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Employing *in vitro* directed molecular evolution for the selection of α-amylase variant inhibitors with activity toward cotton boll weevil enzyme☆

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ABSTRACT

Numerous species of insect pests attack cotton plants, out of which the cotton boll weevil (*Anthonomus grandis*) is the main insect in Brazil and must be controlled to avert large economic losses. Like other insect pests, *A. grandis* secretes a high level of α-amylases in the midgut lumen, which are required for digestion of carbohydrates. Thus, α-amylase inhibitors (α-AIs) represent a powerful tool to apply in the control of insect pests. Here, we applied DNA shuffling and phage display techniques and obtained a combinatorial library containing 10^8 *α-AI* variant forms. From this library, variants were selected exhibiting *in vitro* affinity for cotton boll weevil α-amylases. Twenty-six variant sequences were cloned into plant expression vectors and expressed in *Arabidopsis thaliana*. Transformed plant extracts were assayed *in vitro* to select specific and potent α-amylase inhibitors against boll weevil amylases. While the wild type inhibitors, used to create the shuffled library, did not inhibit the *A. grandis* α-amylases, three α-AI mutants, named α-AIC3, α-AIA11 and α-AIG4 revealed high inhibitory activities against *A. grandis* α-amylases in an *in vitro* assay. In summary, data reported here shown the potential biotechnology of new *α-AI* variant genes for cotton boll weevil control.

1. Introduction

The insect cotton boll weevil *Anthonomus grandis* is responsible for causing several types of damage to the cotton crop (*Gossipium sativum*), both in its native countries and in countries where it was introduced (Haynes and Smith, 1992; Almeida and Silva, 1999). Boll weevil attacks have been responsible for a severe reduction in cotton productivity, particularly in Brazil (Ramalho, 1994). The adult insects attack preferentially bolls and floral buds; chemical control is thus inefficient since larval development occurs inside (Carvalho, 2006; Miranda, 2010). In previous studies, it was demonstrated that pollen grains and the ovary of the cotton plant contain a large amount of starch (Oliveira-Neto et al., 2003). In addition, α-amylase activity was also reported to be high in the intestinal tract of adult insects and larvae in the field (Oliveira-Neto et al., 2003). Accordingly, Dias et al. (2005) suggested that α-amylase enzymes could be an important target for biotechnological strategies to be applied in the control of cotton boll weevil.

Several proteins have been identified and characterized for their involvement in plant defense against insect attacks. In particular, they include inhibitors of insect hydrolytic enzymes that act in the breakdown of macromolecules (Grossi-de-Sa et al., 1997; Haq et al., 2004; Payan, 2004; Franco et al., 2005; Gomes et al., 2005).

Abbreviations: AgA, *Anthonomus grandis* α-amylases; α-AI, α-Amylase Inhibitor; MS, Murashide Skoog.

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Different reports show the detrimental effects of α-amylase inhibitors (αAIs) on enzymes of insect pests (Valencia et al., 2000; Franco et al., 2004; Dias et al., 2005; Dunaevsky et al., 2005; Valencia-Jimenez et al., 2008; Wisessing et al., 2010; Barbosa et al., 2010). In insects, α-amylase enzyme isoforms are few, in lesser number than those of proteases, and insects can develop mechanisms to over-express different proteases to compensate for inhibition of another (Gatehouse, 2011). The use of α-amylase inhibitor (reviewed in Ahmad et al., 2012) thus has advantages as a control strategy for the generation of transgenic plants capable of interfering with digestive enzymes from insect pests.

α-Amylase inhibitors (α-AIs) isolated from common bean seeds (*Phaseolus vulgaris*) – denominated α-AI1 and α-AI2 and members of the lectin-like class – have been extensively characterized as insecticidal proteins. The α-AI isoforms share 78% amino acid identity yet their inhibitory activities are specific. The α-AI1 is able to inhibit α-amylases from coleopteran insects, cowpea weevil (*Callosobruchus maculatus*) and azuki bean weevil (*Callosobruchus chinensis*), as well as inhibiting human salivary α-amylase and pancreatic porcine α-amylase (Ishimoto et al., 1996). The α-AI2 inhibits amylases from Mexican bean weevil (*Zabrotes subfasciatus*) and *Bruchus pisorium*, but does not inhibit amylases from mammals (Ishimoto and Kitamura, 1989; Grossi-de-Sa et al., 1997; Solleti et al., 2008). However, neither of these α-AI isoforms inhibited α-amylases from the coleopteran cotton boll weevil (*A. grandis*) in *in vitro* assay studies (Oliveira-Neto et al., 2003).

The protective effects of α-AI1 against weevils have been shown by its expression in transgenic plants of pea (Shade et al., 1994; Schroeder et al., 1995; Grant and Cooper, 2006); azuki bean (Ishimoto et al., 1996), cowpea (Solleti et al., 2008); chickpea (Sarmah et al., 2004; Ignacimuthu and Prakash, 2006) and coffee (Barbosa et al., 2010). Assays using recombinant α-AI1 derived from these plants showed reduced damage caused by weevils that attack leguminous plants. In the case of transgenic pea plants, complete protection against the weevil *B. pisorium* in field conditions was found (Morton et al., 2000).

Construction of combinatorial protein libraries is a proven method for the development of proteins containing novel or improved binding properties (Yuan et al., 2005; Grönwall and Ståhl, 2009). Such strategies applying *in vitro* molecular evolution have been successful in deriving improved insecticidal proteins. For example, our research group recently generated combinatorial libraries for *cry* variants using *cry* genes and applying DNA shuffling and phage display techniques. Using receptors present in the insect midgut membrane, novel Cry toxins with potential for use in control of insects such as giant borer sugarcane (*Telchin licus licus*) and cotton boll weevil (*A. grandis*) were selected from these libraries (Craveiro et al., 2010; Oliveira et al., 2011).

