**Bean Abscission Cellulase**

**CHARACTERIZATION OF A cDNA CLONE AND REGULATION OF GENE EXPRESSION BY ETHYLENE AND AUXIN**

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**ABSTRACT**

The physiology and anatomy of abscission has been studied in considerable detail; however, information on the regulation of gene expression in abscission has been limited because of a lack of probes for specific genes. We have identified and sequenced a 595 nucleotide bean (*Phaseolus vulgaris* cv Red Kidney) abscission cellulase cDNA clone (pBACI). The bean cellulase cDNA has extensive nucleic and amino acid sequence identity with the avocado cellulase cDNA pAV363. The 2.0 kilobase bean mRNA complementary to pBACI codes for a polypeptide of approximately 51 kilodalton (shown by hybrid-selection followed by *in vitro* translation). Bean cellulase antisera is shown to immunoprecipitate a 51 kilodalton polypeptide from the *in vitro* translation products of abscission zone poly(A)⁺ RNA. Ethylene initiates bean leaf abscission and tissue-specific expression of cellulase mRNA. If ethylene treatment of bean explants was discontinued after 31 h and then 2,5-norbornadiene given to inhibit responses resulting from endogenously synthesized ethylene, polyclonal cellulase mRNA hybridizing to pBACI decreased. Thus, ethylene is required not only to initiate abscission and cellulase gene expression but also to maintain continued accumulation of cellulase mRNA. Explants treated with auxin 4 hours prior to a 48 hour treatment with ethylene showed no substantial accumulation of RNA hybridizing to pBACI or expression of cellulase activity.

Abscission is the process by which plants shed organs. A fundamental event in abscission is the secretion of cell wall hydrolases in a discrete zone of cells at the base of the organ to be shed. An increase in activity of cellulase (β 1:4 glucan 4-glucanohydrolase) in abscissing tissue has been reported for several plants (26). However, in some abscission systems interpretation of changes in cellulase enzyme activity is complicated by the presence of celluase activity prior to induction of abscission. Lewis and Varner (17) used low ionic strength versus high ionic strength buffer to differentially extract two forms of cellulase from bean leaf abscission zones. They further showed that the cellulase extracted by high ionic strength buffer was synthesized de novo during abscission. The high ionic strength buffer extractable cellulase was further distinguished by its basic isoelectric point (pI 9.5, subsequently referred to as 9.5 cellulase) (6). This 9.5 cellulase was purified and used to prepare cellulase specific antisera (6). Durbin *et al.* (6) used a sensitive radioimmune assay to show a close correlation between the appearance of the 9.5 cellulase protein and the onset of abscission. Sexton *et al.* (27) extended this correlation to show that 9.5 cellulase is predominantly associated with a two to three cell wide separation layer though low levels of activity were also found in adjacent stem and petiolar tissue.

Leaf abscission can be induced in the petiole by removal of the subtending leaf blade. Jackson and Osborne (13) reported that an increase in endogenous levels of ethylene correlates with the onset of abscission and proposed that ethylene might be the natural regulator. More recently, several laboratories have shown that inhibitors of ethylene synthesis or action can delay abscission (3, 14, 22, 28). As a result of a series of experiments involving inhibition of RNA and protein synthesis, Abeles *et al.* (1) speculated that the ability of ethylene to regulate bean leaf abscission involved the synthesis of specific mRNAs including that of cellulase. They further proposed that ethylene is ineffective if high levels of auxin are present in the abscission zone. We have identified a bean abscission cellulase cDNA clone (pBACI) and have used this cDNA probe to confirm and extend our understanding of the hormonal and tissue-specific regulation of gene expression in bean leaf abscission.

