos onto fresh MSMK media with carbenicillin + hygromycin + charcoal (Fig. 3A).
•Transferred plantlets at 2-4 leaf stage to MSM in magenta boxes (Fig. 3B).
•Established and acclimatized plants to soil and then moved them to the greenhouse for seed production. Following these methods we were able to move the T0 transgenics to the greenhouse as soon as 6 months and harvest the T1 seeds within 8 months of co-cultivation.

Generation of Embryogenic Cell line (ECL) and Production of Transgenic Cotton Plants via *Agrobacterium*-Mediated Transformation

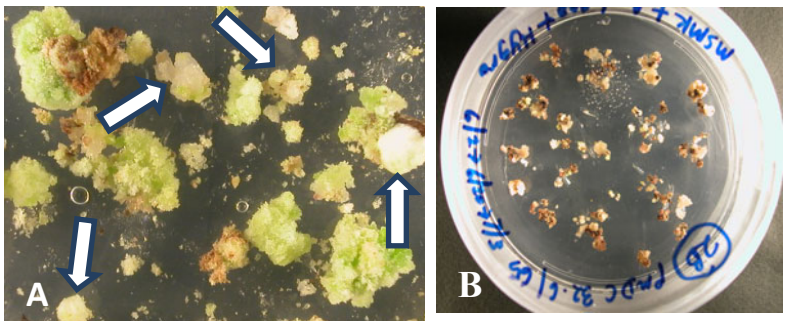

Figure 2. A. MSMK plate with callus/embryogenic cells (EC). Arrows show mass of actively dividing pale green colored embryogenic cells B. *Agro* co-cultured SECs on MSMK + carbenicillin + hygromycin B selection media.

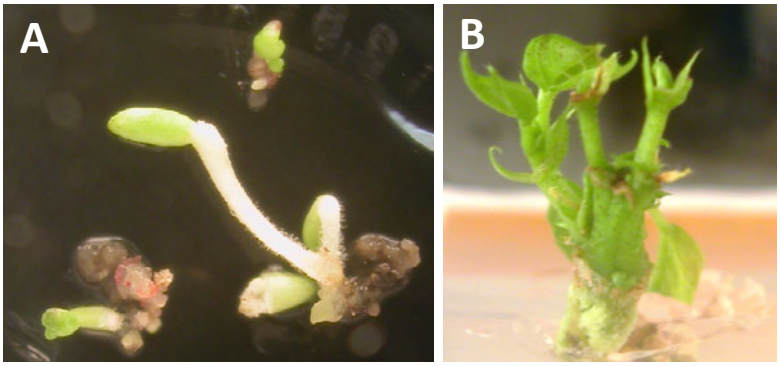

Figure 3. A. Young transgenic plantlet on MSM + charcoal + hygromycin B media. B. Young transgenic plantlet in magenta box with MSM

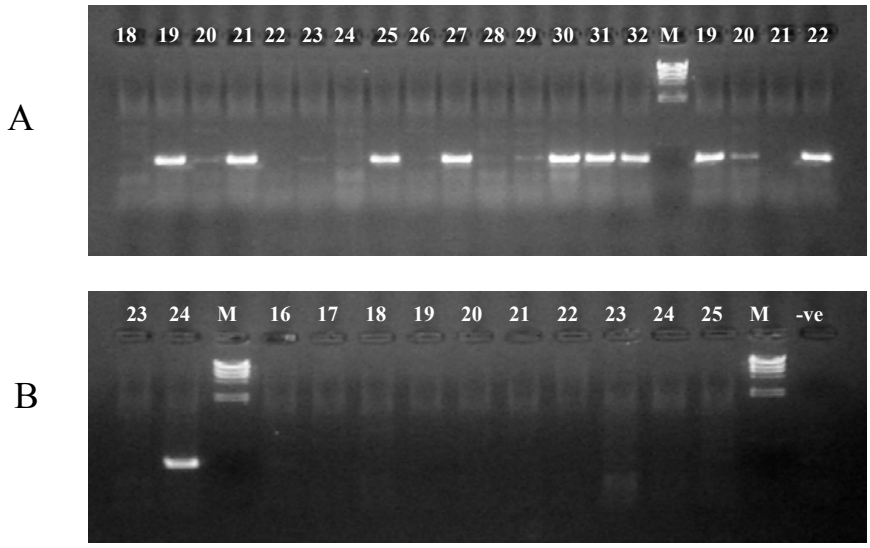

Figure 4. From T1 cotton seeds harvested, about 100 seeds from each of T0 plants (i.e., 2-*OSMII* and 4-*OSMII* progeny) and wild type Coker plant were germinated, seedlings raised and transplanted in to soil. DNA was isolated from youngest leaf punch discs of individual plants using Sigma REDExtract-N-Amp DNA kit. PCR confirmation of transgenics with CaMV35S promoter specific primers (CaMV35SFWD 5'-ACA GTC TCA GAA GAC CAA AGG GCT-3' and CaMV35SREV 5'-GGG TCT TGC GAA GGA TAG TGG GA-3') was carried out (Gel A and B above). Gel A: 2-OSMII progeny #18-32 and 4-OSMII progeny #19-22. Gel B: 4-OSMII progeny #23-24 and Coker wild type plant #16-25. These genetically characterized transgenics and WT Coker plants were subsequently used in plant and aphid interaction studies.

