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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

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

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et al., 2005). 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 quantitatively 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 SfiIAI1R; SfiIAI2R (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

Table 1
Primers.

Primer	Sequence	Restriction site (underlined)
SfiIAI1F	5′CCCGGCCCAGGCGGCCGCCACCGAAACCTC	*Sfi* I
SfiIAI2F	5′CCCGGCCCAGGCGGCCGCCAGCGACACCTC	*Sfi* I
SfiIA1R	5′CCGGCCGGCCTGGCCGAGGATCTTGTTGAG	*Sfi* I
SfiIAI2F	5′CCGGCCGGCCTGGCCGAGGATATTGTTGAG	*Sfi* I
pCOMB F	5′ GCTTCCGGCTCGTATGTTGTGT	
pCOMB R	5′ CGTCCATTGCATTCTTTTAAT	
XmaPSAI1	5′CCCCCGGGATGGCTTCCTCCAACTTACTCTC CCTAGCCCTCTTCCTTGTGCTTCTCAACCAC GCAAACTCAGCCACCGAAACCTCC	*Xma* I
AI2SacI	5′CCCGAGCTCTTAGAGGATCTTGTTGAGGAC	*Sac* I

pH 7.5, 1 mM $MnCl_2$, 0.1 mg L^{-1} de BSA) was incubated at 15 °C for 20 min and the digestion reaction was blocked by addition of 5 μL 0.5 M EDTA. Fragments of 50–300 bp separated by electrophoresis were jointly purified from a 2.5% agarose gel using PCR product purification kit (QIagen). Ten microliters of the pool of purified fragments were used as template in a 25 μL final volume PCR containing 0.4 mM dNTPs, 1 mM $MgSO_4$ and 2.5 U *Pfu* Taq DNA polymerase in 5× *Pfu* Tap polymerase buffer. No primers were added at this point. A PCR program was applied with 5 min 95 °C; 44 cycles: 1 min 95 °C, 1 min 42 °C and 1 min 72 °C (with a 5 s increase in extension time per cycle); with a final extension step of 7 min at 72 °C. The products of the primerless PCR (1.5 μL) were used as template for a second PCR, containing the primers SfiIAI1F and the SfiIAI1R (Table 1). The second PCR, performed in a 100 μL final volume, contained 0.2 mM dNTPs, 2 mM $MgSO_4$, 0.8 μM each primer, 2.5 U *Pfu* Taq DNA Polymerase and Platinum Taq DNA Polymerase recombinant (Invitrogen) (1:1) (v/v) in 1× *Pfu* Taq buffer. The conditions for the second PCR were: 2 min 95 °C; 10 cycles: 30 s 95 °C, 1 min 55 °C and 1 min 72 °C; followed by 14 cycles: 1 min 95 °C, 1 min 55 °C and 1 min 72 °C (with 20 s increase in extension time per cycle); with a final extension step of 10 min at 72 °C. The second PCR product of DNA shuffling procedure was analyzed by 0.8% agarose gel electrophoresis and a single product of approximately 660 bp (containing variant genes) was gel purified using Geneclean II Kit (Bio 101).

The DNA shuffling product was digested with enzyme *Sfi* I and gel purified. The reassembled fragments (1 μg) were ligated into pCOMB3X phagemid (Andris-Widhopf et al., 2000) also digested with *Sfi* I enzyme. The vector constructed was used to transform *Escherichia coli* XL1-Blue (Stratagene) cells by electroporation and the construction of the combinatorial M13 phage library displayed followed exactly as reported by Craveiro et al. (2010).

2.3. Biopanning and analysis of selected variant genes

The combinatorial library was screened using the biopanning procedure as described by Rader et al. (2001) including modifications reported by Craveiro et al. (2010). The microtiter plate wells were coated using 100 μg of purified enzyme AgA. The biopanning was performed in five rounds. In each cycle the number of washes increased. In the first cycle seven washes were performed using PBS (137 mM NaCl, 2.7 mM KCl, 12 mM Na_2HPO_4 and 1.2 mM KH_2PO_4) containing 0.1% Tween 20. A presence of 660 bp fragments (size of parental genes) in several of the clones isolated from the biopanning enriched cycle was checked by PCR colonies using SfiIAI1F and SfiIAI1R primers.

