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# An α-amylase inhibitor gene from *Phaseolus coccineus* encodes a protein with potential for control of coffee berry borer (*Hypothenemus hampei*)

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**Abstract**

Plant α-amylase inhibitors are proteins found in several plants, and play a key role in natural defenses. In this study, a gene encoding an α-amylase inhibitor, named α*AI-Pc1*, was isolated from cotyledons of *Phaseolus coccineus*. This inhibitor has an enhanced primary structure to *P. vulgaris* α-amylase inhibitors (α-AI1 and α-AI2). The α*AI-Pc1* gene, constructed with the PHA-L phytohemaglutinin promoter, was introduced into tobacco plants, with its expression in regenerated (T0) and progeny (T1) transformant plants monitored by PCR amplification, enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis, respectively. Seed protein extracts from selected transformants reacted positively with a polyclonal antibody raised against αAI-1, while no reaction was observed with untransformed tobacco plants. Immunological assays showed that the α*AI-Pc1* gene product represented up to 0.05% of total soluble proteins in T0 plants seeds. Furthermore, recombinant α*AI-Pc1* expressed in tobacco plants was able to inhibit 65% of digestive *H. hampei* α-amylases. The data herein suggest that the protein encoded by the α*AI-Pc1* gene has potential to be introduced into coffee plants in order to increase their resistance to the coffee berry borer.
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*Keywords:* Coffee berry borer; *Phaseolus coccineus*; Digestive enzymes; Insect

## 1. Introduction

The coffee berry borer, *Hypothenemus hampei* (Coleoptera: Scolytidae), is a serious economic pest of commercial coffee (*Coffea* spp.) in many countries of the world (Murphy and Moore, 1990). Coffee seeds are severely attacked by both larvae and adults of the coffee berry borer, which are thus capable of decreasing the quality and the flavor of the grain coffee and consequently, reducing the commercial grain value (Clifford and Wilson, 1985). Control of the coffee berry borer is notoriously difficult since the insects live their entire life cycles within the coffee berries. Insect-pest control depends largely on application of organochloro insecticides, which have low efficiency and are extremely hazardous not only to farmers and consumers, but also to the environment (Sponagel, 1994). Other methods such as biological control are also used for control of this

*Abbreviations*: αAI, α-amylase inhibitor; αAI-1, α-amylase inhibitor 1 from *Phaseolus vulgaris*; PPA, porcine pancreatic α-amylase; αAI-Pc1, α-amylase inhibitor from *Phaseolus coccineus*.

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insect-pest, although these methods currently are of low efficiency (Damon, 2000). As an alternative, particular attention has been focused on transgenic coffee plants, which could express proteinaceous factors to produce resistant coffee plants to the coffee berry borer.

For insects that grow and feed on starch-containing seeds, α-amylolytic enzymes are required (Grossi de Sa and Chrispeels, 1997; Franco et al., 2002). On the one hand, these enzymes catalyze the initial hydrolysis of starch into shorter oligosaccharides that could be assimilated by organisms. On the other hand, previous reports demonstrated the involvement of plant α-amylase inhibitors (α-AI) in the growth, development and reproduction of these insect-pests (Franco et al., 2005; Kluh et al., 2005) due to a remarkable reduction in metabolic energy. Plant α-amylase inhibitors, which are particularly abundant in legumes (Franco et al., 2002) and cereals (Iulek et al., 2000), represent a potent tool in engineering crop plants due to their role as defense factors against insect-pests and pathogens (Svensson et al., 2004). Valencia et al. (2000) have described the presence of several α-amylases in the intestinal tract of larvae and adult insect of *H. hampei*, showing that the enzymatic activity was inhibited by purified αAI-1 from *P. vulgaris* and by a purified α-AI from *Amaranthus*.

This paper focuses on the molecular characterization of a new gene that encodes an α-AI named αAI-Pc1. The corresponding protein inhibitor was isolated and the recombinant protein expressed in tobacco plants, which seemed to confer satisfactory *in vitro* activity by inhibiting coffee berry borer α-amylases at low concentration. The expression of this α-AI in tobacco seeds is an initial part of a larger project to create transgenic coffee plants, which currently needs additional time and effort. Tobacco was thus investigated first to demonstrate the feasibility of this approach.

