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ORIGINAL PAPER

Assessment of the importance of α-amylase inhibitor-2 in bruchid resistance of wild common bean

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Abstract Both α-amylase inhibitor-2 (αAI-2) and arcelin have been implicated in resistance of wild common bean (*Phaseolus vulgaris* L.) to the Mexican bean weevil (*Zabrotes subfasciatus* Boheman). Near isogenic lines (NILs) for arcelin 1–5 were generated by backcrossing wild common bean accessions with a cultivated variety. Whereas seeds of a wild accession (G12953) containing both αAI-2 and arcelin 4 were completely resistant to *Z. subfasciatus*, those of the corresponding NIL were susceptible to infestation, suggesting that the principal determinant of resistance was lost during backcrossing. Three independent lines of transgenic azuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi] expressing αAI-2 accumulated high levels of this protein in seeds. The expression of αAI-2 in these lines conferred protection against the azuki bean weevil (*Callosobruchus chinensis* L.), likely through inhibition of larval digestive α-amylase. However, although the seed content of αAI-2 in these transgenic lines was similar to that in a wild accession of common bean (G12953), it did not confer a level of resistance to *Z. subfasciatus* similar to that of the wild accession. These results suggest that αAI-2 alone does not provide a high level of resistance to *Z. subfasciatus*. However, αAI-2 is an effective insecticidal protein with a spectrum of activity distinct from that of αAI-1, and it may prove beneficial in genetic engineering of insect resistance in legumes.

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Introduction

Genetic resources in the wild relatives of crop plants have been exploited for modification of important agronomic traits such as insect or disease resistance. Among grain legumes, for example, high levels of resistance to the Mexican bean weevil (*Zabrotes subfasciatus* Boheman) have been detected in wild populations of common bean (*Phaseolus vulgaris* L.) in Mexico (Schoonhoven et al. 1983). Seeds of the common bean are a staple source of dietary protein in many countries, but cultivated common bean is subject to postharvest losses by larvae of bruchids (Coleoptera: Bruchidae).

The resistant phenotype of the wild common bean is associated with and has been attributed, at least in part, to the presence of the protein arcelin (Osborn et al. 1988). Arcelin genes are highly similar to and tightly linked with those encoding the lectins (PHA: phytohemagglutinin) and α-amylase inhibitor (αAI), and they are considered as members of the lectin gene family (Chrispeels and Raikhel 1991; Nodari et al. 1993; Mirkov et al. 1994). Arcelin is a highly abundant storage protein in the seeds of wild common bean accessions

resistant to *Z. subfasciatus*, but it is not present in cultivated common bean (Andreas et al. 1986; Osborn et al. 1988; Kornegay et al. 1993). Electrophoretic banding patterns suggest that arcelin exists in at least seven allelic variants that exhibit a codominant pattern of inheritance (Osborn et al. 1986; Lioi and Bollini 1989; Santino et al. 1991; Acosta-Gallegos et al. 1998). Levels of resistance to *Z. subfasciatus* differ according to the genotype of arcelin variants. Feeding tests with insects have shown that accessions containing arcelin 1 or arcelin 5 are more resistant than are those expressing the other variants (Cardona et al. 1990; Acosta-Gallegos et al. 1998), and these accessions have been used in a breeding program to incorporate resistance into commercial bean cultivars (Kornegay et al. 1993). Recently, however, the presence of arcelin was shown not to account sufficiently for insect resistance, given that lines of transgenic tepary bean (*Phaseolus acutifolius* L. Gray) that express either arcelin 1 or arcelin 5 at a high level compared with that in nontransgenic plants were found not to be resistant to *Z. subfasciatus* (Goossens et al. 2000; Zambre et al. 2005). These observations thus suggested that other factors genetically linked to the arcelin locus might contribute to resistance.

Like phytohemagglutinin, αAI is widely distributed among wild and cultivated common bean accessions (Ishimoto et al. 1995). The common αAI isoform αAI-1 inactivates α-amylase activity of some bruchids and mammals and is highly toxic to the cowpea weevil (*Callosobruchus maculatus* F.) and the azuki bean weevil (*C. chinensis* L.), neither of which is able to develop on common bean (Ishimoto and Kitamura 1989; Shade et al. 1994). Transgenic leguminous plants that accumulate αAI-1 induce complete mortality of *C. maculatus* and *C. chinensis*, but αAI-1 is ineffective against *Z. subfasciatus* (Schroeder et al. 1995; Ishimoto et al. 1996). Wild common bean accessions containing arcelin 3 or arcelin 4 were found to manifest an inhibitory activity specific for larval digestive amylase of *Z. subfasciatus* (Minney et al. 1990). However, the observations that resistance was lost in breeding lines exhibiting high levels of *Z. subfasciatus* α-amylase inhibition and that αAI was not detected in the most resistant wild accessions containing other arcelin variants (Suzuki et al. 1995; Fory et al. 1996) suggested that resistance to *Z. subfasciatus* is largely independent of both αAI-1 and the second αAI isoform αAI-2. Nevertheless, αAI-2 remains a potential candidate for enhancing insect resistance in crops because of its wide spectrum of inhibitory activity specific for insect α-amylases (Suzuki et al. 1993; Yamada et al. 2001a).

