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Accumulation, assembly, and digestibility of amarantin expressed in transgenic tropical maize

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Abstract An amaranth (*Amaranthus hypochondriacus*) 11S globulin cDNA, encoding one of the most important storage proteins (amarantin) of the seed, with a high content of essential amino acids, was used in the transformation of CIMMYT tropical maize genotype. Constructs contained the amarantin cDNA under the control of a tissue-specific promoter from rice glutelin-1 (*osGT1*) or a constitutive (*CaMV 35S*) promoter with and without the first maize alcohol dehydrogenase intron (*AdH*). Southern-blot analysis confirmed the integration of the amarantin cDNA, and copy number ranged from one to more than ten copies per maize genome. Western-blot and ultracentrifugation analyses of transgenic maize indicate that the expressed recombinant amarantin precursors were processed into the mature form, and accumulated stably in maize endosperm. Total protein and some essential amino acids of the best expressing maize augmented 32% and 8–44%, respectively, compared to non-transformed samples. The soluble expressed proteins were susceptible to digestion by simulated gastric and intestinal fluids, and it is suggested that they show no allergenic activity. These findings demonstrate the feasibility of using genetic engineering to improve the amino acid composition of grain crops.

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Introduction

An important feature of seed development is the synthesis and accumulation of storage proteins. In dicotyledonous species, these proteins are predominantly in the form of saline-soluble globulins which, based upon their structure similarities and sedimentation coefficients, have been classified as either 7S (vicilin-like) or 11S (legumin-like) proteins. Seed globulins are encoded by multigene families which have been most extensively studied in a number of grain legumes such as pea, soybean, broad bean, common bean, and in some non-legume species (Shewry and Halford 2002). On the other hand, cereal prolamins are normally deficient in lysine and tryptophan, such as in maize and rice, whereas legume globulins show deficiency in sulfur-containing amino acids, such as methionine and cysteine. Consequently, diets based on a single cereal or legume species result in amino acid deficiencies (Habben and Larkins 1995; Wohlfahrt et al. 1998).

Amaranth (*Amaranthus hypochondriacus*) was greatly appreciated by the advanced Meso-American civilizations of the New World as a basic food in their diets. However, its association with religious rites resulted in its cultivation being discouraged after the Spanish conquest (Guzmán-Maldonado and Paredes-López 1998). This pseudo-cereal has been identified as a crop comparable with most potential food and feed resources, because of the exceptional nutritional-functional quality of its seed storage proteins (Guzmán-Maldonado and Paredes-López 1998). Amarantin, one of the most important proteins, contains a very good balance of essential amino acids which nearly meets the needs of human protein nutrition, in reference to protein requirements established by international health organizations (FAO/WHO 1991; Barba de la Rosa et al. 1996). Previous studies on amarantin showed that its cDNA can be expressed in *Escherichia coli*, exhibiting electrophoretic, immunochemical, and surface hydrophobicity properties similar to those of native amarantin from amaranth seed (Segura-

Nieto et al. 1994; Chen and Paredes-López 1997; Osuna-Castro et al. 2000).

A promising strategy is the introduction, through genetic engineering, of genes encoding proteins with high nutritional value into food crops with agronomic importance (Katsube et al. 1999; Stöger et al. 2001; Yang et al. 2002). Thus, it would be advantageous to express novel seed proteins such as amarantin in maize grains with the objective of improving amino acid composition. Moreover, the molecular and functional characterization of the expressed amarantin would facilitate understanding of the mechanisms of expression, processing, and deposition of amarantin in transgenic plants. In this study, we examined whether amarantin can be synthesized, processed correctly, and accumulated specifically in the seeds of tropical maize, as well as its impact on the total protein content and on essential amino acid composition of this crop. Protein digestibility studies were also assessed.

Materials and methods

Constructs carrying the amarantin gene

The amarantin gene (1.7-kb amarantin cDNA) was purified from pSPORT-*Amar* (Barba de la Rosa et al. 1996). The rice glutelin-1 promoter was kindly supplied by N. Murai (Louisiana State University, USA). A 1.8-kb *Eco*RI-*Bgl*II fragment, containing a functional promoter gene (*osGT1*, 5.1 kb) from rice, was used to drive amarantin cDNA expression in maize endosperm (pAmar 1.7k). A PCR-site-modified first maize alcohol dehydrogenase intron (*AdH*) fragment was produced and cloned into a *Bcl*I-*Bam*HI site between the promoter and amarantin gene (pAmar2k). Both constructs were assembled into a pSPORT-derived backbone plasmid with a blunt-ended 1.5-kb cauliflower mosaic virus (*CaMV 35S*) promoter driving a *bar* gene encoding resistance to herbicide BASTA. The expression of amarantin was also under the control of tandem *CaMV 35S* promoter contained in pUC derived plasmid, cloned into *Bam*HI-*Sma*I sites, containing an amarantin cDNA and a 3′ end (pAmar) and (padhAmar); the latter contains an *AdH* intron introduced as above (Fig. 1). The *bar* gene (pAHC20) was under the control of the 5′ untranslated region of the *Ubi*-1 gene, a *Ubi*-1 5′ intron and nopaline synthase 3′ untranslated sequence (Christensen and Quail 1996).

