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Research article

Ethylene induced cotton leaf abscission is associated with higher expression of cellulase (*GhCel1*) and increased activities of ethylene biosynthesis enzymes in abscission zone

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Abstract

Ethylene induced cotton (*Gossypium hirusutum* var RST-39) leaf abscission has been characterized by measuring the activities of ACC synthase (ACS, E.C. 4.4.1.14), ACC oxidase (ACO, E.C. 1.14.17.4) and cellulase (E.C. 3.2.1.4). In addition, a leaf abscission specific cDNA (*GhCel1*) has been cloned from cotton, which belongs to the α_2 subgroup of cellulases that possess a C-terminus carbohydrate-binding domain. Measurement of enzyme activity in the abscission zones of cotton leaf explants exposed to ethylene for 48 h compared to non-treated controls indicated a more than 5-fold increase in the activity of ACS, 1.2-fold increase in the activity of ACO and about 2.7-fold increase in the activity of cellulase in the ethylene treated explants. This increase was accompanied by a substantial decrease in the force required to separate the petiole from the stem (break strength) and an increased accumulation of cellulase transcript in the abscission zone. Treatment of explants with 1-Methylcyclopropene (1-MCP) prior to ethylene resulted in significant inhibition of enzyme activities and transcript accumulation. It is concluded that ethylene response of cotton leaf abscission leads to higher cellulase expression and increased activities of ethylene biosynthesis enzymes in the abscission zone.

Keywords: 1-Methylcyclopropene; ACC oxidase; ACC synthase; Cellulase; Ethylene; *Gossypium hirusutum*; Leaf abscission

1. Introduction

Abscission is the process by which plants shed organs from the parent body in response to developmental cues or to adapt to various environmental stresses including pathogen attack [26,31]. The signal that promotes abscission has been widely recognized as the gaseous plant hormone ethylene [1,37]. The role of ethylene in abscission was deduced by the use of chemical agents such as norbornadiene, silver ions or 1-MCP that block ethylene action [10,40,41] or mutants, which are deficient in ethylene perception and response [7]. Organ separation is accomplished by degradation of the cell wall and middle lamella to form a fracture plane between the abscising organ and the parent plant. Although the precise sequence of events that bring about cell wall degradation is unclear, biochemical and molecular studies indicate that an increase in the activities of hydrolytic enzymes such as glucanases [5,38] and polygalacturanases [14,18] and proteins such as expansins [4] play a major role in the process of abscission.

An increase in cellulase (endo-1,4-β-D-glucanase) activity has been noted in leaf abscission zones (LAZ) [20,40], fruit abscission zones [15,17], ripening fruits [24] senescing styles and anthers [34] and during adventitious root initiation [23]. The isoelectric points (p*I*s) of many of these cellulases differ from each other, which suggest that each may have distinct

Abbreviations: 1-MCP, 1-Methylcyclopropene; ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase; ACC synthase, 1-aminocyclopropane-1-carboxylate synthase; LAZ, leaf abscission zone.

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cellular function. Although cellulases have been identified and cloned from the abscission zones of several different sources, information relating to its role in leaf abscission is limited [31].

In the present study we have investigated petiole abscission in cotton using biochemical and molecular tools. The activities of cellulase and ethylene biosynthesis enzymes have been measured in LAZ and correlated with the progression of petiole separation. A leaf abscission specific cDNA has been cloned and characterized for the first time from cotton and accumulation of mRNA in abscission zones studied in the presence and absence of 1-MCP, an ethylene action inhibitor. The results of this study gain significance due to commercial importance of cotton leaf abscission.

