Unique genetic compartmentalization of the SUF system in cryptophytes and characterization of a SufD mutant in *Arabidopsis thaliana*

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Received 11 October 2004; revised 20 December 2004; accepted 30 December 2004

Available online 19 January 2005

Edited by Julian Schroeder

Abstract The mobilization of sulfur (SUF) system is one of three systems involved in iron–sulfur cluster biosynthesis and maintenance. In eukaryotes the SUF system is specific for the plastid and therefore of symbiotic origin. Analyses in cryptophytes showed a unique genetic compartmentalization of the SUF system, which evolved by at least two different gene transfer events. We analyzed one of the components, SufD, in the cryptophyte *Guillardia theta* and in *Arabidopsis thaliana*. We demonstrated that SufD fulfils house keeping functions during embryogenesis and in adult plants in *A. thaliana*.

Keywords: SUF system; SufD; Cryptophytes; *Arabidopsis thaliana*

1. Introduction

Iron–sulfur (Fe–S) clusters play an essential role as cofactors of Fe–S proteins in many biological processes and are thought to be key players in the origin of life [1]. Three systems involved in Fe–S cluster biosynthesis, assembly, maturation and repair are known, the nitrogen fixation (NIF) [2], ISC [3,4] and the mobilization of sulfur (SUF) [5,6] system. The name of the NIF system traces back to its involvement in Fe–S cluster assembly and maturation of nitrogenase proteins. The ISC system, best studied in bacteria [7,8], is an essential mitochondrial Fe–S cluster forming machinery in eukaryotes and provides Fe–S clusters for mitochondrial and cytoplasmatic Fe–S proteins [9]. The SUF system is found in prokaryotes and plastids. Its role is still unclear and some reports mentioned that, it is responsible for maintenance and repair of Fe–S clusters under conditions of oxidative stress and iron limitation [10]. A recent study showed that under the conditions tested, SUF provides cluster-assembly activity in *Escherichia coli*, but argues that it most likely does not function in cluster repair [7]. Both the ISC and the SUF systems are composed of a scaffold protein for Fe–S cluster assembly (IscU and SufA) and a cysteine desulfurase (IscS and SufS) [11–16]. The SUF system contains a group of three additional proteins (SufBCD). SufC is an ATP binding cassette (ABC)/ATPase [17,18] that has been shown to interact with SufB and SufD in *Erwinia crysanthemi* [19] and *Arabidopsis thaliana* [20]. From bacteria it is reported that the SufBCD complex is involved in repair of oxidatively damaged Fe–S clusters by enhancing SufS cystein desulfurase activity [16]. In *E. coli* SufD has been shown to be necessary for the stability of the [2Fe–2S] FhuF protein [14]. Homologs of the bacterial suf genes were also found in the *A. thaliana* genome. Recently, it was shown that AtNAP7 of *A. thaliana* is a SufC-like ABC/ATPase and it was suggested that it plays an important role for maintenance and repair of plastidic Fe–S cluster proteins. Most striking is the finding that AtNAP7 is essential for embryogenesis [20]. On the other hand, SufB was shown to be important for intracompartmental communication between cell nucleus and plastids in *A. thaliana* [21].

In the paper at hand we show that in cryptomonads, the subunits of the SUF system are compartmentalized in three different genomes, in the nucleus, the nucleomorph and the plastid. We demonstrate that one of the most conserved subunits of the SUF system, SufD, is a plastid protein in cryptomonads and *A. thaliana*. We show furthermore that an *A. thaliana* transferred DNA (T-DNA) insertion mutant in the SufD homologous gene shows decreased viability. Thus, our results emphasize the importance of the plastid located SUF system and indicate that it fulfils general housekeeping functions.

2. Materials and methods

2.1. Expression of Guillardia theta SufD (ORF467) recombinant protein

Orf467 was amplified by PCR with primers 467_1 (5′-GAATTCAGTTGAAGACATGCATCTATC-3′; *Eco*RI) and 467_2 (5′-CGTCGACTTATAAGATAAAACATTTG-3′; *Sal*I). The amplified fragment was cloned into pGEX-5X-3 (Amersham) and expressed in *E. coli* as a C-terminal fusion to glutathione *S*-transferase (GST). Protein expression and purification was done according to standard protocols.