Transformation and regeneration of tropical maize

Immature embryos were taken from maize (*Zea mays*) plants at CIMMYT's greenhouse; immature embryos were obtained by crossing CML72 with CML216 and used for the transformation experiments. For transformation plasmids pAmar1.7k, or pAmar2k (10 μg each) were used, and for co-bombardment equimolar amounts of plasmids pAmar and pAHC20, or padhAmar and pAHC20, and 580 μg gold particles were bombarded using the PDS-1000 particle device (Bio-Rad, Hercules, Calif., USA) as described in Bohorova et al. (1999). Selection of transformed cells was achieved using either phosphinothricine (3 mg/l) or bialaphos (5 mg/l) on a modified N6 medium (N6C1). All herbicide-resistant callus tissue that grew on selective medium was transferred to regeneration medium (N6R) (Bohorova et al. 2001). Plantlets developing in this medium were transferred to soil in environmentally controlled growth chambers and greenhouse conditions for further analyses. Plants were selected by spraying a 2% BASTA solution and assessed for damage 1 week after the herbicide application.

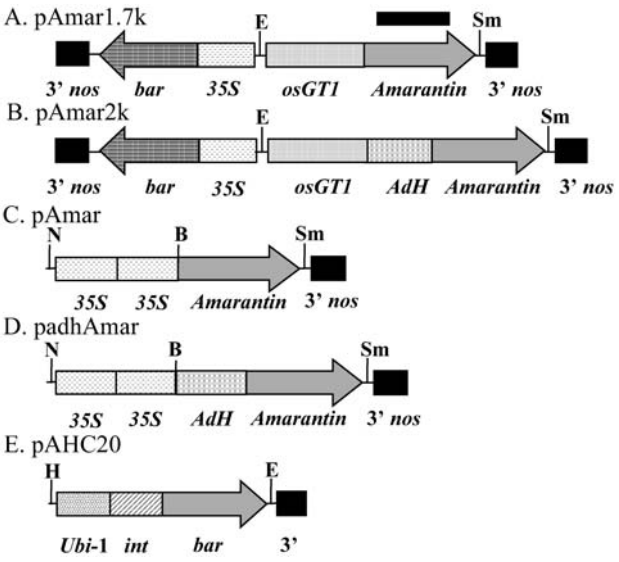

Fig. 1A–E Schematic representation of maize transformation/expression constructs. **A** pAmar1.7k diagram shows the 1.8-kb *osGT1* promoter fused to the translation start ATG from amarantin cDNA including signal peptide and 3′ *nos* end. **B** pAmar2k, same as pAmar1.7k, except that it also contains the maize alcohol dehydrogenase 1 (*AdH*) intron localized between the *osGT1* promoter and amarantin cDNA. Both plasmids are fused to the *bar* coding region driven by the *CaMV 35S* promoter. **C** padhAmar; amarantin cDNA included with the *AdH* intron gene and a signal sequence under the control of doubled *CaMV 35S*. **D** pAmar; amarantin cDNA under the control of doubled *CaMV 35S* promoter. **E** pAHC20; *bar* coding region includes ubiquitin1-intron gene under the control of the ubiquitin promoter. For Southern analysis, amarantin cDNA probe labeled by PCR with digoxigenin is indicated by the *filled box*. *B Bam*HI, *E Eco*RI, *H Hin*dIII, *Sm Sma*I, *N Not*I

Southern-blot analysis

Genomic DNA was isolated from approximately 1 g of leaf tissue ground to fine powder in liquid N_2 and mixed thoroughly with extraction buffer (Shure et al. 1983). The analysis was performed with 30 μg of genomic DNA, digested with a threefold excess of *Eco*RI or *Bam*HI, and *Sma*I restriction enzymes, electrophoresed through 0.7% agarose, and transferred to a nylon membrane using 25 mM sodium phosphate buffer, pH 6.5 (Southern 1975). Filters were prehybridized at 65°C in 5×SSC containing 0.01% lauroyl-sarcosine, 0.02% SDS, and 1% blocking reagent (Boehringer Mannheim, Indianapolis, Ind., USA) for 4 h. Filters were hybridized overnight at 65°C with the aforementioned buffer containing 30 ng/ml of a PCR digoxigenin-labeled amarantin probe.