We have now developed near isogenic lines (NILs) for the arcelin-αAI locus in common bean as well as transgenic lines of azuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi] expressing αAI-2 in order to assess the contribution of αAI-2 to *Z. subfasciatus* resistance.

Materials and methods

Plants and insects

The common bean cultivar and breeding lines used in the present study are listed in Table 1. The wild common bean accessions G12882, G12866, G12922, G12953, and G02771, each of which contains a different arcelin variant, were obtained from the Centro International de Agricultura Tropical (CIAT) in Cali, Columbia. The six NILs TAr1, TAr2, TAr3, TAr4, TAr5, and TO were established by backcrossing each of the wild accessions or an αAI-deficient cultivar (cv. Ofuku-5) (Ishimoto and Kitamura 1991) six times with the Japanese commercial variety Taisho-kintoki (TK).

A laboratory colony of *C. chinensis* was obtained from the National Institute of Agro-Environmental Sciences (Tsukuba, Japan) and was maintained on

Table 1 Effects of arcelin and αAI genotypes on the resistance of common bean seeds to *Z. subfasciatus*

Variety or line	Genotype		Seed weight (mg)	AE[1] (%)	NA[2]
	Arcelin	αAI			
Cultivar					
TK	N[3]	αAI-1	742[a]	44.4[ab]	60.5[a]
NILs					
TO	N	N	451[b]	41.9[ab]	68.0[a]
TAr1	Arc1	N	411[bc]	4.4[c]	4.0[de]
TAr2	Arc2	N	337[cd]	20.0[bc]	30.2[bc]
TAr3	Arc3	αAI-2	404[bc]	45.3[a]	51.0[ab]
TAr4	Arc4	αAI-2	460[b]	50.5[a]	52.1[ab]
TAr5	Arc5	N	286[d]	49.1[a]	28.4[c]
Wild accessions					
G12882	Arc1	N	50[ef]	6.5[c]	5.2[de]
G12866	Arc2	N	50[ef]	10.2[c]	15.8[cde]
G12922	Arc3	αAI-2	119[e]	44.3[ab]	24.8[cd]
G12953	Arc4	αAI-2	84[ef]	0[c]	0[e]
G02771	Arc5	N	40[f]	0[c]	0[e]

Superscript letters indicate statistically significant ($P < 0.05$) differences among the mean values within each column (Tukey–Kramer HSD test)

[1] Adult emergence: percentage of insects developed from hatched eggs within 50 days after first oviposition

[2] Number of adults: number of adults emerging within 50 days after first oviposition per gram of seed weight

[3] Null variant

azuki bean seeds of cv. Beni-dainagon. A colony of *Z. subfasciatus* was obtained from the University of Tsukuba (Tsukuba, Japan) and reared on common bean seeds of TK.

Construction of a transformation vector and its introduction into *Agrobacterium*

A *Hin*dIII site and an *Xba*I site were introduced upstream and downstream, respectively, of the promoter region of *cgy*α′, which encodes the α′ subunit of soybean β-conglycinin (Nishizawa et al. 2003), with the use of the polymerase chain reaction (PCR) and the primers 7SF-HindIII (5′-CGCAAGCTTCAAA TTTGAATTTTAATGTG-3′) and 7SR-XbaI (5′-CGC TCTAGAGGTTCTTGATGATGAAAACT-3′). The amplified DNA fragment was inserted between the *Hin*dIII and *Xba*I sites of pUC19 (Takara Bio, Otsu, Japan), to generate pPcgyα′. The αAI-2 cDNA in pUC19 (Suzuki et al. 1994) was amplified by PCR with the primers AI2F-BamHI (5′-GATGGATCCATGATCAT GGCTTCCTCCAA-3′) and AI2R-SacI (5′-TCT GAGC TCCTAGAGGATATTGTTGAGGA-3′), digested with *Bam*HI and *Sac*I, and then ligated in pPcgyα′, yielding pPcgyα′ai2. The resulting construct was digested with *Hin*dIII and *Sac*I, and the released fragment was inserted into the corresponding sites of pBI121 (Clontech, Franklin Lakes, NJ) to yield pBcgyα′ai2 (Fig. 2).

The binary vector pBcgyα′ai2 was introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by electroporation (Duke-Ras and Hookyaas 1995). Transformed *A. tumefaciens* cells were maintained on solid LB medium supplemented with rifampicin (10 mg/l) and kanamycin (50 mg/l).

