Effect of ethylene, 1-MCP, ABA and IAA on break strength, cellulase and polygalacturonase activities during cotton leaf abscission

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Abstract

Organ abscission in higher plants has invariably been characterized by higher activities of cell wall hydrolases in the abscission zone of the abscising organ, which facilitates degradation of middle lamella and loosening of cell wall of separation layers. The plant hormone ethylene has been implicated in the induction and progression of the abscission process. In the present study, we have measured activities of cellulase and polygalacturonase (PG) in the abscission zone of cotton (Gossypium hirsutum var RST-39) leaf explants in the presence of ethylene. The effects of abscisic acid (ABA) and indole acetic acid (IAA) were monitored to elucidate the role of other phytohormones in the process of abscission. A several fold increase in cellulase and PG activities and decrease in break strength were observed in the LAZ of ethylene-treated explants. The increase in enzyme activities and decrease in break strength were strongly suppressed in the presence of 1-methylcyclopropene (1-MCP) and IAA. ABA alone was found to stimulate enzyme activities and decrease the break strength though not to the extent of ethylene. 1-MCP pretreatment of ABA and ethylene-treated explants showed significant inhibition in enzyme activities. It is concluded that cotton leaf abscission is ethylene regulated and characterized by increased activities of cellulase and PG in its abscission zone. ABA can induce abscission. However, it appears that ABA induced abscission may be mediated through ethylene.

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1. Introduction

Abscission is an important developmental event in the life cycle of higher plant facilitating detachment of plant organs from the main body (Patterson and Bleecker, 2004). Several factors including growth and development, phytohormones, wounding and environmental stress provide important cues to the process of abscission. Plant hormone ethylene has been shown to play an important role in regulating abscission in many cases and often used exogenously to initiate and complete the abscission in experimental/model plants in order to understand the mechanism of abscission (Jackson and Osborne, 1970; Abeles and Leather, 1971). Phytohormones, other than ethylene, such as indole acetic acid (Sexton and Roberts, 1982) and abscisic acid (Suttle and Hultstrand, 1993) have also been shown to modulate abscission. During most abscising events higher synthesis and activities of cell wall hydrolases has been observed which could be responsible for the degradation of middle lamella and the loosening of primary cell wall of separation layers (Taylor et al., 1993; Taylor et al., 1994). Two major cell wall hydrolases viz. cellulase and polygalacturonase (PG) have been widely studied in various plants and their roles documented during organ abscission (Brummel et al., 1999; González-Carranza et al., 2002). Apart from these, the role of expansin proteins and other hydrolases has also been examined during abscission (Wu and Burns, 2004; Belfield et al., 2005). Though there are several reports on the process of fruit abscission (Ruperti et al., 2001; Perin et al., 2002; Rasori et al., 2002), limited information is available related to the mechanism of leaf abscission in species other than soybean (Kemmerer and
Tucker, 1994), bean (Koehler et al., 1996) and Sambucus nigra (Taylor et al., 1993). Though effect of ABA on leaf abscission has been studied to some extent, the effect of IAA has not been well documented. In the present study, we have measured ethylene induced cotton leaf abscission in relation to activities of cellulase and PG enzymes. We have also studied the effect of IAA, ABA and the ethylene action inhibitor 1-MCP (Sisler and Serek, 1997) on the process of leaf abscission in cotton. The study is important because defoliation is an important agronomic practice in cotton cultivation.

2. Materials and methods

2.1. Plant material and treatments

Seeds of cotton (Gossypium hirsutum var RST-39) were germinated in a greenhouse under controlled temperature (32 ± 3 °C) and grown for 12 to 15 days until the primary leaves were fully expanded and the secondary pinnate leaf just began to open. At this time, the blades for the primary leaves were removed and plants harvested by cutting 1 cm above the soil. Explants were surface sterilized, placed in water and exposed to 10 μL/L ethylene in air for the time intervals indicated in the figure legends. For 1-MCP treatment, explants were exposed to 1-MCP (10 μL/L) for 1 h before exposure to ethylene. In order to study the effect of other hormones in the presence and absence of ethylene, the explants were treated with IAA and ABA. The explants were kept in 100 μM IAA or ABA for 2 h prior to treatment with ethylene or 1-MCP. For enzyme assays, leaf abscission zone (LAZ) tissue (1–2 mm on either side of fracture plane, which develops at the junction of stem and petiole), was collected, frozen in liquid nitrogen and stored at −70 °C till further use. Other vegetative tissues were also collected, after exposure to ethylene for 24 h for the purpose of enzyme assays.

