Bean α-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions

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Two α-amylase inhibitors, called αAI-1 and αAI-2, that share 78% amino acid sequence identity and have a differential specificity toward mammalian and insect α-amylases are present in different accessions of the common bean (*Phaseolus vulgaris*). Using greenhouse-grown transgenic peas (*Pisum sativum*), we have shown previously that expression of αAI-1 in pea seeds can provide complete protection against the pea weevil (*Bruchus pisorum*). Here, we report that αAI-1 also protects peas from the weevil under field conditions. The high degree of protection is explained by our finding that αAI-1 inhibits pea bruchid α-amylase by 80% over a broad pH range (pH 4.5–6.5). αAI-2, on the other hand, is a much less effective inhibitor of pea bruchid α-amylase, inhibiting the enzyme by only 40%, and only in the pH 4.0–4.5 range. Nevertheless, this inhibitor was still partially effective in protecting field-grown transgenic peas against pea weevils. The primary effect of αAI-2 appeared to be a delay in the maturation of the larvae. This contrasts with the effect of αAI-1, which results in larval mortality at the first or second instar. These results are discussed in relationship to the use of amylase inhibitors with different specificities to bring about protection of crops from their insect pests or to decrease insect pest populations below the economic injury level.

The use of genes that encode insecticidal proteins in transgenic crops has the potential to benefit agricultural crop production, the environment, and the consumer. The benefit to the environment and the consumer will come from the reduced use of chemical sprays. Insecticidal proteins delivered in an organ-specific fashion allow only the pests of the crop to be targeted, thereby reducing the collateral damage often associated with broad-spectrum chemical insecticides. The elimination of chemical sprays also provides a benefit to agriculture because of the removal of the costs associated with their application.

Most attention in this field has been focused on the *Bacillus thuringiensis* (Bt) toxin, and crops that express the Bt gene are now in production in a number of countries. Alternatives to Bt toxins are needed because, just as with chemical pesticides, resistance to some Bt toxins is emerging and eventually will become widespread (1). Another class of genes that holds promise for genetic engineering of crops are those that encode inhibitors of insect digestive enzymes and considerable progress has been made with inhibitors of protease (2) and amylase (3). Unlike Bt toxins, these proteins have been in the human food chain for millennia because plants contain both types of inhibitors as part of their natural defense mechanisms. These inhibitors often display narrow specificities: a given inhibitor may inhibit the major digestive enzyme of one insect species but not of another. A case in point is provided by the inhibitors of α-amylases found in the common bean, *Phaseolus vulgaris*. Bean seeds contain at least two different α-amylase inhibitors called αAI-1 and αAI-2. They have distinct specificities: αAI-1, which is found in most cultivated common bean varieties, has been characterized extensively (4, 5). It inhibits several mammalian α-amylases and the larval midgut amylases of the Azuki bean weevil (*Callosobruchus chinensis*) and the cowpea weevil (*C. maculatus*), but not of the Mexican bean weevil (*Zabrotes subfasciatus*) (6). The latter insect is a pest of cultivated *P. vulgaris*. Seeds of certain wild accessions of *P. vulgaris* that are rich in the protein arcelin contain the homologue αAI-2, which shares 78% amino acid identity with αAI-1. αAI-2 does not inhibit mammalian amylases (7, 8) but does inhibit the midgut α-amylase of *Z. subfasciatus* (7, 9). The αAI-2-containing beans are resistant to the Mexican bean weevil. Thus, there appears to be a correlation between inhibitor specificity and insect resistance, although the αAI-2 protein is not the sole determinant of resistance to Mexican bean weevil in beans (10).

The pea weevil (*Bruchus pisorum*) is a pest of the field pea (*Pisum sativum*) with a worldwide distribution. *B. pisorum* adults emerge from hibernation in spring and feed on pea pollen before mating and laying eggs on immature pea pods. The larvae, once hatched, burrow through the pod wall and into the seed creating a small, dark "entry hole" approximately 0.2 mm in diameter. The larvae develop through four instars inside the seed, consuming cotyledon contents and creating a cavity with a circular "window" of testa at one end of the seed (11). The larva pupates behind this window. The resulting adult either remains dormant or pushes the window open and leaves the seed, creating a 5-mm "exit hole." The adults survive until the following spring by hibernating in available shelters including pea straw, buildings, and woodlands (12, 13). Pea weevil infestation causes economic loss because of the direct loss of seed contents consumed by the pest and because weevil-damaged seed has lower germination rates and fetches a lower unit price. Currently, this pest is controlled by using chemical insecticides.