RNA analysis

Flag leaf and seeds at 20 days after pollination (DAP) were cut longitudinally and frozen in dry ice. RNA was purified from five endosperm halves or flag leaf by extraction in 100 mM Tris-HCl pH 9.0, 200 mM NaCl, 5 mM DTT, 1% Sarcosyl, 20 mM EDTA, followed by phenol/chloroform extraction. Aqueous phase was precipitated with 2 M LiCl, and re-precipitated in Na acetate/ethanol (Russell and Sachs 1991). Formaldehyde gels of 1.2% agarose in MOPS buffer were run, transferred, and amarantin mRNA analyzed by using a PCR digoxigenin-labeled amarantin probe.

Protein extraction and Western-blot analysis

Salt-soluble fractions (globulins) from transgenic T_1 leaf and seeds and from non-transgenic maize (CML72×216×216) were prepared. The pericarp and embryo from each seed were removed for protein fractionation as described with minor modifications (Chen and Paredes-López 1997). Protein fractions were obtained by grinding 250 mg of vegetative tissue or from 50 μg of defatted endosperm meal, and fractions were then separated by SDS-polyacrylamide gel electrophoresis (Laemmli 1970). The gel was equilibrated in transfer buffer and then proteins were transferred onto a PVDF membrane (Fido et al. 1995). Rabbit polyclonal antibodies (kindly provided by J. Calderon, CINVESTAV-IPN) were raised against the 53-kDa amarantin purified from amaranth seeds. IgG immunoglobulins were purified from serum with an ImmunoPure column (Pierce, Rockford, Ill., USA) and to reduce background anti-amarantin IgGs were treated with a non-transformed maize endosperm extract polypeptide-Sepharose column. Membranes were developed with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (*p*-nitrotetrazolium blue) substrates, until color appeared.

Analysis of assembly by sucrose density gradient ultracentrifugation

Protein samples (3 mg) were concentrated and dissolved in 1 ml of globulin extraction buffer (0.1 M Tris-HCl pH 8.0, 0.1 M NaCl); ultracentrifugation was carried out according to Chen and Paredes-López (1997) without 2-mercaptoethanol. The standard proteins catalase (11.2S), γ-globulin (7S), and lysozyme (1.9S) were run in parallel as size markers. Fractions (1 ml) were collected and used for Bradford analysis, and 30 μg of each fraction was concentrated and then subjected to SDS-PAGE. The electrophoresed proteins in the gels were transferred onto a PVDF membrane for the detection of amarantin, as described above.

Tissue printing

Seeds at 35 DAP were dissected longitudinally without disrupting embryogenic tissues. Halves were dipped using forceps into hexane for 20 s. After the prints had been made, the membrane was baked for 30 min at 60°C (Woo et al. 2001). Amarantin was detected with anti-amarantin IgG (cured with non-transformed protein extract), followed by anti-rabbit IgG-alkaline phosphatase conjugate (Promega). For in situ localization of amarantin, a grain from a maize plant expressing amarantin at the highest level was used and treated as described above.

Amino acid determination

Total protein and essential amino acid composition of seeds from transgenic T_1 maize (pAmar1.7k-1041, pAmar2k-8, pAmar-49 and padhAmar-7) and non-transgenic maize hybrids (CML72×216×216) were analyzed by near infrared transmittance (Foss NIT System, Tecator 1275 Food Analyzer, Denmark) following supplier instructions.

In vitro digestion of heterologous amarantin

Salt soluble fractions (225 μg) from the transgenic maize endosperm extracts were incubated at 37°C with simulated gastric fluid (SGF) during 60 min and then adjusted to pH 8.0 with 100 mM Tris-HCl pH 9.5, 2 mM $CaCl_2$ for intestinal fluid digestion at a 1:100 ratio. At the desired times, 20-μg aliquots were taken and reaction mixtures subjected to SDS-PAGE and then to Western blotting as already described (Momma et al. 1999; Roesler and Rao 2001).

Results

Selection and regeneration of transgenic maize plants

All selected calli were highly regenerable, and a large number of plants per construct regenerated. Within 3–4 months, putatively transformed maize plants were transferred to soil and grown under greenhouse conditions. Herbicide-selected plantlets were healthy and successfully grew into fertile plants. A total of 186 healthy herbicide-resistant T_0 plants were transformed; 91 plants were recovered with either plasmids pAmar1.7k or pAmar2k. Ninety-five plants were recovered with plasmids pAmar and pAHC20 or padhAmar and pAHC20. All plants were back-crossed with CML216 and successfully grew into fertile plants in a containment greenhouse.

