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Tobacco Plants Transformed with the Bean αai Gene Express an Inhibitor of Insect α-Amylase in Their Seeds[1]

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ABSTRACT

Bean (*Phaseolus vulgaris* L.) seeds contain a putative plant defense protein that inhibits insect and mammalian but not plant α-amylases. We recently (J Moreno, MJ Chrispeels [1989] Proc Natl Acad Sci USA 86:7885–7889) presented strong circumstantial evidence that this α-amylase inhibitor (αAI) is encoded by an already-identified lectin gene whose product is referred to as lectin-like-protein (LLP). We have now made a chimeric gene consisting of the coding sequence of the lectin gene that encodes LLP and the 5′ and 3′ flanking sequences of the lectin gene that encodes phytohemagglutinin-L. When this chimeric gene was expressed in transgenic tobacco (*Nicotiana tabacum*), we observed in the seeds a series of polypeptides (M_r 10,000–18,000) that cross-react with antibodies to the bean α-amylase inhibitor. Most of these polypeptides bind to a pig pancreas α-amylase affinity column. An extract of the seeds of the transformed tobacco plants inhibits pig pancreas α-amylase activity as well as the α-amylase present in the midgut of *Tenebrio molitor*. We suggest that introduction of this lectin gene (to be called αai) into other leguminous plants may be a strategy to protect the seeds from the seed-eating larvae of Coleoptera.

Seed development in legumes is accompanied by the accumulation of two classes of abundant proteins, the seed storage proteins and proteins such as lectins and inhibitors of digestive enzymes of mammals and insects that can be classified as plant defense proteins. The molecular basis of the toxic activity of enzyme inhibitors is clearly understood, while that of the lectins remains to be elucidated. The toxic activity of lectins (15, 21), nevertheless, allows us to classify them as plant defense proteins.

It may be possible to obtain increased plant protection against insects by incorporating genes for these proteins into plants, directing the expression of the genes for these toxic proteins in the plant organs that need protecting (leaves, roots, ovaries, seeds), and making sure that the proteins accumulate in subcellular organelles where they are properly processed, active, and stable. The feasibility of this approach has been demonstrated by Hilder *et al.* (11) who transferred the gene for cowpea trypsin inhibitor to tobacco and showed that the transgenic plants had enhanced resistance to the tobacco budworm *Heliothis virescens*. Similarly, Johnson *et al.* (16) recently showed that the expression of potato or tomato proteinase inhibitor II in tobacco plants severely inhibited the growth of *Manduca sexta* larvae.

An αAI[3] has been described in the seeds of several varieties of the common bean, *Phaseolus vulgaris*. The inhibitor is a thermostable glycoprotein with a molecular mass of 45 kD (gel filtration) and is composed of subunits of 15 to 18 kD (SDS-PAGE) (17, 22, 23). The αAI forms a 1:1 complex with insect and mammalian α-amylases, but is inactive against plant and bacterial α-amylases (24, 30). We recently (19) presented strong circumstantial evidence that the αAI is encoded by an already-identified lectin gene (12, 14) whose gene product has been identified as LLP (27). We have now made a chimeric gene consisting of the coding sequence of this αai gene with the 5′ and 3′ flanking sequences of the gene that encodes PHA-L. Phytohemagglutinin is the major seed lectin of the common bean and its two polypeptides, PHA-E and PHA-L, are encoded by two tandemly linked genes (13). Its promoter directs the seed-specific expression of transgenes in tobacco (29). The chimeric gene was introduced in tobacco and gave rise to a protein that inhibits the α-amylase activity extracted from the midgut of the mealworm, *Tenebrio molitor*, which we chose as a representative of the order Coleoptera.

MATERIALS AND METHODS

Bacterial Strains and Vectors

The helper strain *Escherichia coli* HB101 harboring pRK2013, the *Agrobacterium tumefaciens* strain LBA4404, and the vector Bin19 were originally obtained from M. Bevan (2). The *E. coli* DH5α was provided by BRL (Bethesda, MD).