## 2. Results and discussion

### 2.1. Cloning an α-amylase inhibitor from *P. coccineus*

Previous work by Valencia et al. (2000) demonstrated that crude extracts from two different seeds of *P. vulgaris* were able to inhibit up to 80% of *H. hampei* α-amylase activity. Furthermore, the inhibition efficiency was variable among the different cultivars, suggesting that different variants of inhibitors could account for the observed differences. To determine if the *P. coccineus* seeds contained different isoforms of this α-AI, both a preliminary zymogram and an *in vitro* spectrophotometric assay were carried out. We observed different α-AI isoforms in the crude seed extracts of *P. coccineus*, inhibiting 80–100% of the α-amylase activity of *H. hampei* adults (Fig. 1).

With the aim to identify novel inhibitors for potential use on the control of this important coffee pest, these findings prompted us to isolate the gene encoding the α-AI

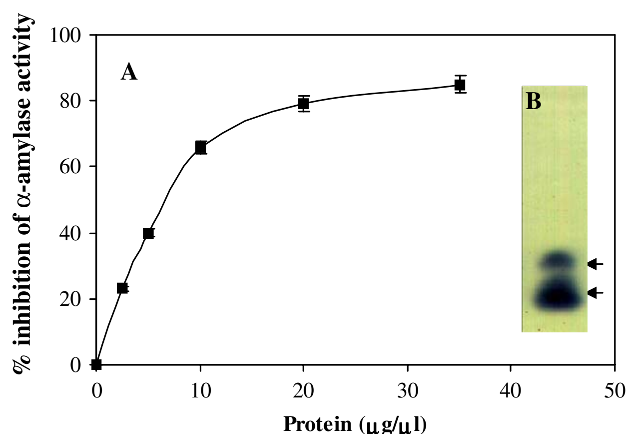


Fig. 1. (A) Inhibition of *H. hampei* α-amylases by a crude inhibitor preparation of *P. coccineus*, accession 35590, and (B) detection of αAIs isoforms present in the crude seed extracts of *P. coccineus*, accession 35590 by using inhibitor zymogram. Arrows in the right indicate the two major inhibitor bands.

from *P. coccineus*. In this way, using specific primers corresponding to the 5′ and 3′ ends of the αAI-1 proprotein, a fragment corresponding to the α*AI-Pc1* gene was amplified by PCR from *P. coccineus* total DNA.

The cloned sequence of α*AI–Pc1* corresponds to an open reading frame of 669-bp, coding for a polypeptide of 223 amino acids (Fig. 2). Similarly to other lectin-like α-AI's, its the genomic sequence has no introns. The deduced amino acid sequence of αAI-Pc is also 97% identical to αAI-1 from *P. vulgaris*, while the protein sequence among other αAIs from different cultivars of *P. vulgaris*, has a surprisingly lower identity (Sawada et al., 2002). The high amino acid identity between αAI-Pc1 and αAI-1 is not surprising, however, in molecular phylogeny studies of *Phaseolus* genus, *P. coccineus* has been placed as a sister species to *P. vulgaris* (Vekemans et al., 1998), or alternatively, the clade formed by *P. coccineus* and *P. polyanthus* as a sister group of *P. vulgaris*, indicating that *P. coccineus* and *P. vulgaris* are closely related species. The high identity with αAI-1 and the detection of complete α and β domains in αAI-Pc1 suggest that the missing amino terminal part of αAI-Pc1 should not differ significantly from other bean α-AI's with similar sequences.

