A Nucleus-Encoded Chloroplast Protein Regulated by Iron Availability Governs Expression of the Photosystem I Subunit PsaA in *Chlamydomonas reinhardtii*

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The biogenesis of the photosynthetic electron transfer chain in the thylakoid membranes requires the concerted expression of genes in the chloroplast and the nucleus. Chloroplast gene expression is subjected to anterograde control by a battery of nucleus-encoded proteins that are imported in the chloroplast, where they mostly intervene at posttranscriptional steps. Using a new genetic screen, we identify a nuclear mutant that is required for expression of the PsaA subunit of photosystem I (PSI) in the chloroplast of *Chlamydomonas reinhardtii*. This mutant is affected in the stability and translation of *psaA* messenger RNA. The corresponding gene, *TRANSLATION OF psaA* (TAA1), encodes a large protein with two domains that are thought to mediate RNA binding; an array of octatricopeptide repeats (OPR) and an RNA-binding domain abundant in apicomplexans (RAP) domain. We show that as expected for its function, TAA1 is localized in the chloroplast. It was previously shown that when mixotrophic cultures of *C. reinhardtii* (which use both photosynthesis and mitochondrial respiration for growth) are shifted to conditions of iron limitation, there is a strong decrease in the accumulation of PSI and that this is rapidly reversed when iron is resupplied. Under these conditions, TAA1 protein is also down-regulated through a posttranscriptional mechanism and rapidly reaccumulates when iron is restored. These observations reveal a concerted regulation of PSI and of TAA1 in response to iron availability.

PSI is a remarkable biological nanodevice capable of using light energy to drive electron transfer reactions with a quantum yield that is close to 100% (Amunts and Nelson, 2009). The core complex of PSI comprises 12 to 19 polypeptides (depending on the organism) that assemble together with up to 200 cofactors that include chlorophylls, carotenoids, iron-sulfur clusters, and phylloquinones. Some subunits are encoded in the chloroplast genome, while others are encoded in the nuclear genome and imported into the chloroplast. The biogenesis of PSI thus requires a tight coordination of gene expression in the nucleus and the chloroplast.

In *Chlamydomonas reinhardtii*, the core complex of PSI is composed of four chloroplast-encoded polypeptides, the two large subunits PsaA and PsaB and two smaller subunits, PsaC and PsaI. The complex further comprises 10 nuclear-encoded subunits. The coordinated biogenesis of PSI also marshals numerous nuclear genes that intervene in posttranscriptional steps of chloroplast gene expression and in the assembly of the complex. The *psaA* gene is split in three separate exons scattered in distant loci of the chloroplast genome (Kück et al., 1987). The exons are transcribed separately, and the three precursors are assembled with the short *trans-splicing in the chloroplast A* (*tscA*) RNA to form the structures of two split introns of group II (Goldschmidt-Clermont et al., 1991). The introns are removed in two steps of trans-splicing to form the mature *psaA* messenger RNA (mRNA; Choquet et al., 1988). Genetic analysis of PSI-deficient *C. reinhardtii* mutants has revealed that at least 14 nuclear loci are required for trans-splicing of *psaA* (Goldschmidt-Clermont et al., 1990). To date, six of the genetically defined factors have been characterized in more detail. The processing of *tscA* from a polycistronic precursor requires RNA MATURATION OF *psaA* (RAA1), RNA MATURATION OF *tscA* (RAA1), RAA2, and RAA4. RAA1 is also required for the trans-splicing of both the introns (Hahn et al., 1998; Balczun et al., 2005; Merendino et al., 2006; Glanz et al., 2012). RAA3 is involved in trans-splicing of the first intron while RAA2 is required for trans-splicing of the second intron (Perron et al., 1999, 2004; Rivier et al., 2001). Biochemical studies have revealed several other proteins that bind to the *psaA* introns (Balczun et al., 2006; Glanz et al., 2006; Jacobs et al., 2013).

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The assembly of PSI is assisted by two chloroplast-encoded proteins, Hypothetical chloroplast reading frame3 (Ycf3) and Ycf4, which are required at early steps in the formation of the functional complex (Boudreau et al., 2000; Naver et al., 2001; Onishi and Takahashi, 2009; Ozawa et al., 2009). The assembly begins with PsbB, which is produced under the control of TRANSLATION OF psbB1 (TAB1) and TAB2. These nucleus-encoded proteins are necessary for translation initiation of psbB mRNA (Stampacchia et al., 1997; Dauvillée et al., 2003; Rahire et al., 2012). The synthesis of stoichiometric proportions of two other chloroplast-encoded polypeptides, PsA and PsC, is ensured through Control by Epistasy of Synthesis: unassembled PsA or PsC subunits exert negative feedback regulation on the translation of their respective mRNAs when they are produced in excess of PsB (Wostrikoff et al., 2004). The stability of psaC mRNA depends on a newly discovered nucleus-encoded factor, MATURATION OF psaC1 (MAC1; D. Douchi and M. Goldschmidt-Clermont, unpublished data). As described above, trans-splicing of psaA requires at least 14 nucleus-encoded proteins. However, psaA trans-splicing can be bypassed, without any apparent phenotypic consequence, by introducing an intron-less copy of the gene in the chloroplast genome (Lefebvre-Legendre et al., 2014). This suggests that the complex trans-splicing pathway does not play a predominant role in the regulation of PSI assembly, at least under a number of different growth conditions that were investigated. By analogy with psbB and psaC, and also with numerous subunits of the other photosynthetic complexes, it could be expected that other specific nucleus-encoded factors control the stability and translation of psaA mRNA. Genetic screens to identify such factors have been hindered by the large number of genetic loci that are required for psaA trans-splicing and constitute the prevalent mutational target. Here, we present a genetic screen designed to identify among a collection of PSI mutants those that would affect expression of psaA at a step other than trans-splicing. This led to the identification of TRANSLATION OF psaA1 (TAA1), a nucleus-encoded factor required for the stability and translation of psaA mRNA. TAA1 turns over rapidly, and its levels are down-regulated under conditions of iron limitation.

