Molecular Cloning and Phylogenetic Analysis of Two Plant-Parasitic Nematode 14-3-3 Genes

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Abstract

Full-length cDNA sequences of highly conserved ubiquitous 14-3-3 proteins were cloned from plant parasitic Heterodera glycines and Meloidogyne incognita using RT-PCR and RACE methods. The two genes were named Hgly2 and Minc3, respectively. Hgly2 consisted of nucleotide sequence of 1027 bp and Minc3 of 1525 bp. And the open reading frames (ORF) encode peptide of 251 and 261 amino acids separately. Homology analysis showed that the deduced amino acid sequences shared the high homology with different nematode species. The phylogenetic analysis indicated that the proteins from plant parasitic nematode were more similar to insect proteins than plant and other animal proteins reported by previous research.

Keywords: Heterodera glycines, Meloidogyne incognita, 14-3-3 proteins gene, Race, Phylogenetic analysis

1. Introduction

A highly conserved family of regulatory proteins formed by 14-3-3 proteins is seemed to be specific to eukaryotic organisms (Fu et al., 2000; Jaubert et al., 2004). The 14-3-3 monomers have a molecular weight of approximately 30 kDa and an isoelectric point of about 5, but functional 14-3-3 exists as a dimer (Wang and Shakes, 1996). First identified in a survey of mammalian brain proteins, 14-3-3 proteins were named on the basis of their separation properties in two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis (Moore and Perez, 1967). 14-3-3 proteins putatively participate in many vital movements. For example, they may be central to integrating the regulation of biosynthetic metabolism, cell proliferation, survival, and other processes in human cells (Rubio et al., 2004). Drosophila 14-3-3 proteins have been shown to function in RAS/MAP kinase pathways that influence the differentiation of the adult eye and the embryo even regulation the entry into mitosis in the undisturbed cell cycle. These proteins also function in normal cell cycle progression, in addition to checkpoint regulation (Su et al., 2001). Moreover in C. elegans, 14-3-3 proteins were reported as interacting proteins of a major life span regulator. They could regulate life span and possibly provide the missing link to connect two well known signaling pathways that control longevity: insulin/IGF-1 and caloric restriction (Wang et al., 2006; Araiz and Château, 2008). Then another research indicated that 14-3-3 proteins binds to and regulates DAF-16 by sequestering it in the cytoplasm (DAF-16 was another important life span regulator in the insulin/IGF-1 signaling pathway). And the mechanism was similar to the regulation has been reported in mammalian cells (Li et al., 2007). 14-3-3 gene even plays a crucial role in the early events leading to polarization of the C. elegans zygote (Morton et al., 2002).

Study in the plant-nematode interaction, 14-3-3 proteins are thought to be pathogenicity factors involved in the invasion of the root tissue and in the induction and maintenance of feeding cells (Klink et al., 2009). A 14-3-3 protein was isolated through direct qualitative analysis of proteins secreted from M. incognita J2 (Jaubert et al., 2002). Next year, 14-3-3 protein was found in the oesophageal glands of second stage juveniles (Abad et al., 2003). In 2008, 486 proteins were identified from M. incognita secretome include 14-3-3 (Bellafiore et al., 2008). In addition 14-3-3 proteins can bind to and affect a wide variety of plant proteins, such as chaperones that
prevent proteolysis; adaptors for mediating interactions between proteins; regulators of intracellular protein distribution and transcriptional regulators (Davis et al., 2009). Hassan et al. revealed that 14-3-3 proteins may have a key role in co-ordination of mitosis, metabolism, stress response and organelle trafficking as the feeding site develops (Hassan et al., 2010).

The interaction between the nematode and its host plant has been concerned for several decades, especially in damage severely plant-parasite nematode. Here we researched two plant parasite nematode 14-3-3 genes, would lead to a better understanding of the molecular events and regulatory mechanisms involved in plant parasitism and allow the development of target-specific strategies to limit crop damage by these pathogens.

2. Materials and Methods

2.1 Collection of nematode

The nematodes were reared by their host plants in sunlight greenhouse. Cysts of H. glycines and oocysts of M. incognita incubated at 25 °C in the laboratory as described by Nitao et al. (1999). Suspension (including J2 hatched in 24 h) was collected into Eppendorf tubes, and then centrifuged at 12000 rpm, 15 min. Repetition until the weight up to 100mg, immersed in liquid nitrogen immediately. J2 were stored at -75°C until further use.

2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from the frozen materials by RNAiso Plus (TaKaRa, China) following the manufacturer’s instructions. The RNA was treated by an RNase-free DNase I for eliminating the residual genomic DNA present in our preparation. The quality of RNA was tested by 1% (w/v) agarose gel electrophoresis. The first-strand cDNA for reverse transcriptional PCR (RT-PCR) was synthesized by following 3′-Full RACE Core Set Ver.2.0 (TaKaRa, China) with 3.5 μL of total RNA as the template.

2.3 Internal amplification

2 pairs of Gene-specific primer (GSP) for amplification H. glycines and M. incognita 14-3-3 proteins gene were designed by primer 5.0.

