THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 43, Issue of October 22, pp. 44407–44416, 2004
Printed in U.S.A.

Light and Redox Control of Photosynthesis Gene Expression in *Bradyrhizobium*

DUAL ROLES OF TWO PpsR*

Received for publication, July 16, 2004
Published, JBC Papers in Press, August 10, 2004, DOI 10.1074/jbc.M408039200

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The two closely related bacteria *Bradyrhizobium* and *Rhodopseudomonas palustris* show an unusual mechanism of regulation of photosystem formation by light thanks to a bacteriophytochrome that antirepresses the regulator PpsR. In these two bacteria, we found out, unexpectedly, that two *ppsR* genes are present. We show that the two *Bradyrhizobium* PpsR proteins exert antagonistic effects in the regulation of photosystem formation with a classical repressor role for PpsR2 and an unexpected activator role for PpsR1. DNase I footprint analysis show that both PpsR bind to the same DNA $TGTN_{12}ACA$ motif that is present in tandem in the *bchC* promoter and the *crtED* intergenic region. Interestingly, the *cycA* and *aerR* promoter regions that contain only one conserved palindrome are recognized by PpsR2, but not PpsR1. Further biochemical analyses indicate that PpsR1 only is redox sensitive through the formation of an intermolecular disulfide bond, which changes its oligomerization state from a tetramer to an octamer under oxidizing conditions. Moreover, PpsR1 presents a higher DNA affinity under its reduced form in contrast to what has been previously found for PpsR or its homolog CrtJ from the *Rhodobacter* species. These results suggest that regulation of photosystem synthesis in *Bradyrhizobium* involves two PpsR competing for the binding to the same photosynthesis genes and this competition might be modulated by two factors: light via the antagonistic action of a bacteriophytochrome on PpsR2 and redox potential via the switch of PpsR1 oligomerization state.

In anoxygenic photosynthetic bacteria, the conversion of light energy into chemical energy requires several specialized membranous complexes, the light-harvesting complexes, the photosynthetic reaction center, and the cytochrome bc_1 complex. After absorption of light by the bacteriochlorophyll and carotenoid molecules associated with the light harvesting complexes, the energy is transferred to the reaction center where a charge separation occurs, leading to an electron transfer that generates a transmembrane proton-motive force ultimately used for ATP synthesis. The synthesis of this photosynthetic apparatus is highly regulated in response to oxygen tension and light intensity (for reviews, see Refs. 1–3). Under aerobic conditions, the expression of photosynthesis genes is low, and the bacteria develop by drawing on the energy derived from aerobic respiration, whereas under semiaerobic or anaerobic conditions and in the light, the photosynthetic apparatus is synthesized in large amounts permitting growth by utilization of light energy.

The genes necessary for the formation of the photosynthetic apparatus are clustered in a 45-kb region designated as the photosynthesis gene cluster (PGC).[1] The PGC includes several operons involved in 1) the photopigment synthesis (bacteriochlorophyll (*bch*), and carotenoid (*crt*)); 2) the light-harvesting polypeptides (*pucBAC* and *pufBA*); 3) the reaction center subunits (*puhA*, *pufLM*); and 4) various regulators (*ppsR*, *tspO*, and *aerR*) (4, 5). The fine control of photosystem synthesis by oxygen relies on the combined action of several transcription factors that activate or repress the expression of photosynthesis genes in response to redox conditions (2, 6). One well characterized regulator is the aerobic repressor PpsR in *Rhodobacter sphaeroides* or its homolog CrtJ in *Rhodobacter capsulatus* (7, 8). Both proteins share the same mode of action: under oxidizing conditions, PpsR/CrtJ blocks transcription by binding as a tetramer to a palindromic ($TGTN_{12}ACA$) motif, which is found in tandem in the *bch*, *crt*, and *puc* promoters (9–11). In addition, biochemical studies have recently shown that CrtJ/PpsR have redox sensing capabilities thanks to the formation of an intramolecular disulfide bond promoted by oxygen, which stimulates their binding to target promoters (12, 13).

Light intensity also controls the expression of photosynthesis genes with repressive effects less drastic than oxygen. The molecular mechanism of this regulation, described in *R. sphaeroides*, implicates the antagonistic actions of the repressor PpsR and the flavoprotein AppA (14). Masuda and Bauer (13) have recently shown that AppA is a blue light photoreceptor that modulates the DNA binding activity of PpsR in response to

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§ Supported by a French Minister of National Education, Higher Education and Research doctoral grant.

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[1] The abbreviations used are: PGC, photosynthesis gene cluster; DTT, dithiothreitol; TMPD, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine dihydrochloride; *Br*BphP, *Bradyrhizobium* bacteriophytochrome photoreceptor; *Rps*., *Rhodopseudomonas*.

oxygen and light signals, using two distinct mechanisms: it may break the disulfide bond in oxidized PpsR, or form an inactive AppA-$PpsR_2$ complex.

A new type of light regulation has been described for the two closely related species *Bradyrhizobium* strain ORS278 and *Rhodopseudomonas palustris* (15). In these two bacteria, depending on the oxygen tension, far-red light is necessary to trigger the expression of photosynthesis genes. The mechanism of this light regulation has been partially elucidated and involves a bacteriophytochrome. This chromoprotein exerts its regulatory activity by switching between a red light absorbing form (Pr) and a far-red light absorbing form (Pfr) (16). It is noteworthy that the bacteriophytochrome genes (*bphP*) responsible for this regulation are found in the PGC region contiguous to a *ppsR* gene in both bacteria. Mutation in *bphP* of *Bradyrhizobium* strain ORS278 results in the absence of photosystem formation irrespective of the light conditions, whereas mutation in *ppsR* leads to the opposite phenotype. Based on phenotypes of these *bphP* and *ppsR* mutants and the action spectrum of the photosystem synthesis, it was proposed that the Pr form of the bacteriophytochrome antagonizes the repressive effect of PpsR. More recently, similar results have been obtained for *Rps. palustris* strain CEA001 (17).

In this study, we reveal the presence of a second *ppsR* gene linked to the PGC of *Bradyrhizobium* strain ORS278. Examination of the genome sequence of *Rps. palustris* (available at http://spider.jgi-psf.org/JGI-microbial/html/) also reveals the presence of a second *ppsR* gene located in the PGC region. The unexpected presence of two *ppsR* genes in the same bacterium raises several questions. Why two different PpsR? Do both of them exert the same repressive effect as usually observed in purple bacteria? On the same photosynthesis genes? Are both PpsR involved in redox and light regulation circuit? Here, we specify the roles and the mechanisms of action of both PpsR from *Bradyrhizobium*, by combining genetic and biochemical approaches.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Bradyrhizobium strain ORS278 (wild type strain) and isogenic mutants were grown in a modified YM-agar medium with addition of appropriate antibiotics when required (18). All the strains were grown at 35 °C for 7 days under either semiaerobic (Petri dishes filled at 40% with YM medium and sealed with a double adhesive tape) or aerobic conditions (Petri dishes with ventilated ergot). Illumination of the cultures was provided by light emitting diodes of different wavelengths between 590 and 870 nm with an irradiance of 6.6 μmol of photons/m^2/s. *Escherichia coli* was grown in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics.

Determination of Photosynthetic Activity—The amount of the photosynthetic apparatus in intact cells was estimated as previously described (18).

