ISOLATION OF cDNA CLONES FROM PETIOLE ABSCISSION ZONES OF COTTON

Liwei Chen, Curt M. Peterson and Narendra K. Singh
Department of Botany and Microbiology
Auburn University
Auburn, AL

Abstract

Abscission is an important physiological process that limits yield and indirectly affects fiber quality in cotton. In an attempt to identify genes associated with abscission in cotton, a cDNA library with a titer of 10^7 pfu/ml was established in the Uni-Zap XR vector. The cDNA library was produced using mRNAs extracted from petiole abscission zones of ethephon treated seedlings. An additional abscission subtracted cDNA library with 2124 individually isolated recombinant cDNA clones was constructed. Randomly selected clones from the subtracted cDNA library were excised in vivo and plasmid DNAs of 42 clones have been analyzed by dot blot. The nylon membranes containing DNAs from the 42 different isolates were probed with ^{32}P labelled first strand cDNA prepared from abscission zone mRNAs of both treated and control tissues. DNA dot blot hybridization showed that all 42 clones are induced in the abscission zone. Northern analysis and DNA sequencing are in progress to identify the cDNA clones and study expression of these genes in abscission zone tissues.

Introduction

Abscission is a process whereby plants shed their organs such as leaves, flowers and fruits. The process of leaf abscission involves a series of physiological and biochemical changes that lead to the breakdown of the middle lamella and primary cell wall of target cells in the abscission zone. Hydrolytic enzymes that cause cell wall degradation have been studied in some plants at both the biochemical and molecular level (Kalaitzis et al, 1995; Coupe et al, 1995; Taylor et al, 1994; Webb et al, 1993; Tucker et al, 1988).

Cotton is an important agricultural crop worldwide. Flower and boll abscission limit yield potential and delayed abscission of later formed leaves in the upper part of the canopy contributes to increased staining and trash in lint during harvesting. However, not much is known about cotton abscission at the biochemical and molecular level. Studying the mechanism of abscission in cotton may permit genetic manipulation of this crop for increased yields and improved lint quality. Thus, the objective of this research is to isolate and characterize genes associated with abscission in cotton and to provide molecular information necessary to study regulation of gene expression within abscission zones.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:1243-1246 (1996)
National Cotton Council, Memphis TN

Materials and Methods

Isolation of RNA

Cotton plants (Gossypium hirsutum L.) 'Deltapine' were grown under natural lighting in a greenhouse. Two week old seedlings were sprayed with 2% ethephon to induce cotyledonary petiole abscission or control seedlings were sprayed with water. After 48 hours of treatment, petiole abscission zone tissues were collected from control (untreated) and ethephon treated seedlings. Total RNAs were extracted from the tissues using the method of Hughes and Galau (1988). Poly $(A)^+$ RNAs were isolated from the total RNAs by oligo-dT chromatography (Sigma Chemical Company, St. Louis, MO).

Subtractive Hybridization

Subtracted cDNAs from ethephon treated abscission zone tissues were obtained following the manufacturer's instructions (Invitrogen Corporation, San Diego, CA). First strand cDNAs were synthesized from poly $(A)^+$ RNAs of the ethephon treated cotton petiole abscission zone tissues, followed by treatment with alkali to remove the template poly $(A)^+$ RNA. The single strand cDNAs were hybridized to the poly $(A)^+$ RNAs from control abscission zone tissues. Before hybridization, the control poly $(A)^+$ RNAs were photobiotinylated. The resulting photobiotinylated RNA-cDNA hybrids and excess photobiotinylated RNAs were incubated with streptavidin to form complexes with biotinylated RNA. Selective phenol/chloroform extraction of the mixture separated streptavidin RNA and streptavidin RNA-cDNA complexes into the phenolic phase leaving the single stranded cDNA behind in the aqueous phase. The subtracted cDNAs were used to established a subtracted cDNA library for cotton abscission.

λ ZAP cDNA Library

Both the subtracted abscission cDNA library and the complete cDNA library obtained directly from the ethephon-treated mRNA were constructed using the manufacturer's kits and instructions (Stratagene, La Jolla, CA). cDNAs were synthesized using MMLV-RT for the first strand, followed by DNA polymerase for second strand synthesis. With the help of adaptors/linkers, the cDNAs were directionally cloned into λ Uni-ZAP XR vectors digested with EcoRI and XhoI. The recombinant vectors were packaged using Gigapack gold packaging protein. Packaged λ Uni-ZAP XR was used to infect XL1-Blue MRF' bacteria. From the subtracted cDNA library, recombinant phage clones were selected on IPTG/X-gal plates by blue-white color selection.