Using seeds produced by transgenic, greenhouse-grown peas that express αAI-1 cDNA from a highly active, seed-specific promoter, we demonstrated previously that low levels of αAI-1 protein are sufficient to make these seeds resistant to the Azuki bean weevil; higher levels of the protein make the seeds resistant to the cowpea weevil and the pea weevil (14, 15). Here, we report that transgenic peas containing αAI-1 were resistant to damage by the pea bruchid under field conditions at a number of sites in Australia and over several seasons. αAI-1 caused larval mortality at the first or second instar stage. We also report field experiments with peas that express αAI-2 and show that this protein was less effective at protecting peas in that it delayed

Abbreviations: αAI, α-amylase inhibitor; Bt, *Bacillus thuringiensis*; DPH, days postharvest.

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larval maturation by around 30 days without affecting overall insect mortality. *In vitro* measurements of the activity of the two inhibitors toward pea bruchid α-amylase over a pH range (4.0–6.5) suggest a basis for the differential effects of the two α-amylase inhibitors.

Materials and Methods

Plasmids. pMCP3 is based on the binary plasmid pGA492 (16), and its construction has been described (14). The αAI-1 gene in pMCP3 is a *Hin*dIII fragment from pTA3 (17) and is an αAI-1 cDNA (GenBank accession no. J01261) flanked by the 5′ and 3′ control regions of the bean phytohemagglutinin gene. The same pTA3 *Hin*dIII fragment was inserted into *Hin*dIII-digested pKSB10.MCS.ori2 (18). The resulting plasmid, pKSBαAI1, is similar to pMCP3 but lacks the GUS, nptII, and CAT genes. pKSBαAI2 is identical to pKSBαAI1 except that the αAI-1 cDNA was replaced by the αAI-2 cDNA (GenBank accession no. U10348).

Plant Lines and Transformation. The line F10 is a pea (*P. sativum*) cultivar Greenfeast transformed with pMCP3 that has been described (15). The pea cultivar Laura was transformed separately with pKSBαAI1 and pKSBαAI2 by using an Agrobacterium-based method as described (18, 19). Two αAI-1 lines and one αAI-2 line of Laura peas were used in the field experiments. The αAI-1 lines (6–23 and 10–40) were derived from two individual pKSBαAI1-transformed plants selected for high seed-specific expression of αAI-1 as determined by Western blot analysis. The αAI-1 lines were derived from single T_1 plants selected as homozygous for the αAI-1 gene by immuno-dot-blot analysis of T_2 seed. T_2 seeds of these homozygous lines were grown in the greenhouse to produce T_3 seed for sowing in the field. The αAI-2 Laura line was a composite line from three different pKSBαAI2-transformed Laura plants, each of which expressed similarly high levels of αAI-2 in the seed. The line was made by pooling seed from three putatively homozygous T_1 plants as determined by immuno-dot-blot analysis of T_2 seed.

Field Experiments. Field trials were conducted in 1996 and 1997. The 1996 trial was performed in Wagga Wagga, New South Wales. The 1997 trial was conducted over three sites: Wagga Wagga, New South Wales; Horsham, Victoria; and Katanning, Western Australia. The sites are in regions in which peas are grown each year. The bruchids overwinter in the surrounding areas and reinfest the trial plots. Each year the severity of this natural infestation depends on many variables. In our trials the degree of infestation (percentage of seed with larval entry hole—see below) was, respectively, 32%, 20%, and 42% at Wagga Wagga, Horsham, and Katanning in 1997 and was 80% at Wagga Wagga in 1996. In 1996, the genotypes tested were F10 and nontransgenic Greenfeast. In 1997, the genotypes tested were nontransgenic cv Laura, Laura transgenic αAI-1 lines 6–23 and 10–40, and the Laura αAI-2 line. The trials were conducted as a "randomized complete block" design with three replicates of each genotype. Each plot consisted of peas sown in two rows, 4 m long, 20 cm apart, at 40 seeds per row, and a 1.5-m border between each plot. The plants were hand-harvested at maturity, threshed, and cleaned.