Molecular analyses of transgenic maize plants carrying the amarantin gene

The presence of the amarantin gene in all 186 primary T_0 transgenic plants was assayed in herbicide-resistant plantlets at the six-leaves stage by PCR (data not shown) and 20 plants per construct were analyzed by Southern-blot analysis. More than 80% of PCR-positive plants showed the presence of the amarantin gene by Southern blot. To identify plants that carried amarantin cDNA by Southern blot, plant DNA was digested with *Eco*RI or *Bam*HI and *Sma*I, restriction enzymes that flank the amarantin cDNA-containing fragment (Fig. 1). This analysis demonstrated that the regenerated maize plants showed the expected hybridization fragment of ≈3.5 kb and ≈2 kb (Fig. 2). Southern analysis showed from one to more than ten insertions in different transgenic lines, as determined using a copy number reconstruction (Fig. 2A, lanes 10C and 1C), and some showed no hybridization signal (Fig. 2A, lanes 2k-192, 1.7k-1425 and 2B, 25–48, 35–421). Each transgenic plant showed a characteristic hybridization pattern, indicating that they are independent transformation events. Transgenic plants containing the amarantin gene, together with non-transformed plants, were subsequently transferred to the containment greenhouse to reach maturity.

Transgenic expression of amaranth 11S globulin (amarantin) in maize

Northern hybridization and Western-blot analyses showed expression of amarantin cDNA in tropical maize lines; the recombinant protein was directed towards seed endosperm for most constructs. Northern hybridization with total RNA samples from endosperms and leaf tissues of independent transgenic lines showed accumulation of the expected amarantin transcript at variable levels.

Representative results are shown for independent transformants (Fig. 3A). RNA and protein were analyzed; the presence of amarantin was associated with the

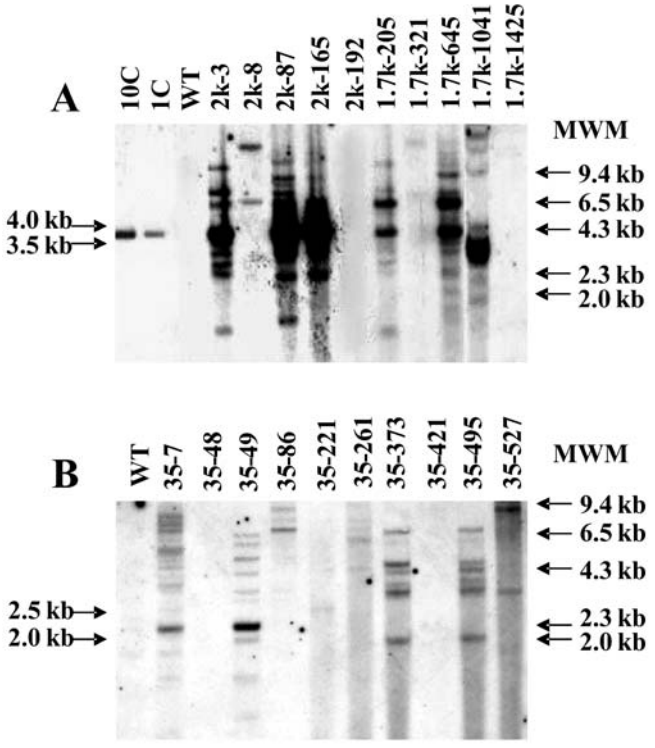

Fig. 2A, B Southern-blot analysis of genomic DNAs from plants transformed with the amarantin constructs. **A** Maize plants transformed with pAmar1.7k and with pAmar2k constructs were digested with *Eco*RI and *Sma*I. Hybridization fragment (1.8 kb *osGT1* + amarantin gene) are indicated. *Numbers* on the lanes are representative of independent transgenic lines. On the *left*, a copy number reconstruction is presented: *10C* 10 copies, *1C* one copy. **B** Maize plants transformed with padhAmar and with pAmar constructs. Hybridization fragments (35S:35S + amarantin gene) are indicated. *MWM* Molecular weight marker, *WT* non-transformed control

corresponding mRNA showing differences in post-transcriptional processing and accumulation in leaf tissues. The corresponding Western-blot analysis demonstrated the presence of amarantin in endosperms of transgenic maize plants bearing the amarantin transgenes. The expression of heterologous amarantin obtained from endosperm of the highest amarantin-expressing transgenic line compared with purified amarantin protein is shown (Fig. 3B, lines 1.7k-1041 and Amarantin), whereas heterologous amarantin was not detected in leaf tissues when compared to purified amarantin protein (Fig. 3B, lines 35–49 and Amarantin). Differences in protein level accumulation in leaf tissues in transgenic plants may be attributed to tissue-specific post-transcriptional effects of amarantin gene transcripts.