Plasmid Construction

A *Hin*dIII fragment excised from the insert of the pTV562 plasmid (29), containing the dlec2 gene that encodes PHA-L, was inserted into the *Hin*dIII site of the PUC8 vector and cloned in *E. coli* DH5α. DNA of this plasmid and the pPVL134 plasmid (14), that contains the coding sequence for αai, was digested with *Bsm*I and *Pst*I, and the fragments of 3240 bp (PUC8 plus a 536 bp 5′ upstream fragment for the

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[3] Abbrevations: αAI, α-amylase inhibitors, LLP, lectin-like protein; PHA, phytohemagglutinin; bp, base pair; kb, kilobase pair.

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PHA-L) and 892 bp (containing the coding sequence) were purified by agarose gel electrophoresis and ligated. The resulting plasmid was introduced in *E. coli* DH5α. DNA of both plasmids was digested with *Xba*I and *Pst*I and the two fragments of interest were purified and ligated. The resulting plasmid, the PUC8 vector with a 5.3 kb insert, containing the coding sequence for *αai*, a 536 bp PHA-L 5′ upstream fragment, and the 3′ flanking sequence for the PHA-L, was cloned in *E. coli* DH5α. This chimeric gene was transferred to the plant vector *Bin*19 as an *Hin*dIII fragment (Fig. 1) and maintained in *E. coli* DH5α.

Transformation of Tobacco

The *Bin*19 construct was mobilized from *E. coli* DH5α to *A. tumefaciens* LBA4404 by triparenteral mating with *E. coli* HB101 containing pRK2013 (2) and used to transform leaf discs of *Nicotiana tabacum* (cv Xanthi). Transformants were selected by their resistance to kanamycin, and transformed plants were regenerated from shootlets by transfer to a root-inducing, kanamycin-containing, agar medium as described in Voelker *et al.* (29). The kanamycin-resistant plants were transferred to soil and grown in the greenhouse.

Protein Extraction

Proteins were extracted from tobacco seeds by grinding in an ice-cold mortar with a buffer containing 50 mM Tris (pH 8), 30 mM NaCl, 0.1% Triton X-100, and 2% β-mercaptoethanol (100 mg of seeds per mL of buffer). The supernatant obtained after centrifugation at 15,000 *g* for 5 min was termed low-salt extract.

Immunoblot Analysis

For immunoblotting, appropriate quantities of protein (determined according to Lowry *et al.* [18]) were fractionated by SDS-PAGE, transferred to nitrocellulose, and the αAI detected using a rabbit anti-αAI serum obtained as described (19). We used goat-rabbit IgG coupled to horseradish peroxidase (Bio-Rad) as secondary antibody.

αAI Extraction and Partial Purification

Tobacco seeds were homogenized in an ice-cold mortar with 1 mL of 10 mM 2-mercaptoethanol per 100 mg of seeds. The homogenate was centrifuged at 35,000 *g* for 1 h and the supernatant (after removal of the lipid pelicle) acidified to pH 4, by addition of 0.2 M succinate, 0.1 M $CaCl_2$ (pH 3.8), and heated to 70°C for 10 min. The heavy protein precipitate was removed by centrifugation (12,000 *g* for 15 min) and the clear supernatant brought to pH 5.6 with NaOH. This extract, referred to as acid- and heat-treated extract, was used for α-amylase inhibition assay or for further purification with an affinity column, consisting of porcine-pancreas α-amylase coupled to CNBr-activated Sepharose 4B (Pharmacia) as described in Moreno and Chrispeels (19).

α-Amylase and Inhibitory Activity

Porcine pancreatic α-amylase (type U11-A, crystalized) was from Sigma Chemical Co. *Tenebrio molitor* α-amylase was a crude extract prepared by homogenization of larval midguts with buffer (15 mM succinate, 20 mM $CaCl_2$, 0.5 M NaCl [pH 5.6], containing 1 mM phenylmethylsulfonylfluoride), followed by centrifugation at 10,000 *g* for 20 min at 4°C.