Between 200 and 500 randomly selected seeds from each plot were scored and the "percent adult emergence" was calculated as the percentage of the seeds containing larval entry holes ("infested seed") that also had windows or exit holes. In some cases we also monitored the time taken for adult emergence. In these cases, infested seeds were stored at 21°C and the percent adult emergence was calculated at various times postharvest.

Immunoblot Analysis. An antibody raised against αAI-1 in rabbits reacts with both amylase-inhibitor types. Purified αAI-2 (kindly supplied by M. Ishimoto, National Agriculture Research Center, Tsukuba, Japan) and αAI-1 were used to create standard curves to quantify the levels of the two inhibitors. The level of inhibitor in the transgenic lines was inferred from an immunoblot, where the signal from the transgenic lines fell in the linear portion of the standard curve.

Amylase and Amylase-Inhibitor Activity Analysis. *B. pisorum* larvae were obtained from greenhouse-grown peas infested with the insect as described (15, 20). To prepare larval extracts, 30 larvae (1.5–3 mm long) were removed from seeds between 40 and 60 days after inoculation and ground in 200 μl of buffer B (0.1 M phosphate buffer, pH 5.8/0.1 mM $CaCl_2$/20 mM NaCl). The soluble fraction was passed through a 0.45-μ filter and stored at 4°C. Amylase activity was measured by quantifying the amount of reducing sugars released from a starch substrate. Amylase reactions were performed in 200 μl of 0.5× buffer B at 37°C by using 0.5% starch (Sigma S2630) as the substrate. It was found that heating of the starch solution to 65°C for several hours before use was required for maximal amylase activity. The enzyme activity was monitored by removing 20-μl aliquots from the reaction at various time points and adding these to 40 μl of dinitrosalicylic acid reagent (21) in a microtiter plate. At the end of the reaction period the plate was floated in a water bath at 97°C to develop the color. After 5 min of incubation, 100 μl of water was added to the samples and the OD read at 540 nm. A standard curve was constructed from a range of maltose concentrations on the same microtiter plate. One microliter of the *B. pisorum* larval extract preparation had an activity approximately equivalent to 0.6 Sigma units of porcine amylase (Sigma A6255) when assayed under these conditions (one Sigma unit is the amount of enzyme that liberates 1 mg of maltose from starch in 3 min at pH 6.9 at 20°C).

For α-amylase-inhibitor measurements, the purified αAI was preincubated in buffer B with 0.6 units of *B. pisorum* α-amylase at 37°C. After 1 h, substrate was added and amylase activity was measured as described above. For determination of the pH dependence of the inhibitor activity, 2.5 μl of pea seed proteins at 10 μg/μl (extracted in 20 mM phosphate buffer, pH 6.1) was preincubated in 100 μl of 13 mM phosphate/citrate buffer (22) with 0.2 mM $CaCl_2$ at the indicated pH. A 1% starch solution (100 μl) in H_2O was added to start the amylase reaction. The initial rate of reaction was measured in samples containing protein from transformed and untransformed plants (R_t and R_{ut}, respectively). The percent inhibition was calculated [% inhibition = $100(R_{ut} - R_t)/R_{ut}$] at each pH point.

Results

αAI-1 Provides Protection Against Pea Weevil in the Field. The field trials were sown at sites at which peas had been grown in the previous season. The previous crops provided a source of pea weevil that naturally infested the field plots. The results obtained in the 1996 trial in Wagga Wagga with the cultivar Greenfeast are illustrated in Fig. 1. Pea weevil larvae had entered 80% of the seed harvested from this trial as evidenced by larval entry holes. At 75 days postharvest (DPH), an average of 98% of these larvae had developed into adults in the nontransgenic pea seeds, whereas only 7% of the larvae developed to adulthood in seeds from plants transformed with the αAI-1 gene. Examination of the infested seeds showed that the remaining larvae had died at the first or second instar.

