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Arabidopsis thaliana plastoglobule-associated fibrillin 1a interacts with fibrillin 1b in vivo

Francisco Manuel Gámez-Arjona, Juan Carlos de la Concepción, Sandy Raynaud, Ángel Mérida *

Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC-US, Avda Américo Vespucio, 49, 41092 Sevilla, Spain

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ABSTRACT

Plant fibrillins are a well-conserved protein family found in the plastids of all photosynthetic organisms, where they perform a wide range of functions. A number of these proteins have been suggested to be involved in the maintenance of thylakoids and the formation of plastoglobules, preventing their coalescence and favoring their clustering via an as-yet unidentified cross-linking mechanism. In this work we show that two members of this group, namely fibrillin 1a and 1b, interact with each other via a head-to-tail mechanism, thus raising the possibility that they form homo- or hetero-oligomers and providing a mechanism to understand the function of these proteins.

Structured summary of protein interactions:
FBN1b physically interacts with **FBN1a** by two hybrid (View interaction)
FBN1b physically interacts with **FBN1b** by two hybrid (View interaction)
FBN1b physically interacts with **Ribosomal protein L22** by two hybrid (View interaction)
FBN1b physically interacts with **F-ATPase gamma subunit 1** by two hybrid (View interaction)
FBN1b physically interacts with **Ribose 5-P isomerase** by two hybrid (View interaction)
FBN1b and **FBN1a** physically interacts by bimolecular fluorescence complementation (View interaction)
FBN1b and **FBN1b** physically interact by bimolecular fluorescence complementation (View interaction)

1. Introduction

The term fibrillins (FBNs) in plants designates a large protein family that is present in all types of plastids, such as chloroplasts, chromoplasts, and leucoplasts [3]. These proteins were named fibrillins as they were first identified in fibrils, the thread- or tube-like structures found in bell pepper (*Capsicum annuum*) fruit chromoplasts [1], which are the main sites of chromoplasts for pigment accumulation [15]. Fibrillins are found in all photosynthetic organisms, ranging from cyanobacteria to plants [5]. Most of them are located in plastoglobules [17], although the presence of FBNs in the stroma and stromal lamellae thylakoids has also been shown [4]. FBNs can be divided into 12 phylogenetic groups and appear to be involved in a wide range of functions, such as abiotic stress tolerance, growth and development, hormone signaling, or lipid transport between thylakoid membranes and plastoglobules [11,12]. Members of group 1, which includes *Arabidopsis* FBN1a and FBN1b, have been reported to be involved in plastoglobule formation and thylakoid maintenance [8,11]. Indeed, a recent proteomics study has indicated that FBNs are the most abundant proteins in the plastoglobules of *Arabidopsis* leaf rosettes, and six FBNs, including the four major ones (FBN1a, 1b, 2 and 4), account for 53% of the plastoglobule protein mass in *Arabidopsis* [6]. The addition of a bell pepper FBN orthologous to *Arabidopsis* FBN1a to carotenoids and polar lipids in the same stoichiometric ratio found in vivo reconstituted the fibrils observed in bell pepper fruit chromoplasts [1], and over-expression of this FBN led to an increase in the number of plastoglobules organized into clusters in tobacco chloroplasts [8]. In light of these results, it has been hypothesized that FBN may prevent plastoglobule coalescence and favor their clustering by acting as an interface between the aqueous environment and lipids as well as by mediating cross-linking via an unknown mechanism [8]. In this work we demonstrate an interaction between *Arabidopsis* FBN1a and FBN1b proteins, as well as FBN1a–FBN1a and FBN1b–FBN1b interactions. Yeast Two-Hybrid analyses indicate that these interactions involve the N-terminal

Abbreviations: FBN, fibrillin; Y2H, yeast Two-Hybrid; BiFC, bimolecular fluorescence complementation; CTP, chloroplast transient peptide

* Corresponding author. Address: Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC-USE, Avda Américo Vespucio, 49, 41092 Sevilla, Spain.
E-mail address: angel.merida@csic.es (Á. Mérida).

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part of one protein and the C-terminal part of the other in a head-to-tail mechanism, thereby suggesting the possibility that these proteins form filaments in vivo. These interactions provide a mechanism to explain the function of these FBNs in fibril formation or the maintenance of plastoglobule clusters.

2. Materials and methods

2.1. Plasmid construction

Full-length ORFs from FBN1a and FBN1b were cloned (without the stop codon) into the pDONR221 entry vector (Invitrogen) by a BP reaction (Invitrogen). After sequence verification, the inserts were transferred into the binary vectors pXNGW (-*n*YFP) and pXCGW (-*c*CFP) [18], respectively, for the BiFC assay using LR Clonase II (Invitrogen). This resulted in translational fusions between the ORFs and the YFP/CFP moieties driven by the CaMV 35S promoter.

For Y2H screening, a cDNA fragment coding for amino acids 71–144 of FBN1b was amplified from *Arabidopsis* cDNA using the primers FBN1B_Y2H_F and FBN1b_Y2H_R (see list of primers in Table S1). These primers introduced *Nde*I and *Eco*RI restriction sites at the 5′- and 3′-ends of the fragment, respectively. The fragment amplified was cloned into the yeast vector pGBKT7 (Clontech) by restriction with *Nde*I and *Eco*RI and subsequent ligation.