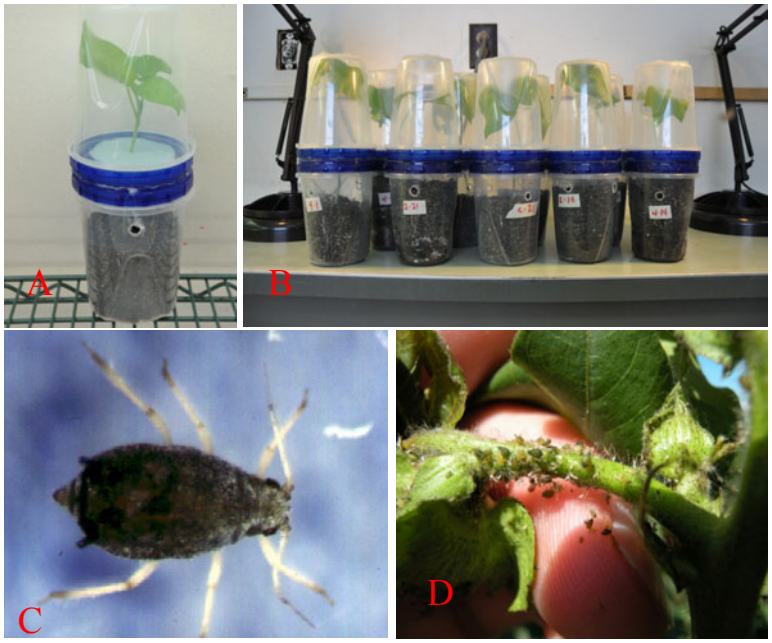

Figure 5. Wild type and transgenic plants (n=8) with 2 to 4 true leaves were transplanted singly into enclosed cages (Fig 5A) and placed in an environmental growth cabinet with a 14:10 L:D photoperiod and a temperature of 20 ± 2°C (Fig 5B). Five reproductively mature cotton aphids, *Aphis gossypii* (Fig 5C), were placed on each plant. After 24 h, all of the adults were removed from the cages, and after 48 h, all but one nymph were removed from each cage (Fig 5D). The remaining nymph was monitored daily and its progeny removed. From these data demographic data was produced as indicators for resistance. Antibiosis was assessed by computing longevity (L), the natural rate of increase (rm), generation time (T), finite daily increase (λ), and doubling time (DT) (Wyatt and White 1977, DeLoach 1974).

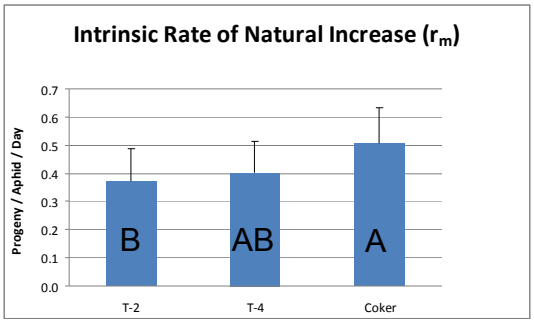

Figure 6. The natural rate of increase (rm) was significantly delayed compared to wild type (Coker 312) for T1 plants of one transgenic line. The other transgenic line trended in the same direction, but was not significantly different from wild type. Bars containing the same letters are not significantly different based on an F protected LSD ($P > 0.10$).

Conclusions

1. Four independent transgenic lines of cotton carrying its own *Osmotin II* gene were produced via *Agrobacterium* – mediated transformation. Plants resistant to hygromycin selection were grown to maturation in the greenhouse. Transgenic nature of T0 plants was confirmed through PCR analysis of genomic DNA.

2. Vegetative growth and development, flowering, fruiting/boll formation and fiber production were absolutely normal in two (2-*OSMII* and 4-*OSMII*) T-zero transgenics, whereas the remaining two T-zeros (1-*OSMII* and 3-*OSMII*) were infertile, and consequently there was no cotton boll formation and/or fiber production.