This dramatic reduction in weevil emergence in αAI-1 transformed lines also was observed in the 1997 field trials, over three different sites, with the cv Laura. The quantitative data for the three trials are shown in Fig. 2. Examination of this indicates that the only adults emerging from the αAI-1-transformed peas came from a single plot at a single site (Wagga Wagga). We germinated four seeds harvested from this plot from which adults had

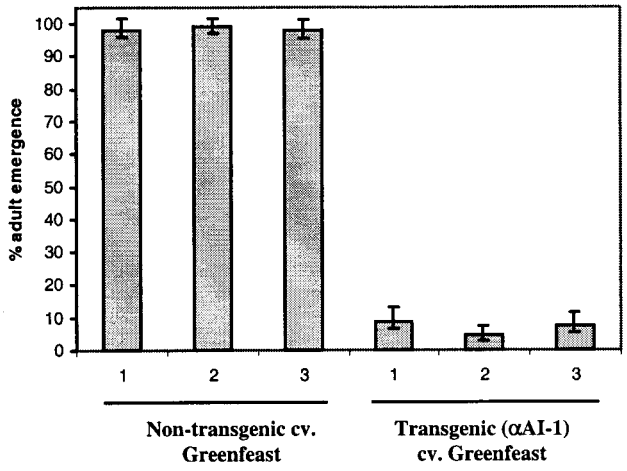

Fig. 1. Pea weevil emergence in nontransgenic and transgenic peas (cv Greenfeast) at a field trial in Wagga Wagga in 1996. The extent of infestation, as determined by larval entry, was 80% of the seed. The percent adult emergence reflects the number of these larvae that matured into adults and was measured at 75 DPH. Error bars are 95% binomial confidence intervals (23). Results are shown for three replicate plots of nontransgenic and transgenic pea containing αAI-1.

emerged and analyzed the DNA from the plants by Southern blotting. The results showed that these plants did not contain the αAI-1 transgene. The transgene was found to be present in four seeds from this plot in which adults did not develop (data not shown). We believe, therefore, that the anomalous result in this plot was caused by contamination of the seed lot with some untransformed pea seeds. Excluding the data from this plot from the analysis indicates that both of the αAI-1 pea lines tested (10–40 and 6–23) provided complete protection from pea weevil damage at the three sites tested. The improved level of protection afforded by the Laura lines relative to the Greenfeast lines may be related to the higher level of αAI-1 expression in the Laura lines (data not shown). Preliminary yield data show that the expression of αAI proteins in peas does not result in a yield penalty.

The different sites showed different degrees of adult emergence in the nontransgenic pea lines. For example, conditions in Wagga Wagga in 1997 resulted in 45% mortality of larvae in control peas compared with 7.2% and 14% mortality, respectively, in Horsham and Katanning. The mortality rate in control peas in Wagga Wagga the previous year was only 2%. Seasonal variation in pea weevil mortality has been observed previously (24) and is probably due to influences of temperature and humidity. Examination of the climatic data collected at the Wagga Wagga site shows that, in the month before the plants were harvested, there was a 5.1°C warmer average maximum temperature, 91 mm more evaporation, and 27 mm less rainfall in 1997 than in 1996. Seasonal variation in *B. pisorum* mortality also may be due to seasonal variation in the incidence of parasitoid infestations (D. Hardie, personal communication).

αAI-2 Retards Development of Larvae and Offers Partial Protection. Pea weevil larvae had entered 32% of the seed harvested from the αAI-2 field trial (Wagga Wagga, 1997). Our initial scoring of these seeds indicated that the degree of adult emergence from the seeds expressing αAI-2 was less than from the nontransgenic line, but higher than the zero emergence from the lines transformed with αAI-1. Thus, the gene that encodes αAI-2 appeared to give less protection than the gene that encodes αAI-1. We noticed, however, that, in contrast to those in the αAI-1 peas, the larvae in the αAI-2 peas were still alive. Therefore, we kept the seeds in storage at room temperature and examined them at

