Gardenia jasminoides Encodes an Inhibitor-2 Protein for Protein Phosphatase Type 1

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Abstract: Protein phosphatase-1 (PP1) regulates diverse, essential cellular processes such as cell cycle progression, protein synthesis, muscle contraction, carbohydrate metabolism, transcription and neuronal signaling. Inhibitor-2 (I-2) can inhibit the activity of PP1 and has been found in diverse organisms. In this work, a Gardenia jasminoides fruit cDNA library was constructed, and the GjI-2 cDNA was isolated from the cDNA library by sequencing method. The GjI-2 cDNA contains a predicted 543 bp open reading frame that encodes 180 amino acids. The bioinformatics analysis suggested that the GjI-2 has conserved PP1c binding motif, and contains a conserved phosphorylation site, which is important in regulation of its activity. The three-dimensional model structure of GjI-2 was built, its similar with the structure of I-2 from mouse. The results suggest that GjI-2 has relatively conserved RVxF, FxxR/KxR/K and HYNE motif, and these motifs are involved in interaction with PP1.

1 Introduction

Protein phosphatase-1 (PP1) is a major protein Ser/Thr phosphatase that is extraordinarily conserved from yeast to human, and Inhibitor-2 (I-2) is the most ancient of specific inhibitory proteins for PP1. 

Protein phosphorylation represents one of the most common post-translational modifications in eukaryotes. It affects 30–70% of all cellular proteins and some cellular processes [18, 22]. PP1 and PP2A, which belong to the phosphoprotein phosphatase (PPP) superfamily of protein Ser/Thr phosphatases, together account for more than 90% of the protein phosphatase activity in eukaryotes [23]. PP1 has barely changed during more than 1 billion years of eukaryotic evolution, have > 80% sequence identity of PP1 from yeast and man [7]. PP1 is ubiquitously distributed and regulates a broad range of cellular functions, including glycogen metabolism, cell-cycle progression and muscle relaxation (Terrak et al. 2004). For vertebrates, close to 200 PP1-interacting proteins (PIPs) have already been identified, which create a vast array of PP1 holoenzymes with a distinct set of substrates and mechanisms of regulation [11], such as glycogen particles, microfilaments, centrosomes, and the nucleus [2].

I-2 is a subset of PIPs, is conserved among all eukaryotes. I-2 was first identified as one of two heat-stable proteins from rabbits that can inhibit the activity of PP1 [9]. I-2 like activity has since been found in diverse organisms, from GLC8 in yeast to I-2 in Rabbit, Caenorhabditis elegans, Drosophila, Plasmodium falciparum, and humans [5, 13, 16]. A plenty of evidence indicated that I-2 function was regulated by phosphorylation. Phosphorylation of I-2 by GSK3 (glycogen synthase kinase-3) was studied for its effects on the PP1 catalytic subunit, and when I-2 (from rabbit) is phosphorylated in an inactive PP1-I-2 complex on Thr-72, the PP1 becomes activated [25].
In yeast, GLC8 encodes a homologue of type 1 protein phosphatase inhibitor-2. The GLC8 kinase is the cyclin-dependent protein kinase. GLC8 function requires Thr118 phosphorylation [20]. Evidence indicates involvement of I-2 in regulation of the cell cycle, modulation of mitosis by reversing phosphorylation of proteins phosphorylated by aurora protein kinase is a critical function for PP1. I-2 localized to the spindle, midzone, and midbody of mitotic human epithelial ARPE-19 cells, knockdown of I-2 by RNA interference produced multinucleated cells, with supernumerary centrosomes, multipolar spindles and lagging chromosomes during anaphase [20]. It was suggested that I-2 acts to enhance Aurora B by inhibiting specific PP1 holoenzymes that dephosphorylate Aurora B substrates necessary for chromosome segregation and cytokinesis. In mammalian cells, the expression level of I-2 fluctuates during the cell cycle, peaking at mitosis, and I-2 is phosphorylated by CDK-cyclinB1 at a conserved PxTP site during mitosis [16]. GLC7 is the sole PP1 in yeast. Mutations of the GLC7 cause mitotic defects, overexpression of GLC7 is lethal. Altogether, these observations indicate that I-2 plays a critical role in achieving successful mitosis and it is apparent that interfering with I-2 functions represents an attractive approach for pharmacological intervention.

