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Iron-sulfur cluster synthesis in plastids by the SUF system: A mechanistic and structural perspective

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ABSTRACT

About 50 proteins expressed in plastids of photosynthetic eukaryotes ligate iron-sulfur (Fe-S) clusters and ensure vital functions in photosynthesis, sulfur and nitrogen assimilation, but also in the synthesis of pigments, vitamins and hormones. The synthesis of these Fe-S clusters, which are co- or post-translationally incorporated into these proteins, relies on several proteins belonging to the so-called sulfur mobilization (SUF) machinery. An Fe-S cluster is first *de novo* synthesized on a scaffold protein complex before additional late-acting maturation factors act in the specific transfer, possible conversion and insertion of this cluster into target recipient proteins. In this review, we will summarize what is known about the molecular mechanisms responsible for both the synthesis and transfer steps, focusing in particular on the structural aspects that allow the formation of the required protein complexes.

1. Introduction

Iron-sulfur (Fe-S) clusters are small inorganic cofactors composed of iron and sulfur atoms that are essential for the function of their associated proteins. By promoting electron transfer, catalyzing redox and non-redox reactions or stabilizing protein structures, Fe-S clusters are present in proteins that support a wide range of essential biological processes such as respiration, photosynthesis, protein synthesis or nucleic acid metabolism [1,2]. The most common forms of Fe-S clusters are the [Fe_2S_2] and [Fe_4S_4] clusters with iron atoms being usually bound to proteins *via* cysteine or histidine residues [3]. In both eukaryotes and prokaryotes, Fe-S cluster-containing proteins are initially synthesized as apo-proteins and the Fe-S cofactors are subsequently incorporated in a controlled manner by specific and complex multi-protein assembly machineries. In addition to the NIF machinery present in nitrogen-fixing bacteria for the maturation of nitrogenases, bacteria express the ISC (Iron Sulfur Cluster) and/or SUF (SUlfur mobilization Factor) machineries, which are also present in eukaryotic mitochondria and chloroplasts respectively [3]. Eukaryotes also have a Cytosolic Iron-sulfur protein Assembly (CIA) machinery for the maturation of cytosolic and nuclear Fe-S proteins [3]. The basic principle of Fe-S cluster incorporation into recipient proteins can be divided into two main steps. First, Fe-S clusters are assembled on so-called scaffold proteins/complexes from iron and sulfur atoms delivered by dedicated systems and using an electron source [3]. The second step consists of the transfer of the pre-formed Fe-S clusters to recipient apo-proteins either directly or *via* dedicated Fe-S cluster transfer proteins, which can also ensure Fe-S cluster conversion from the [Fe_2S_2] to the [Fe_4S_4] cluster type if required. A few other proteins (stabilizing factors, ATP-hydrolyzing proteins, chaperones or sulfur-relay systems) are required [3]. All proteins assisting the synthesis and transfer of the Fe-S cluster are referred respectively to as early- and late-acting maturation factors.

Chloroplasts are the site of photosynthesis and contain the largest number of Fe-S proteins when compared to other subcellular compartments. We have recently identified about 50 Fe-S proteins in *Arabidopsis thaliana* plastids, most of which contain [Fe_4S_4] clusters and the rest either [Fe_2S_2] or [Fe_3S_4] clusters [4]. They are mainly involved in the following processes: linear and cyclic photosynthetic electron transfer, carbon fixation, nitrogen and sulfur assimilation, but also in the synthesis of NAD, vitamins (thiamine, lipoic acid), branched-chain amino

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acids, pigments (chlorophyll, isoprenoid), or phytohormone (strigolactone) [5]. As most of these processes are central to the functioning of photosynthetic cells, the SUF system is essential in plants. In terms of evolution, the SUF system is considered as the most ancient Fe-S cluster biogenesis pathway, probably already present in early anaerobic life forms as a minimal SufBC form, and is nowadays also present in archaea and many bacteria [6,7]. It is thought that the emergence and increased levels of O_2 on Earth led to an increased complexity of the SUF machinery, with the progressive addition of *sufA/D/E/S* genes to the « ancestral » *sufB/C* genes as they exist today in the *Escherichia coli* SUF operon (*sufABCDSE*) [6–8]. The same genes/proteins are present in *A. thaliana* and the role of each protein both from *E. coli* and *A. thaliana* is rather clear. The sulfur atoms are provided by NFS2 (*A. thaliana* ortholog of *E. coli* SufS) and relayed by SUFE (SUFE1 or SUFE2), the SUFBCD proteins form the scaffold complex, which assembles and delivers an Fe-S cluster to the so-called A-type carrier (ATC) protein, SUFA1, even though this remains to be proven for the *A. thaliana* ortholog (Fig. 1) [9,10]. However, other maturation factors are functionally associated with the SUF system. They are named IBA57.2, GRXS14 and GRXS16, BOLA1 and BOLA4, NFU1 to 3 and HCF101 [5]. The Table S1 summarizes the names of the *E. coli* and *A. thaliana* orthologs, as they are sometimes different. Recently, two DNA-J proteins, named DJA5/6, have been proposed to provide iron atoms to the *Arabidopsis* SUF system [11]. This awaits to be confirmed for other organisms. Except for NFU1–3 and HCF101, the exact role of the additional players is not yet known. In addition to recapitulate the information mentioned above, Fig. 1 highlights also current unresolved questions for some molecular steps. The aim, in the next sections, is to summarize our understanding of the molecular and structural details that underlie the specificity and dynamics of complex formation in both the assembly and transfer steps by combining both existing results and structural modelling made using AlphaFold2 [12] and AlphaFold Multimer [13].

2. Synthesis of the iron-sulfur cluster on the scaffold $SUFBC_2D$ complex

It is worth mentioning that the central role of proteins known so far as involved in the Fe-S cluster synthesis in chloroplasts has been validated by genetic studies. The study of *A. thaliana* knock-out lines proved that *NFS2*, *SUFE1*, *SUFE3*, *SUFB*, *SUFC*, *SUFD* and *DJA5–6* genes are essential, the mutant lines being not viable, dying at the embryo or seedling stages [11,14–17]. The use of (inducible)-RNAi lines allowed to demonstrate that NFS2 and SUFBCD are required for the maturation of all plastidial Fe-S proteins tested [16,17]. The developmental and physiological perturbations of these mutants will not be developed in the context of this review and we direct interested readers to other sources [5,9,10].

2.1. Sulfur transfer from NFS2 to SUFE

Previous molecular and structural analyses performed on bacterial and plant SUF systems established that sulfur is mobilized from l-cysteine *via* the action of pyridoxal-l-phosphate (PLP)-dependent cysteine desulfurases, SufS in *E. coli* and NFS2 (formerly referred to as CpNifS) in *A. thaliana*. As class II cysteine desulfurases, the accessibility of the persulfide group in NFS2/SufS is limited by the presence of a so-called β-latch near the catalytic cysteine [18–20]. For this reason, the transfer of sulfur atoms to the scaffold complex relies on additional proteins named SUFEs, themselves containing a conserved reactive cysteine that oscillates between a reduced and a persulfidated form. The β-latch allows also the positioning of SUFE proteins during complex formation [20]. In *A. thaliana*, three SUFE proteins (SUFE1–3) are targeted to the chloroplasts [14,21]. In addition to the SUFE domain, SUFE1 proteins possess a C-terminal BOLA domain, which promotes an interaction with the two chloroplastic class II glutaredoxins (GRXs), GRXS14 and GRXS16 [22]. The role of these interactions is still unknown. SUFE3 proteins possess a bacterial-type quinolinate synthase (NadA) domain at the C-terminus. *Arabidopsis* SUFE3 displays both SufE

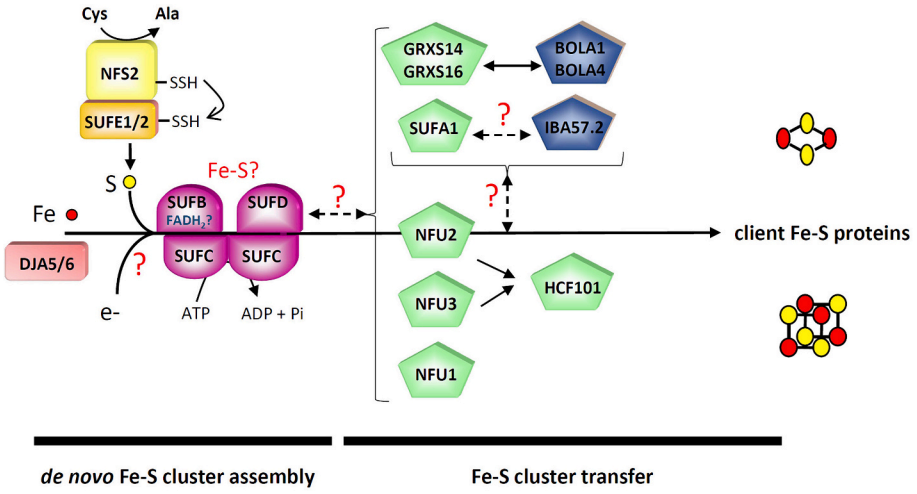

Fig. 1. General model for the assembly of Fe-S clusters by the plastidial SUF system.
For the synthesis of an Fe-S cluster on the $SUFBC_2D$ scaffold complex, the required sulfur atoms are provided by the cysteine desulfurase activity of NFS2 (*A. thaliana* ortholog of *E. coli* SufS) and relayed by SUFE1/2 proteins to the $SUFBC_2D$ scaffold complex. Based on the structural modelling presented here, we consider unlikely that SUFE3 is able to transfer sulfur atoms to the $SUFBC_2D$ scaffold complex. Other molecular steps, identified by question marks, are more uncertain. While iron may be delivered by DNA-J proteins referred to as DJA5/6, there is no evidence yet on how and when iron atoms are inserted. The donor of electrons needed to reduce sulfane sulfur atoms (S^0 oxidation state) present in persulfide (SSH) and/or ferric iron atoms during FeS cluster assembly is unknown. For these reduction steps, a contribution from a bound-$FADH_2$ cofactor was proposed because it was identified in an anaerobically as-purified $SufBC_2D$ complex from *E. coli* but the binding site and exact role could not be identified. The nature of the FeS cluster bound to $SUFBC_2D$ ($[Fe_2S_2]$, $[Fe_4S_4]$ or both) is also uncertain. In the absence of this information and without knowing which late-acting maturation factors interact directly with $SUFBC_2D$, the relative position and exact contribution of most of these late-acting maturation factors remain uncertain. On the one hand, it is documented that the NFU1–3 and HCF101 proteins may be almost uniquely required for the insertion of $[Fe_4S_4]$ clusters, even though NFU2 contributes also to the insertion of $[Fe_2S_2]$ clusters in some proteins. On the other hand, it is unclear whether GRXS14, GRXS16, BOLA1, BOLA4, SUFA1 and IBA57.2 are uniquely required for the insertion of $[Fe_2S_2]$ clusters or for both $[Fe_2S_2]$ and $[Fe_4S_4]$ clusters.

activity (stimulation of NFS activity by a factor 70) and quinolinate synthase activity (quinolinic acid formation) [14]. As shown for the corresponding *E. coli* SufS-SufE couple [23], each *Arabidopsis* SUFE protein enhances the cysteine desulfurase activity of NFS2 by accepting the persulfide group on a catalytic cysteine, thus serving as a sulfur relay towards the scaffold system [14,21]. In the case of SUFE3, the NadA domain likely prevents interaction with the scaffold complex (see below).

