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Bean α-Amylase Inhibitor Confers Resistance to the Pea Weevil (*Bruchus pisorum*) in Transgenic Peas (*Pisum sativum* L.)[1]

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Bruchid larvae cause major losses of grain legume crops throughout the world. Some bruchid species, such as the cowpea weevil and the azuki bean weevil, are pests that damage stored seeds. Others, such as the pea weevil (*Bruchus pisorum*), attack the crop growing in the field. We transferred the cDNA encoding the α-amylase inhibitor (α-AI) found in the seeds of the common bean (*Phaseolus vulgaris*) into pea (*Pisum sativum*) using *Agrobacterium*-mediated transformation. Expression was driven by the promoter of phytohemagglutinin, another bean seed protein. The α-amylase inhibitor gene was stably expressed in the transgenic pea seeds at least to the T_5 seed generation, and α-AI accumulated in the seeds up to 3% of soluble protein. This level is somewhat higher than that normally found in beans, which contain 1 to 2% α-AI. In the T_5 seed generation the development of pea weevil larvae was blocked at an early stage. Seed damage was minimal and seed yield was not significantly reduced in the transgenic plants. These results confirm the feasibility of protecting other grain legumes such as lentils, mungbean, groundnuts, and chickpeas against a variety of bruchids using the same approach. Although α-AI also inhibits human α-amylase, cooked peas should not have a negative impact on human energy metabolism.

The common bean (*Phaseolus vulgaris* L.) contains a family of structurally related seed proteins: PHA-E and -L, arcelin, and α-AI. PHA-E and PHA-L are strong agglutinins, i.e. classical lectins that bind carbohydrate, and arcelin, which is found only in certain wild accessions of the common bean, may be a weak agglutinin (Hartweck et al., 1991). The bean α-AI has 65 to 70% amino acid sequence identity with the other three but lacks at least one of the conserved residues needed for lectin activity. Its biochemical mode of action is to form a one-to-one complex with certain amylases (for reviews, see Chrispeels and Raikhel, 1991; Rouge et al., 1993).

There is good evidence that these four proteins play important and distinctive roles in plant defense. The PHAs are toxic to mammals and birds when added to the diet of experimental animals, causing lesions of the intestinal mucosa and disruptions of nutrient assimilation from the intestine (for review, see Liener, 1986). Arcelin is the major seed protein in certain wild accessions that are resistant to the bean weevils *Zabrotes subfasciatus* and *Acanthoscelides obtectus* (Osborn et al., 1986), and it has been postulated that weevil resistance in these lines is caused by the presence of arcelin (Osborn et al., 1988). It is not clear whether arcelin is toxic or simply an indigestible storage protein. α-AI, when added in low concentrations (1%) to artificial diets, proved to be toxic to the larvae of two major pests of stored legume grain, the *Bruchus* beetles *Callosobruchus maculatus* (cowpea weevil) and *Callosobruchus chinensis* (Azuki bean weevil) (Ishimoto and Kitamura, 1989; Huesing et al., 1991).

The stored grains of peas and other legumes such as chickpea, cowpea, and Azuki bean are highly susceptible to attack by *C. maculatus* and *C. chinensis*. The recent development of procedures for genetic manipulation of peas (*Pisum sativum*) (Schroeder et al., 1993) provided an opportunity to test the insecticidal activity of α-AI protein against these insects in vivo. Transgenic peas that expressed the bean *αai* gene in their seeds at the same level as normally found in bean seeds indeed showed complete resistance to both *C. maculatus* and *C. chinensis* (Shade et al., 1994).

In addition to these insect pests of stored grain, peas are also subject to attack by another Bruchid beetle, *Bruchus pisorum* (pea weevil), during seed development in the growing crop. The pea weevil is one of the major insect pests of peas in the field and is responsible for losses of up to 40% in seed yield (Smith, 1990). The pea weevil lays its eggs on the young pea pod and the hatched larvae burrow through the pod wall and into the immature seed where they feed extensively on the seed contents while developing to fully mature, pre-emerged adults. Apart from losses in yield, pea weevil infestation results in a significant de-

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Abbreviations: α-AI, α-amylase inhibitor; *αai*, α-amylase inhibitor gene; PHA, phytohemagglutinin.

valuation of the harvested grain, which is rendered unsuitable for human consumption and, because of reduced viability, for subsequent re-sowing (Smith, 1990).