It was also found that in Arabidopsis, Type one protein phosphatase1 (TOPP1) inhibited the kinase activity of SNF1-related protein kinase2 (SnRK2), which is a major components in ABA signaling pathway, and this inhibition could be enhanced by I-2 (e.g. At inhibitor-2). Supporting a negative role of TOPP1 and AtI-2 in ABA signaling, topp1 and atI-2 mutant plants displayed hypersensitivities to ABA and salt treatments [12].

The binding of PIPs to PP1 is mediated by docking motifs that are often only four to eight residues long. As PP1 specific inhibitory proteins, I-2 binds and blocks the PP1 catalytic active site. Most PP1 regulatory proteins and some PP1 substrates interact with PP1 via a primary PP1-binding motif (>90%), the RVxF motif, which generally conforms to the consensus sequence [K/R][K/R][V/I][x][F/W], where x is any residue other than Phe, Ile, Met, Tyr, Asp, or Pro [24], functions primarily as an anchoring motif, as RVxF peptide binding does not influence the overall structure nor alter the substrate specificity of PP1. Additional, recently identified docking sites, such as the SILK (KGILK), HYNE, RNYF, FxxR/KxR/K and MyPhoNE (RxxQV/I/LK/RxY/W) motifs [6, 10, 19]. Some Inhibitor-2:PP1 structure was determined in recent years [4, 14]. It was shown in Rabbit PP1: Rodent I-2 structure that I-2 interacts at three sites on PP1: RVxF motif, long ordered α-helix and the SILK motif. Most PIPs contain four to eight residue docking sequences that combine to create a large interaction surface for PP1 (Goldberg et al. 1995).
Figure 1. Alignment of GjI-2 with I-2 (like) and GLC8 amino acid sequence from other organisms. Amino acids are numbered at the right of the sequence. The abbreviation and Genbank accession number are: Gja (Gardenia jasminoides, AIX10938), Rat (Rattus sp., AAB35244), Has (Homo sapiens, CAA55475), Dme (Drosophila melanogaster, CAB57226), Mmu (Mus musculus, PDB:208G), Xla (Xenopus laevis, NP_001091136), Pfa (Plasmodium falciparum, XP_002808630), Sce (Saccharomyces cerevisiae, AAA53673), Ath (Arabidopsis thaliana, BAA97463), Sin (Sesamum indicum, XP_011076197). Underlined: Five binding or phosphorylation motif; Identical residues are shaded; Predicted alpha helices by Swiss-model of GjI-2 are marked with H.

Gardenia jasminoides originates in Asia and has been in cultivation for at least a thousand years. The fruit of G. jasminoides is used in Asian countries as a natural colorant, and as a traditional herbal medicine. Crocin, crocetin and geniposide are the main secondary metabolites in the fruit, and they all exhibit a wide range of pharmacological activities [15]. In this paper, we identified and analyzed a light-harvesting chlorophyll a/b-binding protein (GjI-2) in G. jasminoides.

2 Materials and Methods

2.1 Plant and Growth Conditions

Gardenia jasminoides plants cultivated at Guangdong Pharmaceutical University were used as materials. Fruits were collected at development stage II, closed with yellowish green exocarp and orange mesocarp. The samples were stored at -80°C until required.

2.2 CDNA Library Construction, ESTs Sequencing and Cloning of GjI-2

Total RNA was extracted from Gardenia fruit (stage II), using a modified CTAB (hexadecyl trimethyl ammonium bromide) based extraction protocol [1]. From total RNA, the cDNA library construction and amplification were performed following the users manual of the CreatorTM SMARTTM cDNA Library construction Kit (Clontech, USA). The SMART cDNAs were ligated into SfiI-digested pDNR-LIB vector and transformed into Escherichia coli strain DH5α. Colonies were randomly picked, inoculate each colony to a separated PCR reaction solutions. The colony was lysised by heating the mixed solutions at 95°C in a PTC-200 Thermocycler (MJ Research, USA) for 5 min. After then, went to PCR amplification procedure with M13 primers provided by the CreatorTM SMARTTM cDNA Library construction Kit. The amplified PCR products (ESTs, expressed sequence tags) were analyzed by 1.2% agarose gel electrophoresis. When the amplified PCR products were longer than 1000 bp, in-
We suggest that you use a text box to insert a graph (ideally 300 dpi), with all fonts embedded) because, in an MSW document, this method is somewhat more stable than directly inserting a picture.

