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PREDICTION OF THE POSSIBLE ROLE OF MAIZE TYPE 3 RIBOSOME-INACTIVATING PROTEIN IN ITS ROOT SYSTEM PROCESSING

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Abstract

The relative expression patterns of maize (*Zea mays* L.) ribosome-inactivating protein 1 (RIP1)/type 3 ribosome-inactivating protein transcript were analyzed using reverse transcription and polymerase chain reaction (RT-PCR) with template materials collected separately from mature and immature root, leaf and seed tissues. The presence of RIP transcript was only detected in mature seed and immature root tissues, but not in mature roots or leaves of test plants. The present gene expression results may not only confirm the previous suggestions with regard to the involvement of maize RIP1 in seed tissue development and germination, but also may predict the possible role of same RIP in root processing of maize plant.

Introduction

Ribosome-inactivating proteins (RIP) are a group of proteins with site-specific rRNA N-glycosidase activity (EC number 3.2.2.22). They specifically remove a universally conserved adenine residue from the sarcin/ricin loop of the large ribosomal RNA in both prokaryotic and eukaryotic cells (Barbieri *et al.* 1993, Girbes *et al.* 2004, Stirpe and Battelli 2006). They make susceptible ribosomes impaired in translational elongation processes and so are a group of translational inhibitors (Wool *et al.* 1992). These proteins are frequently found in plant species (Barbieri *et al.* 1993, Stirpe 2013). The first identified RIP were two potent toxins, namely ricin, from the seeds of *Ricinus communis* L., and abrin, from the seeds of *Abrus precatorius* L. (Van Damme *et al.* 2001). Ribosome-inactivating proteins are generally classified into two categories including type 1 and type 2 proteins. Type 1 is composed of a single chain basic proteins while type 2 RIP are heterodimers consisting of an A chain having enzymatic N-glycosidase activity and B chain/lectin with sugar binding domain (Stirpe and Battelli 2006). In some plants such as maize and barley, there are peculiar single polypeptide type 1 RIP without a lectin side chain, but are unusual in being acidic proteins until they are activated by proteolysis. These RIP are considered as type 3 RIP (Hey *et al.* 1995).

Complex biological roles have been ascribed to RIP in different organisms, so far. They have mostly been linked to antiviral, antifungal and insecticidal defense mechanisms (Hong *et al.* 1996, Iglesias *et al.* 2005). The new understandings of the RIP enzymatic activities enhanced their diverse applications in therapeutics and medicine in human beings as well as in plant protection against pathogen attacks (Puri *et al*. 2012, Stirpe 2013).

Ribosome-inactivating proteins have been characterized from several plant tissues such as seeds, leaves, bark and fruits (Barbieri *et al.* 1993, Girbes *et al.* 2004). However, their gene expression patterns were found to be highly differential and variable in different tissues (Bolognesi *et al.* 2002). Plants RIP induction has been suggested to be influenced by different developmental stages such as aging and various environmental clues such as biotic or abiotic factors (Song *et al.* 2000, Iglesias *et al.* 2008, Xu *et al.* 2007, Parente *et al.* 2008, Jiang *et al.* 2008).

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Over the last 50 years, maize is increasingly planted in all of the countries. Due to the commercial importance of this crop, there is significant interest in understanding its expressed sequences and identification of the biological functions of the encoded proteins. There is a wealth of information available from phenotypic descriptions for this plant. This information can be synergistically integrated with well-defined genes at transcriptional and functional levels. To date, the undergoing maize complete genome sequencing project along with large scale expressed sequence tags (EST) and full length cDNA sequencing projects offers a huge advantage toward the main goal (Alexandrov *et al.* 2009).