RESULTS
Isolation of the taa1 Mutant

The taa1 mutant was obtained by screening a collection of PSI-deficient mutants (Girard et al., 1980) to identify those whose genetic target is the 5′ untranslated region (UTR) of psaA. For this screen, the PSI mutants were transformed with two different plasmids bearing aminoglycoside adenyltransferase A (aadA) chimeric genes, which confer resistance to spectinomycin (Goldschmidt-Clermont, 1991). The tester construct psaA-aadA contained the psaA promoter and 5′ UTR fused to aadA (Wostrikoff et al., 2004), while the positive control atpA-aadA contained the promoter and 5′ UTR of adenosine triphosphate synthase A (atpA) fused to aadA (Kuras et al., 1997). Transformation of the taa1 mutant (formerly F23) with psaA-aadA failed to confer spectinomycin resistance, while the control transformation with atpA-aadA allowed growth on the antibiotic, indicating that the promoter or 5′ UTR of psaA is a target of TAA1.

To confirm this conclusion, the taa1,mt(–) mutant was crossed to a transformed strain carrying the psaA-aadA cassette (Wostrikoff et al., 2004). Due to the uniparental inheritance of the chloroplast genome, all the progeny received the psaA-aadA transgene from the mt(+) parent whereas only one-half of the progeny inherited the taa1 mutation from the nuclear genome of the mt(–) parent. In eight complete tetrads, all the PSI-deficient taa1 progeny were unable to grow on spectinomycin, while all the wild-type progeny did show resistance. This is illustrated with the four progeny of a representative tetrad: J11, J12, J13, and J14 (Fig. 1A). In this tetrad, J13 and J14 had the wild-type TAA1 gene, as deduced from the presence of PsA (Fig. 1B) and their growth on minimal medium, while J11 and J12 carried the taa1 mutation. The absence of TAA1 in J11 and J12 leads to a growth defect on photosynthetic medium and on spectinomycin plates. The results thus confirm that the promoter or 5′ UTR of psaA is a target of TAA1. We noted that J14 grew more slowly, suggesting the presence of another mutation in the background of this strain. Therefore, for the further analysis of taa1, the strain was backcrossed three times to the wild type. As shown by immunoblot analysis, the amount of PsA protein was reduced below detection levels in the taa1 progeny, but present in the wild-type progeny (Fig. 1B). There was a concomitant decrease in the level of the chimeric psaA-aadA mRNAs in the taa1 progeny J11 and J12 compared with the wild-type progeny J13 and J14 (Fig. 1C). These data indicate that the absence of TAA1 leads to a defect in either the transcription or the accumulation of the chimeric psaA transcript and an even stronger effect on the levels of the PsA protein.

Identification of the TAA1 Gene

Transformation of the taa1 mutant strain with an ordered cosmid library of genomic DNA allowed the identification of a cosmid (64B4) that could rescue photoautotrophic growth (Zhang et al., 1994). The 9.4-kb EcoRI subfragment of this cosmid (64B4E) was also able to rescue the taa1 mutant (Supplemental Table S1). A BLAST search of the C. reinhardtii genome with the sequence of this subfragment identified a genomic region containing only one gene, Cre06.g262650 (Phytozome version 10.0). The gene comprises 12 exons according to the assembly of EST data and the gene model predictions (Fig. 2A). A partial complementary DNA (cDNA; 3.5 kb) was isolated from a C. reinhardtii CDNA library using as hybridization probe a PCR fragment located in the 3′ UTR of the gene. This cDNA corresponds to the last six exons, and its sequence is in accordance with the annotation of the transcript (Phytozome version 10.0). Sequencing

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of reverse transcription (RT)-PCR fragments from the same region also confirmed the nucleotide sequence. The presence of three in-frame stop codons within 35 bp upstream of the translation initiation codon indicates that the complete coding sequence was identified. The deduced TAA1 open reading frame encodes a protein of 2,083 amino acids (207 kD), which contains seven tandem degenerate repeats of 38 to 40 amino acids belonging to the octatricopeptide repeat (OPR) family (Auchincloss et al., 2002; Merendino et al., 2006; Eberhard et al., 2011; Rahire et al., 2012). The predicted polypeptide (Fig. 2A; Supplemental Fig. S1) shows a group of five OPR repeats (residues 928–1,117) separated from two further repeats (residues 1,448–1,525) and an RNA-binding domain abundant in apicomplexans (RAP) domain (residues 1,978–2,031). The OPR repeats are postulated to form α-helical RNA-binding domains and are also present in other C. reinhardtii chloroplast proteins involved in post-transcriptional steps of mRNA expression (Auchincloss et al., 2002; Balczun et al., 2005; Merendino et al., 2006; Eberhard et al., 2011; Rahire et al., 2012). The RAP domain is thought to be an RNA-binding domain (Lee and Hong, 2004) and is also found in RAA3, which is involved in trans-splicing of psaA in C. reinhardtii (Rivier et al., 2001). When a stop codon was introduced in the TAA1 midigene upstream of the RAP domain (residue 1,906), it lost its ability to rescue the taa1 mutant, an indication that this domain may be functionally important (Supplemental Table S1). Altogether, these observations suggest that TAA1 could be involved in RNA metabolism.

A comparison of the cDNA sequences of the wild-type TAA1 and of the taa1 mutant, obtained by RT-PCR, revealed a single base substitution, which changes Gln_{1277} to a stop codon (Fig. 2A, marked with a star) located between the two OPR domains. The mapping of this mutation confirms that the TAA1 gene was properly identified. Using a different genetic screen, Young and Purton (2014) identified several PSI-deficient mutants that could be rescued by transformation with TAA1 genomic DNA (cosmid 64B4). In one of these mutants, allelism to taa1 was confirmed by the presence of a mutation creating a stop codon in exon 5 of TAA1.