At the structural level, *A. thaliana* NFS2 is organized as a dimeric protein with two distant active sites, which suggests that the functional NFS2-SUFE unit should be a heterotetramer [18]. An amino acid sequence alignment of NFS2 from two representative photosynthetic organisms, the land plant *A. thaliana* and the green microalga *Chlamydomonas reinhardtii*, with a bacterial representative (*E. coli*) reveals that the overall sequences and the key motifs or residues are well conserved (Fig. S1A). The conservation includes the PLP-binding lysine (K226, *E. coli* SufS numbering unless otherwise stipulated), catalytic residues (H123 and the catalytic cysteine C364 which is part of a GHHC motif), residues at the dimer interface (R92, E96) and all four key elements constituting the β-latch: an α_6 helix with three conserved residues, a glycine rich-loop, a β-hairpin and a *cis*-proline [24,25]. The structural superimposition of *A. thaliana* NFS2 homodimer with EcSufS homodimer, both in a persulfidated state, highlights the very high structural similarity (Fig. S1B). Expectedly, the β-latch structural elements of AtNFS2 are perfectly aligned with those of EcSufS, indicating that NFS2/SUFE interactions should be conserved and mediated similarly with the β-latch regulatory motif (Fig. S1B).

Unlike NFS2, the sequence alignment of the SUFE domain present in

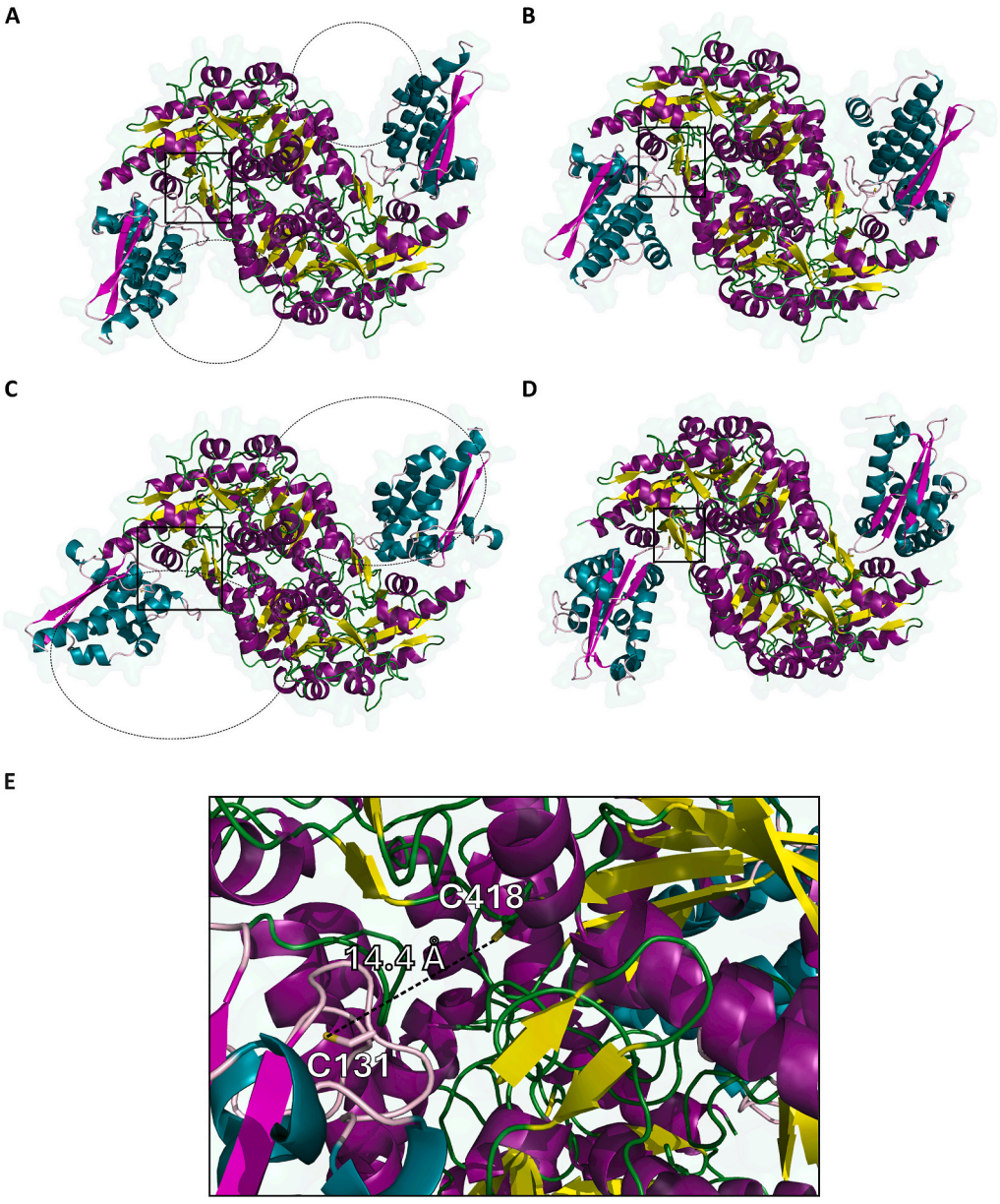

Fig. 2. The NFS2-SUFE interaction in predicted complexes using *Arabidopsis* proteins resembles the interaction between *E. coli* CsdE and CsdA. Best predicted models obtained using AlphaFold Multimer for the AtNFS2–AtSUFE1 complex (A), AtNFS2-AtSUFE2 complex (B), and AtNFS2-AtSUFE3 complex (C) have been represented for comparison with the X-ray crystal structures of *E. coli* CsdA–CsdE (PDB entry 4lw4) (D). The cysteine desulfurase (NFS2 and CsdA)–sulfur transferase (SUFE1–3 and CsdE) complexes are represented as ribbons. Cysteine desulfurases (NFS2 and CsdA) are colored in dark purple, yellow and green, and sulfur-transferases (SUFE1–3 and CsdE) are colored in cyan, purple and pink. For *Arabidopsis* SUFE proteins, only the SUFE domain is shown while the relative positions of the BolA and NadA domains are circled in dotted black line. The position of the catalytic cysteines involved in sulfur transfer from NFS2 to SUFE1–2-3 is outlined with a black box. In (E), a focus is made to show the distance between the catalytic cysteine of AtNFS2 (C131) and of AtSUFE1 (C418).

SUFE proteins from the same distant organisms reveals that only a few residues are strictly conserved (Fig. S2A). They are basically present in the two regions (residues 38–56 and 66–83, *E. coli* SufE numbering) described in EcSufE as interacting with EcSufS and undergoing conformational changes upon the formation of the heterotetramer [26]. The first region encompasses the catalytic cysteine at position 51, accepting the persulfide group from SufS. Despite the relatively low sequence conservation, the superimposition of the predicted structures of the SUFE domain from *A. thaliana* SUFE1, SUFE2 and SUFE3 proteins with EcSufE points to an important structural similarity (Fig. S2B).

Of the three *Arabidopsis* NFS2-SUFE complexes modelled by AlphaFold Multimer using full-length mature proteins, only NFS2-SUFE1 and NFS2-SUFE2 tetramers are predicted with an acceptable confidence score (Table S2). The lower score observed for NFS2-SUFE3 is probably related to the impossibility of predicting the orientation of the NadA domain, which is always different among the five predicted complexes. In comparison, the position and orientation of the BolA domain of SUFE1 is similar in all five predicted models (Figs. 2 & S3). This is likely imposed by the linker present between the BolA and SufE domain although the BolA domain is not in interaction with NFS2. In fact, in all *A. thaliana* NFS2-SUFE heterotetrametric models, including the NFS2/SUFE3 complex for which the confidence score is low, NFS2 interacts with the SUFE domain and the catalytic cysteines of SUFE and NFS2 are situated in a nearby space (Fig. 2). Moreover, the SUFE domain is found at a position similar to the one of the *E. coli* CsdE sulfur-transferase towards the CsdA cysteine desulfurase [27]. This specific positioning of the SUFE domain and the overall constraint due to the presence of other domains in SUFE1 and SUFE3 raise the question of the accessibility of the persulfidated cysteine of SUFE for the sulfur transfer step to the primary acceptor cysteine of SUFB (see below).