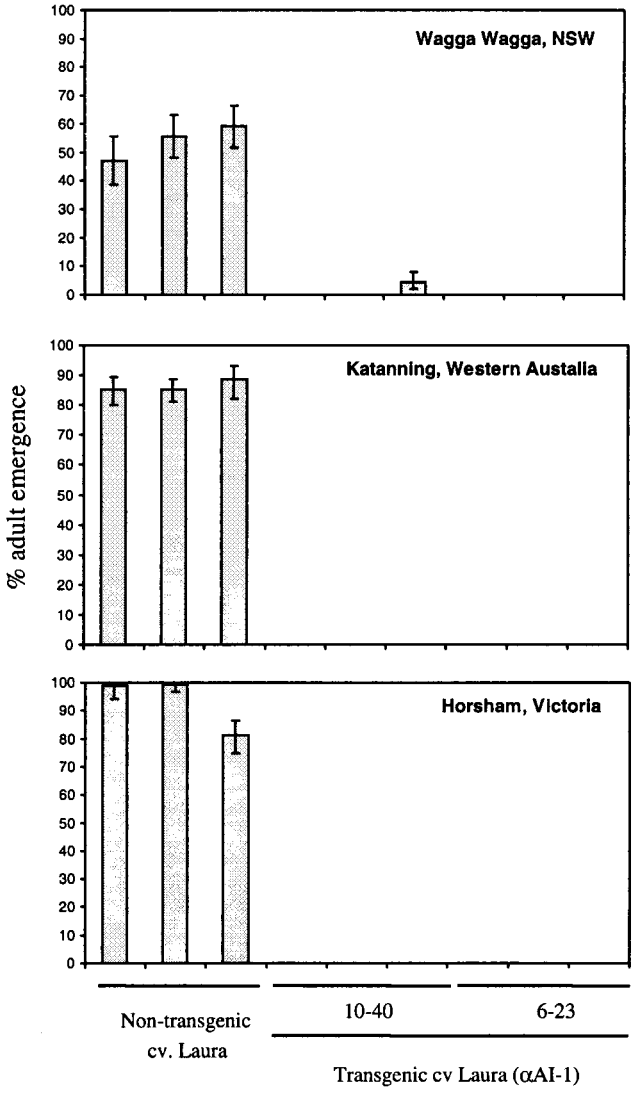

Fig. 2. Pea weevil emergence in nontransgenic and transgenic peas (cv. Laura) at three sites across Australia in 1997. Comparison of percent adult emergence in three replicates each of two different lines of transgenic pea containing αAI-1 and one line of nontransgenic pea. Error bars are 95% binomial confidence intervals (23).

different intervals to determine the rate at which the adults emerged (Fig. 3). In the nontransgenic controls, 30% of the larvae developed into adults by 55 DPH, and this number increased to 55% by 76 DPH with no further increase to 120 DPH. In contrast, in the αAI-2 peas, only 21% of the larvae had developed into adults by 80 DPH, and this number gradually increased to 52% by 110 DPH. These data indicate that αAI-2 had no effect on the final mortality rate of the developing weevil larvae, but had a significant effect on their rate of development, delaying the emergence of the adults by about 1 month.

The Different Degree of Protection Afforded by αAI-1 and -2 Is Not Related to the Inhibitor Content in the Transgenic Lines. The data from the field trial indicated that the αAI-2 seeds did not inhibit weevil development to the same degree as the αAI-1 lines. We measured the level of inhibitor expression in the two lines to determine whether this difference was caused by the expression level or by the nature of the inhibitors—these two inhibitors are

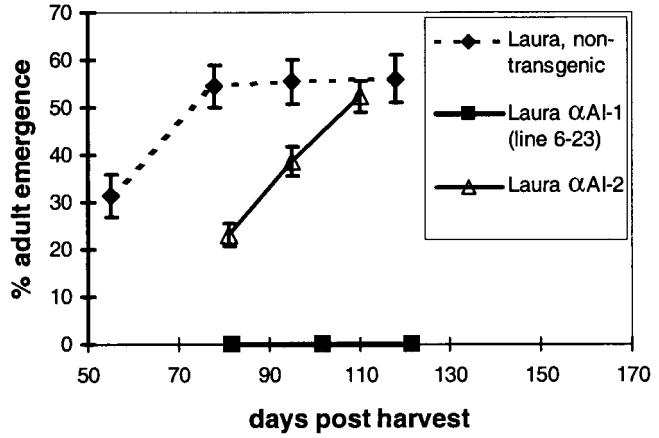

Fig. 3. Emergence of adult pea weevils in nontransgenic and αAI-1 and αAI-2 transgenic peas of cv. Laura. The percent adult emergence in the seed samples was calculated at various times after harvest. Error bars are 95% binomial confidence intervals (23).

known to inhibit different amylases (6, 7, 9). This analysis indicated that the αAI-1 lines contained 0.2 μg of inhibitor per 100 μg of seed protein, whereas the αAI-2 line contained 1.0 μg of αAI-2 per 100 μg of seed protein (Fig. 4). Thus, the level of inhibitor in αAI-2 peas was approximately 5-fold higher than the level in the αAI-1 peas. We concluded, therefore, that the difference in efficacy of the two inhibitors was due to differences in their chemical properties.

