Molecular Cloning of α-Amylases from Cotton Boll Weevil, *Anthonomus grandis* and Structural Relations to Plant Inhibitors: An Approach to Insect Resistance

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*Anthonomus grandis*, the cotton boll weevil, causes severe cotton crop losses in North and South America. Here we demonstrate the presence of starch in the cotton pollen grains and young ovules that are the main *A. grandis* food source. We further demonstrate the presence of α-amylase activity, an essential enzyme of carbohydrate metabolism for many crop pests, in *A. grandis* midgut. Two α-amylase cDNAs from *A. grandis* larvae were isolated using RT-PCR followed by 5’ and 3’ RACE techniques. These encode proteins with predicted molecular masses of 50.8 and 52.7 kDa, respectively, which share 58% amino acid identity. Expression of both genes is induced upon feeding and concentrated in the midgut of adult insects. Several α-amylase inhibitors from plants were assayed against *A. grandis* α-amylases but, unexpectedly, only the BIII inhibitor from rye kernels proved highly effective, with inhibitors generally active against other insect amylases lacking effect. Structural modeling of *Amylag1* and *Amylag2* showed that different factors seem to be responsible for the lack of effect of 0.19 and α-AI1 inhibitors on *A. grandis* α-amylase activity. This work suggests that genetic engineering of cotton to express α-amylase inhibitors may offer a novel route to *A. grandis* resistance.

KEY WORDS: α-Amylase inhibitors; cotton boll weevil; cDNA cloning; plant defense; *Anthonomus grandis*.

1. INTRODUCTION

The cotton boll weevil, *Anthonomus grandis* (Boheman, 1843), is a major insect pest of cultivated cotton, *Gossypium hirsutum* L., being responsible for significant cotton losses in South and North Americas. The adult female moves to the flower bud after alighting on the plant and then proceeds to construct an oviposition hole in which the egg is placed and which is then sealed. Floral buds and bolls are the main *A. grandis* food, in which female insects depend on nutrients from pollen grains and ovules for reproduction and eggs development (Bottrell, 1983). Larval behavior, such as movement into protected places on the bud after egg hatching, has made this insect difficult to control using conventional insecticides and management.

The use of chemical pesticides leads to high production costs as well as causing risks to human health. In the light of these considerations, plant genetic transformation...
with exogenous genes encoding factors of resistance to phytophagous insects is a modern and attractive alternative to synthetic chemical insecticides for the control of several aggressive plant pests (Estruch et al., 1997). Efforts have been focused on the screening of different proteins known to have insect control properties, such as Bt-toxins, proteinase inhibitors, lectins, and α-amylase inhibitors (Boulter, 1993; Gatehouse and Gatehouse, 1998; Carlini and Grossi-de-Sá, 2002). Cotton improvement through genetic engineering has become a reality with the successful testing of insect-resistant transgenic cotton expressing protease inhibitors (Thomas et al., 1995) and Bt-toxin (Jenkins et al., 1995).

α-Amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) catalyzes the hydrolysis of the α-(1,4)-glycosidic linkages of starch components, glycogen and various oligosaccharides that are widespread in nature, being found in animals, microorganisms and plants. Insect and mammalian α-amylases have been characterized from a biochemical, molecular and structural point of view in considerable detail (Franco et al., 2000; MacGregor et al., 2001). Because of their important biochemical roles in insect growth and development, when the action of the α-amylases is inhibited, insect nutrition is impaired. As strategies of control, inhibitors to insect amylase have been already demonstrated to be an important biotechnology system in the control of insect-pests. Pea and azuki transgenic plants expressing α-amylase inhibitors from common beans (α-AI) were completely resistant to the Brachus pisorum and Callosobruchus chinensis weevil (Morton et al., 2000).

Here we show the presence of starch in the floral buds of the cotton plant. Subsequent investigation showed that A. grandis expresses two different amylases. These were both cloned and characterized. Additionally, a range of α-amylase inhibitors was assayed against A. grandis α-amylases. Unusually, only an inhibitor present in rye kernels proved effective toward the digestive amylases from A. grandis.

2. MATERIALS AND METHODS

2.1. Insect Rearing

A population of A. grandis (Coleoptera: Curculionidae) originally obtained from CIRAD (Montpellier, France) was maintained at 27 ± 1°C, 70 ± 10% relative humidity with 14-hr day length. Insects were routinely maintained on standard rearing diet as described by Monnerat et al. (2000). All components were purchased from Sigma (St. Louis, MO). Wild insects were collected in Unaí, Brazil.

2.2. Extraction of Larval α-Amylase and α-Amylase Inhibitory Assays

Midguts from third instar larvae and 10-day-old adults were excised from cold-anesthetized larvae and insect adults and ground in cold 0.15 M NaCl using a 1:10 w/v ratio. The homogenate was centrifuged twice at 10,000 × g for 20 min and stored at −20°C. The cleared homogenates were used for the amyloytic inhibition assays. α-Amylase activity was measured by the Bernfeld method (1955) using a standard concentration at 50 μg/ml. The enzyme was dissolved in 100 mM sodium phosphate buffer (pH 5.8) containing 10 mM NaCl and 1 mM CaCl₂. The enzymatic reaction occurred for 15 min at 37°C using soluble starch as substrate. Protein concentration was determined as described by Bradford (1976). Each assay was done in triplicate, with a maximum difference of 10%.

2.3. Plant α-Amylase Inhibitor Purification

The α-amylase inhibitors 0.19 and 0.53 were purified from BR35 wheat (Triticum aestivum) kernels according to Franco et al. (2000). The inhibitor BII from rye (Secale cereale) kernels was purified according to Iulek et al. (2000). The inhibitor SœAI5 from Sorghum bicolor seeds was purified according to Bloch and Richardson (1991), and the common bean seed α-amylase inhibitors (α-AI1 and α-AI2) were purified according to Grossi-de-Sá et al. (1997). The purification progress was monitored by MALDI-TOF and SDS-PAGE analyses (Laemmli, 1970; Franco et al., 2000).

2.4. RT-PCR, 5' and 3' RACE Amplifications

Reverse transcription of A. grandis total RNA was performed using oligo(dT)-anchor primer and AMV-RT (Boehringer Mannheim) according to the manufacturer’s protocol. For the RT-PCR amplifications, two primers, AmyA (5'-GGGTGTGTTGTTGTCGACGAA-3') and AmyB (5'-GACGTGTGTGTTCAACCATACTGGC-3'), were designed, which correspond to short sequences conserved in a number of insect α-amylases. Amplification was carried out in a PTC-100 Programmable Thermal Controller (MJ Research) using Taq DNA Polymerase (GIBCO) under the following conditions: 2 min at 94°C then 30 cycles of 30 s at 94°C, 45 s at 52°C and 1 min at 72°C plus an extension step for 5 min at 72°C. To obtain the complete cDNA sequences, the 5' and 3' ends were amplified using a 5'/3' RACE Kit (Boehringer Mannheim) according to the manufacturer’s instructions using specific primers designed based on the previous amplified sequence. The amplified cDNAs were cloned into the plasmid vector pGEM-T Easy (Promega, Madison,