Construction of ppsR1, ppsR2, and ppsR2/BrbphP Mutant Strains—Construction of the *ppsR2* mutant has been previously described (15). For the construction of a *ppsR1* mutant, a region of about 2.3 kb containing the *ppsR1* gene was amplified by PCR using the primers 5′-TCAGGATCCTGACCGAACGGCGATAGCTTTAG-3′ and 5′-ATCGGATCCTGCGCCGGACGGCCCCACACAATGAG-3′ and subsequently cloned in the pGEM-T vector (Promega, Madison, WI). The 4.7-kb BamHI *lacZ*-Km^r cassette of pKOK5 (19) was then inserted in the unique BglII site of *ppsR1*. The resulting 7-kb BamHI insert containing the mutated *ppsR1* gene was cloned into the pJQ200mp18 suicide vector linearized by BamHI digestion (20). For the construction of the double mutant *ppsR2/BrbphP*, a region of about 5 kb containing the contiguous *ppsR2* and *BrbphP* genes was amplified by PCR using the primers 5′-TGCGGATCCGCACCCCGTCCTGTGCCAGCGTATC-3′ and 5′-TAGGGATCCATAACCACCGCCGCCTGTGATGATAAAAC-3′ and subsequently cloned into the pGEM-T vector. A 3.2-kb region containing a part of the *ppsR2* and *BrbphP* genes was deleted by XhoI digestion and replaced by the 4.7-kb SalI *lacZ*-Km^r cassette of pKOK5. The resulting 6.6-kb BamHI insert containing the mutated *ppsR2* and *BrbphP* genes was cloned into the pJQ200mp18 suicide vector linearized by BamHI digestion. The pJQ200 derivatives obtained, which encoded a counter selective *sacB* marker, were transformed into *E. coli* S17-1 for mobilization into ORS278 as previously described (18). Double recombinants were selected on sucrose and the insertion was confirmed by PCR.

β-Galactosidase Assay—For analysis of *ppsR1* and *ppsR2* expression, β-galactosidase activity was assayed as previously described (15).

Cloning, Protein Expression, and Purification—The *ppsR1* and *ppsR2* genes from *Bradyrhizobium* strain ORS278 were amplified by PCR using *Pfu* DNA polymerase and the following pairs of primers: PpsR1.pBAD.f, 5′-GGATCCGTGAGGGCGTTCAGAGCTCCGAAAGAG-3′ and PpsR1.pBAD.r, 5′-AAGCTTCTATTCCAACTGACTGTCTTCTTCGCTTG-3′; PpsR2.pBAD.f, 5′-ATGGATCCATGGCCGAGTTTCACGGTCCACGA-3′ and PpsR2.pBAD.r, 5′-ATGGATCCCTAGCTCCCCTTTTCGGTTTCCT-3′. The PCR products were digested (BamHI/HindIII for *ppsR1*; BamHI for *ppsR2*) and ligated into the expression vector pBAD/HisB (Invitrogen). The recombinant PpsR proteins were overexpressed in *E. coli* LMG194 (Invitrogen) by induction with L-arabinose as previously described (17). Purification of His_6-appended PpsR proteins was performed using nickel chelate affinity chromatography as described (17). The purified protein were quantified using the Bradford assay (Bio-Rad) and then aliquoted and stored at −80 °C in the presence of 20% (v/v) glycerol.

Reduction and oxidation of both PpsR were tested using various reagents: 10 mM dithiothreitol (DTT), 0.1 and 1 mM H_2O_2, 0.5 and 5 mM $K_3Fe(CN)_6$ (potassium ferricyanide), or a mixture of 0.5 and 5 mM $K_3Fe(CN)_6$ with 10 μM TMPD (*N*,*N*,*N*′,*N*′-tetramethylphenylenediamine dihydrochloride) diluted in water, and air by exposing the sample to a constant flow of air gas during 3 h. After addition of the oxidizing or reducing reagents, the proteins were incubated overnight on ice and subsequently analyzed by nonreducing SDS-PAGE and gel filtration chromatography, or used for DNase I footprint experiments.

Site-directed Mutagenesis—Single mutations were introduced in PpsR1 and PpsR2 amino acid sequences using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations. To construct the PpsR1-C429S mutant, the plasmid pBAD::*ppsR1* was used as a template with the following primers: PpsR1-C429S sense (5′-GTGATCGAGCGGATGTCGATCGAGACCGCGCTC-3′) and PpsR1-C429S antisense (5′-GAGCGCGGTCTCGATCGACATCCGCTCGATCAC-3′). The Y407C mutation on the PpsR2 sequence was generated using the plasmid pBAD::*ppsR2* as a template and the primers PpsR2-Y407C sense (5′-GTCGAGCAGCATTGTGTCCGCGCCGCG-3′) and PpsR2-Y407C antisense (5′-CGCGGCGCGGACACAATGCTGCTCGAC-3′).

DNase I Footprint Analysis—Four probes corresponding to the promoter regions of *bchC*, *crtE*, *aerR*, and *cycA* were used for studying the DNA binding of PpsR1 and PpsR2. Probes were prepared by PCR using ^{32}P 5′-end labeled oligonucleotide primers, as previously described (17).

The *bchC* promoter region was obtained using the primers: 5′-CTCGAGGATCCGCGGAATTCAAGCTTCTACGACGACCCGCTGCCTAC-3′ and 5′-CTCGAGGATCCGCGGAATTCAAGCTTCTACGACGACCCGCTGCCTAC-3′; the *crtE* promoter region was obtained using the primers: 5′-GCCGATTGCGATCTGGCGCATCTTG-3′ and 5′-AGGGATTTTTCGATCCGGGCCATCAC-3′; the *cycA* promoter region was obtained using the primers: 5′-GAGAGTCTTGAGAATGTCGTTAG-3′ and 5′-GTCGCCGCCGTCCAGCGCGAATG-3′; the *aerR* promoter region was obtained using the primers: 5′-CGGGCACGCACTCGGGGATTTG-3′ and 5′-TGTCGCGCCGGCGCTTTTCCTC-3′. DNase I footprint experiments were performed as previously described (17). PpsR1, PpsR1-C429S, or PpsR2 DNA binding isotherms to the *bchC* promoter were generated by DNase I titration assays using different protein dilutions treated by 10 mM DTT or a mixture of 0.5 mM $K_3Fe(CN)_6$ with 10 μM TMPD. The gel was then analyzed for PpsR binding isotherms using a PhosphorImager (Storm, Amersham Biosciences) to quantify the level of PpsR protection of a single band in the upstream and downstream palindromes. Values were corrected for loading by normalization with a band from an unprotected region.

Gel Filtration Chromatography—The oligomerization states of PpsR1 and PpsR2 were estimated by gel filtration chromatography. The Superdex 200 26/60 column (Amersham Biosciences) was equilibrated with 20 mM HEPES (pH 8.5), 50 mM NaCl in the presence of either 1 mM DTT or 1 mM $K_3Fe(CN)_6$. Before injection of the samples, the purified proteins were previously incubated under different redox conditions as described above. Each experiment was carried out in duplicate. The column was previously size calibrated using commercial gel filtration standards (Amersham Biosciences).