It has been known that maize plants express multiple ribosome-inactivating proteins (Van Damme *et al.* 2001). Among them, seed endosperm type 3 RIP (maize RIP 1) with a molecular weight of about 32 kD is one of the more characterized RIP in this plant. It has been reported that the expression level of this RIP is controlled by the endosperm transcriptional activator "Opaque-2" and it has been suggested to be associated with endosperm development and seed germination processes (Bass *et al.* 1992). Besides these, it has been recently reported that maize seed RIP efficiently exhibit anti-fungal activity *in vitro* (Lanzanova *et al.* 2009). Our literature reviews showed that the expression patterns of this gene has not been studied in different tissues of maize plant, so far. Generally, studies on the tissue specific gene expression profiles enhance the knowledge about the possible biological or developmental functions of a gene product, *in vivo*. Such a study is necessary for the functional analysis of the expressed sequences of commercially important maize plant.

The major objective of the present study was further characterization of maize type 3 RIP/ maize RIP 1 (Accession no. M83926). Analysis of the gene expression profiles in different tissues including mature and immature seed, root and leaf helped us to predict the possible role of type 3 RIP in root system of maize plant.

Materials and Methods

E. coli strain DH5 α was used for bacterial transformation. Plasmid vector pGEM-T easy (Cat. no. A1360; Promega) was used for PCR product cloning. Trizol reagent (Cat. no. RN7713C; RNX^{TM}; CinnaGen) was used for total RNA isolation. mRNA purification kit was provided by QIAGEN, USA (Cat. No.70022). AcessQuick^{TM} RT-PCR System was purchased from Promega (Cat. no. A1701). Fermentas DNA extraction kit (Cat. no. K0513) was used for the purification of the PCR product from the agarose gel. Other chemicals used in this research work were of molecular biology grades.

Seeds of maize (*Zea mays* cv. A188) were provided by Dr B. Baghban Kohnehrouz, (Genetic Engineering Lab., Department of Plant Breeding and Biotechnology, University of Tabriz, Tabriz, Iran). Test plants were allowed to grow completely in greenhouse conditions (28°C, day to night period of 12 hrs : 12 hrs and humidity of about 70%). Experimental materials were collected from fresh root, leaf and seed tissues at immature and mature stages and used for the RNA isolation and RT-PCR reactions.

Total cellular RNA was separately isolated from the root/leaf/seed materials at non-matured and matured stages using Trizol reagent. About 0.2 g of each material was fine powdered using liquid N_2 and 2 ml of Trizol reagent was added to homogenize it at room temperature (RT). 200 µl of chloroform was added to the mixture, mixed for 15 second, incubated on ice for 5 min and centrifuged at 13000 g for 15 min. The upper phase was transferred to another tube and RNA was precipitated with an equal volume of isopropanol. The pellet was washed in 1 ml of 75% ethanol, dried at RT and dissolved in 30 µl RNase-free water. The integrity of the RNA was tested on 1% non-denaturing agarose gel using TBE running buffer. Poly (A^+) RNA was purified from total

RNA using oligo dT-columns according to the provided kit protocol. The integrity of the purified mRNA was also analyzed by electrophoresis using 1% non-denaturing agarose gel. The quantity of the RNA in the starting materials for the next experiments was measured spectrophotometrically (Ausubel *et al.* 1991).

For the amplification by RT-PCR, the specific primers were designed based on already reported RIP cDNA sequence from maize plant (Acc. No: M83926). The primer pairs were designed by Primer 3 software (http://www. Primer3plus.com). The nucleotide sequences of the primers are shown in Table 1.

In order to analyze the expression pattern of the gene, the RT-PCR reactions were separately performed using one-step AcessQuick™ RT-PCR System. About 0.5 µg of each mRNA sample was mixed with 25 µl Master Mix (2x) and 1 µl of primer set (at the final concentration of 0.2 µl). The mixtures were adjusted to a final volume of 50 µl using nuclease-free water. The reaction mixtures were incubated at 45°C for 45 min, the subsequent PCR amplification was carried out after a pre-denaturation stage at 95°C for 3 minutes in 25 cycles. The details of PCR steps performed for each sample were presented in Table 1.

Table 1. Primer set and PCR amplification steps.

Primer sequences	
Forward: 5´TATGGCGCAAACAAACAAAA3´	
Reverse: 5´GTGTCGTTGCATTGATCAGG3´	
PCR amplification steps	
Denaturation	93°C/1 min
Annealing	57°C/1.5 "
Extension	72°C/2 "
Final extension	72°C/10 "

In the next step, amplified products were extracted from the agarose gel, cloned in pGEM-T easy cloning vector (Ausubel *et al.* 1991). The cloned fragments proceeded for the sequencing in Microsynth DNA sequencing center at Switzerland.

