Plant Science 229 (2014) 172–180

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Enrichment and analysis of rice seedling ubiquitin-related proteins using four UBA domains (GST-qUBAs)

Qingshi Meng[a,b,c], Liqun Rao[a,**], Yinghong Pan[b,c,*]

[a] College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha 410128, China
[b] The National Key Facility for Crop Gene Resources and Genetic Improvement, Chinese Academy of Agricultural Science, Beijing 100081, China
[c] Institute of Crop Science, Chinese Academy of Agricultural Science, Beijing 100081, China

ARTICLE INFO

Article history:
Received 15 April 2014
Received in revised form 17 July 2014
Accepted 3 September 2014
Available online 16 September 2014

Keywords:
Oryza sativa
Ubiquitin
Ubiquitin-associated domain
Ubiquitin ligases
Affinity enrichment
Mass spectrometry

ABSTRACT

Protein ubiquitination is a common posttranslational modification that often occurs on lysine residues. It controls the half-life, interaction and trafficking of intracellular proteins and is involved in different plant development stages and responses to environment stresses. Four Ubiquitin-Associated (UBA) domains were sequentially fused with Glutathione S-transferase (GST) tag (GST-qUBA) as bait protein in this study. A two-step affinity protocol was successfully developed and the identification of ubiquitinated proteins and their interaction proteins increased almost threefold compared to methods that directly identify ubiquitinated proteins from crude samples. A total of 170 ubiquitin-related proteins were identified in GST-qUBAs enriched samples taken from rice seedlings. There were 134 ubiquitinated proteins, 5 ubiquitin-activating enzymes (E1s), 5 ubiquitin-conjugating enzymes (E2s), 19 ubiquitin ligases (E3s) and 7 deubiquitinating enzymes (DUBs), which all contained various key factors that regulated a wide range of biological processes. Moreover, a series of novel ubiquitinated proteins and E3s were identified that had not been previously reported. This study investigated a high-efficiency method for identifying novel ubiquitinated proteins involved in biological processes and a primary mapping of the ubiquitylome during rice seedling development, which could extend our understanding of how ubiquitin modification regulates plant proteins, pathways and cellular processes.

1. Introduction

It is well known that plant proteins are subjected to a wide range of posttranslational modifications. The modifications are often genetically predetermined, interconnected, highly dynamic and greatly expand proteome functionality from often limited genomic information [1]. Ubiquitination is one of the most important protein posttranslational modifications in eukaryotes and its biological importance may exceed phosphorylation [2,3]. Ubiquitin contains 76 amino acids and can be covalently attached to a substrate protein lysine residue through the E1, E2 and E3 enzymatic cascades [4]. Furthermore, a family of unique proteases, called deubiquitinating enzymes (DUBs), can release ubiquitin from ubiquitinated substrate proteins by specifically cleaving ubiquitin isopeptide linkages [2,5]. The substrate proteins can either be monoubiquitinated or polyubiquitinated, depending on how many ubiquitin proteins are attached [6]. Since ubiquitin itself contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), ubiquitin proteins can also become substrates for ubiquitin ligation themselves, which means that they can form various types of chains during the polyubiquitination process [7]. The way in which polyubiquitin chains attach themselves is a key mechanism in the regulation of many biological processes, including proteasome degradation, intracellular trafficking, DNA repair and signal transduction [8]. K11-linked and K48-linked polyubiquitination allow the 26S proteasome to target proteins for degradation [9]. Both K27-linked and K33-linked polyubiquitination may be assembled by U-box-type E3 ligases during responses to stress [10] and K29-linked polyubiquitination may participate in ubiquitin fusion degradation [9,11].

Abbreviations: GST-qUBAs, quadruple UBA domains with GST tag; E1s, ubiquitin-activating enzymes; E2s, ubiquitin-conjugating enzymes; E3s, Ubiquitin ligases; DUBs, deubiquitinating enzymes; UBD, ubiquitin-binding domain; MIU, motif interacting with ubiquitin; UBA, ubiquitin-associated domain; UIM, ubiquitin-interacting motif; IPTG, isopropyl-b-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; CAA, chloroacetamide; IAA, iodoacetamide; HCD, high energy collision; FDR, discovery rate.