3. Detailed genotypic analysis of T1 seedlings showed evidence of segregation with reference to transgene. The T1 plants confirmed to be transgenic were tested, in comparison with WT Coker plants, for their susceptibility/resistance to a phloem feeding insect, *Aphis gossypii.*

4. Experimental observations suggest that *OSMII* over-expression confers some tolerance to aphids, slowing their rate of reproduction. Further testing is warranted and will be conducted with subsequent generations of two independent transgenic lines with consistently up-regulated levels of *OSMII* protein.

Acknowledgements

The financial support by Cotton Incorporated (Agreement No. 05-666 and Agreement No.08-395) is gratefully acknowledged.

References

Abad L. R., M. P. D'Urzo, D. Liu, M. L. Narasimhan, M. Reuveni, J. K. Zhu, X. Niu, N. K. Singh, P. M. Hasegava and R. Bressan. 1996. Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. Plant Sci. 118: 11-23.

Ashraf, M. 2009. Inducing drought tolerance in plants: Recent advances. Biotech.Advances 28: 169-183.

Chapman K. D., S. Austin-Brown, S. A. Sparace, A. J. Kinney, K. G. Ripp, I. L. Pirtle and R. M. Pirtle. 2001. Transgenic cotton plants with increased seed oleic acid content. J. American Oil Chemists' Soc. 78: 941-947.

Curtis, M. D. and U. Grossniklaus. 2003. A Gateway Cloning Vector Set for High-Throughput Functional Analysis of Genes in Planta. Plant Physiol. 133:462-469.

Davis, L. C. 2009. Impacts on agrosystems of transgenic insect and herbicide resistance in cotton. *In* Patterson A (ed.) Genetics and genomics of cotton. Vol 3. Springer, New York. pp 451-480.

DeLoach, C. J. 1974. Rate of increase of populations of cabbage, green peach, and turnip aphids at constant temperatures. Ann. Entomol. Soc. Am. 67: 332-340.

Jones, L. A. and C. C. King. 1996. Cotton seed Oil. *In* Y. Hui (ed.) Bailey's industrial oil and fat products. Vol. 2, JohnWiley and Sons, New York. pp 159-240.

Leelavati, S., V. G. Sunnichan, R. Kumria, G. P. Vijaykanth, R. K. Bhatnagar, V. S. Reddy. 2004. A simple and rapid *Agrobacterium*-meidated transformation protocol for cotton (*Gossypium hirsutum* L.): Embryogenic calli as a source to generate large number of transgenic plants. Plant Cell Rep. 22: 465-470.

Liu , Q., S. Singh, K. Chapman, and A. Green. 2009. Bridging traditional and molecular genetics in modifying cottonseed oil. *In* Patterson, A. (ed.) Plant genetics and genomics: crops and models. Vol 3. Springer, New York. pp 353-382.

Liu, D., K. G. Raghothama, P. M. Hasegawa and R. A. Bressan. 1994. Osmotin overexpression in potato delays development of disease symptoms. Proc. Natl. Acad. Scis. 91: 1888-1892.

Parkhi, V., V. Kumar, G. Sunilkumar, L. A. Campbell, N. K. Singh and K. S. Rathore. 2009. Expression of apoplastically secreted tobacco osmotin in cotton confers drought resistance. Mol. Breeding 23: 625-639.

Quim, M. and D. Zilberman. 2003. Yield effects of genetically modified crops in developing countries. Science 299: 900-902.

Quisenberry, J. E., C. W. Wendt, J. D. Berlin.1985. Potential for using leaf turgidity to select drought tolerance in cotton. Crop Sci 25:294-299.

Rajam, M. V., N. Chandola, P. S. Goud, D. Singh, V. Kashyap, M.L. Choudhary and D. Sihachakr 2007. Thaumatin gene confers resistance to fungal pathogens as well as tolerance to abiotic stresses in transgenic tobacco plants. Biol. Plant 51: 135-141.

Rathore, K. S., G. Sunilkumar and L. M. Campbell. 2006. Cotton (*Gossypium hirsutum* L.) In Wang, K. (Ed.) Methods in Mol. Biol., Vol. 343: Agrobacterium Protocols. Humana Press Inc., Totowa, New Jersey. In Wang K (Ed.) Methods in Mol. Biol., Humana Press Inc., Totowa.

Selitrennikoff, C. P. 2001. Antifungal proteins. Applied Environ. Microbiol. 67: 2883-2894.

Wyatt, I. J. and P. F. White. 1977. Simple estimation of intrinsic increase rates for aphids and tetranychid mites. J. Appl. Ecol. 83: 2111-2114.

Zhu, B., T. H. H. Chen, and P. H. Li. 1996. Analysis of late blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. Planta 198: 70-77.