Here we applied DNA shuffling to the genes encoding α-AI1 and α-AI2 and obtained a phage display combinatorial library containing quantity large number of *α-AI* variants. Screening from this library allowed for the selection of diverse genes whose protein products bound to cotton boll weevil α-amylase. Later *in vitro* enzymatic assays revealed three variant molecules capable of inhibiting cotton boll weevil α-amylase enzymes. These *α-AI* variant candidates can be used alone or together (pyramidal gene approach) in transgenic cotton plants, with the aim of achieving cotton boll weevil control.

2. Materials and methods

2.1. Preparation of *A. grandis* α-amylases (AgA)

A. grandis third-instar larvae reared on an artificial diet (Martins et al., 2007) at 25 °C and 55% of relative humidity were obtained from a colony maintained at EMBRAPA Genetic Resource and Biotechnology Center (Brazil). Fifty larvae were macerated in 500 μL of extraction solution (Succinic Acid 0.15 M, NaCl 0.06 M, $CaCl_2$ 0.02 M pH 4.5 containing protease inhibitor cocktail (Sigma)). The larval extract was centrifuged at 10,000 × *g* for 30 min at 4 °C and the supernatant was cleared by filtration using 0.45 μm filter (Millipore). The protein concentration was determined by the Bradford assay (Bradford, 1976), using Bovine Serum Albumin (BSA) as the standard for the curve calibration.

To purify *A. grandis* α-amylases, the volume equivalent to 14 mg of the crude extract was lyophilized, resuspended in the same buffer used to equilibrate column and loaded on a Phenyl-Sepharose CL 4B® (Amersham) column (100 mm × 15 mm) pre-equilibrated with 10 mM imidazole buffer pH 6.0, containing 1 M $(NH_4)_2SO_4$, using a flow rate of 1 mL min^{-1}. The bound proteins were eluted with a linear gradient (1–0 M in the same buffer, 10 mM imidazole pH 6.0). Finally, 30 mL of buffer (10 mM imidazole buffer pH 6.0) were used for isocratic elution. The fractions were monitored by absorbance at 280 nm and α-amylase activity was assayed for each eluted fraction. The α-amylase activity was determined using the method described by Fuwa (1954) with modifications. An aliquot (2 μL) of each fraction was transferred to microplate wells containing 68 μL of activity solution (50 mM Na_2HPO_4 pH 5.8; 20 mM NaCl, 0.1 mM $CaCl_2$ and incubated at room temperature for 30 min with 30 μL of soluble starch (0.125%, w/v) (Sigma) in the same solution. The reaction was blocked by addition of 140 μL of iodine solution (0.01% iodine dye in 125 mM HCl) and the absorbance measured at 630 nm. The fractions presenting α-amylase activity were pooled and dialyzed against water for 24 h. Aliquots containing 50 μg of protein were lyophilized and stored at 4 °C.

The semi-purified AgA was analyzed by 12% SDS-PAGE (Laemmli, 1970) and amylase gel assay (Silva et al., 1999; Campos et al., 1989). To perform the amylase gel assay, the samples were suspended in sample loading buffer (25 mM Tris pH 8.8; 0.01% (w/v) Bromophenol Blue) and separated by electrophoresis. After this, the gel was incubated in substrate solution (1.5%, w/v) Potato Starch (Sigma) for 2 h, 4 °C. Then the gel was washed three times using 0.1% (v/v) Triton X-100, for 30 min at 4 °C. Thereafter, it was incubated in activity solution (50 mM sodium citrate, 2.0 mM NaCl and 0.1 mM $CaCl_2$, pH 5.8) at 37 °C until cleared bands were revealed.

2.2. Combinatorial library construction using DNA shuffling and Phage display

For the generation of the combinatorial library containing the *α-AI* variants, *α-AI1* and *α-AI2* genes (Grossi-de-Sa et al., 1997) were used for the DNA shuffling procedures (Stemmer, 1994; Zhao and Arnold, 1997). *Pfu* Taq DNA polymerase (Promega) was used in all assembly and DNA amplification steps. Firstly, the *α-AI1* and *α-AI2* genes were amplified from original vectors using the primers sense: SfiIAI1F; SfiIAI2F and anti-sense SfiIAIR; SfiIA2R (Table 1). All primers encode a *Sfi* I site (underlined) which is suitable for later cloning into pCOMB3X phagemid (Andris-Widhopf et al., 2000). The PCRs were performed in 50 μL final volume, containing 375 nM each primer, 200 nM de dNTPs, 1 X *Pfu* Taq buffer (Promega®), 3 U *Pfu* Taq DNA polymerase (Promega®) and 400 ng of DNA template. The reactions were carried out in Mastercycler Gradient–Eppendorf thermocycler using the following conditions: 5 min, 95 °C; 29 cycles: 45 s 95 °C; 45 s 55 °C; 90 s 72 °C and final extension 10 min, 72 °C.

The DNAs were gel purified using Geneclean II Kit (Bio 101), mixed and randomly digested using DNAse I enzyme (Invitrogen), as described by Craveiro et al. (2010). The mixture containing 10 μg of DNA and 0.03 U DNAse I enzyme in 80 μL of buffer (50 mM Tris pH 7.5, 1 mM $MnCl_2$, 0.1 mg L^{-1} de BSA) was incubated at 15 °C for

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