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# Inheritance of seed α-amylase inhibitor in the common bean and genetic relationship to arcelin

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**Abstract** The inheritance of seed α-amylase inhibitor in the common bean and the genetic relationships among the variants and six arcelin variants in the common bean were investigated by crossing between accessions containing different αAI and arcelin variants. All seed proteins in parental, $F_1$ and $F_2$ seeds from the crosses were examined by Western-blot analysis. All $F_1$ seeds gave combined αAI banding patterns from parents on the blotting membranes. The segregation of $F_2$ seeds for αAI variants indicated that the polypeptides of αAI variants were inherited as single co-dominant units. Moreover, αAI and arcelin behaved as a single block in crosses, indicating a close linkage relationship between the genes controlling these proteins.

**Key words** *Phaseolus vulgaris* · α-Amylase inhibitor · Inheritance · Arcelin · Linkage

## Introduction

The seed α-amylase inhibitor (αAI), a glycoprotein inhibiting the activity of mammal and insect α-amylases, is widely distributed in the common bean (*Phaseolus vulgaris* L.) and is considered to be responsible for the defense mechanism against insect pests. Eight variants of αAI have been studied by examining α-amylase inhibitory activity against porcine pancreatic α-amylase and larval α-amylase of the Mexican bean weevil (*Zabrotes subfasciatus*) as well as electrophoretic banding patterns (Ishimoto et al. 1995). αAI-1 (1a, 1b, 1c, and 1d), αAI-2 and αAI-3 inhibited the activity of α-amylase of porcine pancreas, that of *Z. subfasciatus*, and the activity of both enzymes, respectively. αAI-0 (0a and 0b) showed no inhibitory activity. The variants listed in alphabetical order in αAI-1 and αAI-0 were identified by the banding patterns of glycopolypeptides. Variants αAI-1 and αAI-2 have been shown to provide protection from bruchid pests. (Ishimoto and Kitamura 1988, Suzuki et al. 1993). Arcelin (arc), a major seed protein in non-cultivated accessions, is also essential to plant defense against *Z. subfasciatus* (Cardona et al. 1990). Five variants of arcelin, arc-1, -2, -3, -4 and arc-5, have been identified and found to be controlled by co-dominant alleles (Osborn et al. 1986; Kornegay et al. 1993). Genetic analysis indicates that the gene for arc-4 is tightly linked to that for αAI-2 (Ishimoto and Kitamura 1993). Genetic studies on αAI and the arcelin variants, should thus provide important data for devising means of enhancing bruchid resistance in legume crops.

In this study, an examination was made of arcelin variants in the cultivated and non-cultivated common bean accessions previously studied in αAI variants. The inheritance of αAI variants, linkage relationships among the genes controlling αAI and the arcelin variants, and their roles in plant defense are discussed.

## Materials and methods

### Plant materials

Seeds of cultivated and non-cultivated accessions studied in αAI variants (Ishimoto et al. 1995) were used to screen arcelin variants.

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Some accessions harboring αAI and arcelin variants were crossed with one of two cultivars, Taishou-kintoki (αAI-1d) or Ofuku-5 (αAI-0b). The two cultivars served as female parents. Individual seeds of the parents, as well as $F_1$ and $F_2$ seeds derived from several crosses, were grown in a greenhouse.

### Seed-protein preparation

The distal portions (5 mg) of individual seeds were extracted with 0.5 ml SDS-sample buffer [50 mM Tris-HCl pH 8.0, 5 M urea, 0.2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue] for 60 min at room temperature. The extracted proteins were examined by Western-blot analysis to identify the variants of αAI and arcelin.

### Electrophoresis and Western-blot analysis

One-dimensional SDS-PAGE was carried out using a 1-mm thick slab gel containing 13.5% acrylamide. Twenty microliters of each seed-protein preparation were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Glycoproteins were stained by reaction with peroxidase-coupled concanavalin A (Con A) (Honen, Japan) according to the method of Kijimoto-Ochiai et al. (1985).