DNA Dot Blot Hybridization

Some positive clones from the subtracted cDNA library were selected randomly, and the pBluescript phagemids from the Uni-ZAP XR vectors were excised in vivo using

the ExAssist™ helper phage. The plasmid mini-preps of those clones were prepared for DNA dot blot hybridization.

A Bio-Dot SF apparatus (Bio-Rad Laboratories, Inc., Hercules, CA) was used to transfer the recombinant plasmid DNA of different clones onto nylon membranes for dot blot analyses. Prior to transferring, the DNAs were denatured by adding 0.4M NaOH and 10mM EDTA (final concentration) and heating at 95^0C for 10 min to ensure complete denaturation. Samples were then neutralized by adding an equal volume of cold 2M ammonium acetate to pH 7.0 and applied to the dot-blot apparatus. Nylon membranes were rinsed with 2x SSC and crosslinked with UV (Stratalinker, Stratagene, La Jolla, CA).

One DNA blotted membrane was probed with first strand ^{32}P labelled cDNAs prepared from abscission zone mRNAs treated with ethephon. For comparison, another membrane was blotted with the same amount of DNA and hybridized with first strand ^{32}P labelled cDNAs prepared from control abscission zone mRNAs. The first strand cDNAs were synthesized using the manufacturer's Kits (Invitrogen Corporation, San Diego, CA). Equal amounts of radioactivity for both probes were used. Hybridizations were performed in 6x SSC, 5x Denhardt's solution, 0.1% SDS and 100 ug/ml denatured herring sperm DNA at 68^0C overnight. Membranes were washed three times in 2x SSC, 0.1% SDS at room temperature for 15 minutes each and twice with 0.1x SSC, 0.1% SDS at 68^0C for 30 minutes each. Cross hybridizations to place cDNA clones in smaller subgroups were conducted using the ^{32}P-labelled cDNA inserts obtained from recombinant clones. Hybridization conditions were the same as those used for DNA dot blot analysis.

Results and Discussion

We have used ethephon, an ethylene releasing agent, to induce cotyledonary leaf abscission in cotton. The natural process of leaf abscission takes place within a restricted region at the base of the petiole referred to as the abscission zone (Chen et al, 1996). Complex biochemical and physiological changes occur during abscission. Ethylene can induce leaf abscission effectively in many plants (Tucker et al, 1988; Webb et al, 1993), and it has been demonstrated that ethylene-induced abscission shares many features in common with the natural process.

The process of leaf abscission is consists of a series of developmental steps. First, specific "signals" such as ethylene are produced that induce specific RNA synthesis. Then, under the direction of the newly formed RNA, abscission specific enzymes including cell wall hydrolases such as cellulase are synthesized. Cell wall degradation or dissociation occurs following secretion of these enzymes into cell walls, and cells dissociate from one another leading to cell separation and finally abscission of the leaf.

Using all poly $(A)^+$ RNAs extracted from the abscission zone tissues treated with ethephon, a cDNA library was constructed in Uni-ZAP XR vectors. The size of the library is about 1 x 10^7 pfu/ml, which corresponds to transcripts expressed in abscission zone tissues at that stage in the treatment process that leads to abscission. In addition, subtracted cDNAs from the ethephon treatment were generated by removal of messages common to control tissues. These subtracted cDNAs were used to established a subtracted cDNA library in Uni-ZAP XR vectors. A total of 2124 recombinant cDNA clones representing genes present in cotton petioles induced to abscise were individually isolated. Compared to the non-subtracted abscission cDNA library, the subtracted abscission cDNA library is much smaller in size, and the clones are presumed to be more specific to abscission as demonstrated by dot blot hybridization (Figure 1 and 2).

We have randomly selected 42 cDNA clones for further analysis. λ ZAP cDNA clones were converted into phagemids using the ExAssist helper phage. The recombinant DNAs from the phagemids were isolated and used in dot blot analysis. The DNA dot blot hybridization in Figure 1A shows that different clones initially selected from the subtracted cDNA library have strong signals. In Figure 1B, however, when the blot was probed with the ^{32}P-labelled first strand cDNAs from control mRNAs, much weaker signals were evident, and several clones appeared to have virtually no signals, even though radioactivity strength of the probes was identical. Probes used in the hybridization in Figure 1A were ^{32}P-labelled first strand cDNAs prepared from mRNAs of ethephon treated abscission tissues. Therefore, the cDNA inserts in the clones correspond to transcripts which are highly expressed during abscission induced by ethephon. Some clones, which had very weak or no signals in the Figure 1B blot, may correspond to transcripts that are expressed only during abscission.