2.4. Sequence analysis

The DNA of clones showing amplicons of 660 bp by PCR analysis was extracted and its nucleotide sequences were determined using 3130xL Genetic Analyzer automatic sequencer (Applied Biosystems). The sense and anti-sense primers used for sequencing the N- and C-terminal coding ends were: pCOMB F and pCOMB R (Table 1), respectively. The nucleotide sequences were analyzed using the BLASTn and BLASTx algorithms (Altschul et al., 1997), available on the web (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Sequence alignments were performed by the ClustalW2 program (Larkin et al., 2007), available on the web (http://www.ebi.ac.uk/Tools/msa/clustalw2). In order to identify the substituted residues, the sequence alignments, including variant sequences, were compared with the α-AI1 and α-AI2 parental sequences.

2.5. Vector construction containing parental and α-AI variants

The parental and *α-AI* variants were amplified by PCR using the sense primer XmaPSAI1 with restriction site *Xma* I and signal peptide sequence from *α-AI1* gene added to 5′end and AI2SacI with *Sac* I restriction sites added to 3′ sequence end (Table 1). The gel-purified variant genes was subcloned into *Xma* I and *Sac* I restriction sites of plant transformation vector pCambia 2300 (Cambia GPO, Canberra, Australia) modified by insertion of CaMV35S (Cauliflower Mosaic Virus 35 promoter), doubled enhancer element (the −208 to −46 bp upstream fragment) from CaMV 35S promoter and the nopaline synthase transcriptional (NOS) terminator. The recombinant plasmids were entitled pFSpl2300AIs.

2.6. Transformation of Arabidopsis thaliana plants

First, the seeds of *A. thaliana* ecotype Columbia 0 were incubated into distilled water at room temperature for 24 h. After this, the seeds were spread into plates containing MS medium (0.25% MS, 0.6% agar) and incubated in the dark, 48 h, 4 °C. The seeds were germinated in 16 °C, 16 h light. This procedure aimed to obtain seedlings at the same development stage. Later the seedlings were greenhouse transplanted into Bioplant® substrate. The binary vectors containing parental or α-*AI* variants were used to transform *Agrobacterium tumefasciens* strain GV3103 (Koncz and Schell, 1986). Arabidopsis plants of 6–10 cm and with most secondary inflorescences closed were transformed using floral dipping procedure (Clough and Bent, 1998). Mature seeds from the transformation of Arabidopsis Col-0 with the binary vectors were spread in MS medium added to kanamycin (100 mg L^{-1}). A quick DNA extraction from kanamycin-resistant seedling was obtained by crushing one leaf with plastic rod directly against microtube wall containing 300 μL of EB buffer (200 mM Tris–HCl, 25 mM EDTA, 250 mM NaCl, 0.5% SDS). When the solution turned transparent green, 100 μL of Cloroform was added and shaken for 1 min using Vortex. After centrifugation (11,000 × *g*, 5 min), the supernatant was precipitated using Isopropanol (1:1 volume). The pellet was washed using 70% ethanol and dissolved in 100 μL distillated water. In order to identify the transgenes, these genomic DNAs were immediately used