We have now extended our observations on transgenic peas expressing the bean *αai* gene to test the possibility that the presence of the α-AI protein in their seeds may also protect the developing pea seeds in a growing crop from attack by the pea weevil. The expression of the bean *αai* gene in peas was stable for at least five generations and T_5 seed, homozygous for the *αai* gene, contained levels of α-AI as high or higher than that found in bean seeds. These pea seeds were totally protected from major damage by the pea weevil and weevil development was completely blocked at an early larval stage.

MATERIALS AND METHODS

Chimeric Gene Construction

Construction of the plasmid gene (pMCP3) containing the chimeric *αai* gene was described in detail elsewhere (Shade et al., 1994). The protein-coding region of the *αai* gene was equipped with 5′ and 3′ flanking regions from the bean PHA gene *dlec*2. In addition, the construct, based on pGA492 (An, 1986), contained the *bar* gene (White et al., 1991) with the cauliflower mosaic virus 35S RNA 5′ flanking region and the octopine synthase 3′ region. This chimeric gene, coding for phosphinothricin acetyl transferase, conferred resistance to the herbicide Basta (AgroEvo, Melbourne, Australia), which was used as a selectable marker in tissue culture and a screenable marker for developing plants in the glasshouse.

Plant Material

The *Agrobacterium*-mediated transformation and regeneration procedure (Schroeder et al., 1993) was used to introduce the *αai* gene into peas (*Pisum sativum*) cv Greenfeast. Of 18 independent transgenic plants, five (S14, S15, S16, F8, and F10) were raised to produce T_1 seeds. Small amounts of cotyledon tissue were filed off individual dry seeds and these were tested for the presence of α-AI protein using immunoblot procedures. Seeds expressing α-AI protein were grown to produce T_1 plants bearing T_2 seeds for weevil infestation studies (bioassay 1). This bioassay involved six T_1 plants of S14, S15, S16, and F8 and 14 T_1 plants of F10. Nontransgenic control plants were grown and infested with *Bruchus pisorum* eggs in parallel with the transformed peas.

Descendants of the F10 transgenic line were selected for high α-AI protein levels through to the T_4 generation and six of these plants producing T_5 seeds, along with six nontransgenic controls, were used in a second experiment to measure the stability of the *αai* gene expression and the degree of resistance to the pea weevil. Plants were established late in winter so that the time of our infestation assays in the glasshouse would coincide with the time of natural pea weevil infestation in the field. Plants were grown in a biosafety (PH1) glasshouse under natural light with day/night temperatures of 24/12°C.

Pea Weevil Infestation Assays

Two pea weevil infestation assays were carried out at the same time of the year, exactly 1 year apart, using the experimental procedures developed by Hardie (1993). At the same time that both control and transgenic plants began to flower and set pods, pods from other nontransgenic plants were placed in cages with mated pea weevils to obtain eggs. Fresh pods were placed in the cages each day and egg-laden pods were removed and stored at room temperature in ventilated boxes. When eggs developed black spots, indicative of the developing larval head, they were manually transferred to the test pods.

To mimic infestation in the field, eggs were transferred to the immature pods at a stage when the pod wall, seed coat, and seed were soft and did not act as a major physical barrier. There were six to eight seeds per pod in both transgenic and control pods. Two eggs per seed were spaced along one side of the pod. Together with each transgenic pod, a control pod was infested on the same day. Larvae emerged from the eggs up to 1 week after transfer onto the pods.

Several weeks later, pods were harvested as they matured on the plants. Harvested pods were stored in seed envelopes at 25°C and the number of emergent adult weevils was monitored regularly. The testa and cotyledons of every seed were examined for larval entry to ascertain the number of infested seeds. The larger hole left by the emerging adult was readily distinguished from the much smaller entry hole caused by the infesting larva. Seeds that had been scored as infested but from which no adult weevil had emerged after 140 d from the date of egg transfer were split open for more detailed examination.

Monitoring *αai* Gene Expression

DNA was extracted (Shure et al., 1983) from young pea leaves, digested with *Hin*dIII, electrophoresed, blotted onto nitrocellulose (Southern, 1975), and probed with a radiolabeled fragment corresponding to the coding region of the *αai* gene. The total RNA fraction was isolated (Chandler et al., 1983) from pooled, developing T_1 pea seeds (22–25 DAF) from the selected primary transformants, fractionated by electrophoresis, blotted, and probed with *αai* DNA as above (Higgins and Spencer, 1991).