## Results

### Variation in α-amylase inhibitor and arcelin types

All accessions with αAI-2 contained one arcelin variant, arc-3 or arc-4, or else a novel glycoprotein (putative sixth arcelin variant, arc-6). Accessions harboring αAI-1 or αAI-3 contained no arcelin. Those harboring other forms of arcelin always contained αAI-0a or αAI-0b; that is, αAI-0a for arc-5 and αAI-0b for arc-1 or arc-2 (Table 1 and Fig. 1). All arcelin variants were only found among non-cultivated accessions.

### Inheritance of α-amylase inhibitor and arcelin

The mode of inheritance of αAI and arcelin variants was clarified by analyzing the banding patterns of individual $F_1$ and $F_2$ seeds from crosses between accessions on blotting membranes. Estimated genotypes of αAI variants are listed in Table 2. All $F_1$ seeds from crosses with Taishou-kintoki gave a combined banding pattern of

**Table 1** Variation of seed α-amylase inhibitor (αAI) and arcelin (Arc) in common bean accessions

| αAI type | No. of accessions | Arc type[a] |
|---|---|---|
| αAI-1a | 1115 | – |
| αAI-1b | 3 | – |
| αAI-1c | 6 | – |
| αAI-1d | 610 | – |
| αAI-2 | 41 | Arc-3(25), Arc-4(15), Arc-6(1) |
| αAI-3 | 52 | – |
| αAI-0a | 39 | Arc-5(2) |
| αAI-0b | 14 | Arc-1(2), Arc-2(4) |

[a] Parentheses indicate the number of accessions containing arcelin

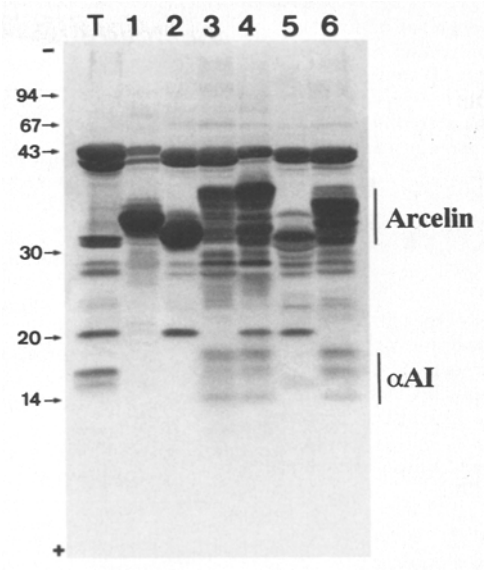



**Fig. 1** Western-blot analysis of seed glycoproteins of one cultivated and six non-cultivated common beans. *Lanes: T* Taishou-kintoki (αAI-1d); *1* G12882 (αAI-0b, arc-1); *2* G12886 (αAI-0b, arc-2); *3* G12922 (αAI-2, arc-3); *4* G12953 (αAI-2, arc-4); *5* G2771A (αAI-0a, arc-5); *6* G20513 (αAI-2, arc-6). *Numbers* in the left margin indicate $Mr \times 10^{-3}$

αAI-1d and the αAI variant of another parent. $F_1$ seeds from crosses with Ofuku-5 (αAI-0b lacking protein bands corresponding to αAI) gave the pattern of the αAI variant of the male parent. All $F_2$ seeds produced from individual $F_1$ plants segregated for the αAI variant (Fig. 2). The segregation ratios for the presence or absence of αAI in $F_2$ seeds from crosses 1, 2, 3, 6 and 8 fit a 3 present: 1 absent ratio. Those in $F_2$ seeds from crosses 4 and 5 fit a 1:2:1 ratio (Table 2). The banding patterns of αAI-1d and αAI-3 overlapped, thus making it quite difficult to distinguish the genotypes of $Ai^3/Ai^3$ and $Ai^3/Ai^{1d}$ in cross 7. The presence or absence of αAI-3 in $F_2$ seeds, however, was consistent with a 3:1 ratio. Each αAI is thus shown to be controlled by a single dominant gene.

The mode of inheritance of genes of arc-1, -2, -3, -4, and arc-5 in this study was monogenic, this being consistent with results reported previously (Andreas et al. 1986; Osborn et al. 1986; Kornegay et al. 1993). A noval glycoprotein, arc-6, which displays major protein bands migrating to regions corresponding to the molecular weights of other arcelin variants, was detected in the non-cultivated accession G20513 (Fig. 1). All $F_1$ seeds from cross 13 between Taishou-kintoki and G20513 contained arc-6. Analysis of $F_2$ seeds in cross 13 (Fig. 3) showed monogenic inheritance of arc-6 ($Arc^6/Arc^6$) ($P > 0.8$) (Table 3).