2.2. Iron-sulfur cluster assembly on the SUFBC$_2$D scaffold complex

2.2.1. General considerations about the mechanisms of how sulfur, iron and electrons are provided to the SUFBC$_2$D scaffold complex

Iron-sulfur cluster assembly on the mitochondrial ISCU scaffold proteins has been described to unprecedented details by structural and very deep biochemical and spectroscopic studies performed mostly with yeast and animal isoforms. The latest evidence indicate that an iron atom binds first to ISCU, which triggers the persulfide transfer from NFS1 to a cysteine of ISCU, a reaction accelerated by the presence of frataxin [28,29]. Upon reduction of the persulfide to sulfide with electrons provided by ferredoxin, and a possible ISCU dimerization, the [Fe$_2$S$_2$] cluster is formed, liberating the holo-ISCU from the NFS1-containing assembly complex [30–32]. Several reasons explain why the characterization of the molecular mechanism of *de novo* Fe-S cluster synthesis used by the SUF system lags behind. First, the SUFBC$_2$D scaffold system is a heterotetrameric complex. Second, the electron donor system, that is required to reduce sulfane sulfur (S^0) atoms in persulfidated cysteines and eventually iron atoms in the course of Fe-S cluster assembly, is still unknown. Third, although structures of most individual SUF components and of an apo-SufBC$_2$D have been elucidated using bacterial proteins (very often *E. coli*) [33], there is no structural data for a holo-SUFBC$_2$D or for a potential assembly complex including SUFBC$_2$D and the sulfur (NFS2 and/or SUFE), iron or electron donors.

The difficulty to work with a protein complex is notably illustrated by the fact that several complexes (SufB$_2$C$_2$ SufC$_2$D$_2$ and SufBC$_2$D) have been isolated upon expression of *E. coli* recombinant proteins depending on the constructs and conditions used [34,35]. Moreover, *E. coli* SufB alone assembles either a [Fe$_2$S$_2$] or a [Fe$_4$S$_4$] cluster *in vitro*. A conversion from the [Fe$_4$S$_4$]-loaded SufB form to a stable [Fe$_2$S$_2$]-loaded form is possible upon exposure to air whereas a conversion from a [Fe$_2$S$_2$] to a [Fe$_4$S$_4$] cluster-bound form is possible upon reduction [36,37]. Nevertheless, all three proteins are required *in vivo* and the SufBC$_2$D complex would be the major or at least the most stable scaffold complex both in *E. coli* and in plants [17,23,34,35]. Still, another problem is that the *E. coli* SufBC$_2$D complex binds several types of Fe-S cluster, a [Fe$_2$S$_2$], a linear [Fe$_3$S$_4$] or a [Fe$_4$S$_4$] cluster depending on the conditions used during *in vitro* reconstitution experiments [34,35,37]. Interestingly, a [Fe$_4$S$_4$]-cluster loaded form of *E. coli* SufBC$_2$D mediates the transfer and incorporation of a [Fe$_2$S$_2$] cluster on SufA or on a ferredoxin in a reaction proceeding through SufA, indicating the possibility of a cluster conversion during the transfer step [38,39]. Based on the structure of an apo-SufBC$_2$D complex, the current view is that both SufB and SufD subunits bind the Fe-S cluster [40]. From the structure and functional studies performed with mutated variants, the residues C405 and E434 of SufB and H360 of SufD have been proposed as potential Fe-S cluster ligands [40,41]. Other residues in the area that may play the role of the fourth ligand are C358 of SufD and E432 or H433 of SufB [40,41].

With regard to the iron delivery mechanism, it was recently proposed that two class A DNA-J proteins from *A. thaliana*, namely DJA5 and DJA6, may serve as iron donors to the SUF scaffold complex [11]. This was rather unexpected because they belong to the HSP40 family whose members bind zinc and act usually as HSP70 co-chaperones. Several lines of evidence have been provided: (i) DJA5 and DJA6 interact *in vivo* and *in vitro* with SUFC and SUFE1 but not with NFS2, SUFB and SUFD, (ii) both recombinant proteins bind ferrous iron *via* conserved cysteine residues in a rubredoxin-like structure with a dissociation constant (K_D) of 8 μM in the case of DJA6, and (iii) a ferrous iron-loaded form of DJA6 seems to mediate the incorporation of an Fe-S cluster into an apo-SUFBC$_2$D complex based on an absorption spectrum but the nature of the species bound has not been characterized. It would be interesting to further analyze the structural determinants that allow the recombinant *Arabidopsis* DJA5 and DJA6 to bind iron. In fact, cytosolic and mitochondrial DNA-J orthologs from yeast do not have critical roles in Fe-S protein maturation or iron regulation [42]. In *E. coli*, the DnaK ortholog has only been reported to bind zinc and no connection with the SUF machinery emerged from the numerous functional studies [43]. Moreover, no interaction was observed with SUFD that has been hypothesized to have a specialized role in iron entry into the complex [34]. Hence, many questions remain about the universality of such an HSP40-mediated iron delivery mechanism.

With regard to the sulfur delivery mechanism to the SUF scaffold complex, it was demonstrated using *E. coli* proteins, that the persulfide intermediate formed on SufE is transferred to the conserved C254 of SufB [23,36,41]. This sulfur atom would then be transferred to another conserved cysteine residue of SufB, the potential ligand C405, despite the distance between the sulfur atoms is too far (~25 Å). However, several residues with hydrophilic side chains (E236, E252, H265, T283, Q285, K303, T326, K328) are present inside the β-helix core domain of SufB and they have been proposed as important for sulfur channeling based on the observation that their mutation led to total or partial loss of SufB function in *E. coli* [41]. Also, it has been proposed from previous biochemical studies that dimerization of SufC in the presence of ATP would generate a large conformational change at the level of SUFB-D thus making the Fe-S cluster binding site more accessible [40].

With regard to the mechanism by which electrons are delivered to the SUF scaffold complex, there is very little information available regardless of whether the *E. coli* or plant SUF systems is considered. A bound-FADH$_2$ cofactor, identified in an anaerobically as-purified SufBC$_2$D complex from *E. coli* was proposed to provide the electrons needed for persulfide and/or ferric iron reduction [34,35], but so far neither the function was determined nor the binding site identified [40]. Noteworthy, while *E. coli* SufB binds small amount of reduced flavin *in vitro* [35], SufC and SufD seem also required *in vivo* [34]. Since FAD is released from the complex upon oxidation, external regeneration and possibly reloading systems would be likely needed but no flavin reductase has been identified yet.

2.2.2. Structural modelling of SUFBC$_2$D from photosynthetic organisms

According to recent phylogenetic studies, the emergence of the SUF machinery in bacteria such as *E. coli* occurred through speciation and

duplication of genes originating from the so-called SUF-like Minimal System (SMS) [6]. For instance, SmsB is thought to have been duplicated to give rise to SufB and SufD proteins, which form a superfamily, while SufC would derive from the ancestral SmsC. Despite having a similar structural fold, SufB protein sequences are highly conserved while SufD protein sequences diverged more (Figs. S4 and S5). The SufB amino acid sequence alignment revealed that residues forming the β-helix core domain, those forming the potential sulfur channeling tunnel and the potential Fe-S cluster ligands (C405 and E434), are conserved in the sequences from organisms of the green lineage. For SufD, even though critical residues of the β-helix core are to some extent conserved, no such sulfur channeling tunnel was visible. However, the putative Fe-S cluster ligating residue (H360) is conserved. Concerning SufC, which is a nucleotide-binding subunit belonging to the family of ATP-binding cassette (ABC) transporters, the key residues, notably those required for the ATPase activity (K40, E171, H203), are conserved (Fig. S6) [40].

Models of the $SUFBC_2D$ complex from *A. thaliana* have been predicted with good confidence scores using Alphafold Multimer. Structural superimposition showed a high similarity with the *E. coli* heterocomplex, notably for SUFB-SUFD protomers (Fig. 3). Indeed, in both models, SUFB and SUFD are predicted to interact through their β19 and β20 strands of the β-helix core forming two anti-parallel β-sheets, as observed for SufB-SufD heterodimer of *E. coli* (Fig. 3) [40]. Strikingly, the majority of the strictly conserved residues of SUFD are found in these β-strands indicating a high-pressure of selection on the terminal region of the β-helix core (Fig. S5). Moreover, SUFB and SUFD are also found to interact through their C-terminal region with the α_2 helix of one SUFC monomer, as in the $SufBC_2D$ complex of *E. coli* (Fig. 3) [40]. The modelling of SUFC subunits raises several questions. First, the SUFC subunits are predicted to interact in the model in the absence of bound ATP, which would be inconsistent with ATP-mediated SUFC dimerization, which has been proposed to promote large structural changes for