A TAA1 midigene (pl30) was constructed as a fusion of a genomic fragment containing the 5’ part of the gene (including 0.6 kb upstream of the initiation codon and the first six introns) to the cDNA of the 3’ part (Fig. 2A; “Materials and Methods”). Transformation of the taa1 mutant with the midigene restored photautotrophic growth (Fig. 2B) at least as efficiently as the genomic fragment 64B4E (Supplemental Table S1). A rabbit polyclonal antibody was produced against a subfragment of TAA1 (amino acids 1,335–1,591). Using this TAA1 antisera for immunoblotting, a weak band at 250 kD was detected in protein extracts from the wild type that was absent in taa1 (Fig. 2C). This is significantly larger than the predicted mass of 207 kD, but slow migration was previously observed for other C. reinhardtii proteins such as TAB1 (Rahire et al., 2012) or RAA1 (Merendino et al., 2006). This could be due to the presence of long hydrophilic stretches and the large size of these proteins. It is noteworthy that different levels of accumulation of TAA1 were observed in different wild-type laboratory strains of C. reinhardtii. Immunoblot analysis of total extracts from the taa1 mutant in the cell wall-deficient 15 (cw15) background transformed with the TAA1 midigene (taa1;cw15;TA1) revealed an overexpression of the TAA1 protein (Fig. 2C). To facilitate the detection of TAA1, a version of the midigene carrying a hemagglutinin (HA) epitope at the C terminus was also constructed (Fig. 2A; “Materials and Methods”). The mutant transformed with this construct, (taa1;cw15;TA1-HA) grew photoautotrophically on minimal medium, showing that the HA-tagged version of the TAA1 midigene is functional (Fig. 2B). This strain also overexpressed the TAA1 protein (Fig. 2C). Northern-blot analysis showed no significant differences in the accumulation of the psaA mRNA in these strains despite the overexpression of TAA1 (data not shown), and PsA accumulated to normal levels (Fig. 2C), suggesting that TAA1 is not limiting for psaA mRNA accumulation in the wild-type strain.

**TAA1 Is a Chloroplast Protein**

To investigate the localization of the TAA1 protein, we took advantage of the taa1;TA1-HA strain to facilitate its detection with the epitope tag. In a first approach,
the subcellular distribution of TAA1 was investigated by confocal immunofluorescence microscopy. To validate the identification of different subcellular compartments, antibodies were used against either the PHOSPHO RIBULO KINASE (PRK) protein, which is located in the chloroplast (Fig. 3A), or the 60S subunit of the cytoplasmic ribosome (Fig. 3B). As a negative control for the HA immunofluorescence signal, the taa1 mutant complemented with the similar midigene construct lacking the HA epitope tag (taa1;TAA1) was used. As expected (Fig. 3, A and B), no HA signal was detected in the latter strain. A colocalization of the TAA1-HA protein with the PRK protein was observed in the chloroplast, while the localization of TAA1 and the cytosolic 60S ribosome subunit were clearly distinct (Fig. 3B). We used Van Steensel’s analysis (for review, see Bolte and Cordelières, 2006), to quantitatively confirm that the TAA1-HA and PRK signals were coincident (green curve in Fig. 3C), whereas the TAA1-HA and the ribosomal 60S subunit signals exhibited complementary patterns (blue curve in Fig. 3C). Accordingly, Manders’ analysis showed that the overlap between the TAA1-HA and the PRK signals was 87% ± 1.2% (average of Manders’ coefficient on n = 43 cells), whereas the overlap between the TAA1-HA signal and the cytosolic 60S ribosomal subunit was only 23% ± 1.8% (n = 53 cells; for review, see Bolte and Cordelières, 2006). These data indicate that TAA1 is a chloroplast protein, as predicted by the WolfpSort and PredAlgo algorithms (Horton et al., 2007; Tardif et al., 2012).

In a second experimental approach, the localization of TAA1 was monitored in cell fractionation experiments. Chloroplasts were prepared by Percoll gradient centrifugation. TAA1 was enriched in the chloroplast fraction, relative to the total cell extract. This enrichment paralleled the chloroplast proteins PsaA and PRK, whereas the cytosolic protein RIBOSOMAL PROTEIN L37 (RP L37) was detectable only in the total extract (Fig. 4). When isolated chloroplasts were further fractionated, TAA1 was found both in the crude membrane pellet together with PsaA and in the supernatant containing soluble proteins such as PRK. It should be noted that the nonspecific bands detected by the anti-RPL37 and anti-PRK sera in the membrane pellet of the chloroplast (marked with a star in Fig. 3) do not correspond in size to RPL37 and PRK. The HA-tagged TAA1 protein is overexpressed compared with the native protein (Fig. 2C), and because the majority is in the soluble fraction (Fig. 3), it cannot be excluded that TAA1 is normally soluble but that its overexpression...
leads to its partial aggregation. Other chloroplast factors involved in RNA metabolism have also been detected in both soluble and membrane fractions of the chloroplast (Boudreau et al., 2000; Vaistij et al., 2000a; Rivier et al., 2001; Auchincloss et al., 2002).

The Stability of psaA mRNA Is Impaired in the taa1 Mutant

To further characterize the taa1 mutation, the mutant was backcrossed three times to the wild-type strain. The phenotypic analysis of the backcrossed strain confirmed the previous results: the absence of TAA1 leads to a defect in photoautotrophic growth due to the absence of the PsA protein (Fig. 5A) and to a large decrease in psaA mRNA (Fig. 5B). To determine whether the reduced accumulation of psaA mRNA in the absence of TAA1 is due to a defect in transcription or to increased degradation, the transcriptional activity of psaA was directly assessed in a run-on transcription assay (Klinkert et al., 2005). In this assay, RNA polymerases that were active on the gene at the time of cell lysis can further extend the nascent transcripts, but transcription initiation does not occur. Thus, the amount of incorporation of radio-labeled nucleotides into specific transcripts reflects the loading of polymerases on the respective genes, and hence transcriptional activity. The radio-labeled RNA was hybridized to DNA probes, which were immobilized on a nylon membrane. The latter included psaA exon 1 and psaA exon 3, as well as a five other chloroplast genes as positive controls and a bacterial plasmid as a negative control (pUC19). No difference in the transcriptional activity of psaA could be observed between the taa1 mutant and the wild-type strain (Fig. 5C). These results indicate that the absence of TAA1 does not lead to decreased transcription of the psaA gene. It can thus be inferred that lack of TAA1 leads to a specific decrease in the stability of the psaA mRNA.
**Figure 5.** Stability of psaA mRNA is impaired in the taa1 mutant. 
A. Immunoblot analysis of PsaA. Total protein extracts were subjected to SDS-PAGE and immunoblotting with antisera against PsaA and the D1 protein of PSII as a loading control. The algae were grown in medium containing acetate (TAP) in the dark. B. RNA-blot analysis of psaA mRNA. Total RNA extracts from the same strains were subjected to denaturing agarose gel electrophoresis, blotted to nylon membranes, and hybridized with radiolabeled probes for exon 3 of psaA or atpB as a control. C. Chloroplast transcriptional activity. Cells were permeabilized through one freeze-thaw cycle and pulse labeled with 32P-UTP for 15 min. Radiolabeled RNA was isolated and hybridized to a filter bearing the indicated gene probes. The two spots for each probe contained 0.6 and 0.3 g of DNA, respectively (except for the short psaA exon1 probe, for which there was a single 0.25-µg spot). WT, Wild type; psaA ex1, psaA exon1; psaA ex3, psaA exon3.