Endosperms from transgenic plants containing the amarantin cDNA accumulated the expected molecular-weight polypeptides, which reacted with the polyclonal antibody specific for the 53-kDa amarantin monomer and its acidic (32-kDa) and basic (21-kDa) subunits. The 53-kDa protein was also detected in the globulin fraction from amaranth seeds (Fig. 3B, lane Amarantin), and was not detectable in extracts from non-transformed plants (Fig. 3B, lane WT). The heterologous amarantin polypeptide comigrates with the subunits of the partially purified protein from amaranth seed.

Processing of amarantin in maize endosperm

No immunoreactive proteins were detected in non-transgenic seed extracts (Fig. 4A). Under non-reducing conditions, a band was detected in the transformed endosperm extracts, with the expected molecular weight of 53 kDa (Fig. 4B, lanes 4–9) and its acidic subunit of

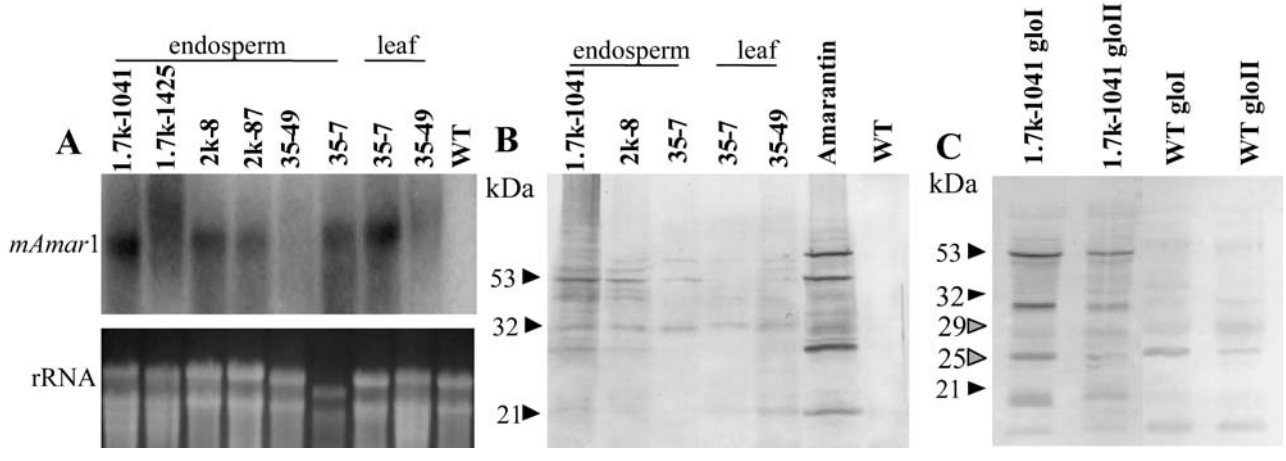

Fig. 3A–C Northern hybridization and Western-blot analysis of expression of amarantin in endosperm and leaf tissues of transgenic maize lines. **A** RNA analysis was performed on 20-DAP maize endosperms and leaves. Seven-microgram aliquots of RNA were loaded per lane. *Numbers* on the lanes are representative of independent transgenic lines. Transcripts were detected using a PCR-digoxigenin-labeled amarantin probe. **B** Western-blot of highly-expressing heterologous amarantin. Aliquots of 30 μg of salt-soluble endosperm protein from plants number 1.7k-1041, 2k-8, 35S-7, 35S-49, and leaf protein from plant number 35S-49 were analyzed. Each *lane* is representative of endosperm expression from five seeds, whereas the *amarantin lane* represents amaranth seed salt-soluble protein (globulins), *WT* represents non-transformed maize endosperm globulins. *Arrows* show the 53-kDa amarantin and the 32-kDa acidic and 21-kDa basic subunit polypeptides

