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Natural and targeted isovariants of the rice actin depolymerizing factor 2 can alter its functional and regulatory binding properties

Sonali Sengupta [a], Kanniah Rajasekaran [b], Niranjan Baisakh [a, *]

[a] School of Plant, Environmental and Soil Sciences, Louisiana State University Agricultural Center, Baton Rouge, LA, 70803, USA
[b] Southern Regional Research Center, USDA-ARS, New Orleans, LA, 70124, USA

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ABSTRACT

Actin depolymerizing factors (ADFs) are ubiquitous actin-binding proteins that play essential roles in maintaining cellular actin dynamics by depolymerizing/severing F-actin. Plant ADF isoforms show functional divergence via differential biochemical and cellular properties. We have shown previously that ADF2 of rice (OsADF2) and smooth cordgrass (SaADF2) displayed contrasting biochemical properties and stress response in planta. As a proof-of-concept that amino acid variances contribute to such functional difference, single amino acid mutants of OsADF2 were generated based on its sequence differences with SaADF2. Biochemical studies showed that the single-site amino acid mutations altered actin binding, depolymerizing, and severing properties of OsADF2. Phosphosensitive mutations, such as serine-6>threonine, changed the regulatory phosphorylation efficiency of ADF_2 variants. The N-terminal mutations had greater effect on the phosphorylation pattern of OsADF2, whereas C-terminal mutations affected actin binding and severing. The presence of introduced mutations in isovariants of monocot ADF suggests that these residues are significant control points regulating their functional divergence, including abiotic stress response.

1. Introduction

Actin depolymerizing factors (ADFs) are a family of ubiquitous conserved eukaryotic proteins. First identified from porcine brain and known for its ability to associate with filamentous actin, this family was historically named as ADF/cofillin [1]. ADFs are low molecular weight proteins that can bind to both G-actin and F-actin and play multiple roles in cell in association with other proteins. Besides severing and depolymerization, ADF is capable of promoting nucleation of actin polymerization and F-actin stabilization at high concentration [2]. With several isoforms available, ADFs have undergone a substantial functional/structural divergence in plants in contrast to other kingdoms. While alga (*Chlamydomomus reinhardtii*) and moss (*Physcomitrella patens*) has a single ADF gene, higher plants show multiple isoforms; 11 in *Arabidopsis* and rice and 27 in banana [3–5]. The divergence in function and expression locale in plant ADFs are attributed to the subtle sequence variations. A single-folded ADF-homology domain (ADF-H) spans almost the entire length of most ADFs, which is shared with other actin binding proteins such as twinfilin [6]. ADFs have a nuclear localization signal at the highly variable N-terminus, but they are also localized in the cytosol and membrane. ADFs are found in cell populations with high actin dynamics, such as growing root tip, meristem, stomatal guard cells, and pollen tube. Since ADF is indispensable for facilitating actin dynamics, ADF protein family in plants expectedly co-evolve with actin [5]. By depolymerizing, severing, bundling and stabilizing actin filaments, plant ADFs directly or indirectly participate in cell division and elongation, signaling, stress response, epigenetic regulation, and defense mechanisms [1,7–9].

ADF activity is regulated by pH, phosphorylation at N-terminal serine, and lipid binding. Biochemical function of ADFs is dictated by its sequence and structure with partially characterized key functional sites. Amino acid mutations in ADF result in considerable changes in their biochemical profiles and structure [10,11]. Differential actin binding may result in changes in depolymerizing/severing activity, whereas altered binding with interacting proteins can change other cellular functions.

Here, we present a case of functional divergence of a constitutively expressed rice ADF isoform (OsADF2) where targeted amino acid mutations resulted in significant biochemical changes. We

* Corresponding author.
E-mail address: nbaisakh@agcenter.lsu.edu (N. Baisakh).

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analyzed the biochemical difference between $OsADF_2$ and its homolog from the halophyte *Spartina alterniflora* ($SaADF_2$) with ~95% sequence similarity and only six amino acids differences. SaADF2 imparts higher drought and salt tolerance in transgenic rice overexpressers as compared to OsADF2 [11]. Based on the amino acid differences, targeted single amino acid mutants of OsADF2 were generated and their biochemical properties, such as actin binding affinity, depolymerization, and phosphorylation pattern were studied in relation to their deduced tertiary structure. The findings suggest that single mutations in $OsADF_2$ could change the structure-function relationship and alter actin binding efficiency and regulatory function such as phosphorylation although the functionally essential predicted actin-binding sites remain intact.

2. Materials and methods

2.1. Sequence analysis

Nucleotide and protein sequences of monocot ADF2 from *Brachypodium distachyon*, *Panicum virgatum*, *Hordeum vulgare*, *Oryza brachyantha*, *Spartina alterniflora*, *Setaria italica*, *Setaria viridis*, *Sorghum bicolor*, *Zea mays*, *Porteresia coarctata*, and several genotypes of rice such as Nipponbare, Nagina 22, IR29, Pokkali, Geungbyeo, Nonabokra, Cocodrie, Vandana and IR64 were retrieved and aligned as described earlier [11] (Supplemental Method 1). Structure analysis and docking was performed in LOMETS or I-TASSER [12], UCSF Chimera [13], ClusPro [14] and Zdock server [15].