**TAAl Is Required for Translation of psaA**

The stability of psaA transcripts is affected in taa1 such that small amounts of mRNA are present, while the accumulation of PsaA protein is more strongly affected (Fig. 5). This raises the question whether TAA1 is also involved in promoting the translation of psaA mRNA. It has been shown previously that some chloroplast transcripts are protected against 5′ to 3′ exonucleolytic degradation by RNA-binding proteins. In some of the corresponding nuclear mutants, stability of the target chloroplast RNA was restored by the insertion of a poly(G) cassette, which can form a very stable secondary structure that impedes the progression of exonucleases (Drager et al., 1998, 1999; Nickelsen et al., 1999; Vaistij et al., 2000b; Loiselay et al., 2008). To investigate the potential role of TAA1 in the protection and in the translation of psaA, a poly(G) tract was inserted by chloroplast transformation in the 5′ UTR of psaA exon 1. The poly(G) tract was introduced in a transformation vector carrying an intron-less version of the psaA gene (psaA-Δi; Lefebvre-Legendre et al., 2014). In this vector, exons 1, 2, and 3 of psaA were fused and placed under the control of the psaA-exon 1 promoter and 5′ UTR to bypass trans-splicing. The flanking sequences in the vector were chosen such that after chloroplast transformation and homologous recombination, the intronless gene replaces the resident psaA-exon3. In this construct (pG-psaA-Δi), 18 G residues were inserted in the 5′ UTR of psaA at position 38 from the 5′ end of the mRNA (~91 relative to the initiation codon). Biolistic transformants of taa1 were selected on spectinomycin-containing medium and subcultured until homoplasy was achieved (Supplemental Fig. S3), giving strain taa1/pG-psaA-Δi. To create a nearly isogenic strain with the wild-type TAA1 gene, the taa1/pG-psaA-Δi strain was rescued by nuclear transformation with the wild-type genomic DNA (cosmid 64B4E) to obtain the strain taa1;TAA1/pG-psaA-Δi, which was, as expected, capable of photoautotrophic growth. Another control strain, taa1/psaA-Δi, containing the intronless psaA gene without the poly(G) tract, was obtained by crossing the taa1 mutant with the wild-type strain WT/psaA-Δi (Lefebvre-Legendre et al., 2014). The level of psaA mRNA in the different strains was determined by quantitative RT-PCR (Fig. 6A), and the accumulation of psaA mRNA in the rescued strain, taa1;TAA1/pG-psaA-Δi, was set to 100%. As expected, the level of psaA was barely detectable in the taa1 mutant and was similarly low in the taa1/psaA-Δi strain, showing that the absence of introns in the psaA transcripts does not restore psaA mRNA accumulation. By contrast, psaA RNA carrying the poly(G) did accumulate in the taa1 mutant background (taa1/pG-psaA-Δi) to 70% of the levels in the rescued control strain. These results provide evidence that TAA1 protects the psaA mRNA against 5′ to 3′ degradation (Fig. 6C).

A further role of TAA1 in the translation of the psaA mRNA was investigated by determining the level of PsaA protein in the same strains (Fig. 6B). As expected, we could not detect any PsaA in the taa1 and taa1/psaA-Δi strains, while the protein was present in the rescued strain (taa1;TAA1/pG-psaA-Δi). The normal accumulation of PsaA in the latter strain shows that the poly(G) tract does not interfere with translation. Interestingly, the PsaA protein was undetectable in the taa1/pG-psaA-Δi strain, even though a nearly wild-type level of the psaA transcript was present (Fig. 6A). These data strongly suggest that TAA1 is required for psaA translation in addition to its role in the stability of the psaA transcript (Fig. 6C). This could be a direct role in promoting translation initiation or an indirect role by ensuring the stability of sequences in the 5′ UTR that would contain the binding site of another unidentified translation factor.

The role of TAA1 in RNA stability and translation suggested that it might associate with other factors and with the psaA mRNA. To investigate this possibility, an extract of taa1/cw15/TAA1-HA was fractionated by centrifugation in a Suc gradient, and the fractions were analyzed by immunoblotting (Supplemental Fig. S2). TAA1-HA sedimented with an apparent mass of 270 kD, while when the sample was treated with ribonuclease (RNase) prior to fractionation, TAA1-HA sedimented with an apparent mass of 200 kD. Mock treatment without added RNase gave a similar shift, probably because of endogenous RNase activity in the extract. Because the molecular mass of TAA1 is approximately 200 kD, these data could suggest that TAA1 associates with RNA but is not part of a large multimolecular complex, unlike other C. reinhardtii
chloroplast proteins of similar function (Vaistij et al., 2000a; Boulouis et al., 2011).

**Regulation of TAA1 in Response to Iron Limitation**

It has been shown previously that PSI is a prime target of protein degradation under iron deficiency in mixotrophic conditions, probably because of its high iron content (Moseley et al., 2002). Thus, we investigated the behavior of TAA1 in response to iron limitation in three different C. reinhardtii lines (Fig. 7). In our standard wild-type strain WT-8B, the analysis of TAA1 was hampered by a band that was detected in immunoblots migrating just ahead of TAA1 (Fig. 7A, labeled with an asterisk). However, this band was not detected in another wild-type strain, 137AH, or in the HA-tagged strain (Fig. 7B). The treatment was initiated 2 h after the beginning of iron starvation. In parallel, a control experiment was performed in the presence of normal levels of iron. Interestingly, in the presence of iron, the content of TAA1 strongly decreased after addition of cycloheximide (approximately 10-fold in 6 h), indicating that TAA1 protein is rapidly degraded under normal conditions (Fig. 7C, lane 6 versus lane 5). In the absence of iron, TAA1 was degraded to even lower levels in the presence of cycloheximide (Fig. 7C, lane 8 versus lane 6). These data suggest that TAA1 always has a constitutively short half-life and that under iron deficiency, its high rate of degradation is no longer counterbalanced by a matching rate of translation.