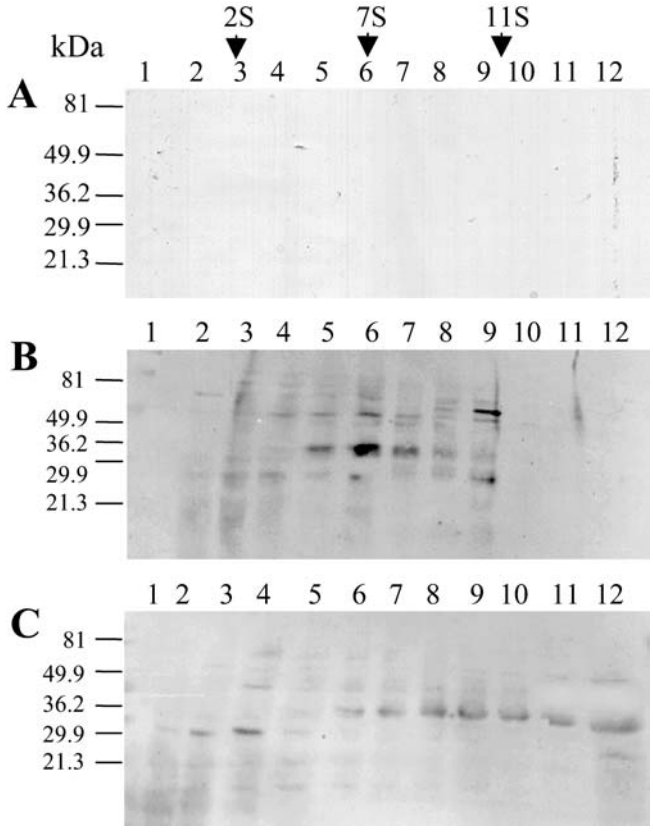

Fig. 4A–C Ultracentrifugation analysis of the size and assembly of amarantin expressed in the endosperms of transgenic maize plants. Endosperm extracts (~3 mg) from non-transgenic maize (**A**) and amarantin-expressing maize (**B**) and (**C**) are shown. Samples were subjected to ultracentrifugation on 12 ml 5–20% (w/v) linear sucrose density gradient centrifugation and analyzed by SDS-PAGE and immunoblot. Fractions from 1 to 12 of endosperm proteins (30 μg) were separated under non-reducing conditions (**B**), or in the presence of reducing agent 5% (w/v) 2-mercaptoethanol (**C**). Sedimentation coefficient standards are indicated by *arrows*

32-kDa. Under reducing conditions two polypeptides of 21 (very faint) and 32-kDa were observed (Fig. 4C, lanes 6–11). Another very light band in the partially purified amarantin was also detected of 53 kDa (Fig. 4C, lane 12). Anti-amarantin antibody reacts weakly against amarantin basic subunit of 21-kDa; this is in agreement with the behavior of soybean glycinin expressed in rice (Katsube et al. 1999). The 53-kDa protein corresponds to the expected size of the amarantin precursor molecule, and the 21- and 32-kDa bands to the basic and acidic amarantin polypeptides, respectively. However, considerable amounts of the unprocessed precursor molecule were also present in maize endosperm extracts (Fig. 4B lanes 5–8), indicating that the efficiency of processing may be lower in the heterologous system.

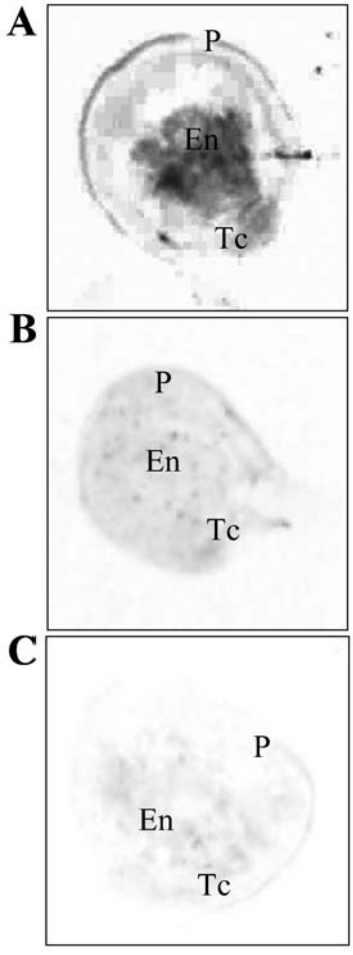

Fig. 5A–C Immunological tissue print localization of heterologous storage protein in transformed maize seeds. Median-longitudinal section showing the distribution pattern of amarantin. Section imprints from tissue specific expression (pAmar1.7k-1041) (**A**), constitutive expression (pAmar-49) (**B**), and tissue section of non-transformed maize seed (**C**). Seeds on nitrocellulose were visualized using a cured anti-amarantin IgG and anti-rabbit IgG-alkaline phosphatase conjugate. *En* Endosperm, *P* pericarp, *Tc* transfer cell layer of endosperm

Histochemical localization of amarantin in maize seed

Immunological analysis of tissue prints prepared from longitudinally sectioned maize seed revealed that amarantin was confined to an area of the transgenic seed when the *osGT1* tissue-specific promoter was used. This promoter directs amarantin expression to starchy endosperm; however, constitutive expression (*35S CaMV*) of amarantin in endosperm is not restricted only to starchy endosperm (Fig. 5). The accumulation pattern driven by the *osGT1* promoter was retained in sections of the maize endosperm. In contrast, amarantin expressed under the control of *35S CaMV* promoter was distributed along the maize endosperm.