2.2. Cloning, protein expression and purification

SaADF2 and *OsADF2* cDNA were cloned in pET200 (Invitrogen, Carlsbad, CA). Mutants (6α, S > T; 19α, D > N; 25α, L > H; 118α, Q > H; 132/133α, PT > SS) were generated in pET200/*OsADF2* vector with respective site-specific primers (Supplemental Table 1) using in-fusion mutagenesis technology (Clonetech, Paolo Alto, CA). The expression of recombinant his-tagged proteins, purification, and immunodetection were performed as described previously [11]. His-tag was removed from recombinant proteins by thrombin digestion for all downstream assays except immunoprecipitation.

2.3. Actin preparation, polymerization, F-actin depolymerization, and actin single filament severing/depolymerization

Actin preparation and polymerization, and binding and F-actin deplymerization assays were performed as described earlier [11,16]. For binding assays, recombinant proteins were incubated with polymerized actin at RT for 2−3 h and then centrifuged at 100,000 g (high) or 25,000 g (low), 4 °C for 1 h. Protein electrophoresis and gel documentation were performed as described earlier [11] (Supplemental Method 1). Actin filaments disassembly and severing by ADF proteins were observed by TIRF (total internal reflection fluorescence) microscopy [11,17].

2.4. Plant growth, stress treatment and protein extraction

One-week-old homozygous rice transgenics overexpressing *SaADF2* and *OsADF2* [11] and WT were transferred to ½ strength Hoaglands solution without (control) and with mannitol (−0.3 MPa) and allowed to grow in hydroponics for two weeks. Total soluble protein was extracted, quantified, and treated with CIP prior to reaction and step-dialyzed against a low-salt buffer (Supplemental Method 1).

2.5. Immunoprecipiation and phosphorylation assay

Pull-down was performed with anti-his antibody conjugated protein-A/G sepharose. As phosphorylation agent, 50 μg plant protein was added to the recombinant epitope-tagged ADF proteins and incubated at 4 °C with gentle shaking O/N in the presence of ATP. Protein was eluted from the beads under acidic condition and probed for phosphorylation with anti-phosphoserine antibody (Supplemental Method 1).

3. Results

3.1. Sequence comparison of ADF2 homologs within monocots

Comparative sequence analysis showed that ADF2 of *Spartina alterniflora*, *Oryza brachyantha*, *Hordeum vulgare*, and *Triticum aestivum* harbor a common substitution at serine-6>threonine along with other mutations (Supplemental Fig. 1). Deduced tertiary structures showed alterations, which could be important for actin binding and/or other regulatory functions of ADF that involve change in the binding domain structure and residues. The F-actin binding domain analysis of the three-dimensional structures of SaADF2 and OsADF2 revealed a noticeably different spatial projection pattern (Fig. 1A) and the N-terminal binding regions showed involvement of different residues, including the substituted threonine-6 (Fig. 1B,C). With the introduction of point mutations the projected three-dimensional structure of the protein changed variously as discussed later (Fig. 1D).

Except the crystal structure of *Arabidopsis thaliana* ADF1 in solution (PDB: 1F7S), no plant ADF structure in complex with actin or other regulatory proteins has been reported yet. Two putative actin-binding sites (ABS) were predicted on ADF/cofilin tertiary structure [18]. The N-terminal end and the long α-helix 3 together form ABS1, and the β-strand 4 and 5 together with the projected F-loop and α-helix 4 form ABS2 in the predicted tertiary structure of Sa/OsADF2 (Fig. 1E). Basic residues exposed on the F-loop are important for F-actin binding.

3.2. OsADF2 mutants exhibited differential actin binding and depolymerizing efficiency

While SaADF2 shows a higher actin-binding affinity compared to OsADF2 [11], substitution mutations in OsADF2 altered the biochemical properties of the protein. The major property of ADFs is to promote actin filaments disassembly, which is orchestrated by other functional activities, such as severing, pointed-end depolymerization, bundling, and maintenance of G-actin pool. ADFs bind to both G-actin and F-actin in different capacities depending on the isoforms and the cell status. The three important stretches of ADF functional domain for G-actin binding are the (a) long central helix, (b) beta strands in between the long central helix, c-terminal helix with the adjacent loop, and (c) N-terminal extension, which is typically disordered in solution without a binding partner (Fig. 1E). The N-terminal hexapeptide in yeast cofilin has been shown to be essential for the survival and its deletion mutation is lethal [19]. Substitution of serine-6 by threonine led to the introduction of a small helix at the N-terminal end (Fig. 1F), which may provide steric hindrance to the protein in accessing G-actin and/or inhibitory substrates such as the interacting protein calcium-dependent protein kinase (CDPK). Indeed, docking of the predicted mutant protein structure showed that the distance between lysine-80 (K80) in CDPK and threonine-6 (T6) in mutant protein 6α does not support the formation of hydrogen bonds, salt bridges or other interactions between these two proteins (Fig. 1G). On the other hand, ABS2 consisting of the F-loop and α-helix 4 in 6α displayed a

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