Transformation of azuki bean and establishment of fixed transgenic lines

Agrobacterium-mediated epicotyl transformation of azuki bean (cv. Beni-dainagon) and kanamycin selection were performed as described previously (Yamada et al. 2001b). After at least three leaves had emerged from regenerated shoots, each shoot was excised and transferred to rooting medium. Rooted shoots were subsequently excised, subjected to repeated selection on the same medium, and screened for the presence of transgenes by PCR analysis. After substantial root growth, plants were directly transferred to soil in a greenhouse. T_2 seeds were harvested from individual T_1 plants derived from self-pollinated T_0 plants (original transgenic plants). All plants were grown under natural light conditions and at a controlled temperature of 25°C in a greenhouse. The presence or absence of αAI-2 in seeds of the T_1 or T_2 generation was determined by immunoblot analysis. T_2 seeds from homozygous (fixed) T_1 plants were analyzed for α-amylase inhibitory activity and bruchid resistance.

PCR and Southern blot analysis of transgene incorporation

Total DNA for PCR analysis of transgene incorporation was extracted from putative transgenic plants with the use of a Kurabo PI-50α instrument (Kurabo, Osaka, Japan) and the plant DNA version 2 method. In brief, the reaction mixture (20 μl) contained 10 ng of genomic DNA, 0.2 μM of each deoxynucleoside triphosphate, 0.2 μM of each primer (7SF-HindIII and 7SR-XbaI, or nptIIF and nptIIR), and 2.5 U of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) in polymerase buffer. The amplification protocol comprised an initial incubation for 9 min at 94°C; 30 cycles of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C; and a final extension for 6 min at 72°C. The sequences of the primers nptIIF and nptIIR are 5′-AT ACCGTAAAGCACGAGG-3′ and 5′-ATCTCACC TTGCTCCTGC-3′, respectively.

The pattern of transgene incorporation was evaluated by Southern blot analysis with total DNA that was isolated from young leaves (2 g) of transgenic plants in the T_1 generation as described (Murray and Thompson 1980). The DNA (5 μg) was digested with *Hin*dIII, and the resulting fragments were separated by electrophoresis through a 1% agarose gel and then transferred to a Hybond N+ membrane (Amersham Biosciences, Little Chalfont, UK). The probe was amplified by PCR from pPcgyα′ai2 with the primers AI2F-BamHI and AI2R-SacI. Labeling of the probe and detection of hybridization were performed with an ECL kit (Amersham Biosciences).

Preparation of seed extracts

The distal portion of individual mature seeds or seed flour (50 mg of seeds per milliliter of buffer) was ground in a mortar with 20 mM sodium phosphate buffer (pH 6.7) and subjected to extraction for 60 min at room temperature. The mixture was centrifuged at 15,000 *g* for 10 min at 4°C, and the protein concentration of the resulting supernatant was determined with a BCA protein assay kit (Pierce, Rockford, IL) and adjusted by dilution to a value appropriate for detection of seed proteins or assay of α-amylase inhibitory activity.

Detection of arcelin and αAI polypeptides

Seed extracts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a 13.5% gel as described (Ishimoto et al. 1996), and the gel was stained with Bio-Safe CBB G-250 (Bio-Rad, Hercules, CA). Alternatively, the proteins separated by SDS-PAGE were transferred electrophoretically to a polyvinylidene difluoride membrane (Amersham Biosciences) and subjected to immunoblot analysis with rabbit polyclonal antibodies to αAI-2 (Suzuki et al. 1994). Immune complexes were detected with horseradish peroxidase–conjugated goat antibodies to rabbit immunoglobulin G (Cappel, West Chester, PA) and 4-chloro-1-naphthol (Bio-Rad). The abundance of αAI in fixed transgenic azuki bean lines was estimated by densitometric analysis in comparison with αAI-1 and αAI-2 standards that were purified from TK and TAr4, respectively, as described Suzuki et al. (1993), and then mixed with seed extracts of nontransgenic azuki bean.