The nucleotide and deduced amino acid sequences of the isolated cDNA were analyzed by computing at BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.blast.com/) and Multalign sequence alignment software at Expasy proteomic tools at http://www.expasy. org/tools/.

Results and Discussion

Maize seed ribosome-inactivating protein gene had been originally identified as an abundant and opaque-2-regulated gene and it had been reported to be associated with endosperm tissue development (Soave *et al.* 1981). Later on, this RIP was shown to be a zymogen that is activated by proteolysis in vivo during seed germination and so it was designated as proRIP (Bass *et al*. 1992).

The homology search data using BLAST server showed that maize proRIP is more similar to the corresponding proteins of monocotyledonous plants investigated hitherto. Multiple amino acid sequence alignments between the maize proRIP and other plant RIP using MultAlin program at Expasy proteomic tools is shown in Fig. 1. In compare to monocotyledonous plants RIP, the homology scores between the sequences of maize proRIP and those of dicotyledonous plants were

found to be very low and not significant. Maize RIP shares highest homology with *O. sativa* and *H. vulgare* (26 and 24%, respectively). Proteins resembling the maize RIP have been previously identified in several close relatives of maize and barley (Chaudhry 1994, Hey *et al.* 1995). Our results also confirmed the close identity of maize RIP with a corresponding protein from barley.

In general, the total sequence similarity among different RIP has been observed to be low (about 15 - 30%), but there is greater homology in regions that correspond to the proposed active site in different RIP (Stripe 1992). Glutamic acid, alanine, and arginine in the sequence order "E*-A*-X-R*" are three functionally important amino acids which are highly conserved in the active sites of the all RIP investigated, so far. The homologies between the functionally important sequences of maize proRIP and others are shown in Fig. 1.

A number of the physico-chemical characteristics of maize and other RIP were theoretically calculated using computer tools available at http//www.expasy.org. Computed data are presented in Table 2. Analysis of the data revealed that despite to the most of plant RIP, maize proRIP is an acidic protein at physiological conditions. This is in agreement to the unique characteristics of type 3 RIP that are found to be unusual in being acidic proteins until they are activated by limited proteolytic removal of both termini as well as internal acidic residues (Hey *et al.* 1995, Bass *et al.* 2004). However, data revealed that *Populus* and *Ricinus* RIP also resemble to maize proRIP and are classified as acidic proteins. Since there are no reports to our knowledge concerning the *in vivo* activities of *Populus* and *Ricinus* RIP, they need to be investigated further for detailed explanations.

Computed data analysis also revealed that maize proRIP is similar to other plant RIP in its amino acid composition and its stability (Table 2). Alanine and leucine are contributing the highest amino acid composition at about 80 and 20% of the plant RIP, respectively. Analysis showed that almost all of the plants RIP are structurally and functionally grouped into the stable proteins with high aliphatic indices.

Large scale surveys for plant RIP have been previously revealed that wide species of plants express RIP (Barbieri *et al.* 1993, Stirpe 2013). Because of their broad distribution and conserved enzymatic activity, they have been generally accepted to make important contributions to the plants life (Veronese *et al.* 2003). However, their roles have not been fully understood in details, so far.

Maize RIP1 (type 3 RIP) is one of the earliest characterized plants RIP in terms of its expression and regulation in seed tissues (Soave *et al.* 1981). To study its expression pattern during plant growth and development, the presence of its transcript as well as its relative expression level were analyzed at three different parts including seed, leaf and root at mature and immature tissues. In overall, six samples each with two replications were considered for this experiment. Analysis of the expression was performed by RT-PCR method using the same amounts of the starting mRNA and a specific primer set designed based on the previously reported sequence (Acc. No. M83926). Results of the separating gel revealed the presence of detectable bands only in lines belong to mature seed and immature root tissues (Fig. 2). No detectable bands were observed in immature seed, mature root, immature and mature leaf tissues. The expression level of maize RIP1 gene was compared according to the relative abundance of the RT-PCR products on the separating gel. As seen on the photograph, there is no considerable difference between the levels of maize RIP1 gene expression in matured seed and immature root tissues.