α-Amylase activity was measured by the decrease of iodine staining after action of the enzyme on soluble starch. Iodine staining assays were performed by adding 0.5 mL of potato starch in 50 mM sodium maleate, 10 mM $CaCl_2$ (pH 6.9) to solutions containing different amounts of α-amylase in a total volume of 0.5 mL of the α-amylase extraction buffer with 1 mg/mL of bovine serum albumin. The concentration of starch was adjusted to give a zero time A_{620} about 1.3 after reaction with the iodine reagent. Reactions were stopped, after 5 min, with 0.5 mL of iodine reagent (26). The blue color was diluted by adding 2.5 mL of water and the A_{620} determined. Appropriate blanks were prepared without α-amylase present. An amount of α-amylase that lowered the A_{620} to 0.3 to 0.4 was selected for the inhibitory assay.

Assays for the α-amylase inhibitory activity were performed as described above, except that an appropriate amount of α-amylase (50 μg of porcine-pancrease α-amylase or 10 μL of *T. molitor* extract) and different volumes of inhibitor extract were preincubated for 30 min at 37°C before the addition of the starch.

Inhibitory units were defined as the amount of αAI that completely inhibits 1 ng of porcine pancreas α-amylase in the conditions of this assay.

RESULTS

To obtain definitive proof that the bean α-amylase inhibitor is encoded by the *αai* gene, we made a chimeric gene consisting of the coding region of *αai* and 3′ and 5′ flanking sequences of the gene (dlec2) that encodes PHA-L (13). This gene was transferred to the *Hin*dIII site of the vector Bin19 (Fig. 1) and used to transform tobacco plants via *Agrobacterium tumefaciens*. The PHA-L promoter directs the seed-specific expression of chimeric genes in transgenic tobacco plants (29).

Expression of α-Amylase (αAI) Inhibitor in Tobacco Seed

The αAI polypeptides from bean seeds can be resolved by SDS-PAGE in at least 5 bands (M_r 15,000–19,000) (Fig. 2,

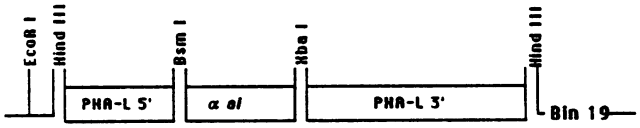

Figure 1. Structure of the chimeric gene. The gene is shown as inserted in the vector *Bin*19. *αai* is a 742 bp fragment from the pPVL134 plasmid that contains the coding sequence for *αai*; PHA-L 5′ and PHA-L 3′ are, respectively, a 536 bp PHA-L 5′ upstream and a 3700 bp PHA-3′ downstream fragments from the insert of the PTV562 plasmid, which contains the dlec2 gene that encodes PHA-L.

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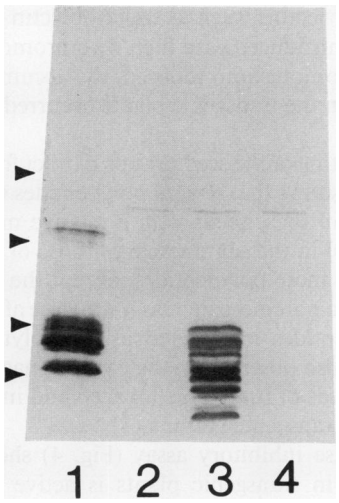

Figure 2. Western blot analysis of transformed tobacco seeds. Lane 1, affinity-purified bean αAI; lanes 2 to 4, aliquots of low-salt protein extract (50 μg per lane) from untransformed (2), transformed (3), and *Bin*19-transformed (4) tobacco seeds. Arrows on the left indicate $M_r \times 10^{-3}$ corresponding to 14, 18, 26, and 43 from bottom to top. Only one transformed plant is shown, but seeds from five other independent transformants showed the same immunostaining pattern.