Hgly2-1: 5´-GCGTTCGATGATGCGATTGCTG-3´,
Hgly2-2: 5´-ATTTACCCAATCAAAGTGCC-3´.
Minc3-1: 5´-TCTTGCGGTGTCTTCTGCTAT-3´,
Minc3-2: 5´-AAAAGACTGCTTACGCAACTGAC-3´.

Both reactions were performed with 50 μL reaction mixture containing 2 μL single-stranded cDNA, 8 μL 1× cDNA Dilution Buffer II, 2 μL of each primer (10 μM), 25 μL 2× GC Buffer, 0.5 μL Tag polymerase (5U/μL), 10.5 μL dH2O. The condition for amplification of cDNA segments were 94°C for 3 min, 30 cycles at 94°C for 30 s, 55°C for 30 s , 72°C for 30 s then 72°C for 10 min.

2.4 RACE

In order to obtain the full-length transcript, a rapid amplification of cDNA ends (RACE) procedure was employed to amplify the 5´ and 3´ end of the coding region according to the instructions (5′-Full RACE Kit and 3′-Full RACE Core Set Ver.2.0, TaKaRa, China). The full-length cDNA sequences from H. glycines and M. incognita were named as Hgly2 and Minc3. In H. glycines, 5′ RACE GSP primers Hgly2-3: 5´-TCGGATGTCCAAGCGTGCA-3´ was applied in outer PCR and Hgly2-4: 5´-GTCCAGCTCAACGCAATGCATC-3´ in inner PCR. 3′ RACE GSP primers were Hgly2-1 (outer PCR): 5´-GCCGATGATGCGATGCTG-3´ and Hgly2-5 (inner PCR): 5´-CTGGGATGAGCAGGAAGG-3´. In M. incognita, the GSP primers of 5′ RACE were Minc3-3 (outer PCR): 5´-AACACCGCAAGAAGACGAC-3´ and Minc3-4 (inner PCR): 5´-TCAGCCTGTTCGCCAGTTGG-3´. Minc3-5: 5´-ATCGTTATTGGCTAGGTTC-3´ used with outer primer and Minc3-6: 5´-CACAAACAGGTACCAAAGGCGC-3´ used with inner primer in 3′ RACE. The PCR products were electrophoresed on 1% (w/v) agarose gel.

A blast search was completed in NCBI BLAST network server (http://blast.ncbi.nlm.nih.gov/ BLAST.cgi). Deduced amino acid sequences were aligned using DNastar software. Subcellular localization by Signalp 3.0 Server (www.cbs.etic.dk/services/signalp) and proteins analysis in ExPASy: ScanProsite tool (http://www.expasy.ch/tools/scanprosite). Alignment and phylogenetic analysis were performed using the software MEGA 4.1 and Clustal X.
3. Results

3.1 Sequence analysis

The cDNA fragments of 338 bp from H. glycines and 411 bp from M. incognita were attained by RT-PCR approach. The RACE procedure was further employed to obtain full-length sequences of the two genes. And results showed that cDNA clone encoding Hgly2 was 1027 bp with an open reading frame for a polypeptide of 251 amino acids (Fig.1) and a molecular mass of 28.47 kD. The cDNA clone encoding Minc3 was 1525 bp with 261 amino (Fig.2) acids and a molecular mass of 29.41 kD. The isoelectric point (pI) of Hgly2 was 4.48 and Minc3 was 4.36. Both Hgly2 and Minc3 have 14-3-3 gene family conserved regions (Fig.3). In addition no signal peptide and transmembrane regions were found.

Further, Subcellular localization and ScanProsite revealed that Hgly2 and Minc3 possibility be found in cytoplasmic, nuclear, cytoskeletal, mitochondrial, golgi, endoplasmic and plasma membrane. Hgly2 was predicted possession 4 protein kinase C phosphorylation sites, 2 cAMP-and cGMP-dependent protein kinase phosphorylation sites, 5 N-myristoylation sites, 7 casein kinase II phosphorylation sites, 1 Tyrosine kinase phosphorylation site and 2 N-glycosylation sites. Minc3 potentially hold 9 casein kinase II phosphorylation sites, 3 protein kinase C phosphorylation sites, 2 cAMP-and cGMP-dependent protein kinase phosphorylation sites, 4 N-myristoylation sites, 1 Amidation site, 1 Tyrosine kinase phosphorylation site and 1 N-glycosylation site.

3.2 Homology analysis

The full amino acid sequences we got were aligned with other species (Fig.4). We found that Hgly2 share 82.46% identity with Minc3; 94.76% with Bursaphelenchus xylophilus (GU130158.1); 93.59% with Caenorhabditis brenneri (EU726795.1); 93.59% with Caenorhabditis remanei (XM_003109679.1); 87.45% with Culex pipiens (GU227357.1); 88.09% with Drosophila melanogaster (NM_165740.2); 70.69% with Rattus norvegicus (BC089860.1) and 81.97% with Homo sapiens (NM_003404.3).