The α-AI protein in transgenic pea seeds was assayed by western blot procedures. Pea seed flour (20–40 mg) was filed off each mature seed. This was done on the surface opposite the embryonic axis so as to preserve seed viability. The flour was extracted with 500 mL of a solution containing 0.1 M Tes, pH 7.8, 0.5 M NaCl, 1 mM EDTA, 1 mM PMSF, and 2% β-mercaptoethanol by vortexing repeatedly for 1 to 2 min. The suspension was centrifuged at 13,000 rpm for 5 min and the resultant supernatant was used for protein determination by the method of Bradford (1976) and for determination of α-AI levels by electrophoresis on SDS-PAGE following immunoblotting on nitrocellulose membrane. The α-AI protein was detected using a rabbit anti-α-AI serum as the primary antibody and anti-rabbit IgG coupled to alkaline phosphatase (Promega)

as the secondary antibody. Quantitative estimates of the level of α-AI in the total soluble protein extracts were obtained by densitometric analyses of blots containing three, known, different levels of α-AI and 40 μg of pea seed protein from each transgenic line.

Microscopy

Freshly laid eggs and larvae dissected from eye-spot stage eggs were freeze dried. Adult beetles were killed by freezing then dried at 35°C for 24 h. Dead larvae were removed by careful dissection from transgenic seed and cleaned of dust. For scanning EM, the dry samples were mounted on cardboard points before sputter coating with gold and viewing in a JEOL 6400 scanning electron microscope at 15 kV. Mature pea seeds of nontransgenic and transgenic plants were hydrated, cut open to reveal internal damage caused by *B. pisorum*, and photographed with a Leica M8 stereomicroscope.

RESULTS

Expression of the Bean *αai* Gene in Peas

Evidence for the successful integration of the T-DNA of MCP3 into peas and the expression of the *αai* gene was obtained from Southern, northern, and western blot analyses. A total of 18 independent MCP3 transformants were obtained and results are presented for five of these T_0 plants, namely S14, S15, S16, F8, and F10. DNA from leaves of these plants was digested with restriction enzyme, fractionated by electrophoresis, and probed with a radiolabeled fragment corresponding to the coding region of the *αai* gene. A single *Hin*dIII band of 4.7 kb was obtained with all five transformants. This band was not present in DNA from the nontransformed pea plant (data not shown).

The presence of *αai* mRNA was confirmed by northern blot analysis of total RNA extracted from immature seeds harvested from all of the primary transformants 22 to 25 DAF (Fig. 1). The mRNA levels in these immature, T_1 seeds varied over a wide range. Several seeds from individual pods were pooled for preparation of each of these RNA samples. Since the *αai* gene would be segregating in this seed population, the actual variation in mRNA levels in individual seeds would be greater than shown in Figure 1a.

α-AI was readily detected by western blot analysis of extracts from mature T_1 seeds from the primary transformants (Fig. 1b). In all transformants the *αai* gene product is represented by a set of at least three immunoreactive polypeptides with relative molecular masses in the 14- to 19-kD range. This is the same range as naturally occurring α-AI proteins found in the common bean in which they are formed by the posttranslational processing of a pro-protein precursor of 35 kD (Moreno and Chrispeels, 1986). The range of final sizes is probably the result of different levels of glycosylation at the three potential sites within the processed 14-kD polypeptide (Moreno and Chrispeels, 1986).

Nine seeds of the T_2 generation from the F10 line were taken from a single pod and analyzed individually for the α-AI protein. Six of the 9 seeds were clearly positive and no α-AI was detected in the remainder (Fig. 2), indicating that