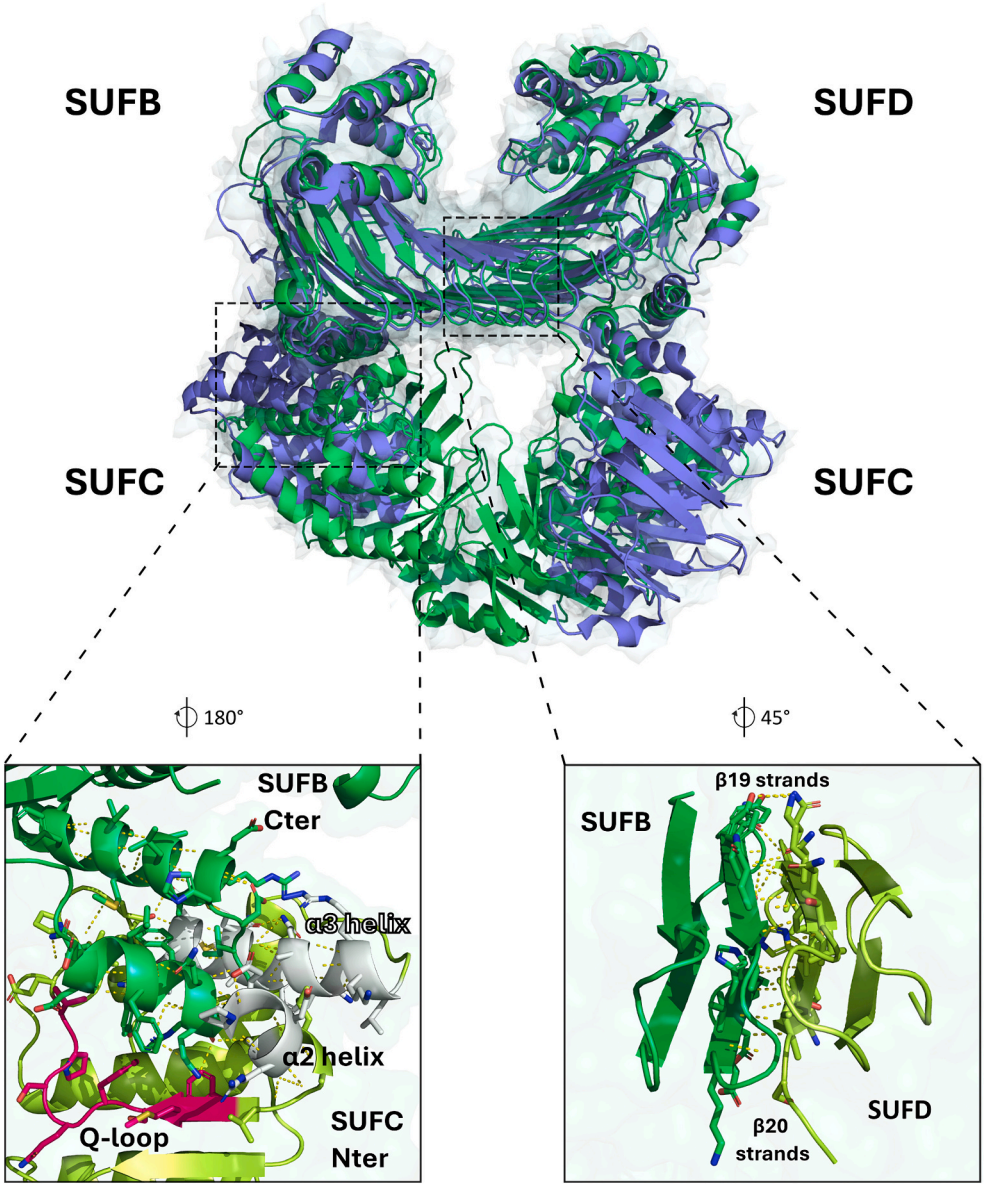

Fig. 3. The predicted structure of an apo-$AtSUFBC_2D$ complex superimposes well with the one of $EcSufBC_2D$.
Structure superimposition of the predicted $AtSUFBC_2D$ complex (in green) with the X-ray crystal structure of $EcSufBC_2D$ (PDB code: 5AWF, in blue). The TM-score, normalized to the $EcSufBC_2D$ complex, is 0.73, highlighting a good overall conservation of the fold. The lower left panel illustrates the involvement of a pair of α-helices (in gray) and Q-loop (in pink) in SUFC for its interaction with SUFB. It is similar to the so-called "transmission interface" typically found between the nucleotide-binding domain and the transmembrane domain of ABC transporters [40,44]. The lower right panel shows the interaction between AtSUFB and AtSUFD through conserved residues of their β19 and β20 strands of the β-helix core to form two anti-parallel β-sheets.

the *E. coli* $SufBC_2D$ complex [40]. Furthermore, they do not form the expected head-to-tail dimer. In this case, the AlphaFold Multimer is unable to make an accurate structural prediction, but this is not so surprising when cofactors are required and/or conformational changes occur. Studies are still needed to assess the ability of the *Arabidopsis* SUFC homodimer to bind and hydrolyze ATP and to unravel its role in the dynamics of the Fe-S cluster assembly mechanism in chloroplasts. Overall, despite these difficulties in modelling SUFC, both sequence alignments and structural predictions provide clues to the similarities between bacterial and plastidial $SUFBC_2D$ complexes.

2.2.3. *Can structural predictions shed light on the formation of a large assembly complex?*

It is currently unclear whether the SufSE and $SufBC_2D$ heterotetramers associate to form a larger complex. It was previously demonstrated that *E. coli* SufE interacts with SufS or $SufBC_2D$ with K_D in the μM range, 0.36 μM or 2.78 μM respectively [36]. So far, there is no convincing evidence that SufE can interact with both proteins/complexes at the same time. As detailed above, the regions of SufE responsible for the interaction with SufS are well described. Concerning the SufE-SufB interaction, it is documented that the sulfur atom is transferred from the cysteine 51 of *E. coli* SufE to the cysteine 254 of SufB [36]. Moreover, the observation that SufC, but not SufD, was required for detecting an *in vitro* interaction, led to the proposal that SufC may act as an allosteric regulator and that the binding to SufB induces a conformation change that would allow SufE binding [36]. Considering the structural models for SufS-SufE and $SufBC_2D$ complexes (Figs. 2 and 3) and the report that SufS does not interact with SufB, it seems rather unlikely that the persulfidated cysteine of SufE could reach the acceptor cysteine of SufB when it is in complex with SufS. Hence, we consider the possibility that SufE separates from SufS once persulfidated to allow sulfur transfer to SufB engaged in the $SufBC_2D$ complex. This contrasts with the hypothesis that a $SufS_2E_2$, a $SufS_2E$ or a SufSE complex could bind to a $SufBC_2D$ heterotetramer forming either octameric, heptameric or hexameric complexes [33,36]. Such a possibility would indeed be supported by the existence of natural fusion proteins containing both SufS and SufE domains in some bacteria, but such an enzyme/system has never been characterized and might be atypical [25]. On the other hand, some organisms equipped with the SUF system have neither SufE orthologs nor SufU orthologs, which play a role similar to SufE in some organisms, highlighting the existence of different scenarios [45]. In terms of prediction, AlphaFold Multimer was unable to propose robust models for hexameric complexes including NFS2, SUFE1/2/3 and $SUFBC_2D$. In all cases, NFS2 and SUFE1/2/3 are not found in interaction. Alternatively, we have tried to predict structural models between SUFE1/2/3 and $SUFBC_2D$. In addition to provide clues about the interactions made by the SUFE domain, this might help to tackle the question of the contribution of the BolA and NadA domains present in SUFE1 and SUFE3 from photosynthetic organisms.

In the case of SUFE1 and SUFE2, the SUFE domain is found to interact with SUFB in all predicted models, but also with SUFC monomers (Fig. 4). This appears consistent with the proposed contribution of SUFC for the SUFB-SUFE interaction. In the best models among the five models generated, the cysteine of SUFE1 or SUFE2 is found oriented towards the primary sulfur-accepting cysteine of SUFB, even though it is still buried in the core of the protein and too distant. Interaction of the

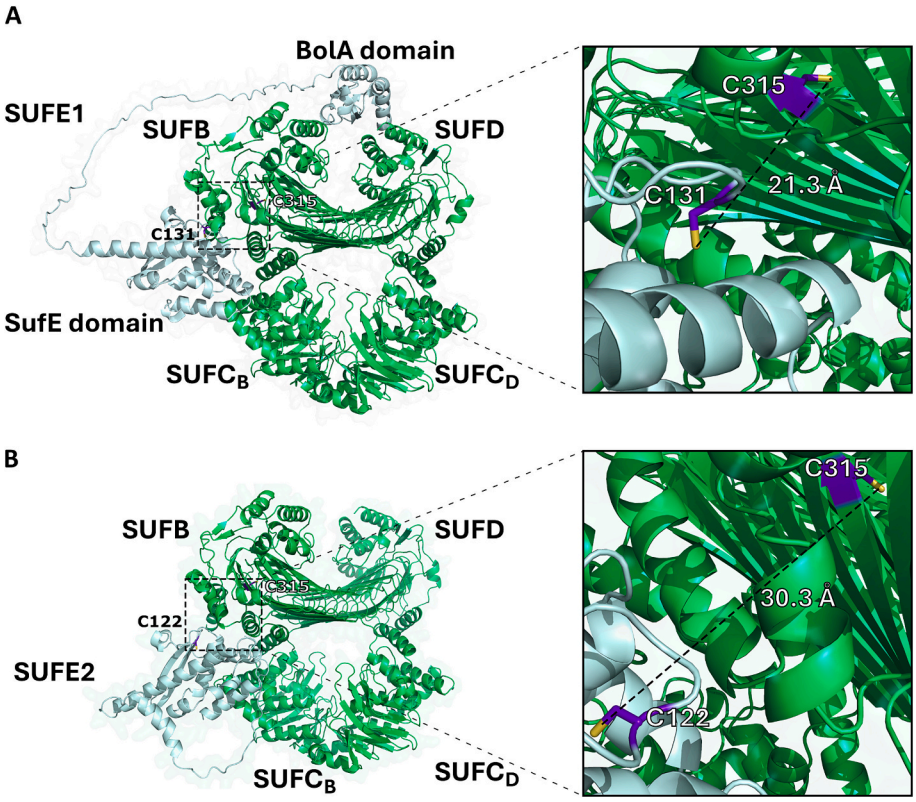

Fig. 4. Predicted structures of complexes between *Arabidopsis* SUFE1/2 and $SUFBC_2D$ point towards the interaction of the SufE domain with the SUFB subunit that is favorable for sulfur exchange.

(A) Structure prediction of an *Arabidopsis* SUFE1-BC_2D complex (confidence score: 78.2). SUFE1 interacts with SUFB and SUFC through the SufE domain and with SUFD through the BolA domain. The catalytic cysteine at position 131 of SUFE1 is oriented towards the cysteine 315 of SUFB, the proposed primary persulfide acceptor in the *E. coli* $SufBC_2D$ complex.

(B) Structure prediction of an *Arabidopsis* SUFE2-BC_2D complex (confidence score: 77.4). The SufE domain of SUFE2 interacts with SUFB and SUFC. The catalytic cysteine at position 122 of AtSUFE2 is oriented towards C315 of AtSUFB, primary persulfide acceptor of $AtSUFBC_2D$.

For each predicted complex, AtSUFE proteins are represented in pale cyan and $AtSUFBC_2D$ complex is represented in lime green.