To have non-visible rules on your frame, use the MSWord pull-down menu, select Format > Borders and Shading > Select "None".

Cubated the isolated colonies and sequenced the ESTs. There are 40 ESTs were sequenced. After sequencing and analysis, the colony containing the predicted pDNR-LIB-GjI-2 was isolated.

### 3 Results and Discussion

We identified novel I-2 homologues in *Gardenia jasminoides* (named GjI-2) by exploiting the fruit cDNA library of *G. jasminoides*. The full-length GjI-2 cDNA (Genbank accession No. KM371230) was obtained. The cDNA was 858 bp in length, contains a predicted 543 bp ORF that encodes 180 amino acids, a 94 bp non-coding region at 5' end and a 221 bp of non-coding region flank at 3' end. The predicted protein sequence of GjI-2 was compared to Genbank database, the best homology was found to inhibitor-2 of sesame (*Sesamum indicum*). The two proteins share 64% identical amino acids. Four particular sequence features are conserved between these two proteins. The first is a glycogen synthase kinase-3 threonine phosphorylation site PxTP motif (PKTP). Phosphorylation at this site results in activation of type-1 protein phosphatase via inactivation of inhibitor [25]. Another three features are conserved binding motifs, RVxF (GRVKW), FxxR/KxR/K(FREHRR) and HYNE(HYDE), these motifs function as PP1 anchoring sites. And compared GjI-2 to the I-2 proteins from *Arabidopsis thaliana* (identity 49%) to *Saccharomyces cerevisiae* (GLC8, 7%) Various species of I-2 have a common PxTP phosphorylation site and three binding motifs, the main difference is exist at the SILK motif, some species (including the *G. jasminoides*) have short N terminal end, lack this motif.

![3D model of GjI-2](image)

The 3D (three dimension) model structure of GjI-2 was predicted using SWISS-PDB software, the X-Ray diffraction at resolutions down to 2.5 Å of rabbit PP1c gamma complexed with mouse inhibitor.
tor-2 (PBD code 2O8G) was used as template [14]. The amino acid sequence of GjI-2 has 20% amino acid identity with mouse I-2. The structure was successfully built as monomer (Fig. 2), it has five α-helical domains, and two α-helices are similar with mouse I-2. I-2 is largely unstructured in solution, except for a single strongly populated α-helix, and that very limited regions of I-2 become ordered upon PP1 binding [3], in the I-2:PP1 crystal structure, only 59 of the 205 residues of I-2 were sufficiently ordered that they could be modeled into the electron density [19]. I-2 residues 130-169 forms a long ordered α-helix which lies directly over the PP1 active site and blocks its access to other substrates. I-2 residues 44-48 (RVxF motif) bind the PP1c, and residues 12-16 (SILK motif) bind the site that is located on the opposite side of the PP1 active site. Mammalian I-2 structure with the SILK motif will mediate PP1 inhibition. The N-terminal sequence SILK (KGILK) has been shown to be essential for PP1 inhibition by low concentrations of I-2. The threonine 72 in rabbit I-2 is phosphorylated by glycogen synthase kinase-3 and is conserved in many I-2 and I-2-like proteins [17]. In the I-2:PP1 structure, the PxTP motif (P72N73T74P75) is not modeled into the electron, while in the GjI-2 structure, it is sited between RVxF motif and FxxR/KxR/K, by compare with the I-2:PP1 structure, suggested that the site is easily approached when the I-2 is bonded to PP1, it is propitious to perform phosphorylation regulation for GjI-2.

4 Conclusions

The ubiquitous Ser/Thr Protein Phosphatase 1 (PP1) regulates diverse, essential cellular processes. The catalytic subunit of PP1 depends on a diverse set of regulatory proteins to confer substrates specificity and activity regulation. PP1 exists in cells as a set of distinctive multisubunit holoenzymes which are made up of PP1 catalytic subunit paired with a regulatory subunit containing an RVxF motif for recognition by PP1C. These subunits control PP1 holoenzyme subcellular localization, catalytic activity, and impact substrate specificity, in part by physical association with substrates.

The result suggests that, GjI-2 has relatively conserved RVxF, FxxR/KxR/K, HYNE motif, and these motifs are involved in interaction with PP1C. As a PP1 inhibitor, the long α-helix (have two adjacent α-helix) which lie directly over the PP1 active site will blocks its access to other substrates.

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