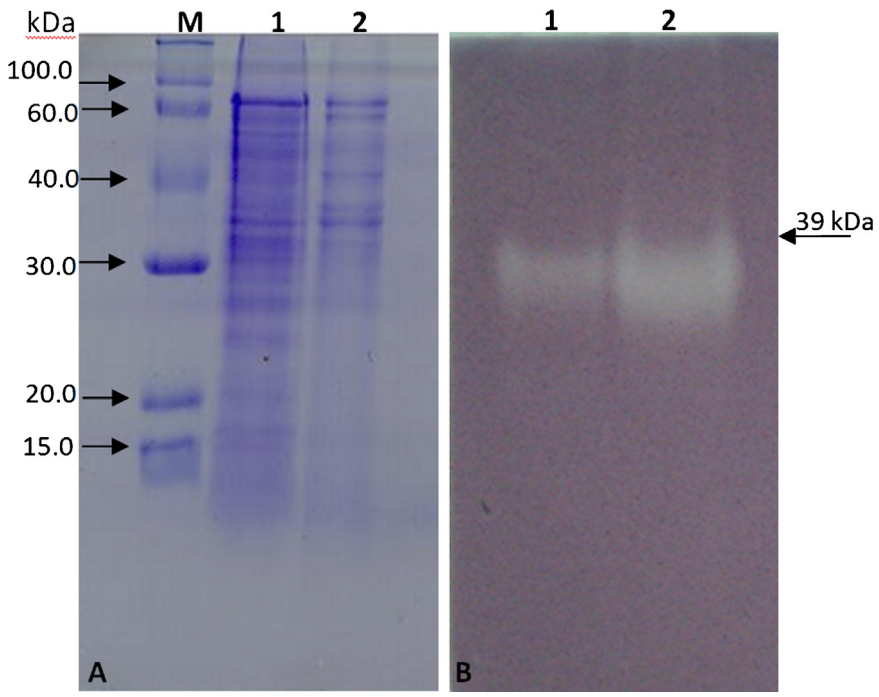

Fig. 1. Analysis of the α-amylase enzymes extracted from *A. grandis* larvae after purification using a Phenyl-Sepharose CL 4B column. (A) SDS-PAGE M: BenchMark protein ladder (Invitrogen); 1: midgut extract; 2: pool of fractions showing amylolytic activity (active peak): (B) Zymogram 1: midgut extract; 2: active peak showing *A. grandis* amylases. The electrophoretic mobility of AgA indicated a molecular mass of approximately 39 kDa.

as template in PCR analysis containing the primers XmaPSAI1 and AI2SacI (Table 1).

2.7. Western blot and ELISA

The α-AI recombinant proteins expressed in *A. thaliana* plants were detected using Western blot and ELISA techniques. Total protein from leaves of transformed and non-transformed plants were extracted using 1:10 (w/v) buffer (sodium succinate 50 mM, pH 4.5; NaCl 150 mM; $CaCl_2$ 0.1 mM; Triton X-100 0.1%). In Western blot, 35 μg of protein determined by Bradford method (Bradford, 1976) was separated using 15% SDS-PAGE (Laemmli, 1970). The proteins in gel were transferred to a nitrocellulose membrane (Hybond C® Amersham) by semi-dry TransBlot Cell Unit (Bio-Rad) using Tris–glycine buffer (Towbim et al., 1979). The primary antibody, polyclonal anti-α-AI1 earlier produced in rabbit (as described in Moreno and Chrispeels (1989), diluted in 1% gelatin in TBS (5 mM Tris–HCl, 15 mM NaCl, pH 7.5) was incubated with the membrane for 2 h. Then the membrane was incubated with conjugated anti-rabbit IgG alkaline phosphatase (Bio-Rad) during 1 h. After rapid washes the signal was visualized using alkaline phosphatase detection kit (Bio Rad). In ELISA analysis, pure α-AI1 inhibitor (range 0.125–8 ng) was used to obtain the standard curve. The wells of microtiter plates were coated with 2.5 μg of total protein extracted from transformed and non-transformed plants (negative control). The same antibodies cited above were used in ELISA procedure and for detection a tablet of p-Nitrophenyl phosphate (Sigma) was used, solubilized in 10% (v/v) Diethanolamine, pH 9.8. The absorbance was measured at 405 nm after incubation period (5 min, room temperature). The assay was carried out in triplicate.

2.8. Cotton boll weevil amylase inhibition assays

The inhibitory activity of α-AI variants against cotton boll weevil amylases (AgA) was assayed by the DNS (dinitrosalicylic acid) method (Bernfeld, 1955). For the inhibitory assays, transformed and non-transformed leaves of *A. thaliana* plants were twice extracted using 1:10 (w/v) buffer solution (sodium succinate 50 mM, pH 4.5, NaCl 150 mM, $CaCl_2$ 0.1 mM). The protein extraction was carried out using vigorous shaking for 3 h, at 4 °C. The centrifuged pellets (10,000 × *g*, 15 min) were dissolved using the same buffer solution (described above) added to 0.1% Triton X-100 (Sigma). The concentration of supernatants (total extracts) was determined by Bradford's method (Bradford, 1976) using BSA as standard. For the *in vitro* α-amylase inhibition assay 85 μg of total protein was added to one unit of cotton boll weevil amylase (AgA). One enzymatic unit was defined as the enzyme quantity to increase OD_{550nm} to 0.1. The inhibitory activity was analyzed by pre-incubation of enzyme and total protein at 37 °C, 30 min. Later 25 μL of substrate (starch solution, 2%, w/v) was added and incubated at 37 °C, 20 min. Then 100 μL of DNS solution was added and the samples were boiled. Following addition of 1 mL distilled water, aliquots of 200 μL were added to microtiter plate and the absorbance measured at 550 nm. Each assay was done in triplicate.