### Linkage relationships among genes for α-amylase inhibitor and arcelin

In crosses 5, 6, 11, 12 and 13, in which the male parent contains αAI-2 ($Ai^2/Ai^2$) and arc-3 ($Arc^3/Arc^3$) or arc-4

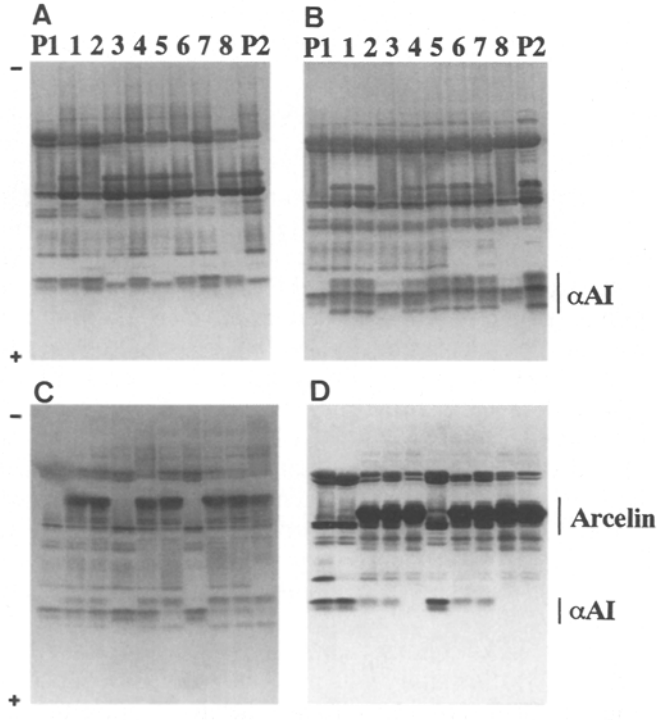
**Table 2** Segregation for the genes controlling α-amylase inhibitor (αAI) variants in $F_2$ seeds from crosses between accessions with different αAI variants and cultivars

| Cross | Parents and their αAI variants | Estimated genotypes and observed segregation of $F_2$ seeds | | | $\chi^2$ | $P$ |
|---|---|---|---|---|---|---|
| 1 | Ofuku-5 × G12851 | *ai/ai*[a] | $Ai^{1b}$/- | | | |
| | αAI-0b αAI-1b | 32 | 87 | | 0.23[b] | > 0.5 |
| 2 | Taishou-kintoki × G2771A | *ai/ai*[a] | $Ai^{1d}$/- | | | |
| | αAI-1d αAI-0a | 34 | 103 | | 0.00[b] | > 0.95 |
| 3 | Ofuku-5 × Taishou-kintoki | *ai/ai*[a] | $Ai^{1d}$/- | | | |
| | αAI-0b αAI-1d | 14 | 61 | | 1.60[b] | > 0.2 |
| 4 | Taishou-kintoki × G12915A | $Ai^{1d}/Ai^{1d}$ | $Ai^{1d}/Ai^{1c}$ | $Ai^{1c}/Ai^{1c}$ | | |
| | αAI-1d αAI-1c | 51 | 113 | 56 | 0.39[c] | > 0.8 |
| 5 | Taishou-kintoki × G12922 | $Ai^{1d}/Ai^{1d}$ | $Ai^{1d}/Ai^{2}$ | $Ai^{2}/Ai^{2}$ | | |
| | αAI-1d αAI-2 | 45 | 78 | 37 | 0.90[c] | > 0.5 |
| 6 | Ofuku-5 × G12922 | *ai/ai*[a] | $Ai^{2}$/- | | | |
| | αAI-0b αAI-2 | 37 | 123 | | 0.30[b] | > 0.5 |
| 7 | Taishou-kintoki × G10018 | $Ai^{1d}/Ai^{1d}$ | $Ai^{3}$/- | | | |
| | αAi-1d αAI-3 | 76 | 201 | | 0.88[b] | > 0.3 |
| 8 | Ofuku-5 × G10018 | *ai/ai*[a] | $Ai^{3}$/- | | | |
| | αAI-0b αAI-3 | 10 | 56 | | 3.41[b] | > 0.05 |