Table 1 Protein content and essential amino acid composition in %/100 g seeds of non-transformed and transgenic T_1 maize seeds. * Methionine + cysteine, † phenylalanine + tyrosine. Protein and amino acid increase (%) from transgenic maize in relation to non-transformed maize is presented in parentheses. Each value is the mean ± SE. *WT* Non-transformed genotype. All data are on dry-weight basis

	WT CML72X216X216	pAmar1.7k-1041	pAmar2k-8	pAmar-49	padhAmar-7
Protein	11.57±0.02	15.32±0.30 (32)	12.89±0.45 (11)	12.26±0.03 (6)	12.29±0.30 (6)
Lys	0.34±0.01	0.40±0.01 (18)	0.38±0.01 (12)	0.35±0.02 (6)	0.36±0.02 (6)
Trp	0.09±0.01	0.11±0.02 (22)	0.10±0.02 (11)	0.09±0.01 (0)	0.09±0.01 (0)
Ile	0.44±0.00	0.60±0.01 (36)	0.50±0.02 (14)	0.45±0.01 (3)	0.45±0.01 (3)
Thr	0.49±0.01	0.53±0.02 (8)	0.51±0.01 (4)	0.50±0.02 (2)	0.50±0.02 (2)
Val	0.54±0.01	0.77±0.01 (42)	0.65±0.02 (20)	0.58±0.01 (7)	0.60±0.01 (11)
His	0.38±0.01	0.46±0.02 (21)	0.41±0.01 (8)	0.39±0.02 (7)	0.39±0.02 (7)
Leu	1.47±0.00	2.12±0.01 (44)	1.84±0.00 (25)	1.63±0.01 (11)	1.64±0.01 (12)
Met*	0.53±0.01	0.68±0.02 (28)	0.58±0.01 (9)	0.53±0.02 (0)	0.55±0.02 (4)
Phe†	1.08±0.01	1.54±0.01 (42)	1.25±0.01 (16)	1.11±0.01 (3)	1.13±0.01 (5)

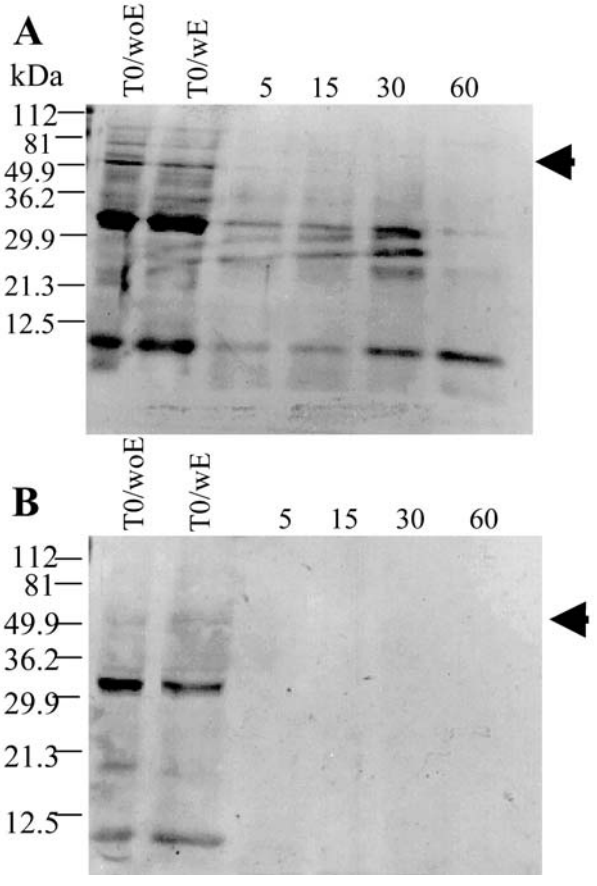

Fig. 6A, B Digestibility of expressed amarantin. A salt-soluble endosperm extract from pAmar1.7k-1041 containing expressed amarantin was incubated with the simulated gastric fluids (SGF) (**A**) and then digested with simulated intestinal fluids (SIF) (**B**). At the subscribed time, samples (30 μg) were taken and subjected to SDS-PAGE followed by immunostaining. *Lane 1* Extract without enzyme at 0 min, *lane 2* extract with enzyme 0 min, *lane 3* 5 min, *lane 4* 15 min, *lane 5* 30 min, *lane 6* 60 min. *Arrow* indicates amarantin precursors

Protein content and limiting essential amino acid profile in transgenic maize

There was a remarkable change in total protein content (6–32% increase) of transgenic maize seeds, which was augmented in close correlation with the increase of essential amino acids; levels of these amino acids in the transgenic maize increased from 0 to 44% (Table 1). The highly expressing maize line pAmar1.7k-1041 showed amino acid increases of 8–44% and specific increases of 18% and 22% in lysine (Lys) and tryptophane (Trp), respectively, which are highly deficient in common maize; the third limiting amino acid, isoleucine (Ile) also increased by around 36% (Table 1). This suggests that the observable increase in lysine, tryptophane and isoleucine are due to the expression of the amarantin protein driven by the *osGt1* promoter in seeds.