To further elucidate this process, the mRNA levels of TAA1 and psaA were monitored by quantitative RT-PCR in the wild-type strain WT-8B. The amount of psaA mRNA was reduced already after 24 h of iron deprivation (Fig. 7B) and declined approximately 2- to 3-fold after 48 h. The levels of the TAA1 mRNA remained essentially constant during iron limitation. Thus, the decrease in the level of the TAA1 protein cannot be attributed to reduced accumulation of the mRNA but rather to a posttranscriptional process. A moderate increase in the level of TAA1 mRNA was observed after iron resupplementation, but a concomitant increase in the level of TAA1 protein was only observed in one of the three strains examined (Fig. 7A).
DISCUSSION

TAA1 Is Involved in psaA mRNA Stability and Translation

Photosynthetic protein complexes have a dual genetic origin, with some subunits encoded in the chloroplast and others in the nucleus. Therefore, a concerted control of gene expression in the two compartments is necessary for the biogenesis of the thylakoid membrane. Coordinated accumulation of the different subunits involves several mechanisms. One of these is the proteolytic degradation of subunits that are not assembled into the photosynthetic complexes. In another mechanism, known as Control by Epistasy of Synthesis, unassembled subunits induce negative feedback regulation of their own translation (Choquet and Wollman, 2009). Furthermore, retrograde signals from the chloroplast to the nucleus control transcription of photosynthesis-related genes (Eberhard et al., 2008). Conversely, the nucleus exerts tight anterograde control on expression of organelle genes. In C. reinhardtii, the expression of chloroplast proteins is generally not limited by transcription, because rates of chloroplast translation are not very sensitive to changes in DNA or transcript levels (Eberhard et al., 2002). Anterograde control mainly occurs at posttranscriptional steps, mediated by nucleus-encoded factors that govern the expression of specific chloroplast genes. Some factors are involved in the stable accumulation of their cognate target transcripts (dubbed M factors, for maturation/stability), while others are primarily required for the translation of specific mRNAs (T factors; Choquet and Wollman, 2002). For example, in C. reinhardtii, MATURATION OF petA1 (MCA1) protects the photosynthetic electron transfer A (petA)
transcripts (encoding cytochrome f) from 5' to 3' exonucleolytic degradation, whereas TRANSLATION OF petA (TCA1) is primarily necessary for the translation of the petA mRNA (Wostrikoff et al., 2001; Raynaud et al., 2007; Loiselay et al., 2008). Likewise, NAC2 is required for the stable accumulation of photosystem II D (psbD) mRNA (encoding subunit D2), while RNA-BINDING PROTEIN40 (RBP40) plays a role in its translation (Kuchka et al., 1989; Schwarz et al., 2007). In these two cases, where both M and T factors for a specific transcript have been identified, they are found associated in a large complex. In fact, it has been shown that the mRNA stability M factor MCA1 is also necessary for the translation of the petA mRNA and that, conversely, in the absence of the translation T factor TCA1, the level of petA mRNA is decreased (Loiselay et al., 2008; Boulouis et al., 2011). These examples illustrate the coupling of RNA stabilization and translation initiation in the C. reinhardtii chloroplast. Similarly, TAA1 may also act both as M and T factor: it is required for the stability of psaA mRNA but is also necessary for its translation. It cannot, however, be excluded that either of these roles could be indirect. That the target of TAA1 is in the 5' UTR of psaA suggests that the role of TAA1 in translation is exerted at the initiation step.

Because TAA1 is required for psaA RNA stability and translation, it is relevant that it contains two sequence features that are ascribed to RNA-binding activity: seven OPRs and a RAP domain. This suggests that TAA1 may have a direct physical interaction with its RNA target in the 5' UTR of psaA. The OPR proteins belong to the superfamily of helical-repeat proteins that also include the pentatricopeptide repeat (PPR), half a tetratricopeptide repeat, and mitochondrial termination factor proteins (for review, see Hammani et al., 2014). Structural studies for some of these proteins have shown that the repeats form superhelical structures made of tandem antiparallel α-helices, and it is believed that all of these proteins have the same kind of structural organization. The best characterized are the PPR proteins of land plants, which constitute a family of more than 400 members required for chloroplast or mitochondrial RNA metabolism, including translation, RNA processing, and RNA editing (Barkan and Small, 2014). Each PPR motif interacts with one base of the RNA target through a few α-amino acid residues that mediate sequence-specific recognition (for review, see Barkan and Small, 2014). C. reinhardtii has only 10 PPR proteins (Toursasse et al., 2013) but harbors a large number of OPR proteins. Forty-four were initially identified but there are probably many more (Eberhard et al., 2011; Rahire et al., 2012; Hammani et al., 2014; Olivier Vallon, personal communication). The OPR motif comprises 38 to 40 amino acids and is repeated between two and 24 times per protein. To date, five OPR proteins have been functionally characterized in C. reinhardtii RAT2 and RAA1 are required for trans-splicing of the psaA transcripts (Balczun et al., 2005; Merendino et al., 2006), while TRANSLATION OF psbC2 (TBC2), TAB1, and TRANSLATION OF atpA1 (TDA1) are involved in the translation of psbC, psAB, and atpA, respectively (Auchincloss et al., 2002; Eberhard et al., 2011; Rahire et al., 2012).

In the taa1 mutant, insertion of a poly(G) tract in the psaA 5' UTR compensated for the role of TAA1 in RNA stability (Fig. 6). Using the same experimental approach, a function in protecting mRNA against 5' to 3' exoribonucleases was demonstrated for other helical-repeat proteins in C. reinhardtii (Drager et al., 1998; Nickelsen et al., 1999; Vaistij et al., 2000; Loiselay et al., 2008). Likewise, the PPR10 protein of Arabidopsis (Arabidopsis thaliana) was shown to protect its cognate RNAs in vivo and in vitro against exonucleolytic degradation from both directions (Pfalz et al., 2009; Prikluy et al., 2011). This is ascribed to the tight binding of the protein to the RNA and leads to the accumulation of a characteristic small RNA footprint (Ruve and Schmitz-Linneweber, 2012). Similarly, in C. reinhardtii, the small RNAs protected by the half a tetratricopeptide repeat protein MATURATION OF psbB1 (MBB1) coincide with the cis-acting elements required for RNA stability that were identified through chloroplast reverse genetics (Loizeau et al., 2014). An ortholog of MBB1 (HIGH CHLOROPHYLL FLUORESCENCE 107) that also has the ability to yield small protected RNA footprints is conserved in maize (Zea mays) and Arabidopsis (Hammani et al., 2012). A footprint reflecting the binding of TAA1 at the 5' end of the psaA mRNA was, however, not detected in the analysis of small chloroplast RNAs from C. reinhardtii (Loizeau et al., 2014).

