Bacteriophytochrome controls carotenoid-independent response to photodynamic stress in a non-photosynthetic rhizobacterium, Azospirillum brasilense Sp7

Santosh Kumar1, Suneel Kateriya2, Vijay Shankar Singh1, Meenakshi Tanwar2, Shweta Agarwal1, Hina Singh1, Jitendra Paul Khurana3, Devinder Vijay Amla4 & Anil Kumar Tripathi1

1School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi-221005, India, 2Department of Biochemistry, 3Department of Plant Molecular Biology, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India, 4National Botanical Research Institute, Rana Pratap Marg, Lucknow-226001, India.

Ever since the discovery of the role of bacteriophytochrome (BphP) in inducing carotenoid synthesis in Deinococcus radiodurans in response to light the role of BphPs in other non-photosynthetic bacteria is not clear yet. Azospirillum brasilense, a non-photosynthetic rhizobacterium, harbours a pair of BphPs out of which AbBphP1 is a homolog of AtBphP1 of Agrobacterium tumefaciens. By overexpression, purification, biochemical and spectral characterization we have shown that AbBphP1 is a photochromic bacteriophytochrome. Phenotypic study of the ΔAbBphP1 mutant showed that it is required for the survival of A. brasilense on minimal medium under red light. The mutant also showed reduced chemotaxis towards dicarboxylates and increased sensitivity to the photooxidative stress. Unlike D. radiodurans, AbBphP1 was not involved in controlling carotenoid synthesis. Proteome analysis of the ΔAbBphP1 indicated that AbBphP1 is involved in inducing a cellular response that enables A. brasilense in regenerating proteins that might be damaged due to photodynamic stress.

Light has been the driving force of life on the planet earth, but it is also a threat to the survival of aerobic organisms due to its photodynamic effect1. Incidental formation of singlet oxygen (‘O2), a highly reactive oxygen species (ROS), occurs in photosynthetic as well as non-photosynthetic cells by the simultaneous presence of light, oxygen and photosensitizers, which causes photooxidative stress to the cells2. The major light-dependent sources of singlet oxygen in a cell include energy transfer to molecular oxygen (O2) from excited triplet state bacteriochlorophyll a (‘Bchl a*) in photosynthetic membranes, or excited endogenous- (e.g. porphyrins or other tetrapyrroles) or exogenous- (e.g. methylene blue, toluidine blue) photosensitizers3. Since light can severely harm living cells, it is important for the organisms to sense and appropriately respond to the light signals4. In both, photosynthetic and non-photosynthetic organisms, carotenoids protect cells from the photooxidative damage by scavenging the singlet molecular species of oxygen produced upon illumination5.

Phytochromes are a widespread family of red/far-red responsive photoreceptors, which sense the quantity and quality of light in the photosynthetic and non-photosynthetic organisms, and transduce the physical signal into the biochemical message for responding to the ambient light conditions6,7. They play important role in adaptive processes in higher plants including seed germination, de-etiolation, neighbor preception and shade avoidance, and the transition from vegetative to reproductive growth (induction of flowering). Phytochromes exist in two spectral forms: red light absorbing form (Pr) and far-red absorbing form (Pfr). The Pr form phototransforms into the Pfr after absorbing red light. Pfr is again converted back to Pr state after absorbing far-red light8. The ratio of these two spectral forms determines the signaling state of the phytochrome.