**MATERIALS AND METHODS**

**Treatment of Plant Materials.** Bean (*Phaseolus vulgaris* cv Red Kidney) plants were grown in a greenhouse and harvested 12 d after germination at which time primary leaves are fully expanded. Explants were prepared by cutting the stem below the cotyledons and then removing the cotyledons. The leaf blades of the primary leaves were removed from explants by cutting 2 cm above the basal pulvinus. The unexpanded trifoliate leaves above the first node were also detached from the explants. Explants were then surface sterilized with a 2 min bath in 0.5% sodium hypochlorite (bleach), rinsed with several volumes of water, and placed upright in styrofoam cups filled with distilled water. Cups containing the explants were placed into a 22 L chamber and the explants exposed to 5 μL/L ethylene in air at a flow rate of 2 L/min. Where indicated, 5 X 10⁻⁶ M IAA in lanolin was applied onto the distal petiolar stump of explants 4 h prior to ethylene treatment. In experiments using NBD as an inhibitor of ethylene action, explants were first exposed to 5 μL/L ethylene in air as described above and then transferred to a 9 L chamber. Liquid NBD was injected into the sealed 9 L chamber through a vaccine seal to achieve a gas concentration of 1,000 μL/L. NBD concentrate is a liquid in a closed bottle, but readily volatilizes in a large

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4 Abbreviations: NBD, 2,5-norbornadiene; kb, kilobase.
volume of air at 25°C (i.e. 40 μL of NBD in 9 L of air). When NBD and ethylene were used together, NBD injection was followed by 0.9 mL of ethylene gas to achieve a concentration of 100 μL/L.

Abscission zones were harvested, frozen in liquid nitrogen, and stored at −80°C. Abscission zone tissue consists of an approximately 2 mm cross-section of the petiole pulvinus. When separation had already occurred, an approximately 1 mm slice was taken from either side of the separation layer.

Preparation of Polysomal RNA, cDNA Synthesis, and Cloning. Frozen abscission zones (−80°C) were combined with an equal or greater volume of dry ice and pulverized in a coffee grinder. After sublimation of dry ice, pulverized abscission zones (2–20 g) were suspended in 5 to 10 volumes (w/v) of polysome extraction buffer (0.5 M sucrose, 200 mM Tris [pH 9.0], 400 mM KCl, 35 mM MgCl₂, 25 mM EGTA, 5 mM 2-mercaptoethanol) (11) and polysomal RNA extracted as previously described (31). The cDNA was synthesized using the procedure of Gubler and Hoffman (9) and cloned into pUC18 as previously described (30). The cloned bean cDNA library was screened (18) with the cDNA insert from the heterologous avocado cellulase clone pAV363 (30). The pAV363 cDNA insert was separated from the vector DNA by the method of Langridge et al. (16). Hybridization conditions used for screening the cDNA library were approximately 35 to 40°C below Tm (i.e. 20% formamide, 0.9 M NaCl, 42°C).

Hybrid-selection and in Vitro Translation. pBACI was digested with EcoRI and HindIII and the intact cDNA subcloned into pUC118 and 119. pUC118 and 119 are pUC18 and 19 containing the origin of replication from M13 (32). cDNA constructs in pUC118 and 119 were used with the helper phage M13K07 to produce single-stranded plasmid DNA (32). Fifteen μg of single-stranded DNA from each of the pUC118, 119, pBACI cDNA constructs were spotted onto separate 0.6 cm² Hybond-N membranes (Amersham nylon membrane). The DNA was fixed to the membranes with a 5 min exposure to UV light (Amersham protocol). The membrane squares were incubated with 80 μg polyosomal poly(A)+ RNA extracted from abscission zones of ethylene treated explants and hybrid-selected RNA released as described by Parnes et al. (20). The protocols for in vitro translation as well as gel electrophoresis and immunoprecipitation have been described elsewhere (31).

Nucleotide Sequence. The single-stranded plasmid DNAs described above were sequenced by the dideoxy chain termination method of Sanger et al. (24) as described in the Amersham DNA sequencing protocol. The primer extension reactions were done at 50°C to melt out secondary structure in areas of high G-C content in the DNA.

Northern and Western Blots. RNAs for the northern blots were denatured at 50°C for 60 min in 1.0 M glyoxal, 10 mM NaPO₄ (pH 6.8). Denatured RNAs were separated by electrophoresis in a 1.5% agarose gel and RNA transferred to Hybond-N nylon membrane (18). Hybridization conditions are described in the text. cDNA inserts used as probes were prepared as described above.