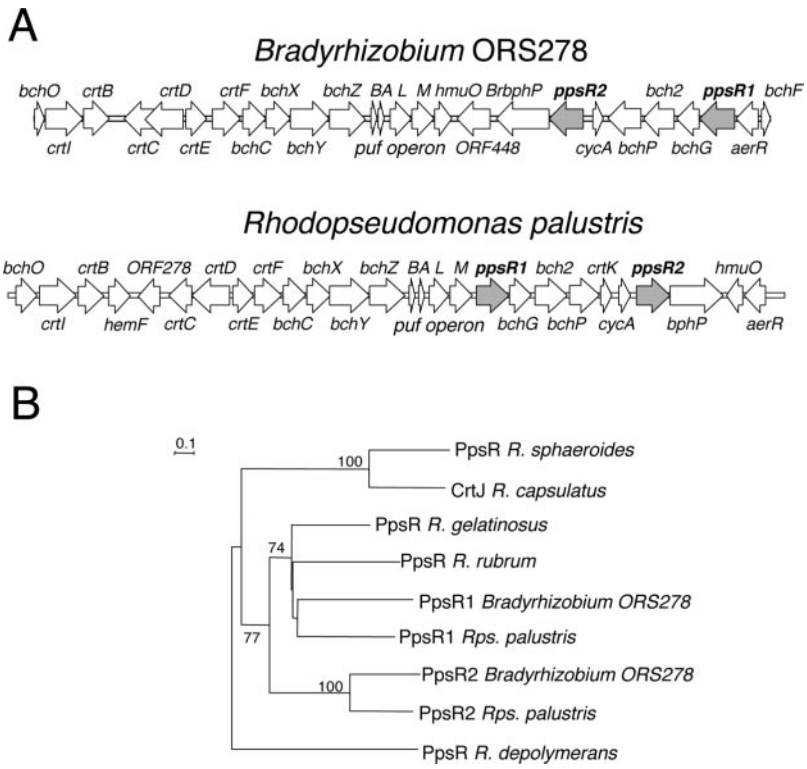

FIG. 1. **Molecular characterization of PpsR1 and PpsR2.** *A*, arrangement of the genes located around *ppsR1* and *ppsR2* in *Bradyrhizobium* ORS278 and *Rps. palustris*. *BrbphP*, *Bradyrhizobium* bacteriophytochrome photoreceptor; *bphp*, bacteriophytochrome photoreceptor. *B*, phylogenetic tree based on the PpsR sequences. This tree was constructed by using the neighbor-joining method. Bootstrap values, expressed as percentages of 1000 replications, are given at the branching points. The GenBank™ accession number are: AF182374, *Bradyrhizobium* ORS278; BX572597, *Rps. palustris*; AF195122, *R. sphaeroides*; Z11165, *R. capsulatus*; AY234385 *Rubrivivax gelatinosus*; AJ310779, *Rhodospirillum rubrum*; AB028938, *Roseateles depolymerans*.

RESULTS

ppsR1 and ppsR2: Identification and Sequence Analysis—We have previously isolated the PGC region of *Bradyrhizobium* strain ORS278 and revealed the presence of a *ppsR* gene contiguous to a bacteriophytochrome gene named *BrbphP*. The sequencing of the downstream region revealed the presence of a second *ppsR* gene located around 5 kb from the first one (Fig. 1*A*). Examination of the PGC region of *Rps. palustris* also revealed the presence of two *ppsR* genes, one of them being also contiguous to a bacteriophytochrome gene (Fig. 1*A*). In the genome annotation of *Rps. palustris,* the *ppsR* gene contiguous to *bphP* is named *ppsR2* and the other one is called *ppsR1*. As the gene organization is similar in both bacteria, we propose to use the same nomenclature for *Bradyrhizobium* and *Rps. palustris*. Interestingly PpsR1 and PpsR2 from both bacteria show a relatively weak amino acid sequence identity (32% between the two PpsR identified in *Bradyrhizobium* and 33% between PpsR1 and PpsR2 from *Rps. palustris*). It is noteworthy that a stronger similarity is observed between the two PpsR1 (54% of identity) and the two PpsR2 (44%). Furthermore, phylogenetic analysis using the available PpsR or CrtJ sequences clearly shows that the two PpsR1 and the two PpsR2 belong to two distinct clusters (Fig. 1*B*). These data suggest functional relationships between the two PpsR1 and between the two PpsR2 identified in *Bradyrhizobium* ORS278 and *Rps. palustris*.

Despite their low sequence similarity, PpsR1 and PpsR2 of both organisms present a similar architecture to the one predicted for *Rhodobacter* PpsR/CrtJ (11), with the presence of two PAS domains and a helix-turn-helix DNA binding motif at the carboxyl-terminal region. One significant feature of both PpsR2 sequences is the absence of the Cys residue. In contrast, the PpsR1 of *Bradyrhizobium* strain ORS278 contains only one Cys residue, whereas three are present in the PpsR1 of *Rps. palustris*. The absence of the Cys residue in both PpsR2 strongly suggests a mode of action different from the "classical" one described in *Rhodobacter* species.

Role of PpsR1 and PpsR2 in Bradyrhizobium Strain ORS278—To specify the role of PpsR1 and PpsR2 identified in *Bradyrhizobium* strain ORS278, we constructed isogenic mutants (278Δ*ppsR1*, 278Δ*ppsR2*) and compared their photosynthetic phenotypes to the wild type strain after growth under different light and oxygen conditions.

The synthesis of the photosynthetic apparatus is highly enhanced by far-red light when the WT strain is grown under semiaerobic conditions (Fig. 2*A*). When the oxygen tension is increased (aerobic conditions), the stimulatory effect of far red light is not observed anymore (Fig. 2*B*) and the photosystem synthesis remains very low irrespective of the light conditions. This suggests that oxygen exerts a repressive effect that dominates the activation by far-red light.

In contrast with the WT strain, we previously observed that the 278Δ*ppsR2* mutant constitutively synthesized the photosystem under semiaerobiosis irrespective of the light conditions (Fig. 2*A*). We therefore concluded that PpsR2 acts as a repressor and that far-red light antagonizes its repressive effect under semiaerobic conditions via the action of the bacteriophytochrome *Br*BphP (15). Under aerobic conditions, the phenotype of the 278Δ*ppsR2* mutant is puzzling. Indeed, we observed that the synthesis of the photosystem is high in darkness and low under far-red light illumination (Fig. 2*B*). To specify the nature of the pigment involved in this light inhibition, we cultivated the 278Δ*ppsR2* mutant in aerobiosis under a series of light-emitting diodes of different wavelengths (between 590 and 870 nm). Bacteriochlorophyll fluorescence was used as a specific marker of the presence of the photosynthetic apparatus (Fig. 2*C*). We observed a clear negative effect of light in the 700–770 nm region, with a maximum close to 750 nm (Fig. 2*D*). The action spectrum of this repressive effect of light under aerobic conditions is very similar to the absorption of the Pfr form of a bacteriophytochrome. To test whether this effect of light under aerobic conditions was controlled by *Br*BphP, we constructed a PpsR2 and *Br*BphP double mutant. The same repressive effect of far red light was observed in this mutant (data not shown) demonstrating that *Br*BphP is not involved in this light inhibition. Therefore, a second bacteriophytochrome is likely involved in this light regulatory circuit. Such an hypothesis