Digestibility of the amarantin

The digestibility of amarantin in the transgenic maize endosperm was confirmed by Western-blot studies, which indicated that high-molecular-weight proteins, including amarantin, were almost completely digested within 20 min in SGF (Fig. 6A). Amarantin was completely degraded within 15 min in simulated intestinal fluid (SIF) (Fig. 6B). These results suggest that this protein may not show allergenic activity (Astwood et al. 1996).

Discussion

A reliable transformation procedure to express an amaranth globulin cDNA in maize was successfully developed. This is a good choice for gene expression analysis of heterologous proteins (Bohorova et al. 1999). Gene transfer studies in plants have become invaluable in furthering our understanding of transcriptional regulation of gene expression in plants (Bellucci et al. 2002; Yang et al. 2002).

We have been able to transform tropical maize with a protein well-balanced in amino acid composition such as amarantin, in order to determine whether this protein would be synthesized and expressed in maize seeds. Tissue-specific expression of the 1.8 kb *osGT1* promoter was previously demonstrated, and it would be possible to enhance gene expression using additional 5′ elements from the 5-kb *osGT1* promoter (Katsube et al. 1999).

Western-blot results suggest that maize endosperm is able to process the 53-kDa precursors encoded by the amarantin cDNA into 34-kDa acidic and the 21-kDa basic polypeptide, as happens in amaranth seeds, meaning that the proamarantin polypeptides present the well-conserved Asn-Gly bond and are specifically cleaved by an asparaginyl endopeptidase (Oliveira et al. 2002).

Approaches for the manipulation of the amino acid composition in plants include the introduction of heterologous genes that encode proteins with balanced amino acid profiles or that are enriched in the amino acid that is under-represented (Chakraborty et al. 2000). For instance, in maize, a methionine-rich γ-zein gene that was expressed increased the accumulation of this protein by mRNA stability (Lai and Messing 2002). However, the three most limiting amino acids in maize as a dietary source for monogastric animal diets are lysine, tryptophane, and isoleucine (in order of importance). Attempts at increasing the most limiting amino acids in grain/tuber crops have had limited success. The transformation of a forage crop such as lupin with a methionine-rich sunflower seed albumin gene, the total sulfur amino acid increased at 19% and no changes in nitrogen level in seed were observed (Molving et al. 1997). In the case of the transformation of soybean with the high-methionine Brazil nut protein, commercialization did not occur because of the protein's allergenic properties (Nordlee et al. 1996). Other examples of improvements are the expression of soybean glycinin in rice and potatoes. In rice, it led to an increase of 20% in lysine, 26% in total sulfur-containing amino acids, and 23% in protein (Momma et al. 1999). In potato, lysine, total sulfur-containing amino acids, and protein did not show any remarkable changes due to the low level expression of the soybean glycinin (Hasimoto et al. 1999).

In our hands, expression of the amarantin gene in maize seeds resulted in increases of 18% in lysine, 28% in sulfur-containing amino acids, a third limiting amino acid in maize, isoleucine, increased by 36%, and protein increased by 32%. The 11S globulin from amaranth (amarantin) is a more promising donor of essential amino acids from two points of view. First, amarantin contains a very good balance of essential amino acids which nearly meets the needs of human protein nutrition, in reference to protein requirements established by international health organizations. Second, a search of the literature has not revealed any reports of allergenicity associated with amaranth seeds. In addition, effective in vitro digestibility has been observed in our lab. Therefore, amarantin would provide a good source of supplementary essential amino acids for both animals and humans.

This is the first report in which an amaranth protein is transferred to maize seeds. The protein and essential amino acid contents in transgenic lines were substantially improved, including the three most limiting amino acids in maize. The use of near-infrared transmittance (NIT) and reflectance spectroscopy (NIRS) to predict chemical composition in plants is nowadays a very useful routine method in plant breeding programs (Cozzolino et al. 2000; Fontaine et al. 2002).

Although the biological function of amarantin is yet to be understood, it has been demonstrated that globulins exhibit a significant hypocholesterolemic effect if the dietary intake of these proteins is above 6 g/d, and some epidemiological studies link their intake with a reduced risk of cardiovascular diseases (Imura et al. 1996). In vitro digestibility suggests that amarantin expressed in maize endosperm may not show allergenic activity (Shirai et al. 1998). To confirm the safety of the transgenic maize obtained in this work, more detailed nutritional and toxicological studies are now being carried out.

Since maize constitutes an important component of the diet for people in many developing countries, the final target of our work is to overexpress amarantin in order to exploit more fully its nutritional potential.

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