A typical (R/FR) phytochrome consists of a conserved N-terminal PAS-GAF-PHY tridomain photosensory core, which is combined with a C-terminal catalytic domain with a histidine kinase (HK) or histidine kinase–related domain (HKRD)9. Phytochromes from different organisms have characteristic variations of this
Figure 1 | (a) Phylogeny of bacteriophytochromes from different bacteria based on amino acid sequences retrieved from NCBI (accession numbers are provided in supplementary file). Neighbor-joining tree was built in MEGA version 5.04 with a 1000 bootstrap replications and Poisson model. The two bacteriophytochromes of \textit{A. brasilense} Sp7 are marked in light blue. (b) Organization of genes around bacteriophytochrome (BphP) encoding gene in \textit{A. brasilense} Sp7 and other bacteria. Direction of arrow indicates the orientation of genes. The nucleotide (nt) shows the distance between overlapping (minus sign) and closely associated gene.
architecture. Based on their distribution, photobiological properties and possible modes of action, phytochromes are divided into several families, which include plant phytochromes (Phys), cyanobacterial phytochromes (Cphs), bacteriophytochromes (BphPs), fungal phytochromes (Fphs) and unorthodox Phys, without apparent relationships. The exact nature of the chromophore varies for different families of phytochromes: plant and cyanobacterial phytochromes covalently attach phytochromobilin (PΦB) and phycocyanobilin (PCB), respectively, to the Cys residues in the PAS domain, whereas BphPs and Fphs attach biliverdin IXα (BV) chromophores to a Cys residue in the PAS domain. The chromophore is autocatalytically assembled within the photosensory core, and the protein-chromophore interactions control the wavelength sensitivities of each phytochrome. Despite their spectral diversity, photoconversion between Pr and Pfr forms of each phytochrome is brought about by a Z-E isomerization about the C15=C16 double bond of the bilin chromophore.

Several bacteriophytochromes from photosynthetic and non-photosynthetic bacteria have been characterized biochemically, spectrosopically and structurally. However, except for controlling the synthesis of photosynthetic machinery in photosynthetic bacteria, their role in the physiology of non-photosynthetic bacteria is not well known. Elucidation of the role of bacteriophytochrome in carotenoid synthesis in Deinococcus radiodurans was the first example of the role of bacteriophytochrome in coupling the photodynamic stress with the induction of carotenoid synthesis in the photobiology of a non-photosynthetic bacterium. Although bacteriophytochromes of A. tumefaciens are among the best characterized bacteriophytochromes, their biological roles are not known yet. BphP of P. aeruginosa has also been characterized in detail, but its knock-out mutant did not reveal any phenotype. However, transcriptomic and proteomic analyses indicated its possible involvement in the quorum sensing.

_Azotobacter vinelandii_ is a non-photosynthetic α-proteobacterium belonging to the family Rhizobiaceae, which lives in the rhizosphere of a large number of non-legume crop plants and grasses, and promotes their growth by producing phytohormones. The genome sequence of _A. vinelandii_ showed the presence of two genes encoding bacteriophytochromes. After the first report of the role of bacteriophytochromes in any non-photosynthetic bacteria in 1999, this is the second report describing a new role of bacteriophytochrome in another non-photosynthetic bacterium, _A. brasilense_, in coping with red light mediated photodynamic stress in dicarboxylate grown cultures by a mechanism that does not involve carotenoid biosynthesis.

**Results**

**Organization of genes in bacteriophytochrome (BphP1) encoding operon in _A. brasilense_ Sp7.** Homology based search of _A. brasilense_ genome database for phytochrome and a phylogenetic analysis of the prokaryotic phytochromes, including the representatives from cyanobacteria (Cphs) and photosynthetic as well as non-photosynthetic bacteria (BphPs), shows that _A. brasilense_ genome harbours two putative genes encoding bacteriophytochromes (Fig. 1a), which were located in two different operons. Both of them were closely related to the two well characterized BphPs from the soil bacteria, _A. tumefaciens_; one showing homology to AtBphP1 (designated as AbBphP1), and the other to AtBphP2 (designated as AbBphP2). Organization of the gene encoding AbBphP1 showed synteny with bphP genes of _Nostoc sp_ PCC7120 and _A. tumefaciens_ in which bphP appears to be the first gene of a tricistronic operon followed by a gene encoding a putative response regulator (bphR), which overlapped with the _bphP_ by 8 bp (Fig. 1b). The third gene encodes a putative hybrid multi-sensor histidine kinase (HK).