Assay of α-amylase inhibitory activity

Midguts dissected from the last instar larvae of *C. chinensis* or *Z. subfasciatus* were homogenized in a solution (50 μl per midgut) containing 20 mM sodium phosphate buffer (pH 5.8), 20 mM NaCl, and 0.1 mM $CaCl_2$, and the homogenate was centrifuged at 10,000 *g* for 20 min at 4°C. The resulting supernatant was passed through a 0.2-μm cellulose acetate filter (Advantec, Tokyo, Japan) to remove small-suspended particles and microorganisms, and the filtrate was used as the preparation of insect α-amylase. Porcine pancreatic α-amylase (type I-A) was obtained from Sigma-Aldrich (St Louis, MO, USA). The α-amylase preparations were diluted with appropriate sample buffer (0.1 M sodium acetate buffer for the pH range of 4–6, or 0.1 M sodium phosphate buffer for the pH range of 6–7.5) to an activity of 0.2 μmol of maltose liberated per minute per 100 μl. α-Amylase activity was measured by a modification of the method of (Bernfeld 1955) as described previously (Ishimoto and Kitamura 1989). The α-amylase preparations (100 μl) were incubated with 150 μl of seed extract or purified proteins for 15 min at 30°C before the addition of 250 μl of substrate solution (1% potato starch in 0.1 M of the appropriate sample buffer containing 20 mM NaCl and 0.1 mM $CaCl_2$) and incubation for an additional 5 min. The reaction was then terminated by the addition of 500 μl of 3,5-dinitrosalicylic acid reagent and boiling for 10 min in a water bath. After the addition of 5 ml of water, the solution was mixed and maintained at room temperature for 15 min. The absorbance of the solution was then measured at 546 nm, and α-amylase activity was expressed as micromoles of maltose liberated per minute. α-Amylase inhibitory activity was expressed as a percentage relative to the α-amylase activity detected in the absence of α-amylase preparation.

Infestation test

At least ten seeds were bioassayed for bruchid resistance by an infestation test with *C. chinensis* or *Z. subfasciatus*. Each seed was exposed to two or three pairs of freshly emerged adult insects. Seven days after initial oviposition, the number of eggs hatched on the seed surface was counted. Petri dishes containing the seeds were placed in a climate chamber maintained at 30°C and 70% relative humidity, and emergence of adult insects was monitored. After 50 days, seeds were dissected under a binocular microscope to evaluate larval development. Insect development on transgenic azuki bean seeds was compared with that on seeds of either common bean or non-transgenic azuki bean.

Statistical analysis

Statistical significance of differences was evaluated by analysis of variance followed by the Tukey-Kramer honestly significant difference (HSD) test for pairwise comparisons with the use of JMP 5.1 software (SAS Institute, Cary, NC). A P value of <0.05 was considered statistically significant.

Results

Bruchid resistance in NILs of common bean

Backcrossing of wild common bean accessions (each expressing a different arcelin variant) six times with cv. TK to generate NILs resulted in a marked increase in seed weight compared with that of the wild parent but a decrease in seed weight compared with that of the recurrent parent (Table 1). In contrast, electrophoretic banding patterns indicated that the protein composition of seeds of each NIL was highly similar to that of the corresponding wild parent (Fig. 1a). The major storage protein phaseolin (~50 kDa) was predominant in seeds of TK and TO but was reduced in abundance in the NILs and wild accessions expressing each arcelin variant. The presence of αAI and arcelin was detected by immunoblot analysis with rabbit polyclonal antibodies to αAI-2 (Fig. 1b), which recognize αAI-1 and arcelin in addition to αAI-2 (Suzuki et al. 1994). The αAI-2 polypeptides (~15 kDa) were detected only in

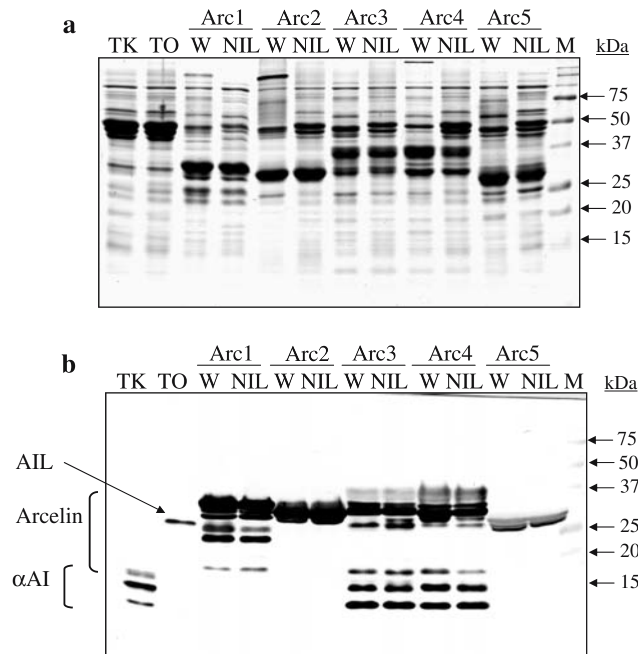

Fig. 1 Seed protein composition of wild accessions, NILs, and a cultivated variety (TK) of common bean. Seed protein extracts (20 μg) were subjected to SDS-PAGE and either staining with Coomassie brilliant blue (**a**) or immunoblot analysis with antibodies to αAI-2 that also recognize αAI-1 and arcelin (**b**). The wild (*W*) accessions included G12882 (*Arc1*), G12866 (*Arc2*), G12922 (*Arc3*), G12953 (*Arc4*), and G02771 (*Arc5*). The *NILs* included TAr1, TAr2, TAr3, TAr4, TAr5, and TO. The positions of αAI, arcelin, and α-amylase inhibitor-like protein (*AIL*) are indicated on the *left* in (**b**). *Lanes M* contain molecular mass standards, which are indicated (in kilodaltons) on the *right* of both panels

