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A PUTATIVE PRECURSOR PROTEIN IN THE EVOLUTION OF THE BEAN α-AMYLASE INHIBITOR

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; common bean; α-amylase inhibitor; arcelin; phytohaemagglutinin.

Abstract—Seeds of the common bean *Phaseolus vulgaris* and the tepary bean (*P. acutifolius*) contain a family of plant defence proteins that includes phytohaemagglutinin (PHA), arcelin and α-amylase inhibitor (αAI). These homologous proteins differ by the absence of short loops at the surface of the protein and by the presence of a proteolytic processing site (Asn^{77}) that allows αAI to be post-translationally cleaved and activated. We now report the derived amino acid sequence of two amylase inhibitor-like (AIL) proteins that are not proteolytically processed, although they have the typical processing site. One protein is from the common bean, and the other from the tepary bean. On a dendrogram, these proteins are grouped with αAIs rather than with the arcelins or lectins. αAI differs from AIL primarily by the deletion of a 15-amino-acid segment from the middle of the AIL sequence. When αAI is expressed in tobacco, it is proteolytically processed to form an active molecule. However, AIL sequences are not processed. We suggest that the AIL proteins may be an intermediate in the evolution of an active αAI. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Beans and other starchy legumes are a major source of dietary protein in many countries, but supplies are diminished by infestation of the crops by bruchid beetles, which damage the seeds when they are ripening on the plant and during storage. The losses are most severe for the resource-poor farmers of developing countries. These losses occur in spite of the presence in the seeds of numerous plant defence proteins. Interspecific transfer by genetic engineering of the genes encoding such proteins can help protect legume seeds against predation by bruchids [1], making the further understanding of these defence proteins of great importance to agriculture.

Beans of the genus *Phaseolus* contain a family of homologous plant defence proteins, encoded at a single locus and referred to as the arcelin-phytohaemagglutinin-α-amylase inhibitor family [2]. The individual proteins have very different defence properties, and their relative abundances vary between species and also between wild accessions of the same species. Phytohaemagglutinin (PHA) is a potent lectin that is toxic to mammals and birds, α-amylase inhibitor (αAI) inhibits the digestive α-amylases in the gut of mammals and larvae of certain bruchid species, and arcelin, which is the major storage protein in certain wild accessions of the common bean, is poorly digestible at least for bruchids.

A number of the genes that encode proteins of this family have been cloned from the common bean (*Phaseolus vulgaris*) and the tepary bean (*P. acutifolius*), and sequence comparisons show that they are all homologous and fall into three groups representing the three proteins [3]. Furthermore, genetic evidence shows that the genes for these three proteins are encoded at the same locus indicating that they may have arisen by duplication of a single ancestral gene [4].

One of the interesting features of αAI is that it must be proteolytically processed to be active [5]. We proposed that processing causes a conformational change that creates the active site necessary for interaction with α-amylase [6]. The amino acid sequence homology (50–90%) among these proteins makes it possible to model their three-dimensional structures based on the three-dimensional structures of legume lectins that have been determined crystallographically [7]. αAI differs from PHA primarily by the deletion of

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three short surface loops from the compact PHA lectin structure, and arcelin differs from PHA by the deletion of two of these loops [8]. However, what makes αAI into an active protein compared to an inactive homologous protein, is processing at Asn^{77} [5]. (The numbering of Asn^{77} refers to the mature protein, not the derived amino acid sequence.) On the other hand, not all proteins that possess this Asn^{77} residue are processed, suggesting that there are other structural requirements for processing [3]. As part of our work on the evolutionary relationships between these proteins, we searched for proteins that are closely related to αAI. We now report the existence of a protein referred to as amylase inhibitor-like (AIL) in the common bean and the tepary bean. We suggest that the protein may be an intermediate in the evolution of αAI, because AIL has the Asn^{77} processing site but it is not processed. AIL has a 15-amino-acid insertion close to the processing site, and this insertion may prevent processing.