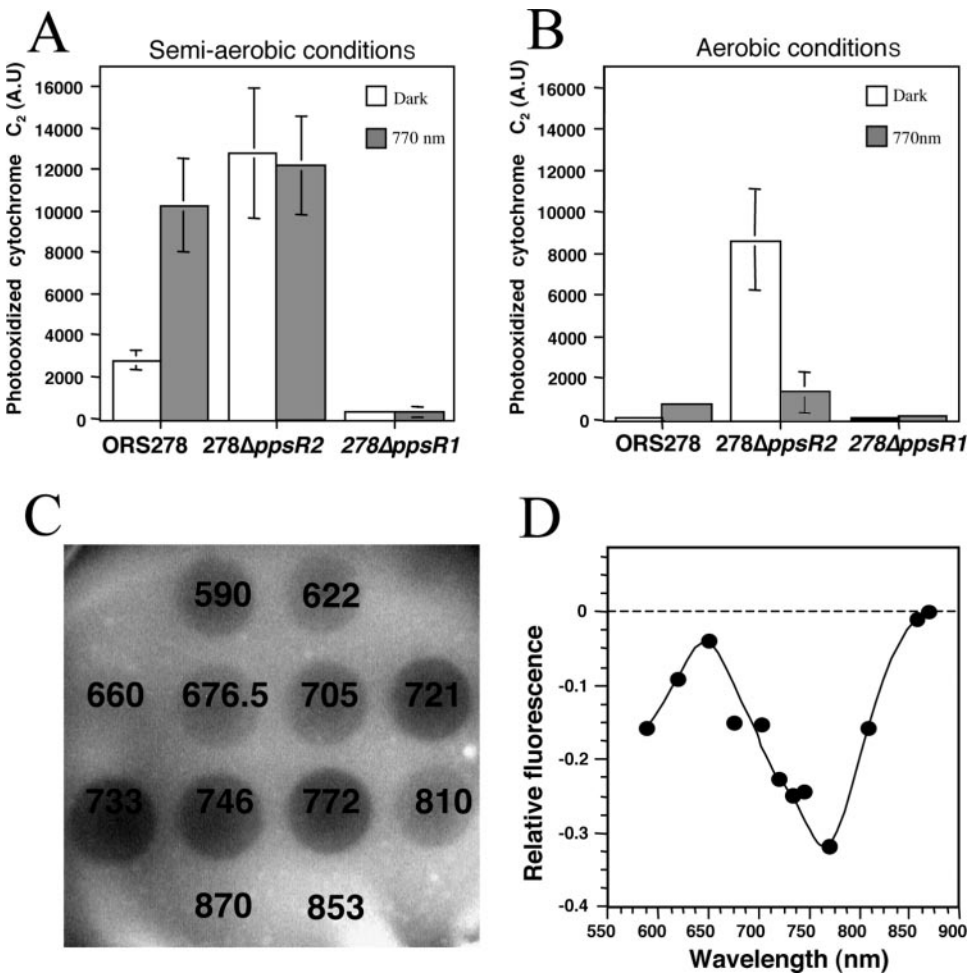

FIG. 2. **Effect of illumination on photosynthetic activity of *Bradyrhizobium* ORS278 wild type strain and mutants 278Δ*ppsR1* and 278Δ*ppsR2*.** *A*, under semiaerobic conditions. *B*, under aerobic conditions. *C*, image of the long-wavelength fluorescence emission of the bacteriochlorophyll of the 278Δ*ppsR2* mutant cultivated under aerobic conditions in the presence of light of different wavelengths (see "Experimental Procedures"). *D*, wavelength dependence of photosystem synthesis of the 278Δ*ppsR2* mutant cultivated in aerobic conditions. The data represent the mean of three experiments (*error bars* indicate ± S.D.)

makes sense because sequence analysis of the closely related *Rps. palustris* genome revealed the presence of 6 genes encoding putative bacteriophytochromes, four of them being located close to photosynthesis genes (21).

The phenotype of the 278Δ*ppsR1* mutant was also unexpected as we observed a very low photosystem synthesis whatever the light and oxygen conditions (Fig. 2, *A* and *B*). This clearly shows that contrary to PpsR2 and PpsR/CrtJ of the *Rhodobacter* species, PpsR1 is an activator of transcription that plays a key role because its presence appears essential for the synthesis of the photosynthetic apparatus in *Bradyrhizobium*.

Oligomerization States of PpsR1 and PpsR2 in Response to Redox Conditions—The His-tagged versions of both PpsR were successfully overexpressed in *E. coli* LMG194 using the pBAD-HisB vector. They were subsequently isolated to a high level of purity (>95% as judged by Coomassie Blue staining) (Fig. 3, *A* and *B*) by affinity binding of the His_6 tag to a Ni^{2+} column.

PpsR/CrtJ from *Rhodobacter* species were shown to be redox sensitive via the formation of an intramolecular disulfide bond between two conserved cysteine residues, which modulates the DNA binding affinity (12, 13). The absence of the Cys residue in PpsR2 as well as the presence of a unique Cys residue in PpsR1 suggest fundamental differences in their response to redox conditions. To assess this hypothesis, the purified proteins were incubated overnight at 4 °C under various oxidizing or reducing conditions and subsequently analyzed by SDS-PAGE under non-reducing conditions. As expected from the sequence analysis, the variation of the redox potential does not influence the oligomerization state of PpsR2. Indeed, a single band around 50 kDa, corresponding to its calculated molecular mass, is observed whatever the redox conditions (Fig. 3*A*). In contrast, the oligomerization state of PpsR1 depends on the redox conditions. Under reducing conditions, PpsR1 migrates as a monomer (Fig. 3*B*, *lane a*), whereas in appropriate oxidizing conditions, an additional band corresponding to a protein of 100 kDa is revealed (Fig. 3*B*, *lanes e*, *h*, and *i*). This molecular mass corresponds to a dimer of PpsR1, suggesting that oxidizing conditions lead to the formation of an intermolecular disulfide bond. Contrary to CrtJ or PpsR, which are oxidized after 5 min of air exposure (9, 13), PpsR1 remains reduced even after several hours of aeration (Fig. 3*B*, *lane c*). The maximal effect on the PpsR1 oligomerization state was only reached when ferricyanide was added in the presence of TMPD (a mediator that facilitates the equilibrium of electrons from the oxidizing reagent to the protein) (see Fig. 3*B*, *lanes h* and *i*). About equal amounts of protein were present in the bands of 50 and 100 kDa when PpsR1 had been incubated with 0.5 mM ferricyanide + TMPD. Higher concentrations of ferricyanide and TMPD up to 10 mM did not increase this ratio. This result suggests that the quaternary structure of the fully oxidized form of PpsR1 corresponds to a complex in which half of the monomers are covalently associated in dimers.

To ascertain that the 100-kDa band results from the formation of an intermolecular disulfide bond, we constructed a mu-