For the BiFC assays of the N- and C-terminal part of FBN1b, the cDNA fragment coding for the FBN1b chloroplast transit peptide (CTP) was fused to the fragment coding for amino acids 71–144 by two sequential steps of PCR amplifications using cDNA as probe and primers FBN1b_CTP-bait_F and FBN1b_CTP-bait_R in the first step, and the products of the first PCR amplification and primers FBN1b_F and FBN1b_CTP-bait_GW_R in the second step (see primers list in Table S1). The C-terminal part of FBN1b (amino acid residues 220 to the end) was fused to the CTP region using the same strategy with the primers FBN1b_CTP-Ct_F and FBN1b_CTP-Ct_R in the first PCR amplification and FBN1b_F and FBN1b_R in the second step. Once the N- and the C-terminal regions of FBN1b had been fused to the CTP, both constructs were cloned into the binary vectors pXNGW (-*n*YFP) and pXCGW (-*c*CFP), respectively, using LR Clonase II (Invitrogen).

The full-length ORF of VTE1 (At4g32770) gene was amplified from leaves mRNA by RT-PCR using the primers described in Table S1, and cloned into the pDONR221 entry vector (Invitrogen) by a BP reaction (Invitrogen). After sequence verification, the insert was transferred into the binary vectors pXNGW (-*n*YFP) and pEarlyGate103 [2], for BiFC assays and fusion to GFP marker respectively.

2.2. Transient expression in Nicotiana benthamiana

The corresponding vectors were electroporated into *Agrobacterium tumefaciens* strain C58 [16]. The saturated overnight bacterial cultures carrying the GFP or the YFP/CFP construct moieties were each adjusted to a final $O.D_{600\ nm}$ of 0.2 and then co-infiltrated with equal amounts of an *Agrobacterium* suspension carrying a p19 suppressor of post-transcriptional gene silencing, following the method of Silhavy et al. [10]. The *Agrobacterium* suspensions were then infiltrated into the leaves of three- to four-week-old *N. benthamiana* plants as described previously [7]. The infiltrated plants were kept in a controlled growth chamber under the above conditions for two days until analysis by confocal microscopy.

2.3. Yeast Two-Hybrid screening

Screening of a commercial, normalized, *Arabidopsis* cDNA library in the yeast expression vector pGADT7-RecAB (Clontech) was performed using the Matchmaker Gold Yeast Two-Hybrid system (Clontech), following the manufacturer's instructions. The bait used in the screening was a fragment corresponding to the N-terminal part of FBN1b (amino acid residues 71–144). The cDNA encoding for the bait was cloned into the yeast cloning vector pGBKT7 as described above.

2.4. Confocal microscopy

A DM6000 confocal laser scanning microscope (Leica Microsystems) equipped with a 63× water-immersion objective was used to examine protein–protein interactions in BiFC assays involving *N. benthamiana* mesophyll cells. YFP/CFP expression and chlorophyll autofluorescence imaging was performed by excitation with a 488 nm argon laser; fluorescence was detected at 500–525 and 630–690 nm, respectively.

3. Results and discussion

3.1. Searching for proteins that interact with FBN1b using the yeast two-hybrid (Y2H) system

Previous studies from our group have identified the interaction of FBN1b with a protein involved in starch metabolism (unpublished results). In order to continue with this analysis, we searched for other proteins that could interact with FBN1b by screening an *Arabidopsis* cDNA library using the Y2H system and a fragment of the N-terminal part of FN1b (residues 71–144) as bait. We selected this fragment because it contains a hydrophilic domain (corresponding to Block 1) defined by Laizet et al. [5] and may therefore be involved in functions distinct from the interaction with the lipids of plastoglobules, such as protein–protein interactions.

The cDNA fragment coding for the FBN1b fragment was fused to the DNA binding domain of the yeast GAL4 transcription factor by cloning into the expression vector pGBKT7 and subsequent transformation into the yeast strain Y2HGOLD (Section 2). Expression of this construct was confirmed by immunoblotting yeast crude extracts employing polyclonal antibodies against the GAL4 DNA binding domain (data not shown). The fragment was used to screen a normalized, commercial *Arabidopsis* cDNA library in the pGADT7-Rec yeast expression vector (Clontech). Although 35 positive clones that grew on selective medium were obtained, 22 of these encoded for proteins with an extra-plastidial localization and were therefore discarded. The remaining proteins found are listed in Table 1. We decided to focus on the study of the positive clones encoding for fragments of FBN1a and FBN1b proteins as these results suggest that FBN1b can interact with another FBN1b polypeptide to form homodimers or with FBN1a to form heterodimers.

3.2. FBN1b interacts with FN1a and FBN1b in vivo

We decided to confirm the interactions observed with the Y2H system using bimolecular fluorescence complementation (BiFC)

Table 1
Plastidial proteins found in Y2H screening of an *Arabidopsis* library using the N-terminal part of FBN1b as bait.

Number of independent clones	AGI code	Protein
1	Atcg00810	Ribosomal protein L22
1	At4g04640	ATPase, F1 complex, gamma subunit protein
1	At3g04790	Ribose 5-P isomerase
2	At4g04020	Fibrillin 1a
4	At4g22240	Fibrillin 1b