The amino acid residues around the cleavage sites and the glycosylated asparagine are conserved between αAI-1 and αAI-Pc1 inhibitors, suggesting that αAI-Pc1 also undergoes proteolytic processing and glycosylation, which are necessary for activation of the inhibitory activity. Residues from 29 to 46 and 171 to 189, which form the inhibitor hairpin N and O-loops that protrude into the active site of the α-amylase ligand, are also conserved in αAI-Pc1, except for the Arg176 residue of αAI-1, which is replaced by serine in the *P. coccineus* inhibitor. Furthermore, all nine residues of αAI-1 that are known to form direct hydrogen bonds with PPA are also conserved in αAI-Pc1.

```
gccaccgaaacctccttcaacatcgatgggttcaacaaaaccaaccttatccttcaaggcgatgccatc   69
 A  T  E  T  S  F  N  I  D  G  F  N  K  T  N  L  I  L  Q  G  D  A  I

gtctcatccaacggcaacttacaactatcctataattcatacgactctatgagcagagccttctactcc  138
 V  S  S  N  G  N  L  Q  L  S  Y  N  S  Y  D  S  M  S  R  A  F  Y  S

gcccccatccaaatcagggacagcaccaccggcaacgtcgccagtttcgacaccaacttcacaatgaat  207
 A  P  I  Q  I  R  D  S  T  T  G  N  V  A  S  F  D  T  N  F  T  M  N

atccgcactcaccgccaagcaaattccgccgttggccttgactttgttctcgtccccgtccagaccaaa  276
 I  R  T  H  R  Q  A  N  S  A  V  G  L  D  F  V  L  V  P  V  Q  T  K

tccaaaggcgatactgtgactgtggagttcgacaccttcctcagccgtattagcatcgacgtgaacaac  345
 S  K  G  D  T  V  T  V  E  F  D  T  F  L  S  R  I  S  I  D  V  N  N

aacgatatcaaaagcgtgccttgggatgtacacgactacgacggacaaaacgccgaggttcggatcacc  414
 N  D  I  K  S  V  P  W  D  V  H  D  Y  D  G  Q  N  A  E  V  R  I  T

tataactcctccacgaaggtcttcgcggtttctctgttaaacccttctacgggaaagagcatcaacgtc  483
 Y  N  S  S  T  K  V  F  A  V  S  L  L  N  P  S  T  G  K  S  I  N  V

tctaccacagtggagctggagaaagaagtttacgactgggtgagcgttgggttctctgccacctcaggg  552
 S  T  T  V  E  L  E  K  E  V  Y  D  W  V  S  V  G  F  S  A  T  S  G

gcttatcaatggagctatgaaacgcacgacgtcctctcttggtctttttcttccaagttcatcaatctt  621
 A  Y  Q  W  S  Y  E  T  H  D  V  L  S  W  S  F  S  S  K  F  I  N  L

aaggaccaaaaatctgaacgttccaacatcgtcctcaacaagatcctctga 669
 K  D  Q  K  S  E  R  S  N  I  V  L  N  K  I  L
```

Fig. 2. Nucleotide and deduced amino acid sequence of αAI-Pc1. The amino acid residues that are part of the active site are underlined. The arrows indicate the possible *N*-glycosylation sites. The residue Asn 77 (N), that is shaded, indicated the cleavage site. The box, gcc, corresponds to the start codon of the mature protein and tga the stop codon of the protein. αAI-Pc1 has been submitted to genebank as accession DQ525689.

### 2.2. Expression of the αAI-Pc1 inhibitor in transformed tobacco plants

Tobacco plants were transformed with the *αAI-Pc1* gene by *A. tumefaciens*-mediated transformation. The *αAI-Pc1* gene was driven by the promoter of phytohemaglutinin, which is a seed-specific promotor (Altabella and Chrispeels, 1990). A total of 14 independent plants were screened for their resistance to hygromycin, and DNA samples from leaves of these plants, growing in the greenhouse, were analyzed by PCR. As expected, the *αAI-Pc1* gene was detected in all primary transformants (T0) and in some of the progeny (T1) plants (Fig. 4). The transgenic tobacco plants were chosen by Northern blot analysis, in which the expression of the recombinant αAI-Pc1 in mature seeds was detected with anti-αAI polyclonal antibodies.

Seed protein extracts from four independent transformants were analyzed to determine the effect of the reducing agent β-mercaptoethanol. All of them showed a similar pattern, containing polypeptides with molecular masses in the 9–17 kDa range (Fig. 5). A similar range, with polypeptides with relative molecular mass of 10–18 kDa, was also observed when the gene encoding *P. vulgaris* αAI-1 was expressed in tobacco seeds (Altabella and Chrispeels, 1990). Two abundant polypeptides recognized by the polyclonal antibody corresponded to the α- and β-subunits,

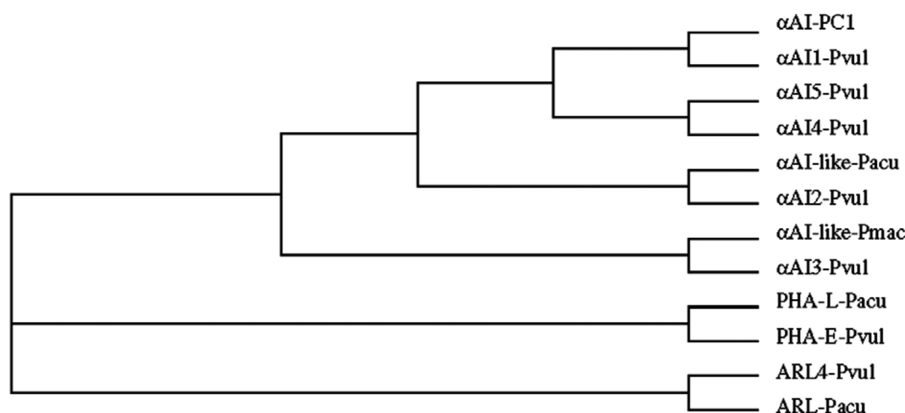


Fig. 3. Dendrogram based on the amino acid sequences of *Phaseolus* α-amylase inhibitors, phytohemagglutinins and arcelins. αAI, α-amylase inhibitor; PHA, phytohemagglutinin; ARL, arcelin; Pvul, *P. vulgaris*; Pacu, *P. acutifolius*; Pmac, *P. maculates*; αAI-PC1, α-amylase inhibitor from *P. coccineus*.