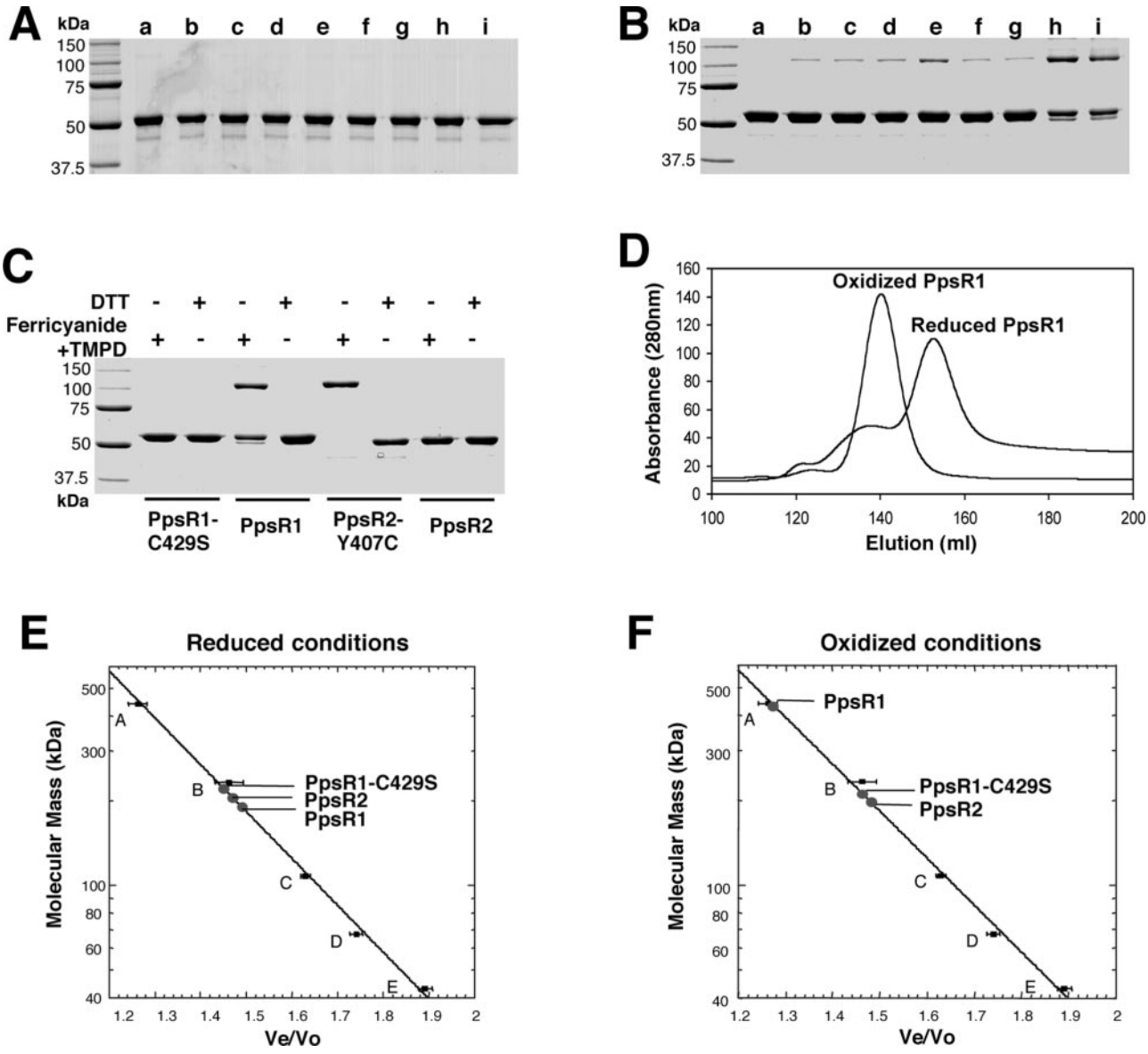

FIG. 3. **Redox dependence of PpsR1 and PpsR2.** SDS-PAGE analysis of PpsR after reduction or oxidation using various reagents. *A*, PpsR2. *B*, PpsR1. *Lane a* shows the effect of exposure of PpsR to 10 mM DTT; *lane b* shows untreated PpsR; *lane c* shows the effect of exposure to air; *lanes d* and *e* show the effect of 0.1 and 1 mM H_2O_2, respectively; *lanes f* and *g* show the effect of 0.5 and 5 mM ferricyanide, respectively; *lanes h* and *i* show the effect of 0.5 and 5 mM ferricyanide, respectively, with 0.01 mM TMPD. *C*, examination of disulfide bond formation in PpsR1, PpsR1-C429S, PpsR2, and PpsR2-Y407C by SDS-PAGE analysis after reduction with DTT (10 mM) or oxidation with 5 mM ferricyanide and 0.01 mM TMPD. *D*, elution profiles of oxidized or reduced PpsR1 that was chromatographed in Superdex 200 column. *E* and *F*, standard curves drawn according to the peak elution volumes (V_e, elution volume; V_0, column volume) of the molecular mass standards (*A*, 440 kDa; *B*, 232 kDa; *C*, 108 kDa; *D*, 67 kDa; *E*, 43 kDa) as detected by absorption at 280 nm. The estimated position of elution of native PpsR1, PpsR2, and PpsR1-C429S are shown after reduced (*E*) or oxidized (*F*) treatments.

tated PpsR1 protein (PpsR1-C429S) in which the unique Cys residue is replaced by a Ser residue. In this case, SDS-PAGE did not reveal any dimeric structure whatever the conditions (Fig. 3*C*).

CrtJ was previously shown to be folded as a tetramer, irrespective of the redox conditions (12). To test whether the formation of an intermolecular disulfide bond in PpsR1 induces a more considerable change in its quaternary structure, we performed size exclusion chromatography of PpsR1 after preincubation under different redox conditions. Under reducing conditions, we observed a single peak elution volume corresponding to a molecular mass of 220 kDa (Fig. 3, *D* and *E*). Because the calculated molecular mass of His-tagged PpsR1 monomer is 54 kDa, we can conclude that PpsR1 is folded in solution as a stable tetramer as previously reported for CrtJ or PpsR in *Rhodobacter* species (12, 13). In contrast, the elution volume is shifted under oxidizing conditions to a lower value that corresponds to a calculated molecular size of ~400 kDa. As a result, the oligomeric state of PpsR1 varies from a tetrameric to an octameric form under oxidizing conditions (Fig. 3, *D* and *F*). This redox-dependent modification of the PpsR1 quaternary structure can be caused by the formation of two Cys^{429}-Cys^{429} intermolecular disulfide bonds connecting two tetramers. In agreement with this interpretation, gel filtration analysis carried out on the PpsR1-C429S mutant revealed a tetrameric structure, irrespective of the oxidizing treatment (Fig. 3, *E* and *F*). The gel filtration procedure was also applied to PpsR2, confirming the results obtained by SDS-PAGE experiments: PpsR2 displayed the same elution volumes under reducing or oxidizing conditions (Fig. 3, *E* and *F*). It is thus folded as a tetramer that is not affected by the oxidizing treatment.

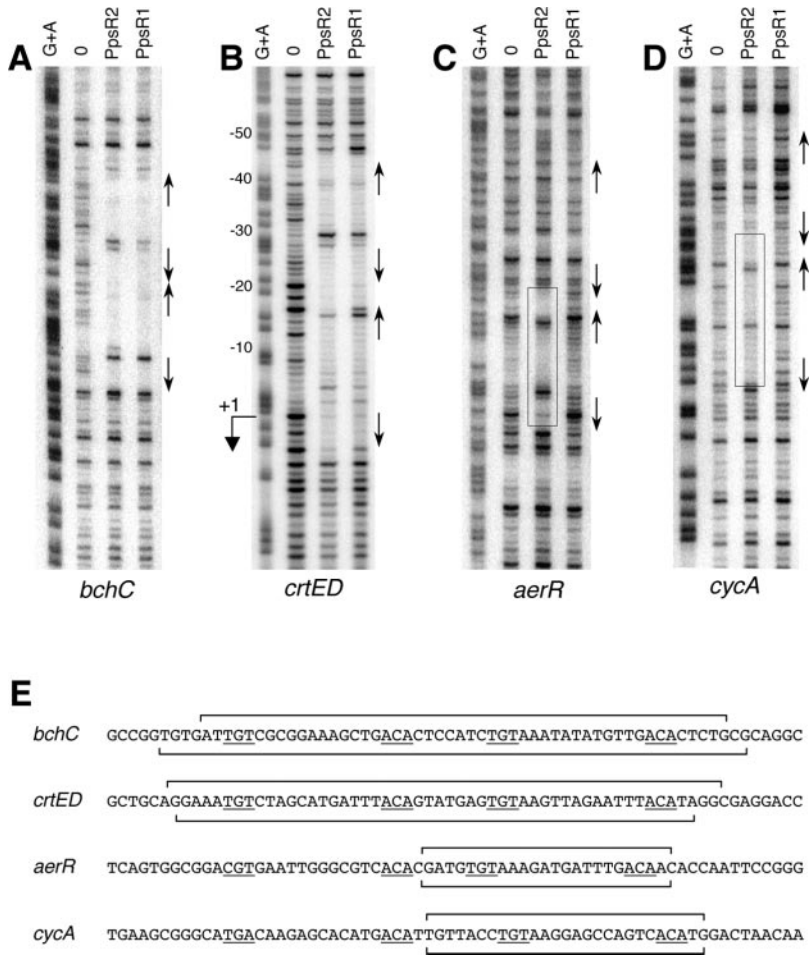

FIG. 4. **DNase I footprint analysis of PpsR2 and PpsR1 binding to the *bchC*, *crtE*, *aerR*, and *cycA* promoter regions.** Gels *A–D* are PpsR2 and PpsR1 protection patterns to the top strand of the *bchC* promoter (*A*), and to the bottom strands of the *crtED* intergenic region (*B*), the *aerR* promoter (*C*), and the *cycA* promoter (*D*). The *second lane* in each gel (0) is DNase I-digested probe incubated without protein. Each of the subsequent lanes are protection patterns generated with PpsR2 and PpsR1 (1 μg). G + A indicates a Maxam and Gilbert reaction that gives a G + A ladder. The *arrows* on the *right* of each gel represent the PpsR palindromic sequences and the *arrow* at *+1* represents the start and direction of transcription of *crtE* gene determined by primer extension analysis. The PpsR2-protected regions for the *aerR* and *cycA* probes are *boxed*. *E*, features of analyzed PpsR-binding promoters. The palindromic sites are *underlined*. The *brackets* indicate the protected regions of both strands.

We further investigated the correlation between the presence of an intermolecular bridge and the redox sensing capacity of both PpsR, by constructing a PpsR2 mutant (PpsR2-Y407C) in which Tyr^{407} was replaced by a Cys residue. Indeed, in the sequence alignment of the proteins, the residue 407 of PpsR2 corresponds to Cys^{429} in PpsR1, the residue responsible for the oligomerization change. As shown by SDS-PAGE analysis (Fig. 3*C*), this single point mutation renders PpsR2 redox sensitive, appearing as a band of 100 or 50 kDa depending on the redox conditions. Nevertheless, two fundamental differences are observed with PpsR1: (i) in its fully oxidized form, PpsR2-Y407C migrates on SDS-PAGE as a single band corresponding to a dimer (100 kDa), whereas PpsR1 was equally partitioned between monomer and dimer; and (ii) PpsR2-Y407C is fully oxidized after the elution step of the purification process, thus much easier to oxidize than PpsR1. These differences clearly indicate that in addition to the crucial role of the Cys residue in redox sensing, other patterns in the primary amino acid sequences define structural specificities of each PpsR.