SUFE domain of SUFE1 with SUFB is not impacted by the presence of the BolA domain and is similar to the one of SUFE2 with SUFB. In fact, the BolA domain is predicted to be in interaction with SUFD subunit in the five models. In contrast, the model confidence for the formation of a SUFE3-SUFBC$_2$D heterocomplex is below our threshold and we consider it unlikely to be relevant. First, depending on the models, the SUFE domain of SUFE3 was not systematically predicted in interaction with SUFB and when an interaction was modelled, the catalytic cysteine of SUFE3 was not oriented towards SUFB. Second, the NadA domain was modelled in interaction with SUFB in the five models, likely preventing sulfur entry into the complex. Overall, coupled with previous experimental data, these structural models reinforce the view that SUFE1 and SUFE2 are the sulfur-transferases required for the general Fe-S cluster assembly in chloroplasts, allowing sulfur relay from NFS2 to SUFBC$_2$D. By making productive interaction with NFS2 but not with SUFBC$_2$D, SUFE3 may be uniquely required for the synthesis of the [Fe$_4$S$_4$] cluster present in the NadA domain as suggested [14], but the mechanism remains to be delineated. The fact that a *Chlamydomonas* mutant for the quinolinate synthase is rescued by adding nicotinamide supports the fact that SUFE3 has no general role in Fe-S cluster synthesis [46].

In conclusion, despite the above-mentioned evidence, many steps of the molecular mechanisms underlying Fe-S cluster synthesis in chloroplasts remain unclear. In particular, the ligands and the type(s) of Fe-S clusters bound by the SUFBC$_2$D complex *in cellulo* remain to be determined. To this end, it is essential to obtain spectroscopic and structural data on as-purified Fe-S cluster-bound SUFBC$_2$D complexes, rather than relying on reconstitution experiments. If this is not possible, it would be imperative to design appropriate *in vitro* procedures and conditions, including in particular the other players in the system, *i.e.* the appropriate cysteine desulfurase and sulfur-transferase, as well as the electron and iron donors. This would possibly allow a detailed dissection of the molecular mechanism of Fe-S cluster assembly by the SUF system, would it be from bacteria, archaea or photosynthetic organisms.

3. Transfer of the iron-sulfur cluster to acceptor proteins *via* a set of late-acting maturation factors

To date, there is no evidence that the SUFBC$_2$D scaffold can directly transfer the cluster to client proteins. Therefore, for the subsequent steps of Fe-S cluster conversion, trafficking and insertion into client proteins, the Fe-S cluster bound to the SUFBC$_2$D complex has to be delivered to specific late-acting maturation factors and in particular proteins that are able to bind and transfer Fe-S clusters (Fig. 1). Interestingly, the late-acting maturation factors from the mitochondrial ISC and chloroplastic SUF systems belong to the same families. However, unlike the model for the mitochondrial ISC system, for which sequential transfer is accepted, the interaction network and hierarchical organization of the corresponding proteins of the SUF system remain largely unknown [5]. In mitochondria, the glutaredoxin S15 (GRXS15) receives a [Fe$_2$S$_2$] cluster from the scaffold protein, and transfers it to an ISCA1/ISCA2 heterodimer or to an ISCA1/ISCA2/IBA57 heterotrimer for the formation of a [Fe$_4$S$_4$] cluster, which is transferred to a homodimer of NFU4/5 [47,48]. The pathway for the insertion of the [Fe$_4$S$_4$] cluster into the MRP-type protein INDH is unknown. The inclusion in this scheme of the BOLA1 and BOLA4 and IBA57.1 proteins as Fe-S cluster binding partners of the GRXS15 and ISCA proteins, respectively, increases the complexity and number of possible pathways, see these recent reviews for more details [49,50].

While characterized *Arabidopsis* T-DNA insertion lines for the genes encoding the late-acting maturation factors of the mitochondrial ISC system or of the early maturation factors of the SUF system (see Section 1) are all lethal at the embryo or seedling stages [5,49], knock-out mutants for the genes encoding the plastidial late-acting maturation factors (GRXS14, GRXS16, NFU1, NFU2, NFU3, SUFA1, HCF101) are all viable and sometimes without growth phenotype [51–55]. Noteworthy, *hcf101* mutants need sucrose to survive their photosynthetic defects [56]. Because many Fe-S proteins in plastids play essential roles, this suggests a certain redundancy between the different Fe-S cluster transfer proteins and/or the existence of multiple pathways for Fe-S cluster insertion in the essential proteins.

As for the synthesis step, this second section is focused on the mechanistic and structural aspects, anticipating that prediction with Alphafold Multimer may bring clue to the interaction network and hierarchical organization among the SUF maturation factors. Indeed, the molecular mechanisms of how Fe-S clusters are transferred from the scaffold to the transfer proteins, or between transfer proteins, have not often been addressed experimentally. In this respect, if it turns out that the SUFBC$_2$D scaffold complex binds both a [Fe$_2$S$_2$] and a [Fe$_4$S$_4$] cluster, this would avoid conversion steps, and in particular the oxidative conversion of a [Fe$_4$S$_4$] cluster to a [Fe$_2$S$_2$] cluster, which is presumably less controllable at the cellular level.

3.1. Insertion of [Fe$_2$S$_2$] clusters and conversion to [Fe$_4$S$_4$] clusters

3.1.1. What is the contribution of GRX homodimers and GRX-BOLA heterodimers?

Based on phylogenetic analyses, glutaredoxins are divided into four classes [57]. While many plant representatives bind a [Fe$_2$S$_2$] cluster, only so-called class II GRXs are directly involved in the maturation of Fe-S proteins [58,59]. The cluster is bridged into homodimers by the cysteine residues (present in a CGFS motif) of two monomers and two glutathione molecules, as exemplified in EcGrx4 (Fig. S7) [60]. The structure of AtGRXS14 and EcGrx4 monomers superimposes very well (Fig. S7). Both plastidial class II isoforms, GRXS14 and GRXS16, bind a [Fe$_2$S$_2$] cluster in homodimers, but also in heterodimers formed with the plastidial BOLA1 or BOLA4 proteins [61–64]. In the case of the heterodimer, the BOLA proteins provide two Fe-S cluster ligands, either two histidines, as in BOLA1 or BOLA4, or one cysteine and one histidine as in BOLA2/3 members (Fig. S8) [22,64]. Noteworthy, GRXS14 interacts also with BOLA proteins without ligating an Fe-S cluster (Fig. 5) [64]. From the position of the respective subunits and of the Fe-S cluster ligands, this may represent an open conformation that is favorable for Fe-S cluster exchange. In Fe-S cluster ligating heterodimers, there is not much contacts between subunits which are basically linked *via* the Fe-S cluster.

The exact role(s) of these homo- or heterodimers is (are) not known. However, the *Arabidopsis* GRXS14 homodimer has been shown to transfer a [Fe$_2$S$_2$] cluster to AtSUFA1 extremely efficiently and rapidly, whereas the reverse reaction was not observed [65]. This suggests that GRXS14 acts upstream of SUFA1, in a sequence similar to the mitochondrial ISC system. In the absence of evidence that AtSUFA1 has the ability to bind a [Fe$_4$S$_4$] cluster, we suggest that cluster conversion may not occur at this step and therefore both proteins are only positioned for the synthesis of [Fe$_2$S$_2$] clusters in Fig. 1. It is possible that additional components (IBA57.2 and/or a suitable electron donor) were missing (see below). Regarding the role of the [Fe$_2$S$_2$] cluster-binding GRX-BOLA heterodimers, it can be hypothesized that this is a starting complex for the formation of [Fe$_4$S$_4$] clusters, analogous to the role of the yeast and human mitochondrial BOLA isoforms, which have been reported to be required for the maturation of proteins that ligate [Fe$_4$S$_4$] clusters but not [Fe$_2$S$_2$] clusters [66]. Further studies in plants are required to clarify this issue.

3.1.2. What is the contribution of SUFA1 homodimers and SUFA1-IBA57.2 heterodimer?

The SufA protein from *E. coli* binds either a [Fe$_2$S$_2$] or a [Fe$_4$S$_4$] cluster, but only a crystal structure of an apo-dimer has been crystallized [67]. Although not all cysteines were visible in the structure, it was proposed that the two cysteines of the CxC motif in the C-terminal part, which are conserved in all A-type carriers (SufA, ErpA or IscA), are the Fe-S cluster ligands (Fig. S9) [67]. To date, *Arabidopsis* SUFA1 was only described for binding a [Fe$_2$S$_2$] cluster in a homodimer [54,65,68].