2. Materials and methods

2.1. Plant material and treatments

Seeds of cotton (*Gossypium hirusutum* var RST-39) were germinated in glasshouse under controlled temperature (32 ± 3 °C) for 12–15 days until the primary leaves were fully expanded and the secondary pinnate leaf was just beginning to open. At this time, the leaf blades for the primary leaves were removed and plants harvested by cutting 1 cm above the soil. Twenty-five explants, each of 8–10 cm long, were surface sterilized using bleach (10% v/v) for 5 min, washed 3–4 times with distilled water and kept in a beaker containing water and exposed to 10 μL/L ethylene in a 10 liter air tight desiccators for the time intervals indicated in the figure legends. Desiccators were opened every 4 h, flushed with air to remove CO_2 accumulation and maintain O_2 concentrations and 10 μL/L ethylene reintroduced fresh each time. For 1-MCP treatment, explants were exposed to 10 μL/L 1-MCP (EthylBlock, BioTechnologies for Horticulture, Inc., Waterboro, USA) for 1 h before ethylene exposure. For 1-MCP application, 1 ml water was added to 1-MCP powder kept in a 10 ml beaker and placed towards sidewall of the desiccators containing explants. Desiccators were closed immediately (within 2–3 s) to prevent diffusion of 1-MCP outside the desiccators. The concentration of 1-MCP was calculated as per manufacturers instructions. LAZs (1–2 mm on either side of fracture plane) were harvested and frozen in liquid nitrogen and stored at −70 °C till further use. Other vegetative tissues were also collected after exposure to ethylene for 24–36 h.

2.2. Break strength measurement

In order to determine the break strength, the petiolar stump of the explant was clamped using an alligator clip to which a string was attached for holding weight. Increasing weights were loaded on the string till the petiolar stump detached at the abscission zone. The distance of the explant from bench top as well as length and angle of the string were kept constant during each measurement to avoid any variations in the force applied. Total weight applied on the petiolar stump was determined and break strength of the abscission zone calculated as force in gram equivalents required to detach the petiole. Break strength values determined for each time point represented an average of at least 20 or more individual measurements.

2.3. Enzyme activity measurements

For ACS activity measurements, LAZs (200 mg) were homogenized in extraction buffer consisting of 100 mM Hepes-KOH, pH 8.5, 4 mM DTT, 10 μM pyridoxal phosphate and 5% PVP 40,000 as described by Kato et al. [19]. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant was desalted using Sephadex G-25 and eluate used for enzyme assay as described by Pathak et al. [29]. ACS activity was expressed as nmoles ACC formed h^{-1} g^{-1} FW.

ACO enzyme was extracted according to Moya-Leon and Jhon [27]. A total of 200 mg frozen tissue was homogenized in 1 ml of extraction buffer consisting of 0.1 M Tris–HCl, pH 7.5, 10% glycerol, 2 mM DTT and 30 mM sodium acetate. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4 °C. Further purification of supernatant and enzyme assay was carried out as described by Pathak et al. [29]. ACO activity was expressed as nmoles C_2H_4 produced h^{-1} g^{-1} FW.

Cellulase activity was measured in 1 g of abscission zones homogenised in 3 ml of 0.1 M sod phosphate buffer (pH 7.8) containing 2 mM EDTA, 0.1 mM DTT and 0.2% Triton X-100. The homogenate was transferred to eppendorf tubes on ice and 0.5 M EDTA (1/500 vol) and 5 M NaCl (1/10 vol) added, mixed thoroughly and kept on ice for 30 min. The homogenate was centrifuged at 15,000 × *g* for 30 min at 4 °C. The pellet was discarded and supernatant was used as enzyme preparation for the cellulase assay. The assay mixture contained 0.5 ml enzyme and 0.5 ml 1.3% (w/v) carboxy methyl cellulose (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 an interval of 30 min from 0 to 2 h at room temperature. Viscosity data was converted to intrinsic viscosity and relative units of activity calculated as described by Durbin and Lewis [12]. Results were expressed as Relative activity h^{-1} g^{-1} FW.

2.4. Scanning electron microscopy

For electron microscopy, abscission zones were fixed in 4% (v/v) gluteraldehyde in 0.5 M potassium phosphate buffer (pH 7.4) for 4 h at 25 °C, rinsed four times in buffer, and then dehydrated in a graded ethanol series. Sputter coated sections were examined at different magnifications with Philips XL20 scanning electron microscope (Philips, Holond).

2.5. Cloning of cellulase gene and nucleotide sequence analysis

Total RNA from abscission zones and other vegetative tissues was isolated according to Asif et al. [3]. In order to clone cellulase cDNA, RT-PCR was carried out using total RNA from 24 h ethylene treated abscission zones. Oligonucleotide primer pairs were designed from regions having maximum sequence