3. Results

3.1. A. grandis α-amylases (AgA) isolation

The fraction of semi-purified sample from the *A. grandis* larval extract of eluting at approximately 0.2 M Ammonium sulfate showed *in vitro* amylolytic activity (data not showed) and was analyzed using SDS-PAGE gel electrophoresis (Fig. 1A). The zymography method showed a clearer band of approximately 39 kDa (Fig. 1B), indicating the presence of cotton boll weevil amylases. The semi-purified AgA enzymes showed affinity for novel inhibitors selected in the biopanning procedure.

3.2. α-AI combinatorial library and biopanning

In order to carry out the DNA shuffling procedure, gel-purified DNA fragments included among 50–300 bp range (DNA product of

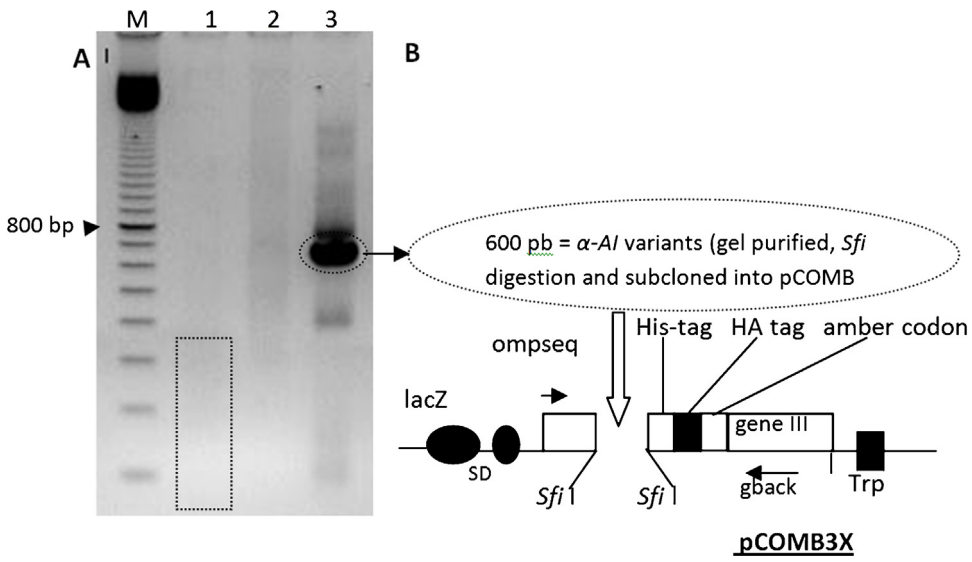

Fig. 2. Schematic representation of DNA shuffling proceeding and of the phagemid construction used to generated the phage combinatorial library (A) analysis in 2.5% gel electrophoresis using products of each step from DNA shuffling proceeding: (1) 100 bp Ladder (Invitrogen®); (2) Product of DNase I digestion using mixture of *α-AI* genes; region of 50–300 bp fragments gel excised are highlighted; (3) Product of PCR without the primers; amplicon corresponding to *α-AI* variant pool is highlighted (B) fluxogram indicating strategy used to subcloning of pool of variants into pCOMB3X phagemid.

the *α-AI* parental genes (660 bp) fragmented with DNase I enzyme) were recombined by primerless PCR. After a subsequent PCR containing suitable primers to recover mutated *α-AI* sequences, a pool of variants presenting 660 bp was obtained (Fig. 2A). The DNA shuffling product was cloned into the pCOMB3X phagemid (which contains a typical phage signal peptide) using *Sfi* I restriction sites (Fig. 2B). *E. coli* cells strain XL-1 Blue transformed with the resulting pCOMB3X-*α-AIs* construct generated a combinatorial phage-display library containing 2.6×10^8 colony-forming units (cfu) mL^{-1}. After the fifth selection round, the biopanning products were analyzed and indicated an increase of 112-fold in the number of colony forming units isolated from the third round when compared to results from the second round. A reduced number of remaining colonies in the fourth and fifth cycles were also observed. Based on that data (Table 2), the third cycle was chosen for further analysis since it indicated a higher production of specific phages. Since that the major aim of present study was to produce α-AI variants to select at least one candidate molecule to be used in the cotton boll weevil control, eighty-six clones, randomly chosen from third biopanning-round were applied for PCR analysis. Amplicons corresponding to the parental gene size of 660 bp were visualized at DNA electrophoresis analysis for all tested clones. Following, the product of the expression of 31 from the 86 clones from the third round showed signal detection of the hemaglutinin epitope (HA) fused to variant molecules (data not showed). Each of 31 clones had their nucleotide sequences determined.