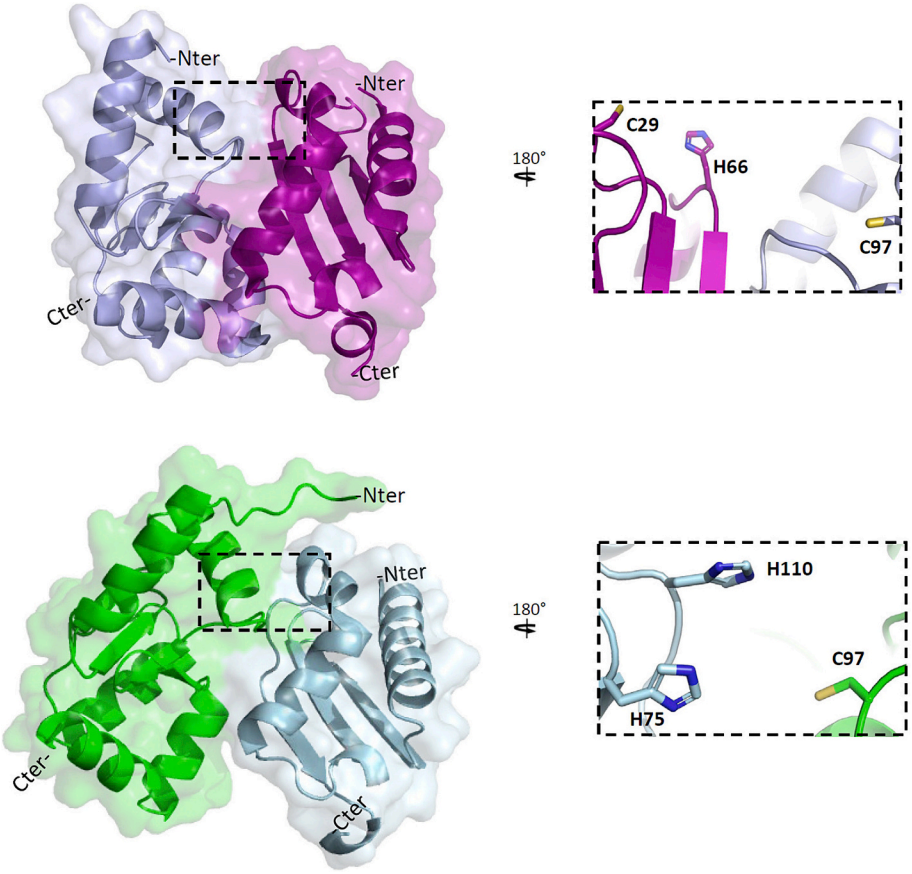

Fig. 5. Structural comparison of the apo-AtGRXS14/BOLA2 heterodimer obtained by NMR (top) with the predicted AtGRXS14/BOLA4 (bottom). On the top, GRXS14 is colored in light purple and BOLA2 in dark purple. Noteworthy, despite they are not localized in the same subcellular compartment, the cytosolic BOLA2 was used in combination with the plastidial GRXS14 for structural studies because all BOLA proteins have a similar structure. On the bottom, GRXS14 is colored in light green and BOLA4 in light blue. The structures have been aligned using TM-align (without the targeting sequences) giving a TM-score of 0.72. A zoom on the area where the Fe-S cluster is known to be bound shows the presence of the cysteine of GRXS14 (C97) and either a cysteine (C29) and a histidine (H66) in BOLA2 or two histidines in BOLA4 (H75, H110).

Hence, a dimeric structure of *Arabidopsis* SUFA1 has been predicted using Alphafold Multimer and compared with the one of *E. coli* SufA (Fig. 6). Although the confidence score is slightly above our threshold (Table S2), the *Arabidopsis* SUFA1 dimer is globally similar to the one of *E. coli* SufA with a TM-score of 0.62. However, a major difference exists in the C-terminal part, where the cysteines are located. In particular, the length and position of the last two β-strands differ and the interface between monomers comprising the cysteines is formed by unstructured regions. As a consequence, the 3 invariant cysteines (C104, C170, C172) are located relatively close to each other in the *Arabidopsis* SUFA1 model (Fig. 6A), which is different from *E. coli* SufA, in which C50 is far from the two other cysteines (C114, C116) (Fig. 6B) [67]. In fact, studies conducted with human ISCA1/2 proteins indicated that all three cysteines are required for receiving the $[Fe_2S_2]$ clusters from human glutaredoxin 5 (GLRX5) and making the conversion to a $[Fe_4S_4]$ cluster [69]. It has been proposed that both C-terminal cysteines bind the first $[Fe_2S_2]$ cluster. Then, another species is formed in which the third cysteine of two monomers acts as ligands, thus liberating two of the C-terminal cysteine residues for accepting the second $[Fe_2S_2]$ cluster. After the reductive coupling, the $[Fe_4S_4]$ cluster is proposed to be ligated by the same cysteines as in the second species. While all these intermediates can hardly be described by static structures because all these cysteines are located in structurally flexible or unstructured regions, which can easily undergo structural rearrangements, it is plausible that a similar reaction mechanism applies for *E. coli* SufA and AtSUFA1.

Concerning IBA57, a crystal structure of the *E. coli* IBA57 ortholog, named YgfZ, has also been solved [70]. It is a monomeric protein formed by three domains. While YgfZ is able to bind folate, its role is currently debated depending on the organisms considered. In *E. coli*, the role of YgfZ is required for the maturation of $[Fe_4S_4]$ clusters and notably those present in radical SAM Fe-S enzymes modifying tRNAs [71,72]. A similar role may be expected for IBA57.1 and 2 isoforms, which are both able to complement an *E. coli ygfZ* mutant [71]. Despite a very low sequence conservation between IBA57.2 from *Arabidopsis* and *E. coli* YgfZ, which is around 18 %, the predicted model of AtIBA57.2 superimposes well with the crystal structure of *E. coli* YgfZ (Fig. S10). The essential cysteine of AtIBA57.2 (C330) is situated exactly in the same region as the corresponding C228 of YgfZ.

Although this has not yet been experimentally demonstrated, it is expected that SUFA1 interacts with the plastidial IBA57.2. By analogy with the evidence obtained for mitochondrial orthologs from yeast and human, different complexes and functions could be proposed. On the one hand, it was reported that IBA57 was mandatory for the FDX2-mediated reductive coupling of $[Fe_2S_2]$ clusters donated by human GLRX5 to a ISCA1/ISCA2 but its precise role is uncertain [73]. On the other hand, evidence exists for a $[Fe_2S_2]$ cluster-bridged ISCA2-IBA57 heterodimer [74,75]. The cluster is ligated by the conserved cysteine (C228, *E. coli* numbering) present in all members of the IBA57 protein family (Fig. S10) and the three conserved cysteines present in all members of the ISCA protein family. This complex can be formed *in vitro* when IBA57 is incubated directly with a $[Fe_2S_2]$-cluster bound form of ISCA2 or with a mixture of a $[Fe_2S_2]$-cluster bound form of GLRX5 and an apo-ISCA2. This $[Fe_2S_2]$ cluster-bridged ISCA2-IBA57 heterodimer may represent an intermediate form towards the formation of a $[Fe_4S_4]$

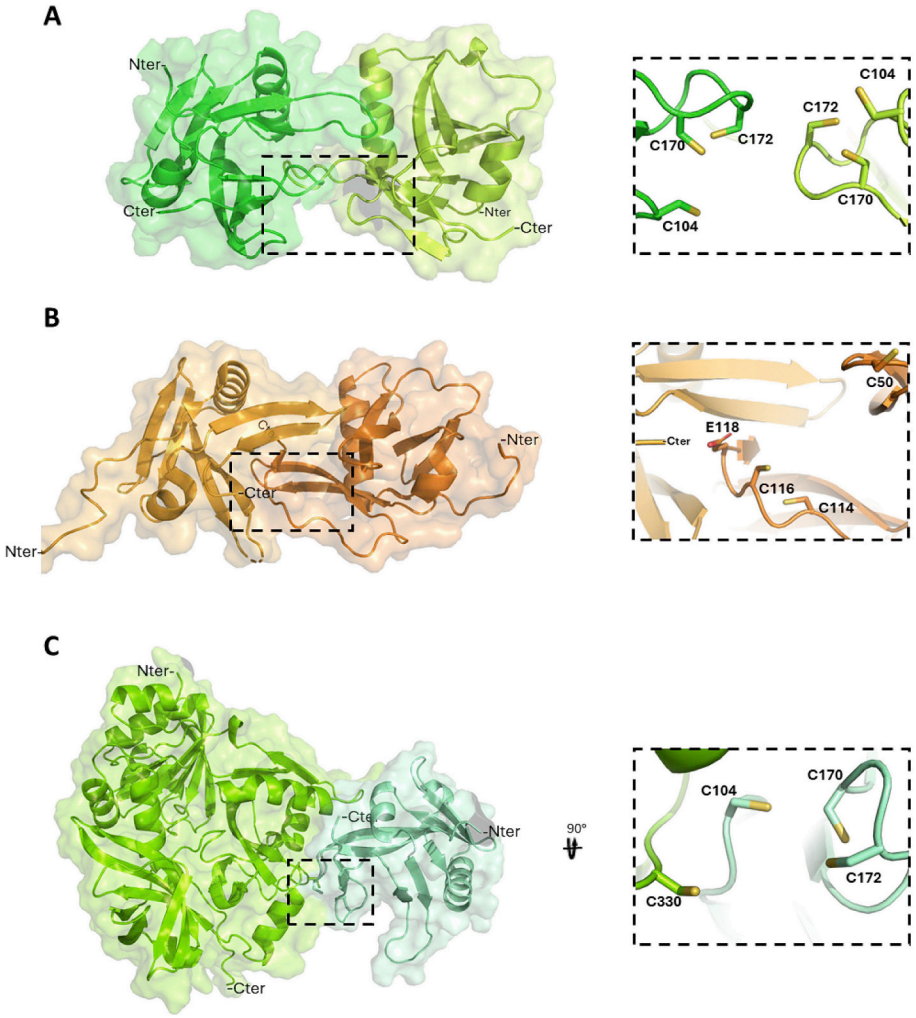

Fig. 6. Structural predictions support Fe-S cluster binding into *Arabidopsis* SUFA1 homodimer and SUFA1-IBA57.2 heterodimer.
(A) Structural model of AtSUFA1 obtained by AlphaFold2. (B) Crystal structure of SufA dimer from *E. coli* (PDB: 2D2A) with a similar orientation. In both structures, a zoom in the potential Fe-S cluster binding site is shown. In EcSufA, the electron density of the C-terminal end of a monomer was partial, but the model proposed favors a role of C114 and C116 from each subunit as ligands because C50 is far away [67]. In AtSUFA1, all three cysteines (C72, C170 and C172) are relatively close in an unstructured region. (C) Structural prediction of *Arabidopsis* SUFA1-IBA57.2 heterodimer obtained with AlphaFold Multimer, IBA57.2 is in green and SUFA1 in turquoise. A zoom in the potential Fe-S cluster binding site indicates that C104, C170 and C172 from SUFA1 and C330 from IBA57.2 could bind the Fe-S cluster.

cluster if ISCA1 comes into play and/or a physiologically relevant complex since a decreased expression and maturation of the [Fe_2S_2] cluster-binding Rieske protein of the cytochrome bc_1 complex was observed in a *S. cerevisiae iba57* mutant [76]. A structural model of an *Arabidopsis* SUFA1-IBA57.2 heterodimer has been obtained with a confidence score of 70.8 (Fig. 6C). The fact that the overall fold and monomer positioning is similar with the heterodimer formed by human ISCA2/IBA57 and modelled by the use of SAXS and docking experiments (Fig. S11) [75], and that the cysteine residues of IBA57.2 and SUFA1 are found in close vicinity suggest that this association is potentially physiologically significant and conserved.