Protein for the Western blot (immunoblot) was prepared from the supernatant after sedimentation of polysomes by ultracentrifugation (31). Protein in 5 mL of this supernatant was precipitated by addition of 0.5 mL of 100% TCA (w/v) to achieve a concentration of 10% TCA and allowed to stand for 10 min at 0°C. The mixture was centrifuged in a SS34 rotor at 12,000 rpm for 15 min. To remove TCA from the protein pellet the pellet was washed with 5 mL cold ethyl ether followed by two washes with 5 mL cold acetone. After each wash the protein was pelleted at 12,000 rpm for 15 min. The washed protein pellet was suspended in 400 μL of 20 mM Tris (pH 8.1), 2 mM EDTA, and 2% SDS. The protein content was determined (21), 20 μg of each sample separated by SDS-PAGE (15), and the protein electroblotted onto a nitrocellulose membrane (29). The nitrocellulose membrane was probed for cellulase protein by incubation with bean 9.5 cellulase antiserum (6) followed with an antibody detection procedure utilizing goat antirabbit antibody conjugated to horseradish peroxidase (Bio-Rad protocol).

Determination of Cellulase Activity. Cellulase activity was measured in a sample from each of the homogenates used for the preparation of polysomal RNA. Activity was determined by monitoring changes in the viscosity of a 1.0% solution of carboxymethylcellulose (pH 6.1) (5). Units for cellulase activity were calculated as described by Durbin and Lewis (5). This method of calculation gives approximately 10-fold greater values for cellulase activity than those previously reported for the same tissue (6). Cellulase activity reported herein is only that activity which could be immunoprecipitated with abscission cellulase antiserum (6). This was done to distinguish between abscission-specific cellulase (9.5 cellulase) and the presence of a nonimmunologically related cellulase which slowly disappears over the duration of an experiment (6).

RESULTS

Identification of a Bean cDNA having Sequence Similarity with that of an Avocado Fruit Cellulase. A cDNA library was prepared from poly(A)+ RNA purified from abscission zones of bean explants treated for 48 h with 25 μL/L ethylene. Two thousand abscission cDNA clones were screened by hybridization to the full-length avocado fruit cellulase clone pAV363 (30). One clone, pBACI, was retained for further characterization. Figure 1 shows that pBACI hybridizes to a 2.0 kb mRNA which accumulates on polysomes of ethylene-treated bean explants. Furthermore, pBACI hybridizes to a 2.2 kb mRNA from ethylene-ripened avocado fruit. The cellulase mRNA complementary to the avocado clone pAV363 is 2.2 kb (30).

Sequence analysis of the 595 nucleotide cDNA insert in pBACI showed it to have sequence similarity with the 3' end of the avocado cellulase clone. The bean cDNA sequence contains a 411 nucleotide open reading frame which begins at the second nucleotide of the pBACI cDNA insert. The nucleotide sequence

![Fig. 1. Northern blot of poly(A)+ RNA from 0 h uninduced (−) and 70 h ethylene-induced (+) abscission zones of bean and ethylene-ripened (+) avocado fruit. The membrane was probed with 32P-labeled cDNA insert from pBACI. Each lane contains 3 μg of poly(A)+ RNA. Hybridization conditions were 30% formamide, 0.9 M NaCl, at 42°C. The numbers to the left and right indicate the mRNA length in kilobases.](image-url)
comprising the open reading frame in the bean clone shows approximately 60% identity with the corresponding region in the avocado cellulase sequence. Figure 2 shows a comparison of the deduced amino acid sequence for the 411 nucleotide open reading frame in pBACI to the corresponding sequence from the avocado cellulase clone pAV363 (30). The two sequences shown in Figure 2 share 48% identically matched amino acids.

The sequence similarity between the avocado and bean cDNAs strongly suggests that the pBACI cDNA is complementary to a cellulase mRNA. However, sequence similarity does not distinguish it as a cellulase involved in leaf abscission. The following experiments were done to show that pBACI is a clone of a cellulase mRNA involved in abscission.

Hybrid Selection. It is common practice to use a denatured double stranded plasmid for use as an immobilized probe for hybrid selection of mRNA (19). We decided to modify this procedure and instead use complementary and anticomplementary single-stranded DNAs as separate probes. This change in protocol improves the efficiency of hybrid selection which is important when investigating mRNA of low abundance, and the anticomplementary single-stranded DNA can be used as a control.