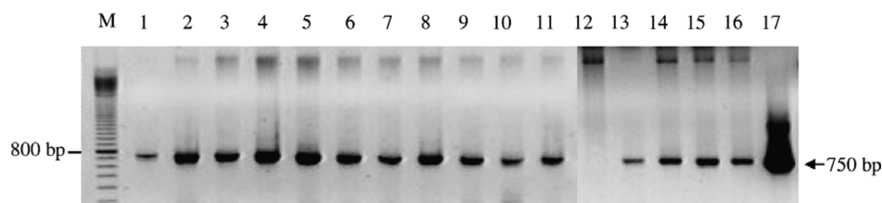


Fig. 4. Detection of the *αAI-Pc1* gene in transformed tobacco plants by PCR. Lanes 1–10 and 13–16: Transformed tobacco plants ($T_0$). Lane 11: Transformed tobacco plant of generation $T_1$. Lane 12: Untransformed tobacco plant (negative control). Lane 17: The plasmid pCAMBIA 1390/*αAI-Pc1* (positive control). M: 100 bp Molecular weight (GIBCO).

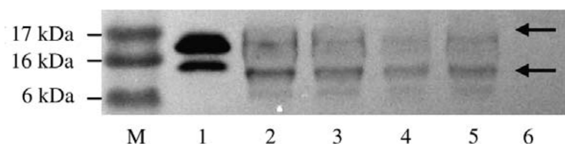


Fig. 5. Detection of the recombinant αAI-Pc1 expressed in transformed tobacco seeds. One hundred and fifty micrograms of total protein from different transformants were separated by SDS–PAGE and submitted to immunoblot analysis using anti-αAI-1 antibody. Lane 1: αAI-1 purified from *P. vulgaris* (positive control). Lanes 2–5: Independent transgenic tobacco plants expressing αAI-Pc1. Line 6: Untransformed tobacco plant (negative control). M refers to the molecular mass marker. The arrows indicate both subunit of the inhibitor with 11.5 and 16.6 kDa.

with relative molecular masses around 16.3 and 11.5 kDa, respectively (arrow in Fig. 4). The proteolytic processing of αAI-1 into α- and β-subunits was reported be essential for conversion of the inactive single-chain precursor into the mature $\alpha_2\beta_2$, the active αAI-1 molecule (Pueyo et al., 1993).

The expression levels of the recombinant αAI-Pc1 inhibitor in mature seeds from 14 transformed tobacco plants were measured using ELISA, revealing expression levels that ranged from 0.02% to 0.05% of the total soluble protein fraction (Fig. 6). The expressed protein levels are considerably lower when compared to the 0.5–1.25% of αAIs

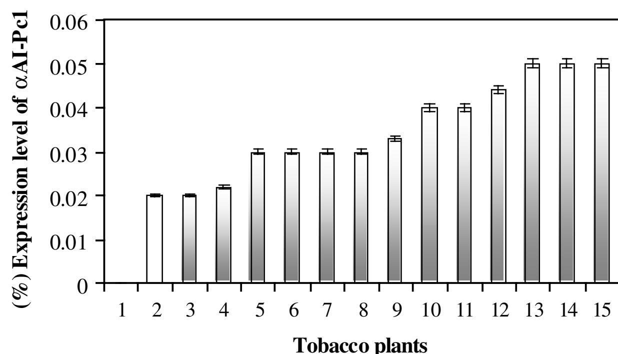


Fig. 6. Determination of the amount of αAI-Pc1 inhibitor expressed in 14 transgenic tobacco plant lines by ELISA. Lane 1, untransformed tobacco plant. Lanes 2–15, transgenic tobacco plant lines expressing the αAI-Pc1 inhibitor. Amounts are indicated as the percent expression relative to the total soluble protein. One hundred micrograms of seed protein extract was used in each analysis. Each measurement was done in triplicate. The bars represent two times the standard error.

normally found in cultivated beans (Shade et al., 1994), or the expression of 1.5–3% of total soluble proteins of αAI-1, expressed in transgenic pea seeds (Schroeder et al., 1995) and 0.82% in transgenic azuki bean seeds (Ishimoto et al., 1996). Another report showed an amount up to 0.2 μg of αAI-1 per 100 mg of total seed protein (Morton et al., 2000), a value of about 4-fold higher than the αAI-Pc protein level found in tobacco reported here.