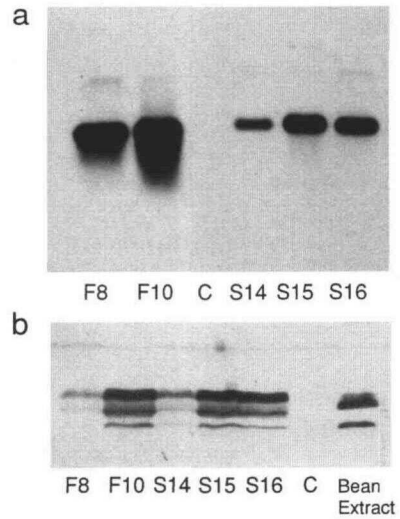

Figure 1. Expression of the *αai* gene in transgenic peas. a, Northern blot analysis of RNA isolated from T_1 seed of control and transgenic peas. Six to eight seeds of each plant were pooled prior to RNA isolation and fractionation; b, western blot analysis of α-AI protein from T_1 seed of control and transgenic peas. Pea meal was filed from dry T_1 seed and the meal from six to eight seeds of each plant was pooled prior to protein isolation and fractionation. Bean seed extract served as a positive control for α-AI protein and an extract from untransformed pea seeds served as a negative control (lane C).

segregation of the *αai* gene was still occurring. However, a similar analysis of more than 50 T_5 generation seeds indicated that, after three further generations of selection, the transgenic F10 line was homozygous for the *αai* gene (Fig. 2b shows 8 such seeds). The T_2 and T_5 generation seeds were used, respectively, in the first and second bioassays of pea weevil development described below. These two bioassays were carried out 1 year apart.

The Insecticidal Activity of α-AI on Pea Weevils

Pea weevil infestation was studied in T_2 generation seeds of five independent pea transformants. Similar observations were made on contemporaneous, nontransgenic, control plants. Bioassay conditions, particularly with respect to pod age, were chosen to mimic as closely as possible conditions in the field under which pea weevil infestation occurs. The level of infestation was similar in all plants (78–94% in transgenic plants and 87% in control plants). However, there was a wide range of responses in pea weevil development to the presence of α-AI (Table I). In the control plants, adult weevils emerged from 94.2% of the infested seeds. The seeds from the five transformant lines fell into two broad groups. In S14, S16, and F8 transformants, there was complete weevil development in 88.0, 70.3, and 87.9% of seeds, respectively. In the case of S15 and F10 transformants, adult weevils emerged in only 33.5 and 45.5% of the seeds, respectively. These results correlated with the levels of α-AI protein in these seed populations. As would be expected of a segregating T_2 population, some seeds in each population contained no detectable α-AI protein (cf. Fig. 2a). In those T_2 seeds of S14, S16, and F8

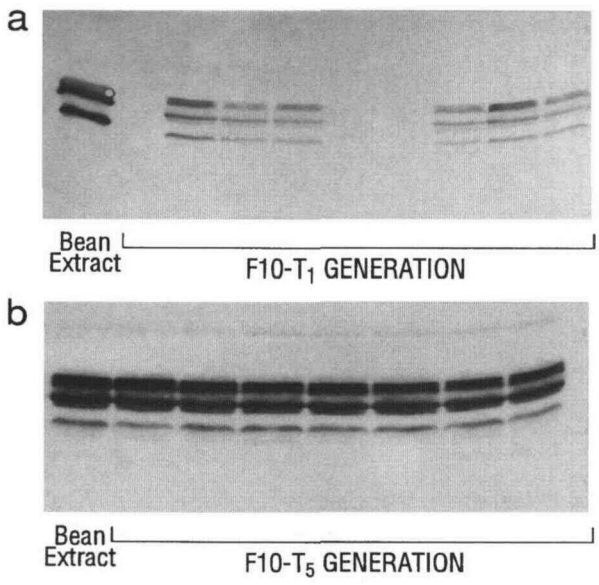

Figure 2. The distribution of α-AI protein in individual transgenic pea seeds. Western blots of α-AI protein in nine T_2 seeds of the F10 line showing segregation of the *αai* product (a) and eight T_5 seeds of the F10 line showing homozygous expression of the *αai* gene (b). Bean seed extract served as a positive control for α-AI protein.

transformants in which α-AI protein was detected, the level was approximately 1.5% of total soluble protein. In the corresponding seeds in the S15 and F10 populations, the level of α-AI ranged from 2.2 to more than 3%. In the S15 and F10 populations, adult weevil emergence was restricted to those seeds that did not contain detectable α-AI protein.