* Corresponding author at: Institute of Crop Science, Chinese Academy of Agricultural Sciences(CAAS), 12 Zhongguancun Nandajie, Beijing, China.
Tel.: +86 10 82105849; fax: +86 10 82105817.
** Corresponding author. Tel.: +86 731 4673600; fax: +86 731 4673600.
E-mail addresses: raoliqun@163.com (L. Rao), panyinghong@caas.cn (Y. Pan).

http://dx.doi.org/10.1016/j.plantsci.2014.09.002
0168-9452/

Furthermore, monoubiquitination and K63-linked polyubiquitination are involved in regulatory signaling, which modulates protein interactions, activity and localization [12,13].

Previous research has pointed to the critical importance of plant protein ubiquitination in the control of plant embryogenesis, hormone signaling, defense and photomorphogenesis [14]. In *Arabidopsis*, the ubiquitin receptor, DA1, can interact with the E3 ubiquitin ligase, DA2, to regulate seed and organ size [15] and many E3s function directly as plant hormone receptors, such as the auxin receptor, TIR1 [16], or indirectly participate in plant hormone signal transduction, such as the GA receptor, GID1 [17]. Arabidopsis RING-type E3 ubiquitin ligase MIEL1 may weaken the plant hypersensitive response by degrading transcription factor, MYB30 [18], and COP1 and COP9 could promote the ubiquitination and degradation of the positive regulators in plant photomorphogenesis [19,20]. Many researchers are currently investigating the enzymology within the ubiquitin system. However, there is still much we do not understand about the process. Approximately 6% of *Arabidopsis* protein-coding genes may be involved in ubiquitin modification [21], but only a few E3s and DUBs have been matched with their substrates.

Clearly, a comprehensive understanding of ubiquitination will require accurate studies of ubiquitin-related proteins, including ubiquitinated proteins and E3s. Unfortunately, to date, ubiquitination studies have been hindered by the low abundance of ubiquitin-related proteins [22] and the fact that ubiquitinated proteins are typically present at transient and low steady state levels. This makes them particularly difficult to identify [23]. Furthermore, the presence of DUBs is also a major complication because their high activities in crude plant extracts releases the ubiquitin moieties from ubiquitinated proteins during the enrichment process [1]. Despite these difficulties, several recent proteomics studies on yeast, mammalian and plant cells have provided some information on the ubiquitylome and a number of studies have focused on ways to improve the enrichment of ubiquitinated proteins.

There are three technical routes for enriching ubiquitinated proteins. The most common are overexpressing the epitope-tagged ubiquitin in the plant followed by affinity purification using immobilized metal (Ni^{2+} or Co^{2+}) affinity chromatography (IMAC) under denaturing conditions [24–26]. Although the latter method is a straightforward procedure, it is limited to samples where the transgenosis lines are hard to obtain. In addition, the heterologous expression of epitope-tagged ubiquitin may subvert endogenous ubiquitin modification pathways, which results in the modification of non-physiological substrates [1,27]. Ubiquitin monoclonal antibodies, such as FK2, have also been used to enrich ubiquitinated proteins [28,29]. However, a limitation of this approach is that IgG and other undesired binding proteins can interfere with the subsequent mass spectrometry (MS) analyses. It should be possible to develop a monoclonal antibody that specifically recognizes diglycine (K-GG)-containing peptides. Using this principle, we could directly isolate the ubiquitin-modified peptides after trypsin digestion and then globally study their ubiquitination profiles [30,31]. However, the same signature diglycine residues are found in other ubiquitin-like protein modifications, such as NEDD8. This can make it hard to distinguish ubiquitination from these other ubiquitin-like modifications.