The low expression level of αAI-1 reported here could be related to the fact that the expression was in tobacco, while in the above mentioned reports the expression of *Phaseolus* αAIs were made in other leguminous plants. It is possible that the PHA-L promoter, from *P. vulgaris*, might work better in closely related plants. Moreover, legumes naturally accumulate higher levels of inhibitors in their seeds than solanaceous plants.

### 2.3. Inhibitory activity of the αAI-Pc1 recombinant protein

In vitro inhibitory effects of the different recombinant αAI-Pc1 expressed in tobacco plants were analyzed against whole-insect extracts of the coffee berry borer and purified porcine pancreatic α-amylases (PPA). Whole-insect extracts were used due to the insect size, which makes dissection of the gut difficult. In addition, the gut of the coffee berry borer contains most of the insect α-amylase activity, so the use of whole-insect extracts should still reflect gut α-amylase activity (Valencia et al., 2000).

Using 250 μg of total protein extracts from seeds of each transformant (50–125 ng of αAI-Pc1), the activities of coffee berry borer α-amylases were inhibited between 32% and 65% under the conditions employed (Fig. 7). When the assays were carried out with seed protein extracts from untransformed tobacco plants, no α-amylase inhibitory activity was detected, indicating that the inhibitory activity results from the presence of the recombinant αAI-Pc1. No significant differences were observed in endogenous α-amylase activity between transformed and un-transformed plants, indicating that the plant α-amylases were not inhibited by αAI-Pc1 (data not shown). As expected, due to the similarity of αAI-Pc1 with αAI-1, the activity of PPA was 100% inhibited with 250 μg of seed protein extract from tobacco transformants expressing 0.05% of recombinant αAI-Pc1 (about 125 ng of the inhibitor) (data not shown) (see Fig. 8).

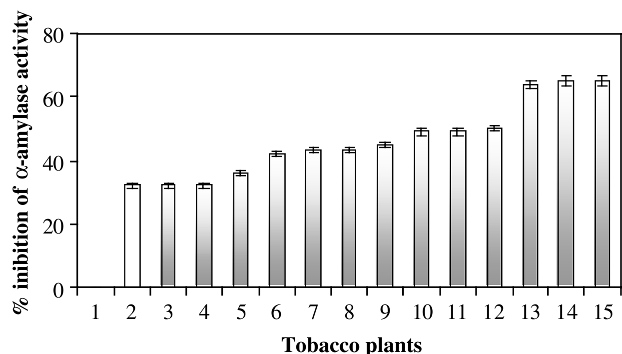


Fig. 7. Inhibition of *H. hampei* α-amylases by recombinant αAI-Pc1 inhibitor expressed in transformed tobacco plant seeds. Enzyme extract and 250 μg of protein extract from seeds containing different concentrations (50–125 ng) of αAI-Pc1 inhibitor were pre-incubated for 20 min prior to the addition of starch. Lane 1: Untransformed tobacco plants. Lanes 2–15: Transgenic tobacco plants expressing αAI-Pc1 inhibitor. The α-amylase inhibition was expressed as a relative α-amylase activity to an α-amylase activity with pre-incubation without the seed protein extracts. Each measurement was done in triplicate. The bars represent two times the standard error.

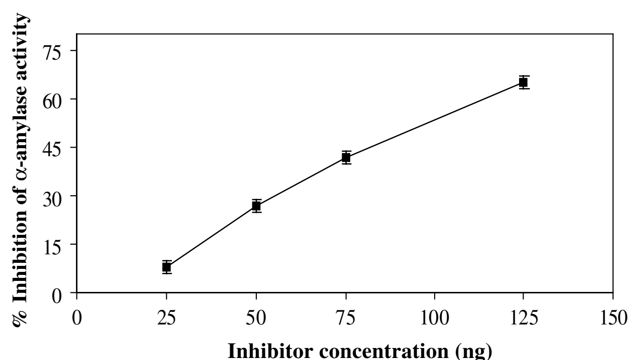


Fig. 8. Inhibition of *H. hampei* α-amylase by αAI-Pc1 inhibitor expressed in tobacco plants seeds. Enzyme extract and several concentrations of seed protein extract containing different concentrations (25, 50, 75 and 125 ng) of αAI-Pc1 inhibitor were pre-incubated for 20 min prior to the addition of starch. The α-amylase inhibition (inhibition %) was expressed as a relative α-amylase activity to an α-amylase activity with pre-incubation without the seeds protein extract. Similar results were obtained in three independent events. Each measurement was done in triplicate. The bars represent two times the standard error.