αAI-1 Is a More Effective Inhibitor of *B. pisorum* α-Amylase than αAI-2. To determine whether the differential effect of the two inhibitors on weevil development may be related to their effectiveness as inhibitors of weevil larval α-amylase, we assayed both pea weevil α-amylase activity and the inhibitory effect of αAI-1 and αAI-2 on this enzyme *in vitro*. From earlier work (9, 26) it is known that both αAI-1 and 2 are maximally active at about pH 5.5. The pH

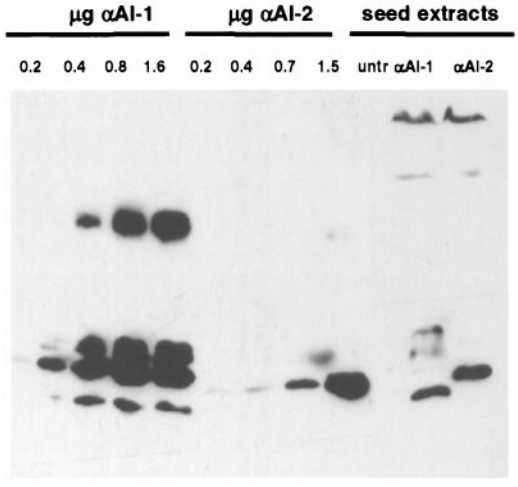

Fig. 4. Quantification of αAI-1 and αAI-2 in transgenic peas by immunoblot assay. Known amounts of purified bean αAI-1 and αAI-2 and 100 μg of protein extracted from representative seeds of nontransgenic Laura (untr), αAI-1 line, and the αAI-2 line were separated an SDS/20% polyacrylamide gel and transferred to nitrocellulose. The α-amylase inhibitors were detected by using an antibody to αAI-1 prepared in rabbit and detected by using chemiluminescence. The multiple bands result from the antibody reacting with the 25-kDa pre-pro-αAI-1 and with the differentially glycosylated isoforms of mature αAI-1 (25).

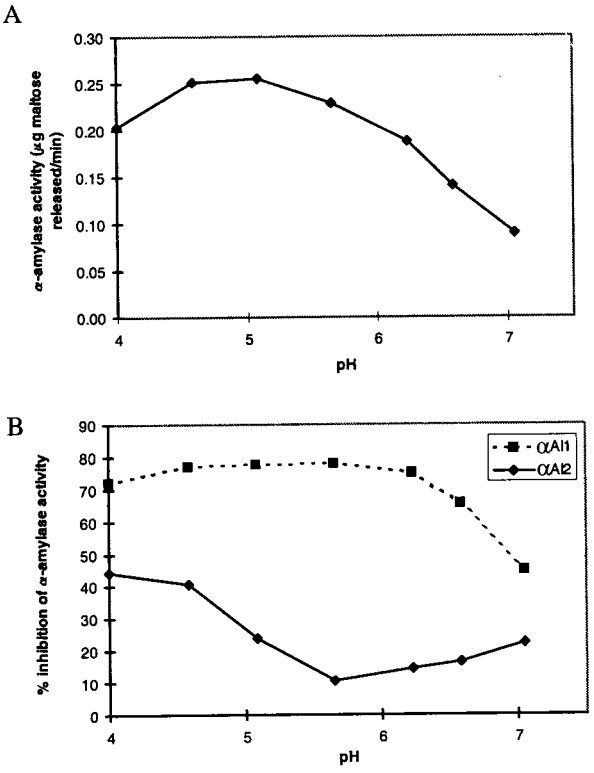

Fig. 5. The influence of pH on the activities of *B. pisorum* α-amylase and the α-amylase inhibitors *in vitro*. (*A*) pH dependence of *B. pisorum* α-amylase activity. (*B*) pH dependence of inhibitor activity from αAI-1 and αAI-2 transgenic pea against *B. pisorum* amylase. Twenty-five micrograms of protein extract was used to determine the percent inhibition of *B. pisorum* amylase at different pH values as described in *Materials and Methods*.