lane 1). The faint bands around 30 kD are undissociated aggregates of the smaller polypeptides. When the protein extracts from transformed tobacco seed were analyzed by SDS-PAGE and immunoblotting with antibodies to the bean α-amylase inhibitor, we observed at least 11 polypeptides (M_r 10,000–18,000) that cross-react with the bean anti-αAI antibodies (Fig. 2, lane 3). We analyzed seeds from six different and independent transformants and all had the same staining pattern shown in Figure 2, lane 3. Variation in the abundance was two- to threefold, as observed previously for PHA expressed in tobacco seeds with this promoter (29). Because we used a seed-specific promoter, we did not examine expression of the transgene in other organs or tissues. In protein extracts of seeds from untransformed and Bin19-transformed tobacco plants (Fig. 2, lanes 2 and 4, respectively), no signal in the range of 10 to 18 kD was observed on the immunoblot.

The bean α-amylase inhibitor is a thermostable glycoprotein that binds to animal α-amylases forming a stable 1:1 complex (24, 30). To characterize the polypeptides that were detected by the antibodies to αAI, we partially purified the putative αAI from mature transformed tobacco seeds using procedures described for bean. Extraction at low ionic strength was followed by heat treatment at pH 4.0. The immunoblot (Fig. 3, lane 1) showed that all polypeptides, with the exception of two smaller, nonabundant polypeptides, survive the acid/heat treatment. Further purification of this extract was carried out by affinity batch adsorption using porcine pancreas α-amylase coupled to agarose beads. The immunoblot of these extracts (Fig. 3) showed that all but the smallest polypeptides (one abundant and two barely visible nonabundant) bound to the α-amylase column and could be eluted at low pH (Fig. 3, lane 2). α-Amylase inhibitor is synthesized as a 28 kD polypeptide which becomes glycosylated to a series of glycoforms (30–35 kD) which are then proteolytically processed (19). There appear to be more processing products in tobacco than in bean. Most of the processed polypeptides bind to the α-amylase affinity column indicating that they must be part of active αAI molecules. The polypeptides which do not bind to the affinity column are probably not contained in active αAI molecules.

Inhibition of Porcine and *Tenebrio molitor* α-Amylases

We used the acid- and heat-treated protein extract from transgenic tobacco seeds to assay α-amylase inhibitory activity. Figure 4 shows that this extract inhibited not only the porcine pancreatic α-amylase (A), but also the α-amylase from *T. molitor* (B). When we added extract from seeds of untransformed tobacco plants, no α-amylase inhibitory activity was detected. The absence of α-amylase inhibitory activity in extracts of control seeds and its presence in the seeds of transgenic plants, demonstrate conclusively that the inhibitory activity results from the presence of the transgene product. In addition, these results show that in spite of the somewhat different processing in tobacco compared to bean, the αAI is stable and active in tobacco seeds.

Accumulation of *αai* during Seed Development

To find out if the expression of this chimeric transgene is developmentally regulated in tobacco seeds, we made extracts of seeds obtained from transformed tobacco plants at different stages of development, ranging from 9 to 30 d after pollination. These extracts were analyzed by SDS-PAGE and immunoblot and for inhibitory activity (Fig. 5). The α-amylase inhibitory activity could be detected in the transgenic seeds

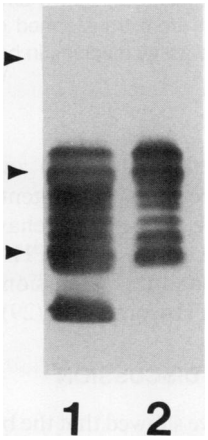

Figure 3. Adsorption of polypeptides from transformed tobacco seeds to porcine pancreas α-amylase coupled to agarose. Immunoblots of total acid- and heat-treated extract (1) and fraction bound to immobilized α-amylase (2). Lane 1 has 50 μg of protein and lane 2 has the proteins that bound to the affinity resin and were eluted when 50 μg of total protein was used. Arrows on the left indicate approximate molecular mass of 14, 18, and 26 kD from bottom to top. Seeds from untransformed plants did not contain immunoreactive polypeptides in this molecular mass range (see Fig. 2, lane 2).