The cDNA insert in pBACI was subcloned into the vectors pUC118 and pUC119 which provided the means for synthesis of the complementary and anticomplementary single-stranded DNAs (32). Lanes 2 and 3 of Figure 3 show the in vitro translations of the mRNA which was hybrid-selected by single-stranded DNA synthesized from the two orientations of the subcloned pBACI cDNA. The most prominent difference in the translation products of the hybrid-selected mRNA is a polypeptide(s) of approximately 51 kD. Translation products other than the 51 kD electrophoretic species are from endogenous wheat germ mRNA in the translation assay (compare to lane 1, Fig. 3). The 51 kD translation product of the hybrid-selected mRNA (Fig. 3, lane 3) is comparable in size to the in vitro translation products of cellulase-specific antisemiser (Fig. 3, lane 5). Cellulase immunoprecipitated from in vivo labeled proteins (Fig. 3, lane 4) has an apparent mol wt of approximately 2 kD less than the in vitro translation product. Cellulase is a secretory protein (2). The in vitro translation product would therefore presumably include a signal peptide which is cleaved in vivo and may account for the 2 kD discrepancy between the in vitro and in vivo immunoprecipitates.

Electrophoresis of immunoprecipitated in vivo labeled and in vitro translated cellulase protein shown in Figure 3, lanes 4 and 5, respectively, each yielded two major electrophoretic species. It is not clear whether the in vitro translation of hybrid-selected mRNA shown in lane 3 is comprised of one or two electrophoretic species. The immunoblot of abscission cellulase shown in Figure 4 shows only a single 51 kD polypeptide. It is not yet possible to conclude whether the two electrophoretic species seen in lanes 4 and 5 of Figure 3 are cellulase isozymes, a degradative artifact, or an artifact of gel electrophoresis.

It may be concluded that pBACI is complementary to a cellulase mRNA coding for a protein of comparable mol wt to that of immunoprecipitated 9.5 cellulase. Unfortunately, the amounts of 51 kD protein produced from the hybrid-selected mRNA were barely detectable and of insufficient quantity to make subsequent immunoprecipitation practical. The following experiments show some aspects of the hormonal and tissue-specific regulation of 9.5 cellulase expression and the mRNA hybridizing to pBACI. The results from these experiments support the conclusion that pBACI is complementary to a cellulase mRNA which is expressed during leaf abscission.

Ethylene-Induced Accumulation of Abscission Cellulase Protein and mRNA. Explants exposed to ethylene free air showed no appreciable increase in abscission cellulase over a 48 h period (Fig. 4). A similar treatment of explants including 5 μL/L ethylene showed a marked increase in cellulase activity and immunodetectable protein beginning approximately 24 h after exposure to ethylene. The temporal accumulation of polysomal RNA

| Fig. 3. Mol wt comparison of immunoprecipitated bean cellulase to the in vitro translation product of pBACI hybrid-selected mRNA. Poly(A) RNA used in hybrid-selection was extracted from abscission zones of bean explants treated with ethylene for 48 h. Hybrid-selection was done using single-stranded plasmid DNA (ssDNA) synthesized from pUC118 and 119 constructs containing the subclone pBACI cDNA insert. Lanes: 1, in vitro translation with no RNA added; 2, in vitro translation of RNA hybridizing to ssDNA from the pBACI, pUC118 construct; 3, in vitro translation of RNA hybridizing to ssDNA from the pBACI, pUC119 construct; 4, cellulase immunoprecipitated from in vivo labeled protein; 5, cellulase immunoprecipitated from the in vitro translation shown in lane 6; 6, in vitro translation of poly(A) RNA from 48 h ethylene-induced abscission zones; 7, mol wt markers. Lanes 1 to 3 contain 25 μL of a [35S]methionine labeled wheat germ in vitro translation assay compared with 2.0 μL in lane 6. |
FIG. 4. Time course for the accumulation of bean abscission cellulase and polysomal RNA hybridizing to pBACl. Cellulase activity shown is only that activity which is immunoprecipitated by 9.5 cellulase specific antiserum. Hybridizable RNA was determined by 3P scintillation counting of the RNA dot blots shown above the bar graph (5 µg polysomal RNA per spot). Explants were treated with either 5 µL/L ethylene in air or ethylene free air.