G12922, G12953, and the corresponding NILs; the banding patterns in these lines were distinguishable from that attributable to αAI-1 in TK. The seeds of TO contained α-amylase inhibitor–like protein (AIL) but not αAI or arcelin (Ishimoto et al. 1999). The content of arcelin or αAI-2 did not differ substantially between each NIL and its wild parent.

To evaluate the importance of the arcelin-αAI locus in bruchid resistance, we exposed seeds of the NILs and their parents to *Z. subfasciatus*. With regard to the percentage or number of adult insects emerging, the wild accessions expressing arcelins 2, 4, or 5 showed higher levels of resistance to *Z. subfasciatus* than did the corresponding NILs (Table 1). The highest levels of resistance were apparent in the wild accessions expressing arcelin 4 or arcelin 5, followed by those expressing arcelins 1, 2, or 3. Indeed, no adults emerged from the seeds of G12953 (arcelin 4) or G02711 (arcelin 5) within 50 days after first oviposition. Comparison of the NILs revealed that those expressing arcelin 3 (TAr3) or arcelin 4 (TAr4) were as susceptible to *Z. subfasciatus* as was the cultivated parent (TK), in spite of the additional presence in these NILs of αAI-2. The resistance of TAr1 did not differ significantly from that of the wild parent (G12882), whereas TAr2 and TAr5 showed low levels of resistance.

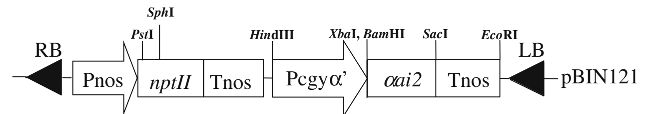

Fig. 2 Structure of the T-DNA region of the transformation vector pBcgyα′ai2. *RB* right border, *LB* left border. Pnos, promoter of the nopaline synthase gene, *nptII*, neomycin phosphotransferase II gene. Tnos, polyadenylation site of the nopaline synthase gene. Pcgyα′, 5′ upstream region (nucleotides −958 to −46, relative to the transcription start site) of the gene for the α′ subunit of soybean β-conglycinin; *αai2*, α-amylase inhibitor-2 cDNA

Expression of αAI-2 in transgenic azuki bean

To investigate directly the possible role of αAI-2 in *Z. subfasciatus* resistance, we introduced the αAI-2 gene (*αai2*) into azuki bean, which is susceptible to *Z. subfasciatus* infestation. Azuki bean explants were cultivated with an *A. tumefaciens* strain (EHA105) transformed with the vector pBcgyα′ai2 (Fig. 2), in which *αai2* is controlled by the promoter region of the gene (*cgyα′*) for the α′ subunit of soybean β-conglycinin and would therefore be expected to be expressed specifically in seed embryos. Three independent transgenic lines were obtained that manifested stable transgene integration and different integration patterns on Southern blot analysis, yielding one or two bands with a probe corresponding to the *αai2* cDNA (Fig. 3). Immunoblot analysis revealed that the seeds of these transgenic azuki bean lines accumulated αAI-2 polypeptides in the same size range as those detected in the common bean NIL TAr4 (Fig. 4), indicating that post-translational cleavage of the αAI-2 precursor occurred in the azuki bean seeds. In addition, given that αAI-2 is a glycoprotein with *N*-glycosylation sites (Nakaguchi et al. 1997), differences in the extent of glycosylation might underlie the generation of more than three bands for αAI-2 in azuki bean.

pH dependence of inhibition of α-amylase by αAI

Given that the azuki bean weevil, *C. chinensis*, is a common insect pest during storage of azuki bean seeds, we examined the effect of αAI expression in seeds of transgenic azuki bean on the α-amylase activity of *C. chinensis* in addition to that of *Z. subfasciatus*. Crude extracts of the midgut were thus prepared from last