2.2. Immunogold labelling analysis

Cells from *G. theta* were embedded in Lowicryl K4M (Chemische Werke Lowi) according to manufacturer's instructions. Ultrathin sections were incubated with a polyclonal antiserum raised in rabbit

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Abbreviations: ABC, ATP binding cassette; EST, expressed sequence tag; Fe–S, iron–sulfur; GFP, green fluorescent protein; GST, glutathione *S*-transferase; ISC, iron–sulfur cluster assembly machinery; NAP, non-intrinsic ABC protein; NIF, nitrogen fixation; SUF, mobilization of sulfur; T-DNA, transferred DNA

0014-5793/$30.00
doi:10.1016/j.febslet.2004.12.084

against the recombinant *G. theta* SufD protein. Gold-coupled goat anti-rabbit IgG was used as secondary antibody to detect the primary antibodies as described by Fraunholz et al. [22].

2.3. Generation of AtNAP6/GFP fusion constructs

AtNAP6 cDNA was amplified by PCR using the primers green fluorescent protein 1 (GFP1) (5′-CGTCTCCCATGGCGGCTGCCACAGTTCTCG-3′; *Esp*3I) and GFP2 (5′-CGTCTCCCATGGCGAGCAAGCCTTTGACGTG-3′; *Esp*3I) for the full length *AtNAP6* construct. Primers GFP1 and GFP3 (5′-CGTCTCCCATGGCAAGATCACT-GATAGCAGC-3′; *Esp*3I) were used to amplify the C-terminal deletion construct. Primers GFP4 (5′-CGTCTCCCATGGCGTCT-GCAAACGCTC-3′; *Esp*3I) and GFP5 (5′-CTGCAGCTTTACATT-AGCTCTAACAC-3′; *Pst*I) amplified the chloroplast transit peptide domain of *cpn60* from cDNA, this was combined with the PCR product obtained from primers GFP6 (5′-CTGCAGATGGTGATTTATGTTCCTG-3′; *Pst*I) and GFP3 for the β-helix construct. The β-helix deletion construct was amplified by using primers GFP1, GFP7 (5′-CTGCAGCAAATCCGGTGCTCC-3′; *Pst*I), GFP8 (5′-CTGCAGGATCTTGAAGAAGACC-3′; *Pst*I) and GFP2. Primers GFP1 and GFP7 were used for the N-terminal construct, primers GFP4 and GFP9 (5′-CGTCTCCCATGGCCTTTACATTAGCTC-3′; *Esp*3I) were utilized for the Cpn60 transit peptide control construct. The cytosolic β-helix construct was amplified with primers GFP10 (5′-CGTCTCCCATGGTGATTTATGTTCCTG-3′; *Esp*3I) and GFP3. *Orf467* from *G. theta* was amplified from genomic DNA by using primers GFP11 (5-CGTCTCCCATGGTCATTAATTGTTAC-3′; *Esp*3I) and GFP12 (5′-CGTCTCCCATGGCTAATAACGAAAAAATTTTC; *Esp*3I). All constructs were cloned into pAVA393 [23] as N-terminal fusions to GFP.

2.4. Transformation of Arabidopsis protoplasts

The preparation and subsequent transformation of protoplast from *A. thaliana* ecotype Columbia (Col-0) mesophyll cell culture were performed according to Altmann et al. [24].

2.5. GFP fluorescence analysis

Confocal laser scanning microscopy was done with the Leica TCS SP2 using HCX PL APO 40×/1.25–0.75 oil CS or PL APO 63×/1.32–0.60 oil Ph3 CS objectives. GFP and chlorophyll fluorescence was excited at 488 nm, filtered with beam splitter TD 488/543/633 and detected by two different photomultiplier tubes with a bandwidth of 500–520 and 625–720 nm for GFP and chlorophyll fluorescence, respectively.

2.6. Plant material, growth conditions and embryo analysis

Seeds of *A. thaliana* ecotype Columbia (Col-0) as well as seeds of *atnap6* knockout mutant (SALK Institute T-DNA insertion line N555996, http://signal.salk.edu) were grown on soil under 16 h light/8 h dark cycles at 22 °C. Viability of embryos and primary root length were examined by germinating seeds on watered Whatman paper in sealed Petri discs for 6 days under same conditions. To analyze the seed phenotype mature siliques were opened, seeds removed, collected and partitioned according to their phenotype: normal (oval, bright brown seeds, filled out with the embryo) and abnormal (shrunken, dark brown seeds). Seedling roots, seeds and embryos were examined under a binocular microscope (Leica MZ 8). To visualize embryos, rehydrated seeds as well as excised embryos were mounted in Hoyers solution (7.5 g arabic gum, 100 g chloral hydrate, 16.7% glycerol, and 30 ml H_2O) and then observed under differential interference contrast optics (Nomarski) using confocal laser scanning microscopy (Leica TCS SP2).