The range of inhibitory effects of αAI-Pc1 on coffee berry borer α-amylases is probably related to different expression levels of αAI-Pc1 in these transformants. Accordingly, transformants with the highest expression levels displayed greatest inhibition of the α-amylase enzymatic activities of *H. hampei*. The maximum inhibition of 65% of the enzymatic activity was achieved by addition of 125 ng of αAI-Pc1, present in 250 μg of seed protein extracts of three transformants with expression levels of 0.05% αAI-Pc1 relative to the total soluble protein (Fig. 3). Larger quantities of the inhibitor were also tested, but beyond 250 μg of the transformed tobacco seed protein extracts no additional differences in inhibitory activity were observed. However, due to low expression level of αAI-Pc1 in transformed tobacco plants, larger quantities of inhibitor could not be tested without a corresponding increase in amounts of seed total protein extracts; it is possible that this may interfere with inhibitory assays.

Otherwise, the remaining α-amylase activity observed in the *H. hampei* total protein extracts could be explained by the presence of enzymes insensitive to αAI-Pc1. Valencia et al. (2000) showed that *H. hampei* contains several α-amylases with digestive activity in the intestinal tract of larvae and whole-insect (adult) total protein extracts, and reported an inhibition of approximately 70% and 40% on the α-amylase enzymatic activity with 100 μg $ml^{-1}$ of αAI-1 purified from *P. vulgaris* and with 1 mg/ml purified inhibitor from *Amaranthus*, respectively. By contrast, the bean inhibitor αAI-2 did not inhibit the *H. hampei* α-amylase enzymatic activity at pH 5.0.

In the work of Valencia et al. (2000) a similar finding of 65% inhibition of coffee berry borer α-amylase activity was achieved with 250 μg of crude inhibitor preparation, from a powdered and dialyzed preparation of *P. vulgaris* beans, which normally contain 0.5–1.25% of αAIs (Shade et al., 1994). In the same work, 100 mg of purified αAI-1 inhibited the *H. hampei* amylase activity by 70%. In other reports, Morton et al. (2000) found that 400 ng of purified αAI-1 inhibited 0.6 units of the α-amylase activity of the pea weevil (*Bruchus pisorum*) by almost 80%. Titarenko and Chrispeels (2000) also reported that 5 μg of purified αAI-1 inhibited 65% of western corn rootworm (*Diabrotica virgifera*) α-amylase activity.

Compared to these reports, the results reported here are significant as αAI-Pc1 has similar inhibition efficiencies at much lower concentrations. Considering that αAI-Pc1 and αAI-1 are 97% identical, the differences in inhibition efficiency between our results and those of Valencia et al. (2000) are surprising. An explanation could be that some of the different residues in αAI-Pc1 are involved in the interaction with the *H. hampei* α-amylases. In addition, a recent work done by our group, shows that *P. coccineus* seeds contains not only a highest concentration of αAIs than *P. vulgaris*, but also a major quantity of iso-inhibitors, which could show different inhibitory potency when they are purified.

These results suggest that αAI-Pc1 represent a promising candidate for engineering resistance in coffee towards *H. hampei*. Although αAI-Pc1 also inhibits mammalian α-amylase, human consumption should not be negatively impacted, since coffee is extensively processed and heated before use. Moreover, experiments using rat feeding with transgenic peas containing αAI-1 had no detrimental effects on weight gain, carbohydrate or nitrogen metabolism or in growth of internal organs when transgenic peas were fed at 30% of the diet (Pusztai et al., 1999). Morton et al. (2000) showed that the influence of αAI-1 on the larval development was caused by partial inhibition of gut amylases activities of *Bruchus pisorum*. Inhibitors that are only partially effective *in vitro* may be useful for genetic engineering,

preferably, in combination with other transgenes that are also partially effective. Since an inhibitor that simply reduces the insect population below the economic damage level may be more desirable in comparison with the use of a transgene that is so effective that may results in a selection pressure, causing a rapid emergence of insect strains that are not affected by the inhibitor (Morton et al., 2000).