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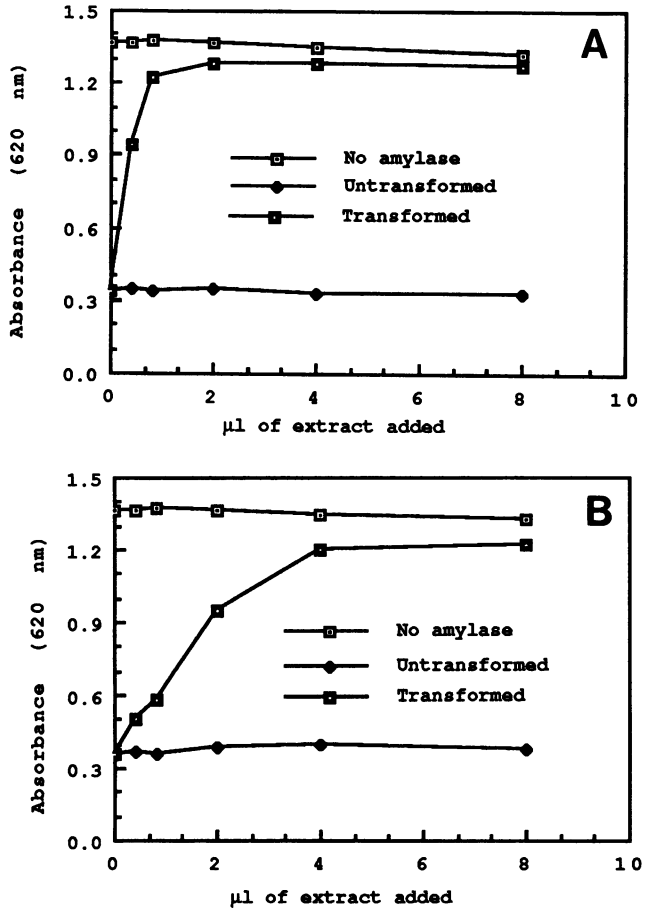

Figure 4. Assay of α-amylase inhibitory activity. The figure represents the amount of starch (measured as A_{620} after reaction with the iodine reagent) remaining after 5 min incubation with porcine α-amylase (A), or *T. molitor* larvae midgut α-amylase (B) *versus* the amount of inhibtor extract (from transformed or untransformed tobacco plants) added to the assay medium. In both cases, we used a blank without α-amylase.

between 12 and 15 d after pollination and reached a maximum level by d 20. The inhibitor content decreased slightly during the drying of the seeds. This behavior is very similar to that observed for the expression of PHA during cotyledon development of beans, and the expression of chimeric genes using the seed-specific PHA promoter (29).

DISCUSSION

In a previous paper, we showed that the bean αAI is encoded by a lectin gene identical with or very closely related to the lectin gene described by Hoffman *et al.* (12, 14). This gene encodes the LLP protein that is known to be proteolytically processed to polypeptides of 15,000 to 18,000 D after its synthesis in the ER as a polypeptide of 35 kD (4, 6). Here we show that seeds of tobacco plants transformed with a chimeric gene containing the coding region of this gene under the control of the PHA-promoter, contain α-amylase inhibitory activity. For this reason, we propose to call this gene *αai*. When genes for lectins, such as soybean lectin (20) and PHA (28, 29), were introduced with their own promoter or another seed protein promoter into tobacco, the accumulation of the gene products in the transgenic plants occurred specifically in the seeds.

Immunoblotting of the seed protein extract from transgenic tobacco plants shows that several polypeptides were produced and processed in these seeds with a relative molecular mass (10,000–18,000) in the same range as those of the bean αAI. In addition, all these polypeptides, except the three smallest ones, bound to a porcine-pancreas α-amylase affinity column. Smaller polypeptides interpreted as proteolytic breakdown products were also observed in the case of phaseolin (25) and PHA (29) in seeds of transgenic tobacco and in the case of β-conglycinin in transgenic petunia (1).