**AbBphP1 is a photochromic bacteriophytochrome.** To elucidate, if AbBphP1 is a photochromic bacteriophytochrome, the holoprotein was produced in _E. coli_ by coexpressing a heme oxygenase along with AbBphP1 apoprotein. The apoprotein was also produced in _E. coli_ cells lacking heme oxygenase expressing plasmid. Both, apo- and holoproteins were expressed in soluble form, and purified using affinity- and gel filtration chromatography. The purified AbBphP1 apoprotein was colorless, whereas the holoprotein had a blue-green color indicative of bound BV and the consequent photochromicity (Fig. 2a). When protein extracts of _E. coli_ expressing AbBphP1 holoprotein were resolved in SDSPAGE (Fig. 2c), stained with a Zn²⁺ solution, and exposed to UV light, Zinc-dependent fluorescence was observed (Fig. 2b) in the holoprotein, but not in the apoprotein, which indicated that BV was bound covalently in the AbBphP1 holoprotein, as observed in typical bacteriophytochromes.

In the dark (Pr state), holoprotein showed an absorbance maximum at 710 nm, which is a characteristic of Pr state of the bacteriophytochrome(s). Upon saturating illumination of 2 min with red light (λmax = 695 nm), Pr state was phototransformed into Pfr state, which was evident by 50% photobleaching, a red shift, and a new broad absorbance of holoprotein at 750 nm. Analysis of the difference (dark minus red light illuminated) spectra of AbBphP1 showed maximum difference at 710 and 750 nm (Fig. 3a). The changes induced by the red light were completely reversible upon incubation in the dark with recovery time of 40 min at room temperature (Fig. 3b). Photo-convertion of Pfr state to Pr state was accelerated with a recovery time of 25 min, when red-light irradiated sample was irradiated with far-red (750 nm) light (Fig. 3c).

**AbBphP1 is a dimer.** Propagation of signal from sensory module to the output module depends upon phytochrome dimerization. Bacteriophytochrome from _D. radiodurans_ dimerizes through PAS-GAF-PHY and HK domain. Size exclusion chromatography profile of the AbBphP1 also indicated dimerization of the holoprotein. Under native condition, the major fraction of the
chromophore-bound AbBphP1 protein was eluted as dimer, whereas monomer and higher oligomer were eluted as minor fractions (Fig. 4a and b). Under native conditions, AbBphP1 had a size of 220 kDa, which was more than the dimer (170 kDa) of monomeric size (85 kDa). Larger than the predicted size of the phytochrome holoproteins have also been observed earlier due to the non-spherical shape of the holoprotein. We observed a more prominent peak of the holoprotein monomers as compared to the apoprotein. This may be due to the limitation of SEC, as the interacting protein subunits, which are diluted during the separation, may dissociate during the analysis. Quaternary structural characteristics of AbBphP1 were further confirmed by chemical cross-linking with glutaraldehyde, which also retained the protein as dimer. The presence of higher oligomeric species could be due to some degree of non-specific cross-linking (Fig. 4c). These experiments indicated that both apo- and holo- AbBphP1 monomers have a site each for dimerization of the photoreceptor in native condition. Modeling of AbBphP1 and protein-protein docking by Cluspro 2.0 also predicted it as a dimer, in which dimerization interface was made up of α helices and β sheets (Supplemental Fig. S1B). Some structural components of the chromophore binding pocket also seem to be involved in the dimerization site (Supplemental Fig.1C).

Figure 3 | Spectral characterization of recombinant AbBph1 protein. (a) UV-Visible spectra of holoprotein in the dark and after irradiation with red light (695 nm) light. (b) Slow reversion to dark state of the recombinant holoprotein after irradiation with red light. (c) Fast reversion to dark state of the recombinant holoprotein after irradiation with red light followed by far-red light irradiation.
AbBphP1 is not involved in light-induced carotenoid synthesis. In order to understand the physiological role of AbBphP1 in *A. brasilense* Sp7, an in-frame deletion of the gene encoding AbBphP1 was constructed in *A. brasilense*. In view of the previous report that bacteriophytochrome is involved in red light-induced carotenoid synthesis in *D. radiodurans*, we first studied the role of AbBphP1 in carotenoid synthesis in *A. brasilense* Sp7. When *A. brasilense* Sp7 and AbBphP1 mutant were grown in dark, red light or white light, the colonies grown in dark did not show pink color. In red light, both parent and AbBphP1 mutant showed very little pink color. However, in white light, there was maximum production of pink color both in parent and the mutant (Supplemental Fig S2). Absorption spectra of the methanolic extracts of the parent and AbBphP1 mutant exposed to white light also showed that carotenoid content in the AbBphP1 mutant was almost same as in the parent (Fig. 5).