2.7. Measurement of chlorophyll content

Chlorophyll was extracted with *N*,*N*-dimethylformamide (DMF) and assayed based on the absorbance of the extract at 647 and 664 nm [25].

2.8. DNA isolation and PCR analyses

Genomic DNA from *A. thaliana* was prepared using REDExtract-N-Amp Plant PCR Kit (Sigma). The genotype of *atnap6* mutant plants was analyzed by PCR using primers specific for the T-DNA-flanking genomic regions (5′-CGTTTGTGAACTGGGTTTCCG-3′ and 5′-TCACGTCTAAACTTTTTGGGCCAT-3′) and for the T-DNA left border (5′-TTTTTCGCCCTTTGACGTTGGAG-3′).

2.9. Southern blot

To verify the number of T-DNA inserts in the SALK line N555996, 10 μg each of genomic DNA were digested with EcoRI, BamHI and HindIII, separated on agarose gel, transferred to Hybond N^+ membrane (Amersham) and hybridized with Digitonin-labeled (Roche) probes specific for the NPTII gene (kanamycin resistance) of the T-DNA.

2.10. RNA isolation and RT-PCR analyses

Total RNA was extracted using the Plant RNeasy Kit (Qiagen). The presence of *AtNAP6* and chimeric T-DNA-*AtNAP6* transcripts in mutant plants were checked by RT-PCR analyses using *AtNAP6* specific primers (5′-ATGGCGGCTGCCACAGTTCTC-3′and 5′-CAAGTTTCCCACC-AGTACTCAC-3′) and T-DNA specific primers (5′-AACCAGCGTGGACCGCTTGCTG-3′, 5′-CGTCCGCAATGTGTTATTAAG-3′).

3. Results

3.1. SUF genes of cryptomonads and the nucleomorph-encoded SufD homolog

Cryptomonads show a unique compartmentalization of the genetic components of the *suf* operon (Fig. 1). Whereas SufB (Ycf24) and SufC (Ycf16) homologs were encoded in the plastid genome as in other non-green phototrophic eukaryotes [26], a *sufS* transcript was detected in our expressed sequence tag (EST) project and the corresponding gene was nucleus-located (Accession No.: AJ821799). We additionally identified a *sufD* homolog on the remnant nucleus of the secondary endosymbiont (nucleomorph) [27]. The cryptophytic SufD homolog shows identical or similar amino acids at conserved positions in the SufD proteins of other organisms (Fig. 2). It is encoded as a pre-protein with an N-terminal transit peptide. In silico predictions for the *G. theta* homolog and the recently resolved crystal structure of the *E. coli* SufD show a remarkable secondary structure in the second half of the protein. It is composed solely of short β-strands followed by three α-helices at the C-terminal end of the protein. The β-strands fold into a structure that can be described as a parallel β-helix, similar to certain β-solenoid proteins [28]. Due to the high degree of amino acid identities, especially in this β-helix region (Fig. 2), we expected a similar 3-dimensional structure for all SufD homologs, including the nucleomorph-encoded one.

We expressed the nucleomorph-encoded SufD in *E. coli* to obtain antigen for immunizations. The serum was used to examine whether the nucleomorph-encoded protein is expressed in *G. theta*. Western blot analyses showed a specific cross reacting band of the predicted molecular mass in protein extracts from *G. theta*. In immunogold-labelling experiments we identified binding of the antibody preferentially in the tips of the plastid (Fig. 3(a)). Control experiments with the pre-immune serum gave no signal in neither the Western blot nor in immunogold labelling (Fig. 3(b)). As such a sub-plastidal localization is unusual, and tools for genetic studies are not yet developed for *G. theta*, we characterized the SufD homolog of *A. thaliana*.

3.2. The SufD homolog of A. thaliana

Searching for a *sufD* homolog in *A. thaliana* led to the identification of *AtNAP6* (Fig. 2), a single-copy gene encoded on chromosome 1. Subcellular localization was determined by transient expression of AtNAP6/GFP fusion protein in leaf cell protoplasts from *A. thaliana*. Interestingly, the fusion protein