The α-amylase inhibitory assay (Fig. 4) showed that the αAI produced in transgenic plants is active and stable in tobacco seeds and accumulated during seed development (Fig. 5). This protein inhibited not only the porcine-pancreas α-amylase, but also the *T. molitor* enzyme.

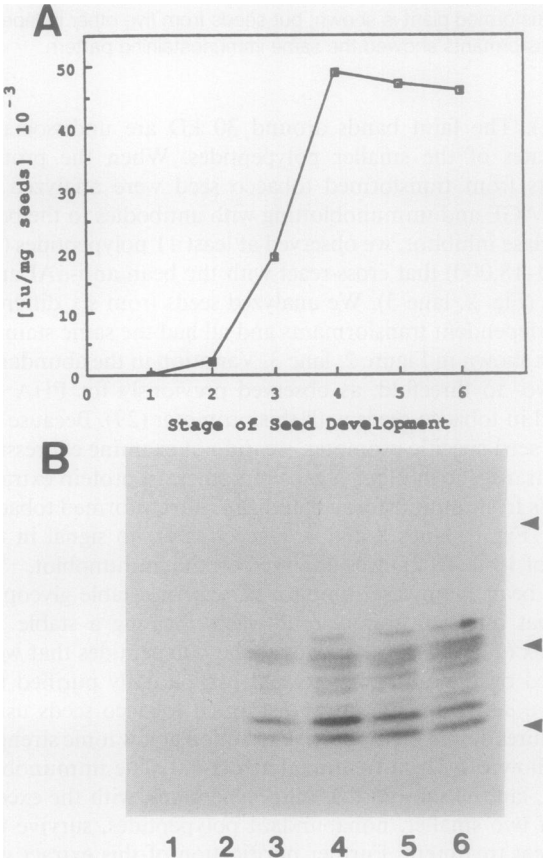

Figure 5. Time course of αAI accumulation during seed development. Samples of transgenic tobacco seeds were taken at 9, 12, 15, 20, 25, and 30 d after pollination (stage 1–6 of seed development), and αAI was determined either by inhibitory activity assay or SDS-PAGE (same amount of seeds loaded) and immunoblot. Arrows on the right indicate molecular mass of 14, 18, and 26 kD from bottom to top.

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Inhibitors of mammalian digestive enzymes (proteases and amylases) are thought to be involved in the defense of plants against pathogens. Most of them, as is the case of the α-amylase, are active against microbial and animal enzymes but not against the endogenous plant enzyme. The addition of purified plant proteinase inhibitors to an artificial diet inhibits the growth and development of diverse insect larvae (5, 8, 31). A toxic effect also has been demonstrated for the bean lectins PHA and arcelin (15, 21), two other plant defense proteins that are homologous to the gene that encodes the αAI. That genes for digestive enzyme inhibitors can be used to control insect larvae, when the proteins are expressed in transgenic plants has been demonstrated by Hilder *et al.* (11) and Johnson *et al.* (16). Our identification of an amylase inhibitor that discriminates between foreign (insect and mammalian) and plant amylase as a product of the *αai* gene and the finding that this inhibitor is stable and active when transferred to transgenic plants opens the possibility to increase plant protection against insects by introducing these lectin genes into plants and directing their expression to specific plant organs. Because αAI is a vacuolar protein and the vacuoles of leaves (3, 10) and probably also of other organs are rich in endoproteolytic activity, it will be necessary to protect the protein from proteolytic degradation. Vacuoles of seeds have a low endoproteolytic activity (9) and are therefore most suited for limited proteolytic processing of proteins.

Although further work is needed to show that αAI retards larvae growth, the finding that the αAI from transgenic tobacco seeds inhibits the α-amylase present in the midgut of *T. molitor* suggests that the introduction of *αai* in other leguminous plants could be a strategy to protect the seeds of these plants from seed-eating larvae. Because the pH optimum for the formation of the protein complex between α-amylase and αAI is five to six (24), the inhibitor may work much better against Coleoptera than against Lepidoptera. The former have an acidic pH, while the latter have a basic pH in their midgut (7).

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