AbBphP1 is required for optimal growth under red light in malate minimal medium. Both, *A. brasilense* Sp7 and ΔAbBphP1 mutant, grew equally well in the dark when their growth was compared in rich medium and in minimal medium (Fig. 6a). In white light also, both, wild type and the mutant, grew equally well, however, the mutant grew slower than the wild type in minimal malate medium. In red light, growth of the mutant was much slower than that of the wild type in minimal medium (Fig. 6a). But, in LB medium, both wild type and the mutant grew almost equally well in red light, indicating that ΔAbBphP1 mutant was sensitive to red light only in minimal malate medium. Although ΔAbBphP1 mutant displayed reduced chemotaxis towards malate in the minimal medium, this difference was observed both in dark as well as in red light. The ΔAbBphP1 mutant carrying a cloned copy of the AbBphP1 gene showed almost as good chemotaxis as the parent, indicating functional complementation (Fig. 6b).

Figure 4 | Characterization of oligomeric states of AbBphP1 by size exclusion chromatography (SEC) of holoprotein (a) and apoprotein (b). FMN was used as a loading indicator, 120 ml-140 ml elution volume indicates FMN fraction. SDS-PAGE with Coomassie (left) and Zn (right) staining of glutaraldehyde (G) cross-linked SEC purified AbBphP1 holoprotein (c).
Figure 5 | Absorption spectra of methanolic extracts of carotenoids showing effect of darkness and white light on carotenoid content of A. brasilense Sp7 and ΔAbBphP1 mutant.

We also examined the effect of red light on the growth of A. brasilense Sp7 and its ΔAbBphP1 mutant, by epifluorescence microscopy, to understand if red light caused killing of ΔAbBphP1 mutant. When A. brasilense Sp7 and its ΔAbBphP1 mutant were grown in minimal medium in the dark, and the cells of the early stationary phase culture (at 15 h) stained with DAPI and propidium iodide, both showed blue color, indicating that all the cells were viable (Fig. 6c). When they were grown in white light, some (~5%) of the cells of ΔAbBphP1 showed yellowish fluorescence, indicating that the yellow fluorescing cells were dead, because propidium iodide can enter only in the dead cells. However, a large proportion of cells (~35%) of the early stationary phase culture of ΔAbBphP1 grown in red light showed yellowish fluorescence (Fig. 6c). The wild type cells exposed to white light or red light did not show yellow fluorescing cells, indicating that they were not killed by red light in the minimal medium. The ΔAbBphP1(pSK5) strain, expressing cloned copy of AbBphP1 gene, was almost as resistant to killing by red light as wild type.

ΔAbBphP1 is sensitive to photodynamic stress by toluidine blue. Since red light is known to cause photodynamic stress, by evolving stressful levels of ROS26, we tested the effect of different oxidative stress agents, viz paraquat (superoxide), hydrogen peroxide (peroxide) and toluidine blue plus light (singlet oxygen) on the growth of A. brasilense Sp7, ΔAbBphP1 mutant and ΔAbBphP1(pSK5). The zone of inhibition observed with toluidine blue plus light in the ΔAbBphP1 mutant was considerably larger than that observed in A. brasilense Sp7 or ΔAbBphP1(pSK5) (Fig. 7; Supplemental Fig. S3). But, the zones of inhibition observed with hydrogen peroxide and paraquat were similar in all the three strains (not shown), indicating that AbBphP1 plays major role in tolerating stress generated by singlet oxygen, but not the stresses caused by peroxide or superoxide.

Identification of differentially expressed proteins in A. brasilense Sp7 and ΔAbBphP1 mutant in response to red light. The comparison of the proteomes of A. brasilense and ΔAbBphP1 mutant grown in red light or dark, revealed at least 9 proteins, which were upregulated in A. brasilense in response to red light, but not in ΔAbBphP1 (Supplemental Fig. S4). The proteins, whose intensity varied more than 1.5 fold in the 2D gel, were identified by MALDI-TOF/TOF. A list of the proteins that appeared to be red light regulated in three out of three independent experiments is given in Table 1. The notable proteins upregulated in response to red light included DnaK, GroEL, AtpD, TuB, RpsA and OmaA.