Interestingly, TAA1 also contains a RAP domain that comprises approximately 60 amino acids and is predicted to be involved in diverse activities that involve RNA binding (Lee and Hong, 2004). If TAA1 has a function both in RNA stability and in translation, the question arises whether one function is performed by the OPR motifs and the other by the RAP domain. However, the single OPR protein present in Arabidopsis (RAP) shows a structure similar to TAA1 with four OPR repeats followed by a RAP domain and is specifically required for chloroplast 16S ribosomal RNA maturation (Kleinknuch et al., 2014) but is apparently not directly involved in translation.

**A Coordinate Response to Iron Limitation**

The restricted bioavailability of iron often limits photosynthesis in the oceans and on land. The electron transfer chains in the chloroplast and the mitochondrion are major sinks for iron. The abundance of other iron-dependent proteins may be comparatively small but they are essential for fitness and stress acclimation (Page et al., 2012). Therefore, plants and algae optimize iron utilization in response to changes in its availability. In C. reinhardtii grown under iron limitation in mixotrophic conditions (i.e. photoheterotrophically in acetate-containing medium under the light), mitochondrial respiration is favored at the expense of photosynthesis (Moseley et al., 2002; Terauchi et al., 2010; Urzica et al., 2012). At the molecular level, PSI degradation and the disconnection of its light-harvesting antenna are early targets of iron deficiency, presumably because of its high iron content (Moseley et al., 2002; Naumann et al., 2005). Under iron deficiency, iron that is released by PSI degradation is bound by ferritin, the
the acclimation response to iron starvation coordinately but availability. However, it cannot be entirely ruled out that in regulating the synthesis of PsaA in response to iron can thus be tentatively concluded that TAA1 is involved psaA concomitant decrease in TAA1, which exerts anterograde of protein accumulation. This correlates well with the the level of its mRNA and even more strongly at the level 1536 Plant Physiol. Vol. 167, 2015 translation of which is strongly and speci...mal iron-replete conditions, TAA1 has a short half-life. This TAA1 is regulated posttranscriptionally. Even under nor...limitation is largely manifested at the transcript level with...mal iron-replete conditions, TAA1 has a short half-life. This TAA1 is regulated posttranscriptionally. Even under normal iron-replete conditions, TAA1 has a short half-life. This is a characteristic of proteins that serve a regulatory function, allowing rapid changes in abundance through proteolytic degradation. The response of TAA1 to iron limitation is very fast, as a large decrease of the protein was observed already 6 h after the transfer to iron-free medium.

A distinction can be made between anterograde control exerted by the nucleus on chloroplast gene expression through factors that are constitutively required and anterograde regulation in a strict sense, where chloroplast gene expression is modified in response to environmental or developmental cues. During iron starvation, the chloroplast-encoded PsaA subunit is down-regulated at the level of its mRNA and even more strongly at the level of protein accumulation. This correlates well with the concomitant decrease in TAA1, which exerts anterograde control on psaA mRNA stabilization and translation. It can thus be tentatively concluded that TAA1 is involved in regulating the synthesis of PsaA in response to iron availability. However, it cannot be entirely ruled out that the acclimation response to iron starvation coordinately but separately regulates the expression of TAA1 and of PsaA.

An interesting parallel can be drawn with the regulation of the cytochrome b₆f complex under conditions of nitrogen limitation. This response involves the degradation of the b₆f complex, a reduction in the level of the corresponding chloroplast mRNAs, and a coordinated down-regulation of the proteins involved in b₆f biogenesis (Wei et al., 2014). The nucleus-encoded proteins MCA1 and TCA1 are required for the stability and translation of petA, the chloroplast-encoded mRNA that encodes cytochrome f. MCA1 is also a short-lived protein that is rapidly degraded during nitrogen starvation (Raynaud et al., 2007) with a half-life (2 h) that is comparable to that of the TAA1 protein under iron deprivation (Fig. 7).

The complexity of chloroplast RNA metabolism raises puzzling question on the evolution of chloroplast gene expression. A surprisingly numerous battery of nucleus-encoded factors intervene in the expression of specific plastid genes at posttranscriptional steps, such as processing, splicing, RNA stabilization, editing, or translation (Stern et al., 2010; Barkan, 2011). Some of this complexity can be bypassed if introns or editing sites are removed from the chloroplast genome (Johanningmeier and Heiss, 1993; Minagawa and Crofts, 1994; Holloway et al., 1999; Schmitz-Linneweber et al., 2005; Petersen et al., 2011; Lefebvre-Legendre et al., 2014). In part, the complexity may reflect a form of genetic drift, an evolutionary ratchet described by the theory of constructive neutral evolution (Gray et al., 2010). During evolution, some of the mutations that arise in the chloroplast genome could be compensated by the recruitment of nucleus-encoded RNA-binding factors capable of suppressing their phenotype and debugging chloroplast gene expression (Maier et al., 2008), a mechanism we dub the spoiled-kid hypothesis (Lefebvre-Legendre et al., 2014). However, such mutations will only become fixed if they have a minimal fitness cost, implying that the suppressors must actually preexist the appearance of the mutations (Lynch, 2007; Gray et al., 2010). The large families of helical-repeat proteins with their modular architecture and simple mode of RNA recognition could contribute to a pool of such suppressors (Barkan and Small, 2014). However, this multiple aspect of complexity does not preclude that some of the nucleus-encoded proteins actually do play a role in anterograde regulation of chloroplast gene expression in response to developmental or environmental factors. The best studied examples are MCA1 and TCA1 and their role in the response to nitrogen supply. TAA1 and its response to iron availability may represent another actor in this type of regulation. Such regulatory activity could have been the function of these factors at early stages of their evolution, but alternatively they could have been coopted for these regulatory roles from the complex pool of factors that had accumulated at later stages.

MATERIALS AND METHODS

Strains and Media

The Chlamydomonas reinhardtii strains were grown in TAP or in high-salt minimal (HSM) medium (Rochaix et al., 1988) to densities of 1 to 2 × 10⁶ cells mL⁻¹ in the dark or under fluorescent lights (6 or 60 μE m⁻² s⁻¹) at 25°C. For growth tests, 10 μL of cell culture at 2 × 10⁶ cells mL⁻¹ was spotted on agar plates and grown under 6 or 60 μE m⁻² s⁻¹ light as indicated. Where necessary, the TAP medium was supplemented with 100 μg mL⁻¹ spectinomycin (Sigma-Aldrich).