DNA Binding Characteristics of PpsR1 and PpsR2—PpsR/CrtJ of *Rhodobacter* species is known to recognize the $TGTN_{12}ACA$ palindromic motif found in tandem in the promoter region of some photosynthesis genes (7, 9, 11). In addition, CrtJ displays a structural flexibility in DNA recognition, because it is also able to bind to the $TGTN_{12}ACG$ sequence located in the promoter region of *crtI* (10). A close examination of the partial PGC sequence available for *Bradyrhizobium* ORS278 revealed the presence of four putative PpsR binding sites (Fig. 4E). Two of them are found in the promoter regions of photosynthesis genes *sensu stricto* (*bchC* and *crtE-crtD* intergenic region). They correspond to a pair of perfectly conserved palindromic $TGTN_{12}ACA$. The two other putative binding sites are located in the promoter regions of *cycA* (encoding the soluble cytochrome c_2) and *aerR* (coding for a regulator of photosynthesis genes in *Rhodobacter* species (22, 23)) and contain only one palindromic motif $TGTN_{12}ACA$ associated to an incompletely conserved pattern.

We investigated the ability of both PpsR to bind to these four distinct DNA regions by DNase I footprint analysis. As shown in Fig. 4, *A* and *B*, both PpsR bind exactly to the same DNA region of the *bchC* and *crtE* promoters. Interestingly, the protected regions that correspond to the pair of palindromic motif $TGTN_{12}ACA$, present the same DNase I-hypersensitive sites, suggesting that both PpsR similarly interact with the DNA. However, the DNase I footprint analysis using *aerR* and *cycA* promoters clearly indicates that the DNA recognition process differs between the two PpsR because PpsR2 only is able to bind to these regions (Fig. 4, *C* and *D*). Interestingly, in these cases, only the downstream palindrome, which is perfectly conserved, is protected.

The DNA binding activity of the PpsR proteins was further investigated by quantifying the footprints obtained with the *bchC* promoter, varying either the amount of added protein at a fixed DNA concentration or the reverse. *Panels A* and *B* in Fig. 5 show typical results obtained for PpsR2. The binding isotherm (*A*) is clearly steeper than would be expected if a single ligand was involved (*dashed line*). A fit of the data with the function $1/(1 + (K/x)^n)$ gives a Hill coefficient $n \approx 2$, suggesting the cooperative binding of (at least) two ligands (*solid curve*). The same conclusion applies to PpsR1 (see *panel D*) and its C429S mutant (data not shown). For all cases (except oxidized PpsR1, see below), the protein concentration (expressed

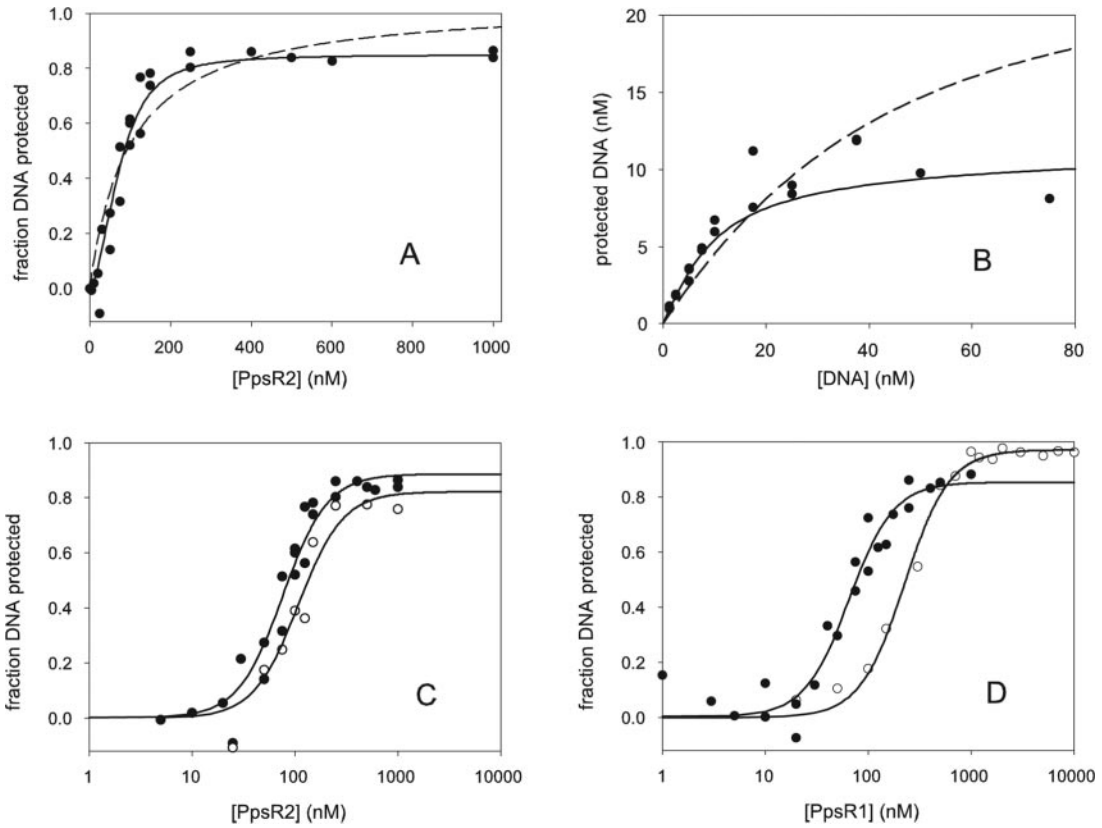

FIG. 5. **DNA binding characteristics of PpsR2 and PpsR1.** *A*, fraction of protected DNA as a function of the concentration of added PpsR2. The DNA concentration was 5 nM. The saturated protection level is somewhat below 1 (0.85). The *solid line* is a fit assuming the cooperative binding of two ligands (*i.e.* the equilibrium $A + 2P \leftrightarrow AP_2$, where *P* stands for the protein and *A* for DNA). The *dashed line* is a best fit when assuming that the protection involves the binding of a single ligand. *B*, the concentration of PpsR2 was kept fixed (100 nM) and a variable concentration of DNA was added. The *ordinate* is the concentration of protected DNA (corrected for the amplitude factor of 0.85 mentioned above). The experimental scatter increases at high [DNA] where the protected DNA becomes a small fraction of the total. The *solid line* was computed assuming the binding of two tetramers, using the dissociation constant estimated from the fit in *A*. The *dashed line* is the predicted behavior for single ligand binding. *C*, fraction of protected DNA as a function of the concentration of reduced (*solid circles*) or oxidized (*open circles*) PpsR2. The *horizontal* scale is logarithmic. *D*, same as *C*, for PpsR1. The lines in *C* and *D* are best fits with the function $a/(1+(K/X)^2)$, yielding $K = 76$ and 107 nM for reduced and oxidized PpsR2, 65 and 226 nM for reduced and oxidized PpsR1, respectively. All protein concentrations are expressed with respect to the amount of the monomeric subunit.

in terms of the monomeric subunit) for 50% of the maximum protection (EC_{50}) was in the range of 60–75 nM.