RESULTS AND DISCUSSION

cDNAs encoding proteins in the PHA-arcelin-αAI family were obtained by reverse transcriptase–polymerase chain reaction (RT-PCR) using oligonucleotides that match the 5′ and 3′ ends of the coding region of this gene family [3] with RNA isolated from mid-maturation seeds of *P. acutifolius* (cv Blue speckled) or *P. vulgaris* (cv Rico 23). From *P. acutifolius* cv Blue specked, we obtained a derived amino acid sequence called AIL-Pa, that matched exactly (except for one substitution of a Leu for an Ile) a sequence we obtained previously by genomic PCR of *P. acutifolius* cv White and that we reported as αAI-like Pacu [3]. For *P. vulgaris* cv Rico 23, we obtained a derived amino acid sequence, called AIL-Pv, that matched exactly the amino acid sequence of a genomic PCR product reported for a wild accession of *P. vulgaris* and previously called αAI-3 Pvul (w) [3, 9]. We assumed at the time [3, 9] that this sequence represented a second αAI isoform present in these seeds. By starting with cDNA rather than with genomic DNA as we did earlier, we can be sure that AIL-Pa and AIL-Pv represent genes that are transcribed in the developing seeds. The derived amino acid sequences of αAI-1, the inhibitor found in cultivated beans, and of αAI-2, the inhibitor found in arcelin-type wild accessions of *P. vulgaris* are compared with the two new sequences in Fig. 1. The new sequences are referred to as AIL with the species specific designation (AIL-Pv and AIL-Pa). The two AIL sequences differ from the two αAI sequences primarily by a 15-amino-acid insert around position 115. Both AIL sequences have several putative glycosylation sites that are conserved between AIL and αAI proteins, and have a putative signal peptide cleavage site indicated by an arrowhead in Fig. 1. The molecular weights of the mature polypeptides are 26 800 for AIL-Pv and 26 000 for AIL-Pa. However, like the other proteins in this family, it is likely that the AIL polypeptides will be glycosylated and that the mature glycoproteins will be somewhat larger. On an alignment dendrogram (see Experimental for details), the AIL sequences fall within the grouping of the amylase inhibitors rather than the PHAs or the arcelins (Fig. 2). It is on this basis that these proteins are referred to as amylase-inhibitor-like rather than PHA-like or arcelin-like.

AIL proteins are not processed in tobacco

Two different forms of αAI (αAI-1 and αAI-2) have been isolated from *P. vulgaris* and both forms are post-translationally processed by proteolysis and are made up of α and β-subunits [10]. It has been shown for αAI-1 that proteolytic processing is essential for the protein molecule to acquire its active inhibitory configuration [11]. Such processing normally occurs in bean cotyledons after the protein has been transported to the vacuoles. When αAI-1 is expressed in tobacco seeds, it is also proteolytically processed, and tobacco seeds that contain active inhibitor always have processed αAI polypeptides [12]. Many vacuolar polypeptides in legume seeds are processed on the carboxyl side of an Asn residue and a specific endopeptidase is found in diverse plant species [13, 14]. Both AIL-Pv and AIL-Pa were cloned in seed expression vectors in which the expression of the gene is driven by the PHA-promoter, and these constructs were used to transform tobacco. The seeds obtained from the kanamycin-resistant primary transformants did not contain αAI-activity (the extracts did not inhibit porcine pancreatic α-amylase) (data not shown). Immunoblot analysis revealed the presence in the tobacco seed extract of a 32 kDa protein that crossreacted with the antiserum made against αAI (Fig. 3, lane 6), and the smaller polypeptides (18–10 kDa) that are typical of αAI were not present (lanes 1, 2 and 3). A comparison with extracts of kidney beans and tepary beans showed that a protein of similar size (32 kDa) was also present in certain bean cultivars. For example, Fig. 3 shows a comparison of extracts from Red kidney (lane 1), Pinto (lane 2), Rico 23 (lane 3), White tepary (lane 4) and the cultivar To (lane 5) with an extract of transgenic tobacco seeds expressing AIL-Pv (lane 6). AIL-Pv is abundantly present in Pinto, Rico 23 and the cultivar To, but is absent from Red kidney. White tepary bean has a low level of AIL-Pv or has a protein that is less immunoreactive with this serum. Similar results were obtained when AIL-Pa was expressed in tobacco. With both the White tepary and the Blue speckled AIL cDNAs, we obtained a 32-kDa protein that was not processed in tobacco and matched in size a similar protein in the tepary bean seeds.