2.2. Break strength measurement

In order to determine the break strength of LAZ, the petiolar stump of the explant was clamped using a small clip to which a string was attached to which a weight was attached. Increasing weights were attached to the string till the petiolar stump detached at the abscission zone. The height of the explant from the bench top as well as length and angle of the string were kept constant during each measurement. The total weight applied on the petiolar stump was determined and the break strength of the abscission zone calculated as gm equivalent required to detach the LAZ. Break strength values determined for each time point represented an average of at least 20 individual measurements.

2.3. Assay of cellulase activity

To measure cellulase activity, 1 g of leaf abscission zones was homogenised in 3 ml of 0.1 M Na-phosphate buffer (pH 7.8) containing 2 mM EDTA, 0.1 mM DTT and 0.2% Triton X-100. To the homogenate 0.5 M EDTA (1/500 vol) and 5 M NaCl (1/10 vol) were added, mixed thoroughly and kept on ice for 30 min followed by centrifugation at 15,000 g for 30 min followed by centrifugation at 4 °C. The pellet was discarded and supernatant was used as enzyme preparation for the assay of cellulase. The assay mixture contained 0.5 ml enzyme and 0.5 ml 1.3% (w/v) carboxyl methylcellulose (CMC) prepared in 0.02 M Tris–HCl pH 8.0. Drainage time of assay mixture through a calibrated portion of 100 μl pipette was used as a measure of viscosity. Viscosity of the mixture was measured at 0 and 60 min at room temperature. Viscosity data was converted to intrinsic viscosity and relative unit of activity was calculated as described by Durbin and Lewis (1988). Results were expressed as Relative activity h⁻¹ g⁻¹ FW.

2.4. Assay of PG activity

To measure PG activity, 250 mg of leaf abscission zones was homogenized in 1 ml of 20 mM Tris–HCl buffer (pH 7.8) containing 20 mM cysteine–HCl, 20 mM EDTA and 0.5% Triton X-100. Homogenate was filtered through 2 layers of muslin cloth to remove cell debris. The homogenate was centrifuged at 15,000 × g for 30 min at 4 °C. The supernatant was used as enzyme preparation for assay of polygalacturonase. The assay mixture consisted of 0.2 ml of 0.2 M Tris–HCl buffer pH 7.8, 0.1 ml of 0.2 M NaCl, 0.3 ml of 1% polygalacturonic acid (PGA) prepared in 0.2 Tris–HCl buffer pH 7.8 and 0.3 ml enzyme suspension. The reducing sugar liberated by enzyme action was measured by the DNS method (Miller, 1959). One unit of enzyme was expressed as the liberation of 1 nmol of reducing sugar in one sec under the enzyme assay conditions. Results were expressed in nmol s⁻¹ mg⁻¹ protein.

2.5. Statistical analysis

Each experiment was carried out under completely randomized design with three replications repeated at least thrice. The

![Fig. 1. Changes in break strength of cotton LAZ after exposure of explants to ethylene, 1-MCP + ethylene, ABA, ABA + ethylene, 1-MCP + ABA + ethylene, IAA, IAA + ethylene, and 1-MCP + IAA + ethylene in comparison to control.](image-url)
data were analyzed by student’s unpaired t-test, and the treatment mean values were compared at \( P \leq 0.05 \) – 0.001.

3. Results

3.1. Changes in break strength of abscission zone

A continuous decrease in the break strength of the leaf abscission zone in ethylene-treated explants was observed. The effect of different phytohormones and 1-MCP on break strength of leaf abscission zone is shown in Fig. 1. A significant decrease \((P \leq 0.001)\) in break strength was observed within 48 h of ethylene-treated explants when compared to 0 h. Though a decrease in break strength was also observed in control and explants pre-treated with 1-MCP, this decrease was significantly lower \((P \leq 0.001)\) than the ethylene-treated samples. In comparison to 0 h \((28.35 \pm 1.92 \text{ gm equivalent})\), a 91% decrease in break strength was observed in ethylene-treated explants after 48 h \((2.4 \pm 1.64 \text{ gm equivalent})\), whereas 60 and 76% decrease in break strength was observed in control \((10.90 \pm 0.84 \text{ gm equivalent})\) and 1-MCP \((8.62 \pm 1.18 \text{ gm equivalent})\) treated explants respectively for the same period of time.

Break strength of abscission zones treated with ABA decreased continuously and reached a minimum \((6.71 \pm 0.84 \text{ gm equivalent})\) after 48 h, which was significantly lower \((P \leq 0.001)\) as compared to control and 1-MCP treated explants but higher than ethylene-treated. Decrease in break strength in explants treated with ABA plus ethylene was comparable to ethylene-treated explants. Treatment with 1-MCP, prior to ABA plus ethylene reduced the decrease in break strength \((10.81 \pm 1.01 \text{ gm equivalent})\), which was equal to control.