Although the resistance level to pea weevil infestation was highest in T_2 seeds of the transformant S15, the level of α-AI protein was highest in some F10 seeds. These were presumed to be homozygous for the *αai* gene and were

Table I. *Pea weevil development in T_2 seeds of five transgenic lines of pea plants (S14, S15, S16, F8, and F10)*

Plants	No. of Seeds Infested	No. of Infested Seeds with Emerged Adults	Infested Seeds with Emerged Adults	α-AI Protein[a]
			%	%
S14	159	140	88.0	1.0 –1.5
S15	161	54	33.5	2.2 –3.0
S16	160	117	70.3	1.5
F8	158	139	87.9	1.0 –1.5
F10	387	176	45.5	1.5 –3.5
Control	198	186	94.2	0

[a] α-AI protein as a percentage of total soluble protein. These values were obtained by analysis of a sample of individual seeds in T_2 populations of each transgenic line. The range of values shown applies to those seeds in which α-AI protein was detected. Each segregating (T_2) population contained some seeds with no detectable α-AI protein.

Table II. *Pea weevil development in T_5 seeds of six transgenic (F10) pea plants*

Plants	No. of Seeds		No. of Seeds Infested		No. of Infested Seeds with Emerged Adults	
	T_5	Control	T_5	Control	T_5	Control
1	50	38	27	26	0	23
2	45	44	42	35	0	32
3	50	42	30	35	0	30
4	48	46	35	30	0	25
5	40	35	26	27	0	21
6	46	39	29	28	0	26
Total	279	244	189	181	0	157

selected for high α-AI protein levels through two further generations. This material (immature pods containing T_5 seeds of the line F10) was used for a second bioassay of the effect of α-AI on pea weevil development (Table II).

The second bioassay experiment involved six control and six T_4 plants of the F10 line, each set carrying approximately 250 seeds (Table II). All of the T_5 seeds from the T_4 plants contained the same high level of α-AI protein (approximately 3.5%). Of these, approximately 70% were invaded by the newly hatched larvae in both control and transgenic plants. Eighty-seven percent of the infested control seeds yielded emerged adult weevils and the mean time to adult emergence was 85.3 d. The experiment was terminated after 140 d and at this time no adult weevils had emerged from any F10 seeds of the T_5 generation. Additional observations more than 200 d after infestation indicated that total control over adult pea weevil development had been achieved.

Pea Weevil and Seed Development in Transgenic Peas

Pea weevil eggs (500–700 μm in length) are laid on, and cemented to, the pea pod (Fig. 3). The larva develops within the egg and burrows through the egg case and the pod wall into the cotyledon of the immature seed. The larva at this stage (first instar) is approximately 400 μm long (Fig. 3b). The larva develops through three more instars and a pupal stage before emerging from the mature seed as an adult beetle (Fig. 3c). As reported above, no adults ever emerged from T_5 seeds (Table II). Careful dissection of these seeds revealed only dead larvae of the first or second instar (Fig. 3d). The presence of the α-AI prevented development to the later, more damaging, developmental stages. The contrast in physical damage caused by the pea weevil in transgenic and nontransgenic plants is shown in Figure 3, e and f. During its development in a nontransgenic seed, the larva creates a large cavity in the cotyledons, causing a reduction of between 12 and 27% in the final seed weight and a concomitant loss in seed viability. Losses of 20 to 40% of seed weight have been reported under field conditions in Australia (Smith, 1990). In transgenic seeds, final weight loss ranged from 1.8 to 3.5%, which is less than single seed weight variation.