3.3. GM Arabidopsis plants and molecular characterization

The *α-AI* variant genes amplified by PCR from selected phagemid DNA were sub-cloned into modified pCambia2300 vectors, producing the constructs denominated pFSpl2300AIs (Fig. 3A). These constructs were used to introduce the *α-AI* variants into *Arabidopsis* plants by the floral dip method. PCR analysis of kanamycin-resistant seedlings grown from seeds deriving from the transformation of *Arabidopsis* with the 26 constructs containing the variant genes indicated that the level of transformation ranged from 0.09 to 0.2% (data not shown). *Arabidopsis* transgenic plants (T1 plants) from each construct were evaluated, considering the quality and quantity of recombinant protein expression. Western Blot analysis using total proteins from plants expressing α-AIC3 and α-AIA11 variants (Fig. 3B) revealed two immunoreactive bands corresponding approximately to 11 kDa and 14 kDa. This profile was reminiscent of the α and β subunits, respectively, found in original α-amylase inhibitors isolated from common beans (Ishimoto and Kitamura, 1989). The quantification of α-AI variants in *A. thaliana* plants based on ELISA assays and using semi-purified αAI-1 protein as a standard indicated a similar level of expression comprising between

Table 2
Determination of enrichment of phages that bind to AGA using plaque-forming units.

Round of panning	Phage output[c]	Phage input[b]	Ratio Phage input/output	Number of washes[e]
0[a]	2.6×10^8			
1	9.7×10^3	1.9×10^{11}	1.9×10^8	7
2	8.8×10^6	1.5×10^{12}	1.7×10^6	9
3[d]	$\mathbf{8.3 \times 10^3}$	$\mathbf{2.3 \times 10^{12}}$	$\mathbf{2.8 \times 10^9}$	**11**
4	1.1×10^4	3.3×10^{12}	3.0×10^8	13
5	1.5×10^3	4.1×10^{11}	2.7×10^8	16
6	3.1×10^3	2.5×10^{11}	0.8×10^8	20

[a] Round 0 is 2.6×10^8 plaque-forming units mL^{-1} phages of original library.
[b] The number of phages selected after each round of panning is indicated (input).
[c] The output of each panning round is shown.
[d] The third round of panning was chosen as indicative of enrichment of specific phages based on input/output data.
[e] In each round the quantities of washes were increased as indicated to improve the specificity.