The lack of proteolytic processing of AIL is not due to the absence of an Asn residue at a position equivalent to Asn^{77}, suggesting that the presence of this Asn residue by itself is not sufficient for processing to occur. Arcelins also contain Asn residues equivalent to the Asn^{77} of αAI, and like AIL they also are not processed [6]. Other features of the protein must be involved in permitting proteolytic processing in seeds.

```
         1                          ↓                          50
AIL-Pv   MASSKFft..   ..vLFLVLLs   HANSATETSF   NiDaFNkTNL   ILQGDATVtS
AIL-Pa   MASSKFcsvL   SLvLFLVLLt   HANSAcDTSF   NfhsFNeTNL   ILQGqATVSS
αAI-1    MASSKL...L   SLaLFLaLLs   HANSATETSF   iiDaFNkTNL   ILQGDATVSS
αAI-2    MASSnL...L   tLaLFLVLLt   HANSAsDTSF   NfysFNeTNL   ILQGDATVSS

         51                                                   100
AIL-Pv   kGyLrLtddT   eDSMgrAFYS   vPIQIRDSTT   GNVASFsTNF   Tfimd...EA
AIL-Pa   nGnsQL..NT   xDSMCsAFYS   APIQIRDSTT   GNVvSFDTNF   TMNITTyRkA
αAI-1    nGnLQLsyNs   yDSMsrAFYS   APIQIRDSTT   GNVASFDTNF   TMNIrThRqA
αAI-2    kGyLQL..hT   vDSMCsAFYS   APIQIRDSTT   GNVASFDTNF   TMNITTqREA

         101                                                  150
AIL-Pv   NStyGLaFAL   VPVgsePKan   GpFLGLFrkP   gYDpeAhTVa   VvFinhWypn
AIL-Pa   NSAVGLDFAL   VPV..QPKSK   GrLLGLFktP   dYDrnAgnVT   VEFDTFR...
αAI-1    NSAVGLDFvL   VPV..QPeSK   G.........   ......dTVT   VEFDTFl...
αAI-2    NSvIGLDFAL   VPV..QPKSK   Gh........   .......TVT   VqFDTFR...
          ↑
         151                                                  200
AIL-Pv   angrhlgIDV   NsilpIeSkP   WyVg..qGkh   AvVqITYvSS   kKVLtVSLly
AIL-Pa     ...rRISIDg NhN.DIeSVP   WDVdDYDGQN   AEVRITYNSS   TKVLAVSLlN
αAI-1    ...sRISIDV   NNN.DIkSVP   WDVhDYDGQN   AEVRITYNSS   TKVFsVSLsN
αAI-2    ...sRISIDV   NNN.DIkSVP   WDeqDYDGQN   AkVRITYNSS   TKVLAVSLsN

         201                                                  250
AIL-Pv   PSTGtmydly   AkkvELEeEv   yDWVSVGFSA   TSGanQWSYE   ThDViSWSFS
AIL-Pa   lSTGKSNnVS   A.RmELEKkl   dDWVSVGFig   TSGvHQYSFE   TrDVFSWSFS
αAI-1    PSTGKSNnVS   t.tvELEKEv   yDWVSVGFSA   TSGayQWSYE   ThDVLSWSFS
αAI-2    PSTGKSNeVS   A.RmEvEKEl   dDWVrVGFSA   iSGvHeYSFE   TrDVLSWSFS

         251                       273
AIL-Pv   SKFSd.dddT   SERSNILLNn   IL*
AIL-Pa   SKFS..QhTT   SERSNILiNQ   IL*
αAI-1    SKFinlkdqk   SERSNIvLNk   IL*
αAI-2    SKFS..QhTT   SERSNILLNQ   IL*
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Fig. 1. Amino acid sequence comparisons of αAI-1 and αAI-2 from *P. vulgaris*, AIL-Pv and AIL-Pa using the program PILEUP with the default parameters. The arrows represent sites of processing for removal of the signal peptides (downward arrow) and the processing site (Asn^{77}) (upward arrow) utilized to yield active αAI.