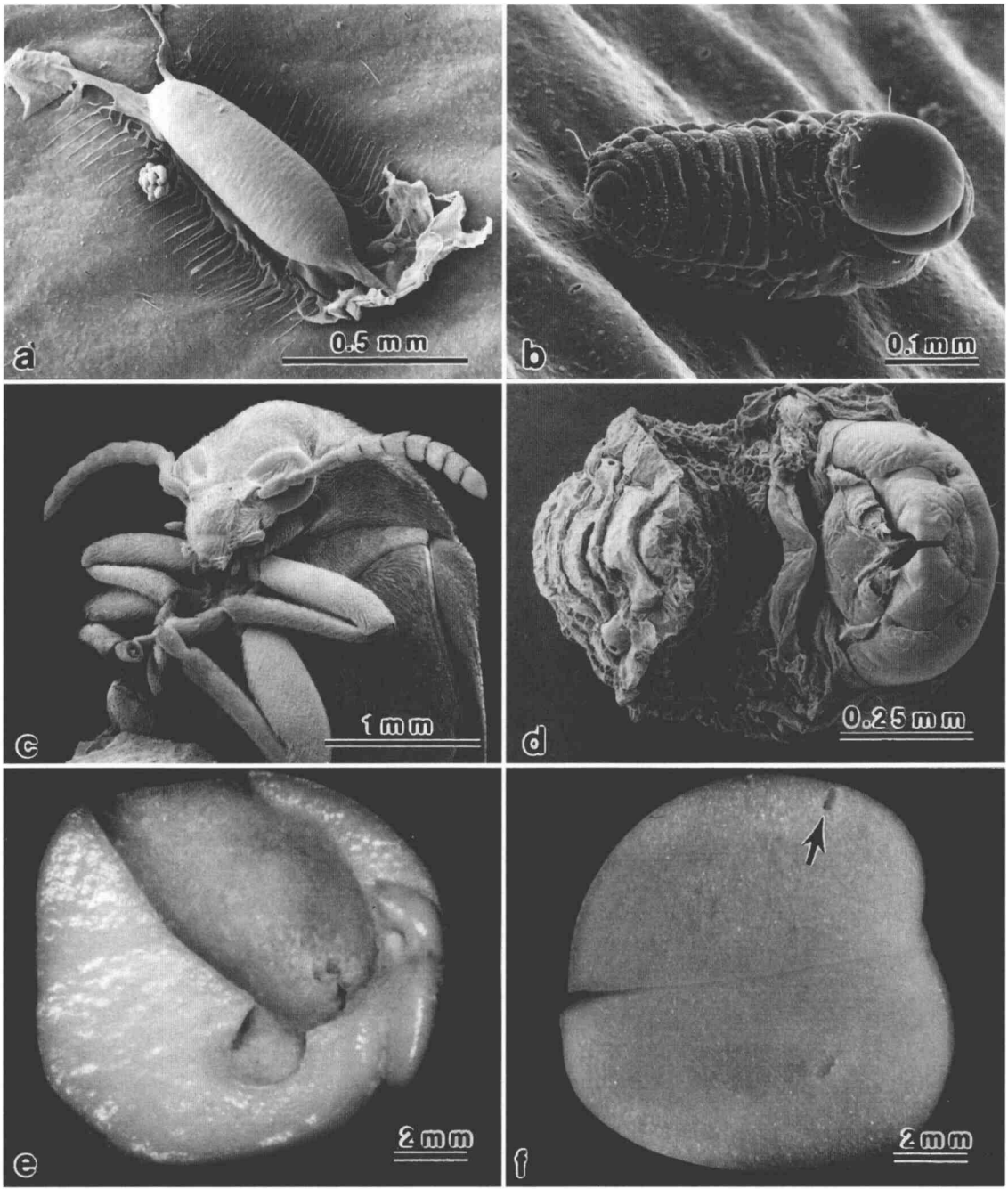

Figure 3. Microscopic images of developing pea weevil and the damage caused to control and transgenic peas. a to d, Scanning electron microscopic images. a, Pea weevil egg attached to pea pod; b, newly hatched (first instar) pea weevil larva; c, adult pea weevil, newly emerged from mature pea seed; d, dead pea weevil larva dissected from a transgenic pea seed similar to that shown in f. e and f, Macrophotographs of hydrated seeds. e. Dissected nontransgenic pea seed showing damage caused by a pea weevil during the weevil's development and maturation; f, sliced cotyledons of infested, transgenic (T_5) pea seed showing minimal damage (arrow) caused by entry of the pea weevil larva.

The homozygous T_4 plants and their T_5 seeds were phenotypically indistinguishable from their nontransgenic parents. For example, seed development in the T_5 generation was equal to that in noninfested, nontransgenic controls. Mean seed weights were 291 and 301 mg, respectively, and the number of seeds per pod was 8.0 in the F10 plants and 7.0 in the controls.

DISCUSSION

Peas are an important grain legume crop in many parts of the world, providing a valuable source of food for human consumption and feed for livestock. In Australia, as elsewhere, the pea weevil is one of the major insect pests of pea crops in the field. The pea weevil eggs are laid on the

immature pea pod soon after flowering and the first instar larva burrows directly through the pod wall and into the developing seed. It develops through a further three larval instars and a pupal stage and finally forms an adult beetle, all within the maturing pea seed. Adults emerge and live in surrounding refuges until mating and laying eggs on the developing pods of subsequent pea crops. The results presented in this report indicate that the presence of bean α-AI protein in the developing pea seeds completely blocks this life cycle at an early instar larval stage. No adult beetles emerged from seeds of the T_4 generation of transformed pea plants (T_5 seeds), all of which had α-AI levels of approximately 3% of total soluble protein.