For growth under iron limitation, glassware was treated with 50 mM EDTA (pH 8) and washed with milliQ water. TAP minus iron (TAP-Fe) was prepared with trace elements lacking iron. For supplementation, iron was added at 18 μM from a stock solution of 50 mM FeSO₄ chelated with 134 mM EDTA. For iron deprivation experiments, C. reinhardtii cells from cultures at 2 × 10⁶ cells mL⁻¹ were harvested by centrifugation and resuspended in TAP-Fe at 0.5 × 10⁶ cells mL⁻¹. After 2 h, cycloheximide at 10 μg mL⁻¹ was added where indicated. After 24 and 48 h, cells were diluted in TAP-Fe to 0.5 × 10⁶ cells mL⁻¹. After the dilution at 48 h, iron was supplemented to 18 μM.

Genetic Analysis

For the genetic screen using chloroplast transformation of PSI-deficient mutants (Girard et al., 1980), the tester construct ppsA-aadA contained the ppsA-exon 1 promoter and 5' UTR fused to aadA (plasmA; Wostrikoff et al., 2004), and the positive control construct atpA-aadA contained the promoter and 3' UTR of atpA fused to aadA (plasmA; Kuras et al., 1997).

Crosses were performed using standard protocols (Harris, 1989). The tau1 mutation, formerly named F23 (Girard et al., 1980), was backcrossed three times to the wild type. For nuclear transformation, the F23 mutant was crossed to the cir15 strain to give cir15tau1. The F23 mutant was crossed to the strain WT::plasmA, mt+ (which carries a ppsA-aadA marker [dubbed akJ]; Wostrikoff et al., 2004). In eight complete tetrad, the two PSI mutant progeny were
unable to grow on 100 μg mL⁻¹ spectinomycin, while the two wild-type progeny grew like the WT:ααK, mt+ parent. The strains J11, J12, J13, and J14 represent one of these tetrads. The taa1/psaA-Δ strain was obtained by crossing a WT/psaA-Δ mt(+) strain with the taa1Δmt5 strain.

Nuclear transformations were achieved using the glass beads/vortex protocol (Kindle, 1990), and transformants were selected on HSM plates for phototrophic growth. Chloroplast transformations were performed by helium gun bombardment, and transformants were selected on TAP plates supplemented with 100 μg mL⁻¹ spectinomycin, subcultured several times to obtain homoplasmic strains, and genotyped by PCR (Supplemental Fig. S2).

Cloning of the TAA1 Gene

By transformation of the taa1 mutant (Shimogawara et al., 1998) with an ordered cosmid library and selection for phototrophic growth, the cosmids 64B4 was identified (Burton and Rochaix, 1994; Depège et al., 2003). This cosmids was digested with EcoRI, and a resulting 9.4-kb fragment, called 64B4L, was also capable of rescuing the taa1 mutant.

The TAA1 gene corresponds to Cr09g262650 (Phytozome v10.0; http://phytozome.jgi.doe.gov/pv/portal.html#info?alias=Org_Creinhardtii), and TAA1 is listed as OP222 (for OCTATRICO PEPTIDE REPEAT22) in Rahire et al. (2012). To identify the mutation in the taa1 mutant, total RNA was extracted from the taa1 and wild-type strains and subjected to RT-PCR (see below) using the following oligonucleotides: E9-down + 5103-up, E9-up + E11-down, 3531-up + 4012-down, and 4012-up + 5103-down. After agarose gel electrophoresis, the PCR fragments were purified and sequenced (Fasteris). Comparison of the mutant and wild-type products allowed the identification of a single base substitution, which changes GlmN to a stop codon in the taa1 mutant.

The cDNA clone number 21 (exons 7–12) was isolated by screening a library constructed in bacteriophage λgt10 (Goldschmidt-Clermont and Rahire, 1986), using a 0.6-kb PCR probe amplified with the oligonucleotides E11-down and 6541-up. The cDNA insert was excised with EcoRI and cloned into pBluescript KS+ to give plasmid pL29. A 2.6-kb EcoRI fragment of pL43 containing the 5' UTR and the beginning of the TAA1 gene (0.66 kb upstream the ATG start codon and the first 5 kb of the TAA1 gene), was cloned into pBluescript KS+ to yield plasmid pL29. A 2.6-kb XhoI fragment of pL28 was inserted at the XhoI site of pL29 to give the midigene pl30.

The HA-tagged midigene pl37 was constructed as follows. The oligonucleotides BspEI-For and Stop-rev and NsiI-Rev and Stop-For were used to generate by PCR using pl30 as the template a modified 1.4-kb BspEI-NsiI fragment containing a StuI site just before the stop codon. This fragment was cloned into the PCRIII-TOP6 vector (Invitrogen) to yield plasmid pL33. A 1.5-kb EcoRV-BamHI fragment of pl33 was cloned into pBluescript KS+ to give plasmid pL35. The StuI-NsiI fragment of pl35 was replaced by a synthetic StuI-NsiI fragment containing a triple XbaI site inserted between the StuI site and the stop codon to yield plasmid pL36. Finally, the BspEI-NsiI fragment from pl30 was replaced by the BspEI-NsiI fragment of pl36 to provide the HA-tagged midigene pl37. The TAA1 midigene carrying a C-terminal deletion lacking the RAP domain (pL57; stop codon at residue 1,906) was obtained in two steps. Plasmid pL36 was digested with XbaI and StuI and religated to yield pl56. The BspEI-NsiI fragment of pl50 was then replaced by the corresponding fragment from pl56 to give pl57.

Insertion of a Poly(G) Cassette in the psaA 5' UTR

To introduce the poly(G) tract in the psaA 5' UTR, the AflII-Xmal fragment of psaA-Δi (pOS20; Lefebvre-Legendre et al., 2014) was inserted into pBluescript KS+ to give plasmid pL43. The Ncol-SphI fragment of pl43 containing the psaA 5' UTR was replaced by a synthetic Ncol-SphI fragment (Blommat), where a tract of 18 G has been introduced 91 bp before the ATG translation initiation codon to yield plasmid pL46. Finally, the modified AflII-Xmal fragment from pl46 was reintroduced into psaA-Δi (pOS200) to give plasmid pL47 (pG-psaA-Δi).