[a] The genotypes for αAI-0a and αAI-0b lacking α-amylase inhibitory activity are expressed by an identical symbol, *ai/ai*

[b] $\chi^2$ tested against an expected 1:3 ratio

[c] $\chi^2$ tested against an expected 1:2:1 ratio



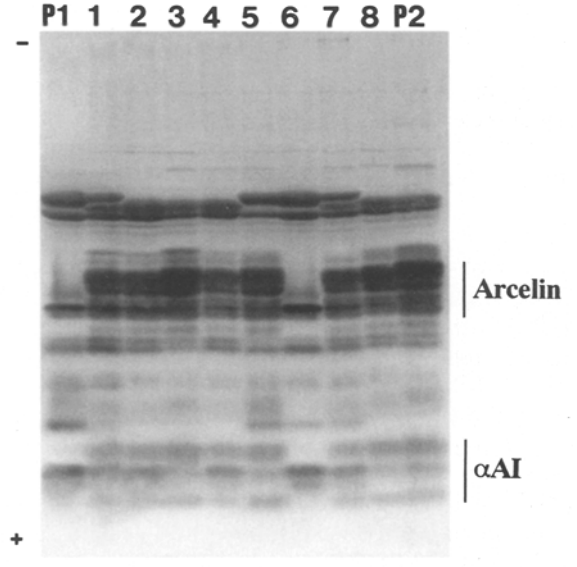




**Fig. 3** Western-blot analysis of seed glycoproteins of parental and $F_2$ seeds. *Lanes: P1* Taishou-Kintoki (αAI-1d); *P2* G20513(αAI-2, arc-6); *1–8* $F_2$ seeds derived from a cross between P1 and P2

**Fig. 2A–D** Western-blot analysis of seed glycoproteins of parental and $F_2$ seeds. **A** Cross 4, Taishou- kintoki(αAI-1d) × G12915A(αAI-1c). **B** Cross 5, Taishou-kintoki × G12922(αAI-2, arc-3). **C** Cross 7, Taishou-kintoki × G10018(αAI-3). **D** Cross 9, Taishou-kintoki × G12882(αAI-0b, arc-1). *Lanes: P1* female parent; *P2* male parent; *1–8* $F_2$ seeds from crosses

($Arc^4/Arc^4$) or arc-6 ($Arc^6/Arc^6$), αAI-2 co-existed with the arcelin variant in $F_2$ seeds (Table 3). Consequently, only $F_2$ seeds with αAI-2 and arcelin or without αAI-2 and arcelin were observed. Chi-square analysis by orthogonal function was made of test pairs of loci for significant deviation from an independent assortment (Mather 1951). Data (69.69-187.78, Table 3) from the analysis indicated the presence of tight linkage between the genes for αAI-2 and arc-3 or arc-4 or arc-6.

$F_2$ seeds from crosses 2, 9, and 10, in which the male parent contains arc-1 ($Arc^1/Arc^1$) or arc-2 ($Arc^2/Arc^2$) or arc-5 ($Arc^5/Arc^5$) but not αAI (αAI-0a or αAI-0b: *ai/ai*), were segregated into a group with a seed composition for αAI and arcelin variants the same as that in the male parents and a group with a composition the same as that in the female parents. Hence, no $F_2$ seeds lacking αAI-1d and arcelin were obtained. The segregation ratios for the presence of αAI-1d and arcelin variants in $F_2$ seeds fit well with a 1 (presence of αAI-1d): 2 (presence of αAI-1d and arcelin): 1 (presence of arcelin) ratio. There would thus appear to be a tight linkage relationship between the loci of αAI and the three arcelin variants.