Because the *αai* gene construct used in these experiments is regulated by flanking sequences from the seed-specific bean PHA (*dlec2*) gene, we assume its expression would be restricted to the cotyledon and embryonic axis of the developing seed. For this reason, the infestation by the pea weevil proceeds normally in the transgenic peas until the larva reaches the cotyledons. Here the larva is exposed to the α-AI protein for the first time and its development rapidly ceases. At this early stage in larval development (first or second instar), little physical damage has been done to the pea seed and no significant weight loss was recorded. These results confirm earlier results of Smith (1990) concerning the limited extent of damage caused by early larval stages.

The PHA regulatory DNA sequences also impose a specific time course on the expression of the genes that they regulate. Thus, in bean, PHA begins to accumulate in early stages of seed development and does not reach a maximum until about midway to seed maturity (Voelker et al., 1987). A similar temporal pattern was seen for the expression in tobacco of the *αai* gene flanked by PHA regulatory sequences (Altabella and Chrispeels, 1990), and we assume this pattern would apply in the transgenic peas in the present study. It is perhaps a measure of the sensitivity of pea weevil larvae to α-AI protein that they are killed at an early stage of their development before α-AI levels have reached their maximum.

In the developing bean seed, the gene for α-AI codes for a pre-proprotein of 35 kD, which is transported through the endomembrane system to the vacuole where it accumulates. During this process, the protein is extensively modified by removal of both the pre- and pro-amino acid sequences and by varying degrees of glycosylation. The end product is a family of three or four polypeptides of relative molecular mass 14 to 19 kD (Moreno and Chrispeels, 1986). Pueyo et al. (1993) have shown that this processing is essential for biological activity; the unprocessed pro-protein does not show α-AI activity. The processing of this pro-protein observed in transgenic peas is thus an important element in conferring pea weevil resistance with the *αai* gene. The processed products of the *αai* gene in transgenic peas are in the same size range as those in bean seed (14–19 kD). They do not all co-electrophorese precisely with their bean counterparts (compare bean extract and pea extracts in Figs. 1b and 2), presumably because of slight differences in posttranslational modification of the pro-protein. Immunolocalization studies with transgenic peas of the F10 line have confirmed that α-AI is accumulated in the protein bodies in the pea seed cotyledons (S. Craig, unpublished observations), as is the case in bean (Moreno et al., 1990).

Given the strong insecticidal activity of α-AI reported in this paper, it is possible that the expression of other *αai* gene constructs containing pod-specific gene promoters could prevent the entry of the newly hatched larva into the seed and minimize still further the physical damage caused by the pea weevil. This approach would depend on the correct processing of α-AI in pod cells. We recently reported that the *αai* gene also renders transgenic peas resistant to two serious pests of stored grain, *C. chinensis* and *C. maculatus* (Shade et al., 1994).

A large number of cultivars, lines and wild accessions among peas and their relatives have been screened for natural resistance to the pea weevil and complete resistance was found in some accessions of *Pisum fulvum* (Hardie, 1993). However, no trace of α-AI protein could be detected in these seeds (A. Moore, unpublished data); therefore, we presume that their pea weevil resistance is the result of some other mechanism. In principle, it should be possible, by conventional plant breeding, to transfer the *P. fulvum* resistance trait into genetically engineered domesticated peas containing α-AI protein and, in this way, generate a two-pronged defense against the pea weevil in one pea line. However, the primitive character of *P. fulvum* would make this a major plant-breeding task.

At present, the pea weevil is controlled by a range of management strategies including use of chemical sprays on adult weevils in the field, the early harvest of mature seed from the crop, and the prompt fumigation of the stored grain to prevent the exit of pre-emerged adult beetles. The results described here, in which the gene for a seed protein from the common bean has been transferred into pea, opens the way for the development of pea weevil-resistant pea cultivars and a concomitant reduction in the use of insecticidal chemical sprays and fumigants. Because the pea weevil does not infest any crop other than peas (Clement, 1992), the addition of the bean *αai* gene to commercial pea cultivars could be a major weapon in completely eliminating this insect pest. We are currently investigating whether the α-AI protein in transgenic pea seeds has any antinutritional effects in mammals.

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