FIG. 5. Northern blot analysis of the tissue specificity of three bean cDNA clones. Letter codes: (a), abscission zone; (p), petiole; (s), stem. Numbers to the left and right of blots indicate the mRNA lengths in kilobases. Each lane contains 2 µg of polysomal poly(A)- RNA.

hybridizing to pBACl correlates with the observed changes in cellulase activity and immunoblotted protein.

Tissue-Specific Expression of RNA Hybridizing to pBACl. Figure 5 shows the expression of three different polysomal mRNAs in three different tissues. Message hybridizing to pBACl, the bean cellulase clone, is expressed most abundantly in abscission zones but is also expressed at a much lower abundance in both petiole and stem tissue. Scintillation counting of the 3P labeled 2.0 kb region of the pBACl northern blot indicated a 10-fold higher concentration of RNA hybridizing to pBACl in ethylene-induced abscission zones than in stems or petioles. The bean clones pBZ30 and pBA32 were taken from the abscission cDNA library and used for comparison to pBACl. pBZ30 is induced by ethylene, however, unlike pBACl, pBZ30 hybridized to ethylene-evoked mRNA expressed approximately equally in abscission zone, stem, and petiole tissue, showing that ethylene responses are not limited to the abscission zone. pBA32 is complementary to a constitutively expressed mRNA and is used as a control for RNA quantity.

Aspects of the Hormonal Regulation of Cellulase Gene Expression. Experiments were undertaken to examine whether the continued presence of ethylene was necessary after cellulase accumulation had commenced. Earlier results showed that removal of explants from ethylene to air slowed the increase in cellulase activity but did not stop it (25). It seemed possible that this could have occurred because endogenously synthesized ethylene was making the complete removal of ethylene impossible. Preliminary experiments showed that NBD, a competitive inhibitor of ethylene action (28), prevented the onset of abscission whereas abscission occurred in explants kept in air alone, presumably because of endogenous synthesis of ethylene. Thus, instead of placing explants in air after ethylene exposure, explants were placed in an atmosphere containing NBD to inhibit ethylene action due to endogenous ethylene. In the experiments shown in Figures 6 and 7 explants were exposed to 5 µL/L ethylene for 31 h to initiate abscission. The 5 µL/L ethylene treatment was then discontinued and the explants treated with either 1000 µL/L NBD alone or 1000 µL/L NBD plus 100 µL/L ethylene as a control. An ethylene treatment of 31 h was sufficient to initiate an increase in cellulase activity and accumulation of cellulase mRNA on polysomes (Figs. 6 and 7). In contrast, explants sequentially exposed to 1000 µL/L NBD alone the accumulation of cellulase activity was inhibited (Fig. 7) and the concentration of polysomal RNA hybridizing to pBACl decreased (Figs. 6 and 7). At the end of a 24 h exposure to NBD, cellulase mRNA hybridizing to pBACl had declined to undetectable levels (Fig. 6). These results show that ethylene is necessary not only to initiate but also to maintain expression of cellulase gene. The ethylene-evoked mRNA hybridizing to pBZ30, which was not specific to the abscission zone, also disappeared after 24 h of NBD (Fig. 6) showing that the expression of this gene too is dependent upon the continued presence of ethylene.

Application of auxin to the distal end of explant petioles, prior to exposure to ethylene, inhibits abscission and accumulation of 9.5 cellulase. IAA at 5 x 10^-6 m in lanolin applied 4 h prior to a 48 h exposure to ethylene completely inhibited the accumulation of mRNA complementary to pBACl (Fig. 6). However, IAA did not act as a general inhibitor of all ethylene-induced responses. This is noted in that 5 x 10^-6 m IAA did not appreciably effect
the accumulation of mRNA hybridizing to pBZ30 (Fig. 6), a nonabscession specific but ethylene-induced mRNA.

**DISCUSSION**

If the primary leaves of bean are debladed and left in air the first signs of weakening in the abscission zone are apparent after 5 to 6 d. Ethylene accelerates this process so that weakening starts between 20 and 30 h and petals fall under their own weight 24 to 30 h later. As has been previously reported this loss of abscission zone integrity is paralleled by a progressive increase in a cellulase having an isoelectric point of 9.5 (9.5 cellulase) (6). The process of cell separation starts near the upper surface of the petiole and spreads across the fracture plane (27). Consequently, this increase in cellulase probably reflects a recruitment of new cells to the expanding separation layer as well as the accumulation of cellulase in individual cells.