Some cDNA inserts from plasmid DNA of recombinant clones were obtained and ^{32}P-labelled for use as probes to hybridize with the DNA blot. Cross-hybridization indicated some clones are very specific. Figure 2 shows that cDNAs in clone pCA39 and pCA67 are unique, and belong to different groups, since hybridization signals could not be observed with any other clones.

A number of hydrolytic enzymes including cellulase, pectinase and polygalacturonase are considered to be involved in cell wall degradation during abscission in different plants (Kalaitzis et al, 1995; Sexton et al, 1989; Tucker et al, 1988). Proteins other than cell wall degrading enzymes also have been shown to increase during abscission. Some of them have been identified as pathogenesis-related proteins possibly formed to protect the exposed fracture surfaces from invasion by bacteria and fungi once cell separation occurred (del Campillo and Lewis, 1992a, 1992b; Rahmatullah et al, 1992), although

that remains to be confirmed. Recently, Coupe et al (1995) found an mRNA of a metallothionein-like protein accumulated during ethylene-promoted abscission of Sambucus nigra leaflets. Proteins involved in programmed cell death, mitotic divisions leading to a cell separation layer, and cell wall lignification and/or suberization associated with the formation of a protective layer also may be involved in this process. Our subtracted library will provide novel genes associated with these unique developmental events occurring during the onset and progression of abscission in cotton plants.

Based on previous studies it seems that cellulase (end-β-1,4 glucanohydrolase) is one of the most important cell wall hydrolyzing enzymes involved in abscission. A particular form of cellulase, PI 9.5, is synthesized de novo, and ethylene either naturally produced or artificially applied is an important regulator of cellulase production (Sexton et al, 1989). Northern analysis and in situ hybridization demonstrated that cellulase mRNA is expressed specifically in abscission zone tissues (Tucker et al, 1988, 1991). We used a soybean PI 9.5 cellulase cDNA to probe a blot of RNAs from soybean and cotton seedling abscission zone tissues of both untreated (control) and ethephon treated seedlings. The PI 9.5 cellulase mRNA was detected only within ethephon treated soybean abscission zone tissues (data not shown). Thus, this Northern hybridization demonstrates that soybean abscission cellulase gene has little or no homology with cotton abscission cellulase. Northern analysis and DNA sequencing are in progress in order to identify the abscission specific cDNA clones and study expression of the genes during abscission in cotton.

Acknowledgements

Authors express appreciate to Drs Brent Nielsen and Herry Daniell for review of the manuscript and Cecilia Mosjidis for technical assistance. AAES Journal No. 6-965151.

References

1. Chen, L., C. M. Peterson and R. R. Dute. 1996. Petiole abscission in cotton: cytological and ultrastructural changes induced by ethephon. Submitted to Journal of Experimental Botany

2. Coupe, S. A., J. E. Taylor, and J. A. Roberts. 1995. Characterization of an mRNA encoding a metallothionein-like protein that accumulates during ethylene-promoted abscission of Sambucus nigra L. leaflets. Planta 197:442-447

3. del Campillo, E. and L. N. Lewis. 1992a. Identification and kineitics of accumulation of proteins induced by ethylene in bean abscission zone. Plant Physiology 98:955-961

4. del Campillo, E. and L. N. Lewis. 1992b. Occurrence of 9.5 cellulase and other hydrolases in flower reproductive organs undergoing major cell wall disruption. Plant Physiology 99:1015-1020

5. Hughes, D. W. and G. Galau. 1988. Preparation of RNA from cotton leaves and pollen. Plant Molecular Biology Reporter 6:253-257

6. Kalaitzis, P., S. M. Koehler and M. L. Tucker. 1995. Cloning of a tomato polygalacturonase expressed in abscission. Plant Molecular Biology 28:647-656

7. Rahmatullah, R. J., N. K. Singh, S. Tuzun, A. K. Handa and C. M. Peterson. 1992. Induction of genes involved in cell wall degradation and hypersensitive response in soybean abscission zones. Plant Physiology Supplement 99:33 (abstract)