In AIL-Pv, Asn^{77} is part of a possible glycosylation site (Asn-Ser-Thr) and if this site is glycosylated, it would surely prevent proteolytic cleavage. The corresponding triad in AIL-Pa is Asn-Ser-Ala, which cannot be glycosylated. Most remarkably, AIL-Pv and AIL-Pa have an insertion of 15 amino acids starting around position 95 (mature protein), relatively close to the Asn^{77} processing site. It is tempting to speculate that the deletion of this loop (and the change in the glycosylation site) in αAI makes proteolytic processing possible and that AIL is an evolutionary precursor to αAI. This deletion is the major difference between the αAI and the AIL sequences. However, the *in vitro* deletion of this loop may not be sufficient to create a protein that can be processed into an active molecule, because AIL-Pv also lacks the equivalent of an Arg^{74} which is part of the active site [6]. AIL-Pa has ArgLys at the equivalent position, and we do not know how the additional positive charge from the lysine would affect the activity of the protein molecule, and its ability to form an αAI-α-amylase complex.

AIL is at the same locus as αAI

To find out if AIL-Pv is at the same locus as αAI, we examined the distribution of the polypeptides in the progeny of a recurrent selection experiment in which the αAI-PHA locus of the cultivar Sanilac was replaced by the equivalent loci in two wild accessions of the common bean that contain arcelin 1 and arcelin 4. Progeny were selected for the presence of the arcelin proteins [15, 16]. These arcelin-containing accessions have αAI-2, rather than the αAI-1 found in bean cultivars, as shown by the different banding pattern of the polypeptides [16, 17].

Fig. 4 shows the pattern of immunocrossreactive polypeptides present in Sanilac, the parent cultivar, in Arc1 and Arc4, two wild accessions that have arcelin 1 and arcelin 4 proteins, and in the progeny, SARC1 and SARC4 obtained by recurrent selection for the arcelin protein. First, with respect to arcelin, it is clear that arcelin has been introduced into SARC1 and SARC4. At the same time, the typical polypeptide pattern for

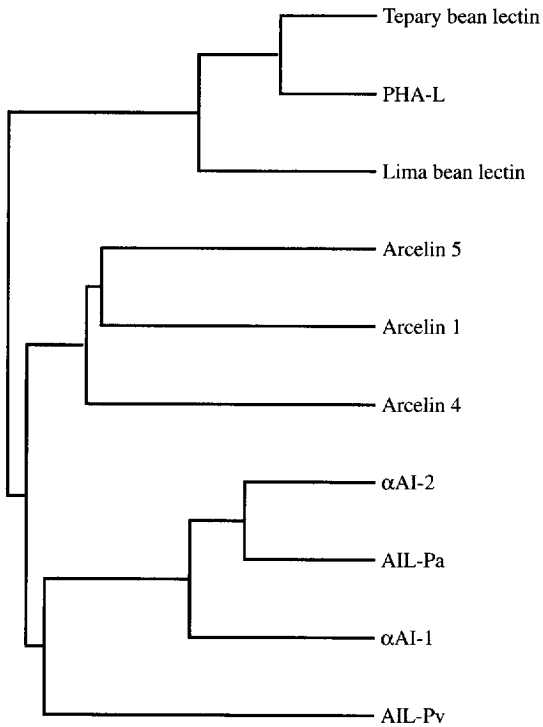

Fig. 2. Dendrogram of lectins, arcelins and αAI sequences created with the programs PILEUP and FIGURE using the default parameters. Sequences with the following accession numbers were used: JO1261 (αAI1), V10348 (αAI2), V10352 (AIL-Pv), U10415 (AIL-Pa). The AIL proteins fall within the αAI group.

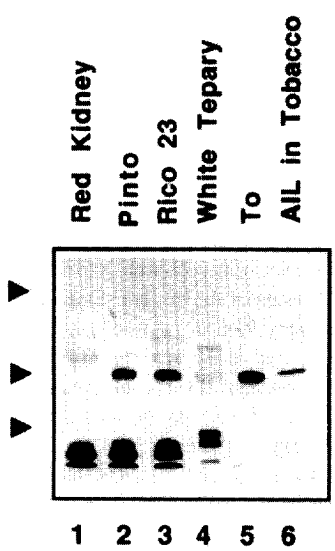

Fig. 3. Immunoblot of beam seed extracts and transformed tobacco seeds. The blot is visualized with a rabbit serum against deglycosylated αAI-1. Lanes 1, 2, 3 and 5 are different cultivars of *P. vulgaris*; lane 4 = *P. acutifolius*; lane 6 = AIL-Pv expressed in tobacco seeds. The arrowheads on the left indicate the molecular weight standards from top to bottom: 27 600, 18 600, 15 200.

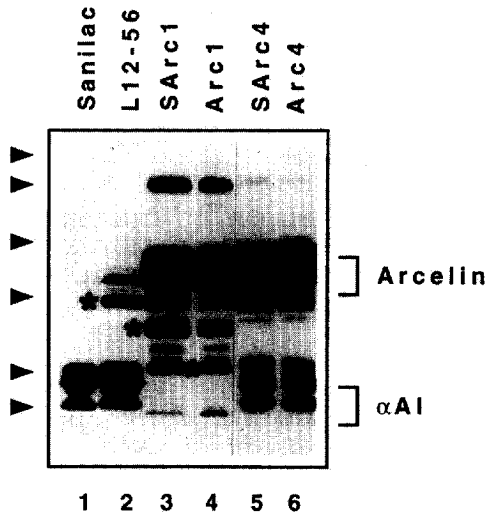