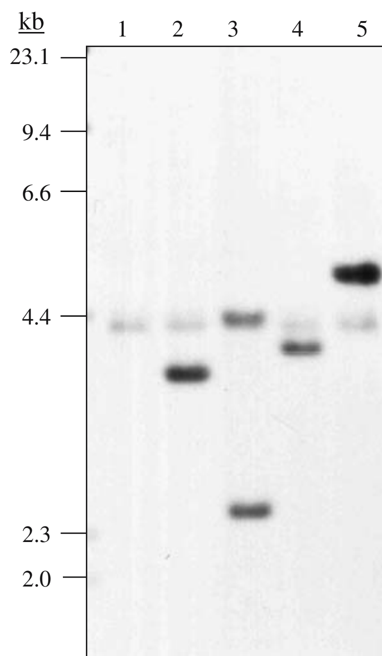

Fig. 3 Detection of the *αai2* transgene in three fixed lines of transgenic azuki bean. Total DNA (5 μg) was digested with *Hin*dIII, and the resulting fragments were fractionated by agarose gel electrophoresis and subjected to Southern blot analysis with a probe corresponding to the αAI-2 cDNA. *Lane 1* Non-transgenic azuki bean (control). *Lanes 2–4* Transgenic azuki bean lines (AI2-1, AI2-2, and AI2-3, respectively) harboring *αai2*. *Lane 5* A transgenic azuki bean line (AR9) harboring *αai1*. The positions of molecular size standards are indicated (in kilobases)

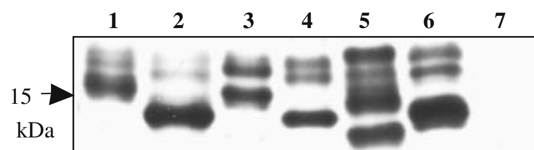

Fig. 4 Detection of αAI-2 in seeds of transgenic azuki bean. Seed protein extracts (20 μg) of common bean cv. TK, common bean NIL TAr4, transgenic azuki bean (AR9) harboring *αai1*, transgenic azuki bean (AI2-1) harboring *αai2*, and non-transgenic azuki bean (*lanes 3–7*, respectively) were subjected to immunoblot analysis with antibodies to αAI-2. *Lanes 1*, *2* contain 5 μg of purified αAI-1 or αAI-2 respectively

instar larvae of both insect species. The maximal amylolytic activities of the larval preparations were apparent at around pH 5.5 for both species, whereas that of porcine pancreatic α-amylase (PPA) was observed at pH 6.5–7.0 (Fig. 5a). At these optimal pH values, the activity of larval preparations was 235.3 ± 30.6 and 112.3 ± 10.6 μmol of maltose liberated per minute per larva (means ± SE, $n = 5$) for *Z. subfasciatus* and *C. chinensis*, respectively.

Seed extracts of the common bean NIL TAr4 and of transgenic azuki bean line AI2-1 as well as purified αAI-2 inhibited the α-amylase activity of *Z. subfasciatus* over a broad pH range (Fig. 5b) but had no effect on that of PPA (data not shown), suggesting that the inhibitory activity of the transgenic azuki bean expressing *αai2* had the same specificity as did that of the common bean (Suzuki et al. 1993). In addition, αAI-2 inhibited the α-amylase activities of *C. chinensis* at around the optimal pH values. Seed extracts of TK and of a transgenic azuki bean line (AR9) harboring *αai1* (Ishimoto et al. 1996) as well as purified αAI-1 inhibited the α-amylase activity of *C. chinensis* and PPA over a wide range of pH (data not shown).

Analysis of bruchid resistance in transgenic azuki bean lines

Densitometric analysis of immunoblots revealed that the amounts of αAI-2 in seeds of the three independent *αai2* transgenic azuki bean lines were similar to that in seeds of the *Z. subfasciatus*–resistant common bean accession G12953 (Table 2), indicating that the promoter region of *cgyα′* conferred a high level of expression of *αai2* in the azuki bean seeds. The abundance of αAI-2 in the seeds of TAr4 tended to be lower than that in those of the wild parent, but this difference was not statistically significant.

In the infestation tests of the transgenic azuki bean seeds with *Z. subfasciatus* and *C. chinensis*, we included a resistant common bean accession (G12953), a susceptible common bean cultivar (TK), and a common bean NIL for arcelin 4 (TAr4) as well as a non-transgenic azuki bean cultivar (Beni-dainagon) as controls. Common bean is resistant to *C. chinensis* in a manner independent of the absence or presence of αAI (Ishimoto et al. 1999), and all the common bean types analyzed here showed complete resistance to this pest (Table 2). The three transgenic azuki bean lines producing αAI-2 as well as AR9 producing αAI-1 (Ishimoto et al. 1996) showed high levels of resistance to *C. chinensis*. Furthermore, both the number and percentage of emergent adults of *Z. subfasciatus* were lower for the transgenic azuki bean lines producing αAI-2 than for the azuki bean cultivar Beni-dainagon or for AR9. However, the similar seed content of αAI-2 in the *αai2* transgenic lines compared with G12953 did not confer as great a level of resistance to *Z. subfasciatus* as observed in G12953.