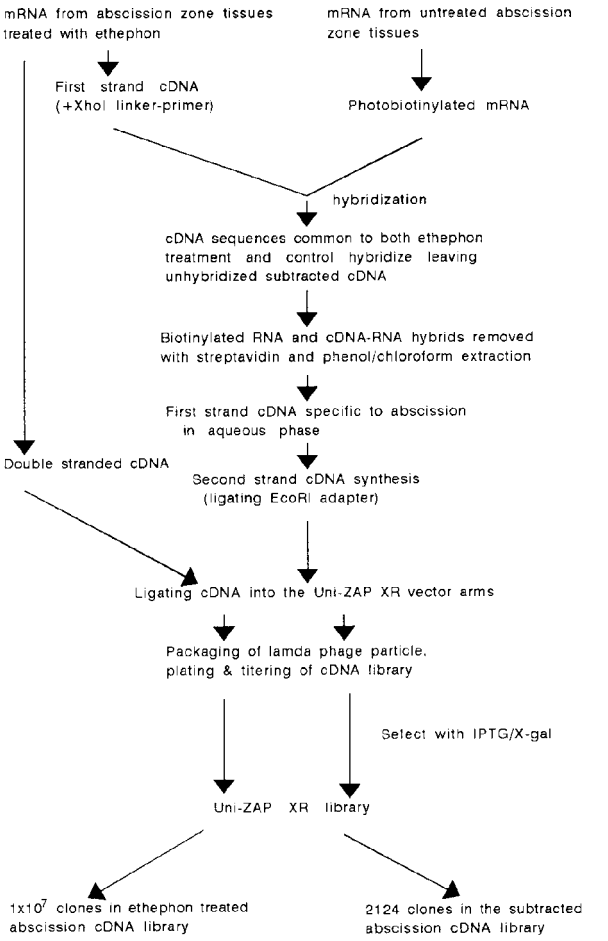
8. Sexton, R., M. L. Tucker, E. del Campillo, and L. N. Lewis. 1989. The cell biology of bean leaf abscission. In Cell Separation in Plants. Edited by D. J. Osborne and M. B. Jackson. Springer-Verlag Berlin Heidelberg, pp 69-78.

9. Taylor J. E., S. A. Coupe, S. Picton and J. A. Roberts. 1994. Characterization and accumulation pattern of an mRNA encoding an abscission related β-1,4-glucanase from leaflets of Sambucus nigra. Plant Molecular Biology 24:961-964

10. Tucker M. L., S. L. Baird and R. Sexton. 1991. Bean leaf abscission: tissue-specific accumulation of a cellulase mRNA. Planta 186:52-57

11. Tucker, M. L., R. Sexton, E. del Campillo and L. N. Lewis. 1988. Bean abscission cellulase, characterization of a cDNA and regulation of gene expression by ethylene and auxin. Plant Physiology 88:1257-1262

12. Webb, S. T. J., J. E. Taylor, S. A. Coupe, L. Ferrarese and J. A. Roberts. 1993. Purification of β-1,4-glucanase from ethylene-treated leaflet abscission zones of Sambucus nigra. Plant Cell Environ. 16:329-333



Construction of cDNA libraries from petiole abscission zones of cottor

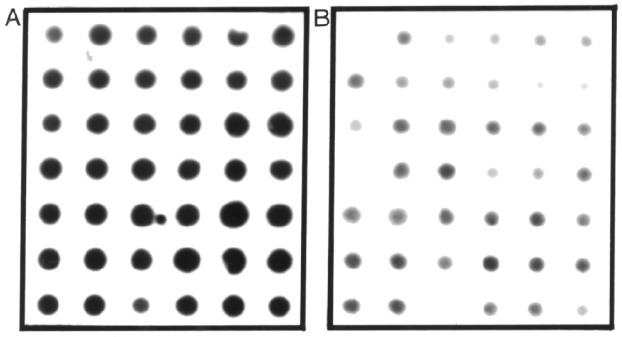


Figure 1. DNA dot blot hybridization. Plasmid DNAs of 42 positive clones from a subtracted abscission cDNA library were dot blotted on nylon membranes, and probed with ^{32}P labelled first strand cDNAs prepared from abscission zone mRNAs of ethephon treated (A) and control (B) seedlings.

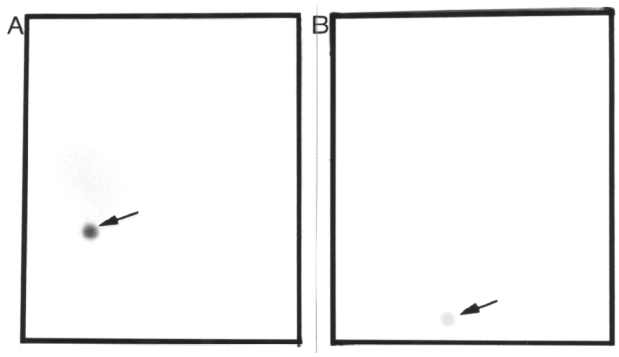


Figure 2. DNA dot blot cross-hybridization. Inserted plasmid DNAs of 42 clones from the subtracted abscission cDNA library of cotton were probed with ^{32}P labelled CA39 (A) and CA67 (B),cDNA inserted in clone pCA39 and pCA67, respectively. Note that pCA39 only hybridized with CA39 (A) and pCA67 with CA67.