As discussed above, PpsR2 and (reduced) PpsR1 appear as tetramers. The present results thus suggest the cooperative binding of two PpsR tetramers to the two palindromic regions of the DNA. This feature (*i.e.* the cooperative binding of two ligands) is quite common for the association of transcription factors to DNA (24), allowing a narrower concentration range for switching the activity on or off. On the other hand, the binding of two tetramers may appear uneconomical, because a dimer is expected to suffice for the association to one palindrome. One may therefore consider the possibility that the tetrameric association found in the 10 μM concentration range (gel filtration conditions) might not reflect the oligomerization state prevailing in the footprints experiments (10–100 nM range) so that dimers would be the effective binding unit to each palindrome. Although it cannot be totally excluded at present, this possibility does not appear very likely, for two reasons. First, we obtained no biochemical indication of the presence of a dimeric form, although it should remain detectable if a dimer-tetramer equilibrium were involved. Furthermore, the binding experiments in which a variable amount of DNA was added to a fixed protein concentration are consistent with the binding of two tetramers. *Panel B* in Fig. 5 shows the results of such an experiment, where the protein concentration was 100 nM (for the monomeric subunit). If two tetramers (8 monomeric subunits) must be bound for protection, the maximum protected amount of DNA should be 100/8 = 12.25 nM. This is the asymptotic value of the *solid line* in *panel B*, which shows the computed behavior for this model, using the dissociation constant value estimated from the fit of the data in *panel A*. The agreement is satisfactory within experimental accuracy. Similar results were obtained with PpsR1 (not shown). On the other hand, if two dimers rather than two tetramers were involved, 100/4 = 25 nM DNA should be protected at saturation. This is not supported by the data, unless one makes the *ad hoc* assumption (9) that about half of the protein is in some damaged form, incompetent for DNA protection.

We next addressed the question whether the DNA binding of both PpsR is redox dependent as observed for PpsR and CrtJ in *Rhodobacter* species. Fig. 5*C* shows that the oxidizing treatment did not affect significantly the binding properties of PpsR2, in agreement with the biochemical results presented previously. In contrast, the redox conditions did modulate the binding of PpsR1 (Fig. 5*D*). The EC_{50} of the oxidized protein was increased more than 3-fold (226 nM). This effect was not observed with the PpsR1-C429S (data not shown), supporting the view that it is caused by the formation of an octameric form because of the establishment of a disulfide bond.

Interestingly, the binding isotherm for oxidized PpsR1 (Fig. 5, *open circles* in *panel D*) is also characteristic of a cooperative two-ligand process, although in this case the ligand species should be an octamer. If one compares the EC_{50} expressed for

the effective oligomerization state of the ligand (16 nM for the tetrameric, reduced PpsR1 and 28 nM for the oxidized octamer), it appears that the overall decrease of the affinity is contributed both by the aggregation of two tetramers to form a single ligand (*i.e.* the protein-DNA complex now consists of 16 rather than 8 monomeric subunits) and by a 1.75-fold lower affinity of this bulkier ligand.

Expression Levels of PpsR1 and PpsR2 in Regard to Oxygen and Light Conditions—Interestingly, the *ppsR1* gene of the *Bradyrhizobium* strain ORS278 is located immediately downstream from the regulator *aerR*, whose promoter is recognized by PpsR2. Such an organization is also found in *R. capsulatus* for which it has been shown that the promoter of *aerR* contributes significantly to the expression of the downstream *ppsR* gene (22). It is therefore tempting to think that PpsR2 could control the level of expression of *ppsR1*. To test this hypothesis, we used 278Δ*ppsR1* and 278Δ*ppsR2* mutants carrying *lacZ* fusions (see "Experimental Procedures") cultivated under various light and oxygen conditions. As shown in Fig. 6*A*, the expression level of *ppsR2* remains constant irrespective of the light and oxygen tension indicating that PpsR2 is constitutively synthesized. In contrast, the expression level of *ppsR1* is enhanced under far-red light and semiaerobic conditions, *i.e.* the optimal conditions for photosystem synthesis. A close examination of the light effect using a series of light-emitting diodes of different wavelengths (from 590 to 870 nm) confirmed that the synthesis of PpsR1 is controlled by a bacteriophytochrome, the action spectrum corresponding to the Pr form of *Br*BphP (Fig. 6*B*). Taken together with the footprint analysis, these results strongly suggest that the expression level of *ppsR1* is controlled by the PpsR2/*Br*BphP repressor/antirepressor system.

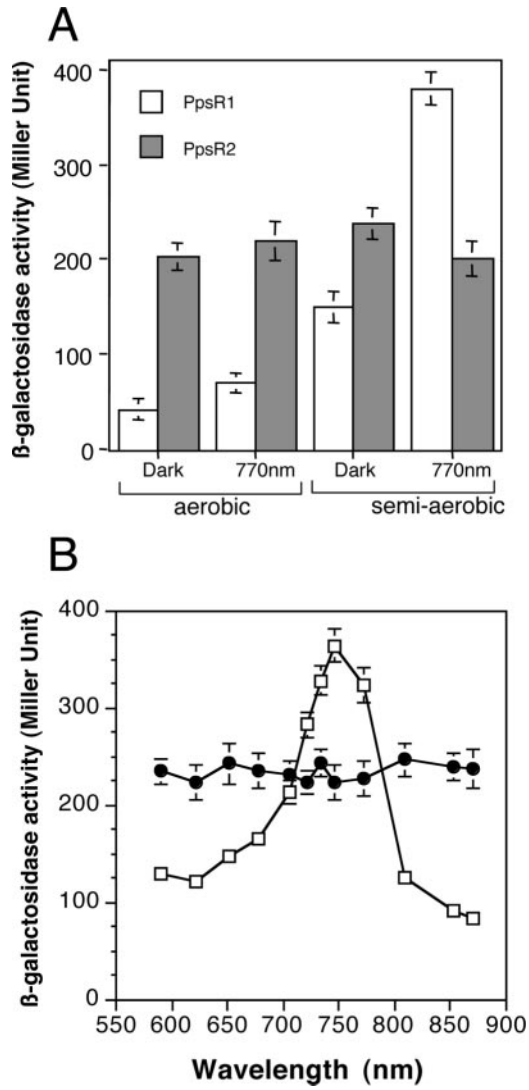

FIG. 6. **Effects of illumination and oxygen on *ppsR1* and *ppsR2* expressions.** *A*, β-galactosidase activity of the mutated 278Δ*ppsR1* and 278Δ*ppsR2* strains harboring, respectively, the *ppsR1*::*lacZ* and *ppsR2*::*lacZ* fusion cultivated under various oxygen and illumination conditions. *B*, light action spectra of *ppsR1* (*open squares*) and *ppsR2* (*filled circles*) gene expression determined using the mutated 278Δ*ppsR1* and 278Δ*ppsR2* strains cultivated in semiaerobiosis under light of different wavelengths (see "Experimental Procedures"). β-Galactosidase activity is expressed in Miller units. The data represent the mean of three experiments (*error bars* indicate ± S.D.).

DISCUSSION

We previously showed that *Bradyrhizobium* and *Rps. palustris* display an unusual behavior for the regulation of photosystem synthesis depending on the light quality and oxygen tension conditions (15, 17). In this paper we further show that this mechanism of regulation involves the unexpected dual action of two different regulators related to the PpsR/CrtJ family, a transcription factor family known to act as aerobic repressors of photosystem synthesis in purple bacteria. Indeed, PpsR1 and PpsR2 present strong similarity with the previously characterized PpsR/CrtJ proteins from *Rhodobacter* species: (i) the same predicted architecture consisting of two PAS domains followed by a COOH-terminal helix-turn-helix domain; (ii) the same DNA binding recognition sequence (*i.e.* the palindrome $TGTN_{12}ACA$); and (iii) the same photosynthesis target genes (*bch* and *crt* genes). However, fundamental differences are also observed, the most striking being: (i) the activator role observed for PpsR1; (ii) a higher DNA binding affinity for the reduced form of PpsR1 than for the oxidized one; and (iii) the redox independent DNA binding affinity of PpsR2.