**Table 3** Segregation for the genes controlling α-amylase inhibitor (αAI) and arcelin variants in $F_2$ seeds from crosses between accessions with different αAI and arcelin variants

| Cross | Parents and their seed-preotein genotypes | | Segregation of $F_2$ seeds | | | $\chi^2$ values of αAI and arcelin linkage[a] |
|---|---|---|---|---|---|---|
| | | | αAI genotype | *Arcelin genotype* *Arc/-* | *arc/arc* | |
| 9 | Taishou-kintoki | × G-12882 | $Ai^{1d}$/- | 67 | 35 | 17.16 |
| | $Ai^{1d}/Ai^{1d}$ | *ai/ai* | *ai/ai* | 36 | 0 | (<0.001) |
| | *arc/arc* | $Arc^1/Arc^1$ | | | | |
| 10 | Taishou-kintoki | × G12866 | $Ai^{1d}$/- | 69 | 43 | 16.46 |
| | $Ai^{1d}/Ai^{1d}$ | *ai/ai* | *ai/ai* | 28 | 0 | (<0.001) |
| | *arc/arc* | $Arc^2/Arc^2$ | | | | |
| 5 | Taishou-kintoki | × G12922 | $Ai^2$/- | 115 | 0 | 187.78 |
| | $Ai^{1d}/Ai^{1d}$ | $Ai^2/Ai^2$ | $Ai^{1d}/Ai^{1d}$ | 0 | 45 | (<0.001) |
| | *arc/arc* | $Arc^3/Arc^3$ | | | | |
| 6 | *Ofuku-5* | × G12922 | $Ai^2$/- | 123 | 0 | 144.4 |
| | *ai/ai* | $Ai^2/Ai^2$ | *ai/ai* | 0 | 37 | (<0.001) |
| | *arc/arc* | $Arc^3/Arc^3$ | | | | |
| 11 | Taishou-kintoki | × G12953 | $Ai^2$/- | 92 | 0 | 109.57 |
| | $Ai^{1d}/Ai^{1d}$ | $Ai^2/Ai^2$ | $Ai^{1d}/Ai^{1d}$ | 0 | 28 | (<0.001) |
| | *arc/arc* | $Arc^4/Arc^4$ | | | | |
| 12 | Ofuku-5 | × G12953 | $Ai^2$/- | 64 | 0 | 131.186 |
| | *ai/ai* | $Ai^2/Ai^2$ | *ai/ai* | 0 | 30 | (<0.001) |
| | *arc/arc* | $Arc^4/Arc^4$ | | | | |
| 2 | Taishou-kintoki | × G2771A | $Ai^{1d}$/- | 73 | 30 | 11.48 |
| | $Ai^{1d}/Ai^{1d}$ | *ai/ai* | *ai/ai* | 34 | 0 | (<0.001) |
| | *arc/arc* | $Arc^5/Arc^5$ | | | | |
| 13 | Taishou-kintoki | × G20513 | $Ai^2$/- | 62 | 0 | 69.69 |
| | $Ai^{1d}/Ai^{1d}$ | $Ai^2/Ai^2$ | $Ai^{1d}/Ai^{1d}$ | 0 | 18 | (<0.001) |
| | *arc/arc* | $Arc^6/Arc^6$ | | | | |

[a] $\chi^2$ values of αAI and arcelin linkage are determined by an orthogonal function (Mather 1951)

## Discussion

Eight variants of the bean seed α-amylase inhibitor (αAI) have been identified based on inhibition of the activity of porcine pancreatic α-amylase and larval α-amylase of *Z. subfasciatus* by seed extracts and the banding patterns of glycopolypeptides in the range of 14 to 20 kDa (Ishimoto et al. 1995). Genes controlling αAI-1d and αAI-2 have been shown to be co-dominant by the segregation of $F_2$ seeds from crosses between an αAI-2-containing accession and cultivars for inhibitory activity against mammal and insect α-amylases (Ishimoto and Kitamura 1993). This study confirmed relationships among these eight αAI variants by analyzing banding patterns in progenies from crosses between accessions with different αAI variants. No $F_2$ seeds from crosses between accessions with different αAI variants and cultivars deviated significantly from the expected 1:2:1 segregation ratio of parental:combination:parental αAI patterns or the expected 3:1 segregation ratio for the presence:absence of αAI. The polypeptides of each αAI variants are thus inherited as single units and are controlled by co-dominant alleles.