3.2. Insertion of [Fe_4S_4] clusters into recipient apo-proteins

3.2.1. Structural modelling supports the dimerization of NFU proteins for Fe-S cluster binding

Plastidial NifU-like (NFU) proteins are classically composed of two NFU domains, a regular domain containing the CxxC motif responsible for Fe-S cluster ligation and a second domain with the same topology, that has lost the motif, referred to as degenerated NFU domain [77]. Three members, namely NFU1/2/3, exist in *A. thaliana* plastids [52]. Most characterized plastidial NFUs bind [Fe_4S_4] clusters into homodimers [77,78]. However, *Arabidopsis* NFU2 is also able to accommodate a [Fe_2S_2] cluster, possibly in a tetramer or in a dimer with a different arrangement [77]. This form is required for the maturation of the [Fe_2S_2] cluster present in the plastidial dihydroxyacid dehydratase (DHAD) [79]. Intriguingly, *C. reinhardtii* and many Chlorophytes possess only two NFUs (NFU1 and NFU2). Moreover, NFU1 is atypical as it comprises an N-terminal GIY-YIG type endonuclease domain, similar to the one present in GRXS16, making this protein much larger. The amino acid sequence alignment of plastidial NFUs from *Arabidopsis* and *C. reinhardtii* with the NFU domain of *E. coli* NfuA confirms that the regular NFU domain exhibits a particularly high level of conservation whereas the degenerated NFU domain has diverged dramatically (Fig. S12). In the structural models built using AlphaFold Multimer, all five proteins are similarly organized as head-to-tail homodimers (Fig. 7). In these models, the N-terminal NFU domain of one monomer interacts with both domains of the other monomer. The two cysteine pairs of the CxxC motif are systematically located at the contact site between the two N-terminal domains, forming a quartet that is undoubtedly the Fe-S cluster binding site. The role of the degenerated domain is unclear as it is absent in cyanobacterial orthologs and replaced by other extensions in bacterial and mitochondrial NFUs [77]. In *E. coli* NfuA, the additional domain, which is a degenerated A-type carrier domain, serves for the

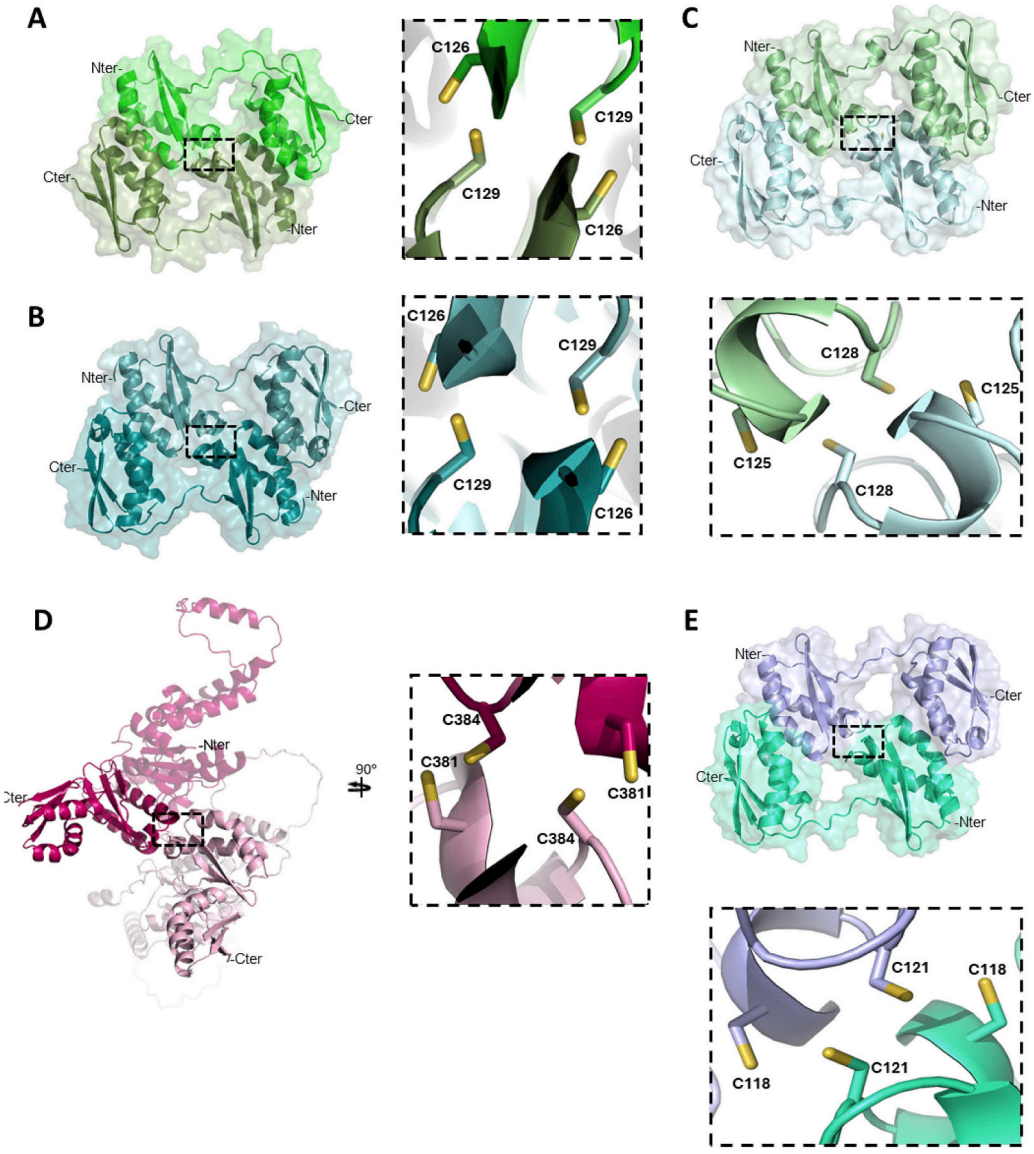

Fig. 7. The position of the cysteines presumably involved in Fe-S cluster ligation is conserved between plant and algal NFU proteins.
Structure predictions of NFU dimers were generated with AlphaFold Multimer for AtNFU1–3 (A-C) and CrNFU1–2 (D-E). CrNFU1 possesses a large additional domain of unknown function at the N-terminal end. In all models, the two cysteines of the CxxC motif of each monomer are always in close vicinity at the center of the dimer, supporting a role in Fe-S cluster binding.

recognition of partner proteins [80]. Hence, a degenerated NFU domain may also aid in recruiting protein partners or in facilitating protein–-protein interactions. Interestingly, a similar role was proposed for bacterial SufT formed by a domain (DUF59) with a αββαβ topology that strikingly resembles the one of the NFU domain (Fig. S12). In fact, the DUF59 domain is sometimes present in natural fusion proteins associated with a regular NFU domain and phenotypic analyses of both *ΔsufT* and *Δnfu Staphylococcus aureus* mutant strains point to partially overlapping functions [81]. Last but not least, this DUF59 domain is present in several other maturation factors, the cytosolic AE7/Cia2 maturation factor and HCF101 (see below) [82]. The presence of a DUF59 domain in HCF101 fits with the current evidence indicating that NFU2 and NFU3 would transfer their [Fe_4S_4] clusters to HCF101, thus acting upstream of HCF101 [52]. Consistently, the set of direct or indirect partners of NFUs is much larger than the one of HCF101, the role of which appears associated with the maturation of Fe-S proteins involved in light-dependent reactions [51,52,83–85].

3.2.2. Binding of the [Fe_4S_4] cluster by HCF101 may differ between photosynthetic organisms

The HCF101 proteins belong to the P-Loop-NTPase or MRP (metG-related protein) family as do the *E. coli* Mrp and *Salmonella enterica* ApbC orthologs but also proteins involved in the ISC and CIA machineries, INDH (iron–sulfur protein required for NADH-dehydrogenase), NBP35 (nucleotide-binding protein of 35 kDa) and cytosolic Fe-S cluster deficient (CFD1) [51,86–88]. However, the HCF101 proteins differ from the others in terms of domain architecture, oligomerization state and Fe-S cluster ligation. Indeed, they possess two domains of unknown functions, DUF59 at the N-terminus and DUF971 at the C-terminus separated by a central P-loop ATPase domain (Fig. 8A). Moreover, while all proteins bind [Fe_4S_4] clusters, *Arabidopsis* HCF101 has been reported to be a monomer whereas other proteins are dimeric [86–89]. Finally, NBP35, INDH/Ind1 or Mrp/ApbC bind the cluster using cysteines present in a CxxC motif in the central domain, whereas 3 cysteines (C128, C347, C419) distributed along the sequence of *Arabidopsis* HCF101 have been proposed to be involved in [Fe_4S_4] cluster binding (Fig. 8A) [86–89]. It is surprising that C347 and C419 are not conserved in HCF101 from