optimum of pea weevil amylase has not been reported. We therefore assayed pea weevil amylase and showed that it has a broad pH optimum over the range of pH 4.5 to 5.5 (Fig. 5*A*). Following a published method (27) that assays inhibitor activity against insect amylases at pH 5.8, we found that 400 ng of purified αAI-1 inhibited 0.6 units of the weevil amylase almost 80%, whereas up to 970 ng of purified αAI-2 had essentially no inhibitory activity (not shown). This suggested that the higher efficacy of αAI-1 relative to αAI-2 against pea weevil in the field is likely to be associated with its higher potency as an inhibitor of the bruchid α-amylase. However, these results do not explain the biological effect of αAI-2 on delaying pea weevil emergence. The inhibitory activity of αAI-1 and αAI-2 against amylases of porcine pancreas and Mexican bean weevil, respectively, have been demonstrated to be pH-dependent (9, 26, 28). Because there are no data on the pH dependency of αAI complex formation with the pea weevil amylase, we examined the influence of pH on the inhibitory activity of pea seed extracts that contain αAI-1 or αAI-2. Extracts of peas containing αAI-1 were active against pea weevil amylase over a broad pH range, stretching from pH 4.0 to 6.5 (Fig. 5*B*). Extracts of peas containing αAI-2 were inactive against pea weevil α-amylase at pH 5.8, as noted previously for the purified protein, but showed considerable (40%) inhibitory activity at pH 4.0 and 4.5. This inhibition curve differs considerably from that obtained previously for αAI-2 against the Mexican bean weevil amylase (9), which has a pH optimum at 5.5.

Discussion

We show here that the gene that encodes αAI-1 can be used to create transgenic peas that are resistant to the pea weevil under

field conditions. We also show that the effectiveness of an amylase inhibitor probably is related to the degree to which it inhibits larval α-amylase and that even partial inhibition of α-amylase still may result in a substantial effect on insect development. We illustrate the need to assay the effect of an inhibitor over a broad pH range.

αAI-1 Provided Total Protection Against Pea Weevil Damage, but αAI-2 Was Less Effective. Both of the cv Laura αAI-1 lines tested in 1997 were immune to damage by pea weevil (Fig. 2). The observation that the F10 line of pea cv. Greenfeast tested in 1996 in Wagga Wagga was incompletely protected from weevil damage (Fig. 1) may be due to the lower level of inhibitor in the latter line (50–70% of the cv Laura lines, data not shown). This interpretation is supported by previous data indicating that the rate of development of Bruchid larvae is inversely related to the levels of αAI-1 in the seed (14).

Seeds into which bruchid larvae have entered can be recognized by the presence of a small, dark larval entry hole. Seeds in which bruchids have developed to the pupal stage or beyond have a circular window that covers the larval development chamber. Empty chambers indicate that the adults have left the seeds. At 80 DPH the αAI-2 seeds had more windows and empty chambers than the αAI-1 seed but less than the control seed. We dissected αAI-2 seed that contained entry holes but in which no adult had developed by 100 DPH. In some of these infested αAI-2 seeds, partially developed, live larvae were found, and in others, dead, first-instar larvae were observed. The live larvae became fully mature if the seeds were stored at 21°C for a further 10 days. It appears that larval development was delayed by 30–40 days in the αAI-2 seeds compared with the control seeds but that the overall mortality rate was unaffected. If pea crops are harvested at the earliest possible harvest date, then it has been shown that losses from pea weevil are usually below 4%, which is the loss equivalent to the ''break-even'' cost for spraying (24). Nevertheless, spraying currently is recommended, because unforeseen events may prevent early harvest and allow economic losses to exceed the cost of spraying. Although the αAI-2 gene could be a used to extend the time before the weevil damage reaches the break-even cost of spraying, and thus remove the need for chemical sprays in the crop, fumigation during storage of the harvested peas still would be required.