Discussion

Since the discovery of high levels of resistance to *Z. subfasciatus* in wild common bean accessions collected

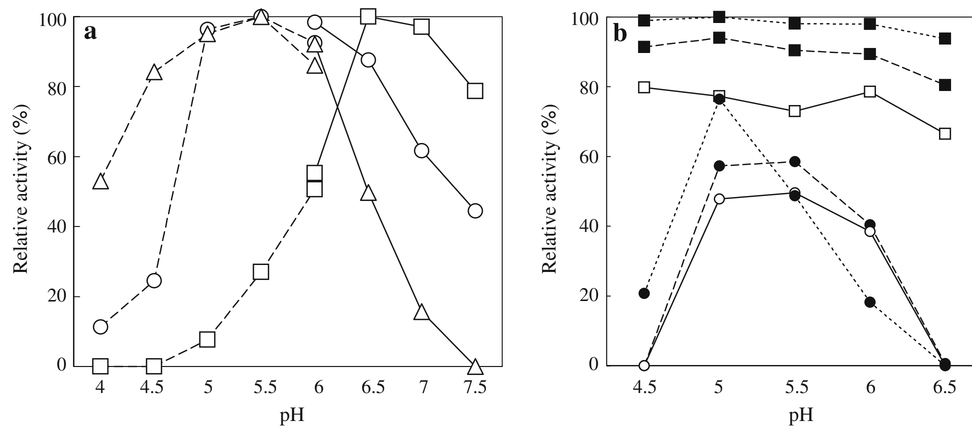

Fig. 5 Effects of pH on amylolytic activity of insect digestive enzyme preparations and on the α-amylase inhibitory activity of seed protein extracts of transgenic azuki bean harboring *αai1* or *αai2*. **a** Activity of α-amylase preparations from *Z. subfasciatus* (*empty triangle*), *C. chinensis* (*empty circle*), or porcine pancreas (*empty square*). The preparations were adjusted to yield an activity of 0.2 μmol of maltose per minute per 100 μl at the optimal pH, and the α-amylase activity at each pH was then expressed as a percentage of this maximal value. Broken and solid lines represent activities determined in sodium acetate and sodium phosphate buffers, respectively. Data are means of triplicates from a representative experiment. **b** Inhibition of α-amylase preparations from larvae of *Z. subfasciatus* [(*empty square*) or(*filled square*)] or *C. chinensis* [(*empty circle*) or (*filled circle*)] by seed extracts or purified αAI-2. The effects of 60 μg of the purified inhibitor protein (*dot lines*) or of seed extracts (750 μg of protein) of TAr4 (*broken lines*) or *αai2* transgenic azuki bean line AI2-1 (*solid lines*) on the α-amylase activities of insect larvae were determined at the indicated pH values. Data are expressed as percentage inhibition and are means of triplicates from representative experiments

Table 2 Bruchid resistance of transgenic azuki bean lines expressing αAI-2

Variety or line	Transgene	αAI type	αAI-2 content[1] (%)	Seed weight (mg)	Insect resistance			
					C. chinensis		*Z. subfasciatus*	
					AE[2]	NA[3]	AE[2]	NA[3]
Transgenic azuki bean								
AR9	*αai1*	αAI-1	0^{b}	132^{c}	0^{b}	0^{b}	42.9^{b}	58.5^{a}
AI2-1	*αai2*	αAI-2	1.33^{a}	114^{c}	5.0^{b}	3.8^{b}	10.3^{c}	27.2^{b}
AI2-2	*αai2*	αAI-2	1.35^{a}	115^{c}	0^{b}	0^{b}	10.3^{c}	25.6^{b}
AI2-3	*αai2*	αAI-2	1.02^{a}	105^{cd}	0^{b}	0^{b}	5.2^{c}	27.6^{b}
Nontransgenic azuki bean								
Beni-dainagon			0^{b}	124^{c}	59.7^{a}	44.6^{a}	37.8^{b}	64.0^{a}
Common bean								
TK		αAI-1	0^{b}	735^{a}	0^{b}	0^{b}	72.9^{a}	21.8^{b}
TAr4		αAI-2	0.85^{a}	483^{b}	0^{b}	0^{b}	62.0^{a}	30.9^{b}
G12953		αAI-2	1.01^{a}	61^{d}	0^{b}	0^{b}	0^{c}	0^{c}

Data are means with *n* values of six seeds for αAI-2 content and at least ten seeds for seed weight and the infestation tests. Superscript letters indicate statistically significant ($P < 0.05$) differences among the mean values within each column (Tukey–Kramer HSD test)

[1] Amount of αAI-2 as a percentage of seed weight

[2] Adult emergence: percentage of insects developed from hatched eggs within 50 days after first oviposition