The cellulase cDNA probe pBACl has been used to show that the increase in 9.5 cellulase protein and activity is accompanied by a concomitant accumulation of cellulase mRNA on polysomes. This partially substantiates the prediction of Abeles et al. (1) that transcriptional control mediates the production of abscission cellulase. On the basis of our data alone, however, we are unable to distinguish between a transcriptional regulation and an increase in the stability of cellulase mRNA.

Dela Fuente and Leopold (7) first showed that the rate of abscission zone weakening was quickly reduced upon ethylene withdrawal. Abeles and Leath (2) confirmed this finding and concluded that there was no change in the rate of cellulase accumulation, even though secretion of the enzyme was halted. In our hands removal of ethylene and addition of NBD (to compete with endogenously generated ethylene) slowed the increase in cellulase activity and lead to the virtual disappearance of the cellulase mRNA on polysomes. This indicates that the continued presence of ethylene is essential both for the accumulation of cellulase mRNA and perhaps also for the recruitment of new cells to the expanding separation layer. This result differs from the findings of Sisler et al. (28) who found that NBD had no effect on the progress of citrus abscission once the process had started.

Ethylene has been shown (26, 27) to induce the accumulation of low levels of cellulase enzyme activity in both stem and petiole tissue adjacent to the abscission zone. Figure 5 corroborates these previous reports showing that, although the bulk of cellulase mRNA hybridizing to pBACl is restricted to the abscission zone, measurable amounts are found in adjacent stem and petiolar tissues.

Although 9.5 cellulase protein predominates in the separation layer (27), this layer dissects the width of the petiole. As a consequence, the separation layer contains cells representative of many cell types. Immunocytochemical localization of 9.5 cellulase suggests that several cell types synthesize cellulase (27). It is an interesting and as yet unanswered question as to whether all of these cells synthesize the same cellulase protein or different closely related isoforms of cellulase.

In this context, it is worth noting that the bean 9.5 cellulase antiserum does not immunoprecipitate the 4.5 cellulase enzyme found in the petiolar pulvinus prior to induction of abscission (6). Similarly, the pBACl clone did not show any significant hybridization to RNA extracted from abscission zones of explants not treated with ethylene (i.e. 0 h abscission zones) (Figs. 1, 4, 5, 6). This shows that the 4.5 cellulase found in the abscission zone of the petiolar pulvinus prior to abscission (6) does not share enough sequence similarity with the pBACl clone as to be detected at the hybridization conditions used in this study. Therefore, changes in the concentration of mRNA coding for the 4.5 cellulase are not reflected in any of the RNA analyses shown.

The addition of auxin to debladed petals prior to ethylene exposure stops weakening and the accumulation of cellulase (1, 12, 19). Our data show that this effect is mediated by an inhibition of cellulase mRNA accumulation. Originally it was thought that auxin addition inhibited abscission only if administered before weakening commenced (1, 26); however, Osborne et al. (19) recently showed using a modified procedure for the application of auxin that auxin negatively regulates cellulase production throughout the entire abscission process. These opposing stimulatory and inhibitory effects of ethylene and auxin are reminiscent of gibberellic and abscisic acid control of amylase synthesis in cereal aleurone (10). This type of antagonistic control may be a common feature in plants.

A striking feature of the accumulation of cellulase mRNA is the long lag between the addition of ethylene and any effect. Other ethylene-induced mRNAs such as that hybridizing to pBZ30 and that which translates into chitinase (8) appear rapidly in the same tissue. The length of the lag is variable in different abscission zones of the bean plant and ethylene markedly reduces its duration (25). It has been suggested that the lag might represent the time taken for the endogenous levels of auxin at excision to fall to nonhibernatory levels. Ethylene has been reported to inhibit auxin transport and possibly influence its destruction (4, 23, 33). Ethylene, therefore, would tend to shorten the lag phase by reducing the concentration of auxin in the abscission zone. Once auxin had decreased to nonhibernatory levels, ethylene would be required to initiate and sustain abscission specific gene expression (e.g. cellulase).

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