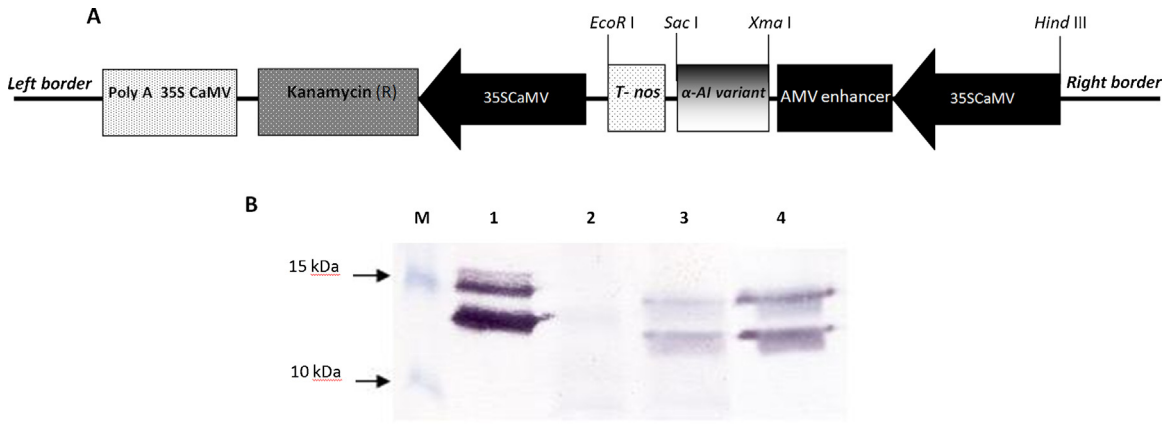

Fig. 3. Protein expression analysis of the α-AI variants in the transgenic *Arabidopsis* plants (A) Linear T-DNA map of the binary vector pFSpl2300AIs used to transform *Arabidopsis* plants. The pCambia 2300 (Cambia) vector basis it was applied including polyadenylation signal (poly A) of 35SCaMV (Cauliflower Mosaic Virus); *ntp II* (neomycin phosphotransferase II conferring kanamycin resistance); double-35SCaMV promoter; Termination sequence of Nopaline Synthase gene (tNOS), the enhancer from Alfalfa Mosaic Virus (AMV) and the *α-AIs* selected variants were subcloned. (b) Western blot analysis using 35 μg of total proteins extracted from leaves of transgenic *Arabidopsis* plants: M: BenchMark protein ladder (Invitrogen); 1: α-AI1 protein isolated from common bean flour (positive control); 2: total extract from untransformed plant; 3: total extract from transformed plants expressing α-AIC3; 4: total extract from transformed plants expressing α-AIA11. The arrows indicate the presence of α (11 kDa) and β (14 kDa) subunits, resulting from post-translational modification producing active inhibitor.

Table 3
Expression of *α-AI* variants in leaves protein extracts from transgenic *Arabidopsis* plants.[a]

Plants	α-AIs concentration (%) (by ELISA)[b]
α-AI1	0.30 ± 0.03
α-AIC3	0.20 ± 0.01
α-AIG4	0.23 ± 0.02
α-AIA11	0.28 ± 0.03

[a] For the tests were used pools of transgenic plants (T1).
[b] Each data represents mean ± SD of three replications.

0.2% and 0.3% for the extracts of α-AIC3, α-AIA11, α-AIG4 and α-AI1 plants (Table 3). Leaf extracts from independent transgenic plants expressing wild type or each α-AI variant were assayed for inhibitory activity *in vitro*. All three variants inhibited cotton boll weevil α-amylase at a concentration of 35 nM. The percentage of inhibition was 77% for α-AIC3 protein, 57% with α-AIA11 protein and 74% for α-AIG4 protein. No AgA inhibitory activity was detected in extracts from tissue of plants transformed with original α-AI1 or from tissue of untransformed plants (Fig. 4).

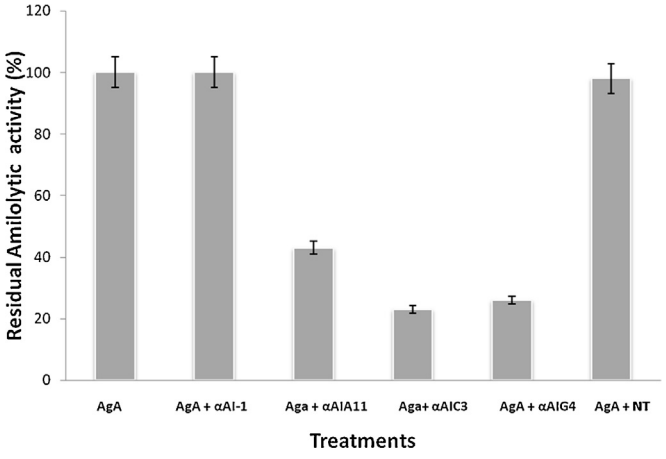

Fig. 4. α-AI variants expressed into transgenic *Arabidopsis* plants are able to inhibit *A. grandis* α-amylases. For the *in vitro* enzymatic assays total extracts (85 μg) obtained of leaves from each transgenic plant (T1 progeny) expressing α-AIC3, α-AIA11 or α-AIG4 variants were used. The assays were done in triplicate and the bars represent standard deviations calculated from three independent experiments. All variants inhibited cotton boll weevil α-amylase at concentration of 35 nM. One unit of AgA activity was utilized in the inhibition assay.

3.4. In silico analysis of the mutations generated in the α-AI variants

After alignment, the variant sequences could be clustered into three different groups: 17 of 31α-AI variants showed at least 65% sequence identity with the α-AI1 amino acid sequence; three had at least 65% identity with α-AI2 while the remaining six shared 40–60% sequence identity with each parent. Finally, among the selected molecules a repetitive sequence was identified for three different variants. A schematic comparison of the α-AIs variants and original α-AIs is shown in Fig. 5A and a sequence alignment in Fig. 5B. The DNA sequences of *α-AI* variants (α-*AIC3*, α-*AIA11* and α-*AIG4*) encoding for active variants against AgA were deposited at the NCBI (Genbank IDs: JQ820021, JQ820022, JQ820023, respectively). The shuffled variant genes encode mature proteins of 222 amino acid residues.