Eukaryotic cells have evolved more than 20 different ubiquitin-binding domain (UBD) families that recognize and bind ubiquitin, such as the motif interacting with ubiquitin (MIU), the ubiquitin-associated domain (UBA) and the ubiquitin-interacting motif (UIM) [32,33] families. Moreover, the UBA domain can recognize both ubiquitin and polyubiquitin chains, which has meant that multiple UBA domains have been previously used to successfully enrich ubiquitinated proteins [34–36]. For example, Do-Young et al. [1] successfully used GST-2xUSU combined with epitope-tagged ubiquitin to analyze and catalog ubiquitinated proteins from *Arabidopsis* seedlings. Despite their potential uses, there are no UBA domains that can be used to enrich whole type ubiquitinated proteins, especially among different species, which has meant that this technique has not been widely used to analyze ubiquitination in important crops, such as rice.

In order to create a high-efficiency method for enriching ubiquitinated proteins in rice, we compared the interactions between polyubiquitin chains and four UBA domains and then fused the four UBA domains to the C-terminal of GST, which we used as a bait protein. Then we developed a two-step affinity protocol for enriching rice ubiquitin-related proteins and removed the high abundance bait protein to reduce the negative impact on subsequent MS identification. The results showed that the two-step affinity method dramatically improved the purification stringency and yield of ubiquitin-related proteins. We identified 134 ubiquitinated proteins and a series of ubiquitin system enzymes in the rice seedling samples and they all contained key factors that regulated a wide range of biological processes. Collectively, this study illustrated the ubiquitination processes in rice seedlings and provided a strategy that could be used to effectively describe the ubiquitylome.

2. Materials and methods

2.1. Collection and bioinformation analysis of rice UBA domains

UBA was used as a keyword to search the *Oryza sativa* cv Nipponbare database in UniProt KB and the whole sequences of proteins containing UBA domains, together with the amino acid sequences for the UBA domains, were collected. These proteins were grouped according to domain function. Then we investigated the evolutionary relationships between the whole UBA domains using neighbor-joining algorithms. The UBA domain characteristics were identified by analyzing six representative UBA domains using Clustal W and WebLogo.

2.2. Cloning, protein expression and purification of GST-qUBAs

Four different types of UBA domain sequences were obtained from the Arabidopsis, Rice and Human cDNA library. They were then cloned in the pGEX-6P-1 vector using the following steps. The first tandem repeat UBA domains were successively constructed using isocaudamer *Bgl* II and *Bam*H I in the pGEX-6P-1 vector. Each consecutive clone of the UBA domains was linked by a polyglycine linker (GGGGSGGGGS). Then each recombinant GST-qUBAs vector was modified to code for 6xHis tags at the 3′ end, respectively. All the proteins were inducibly expressed, using 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG), in *Escherichia coli* B21 (DH3) for 6 h at 16 °C and at 160 rpm. The cells were lysed by sonication in GST loading buffer (10 mM sodium phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4) supplemented with 1 mM PMSF and then the lysates were centrifuged at 15,000 × *g* before being separated on a 5 ml GSTrap HP affinity column (GE. USA). The crude target proteins were eluted by the GST elution buffer (50 mM Tris–HCl, 10 mM reduced glutathione (GSH), pH 8.0). The eluent was subjected to mono Q (GE. USA) to further purify the proteins after changing the GST elution buffer to an anion loading buffer (50 mM Tris–HCl, pH 7.4) using a HiPrep 26/10 desalting column (GE. USA). The target protein was eluted by an anion elution buffer (50 mM Tris–HCl, pH 7.4, 1 M NaCl) and then dialyzed with IP buffer (50 mM Tris–HCl, pH 7.2, 200 mM NaCl and 0.25% Triton X-100).

2.3. In vitro binding abilities of the different UBA domains

IP buffer was added to the K48-linked polyubiquitin (Ub_4) and K63-linked polyubiquitin (Ub_4) to make a final concentration of