Discussion
Although the genes encoding bacteriophytochromes are represented in the genomes of a large number of non-photosynthetic bacteria, the knowledge about their role is rather limited14,17,22. When we analyzed the genome of the non-photosynthetic rhizobacterium, A. brasilense, we found two ORFs encoding bacteriophytochromes, showing homology to a pair of BphPs found in A. tumefaciens. Bioinformatic analysis indicated, that like other phytochromes, AbBphP1 consists of an N-terminal chromophore binding domain (CBD), consisting of PAS, GAF and PHY sub-domains, and a C-terminal effector domain that includes HisKA and HATPase domains. The organization of the genes flanking bphP was also similar to that found in A. tumefaciens and Nostoc PCC7120, in which the gene encoding heme oxygenase (BphO) is not in the vicinity of bphP10. In A. brasilense Sp7, bphP1 is the first gene of a tricistronic operon, a response regulator and a multi sensor HK, being the second and the third genes, respectively. Thus, it is likely that all the three genes coordinate in mounting a response to light.

By cloning and overexpressing the AbBphP1 in E. coli BL21 DE3, expressing a heme oxygenase, we produced the typical blue colored recombinant holoprotein, which was purified and visualized by zinc induced fluorescence, and further confirmed by immunoblotting using 6XHis antibody (data not shown). Recombinant AbBphP1 kept in the dark, showed absorbance maximum at 710 nm, which is similar to that of R. sphaeroides22. Absorbance maximum of bacteriophytochrome at 710 nm has been attributed to the Pr state of these photoreceptors. Illumination of the dark-adapted AbBphP1 with red light showed spectral shift, suggesting photoconversion of Pr (710 nm) state to Pfr (750 nm) state. The spectral changes induced by the red light were completely reversible upon incubation in the dark. Illumination with far red light, however, resulted in very little photoconversion from Pfr state to Pr state. Size exclusion chromatography and protein cross-linking experiments suggest that like other bacteriophytochromes, quaternary structure of AbBphP1 is also dimeric28. These characterizations indicated that AbBphP1 was a photochromic bacteriophytochrome in A. brasilense Sp7.

Although spectral properties and reversion kinetics of AbBphP1 were comparable to that of an unorthodox bacteriophytochrome BphG1 of R. sphaeroides28, the BphG1 of R. sphaeroides has GGDEF and EAL as output domains, but AbBphP1, like most other phytochromes, possesses a histidine kinase as its output domain. Further, the gene encoding AbBphP1 is the first gene of a tricistronic operon, the gene encoding BphG1 seems to be bicistronic, as it shows an overlap of 79 nucleotides with BphO gene located upstream. Both, R. sphaeroides and A. brasilense, are closely related members of α-proteobacteria, the former is photosynthetic whereas the latter is non-photosynthetic.

In order to understand the biological role of AbBphP1 in A. brasilense, we constructed a mutant, in which the wild copy of the bphP1 was replaced by a mutant copy, having an in-frame deletion of 2229 bp in bphP1. Both, A. brasilense Sp7 and ΔAbBphP1 mutant produced almost equal amount of carotenoids in light, but failed to do so under dark. This indicated that although light induces carotenoid synthesis in A. brasilense, AbBphP1 is not involved in controlling carotenoid synthesis. This situation in A. brasilense is in contrast to D. radiodurans, where bacteriophytochrome is known to control carotenoid synthesis; it accumulated higher amounts of carotenoids under white/red light, whereas the synthesis of carotenoids was severely inhibited in the BphP1 mutant. The bacteriophytochrome found in D. radiodurans thus helps in protecting the bacterium from the adverse effects of intense visible light by controlling the synthesis of the deinoxanthin carotenoid29-31. Although AtBphP1 and AtBphP2 of A. tumefaciens are one of the most thoroughly...
investigated bacteriophytochromes\textsuperscript{21,23,24}, their biological role is not known yet.