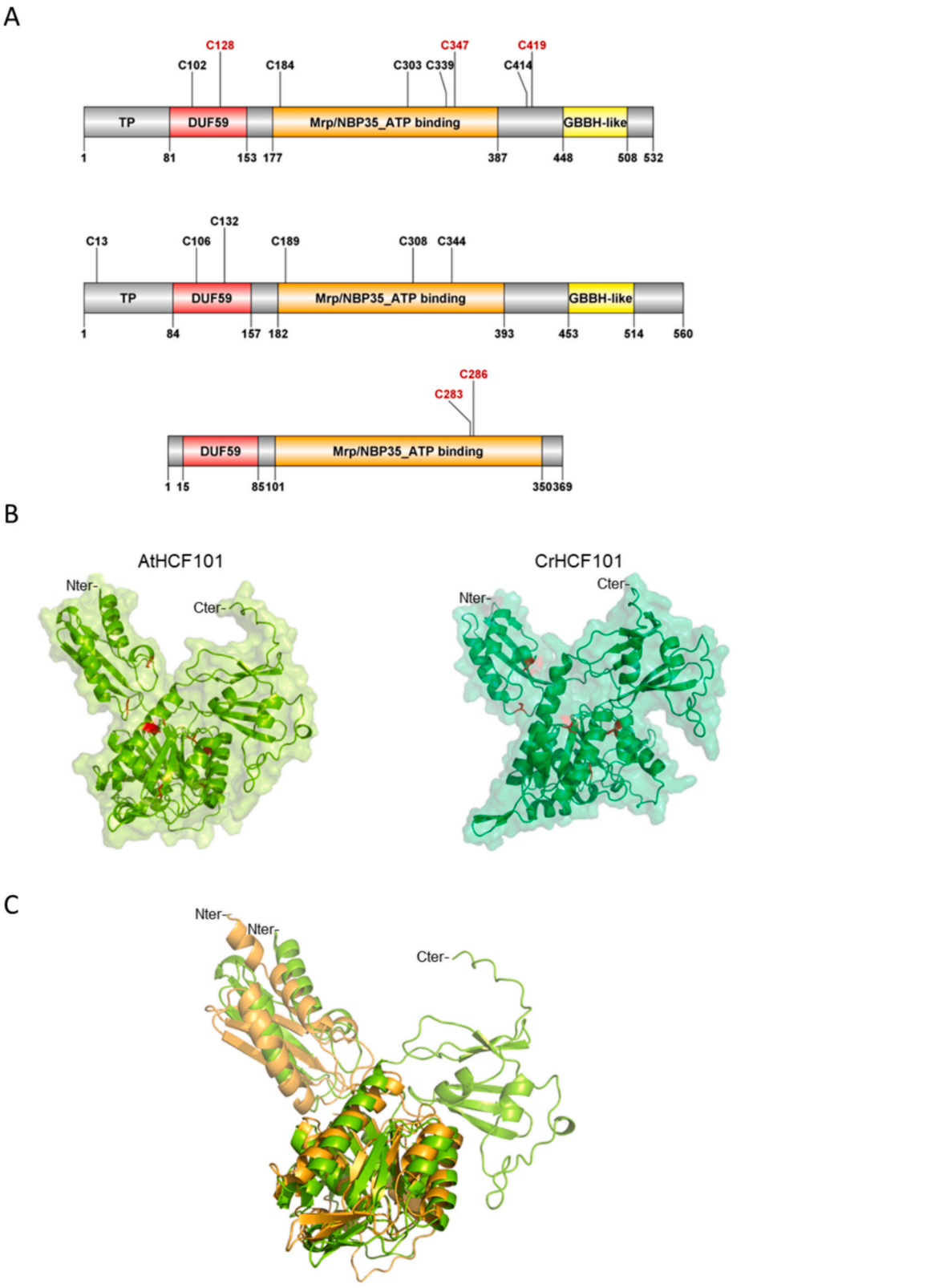

Fig. 8. Cysteine content and structural modelling of HCF101 orthologs from two model photosynthetic organisms.
(A) Representation of the domain organization of *Arabidopsis* and *Chlamydomonas* HCF101 and *E. coli* Mrp including the position of the cysteines. The cysteines proposed to serve as Fe-S cluster ligands in AtHCF101 and in EcMrp, based on their conservation in *Desulfovibrio* orthologs, are in red [89,94]. The positions of the domains (DUF59 = IPR002744, Mrp/NBP35_ATP binding = IPR019591, GBBH-like = IPR010376) were recovered from InterPro.
(B) Structure prediction of *Arabidopsis* and *Chlamydomonas* HCF101 generated with AlphaFold2 showing the position of the 3 domains and of the cysteine residues in red.
(C) Structure superimposition of the predicted models for EcMrp (in orange) and AtHCF101 (in green) using TM-align. Both models are similar with a TM-score of 0.80 when normalized on Mrp length.

Chlamydomonas (Fig. S13) and many other Chlorophytes. In fact, the CxxC motif used for Fe-S cluster binding in other P-loop NTPases is present in some plastidial and mitochondrial HCF101 isoforms from protists (Haptista and Cryptista), but neither in *Arabidopsis* nor in *Chlamydomonas* HCF101, pointing to an extreme diversity among these family members [90]. The C128 is present in the DUF59 domain. It is conserved in all SufT in a DPE-$X_{26–31}$-T-$X_{2/3}$-C motif and is highly reactive [82,91]. Although SufT itself is not able to bind an Fe-S cluster *in vitro*, it could form heterodimers with other proteins such as NFU for instance (see above) [82,92]. Intriguingly, another cysteine (C102, *Arabidopsis* numbering) is conserved in the DUF59 domain of HCF101 proteins, but not in all bacterial SufT (Figs. 8A & S13). Overall, AtHCF101 possesses 8 cysteines of which 5 are present in *Chlamydomonas* but as mentioned already, two proposed ligands in AtCHF101 are absent in CrHCF101. Given these differences in the number and position of cysteines, the structural prediction was performed for both *Arabidopsis* and *Chlamydomonas* HCF101 as monomers (Fig. 8B). One difficulty with this kind of multi-domain proteins is related to the existence of linkers that are potentially flexible and could impact the respective positioning of the domains. Here, their position was relatively constant in the five models generated for each protein. Slight differences in the orientation of the domains exist when comparing *Arabidopsis* and *Chlamydomonas* HCF101 models. The central domain, which is shared with all other P-Loop-NTPase family members, adopts a structure similar to the one in EcMrp, according to a high identity level (Fig. 8C, Fig. S13).

Examination of the structures for identifying relatively close cysteine residues suggests that both cysteines of the DUF59 domain are relatively close from each other and C102 is present in a flexible loop between the second α-helix and the third β-strand. This region could actually represent a suitable environment for the binding of an Fe-S cluster. There are several conserved acidic residues in the area that could eventually serve for the binding of an Fe-S cluster into an HCF101 monomer. Alternatively, dimerization could be required for bringing two DUF59 domains close enough. Such a prediction has also been generated with AlphaFold Multimer but the confidence score was low (55.6) and the DUF59 domains are situated on opposite sides.

Another interesting cysteine is the one present in the otherwise very conserved SCKGGVGKS motif (it replaces the usual glycine), which is present in the central domain in a loop responsible for nucleotide binding (Fig. S13) [89]. Using the yeast NBP35-CFD1 heterodimer as model, it was shown that ATP binding is necessary to induce conformational changes that facilitate Fe-S cluster binding, while ATP hydrolysis may be required for Fe-S cluster transfer [93]. This change in the motif raises the question of the impact of such a substitution in HCF101 for which ATPase activity has not yet been tested.

In conclusion, the P-loop NTPase proteins are globally quite divergent although they seem all required for the binding and transfer of $[Fe_4S_4]$ clusters. While it is not so surprising that Fe-S cluster binding in HCF101 proteins differs from the one in bacterial Mrp and eukaryotic NBP35 and INDH, the divergence in the conservation of cysteine residues among HCF101 from land plants and Chlorophytes is puzzling. Additional studies are needed to decipher whether Fe-S cluster ligation is indeed different. Moreover, the role(s) of the additional domains and of a potential ATP binding and hydrolysis activity of HCF101 has to be investigated as well.

4. Conclusions

Even though the SUF machinery is widespread, *i.e.* it is present in archaea, bacteria including *E. coli* and eukaryotic photosynthetic organisms, the characterization of the molecular mechanisms of Fe-S cluster assembly and transfer lags behind that of the other widespread ISC machinery. For the assembly step, some players, in particular the electron donor, remain to be identified and the nature of the Fe-S cluster bound to the scaffold complex in cells is still elusive. The question of whether a large Fe-S cluster assembly complex is formed as for the ISC machinery needs to be investigated together with the mechanisms regulating the incorporation of iron and sulfur into the scaffold complex. The considerable recent advances in structural modelling may provide some clues. For example, it seems more likely that SufE brings sulfur atoms to SufB after unbinding from SufS rather than in a SufS-SufE complex.

There are also many uncertainties about the steps of Fe-S cluster transfer, conversion and insertion into client proteins. Apart from the evidence showing that SufA proteins can accept a cluster from $SufBC_2D$ and from glutaredoxins and that HCF101 acts upstream of NFU2/3 in *Arabidopsis*, the exact position and partners of the other Fe-S cluster transfer proteins remain largely unknown. For the HCF101/Mrp family, evolution led to significant differences since the domain organization and ligation mode of the $[Fe_4S_4]$ cluster differ between orthologs from bacteria and photosynthetic organisms and even between Chlorophytes and Embryophytes.

Abbreviations

ATC	A-Type Carrier
CFD1	Cytosolic Fe-S cluster Deficient 1 protein
CIA	Cytosolic Iron-sulfur protein Assembly
CpNifS	Chloroplastic NifS-like protein
FDX2	Ferredoxin 2
GLRX5	human Glutaredoxin 5
GRX	Glutaredoxin
HCF101	High-chlorophyll fluorescence 101
HSP40/70	Heat-shock protein 40/70
INDH/Ind1	Iron-sulfur protein required for NADH-dehydrogenase
ISC	Iron-Sulfur Cluster
K_D	Dissociation constant
MRP	metG-related protein
NBP35	Nucleotide-Binding Protein of 35 kDa
NFU	Nitrogen-Fixation-subunit-U
NIF	Nitrogen Fixation
NMR	Nuclear Magnetic Resonance
PLP	Pyridoxal-L-phosphate
SAM	*S*-Adenosyl methionine
SAXS	Small Angle X-ray Scattering
SMS	SUF-like Minimal System
SUF	SUlfur mobilization Factor

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CRediT authorship contribution statement

Antoine Kairis: Writing – review & editing. **Benjamin Das Neves:** Writing – review & editing. **Jérémy Couturier:** Writing – review & editing. **Claire Remacle:** Writing – review & editing. **Nicolas Rouhier:** Writing – review & editing.

Declaration of competing interest

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Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2024.119797.

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