## 3. Conclusions

The results described here indicate that αAI-Pc1 is correctly processed into a biologically active α-amylase inhibitor in transgenic tobacco seeds. In vitro inhibitory activity of αAI-Pc1 against the α-amylase activity of *H. hampei* is an important further step towards the selection of an inhibitor with potential use to engineering coffee plants, which might exhibit increased resistance against the coffee berry borer. Further studies are now being directed to validate the in vivo effect of the αAI-Pc1 over *H. hampei* in coffee plants transformed with αAI-Pc1 and using the recombinant inhibitor in the dietary composition of laboratory reared insects.

## 4. Experimental

### 4.1. Insect and Phaseolus coccineus sources

Wild accessions (35590) of *P. coccineus* were obtained from the University of Caldas in Manizales, Colombia. Adults insects of *H. hampei* were obtained from a laboratory reared colony of the Instituto Agronômico do Paraná (IAPAR), Londrina, Paraná, Brazil.

### 4.2. Cloning of αAI-Pc gene

Genomic DNA was extracted from *P. coccineus* seeds by using the DNeasy Plant Mini Kit (Quiagen) according to the manufacture's instructions. The amplification of the αAI-Pc gene was done using genomic DNA and primers corresponding to the 5′ (5′-GCCACCGAAACCTC-3′) and 3′ (5′-TCAGAGGATCTTGTTGAG-3′) ends of the αAI-1 gene from *P. vulgaris*. Amplifications were done in a Mastercycle gradient (Eppendorf) in 25 μl containing 0.5 μmol of each primer and 2 units of Taq DNA polymerase (Gibco) under the following conditions: 30 cycles of 30 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C. The amplified products were excised from agarose gels, purified, ligated into the plasmid vector pGEMT-easy (Promega) and recombinant clones were sequenced in both strands. Computer analysis of the sequences was done using the bioinformatics resources of the NCBI homepage (www.ncbi.nlm.nih.gov), the ExPASy Molecular Biology Server (http://bo.expasy.org) and CLUSTAL W software (www.ebi.ac.uk/clustalw). The sequence has been submitted to Genbank as Accession DQ525689.

### 4.3. Plasmid construction for expression of αAI-Pc1 in transformed plants

The *αAI-Pc1* gene was PCR amplified using a 5′ end primer containing a 5′ *Bsm*I site followed by the 60-bp signal peptide of αAI-1 and a 3′ end primer with a stop codon followed by a *Xba*I site. The amplified product was digested with *Bsm*I and *Xba*I, and cloned into the corresponding sites of the vector pTA-2 (Grossi de Sa and Chrispeels, 1997), downstream of the phytohemaglutinin PHA-L 536-bp promoter. The resulting construction containing the PHA-L promoter, the αAI-1 signal peptide and the αAI-Pc1 proprotein sequence, was cloned into the *Hin*dIII and *Eco*RI sites of the plasmid vector pCAMBIA 1390 (Cambia GPO, Canberra, Australia), originating the binary vector pCAMBIA 1390/*αAI-Pc*, which was used for plant transformation.

### 4.4. Transformation of tobacco

The pCAMBIA 1390/*αAI-Pc* binary vector was transferred to *Agrobacterium tumefasciens* EHA 105 by electroporation. Sterilized leaf explants of tobacco plants (*Nicotiana tabacum* cv. Xanthi) were transformed by a simplified version of the leaf-disc method (Horsh et al., 1985). Five different cultures of *Agrobacterium* were grown in 5 ml YEB medium (Vervliet et al., 1975) containing rifampicin (100 μl/M) and kanamycin (50 μl/M), for 16 h, 28 ± 2 °C. For co-transformation experiments, 800 μl of the *Agrobacterium* culture ($A_{600\ nm} = 0.1$) was added to 20 ml MS liquid medium (Murashige and Skoog, 1962). Twenty leaf explants were incubated with the *Agrobacterium* culture in a Petri dish for 5 min, at room temperature. Explants were then immediately placed on MS solid medium (0.7% agar) for two days in darkness at 28 ± 2 °C. For regeneration and selection, the explants were transferred to MS solid medium (0.65% agar) containing 1 mg/ml benzylaminopurine (BAP), 500 μg $ml^{-1}$ cefotaxime and 30 μg $ml^{-1}$ hygromycin and maintained under a 16 h photoperiod at 25 ± 2 °C. Untransformed explants were placed onto the same medium with or without hygromycin as the negative and positive controls, respectively. Shoots regenerated on selection medium were excised at the base and placed in Magenta GA7 boxes containing rooting medium (the same medium as the regeneration medium but without BAP). The hygromycin-resistant and PCR positive plants were transferred to soil and grown in a greenhouse at 25 ± 10 °C and 50% humidity. The mature seeds were collected after 4 months.