Though IAA treated samples did show some decrease in break strength, this decrease was more or less same as in control or 1-MCP. However, when IAA treated plants were treated with exogenous ethylene, IAA inhibited the decrease in break strength caused by ethylene and was similar to 1-MCP plus ethylene-treated explants \((13.76 \pm 2.96 \text{ gm equivalent})\).

3.2. Changes in activities of cell wall hydrolases during ethylene induced abscission

The distribution of cellulase activity in different tissues as well as LAZ during ethylene induced abscission is shown in Fig. 2. The cellulase activity continuously increased in LAZ.
after ethylene treatment of explants (Fig. 2A). A substantial increase in activity of cellulase was observed after 12 h (33.41 RA×10^3 h^−1 g^−1). The activity increased to 63.35 RA×10^3 h^−1 g^−1 after 36 h. The maximum activity (93.50 RA×10^3 h^−1 g^−1) was observed at 48 h. In 1-MCP pre-treated abscission zones, cellulase activity did not increase with ethylene exposure. The cellulase activity in 1-MCP pre-exposed abscission zones was 1.43 RA×10^3 h^−1 g^−1 48 h after ethylene treatment, which was negligible compared to ethylene-treated samples. Cellulase activity was also observed in other vegetative tissues such as the petiole (26 RA×10^3 h^−1 g^−1), leaf (16.6 RA×10^3 h^−1 g^−1), stem (15.1 RA×10^3 h^−1 g^−1), root (9.5 RA×10^3 h^−1 g^−1), flower (6.3 RA×10^3 h^−1 g^−1) and control abscission zone (11.21 RA×10^3 h^−1 g^−1). No significant effect of ethylene exposure for 24 h was observed on cellulase activity in any vegetative tissues other than LAZ (Fig. 2B). For this set of experiments the LAZ cellulase activity was more than three fold higher in ethylene-treated explants (39.12 RA×10^3 h^−1 g^−1) in comparison to control explants (11.21 RA×10^3 h^−1 g^−1). Root and Flower tissues were not treated with exogenous ethylene (Fig. 2B).

The changes in PG activities in different vegetative tissues and in LAZ are shown in Fig. 3. During a time course study in LAZ, the PG activity increased more than 5 fold during 48 h period from 3.9 nmol s^−1 mg^−1 protein to 20.2 nmol s^−1 mg^−1 protein. In 1-MCP treated explants, no appreciable PG activity was observed even after 48 h (Fig. 3A). PG activity was higher in petiole (7.9 nmol s^−1 mg^−1 protein) and flower tissue (11.2 nmol s^−1 mg^−1 protein) compared to leaf (5.19 nmol s^−1 mg^−1 protein), stem (1.78 nmol s^−1 mg^−1 protein) and root (0.5 nmol s^−1 mg^−1 protein) tissues not exposed to ethylene (Fig. 3B). When these tissues were exposed to ethylene for 24 h no significant increase in PG activities were observed in tissues other than the LAZ where an almost two fold increase in PG activity was observed (3.9 nmol s^−1 mg^−1 protein at 0 h to 7.35 nmol s^−1 mg^−1 protein).

3.3. Effect of ABA on cellulase and PG activities

The effect of ABA on the activities of cellulase and PG in leaf abscission zones is shown in Fig. 4. Our objective was to study effect of ABA on cellulose and PG activities during ethylene induced abscission; hence activities were not measured in untreated plants at different time points. The activities of both enzymes increased continuously after ABA treatment when compared to 0 h. The maximum activity was observed 48 h after post ethylene treatment (24.23 RA×10^3 h^−1 g^−1 for cellulase and 14.58 nmol s^−1 mg^−1 protein for PG). When ABA treatment was combined with ethylene there was a significant increase in the in the activities at all time point. The activities after 48 h in such cases were 89.22 RA×10^3 h^−1 g^−1 for cellulase and 18.1 nmol s^−1 mg^−1 protein for PG. Treatment of ABA+ethylene with 1-MCP explants inhibited the increase in cellulase and PG activities.

3.4. Effect of IAA on cellulase and PG activities

The effect of IAA on the activities of cellulase and PG with or without ethylene is presented in Fig. 5. Our objective was to study
effect of IAA on cellulose and PG activities during ethylene induced abscission; hence activities were not measured in untreated plants at different time points. The explants treated only with IAA did not show any appreciable increment in cellulase activity even after 48 h. A moderate increase in PG activity before 36 h (7.45 nmol s\(^{-1}\)mg\(^{-1}\) protein) and 48 h (9.5 nmol s\(^{-1}\)mg\(^{-1}\) protein) was observed when compared to 0 h (4.2 nmol s\(^{-1}\)mg\(^{-1}\) protein). After 48 h, cellulase activity in ethylene and IAA plus ethylene-treated samples was 97 RA×10\(^{-3}\) h\(^{-1}\)g\(^{-1}\) and 32 RA×10\(^{-3}\) h\(^{-1}\)g\(^{-1}\) respectively. At the same time, PG activity in ethylene and IAA plus ethylene-treated was 20.2 nmol s\(^{-1}\)mg\(^{-1}\) protein and 11.82 nmol s\(^{-1}\)mg\(^{-1}\) protein respectively. This suggests that treatment of explants with IAA before exposure to ethylene strongly inhibited cellulase activity and moderately inhibited PG activity. When 1-MCP was included along with IAA before exposure to ethylene it had an additive effect on inhibition both for cellulase and PG.