Fig. 4. Immunoblot of seed extracts of domesticated beans, wild accessions and the progeny obtained by recurrent selection for arcelin. Sanilac and L12-S6 are cultivars. The latter has no phytohaemagglutinin. Arc1 and Arc4 are wild accessions of *P. vulgaris* with arcelin 1 and arcelin 4. SARC1 and SARC4 are the progeny obtained by recurrent selection for arcelin. The arrowheads on the left indicate the molecular weight standards from top to bottom: 105 700, 74 700, 46 300, 27 600, 18 600, 15 200. The asterisks indicate cross-reacting protein at the PHA-arcelin-αAI locus not previously identified. The largest one corresponds to AIL.

αAI-1 in Sanilac (3 bands) has been lost and been replaced by the two-banded pattern of αAI-2 in SARC4 or the absence of bands in SARC1. (Beans that have the arcelin 1 protein do not have αAI activity or polypeptides [16].) Remarkably, the recurrent selection also resulted in the transfer of 3 other cross-reactive polypeptides (asterisks) with M_r 32 000, 23 000 and 18 000. The largest of these corresponds to AIL (arrow). These data indicate that AIL, and other proteins yet to be described, are encoded at this locus.

It therefore appears that AIL and the 23 kDa and 18 kDa proteins are encoded by the same locus as PHA, arcelin and αAI. It seems likely that all these genes arose by tandem duplication of an ancestral lectin-like gene. Legume lectins, and by extension arcelins and αAIs, are β-barrel proteins in which each monomer consists of two antiparallel β-pleated sheets; one sheet has five strands and the other one seven, and the strands are connected by short loops. Arcelins differ from lectins (PHA) by the deletion of one loop segment and αAIs differ from arcelins by the deletion of two additional loop segments. AIL appears to be the intermediate as it is one segment shorter than arcelin and still one longer than αAI (Fig. 5). The AIL genes may thus be an evolutionary intermediate between arcelins and αAIs. Both arcelin and αAI are known to inhibit the development of bruchid larvae. Whether AIL also has such a biological activity, perhaps less potent than αAI but stronger than arcelin, still needs to be established.

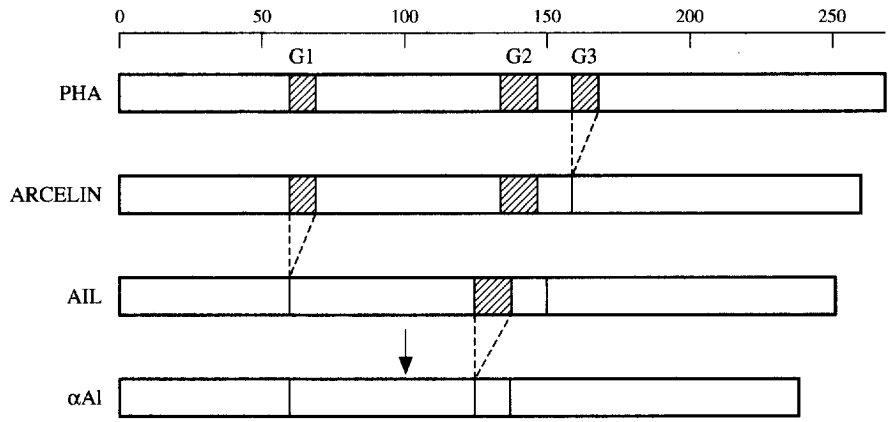

Fig. 5. Schematic representation of the 4 types of genes at the PHA/Arcelin/αAI locus, G1, G2 and G3 refer to gaps in the amino acid sequence alignments of αAI, arcelin and PHA (see Mirkov *et al.* [3]). The scale at the top indicates amino acid position. Only αAI is proteolytically processed at the site indicated by the arrow.

EXPERIMENTAL

Plant materials. P. acutifolius cv Blue speckled and White were obtained from Native Seeds/Search in Tucson, AZ. Cv. Rico 23 is a *P. vulgaris* cultivar from Brazil. Cv. To is a *p. vulgaris* line from Japan provided by M. Ishimoto (National Agriculture Research Center, Tsukuba, Japan).

