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Detection of the antimicrobial peptide gene in different *Amaranthus* species

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Abstract: Using primers to amplify the gene *AMP2* in *Amaranthus caudatus*, we found the gene to be present in seven other species of the *Amaranthus* genus (*A. albus*, *A. cruentus*, *A. blitum*, *A. hybridus*, *A. hypochondriacus*, *A. retroflexus* and *A. tricolor*), in which it had not been described previously. The PCR products were sequenced and it was established that all the sequences were identical, except for two polymorphisms. These single nucleotide polymorphisms occurred at nucleotide positions 45 and 246. This exchange of one nucleotide for another was manifested in an amino acid change in both cases. Due to the fact that both polymorphisms lay outside the region encoding the chitin-binding peptide domain, which is crucial for antimicrobial peptide function, they will not likely affect the proper functioning of the peptide. With the exception of the above-mentioned polymorphisms, all sequences were identical to the sequence of the *AMP2* gene that codes for the *A. caudatus* Ac-AMP2 (antimicrobial peptide isolated from *Amaranthus caudatus* seeds). The detection of sequences with high degree of sequence similarity to *A. caudatus AMP2* gene leads us to the assumption that an antimicrobial peptide could also be produced by other amaranth species.

Key words: amaranth; PCR; internal transcribed spacer; chitin-binding domain.

Abbreviations: Ac-AMP peptide, antimicrobial peptide isolated from *Amaranthus caudatus* seeds.

Introduction

Antimicrobial substances of different molecular weights are used by plants for defence against a variety of pathogens and pests. Antimicrobial agents include the products of plant secondary metabolism (phytoalexins), high-molecular defence proteins and antimicrobial peptides rich in cysteine (Cammue et al. 1994). Plant defensins, thionins, lipid transfer proteins or hevein-type peptides are categorised as belonging to the cysteine-rich peptide group (Broekaert et al. 1995, 1997; Thomma et al. 2002).

Antimicrobial peptides isolated from *Amaranthus caudatus* seeds (Ac-AMPs) belong to the latter group. The characteristic feature of hevein and hevein-like peptides is their ability to bind to chitin and thus provide defence against pathogenic fungi (Broekaert et al. 1992, 1997). Although the exact mechanism of protection still remains unclear, it will most probably be similar to that of plant defensins. The salient feature of defensins is their capacity to bind to the fungal cell wall resulting in a change to the ion permeability of membrane (Thevissen et al. 1999). As well as being effective against plant fungal pathogens, Ac-AMPs have also been shown to inhibit the growth of some G^+ bacteria (Broekaert et al. 1992).

Genes encoding the antimicrobial peptides have not only been identified in *A. caudatus*, but also in *A. retroflexus*; these have been named as Ar-AMP (Lipkin et al. 2005). However, among the amaranths, most research has been concentrated on the *A. caudatus* antimicrobial peptide Ac-AMP2 (Broekaert et al. 1992; De Bolle et al. 1993, 1996). With the exception of the above-mentioned peptide, the presence of an antimicrobial peptide has until now not been proven in other amaranth species. Due to the antimicrobial qualities of Ac-AMP2, the *AMP2* gene has been used for the production of transgenic tobacco (De Bolle et al. 1996) and transgenic potatoes (Liapkova et al. 2001). In the case of transgenic potatoes a novel PCR method based on *A. caudatus AMP2* gene detection was developed (Pribylova et al. 2006).

The detection of the transgenic potatoes mentioned above was done using primers specifically amplifying the gene for *A. caudatus* Ac-AMP2 antimicrobial peptide. As there is a lack of information regarding the presence of the "*AMP2*" gene in other amaranth species, we decided to investigate this phenomenon using the above primers. The purpose of the present study was therefore to test several economically significant species and cultivars of the *Amaranthus* genus and to establish whether they contain the "*AMP2*" gene encoding the Ac-AMP2 antimicrobial peptide.

Table 1. The list of tested members of *Amaranthus* genus.