4. Discussion

Organ abscission is believed to be a highly coordinated process where the plant hormone ethylene plays a major role in regulating activities of cell wall hydrolases that are ultimately responsible for the loosening of abscising organs from the parent body (Sexton and Roberts, 1982). Cotton is an important cash crop and defoliation before cotton boll harvesting is an important agronomical practice. However, the studies on the process of leaf abscission have been limited at physiological, biochemical and molecular levels in order to utilize it for biotechnological purposes. We have monitored the activities of two major cell wall hydrolases viz. polygalacturonase and cellulase as well as the break strength in the leaf abscission zones of ethylene exposed cotton explants over a period of 48 h. A decrease in break strength of LAZ was observed after ethylene exposure, which was inhibited in the presence of ethylene perception inhibitor 1-MCP. This decrease was visible as early as 12 h post ethylene treatment and continued up to 48 h. Break strength of the flower petals has already been taken as a measure of ethylene dependent abscission in Arabidopsis (Patterson and Bleeker, 2004). This suggests that cotton leaf abscission is ethylene dependant. Though ethylene dependent organ abscission has been shown in other crops, the break strength data was not available for most crops. Our data suggests that ABA treatment alone can induce abscission process whereas IAA completely inhibited this (Fig. 1). ABA has already been reported to induce leaf abscission in cotton (Suttle and Hulstrand, 1993) and in cocoa flowers (Aneja et al., 1999).

In our study, an early induction followed by continuous increase in cellulase and PG enzyme activities after ethylene treatment has already been observed in LAZ, which could be detrimental for the abscission of cotton leaf. Similar observations have been made in peach fruit abscission zones in which activity of these cell wall hydrolases increased after ethylene treatment (Ruperti et al., 2001). Tucker et al. (1988) had shown that the continuous presence of ethylene is required for the accumulation of cellulase mRNA in the abscission zones by recruiting more and more cells to synthesize cellulase. In our experiment, some activities of cellulase and PG were also observed in different vegetative tissues with or without ethylene exposure. However, no significant increase was observed in response to ethylene except in abscission zones (Figs. 2B, 3B). This shows that response to ethylene is tissue specific and developmentally regulated.

We have measured the effect of other phytohormones such as ABA and IAA on break strength and cellulase and PG activities of cotton leaf explants. We have observed that ABA alone could initiate the abscission process. The ABA treated LAZ showed a decrease in break strength greater than the control, suggesting that the ABA effect was over and above the wounding effect. An appreciable increase in cellulase and PG activity in the LAZ of ABA treated explants compared to 1-MCP + ABA + ethylene-treated explants suggested that ABA could be responsible for this increment. However, in the presence of ethylene, this effect could be masked. It has been suggested that in aged or injured detached tissues it is possible to stimulate ethylene synthesis and promote abscission with high amounts of ABA (Goren, 1993). Environmental stress can also induce in vivo ABA accumulation in intact plants and induce leaf abscission to occur (Gomez-Cadenas et al., 1996; Gomez-Cadenas et al., 1998). Ethylene and auxin are important regulators of abscission (Sexton and Roberts, 1982). It has been shown that IAA retards while ethylene accelerates the abscission process (Abeles, 1968). The IAA completely inhibited ethylene induced cotton leaf abscission in the present study as measured by break strength. The activities of cell wall hydrolases remained close to control values. It has been suggested that the flux of IAA to the abscission zone inhibit cell separation causing inhibition of the abscission process (Addicott, 1983). The activities of cellulase and PG in IAA + ethylene-treated samples do not show much difference in comparison to the IAA treated samples. It can be concluded that pretreatment with the IAA to cotton explants abolishes the abscission process caused by ethylene. Similar to our results, pretreatment with 2, 4-Dichlorophenoxyacetic acid in bean explants completely blocked the abscission and reduced cellulase activity as much as 90% when compared to ethylene-treated explants (Koehler et al., 1996). It is concluded that cotton leaf abscission is initiated and promoted by the plant hormone ethylene through induction of at least cellulase and polygalacturonase activities in its abscission zones. The process of abscission is inhibited by 1-MCP and IAA and facilitated by ABA.

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