Relationship Between Inhibitor Effectiveness in the Field Trial and Its Inhibition of Larval α-Amylase. It generally is assumed that amylase inhibitors are effective inhibitors of larval development because they inhibit the larval digestive amylases. Larvae feeding on αAI-1 seeds die at a very early stage, probably because they are unable to hydrolyze the starch in these peas. We observed greater than 80% inhibition of larval amylase by an extract of αAI-1 seeds over a broad pH range (Fig. 5*B*). To be able to predict whether a particular inhibitor will be effective it would be useful to know the pH of the larval gut and to assay the effect of the inhibitor at that pH. A survey of the literature did not reveal any data on the pH of *B. pisorum* gut contents, but recent work (29) shows that the pH of the midgut of the related weevil, *B. affinis,* is in the pH range of 5.5 to 6.5 and that the amylase of this species has a pH optimum of 5.5.

αAI-2, the less effective inhibitor in the field trial, weakly inhibited the pea bruchid amylase in the pH range of 5.5 to 6.5, whereas it inhibited amylase activity substantially (40%) at pH 4.0 and 4.5 (Fig. 5*B*). We postulate that its effectiveness in slowing down larval development is caused by this partial inhibition of amylase activity at midgut pH values. It is possible that, like other insects (30–33), the pea weevil possesses multiple α-amylase enzymes and that not all are inhibited by the αAI-2 protein. We interpret these data to mean that an inhibitor that is only partially effective *in vitro* may nevertheless be useful for genetic engineering either singly or, preferably, in combination with other transgenes that are also partially effective. These results show that it is important to assay the effect of an inhibitor over a pH range. One study (7) showed that αAI-2, when present in artificial seeds, increased both the development time and mortality of Azuki bean weevil (*C. chinensis*), although the inhibitor had no effect on the α-amylase obtained from the last larval instar of this insect species, when inhibitory activity was determined at pH 6.7. In light of the results presented here, it would be interesting to determine the pH dependence of αAI-2 activity against *C. chinensis* α-amylase.

Prospects for a Weevil-Resistant Transgenic Pea. The primary reason for producing insect-resistant transgenic crops is to reduce the use of chemical pesticides and, thereby, the cost to the farmer and the consumer and to reduce the insecticide load on the environment. The control of pea weevil in the field requires at least two pesticide applications: one or two field sprays when the plants are in the flowering stage to prevent infestation and a fumigation of the harvested seeds to kill any live insects (Australian export standards have a zero tolerance for live insects). The presence of αAI-1 confers protection against pea bruchid damage and would eliminate the need for both the field and the postharvest chemical pesticide applications. However, the use of a transgene that is so effective may result in a selection pressure that causes the rapid emergence of bruchid strains that are not affected by the inhibitor. In this respect, an inhibitor that simply reduces the bruchid population below the economic injury level may be more desirable. Another strategy to slow the rate of resistance development is to introduce two insecticidal proteins that act at different sites in the insect. In this regard, αAI-2 may not be a useful gene to combine with αAI-1 because it also appears to function as an α-amylase inhibitor. A third strategy, which we are investigating currently, is the use of mixed populations of αAI-1 and wild-type peas to reduce the selection pressure but still afford good crop protection.

In recent rat feeding experiments with transgenic peas containing αAI-1 we have shown that there is no detrimental effect on weight gain, carbohydrate or nitrogen metabolism, or the growth of internal organs when these peas were fed at 30% of the diet (34). The consumption of αAI-1 protein (marketed as a weight-loss aid in the United States in the 1980s) by humans (35, 36) or rats (37) also has been shown to have no effect on their carbohydrate metabolism. Several factors probably contribute to the lack of inhibition in mammals: the inhibitor may be inactivated by gastric juices (35), the pH optimum for inhibition is lower (pH 4.5–5.0) than the pH that prevails in the duodenum (pH 6–7) (38), and α-amylase is produced in vast excess in the human gut (39). We therefore are proceeding with the further development of pea weevil-resistant peas containing the bean αAI-1 protein. The final release of such a line as a commercial cultivar must await further nutritional tests. Widespread use of such a cultivar would be expected to result in the reduced usage of insecticidal sprays and fumigants.

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