No.	*Amaranthus* sp.	Further specification	*AMP2*[a]	45[b]	246[b]	GenBank[c]
01	*A. caudatus* L.	pale seeds of *A. c.*	+	A	A	EF066732
02	*A. caudatus* L.	dark seeds of *A. c.*	+	A	A	
03	*A. cruentus* L.	cultivar Olpir	+	A	A	EF066733
04	*A. hybridus* L.	cultivar K432	+	A	A	EF066735
05	*A. hypochondriacus* L.	cultivar KONI2	+	A	A	EF066736
06	*A. caudatus* L.		+	T	A	–
07	*A. hypochondriacus* L.		+	A	C	EF066736
08	*A. cruentus* L.		+	A	C	EF066733
09	*A. albus* L.		+	A	C	EF066731
10	*A. blitoides* S. Watson		–	–	–	–
11	*A. blitum* L.		+	A	C	EF066734
12	*A. blitum* L.	subsp. *oleraceus* L.	+	A	C	EF066734
13	*A. lividus* L.		–	–	–	–
14	*A. retroflexus* L.		+	A	C	EF066737
15	*A. graecizans*	subsp. *silvestris* (Vill.) Brenan	–	–	–	–
16	*A. tricolor* L.		+	A	C	EF066738

[a] *AMP2* gene was either detected (+) or not (-).
[b] Type of nucleotide at the position 45 or 246 of the *AMP2* sequence; not examined (-).
[c] GenBank Accession Nos.

Table 2. Primers used for the amplification of amaranth DNA.

Primer	Sequence	PCR product
OWB40	5'-AATTGGTACCAGTCAAGAGTATTAATTAGG-3'	306 bp
OWB217	5'-CTACTTTCATGGACTACCAGC-3'	
A–ITS1–F	5'-GCCTTACGGACGAGCTATTG-3'	124 bp
A–ITS1–R	5'-CACGCTCAAGTTCATGTTCC-3'	

Material and methods

A total of 16 *Amaranthus* sp. members were tested (Table 1). Among these samples, *A. caudatus* species was examined three times, *A. cruentus* (*A. cruentus* and *A. cruentus* cultivar Olpir) and *A. hypochondriacus* (*A. hypochondriacus* and *A. hypochondriacus* cultivar KONI2) were tested twice. The amaranth seeds were obtained from the Botanical Garden of Masaryk University in Brno, Czech Republic, as well as from the Department of Food and Feed Safety at the Veterinary Research Institute in Brno. Amaranth seeds were cultivated on wet cotton wool in Petri dishes for 4–7 days at 22–24 °C. One hundred mg of plant sprouts were homogenised in a mortar using liquid nitrogen. The DNA was subsequently isolated using the DNeasy Plant Kit (Qiagen, Germany) according to the manufacturer's instructions.

The resulting DNA solution was used for a PCR reaction performed in a PTC-200 thermocycler (MJ Research Inc., USA). The reaction mixture contained 1U Taq-Purple DNA Polymerase PCR Master Mix (Top-Bio, Czech Republic), primers (OWB40, OWB217, A-ITS1-F and A-ITS1-R) and 2 μL of isolated DNA. The total volume of the reaction mixture was 20 μL and proceeded according to the following PCR step-cycle programme: pre-incubation at 96 °C for 15 min, denaturation at 96 °C for 10 s, annealing at 45 °C for 20 s, and extension at 72 °C for 40 s. The cycle was repeated 40 times followed by a final extension at 72 °C for 2 min.

The PCR employed a duplex system. Primers OWB40 and OWB217 specific to the sequence of the *AMP2* gene encoding antimicrobial peptide Ac-AMP2 in *A. caudatus* have been described previously (De Bolle et al. 1996). An amaranth gene encoding internal transcribed spacer (*ITS*) 1 and 2 of 5.8 S rRNA was used as an internal amplification control. Primers A-ITS1-F and A-ITS1-R were suggested by the Primer3 software for its amplification (Table 2).

Plasmids with cloned PCR products (PCR Cloning Kit, Qiagen, Germany) of *AMP2* and *ITS* genes were used as positive controls. The isolation of plasmids was performed using the QIAprep Spin (Qiagen, Germany). The presence of the inserts was ascertained by sequencing (MWG Biotech, Germany).

The PCR products were visualised by electrophoresis (voltage: 5V/cm, time: 30–40 min) using 2.0% agarose gel. Agarose (Amresco, USA) and 0.5 × TBE buffer were used for the preparation of the gel. Ethidium bromide (Amresco, USA) of final concentration 0.075 μg/mL was added. A 100 bp ladder (Malamité v.o.s., Czech Republic) was used as a weight standard. Samples with amplified *AMP2* genes were shipped for sequencing (MWG Biotech, Germany).