In a previous study (Ishimoto et al. 1995) the electrophoretic banding pattern of αAI-3 appeared to be derived from the combined bands of αAI-1 (one of 1a, 1b, 1c, or 1d) and αAI-2. It is shown by the present results that $F_2$ seeds from crosses between an accession containing αAI-3 and cultivars with no αAI-3 are segregated to one group containing αAI-3 and one containing no αAI-3 (Table 2). The segregation of αAI-3 followed the pattern expected for single dominant gene inheritance. Two possible explanations for αAI-3 may be proposed. αAI-3 may consist of a single protein molecule which inhibits the activity of the α-amylases of porcine pancreas and *Z. subfasciatus*. Or αAI-3 may require the presence of αAI-1 and αAI-2 proteins whose genes are tightly linked to each other. The purification and characterization of the αAI-3 protein(s) may show which of these possibilities is valid.

Arcelin variants were previously shown to be controlled by co-dominant alleles inherited as single genes (Andreas et al. 1986; Osborn et al. 1986; Kornegay et al. 1993). In the present study the genes of arc-1, -2, -3, -4 and arc-5 were confirmed to be inherited monogenically and controlled by co-dominant alleles. The sixth arcelin variant (arc-6) was detected in a non-cultivated accession and found to be controlled by a dominant gene in a manner similar to that of other variants.

Analysis of seed extracts of the accessions indicated that there were specific combinations for the presence or absence of αAI and arcelin variants (Table 1). Ishimoto and Kitamura (1993) have already confirmed tight linkage between αAI-2 and arc-4. The results in Table 3 indicate that the gene controlling αAI-2 expression is tightly linked to those controlling arc-3, arc-4 and arc-6, and that the gene controlling the absence of αAI is tightly linked to arc-1, arc-2 and arc-5. The genes of phytohemagglutinin (PHA) and those of arcelin (arc-1, 2, 3, or arc-4) and of αAI-1d are all tightly linked to each other (Osborn et al. 1986; Ishimoto and Kitamura 1991;

Nodari et al. 1993). The genes controlling PHA, αAI, and arcelin and those belonging to the phytohemagglutinin family (Chrispeels and Raikhel 1991) are also tightly linked to each other. Based on the similarities of the amino-acid and nucleotide sequences in this family (Hoffman et al. 1983; Hoffman and Donaldson 1985; Osborn et al. 1988; Hartweck et al. 1991; Suzuki et al. 1994), the present study indicates the genes of this family to possibly have evolved through the duplication or divergence of a common ancestral gene (Osborn et al. 1988; Chrispeels and Raikhel 1991; Ishimoto and Kitamura 1993).

αAI-1 strongly inhibits the growth of the adzuki bean weevil (*Callosobruchus chinensis*) at physiological levels (0.4–0.5%) in seeds of the common bean, but not that of *Z. subfasciatus* even at a 2.0% level (Ishimoto and Kitamura 1989). αAI-2 inhibits the growth of *Z. subfasciatus* as well as that of *C. chinenis* at levels of more than 1% (Suzuki et al. 1993). Arc-1, -2, -4 and arc-5 are associated with protection against *Z. subfasciatus* (Cardona et al. 1990; Kornegay et al. 1993), although accessions containing arc-3 or arc-6 confer a susceptibility (Cardona et al. 1990; Toro et al. 1990). In feeding tests using artificial beans, a high arc-1 (10%) and arc-4 (7.1%) content, corresponding to that of seeds of the non-cultivated bean, strongly inhibit the growth of *Z. subfasciatus*, indicating a major role in providing protection from the bruchid pest (Osborn et al. 1988; Minney et al. 1990).

The sufficient accumulation of αAI and arcelin in leguminous seeds should greatly facilitate the control of bruchid pests. The incorporation of genes controlling αAI and arcelin variants in desirable combinations, such as αAI-2 and arc-1, cannot be readily achieved by conventional breeding methods, since the loci of αAI and arcelin are tightly linked. αAI-1 and αAI-2 inhibit the growth of bruchid pests at lower levels (0.4–1%) than arcelin (7–10%) and thus are useful in breeding programs not attended with major changes in seed composition. Accumulation of αAI at low levels, such as 1%, in seeds should be possible by genetic engineering techniques. Molecular cloning of αAI-1 ad αAI-2 has already been achieved (Hoffman et al. 1982; Suzuki et al. 1994), consequently genes coding for αAI-1 and αAI-2 should be transferable to legume crops by plant transformation methods (Russell et al. 1993; Schroeder et al. 1993).

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