4. Discussion

In molecular evolution strategies, mutational techniques (*e.g.* DNA shuffling, site directed mutagenesis, and others) combined with the phage display technique have been widely used to generate molecules containing new or improved functions (bioactivities). According to the basic theory of phage display, millions or even billions of variant molecules can be accessed through the use of phage system libraries. Therefore, molecules presenting specific interactions with a target protein can be selected. For example, expression of proteinase inhibitors on the phage surface allowed the selection of molecules with greater specificity to the target enzymes (Kiczak et al., 2001; Volpicella et al., 2001). Aphids were controlled by mustard trypsin inhibitors (MTI) selected against α-chymotrypsin from a combinatorial library of MTIs generated by mutation in five codons of the active site (Ceci et al., 2003). These results demonstrated that this technology could provide new molecules effective in the control of insect pests and pathogens and that it could be used in breeding crops. Previously, we have generated novel insecticidal molecules, using *cry1Ia12 and cry8ka1* and genes that encode Cry toxins with specificity to lepidopterans and coleopterans (Craveiro et al., 2010; Oliveira et al., 2011, respectively). In those approaches, the *cry* genes were separately shuffled resulting in two different phage display libraries. From the *cry1Ia12* combinatorial library, variant molecules were selected with different levels of toxicity against *T. licus licus*, an insect that causes harm to sugarcane crops

Fig. 5. Identification of amino acid substitutions in the α-AI variant sequences. (A) Diagrammatic representation of amino acid sequence comparison of α-AI1, α-AI2 and the variants selected from the combinatorial library using cotton boll weevil α-amylase. Conserved amino acids are green, blue represents residues from α-AI1 sequence and yellow residues from α-AI2 sequence. (B) Sequence made with ClustalW2 (Larkin et al., 2007). The residues doubly underline form the two main loops (N and C-terminal) involving in the enzyme-inhibitor interactions (Grossi-de-Sa et al., 1997; Silva et al., 2000, 2004). (*) Indicates the N77 involved in post-translational proceeding (Young et al., 1999).

(Craveiro et al., 2010). The panning procedure from the *cry8ka1* variant phage library resulted in a novel Cry toxin, entitled Cry8ka5, with 3-fold improved toxicity against *A. grandis* (Oliveira et al., 2011). The present study reports, for the first time, the construction of a combinatorial phage library using shuffled products from a mixture containing two genes that encode α-amylase inhibitors. By comparing the molecules selected from the *α-AI* phage library and wild-type inhibitor isoforms, it was possible to clearly identify distinct segments in the novel sequences deriving from both parents. Thus, DNA shuffling and phage display was an efficient approach to generate and select a number of diverse sequences encoding for novel α-AI molecules. Although we report only the selection of variant molecules active against AgA, the phage library constructed can be used in future to select novel molecules with entomotoxic activity against other insect pests of agricultural importance.

In the present work the overexpression system in model plants was chosen to validate the variants because it is well known that bean α-AIs become active after the post-translational process. In Young et al. (1999) it was reported that α-AIs mature proteins from common bean (*P. vulgaris*) are composed of two α and two β subunits (approximately 11 kDa and 16.0 kDa, respectively) forming a heterotetrameric unit that interacts through non-covalent bonds. The post-translational modifications occur by proteolytic cleavage of the carboxyl group of Asn77 using an Asn-specific protease followed by the removal of Asn 79 using a carboxypeptidase. In addition, residues in the C-terminus from the β subunit are removed. Evidence of correct post-translational processing was previously reported for several species of transgenic plants (tobacco, peas, cowpea, chickpea and coffee) expressing active α-AI1 or α-AI2 (Morton et al., 2000; Pereira et al., 2006; Nishizawa et al., 2007; Sousa-Majer et al., 2007; Barbosa et al., 2010). In addition, differences in α and β subunits caused by modifications originated from glycosylation were discussed in the studies concerning expression of bean *α-AI* genes (Prescott et al., 2005; Barbosa et al., 2010; Campbell et al., 2011). Western immunoblot detection obtained in the present study showed a similar profile compared with results from the expression of α-AIs obtained for other transgenic plants, such as peas (Morton et al., 2000; Sousa-Majer et al.,

2007), chickpea (Sarmah et al., 2004), beans (Nishizawa et al., 2007) and tobacco (Altabella and Chrispeels, 1990; Pereira et al., 2006). Also, the ELISA data here reported are in accordance with expression levels obtained by Clemente et al. (2007) in their study also using the 35S promoter (Cauliflower Virus CaMV with enhancer element).