### 4.5. Protein extraction

Proteins were extracted from untransformed and transformed tobacco seeds as previously described (Grossi de Sa and Chrispeels, 1997) by grinding 250 mg of dry tobacco seeds, in an ice-cold mortar with 1 mL of 50 mM Tris pH 8.0 containing 30 mM NaCL, 0.1% Triton X-100 and 2%

β-mercaptoethanol. For inhibitory enzyme assays, proteins were extracted in the same buffer in the absence of β-mercaptoethanol. Extraction was carried out for 2 h at 4 °C with agitation. The extract was cleared by centrifugation at 12,000*g* for 10 min and the supernatant was used as a source of inhibitor for α-amylase assays. Protein concentration was measured by the Bradford (1976) procedure. Bovine serum albumin was used as standard.

### 4.6. Immunoanalysis

Denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method described by Schagger and Jagow (1987). Protein extracts (150 μg) were precipitated with 12% trichloroacetic acid according, solubilized in SDS-containing buffer and run into an electrophoresis unit. Immunoblots were performed as described by Sambrook et al. (1989). Proteins were transferred to nitrocellulose membranes using a semi-dry TransBlot Cell Unit (Bio-Rad) and Tris-glycine transfer buffer (Towbim et al., 1979). Membrane was treated with anti-α-AI1 polyclonal antibody from rabbit (Grossi de Sa and Chrispeels, 1997), which was utilized as primary antibody, followed by secondary anti-rabbit IgG antibody from goat conjugated to horseradish peroxidase HRP (Bio-Rad). Furthermore, procedures for sandwich enzyme-linked-immunosorbent assay (ELISA) were done following the procedure described by Ausubel et al. (1989). A calibration curve, previously prepared with α-AI1 (20 and 100 ng) was used to quantify the correlate αAI-Pc1 inhibitor in tobacco plants. Microtiter plates were coated with 100 μg of protein extract of transformed and untransformed (negative control) tobacco seeds. A rabbit polyclonal antibody serum raised against αAI-1 was used as primary antibody and anti-rabbit IgG antibody from goat conjugated to alkaline phosphatase as secondary antibody. A 0.1% (w/v) *p*-nitrophenyl phosphate (Sigma) in 9.7% diethanolamindé pH 9.8 was use as substrate. The reaction was stopped after 15 min and the absorbance was determined at 405 nm. Each assay was carried out in triplicate.

### 4.7. Inhibitory activity assay

The α-amylase activity was measured by using the dinitrosalicylic acid (DNS) method adapted by Ishimoto and Chrispeels (1996) using 1% soluble starch as substrate. The assays were conduced with guts from coffee berry borer (CBB) adult insects which were macerated in 0.1 M phosphate buffer pH 5.0 containing 200 mM NaCl and 0.1 mM $CaCl_2$. Crude extract was cleared by centrifugation at 12000*g* for 20 min. After centrifugation, the supernatant was stored at −20 °C and used as a source for both α-amylase and inhibitor assays. For the inhibitory assays, 250 μg of crude protein extract from tobacco seeds expressing the αAI-Pc1 inhibitor were pre-incubated for 20 min at 37 °C with one unit of α-amylase activity of PPA (porcine pancreatic α-amylase) or CBB (coffee berry borer). One unit of amylase activity was defined as the amount of enzyme required to increase the absorbance at 546 nm by 0.1 absorbance unit after 20 min reaction prior to the addition of 250 μl of the substrate solution followed by incubation for 10 min at 37 °C. The reactions were stopped by the addition of 500 μl of DNS reagent followed by color development placing the tubes in boiling water for 10 min. After addition of 5 ml distilled water, the absorbance was read at 546 nm. Assays were carried out in triplicate.

### 4.8. Inhibitor zymogram

Proteins from *P. coccineus* crude extracts, were separated on a IEF (pH range 3–9) gel by using a PhastSystem electrophoresis unit. To detect the inhibitory activity band, the IEF gel was incubated for 1 h with 1.5% starch solution, rinsed and incubated at 30 °C for 20 min with human salivary amylase dissolved in 0.1 M phosphate buffer pH 5.0 containing 200 mM NaCl and 0.1 mM $CaCl_2$.

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