[3] Number of adults: number of adults emerging within 50 days after first oviposition per gram of seed weight

in Mexico (Schoonhoven et al. 1983), the factors responsible for this resistance have been the subject of many investigations. The resistant accessions are characterized by the presence of the seed protein arcelin, which is not found in cultivated common bean (Andreas et al. 1986; Osborn et al. 1988; Kornegay et al. 1993; Acosta-Gallegos et al. 1998). Furthermore, the presence of either arcelin 1 or arcelin 5 was shown to cosegregate with resistance in the wild accessions (Kornegay et al. 1993). We have now developed five common bean NILs for arcelins 1–5. Among these lines, only TAr1 exhibited a high level of resistance to *Z. subfasciatus* similar to that of its wild parent. This finding is consistent with the previous observations that some breeding lines of common bean that accumulate arcelin 5 to a high level are not resistant to *Z. subfasciatus* and that the resistance levels of transgenic lines of tepary bean that express arcelin 5 are lower than are

those of the resistant common bean accessions (Goossens et al. 2000; Zambre et al. 2005). In addition, introduction of the arcelin 1 gene into tepary bean revealed that a high level of expression of arcelin 1 did not confer a high level of resistance to *Z. subfasciatus*, although adult insect emergence was reduced in most of the transgenic lines compared with that in non-transgenic lines (Zambre et al. 2005). These results suggest that arcelin 1 and arcelin 5 are not principal determinants of resistance to *Z. subfasciatus* and that a factor (or factors) encoded by a gene linked to the arcelin locus might be important in such resistance. TAr1 could be one of the adequate materials to elucidate the factor responsible for the resistance.

The NIL TAr4, selected for the presence of arcelin 4, was not resistant to *Z. subfasciatus*. Thus, in spite of the presence of arcelin and αAI-2, TAr4 as well as TAr3 was as susceptible to this pest as was cultivated common bean. These results are consistent with those of Cardona et al. (1990) showing a lack of resistance in 25 lines obtained from crosses with resistant accessions expressing arcelin 4. The amounts of arcelin 4 and αAI-2 in TAr4 appeared similar to those in the resistant wild parent by Coomassie blue staining and immunoblot analysis. Cardona et al. (1990) showed that the arcelin/phaseolin ratio correlated with the level of resistance and that this ratio was lower in arcelin 4–containing progeny than in the resistant donor. The ratio in TAr4 may thus be insufficient to confer resistance, or quantitative trait loci independent of the arcelin-αAI locus may largely be responsible for resistance (Kornegay et al. 1993).

To assess directly the contribution of αAI-2 to resistance, we introduced the αAI-2 gene into azuki bean, which is susceptible to *Z. subfasciatus* infestation. Proteolytic cleavage of the αAI precursor gives rise to the two subunits that form the active inhibitor (Pueyo et al. 1993; Young et al. 1999). The *αai2* transgene in azuki bean gave rise to several polypeptides of ~15 kDa that were similar to those detected with the same antibodies to αAI-2 in TAr4 and its wild parent. In addition, the specificity and pH stability of the α-amylase inhibitory activity in seed extracts of *αai2* transgenic azuki bean were similar to those of αAI-2 in the common bean. These results indicated that the posttranslational processing of αAI-2, like that of αAI-1 and the tepary bean inhibitor αAI-Pa2 (Ishimoto et al. 1996; Yamada et al. 2005), occurs normally in transgenic azuki bean seeds. However, whereas the seed content of αAI-2 in the transgenic azuki bean lines was similar to that in a resistant common bean accession, the resistance of the transgenic lines to *Z. subfasciatus*, although greater than that of nontransgenic azuki bean, was significantly lower than that of the resistant common bean accession. In contrast, the content of αAI-2 in the transgenic lines was sufficient to confer a high level of resistance to *C. chinensis*. Larvae of *Z. subfasciatus* modulate their production of α-amylase in response to the ingestion of seed constituents such as αAI (Silva et al. 1999, 2001). It is therefore possible that the induction of digestive α-amylase in young larvae of *Z. subfasciatus* overcomes the deleterious effect of αAI-2.

Our data thus suggest that neither αAI-2 nor arcelin alone confers a high level of resistance to *Z. subfasciatus*. However, αAI-1, αAI-2, αAI-Pa1, and αAI-Pa2 are all bean insecticidal proteins with different specificities that have been successfully used to confer insect resistance by genetic transformation (Shade et al. 1994; Schroeder et al. 1995; Ishimoto et al. 1996; Morton et al. 2000; Sarmah et al. 2004; Yamada et al. 2005). Up-regulation of the expression level of αAI genes in the common bean may thus prove sufficient to provide a beneficial level of resistance to *Z. subfasciatus*. Furthermore, azuki bean exhibits a relatively high competence for transformation by *Agrobacterium* and for regeneration under selection with kanamycin or hygromycin B (Ishimoto et al. 1996; Yamada et al. 2001b, 2005; Chen et al. 2005; Hanafy et al. 2006). These properties may facilitate functional characterization of genes of interest in grain legumes.

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