In this study, we have demonstrated that only PpsR1 is redox sensitive. As it is observed for PpsR/CrtJ from the *Rhodobacter* species, the Cys residues play a key role in the redox response of PpsR1 via the formation of a disulfide bond. Nevertheless, contrary to PpsR/CrtJ, disulfide bond formation in PpsR1 is intermolecular and leads to a global change of the quaternary structure, switching from a tetramer to an octamer. The mechanism of PpsR1 oxidation also appears different from that of PpsR/CrtJ proteins because oxidation of PpsR/CrtJ occurs mainly in response to oxygen, whereas oxidation of PpsR1 remains very limited in such a condition. A mediator (TMPD) appeared necessary in our different assays to oxidize efficiently the protein in the presence of ferricyanide. Bauer's group has also studied the oxidation state of PpsR/CrtJ *in vivo* (12, 13) and showed that they formed a disulfide bond specifically under aerobic conditions. It has been also observed that the *R. sphaeroides* cytoplasmic redox poise remains constant, around −220 mV, under both aerobic and anaerobic conditions. Because this is lower than the midpoint potential for oxidation of CrtJ (−180 mV); it was proposed that molecular oxygen itself stimulates disulfide bond formation in CrtJ (12). The absence of an oxygen response on the redox state of PpsR1 *in vitro* leads us to suggest that PpsR1 senses *in vivo* another signal redox than the oxygen tension. The redox state of the quinone pool that has been shown to play a key role in the regulation of photosystem synthesis in *R. sphaeroides* (25) could be this signal. As observed in PpsR/CrtJ (12, 13), disulfide bond formation affects the DNA binding affinity of PpsR1. However, contrary to PpsR/CrtJ, the reduced form presents the higher affinity. PpsR1 exhibits a 3.5-fold modulation of its affinity for DNA, depending on the redox conditions, which is in the same range as the difference determined for CrtJ (5-fold) or PpsR (2.2-fold).

Contrary to PpsR1, PpsR2 contains no Cys residue and is thus not redox sensitive. Interestingly, a single point mutation (PpsR2-Y407C) induces a redox sensitivity of PpsR2. This result raises the possibility of a spontaneous mutation of PpsR2 in the ORS278 strain, rendering it redox insensitive. In the same way, a double spontaneous mutation in the *Rps. palustris* CGA009 has been recently revealed: one frameshift mutation in the bacteriophytochrome *rpa1537* gene, and one single point mutation in the *ppsR2* gene had rendered the two corresponding proteins inactive (17). To assess the possibility that the absence of a Cys residue in PpsR2 might result from the accidental selection of a spontaneous mutant, we sequenced the corresponding gene from two other strains (*Bradyrhizobium* Btai1 and *Bradyrhizobium* ORS285). Both strains have been isolated from different regions and different host plants. A slight polymorphism is observed between the various amino acid sequences of PpsR2, the identity percentage ranging from 95.4 to 96.5%. However, in all three photosynthetic strains, the PpsR2 contains no Cys residue. In addition, sequencing the *ppsR1* genes of the *Bradyrhizobium* Btai1 and *Bradyrhizobium* ORS285 strains confirmed the presence of only one conserved Cys residue in the corresponding protein. These data clearly indicate that the presence of two PpsR that differ in their Cys content and, in all likelihood, in their redox sensing behavior, is a typical feature of photosynthetic *Bradyrhizobium* strains.

The opposite phenotypes observed for the *278ΔppsR1* and *278ΔppsR2* mutants suggest that both PpsR play dual roles in the regulation of photosystem formation in *Bradyrhizobium*, with a classical repressor role for PpsR2 and an unexpected activator role for PpsR1. Two other pieces of evidence are in agreement with the activator role of PpsR1: 1) its expression level is correlated with the amount of photosynthetic apparatus and 2) its DNA binding affinity is higher under reducing conditions, as expected for promoting photosynthesis under low oxygen tension conditions. Such an activator role has been very recently described in *Rubrivivax gelatinosus* (26). In this bacterium, a single PpsR displays both a negative and a positive activity: it represses the expression of the *crtI* gene on the one hand and it stimulates the expression of *pucBA* genes on the other hand. It is believed that the difference of spacing observed between the two putative PpsR binding boxes of the *crtI* and *pucBA* promoters could be responsible for this modulation of PpsR behavior. In the case of *Bradyrhizobium,* the situation is different because the activator PpsR1 and the repressor PpsR2 protect exactly the same DNA region of the *bch* and *crt* promoters. Interestingly, primer extension analysis of the *crtE* gene (data not shown) indicates that the two PpsR binding palindromes overlap the −35 and −10 promoter regions (see Fig. 4*B*). PpsR2 is then likely to exert its repressive effect by preventing the RNA polymerase from binding to the DNA, or by blocking another step of the transcription initiation, like, for instance, the formation of the open complex. The PpsR1 activator role, when bound to the same sites, could also be explained by different mechanisms. PpsR1 could be a class II activator, activating transcription by recruiting the RNA polymerase via its interaction with the σ subunit. Alternatively, it might accelerate promoter clearance or alter the promoter conformation leading to a better recognition by the RNA polymerase, as observed for the transcriptional regulators of the MerR (27). Further experiments are required to understand the molecular mechanisms used by PpsR1 and PpsR2 to control transcription, in particular for assessing the probable role of their PAS domains, which is likely to be a determinant in their repressor/activator behavior toward RNA polymerase.

The two PpsR interact with the same photosynthesis promoters while they play antagonistic roles. This implies that the regulation of photosystem formation in *Bradyrhizobium* results from a competition between the two PpsR for the binding to these target genes. The EC_{50} observed under reducing conditions are similar for the two PpsR proteins (65 ± 5 nM for PpsR1 and 76 ± 5 nM for PpsR2). However, redox and light conditions modulate their efficiency, via the change of the oligomerization state of PpsR1 and via the action of *Br*BphP, which antirepresses PpsR2, respectively. The latter mechanism is still unknown, but one may suppose that it is similar to that of AppA (13), involving the formation of an inactive, light-dependent, *Br*BphP-PpsR2 complex. An essential parameter to consider in this competition process is the relative amounts of PpsR1 and PpsR2. Using *ppsR*::*lacZ* fusions, we demonstrated that *ppsR2* is constitutively expressed, whereas the *ppsR1* expression level is strongly dependent on oxygen and light. The light control of *ppsR1* expression surely involves the antagonistic action of PpsR2/*Br*BphP. In contrast, the aerobic circuit remains unknown and may involve an aerobic repressor not yet identified.

Besides the fact that the two PpsR display similar binding properties toward the photosynthesis genes, we showed that PpsR2, but not PpsR1, recognized the *aerR* and *cycA* promoters. This indicates that the DNA recognition process is more flexible for PpsR2 than for PpsR1 leading to a broader panel of target genes. In the *cycA* and *aerR* promoters, the upstream palindrome is not perfectly conserved and we observed that it was not protected by PpsR2 from DNase I digestion. Its role in the process of recognition of PpsR2 is therefore debatable. If this region is not able to interact tightly with the protein, one may hypothesize that the binding of PpsR2 on these promoters is weaker and will exert a weaker repressive effect on the downstream gene. This could be beneficial in the case of the *cycA* gene, which encodes the cytochrome c_2. This soluble electron carrier is implicated in both photosynthesis and respiratory processes. A not too drastic control of the *cycA* gene by PpsR2 would permit the synthesis of cytochrome c_2 to a basal level sufficient for the functioning of the respiratory chain and also boost its production when photosynthesis takes over. On the other hand, the absence of control of the *cycA* gene by the activator PpsR1 makes sense, because in aerobic conditions, where PpsR1 is probably inactive, the synthesis of cytochrome c_2 is still indispensable for the respiratory process.

The regulator PpsR has been identified in all purple bacteria studied so far, suggesting that it is a common regulator for this bacteria family. However, as illustrated in this article, its mode of action has evolved to respond to specific needs in various species. Indeed, the mechanism of regulation of photosynthesis, implicating the combined action of two PpsR in *Bradyrhizobium* appears more sophisticated than in *Rhodobacter* species. Presumably, this sophistication, which integrates both light and redox signals permits better control of photosystem formation. This fine control seems particularly adapted to *Bradyrhizobium*, which contrary to the *Rhodobacter* species is a strict aerobic bacterium and therefore must carry out photosynthesis under conditions where harmful reactive oxygen species are generated.

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