While Minc3 identity to B. xylophilus was 82.74%; C. brenneri, 82.89%; C. remanei, 82.89%; C. pipiens, 87.45%; D. melanogaster, 88.09%; R. norvegicus, 70.69% and Homo sapiens, 81.97%.

Comparison of protein sequences was also revealed that the N terminal 10 amino acids and the C terminal 20 amino acids showed more variations than the other parts, indicating that these amino acids may be responsible for the isoform specificities.

3.3 Phylogenetic analysis

Based on the deduced amino acid sequences, a phylogenetic tree was constructed (Fig.5). All the sequences used in our analysis got from NCBI but 14-3-3 proteins from Entamoeba histolytic were approved distant related (Wang and Shakes, 1996) and other protozoa not employed. 14-3-3 proteins from 24 species could be divided into 4 groups through the comparison. Nematode proteins except Trichinella spiralis were clustered together and appeared to be more closely to insect, distantly to animal. T. spiralis was a human parasite more similar to insect than plant parasite nematode. The plant lineage formed a distinct grouping in N-J trees, suggested an early divergence from the other species. Invertebrate proteins group including nematode and insect with the nonepsil on mammalian and the result basically consisted with former research (Wang and Shakes, 1996).

4. Discussion

Protein 14-3-3 was also named tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein. Since first reported about 14-3-3 proteins, the researchers have done a lot of work in animal and human parasite (Siles-Lucas et al., 2003; Joshua et al., 2009) but on the contrary for plant parasite nematodes. We know H. glycines and M. incognita were two important pathogens for crops and vegetables in the world. As early as 1987, Meloidogyne spp. was reported responsible for a large part of the annual 100 billion $ losses attributed to nematode damage in US (Sasser et al., 1987). Soybean cyst nematode suppressed US soybean yield more than any other disease during 2003 to 2005, soybean yield suppression was 2.9 million ton in 2003 and 1.9 million ton in 2005 (Wraether and Kenning, 2006). Unfortunately we hadn’t high efficiency and environmental friendly control methods presented due to nematodes body was extremely small, physiology and metabolism almost incomprehension for us.

Protein 14-3-3 was speculated to participate in the process nematode infect host plant. And two 14-3-3 isoforms isolated from M. incognita infective larvae (Jaubert et al., 2004; Curtis, 2007; Dubreuil et al., 2007) maybe involve in infection. Expression 14-3-3 gene in H. schachtii has also been localized within genital primordia of infective J2 (De et al., 2001). And we could obtain the partial sequences of H. glycines 14-3-3 gene from NCBI (article unpublished) and the full long still not reported. In our study we cloned the full cDNA sequence of
14-3-3 gene from *H. glycines* and *M. incognita* further alignment amino acid sequences, the results showed that the sequence of *H. glycines* 14-3-3 gene was 100% homology with the fragment in GenBank (GenBank: AF402309.1) and *M. incognita* 14-3-3 gene was 97% similar to that previously reported (GenBank: AF070225.1).

The ubiquity of 14-3-3 gene was validated again by our subcellular localization analysis. Multiple sequence alignment and phylogenetic analysis showed 14-3-3 genes were the highly conserved this result was consistent with former research. We supposed the functions of these proteins be conserved between plants and nematodes. The important function was phosphatases. The 14-3-3 proteins bound to a wide array of target proteins and then modulation many proteins involved in phosphatases and protein kinases (Yaffe *et al*., 1997; Masters *et al*., 1999; Sehnke *et al*., 2002). Other functions of 14-3-3 proteins also reported. For example, cell regulatory pathways, including signal transduction, apoptosis, stress response and transformation, could be targeted by one or several 14-3-3 gene isofrom products in organisms ranging from yeast to human (Finnie *et al*., 1999; Van Hemert *et al*., 2001).

Therefore studies on these highly conserved proteins may allow novel strategies of plant-parasite nematode control. Maybe a potential strategy by interfering with the expression of 14-3-3 genes would be used to control the two nematodes.

References


Figure 1. cDNA sequence and predicted amino acids sequence of \( Hgly2 \)

The lower-case characters indicate noncoding regions; the stop codon is indicated with an asterisk; the shadow areas indicates poly (A); underline sequences were proteins motif.
Figure 2. cDNA sequence and predicted amino acids sequence of Minc3

The lower-case characters indicate noncoding regions; the stop codon is indicated with an asterisk; the shadow areas indicates poly (A); underline sequences were proteins motif.
Figure 3. Conserved domains of amino acid sequences of Hgly2 (A) and Minc3 (B).

Figure 4. Alignment of deduced amino acid sequences of Hgly2 and Minc3 with other species. HEG, MEI, BUX, CAB, CAR, CUP, DRM, RAN, HOS denote 14-3-3 protein from H. glycines, M. incognita, B. xylophilus, C. brenneri, C. remanei, C. pipiens, D. melanogaster, R. norvegicus, Homo sapiens respectively; Dots indicate gaps introducing to facilitate the alignment; Identical and similar amino acid residues are shaded in black and gray.
Figure 5. A phylogenetic tree analysis with Hgly2, Minc3 and 22 species 14-3-3 proteins using Neighbor-Joining method.