Protein extraction. Proteins were extracted from seeds by grinding in an ice-cold mortar with a buffer containing 10 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.1% Triton X-100 and 1% 2-mercaptoethanol supplemented with 1 μg ml^{-1} of leupeptin, aprotinin, and pepstatin A (125 mg ml^{-1} buffer). The supernatant obtained after centrifugation at 15,000 *g* for 5 min (twice) was frozen at −20° until further use.

Immunoblot analysis. For immunoblotting, appropriate quantities of protein (determined according to [18]) were fractionated by SDS-PAGE, transferred to nitrocellulose, and the αAI detected using a rabbit anti-αAI serum obtained as described [19]. Goat anti-rabbit IgG coupled to horseradish peroxidase (BioRad) served as the secondary antibody.

RT-PCR analysis. Dry beans were ground to a fine powder in liquid nitrogen and total RNA was purified by the procedure of [20]. Total RNA (5 μg) was used in the following 100-μl one-tube RT-PCR reaction: 200 ng of each primer, 10 mM Tris, pH 9.0, 50 mM KCl, 3.0 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each dNTP, 40 units RNasin (Promega, Madison, WI) 15 units AMV reverse transcriptase (Promega) and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). Conditions for RT-PCR were 42° for 1 hr, 95° for 5 min, and then 40 cycles of 55° for 1 min, 72° for 1 min, and 94° for 1 min. The resulting RT-PCR products were cloned directly in pCRII (Invitrogen, San Diego, CA), and sequenced by the Sequenase dideoxy chain termination method (United States Biochemicals, Cleveland, OH). The design of our PCR primers was based on the conserved amino-terminal (MASS^{K}/$_{N}$) and carboxy-terminal (LN^{Q}/$_{K}$IL) amino acids of PHA-E, PHA-L, αAI and arcelin [2]. The sequences were as follows: P1; 5′GGCTCGAGTCTAGAGGAT^{T}/cT^{G}/$_{T}$GTTG-3′,P2; 5′CCATCGATAGATGGCTTCCTCC-AA^{C}/$_{G}$T-3′.

Sequence analysis. Deduced amino acid sequences were aligned using the PILEUP and PRETTY programs of the University of Wisconsin Genetics Computer Group [21] and the dendrogram was created using the program FIGURE, using the default parameters of each program.

Tobacco transformation. A *Bin* 19-based vector was used to introduce αAI-1 into tobacco and express the protein in the seeds [12]. The coding sequence of αAI-1 was replaced by the coding sequences of AIL-Pv or AIL-Pa. The *Bin* 19 construct was mobilized from *Escherischia coli* DH5α to *Agrobacterium tumefaciens* LBA4404 by triparenteral mating with *E. coli* HB 101 containing pRK2013 [22] and used to transform leaf discs of *Nicotiana tabacum* (cv Xanthi). Transformants were selected by their resistance to kanamycin, and transformed plants were regenerated from shootlets by transfer to a root-inducing, kanamycin-containing, agar medium as described in [23]. The kanamycin-resistant plants were transferred to soil and grown in the greenhouse.

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