The putative genes for the *A. albus*, *A. caudatus*, *A. cruentus*, *A. blitum*, *A. hybridus*, *A. hypochondriacus*, *A. retroflexus*, and *A. tricolor* antimicrobial peptide were deposited with the GenBank database (Benson et al. 2007) under the accession numbers EF066731, EF066732, EF066733, EF066734, EF066735, EF066736, EF066737, and EF066738, respectively (Table 1).

Results and discussion

To avoid false negative results, the presence of the *ITS* gene was determined simultaneously with the *"AMP2"* gene. False negatives can appear as the result of PCR inhibitors that might be present in the DNA solution, for example, certain buffer components, SDS or laboratory gloves talc (Wilson 1997). The *ITS* gene en-

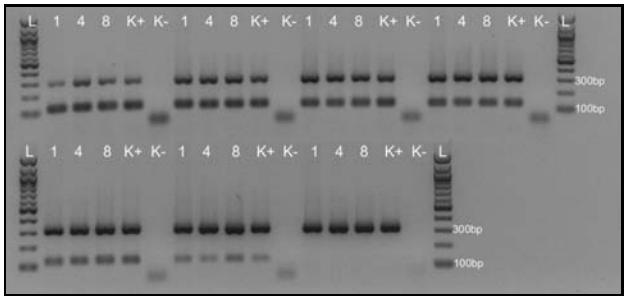

Fig. 1. Testing of different concentrations of primers OWB40/217 and A-ITS1-F/R. One hundred bp ladder (lane L), sample No. 1, 4 and 8 (lane 1, 4, 8), positive PCR control (lane K+), negative PCR control (lane K-). Concentrations of OWB40/217 primers were always 10 pM; the concentrations of A-ITS1-F/R primers were (from the upper left corner): 6 pM, 5 pM, 4 pM, 3 pM, 2 pM, 1 pM and 0.5 pM (lower right corner).

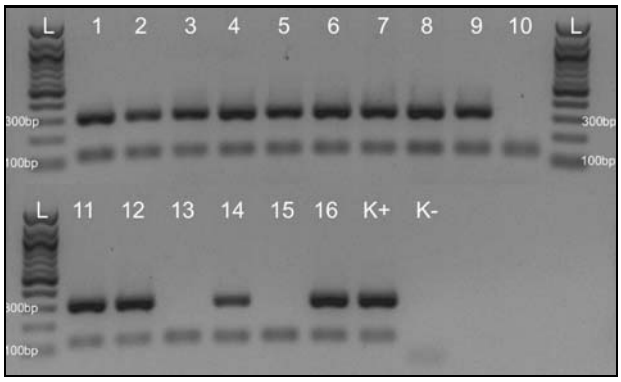

Fig. 2. Testing of *Amaranthus* sp. for the presence of the gene encoding an antimicrobial peptide. One hundred bp ladder (lane L), amaranth sample No. 1–16 (lanes 1–16), positive PCR control (lane K+), negative PCR control (lane K-).

codes the internal transcribed spacer 1 and 2 of 5.8S ribosomal RNA and was described in GenBank for *A. caudatus* (GenBank AF210907), *A. cruentus* (GenBank AF210912) and *A. hypochondriacus* (GenBank AF210917). The locus sequences are identical for all the mentioned amaranths except for the *A. hypochondriacus*, which has an adenine insertion at position 201. Therefore, the sequence of nucleotides 1 to 200 was selected for the design of A-ITS1 primers.

As both genes were identified in one reaction (duplex system) it was necessary to support the amplification of the *"AMP2"* gene against the *ITS* gene. Therefore, it was necessary to determine the most appropriate concentrations of both sets of primers (OWB-40/217 and A-ITS1-F/R). In all cases the concentration of OWB-40/217 primers was 10 pM, while the concentration of A-ITS1-F/R primers decreased from 6 pM to 0.5 pM. The concentrations of primers selected as the best were 10 pM and 2 pM for OWB-40/217 and A-ITS1-F/R, respectively (Fig. 1).