The original α-AI1 and α-AI2 inhibitors have received much attention for their distinct specificities. In present study, a crude extract using a pool of *Arabidopsis* plants expressing similar quantities of α-AI variants showed a varied inhibition level (57–75%) against AgA. Other reports demonstrated α-amylase inhibitory activity in *in vitro* assays against enzymes from diverse insect pests: the bean α-AI1 inhibited the activity from *Cryptolestes ferrugineus* and *Tribolium castaneum* α-amylases by 50% (using concentrations of 10.9 and 4.2 nM and 2.4 and 4.8 nM, at pH 4.5 and 6.0, respectively) (Kluh et al., 2005). Dayler et al. (2005) demonstrated that PvAIC protein isolated from common bean (*P. vulgaris*) inhibited by 90% the ZSA (*Z. subfasciatus α-amylase*) activity when assayed at a concentration of 5.5 or 9 μM. Likewise, the inhibition by 60% of *Acanthoscelides obtectus* amylases activity was reached in concentrations of 2.5 and 3.75 μM using wheat inhibitors 0.19 and 0.53, respectively (Franco et al., 2005). It was also reported that α-AI1 expressed in genetically modified peas completely prevented the infestation of *B. pisorum* in the field and storage conditions (Morton et al., 2000). Peas expressing α-AI2 do not prevent infestation on seeds, but the development of insects was delayed by thirty days (Morton et al., 2000). Further, it was demonstrated that α-AI1 produced in chickpea plants conferred protection against the bruchid weevil *C. maculatus* (Sarmah et al., 2004; Ignacimuthu and Prakash, 2006). Nishizawa et al. (2007) in an interesting study showed that the resistance of bean accessions to bruchid *Z. subfasciatus* is not only due to α-AI2, but also to other factors present in seeds, acting synergistically as inhibitors and collectively promoting insect control. These results reinforce the idea of pyramiding genes to improve resistance in plants (Tarver et al., 2007).

Structural flexibility has previously been identified as an important factor in the binding of amylase to inhibitor (Payan, 2004; Nahoum et al., 1999; Strobl et al., 1998). Several papers have reported diverse conformations adopted by the two main interaction loops (the N- and C-terminal) of the inhibitor, depending for example on whether the inhibitor was crystallized alone or in complex with enzyme (Bompard-Gilles et al., 1996; Silva et al., 2000; Franco et al., 2002; Payan, 2004; Silva et al., 2004). Interestingly, these loops well known to be involved in enzyme-inhibitor interactions (Grossi-de-Sa et al., 1997; Silva et al., 2000, 2004) are not shuffled in the variant inhibitors presented here. The N-terminal loop the α-AIC3 variant is identical in sequence to that in α-AI1, while those of the α-AIA11 and α-AIG4 variants were identical to the equivalent loop in α-AI2. Conversely, the C-terminal loop of the α-AIC3 variant is sequence identical to that of α-AI2 while the α-AIA11 and α-AIG4 variants have the C-terminal loop sequence of α-AI1 (Fig. 5B). Unexpectedly, therefore, the main interacting loops from the three variants with activity against AgA, have sequences found either in α-AI1 or α-AI2, both inactive against AgA. As a result, the molecular basis of AgA inhibition by the variants remains obscure for the moment, but molecular dynamics experiments, currently underway, may shed light on the question.

5. Conclusion

Our results demonstrate that the DNA shuffling and Phage display strategy we adopted successfully generated functional novelty in the α-AI family, enabling isolation of variants with activity against cotton boll weevil α-amylases. Based on the capacity of the selected variants to inactivate amylolytic activity of insect enzymes, we conclude that these candidate genes present high potential for biotechnology applications. We are currently using these, separately or in pyramid constructions, in the generation of transgenic cotton plants.

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