This experiment results may not only confirm the suggestions of Bass group (1992) with regard to the involvement of maize RIP 1 in seed endosperm development and seed germination processes, but also may predict its possible role in root system of maize plant. The absence of the expression signals in the leaf samples may also predict that maize RIP 1 protein not involved in maize plant leaf processing.

Fig.1. Multiple amino acid sequence alignment. 1= *Zea mays* (M83926); 2 = *Oryza sativa* (NP_001042090); 3 = *Hordeum vulgare* (B5TWK6); 4 = *Secale cereale* (Q7M1Z3); 5 = *Triticum aestivum* (1919435A); 6 = *Muscari armeniacum* (Q8L5M4); 7 = *Hyacinthus orientalis* (Q677A1); 8 = *Populus trichocarpa* (XP_002328056); 9 = *Sorghum bicolor* (XP_002463678); 10 = *Ricinus communis* (0408164A); 11 = *Brachypodium distachyon* (C3SA73). The identical sequences and the functionally important conserved amino acids are shown in light and dark gray boxes, respectively.

Table 2. Predicted physic-chemical characteristics of maize and number of closely related plant RIP.

	Zea mays	*Oriza sativa*	*Hordeum vugare*	*Secale cereale*	*Triticum aestivum*	*Muscari armeniacum*	*Populus trichocarpa*	*Sorghum bicolor*	*Brachypodium distachyon*	*Hellebonus orientalis*	*Ricinus communis*
Number of amino acids	300	282	277	280	275	298	330	303	197	258	265
Molecular weight (kDa)	33	30	29	30	29	33	37	33	21	29	29
Isoelecteric point (pI)	**5.53**	9.30	9.67	9.77	9.69	9.94	**4.70**	9.37	7.90	9.68	**6.49**
Highest amino acid composition	Ala: 11.1%	Ala: 12.1%	Ala: 12.3	Ala: 11.8%	Ala: 10.5%	Ala: 10.7%	**Leu: 8.8%**	Ala: 13.9%	**Leu: 10.2%**	Ala: 10.1%	Ala: 9.1%
Instability index	29.42 stable	26.44 stable	28.21 stable	32.85 stable	31.76 stable	38.07 stable	28.93 stable	**41.29 unstable**	38.72 stable	32.72 stable	35.71 stable
Aliphatic index	80.03	86.88	84.95	79.54	78.80	91.38	82.70	82.54	92.54	78.33	90.23

Data were computed by Protoparam at http//www.expasy.org/tools/.

It had been previously reported that some plants such as *Luffa cylindrica* and *Phytolacca americana* produce RIP in their hairy roots and secret them as a part of root exudates to the rizosphere to prevent pathogen infection (Poma *et al.* 1997, Park *et al.* 2002). Based on the presence or absence of the RIP 1 gene expression signals at two different developmental stages of maize roots, we predict that this RIP may be possibly involved in the developmental processing of root system in maize plant. However, this needs further investigation to be clarified.

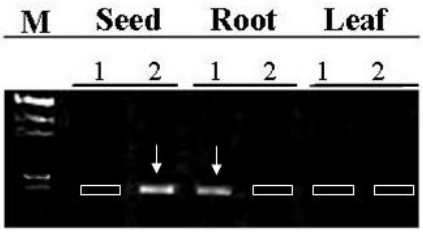

Fig. 2. RT-PCR analysis of RIP 1 gene expression in maize M = *Eco*RI and *Hind*III double digested lambda DNA marker; Lines 1 = Immature tissues, lines 2 = Mature tissues, Closed boxes = No detectable band.

To date, due to the commercial importance of maize, there is a significant interest towards understanding of its expressed sequences and their roles that may be required for genetic improvement of this plant. Present results could help us to initially predict the possible role of maize type 3 RIP in root system processing.

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