Sixteen different amaranth species seeds (Table 1) were subsequently tested by the suggested duplex PCR system using the above-mentioned primer concentrations (Fig. 2). Amplification of the 124 bp fragment corresponding to the *ITS* gene was observed in all samples. The amplification of the *ITS* gene indicated that no PCR inhibitors were present in any of the samples. The 306 bp long PCR product of the *"AMP2"* gene was detected in all samples, except for samples No. 10 (*A. blitoides*), 13 (*A. lividus*) and 15 (*A. graecizans*, subsp. *silvestris*).

Sequencing of 13 isolates positive for the *"AMP2"* gene (Table 1, Fig. 2) resulted in a sequence 306 nucleotides long, bordered by primers OWB40 and OWB217. A sequence identical with the sequence of the *"AMP2"* gene from *A. caudatus* (GenBank X72641) published previously was obtained after the subtraction of 10 nucleotides, due to the OWB40 primer binding to the *"AMP2"* gene up to the 11th nucleotide. All sequences, except the one from the sample No. 6 (*A. caudatus*), which was completely identical to the previously published sequence (GenBank X72641), were submitted to the GenBank and were assigned the accession numbers (Table 1). The sequences deposited with GenBank originated from two independent PCR reactions and two independent sequencing processes.

Two significant polymorphisms were detected by the sequencing of otherwise completely identical sequences (Table 1). At the nucleotide position 45 in the sample No. 6 thymine was present (*A. caudatus*), whereas adenine was found at this position in all remaining samples. The exchange of thymine for adenine will be manifested in the amino acid substitution: cysteine (TGT) will be replaced by serine (AGT). The sequence of the *A. caudatus "AMP2"* gene described previously in GenBank has thymine at position 45, which is in accordance with our *A. caudatus* sequence in the sample No. 6, but not in samples No. 1 and 2. The occurrence of different nucleotide at the same nucleotide position within one amaranth species (nucelotide 45: samples No. 1, 2 and 6 and also nucleotide 246: samples No. 3 and 8 and 5 and 7) could be a consequence of different cultivars within one species (Table 1).

The second polymorphism was found at the nucleotide position 246. Adenine was found to be here in the first six samples (No. 1–6), while cytosine was present at the same position in the remaining seven samples (No. 7, 8, 9, 11, 12, 14 and 16). This exchange of adenine for cytosine will result in replacing the lysine encoded by the AAG triplet (amaranths No. 1–6) by glutamine encoded by a CAG triplet (samples No. 7, 8, 9, 11, 12, 14 and 16). The polymorphic site is located in the region of the C-terminal domain of the *"AMP2"* gene (De Bolle et al. 1993), whose function remains unclear at present. It has been suggested that it may function as a target signal but this hypothesis has not been confirmed (De Bolle et al. 1996). In the close proximity to the site of replacement an asparagine was found as a most probable target for N-glycosylation (Faye et al. 1989). No variation in the nucleotide sequence was detected in the chitin-binding domain of any of the 13 amaranths investigated in our study; it is critical for peptide function.

Amaranth is characterised by a wide range of ge-

netic variability and a high degree of polymorphism among populations; this is particularly evident in domestic versus wild species. A close relationship has been established among the *A. caudatus*, *A. cruentus* and *A. hypochondriacus* (grain species) and also between them and their putative antecedents *A. hybridus* and *A. quitensis* (Kunth). All three grain amaranths, including *A. hybridus,* have been classified in one evolutionary clade and it is supposed that their origin is monophyletic (Xu & Sun 2001).

The nucleotide sequence of the *"AMP2"* gene was almost identical in all 13 investigated isolates, including *A. hybridus*, thought to be the progenitor of grain amaranths. As we detected merely two polymorphisms among all the sequences, we can suppose that no significant changes occurred during the evolution of this gene sequence, and that it is highly conserved. The preservation of a particular gene during the evolution indicates the significance of this gene and as a result its protein, for the species in question. Therefore, it is logical to suppose that amaranth antimicrobial peptide carries out important tasks for the plant and for this reason it has been maintained during the evolution of *Amaranthus* genus.

The translation of the *"AMP2"* gene to antimicrobial peptide was expected in all 13 amaranths tested in our study. It was confirmed by the fact that all tested sequences were identical with the sequence of the previously described *A. caudatus* gene (GenBank X72641), in which translation into antimicrobial peptide was described (De Bolle et al. 1996). The two polymorphisms described above should not affect the formation of antimicrobial peptide because they do not lie in the chitin-binding domain crucial to the function of the peptide.

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