RT-PCR Analysis of TAA1 Transcripts

Total RNA was extracted from cells using RNeasy plant mini kit (Qiagen), treated with DNase I (Qiagen) for 20 min at 25°C, and reprocessed using the RNeasy mini kit (Qiagen). The RNA concentration was determined by spectrophotometry using a NanoDrop (Thermo Scientific), and the lack of genomic-DNA contamination or degradation was checked by gel electrophoresis. Reverse transcription was performed with 1 μg of total RNA using PrimeScript First-Strand cDNA Synthesis (Takara) following the manufacturer's instructions with random hexamer primers. For each experiment, a no-reverse transcription control was performed.

Quantitative PCR reactions were performed using LightCycler480 SYBR Green I Master (Roche) following the manufacturer's instructions. Water controls were included in each 96-well PCR plate, and dissociation analysis was performed at the end of each run to confirm the specificity of the reaction. The specificity of PCR products was checked by gel electrophoresis and DNA sequencing. Cycle threshold (Ct) values were obtained through LightCycler480 software release 1.5.0, and relative changes in gene expression were calculated using the ΔΔCt method using RPL13 for normalization. Experiments were performed in biological duplicates and technical triplicates. The following oligonucleotides (Supplemental Table S2) were used for the quantitative PCR reactions: for psaA exon 1, qPCR-psaAEx1-For + qPCR-psaAEx5-Rev; for TAA1, qRT-TAA1Fw1 + qRT-TAA1Rv1, and for RPL13, qRT-RPL13Fw1 + qRT-RPL13Rev1.

RNA Analysis

C. reinhardtii cells from 50-mL cultures (2 × 10⁸ cells mL⁻¹) were harvested by centrifugation and washed once with 10 mL of 20 mM Tris (pH 7.9), and the pellets were stored at −70°C. For RNA extraction, 3 mL of Tri Reagent (Sigma-Aldrich) and 500 μL of glass beads (G8772; Sigma-Aldrich) were added to the pellets, and the suspensions were vigorously agitated for 2 min. The homogenates were split into 3 Phase Lock Gel tubes (5 PRIME). Four hundred microliters of chloroform was added followed by thorough mixing. After 10-min centrifugation at 14,000 rpm at 4°C, 200 μL of chloroform was added, followed by thorough mixing. After 10-min centrifugation at 14,000 rpm at 4°C, the RNA was precipitated from the aqueous phase with 1 mL of isopropanol, collected by centrifugation, and washed with 70% (v/v) ethanol. Total RNA (5 μg) was analyzed by formaldehyde agarose gel electrophoresis, transfer to nylon membranes, and hybridization using probes labeled with ³²P-dATP by random priming (Ausubel et al., 1998).

The chloroplast run-on transcription assay was performed as described (Klinkert et al., 2005).

Protein Analysis

C. reinhardtii cells (5 mL, 2 × 10⁶ cells mL⁻¹) were collected by centrifugation, washed with water, and resuspended in lysing buffer (100 mM Tris-HCl, pH 6.8, 4% [w/v] SDS, 20 mM EDTA, and protease inhibitor cocktail [Sigma-Aldrich]). After 30 min at room temperature, cell debris was removed by centrifugation, and total protein was analyzed by SDS-PAGE (15%, 12%, or 4% gel), and immunoblotting. Labeling of the membranes with antisera against PsaA, D1, PRK, anti-cytochrome f, and RPL13 (Ramundo et al., 2013) was performed as described by Ramundo et al. (2013). The plasmid L34, consisting of a fragment of TAA1 (amino acid residues 1,335–1,591) amplified with the oligonucleotides Ab-F23-2-For and Ab-F23-2-Rev and cloned between the BamHI and XhoI sites of vector pET28a (Novagen), was introduced into Escherichia coli BL21.

Cell Fractionation

Cells were lysed with a nebulizer, and chloroplasts were purified on Percoll gradients as described previously (Rivier et al., 2001) in the presence of 0.1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride. Chloroplasts were resuspended in hypotonic buffer (10 mM HEPES, pH 7.2, 5 mM MgCl₂, and 1 mM dithiothreitol) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Two hundred fifty microliters of chloroplasts was lysed by sonication, and the solution was adjusted to 20 mM HEPES (pH 7.2), 50 mM KCl, and 10 mM MgCl₂ and subjected to centrifugation at 100,000×g for 30 min at 4°C in a TLA45 rotor (Beckman). The supernatant was recovered for analysis, and the pellet was washed by resuspension in 20 mM HEPES (pH 7.2),...
Immunofluorescence Microscopy

Slides were pretreated with a drop of a solution of 10% (w/v) poly-Lys (Sigma) for 10 min and air dried overnight. A sample of cells (1.5 × 10⁴) was deposited and left to sediment and attach during 5 min. Fixation was done by incubating the slides for 5 min in a vertical side slide holder filled with methanol precooled to –20°C. After drying, the slides were incubated in blocking solution (phosphate-buffered saline plus Tween [PBST; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, and 0.1% [w/v] Tween 20], 1% bovine serum albumin, and 3% [w/v] fish skin gelatin) for 40 min, followed by 2 h of incubation with a mix of two primary antisera (1:1,000 dilutions in blocking buffer): either rabbit anti-PRK with mouse monoclonal anti-α-tubulin or rabbit anti-60S with anti-AHA-11. Anti-60S detects the ribosomal proteins of the 60S subunit of the cytoplasmic ribosome (gift of William Zerges; Unicake and Zerges, 2007). After three washes of 10 min in PBST, cells were incubated for 2 h in blocking buffer with secondary antibodies (goat anti-rabbit and goat anti-mouse antibodies coupled to Alexa488 or Alexa546 fluorophore, respectively [Life Technologies]). After three washes of 10 min in PBST, cells were finally washed one time in phosphate-buffered saline and mounted in phosphate-buffered saline plus 50% (v/v) glycerol. Imaging was performed on an SP5 confocal laser-scanning microscope (Leica) equipped with a resonant scanner and a 63× oil numerical aperture 1.4 PlanApo lens. The pinhole was opened at 1 Airy, and the zoom was set so that the pixel size was comprised between 80 and 120 nm. Alexa488 and Alexa546 were imaged sequentially; they were respectively excited using the 488- and 610 nm, respectively. Maximum intensity projections were subsequently performed and displayed.

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