Phenotypic characterization of the ΔAbBphP1 mutant showed that its growth was sensitive to red light, when it was grown on minimal medium containing malate as carbon source. The reduced growth of ΔAbBphP1 was due to the lethal effects of red light. This indicated an involvement of AbBphP1 in coping with the photodynamic stress, probably by sensing the light signal, and transducing it through response regulator to the downstream regulatory cascade to induce the proteins, which might be involved in responding to photodynamic stress, created by the red light. Why red light is more lethal to ΔAbBphP1 in minimal medium than in the rich LB medium, needs to be investigated further. We also observed that ΔAbBphP1 showed reduced swarming ability as compared to its parent. This difference in chemotaxis was observed in dark as well as under white or red light. This observation is in contrast to that found in \textit{A. tumefaciens} where the swarming motility of AbBphP1 mutant on minimal medium did not differ from its parent under any light condition\textsuperscript{21}.

Our observation, that ΔAbBphP1 mutant was more sensitive to toluidene blue plus light than its parent, also indicated that AbBphP1 might be important in coping with the stress generated by ROS including \textsuperscript{1}O\textsubscript{2}, which damages a large variety of biological molecules such as DNA, proteins and lipids\textsuperscript{32}. Toluidene blue is a well known photosensitizer, which shows maximum phototoxicity to the Gram negative bacteria upon red light irradiation\textsuperscript{33,34}. After the absorption of light, part of the energy is transferred to the triplet state, and then to the molecular oxygen to generate ROS. In this photodynamic action, singlet oxygen (\textsuperscript{1}O\textsubscript{2}), the most reactive ROS, causes damage to the bacterial cell wall, membranes, proteins and nucleic acids\textsuperscript{35,36}. The photooxidative stress in the cell induces pathways for the repair of DNA, and refolding of misfolded proteins\textsuperscript{2,37}. The correct conformation of the misfolded proteins, which accumulate during heat shock in \textit{E. coli}, is restored by the chaperones like GroEL, DnaK, DnaJ and GrpE\textsuperscript{38,39}.

Comparison of the effect of red light on the proteome of \textit{A. brasilense} Sp7 revealed 3 fold, and 8 fold increase in the levels of DnaK (Hsp70) and GroEL (Hsp60) proteins, respectively, as compared to
the dark. Such a response was not seen in the ΔAbBphP1 mutant. This indicated that these proteins might be involved in refolding of the misfolded, unfolded or aggregated proteins inside the cell, due to the photodynamic damage caused by the red light. Chaperonins, like DnaK and GroEL, which possess N-terminal ATPase domain and a C-terminal substrate binding domain, work in association with other co-chaperones in an ATP dependent manner. The enhanced expression of the beta chain of F-type, H+ transporting ATPase, observed in the wild type A. brasilense (but not in ΔAbBphP1 mutant) cells exposed to red light, indicates an increased demand for ATP to facilitate protein refolding by such HSPs. Induction of GroEL and DnaK was also reported in E. coli in response to photodynamic therapy-mediated oxidative stress. Accumulation of these HSPs along with other stress-associated proteins was also observed when Synechocystis and Prochlorococcus were exposed to high light. In R. sphaeroides too, expression of the three alternative sigma factors rpoE, rpoHII as well as rpoHI is upregulated in response to photooxidative stress. The observation, that several of the genes responding to O2 are controlled by RpoHII as well as RpoHI, indicated that the responses to O2 and heat partially overlap in R. sphaeroides.

The discovery of photoreceptor proteins in non-photosynthetic bacteria, and their ability to sense and respond to light have opened many as yet unexplored issues in the photobiology of bacteria. Since blue and, to a lesser extent, red light can cause photooxidative stress in aerobic bacteria, it is useful for non-photosynthetic bacteria to harbour a light sensing system, which can enable them to activate an appropriate defense response to prepare them for damage by light. Although the effect of red light on the physiology of photosynthetic prokaryotes is well known, there is very little information about the red light effects in non-photosynthetic bacteria. Unlike D. radiodurans, where bacteriophytochrome is involved in coping with photooxidative stress by inducing carotenoid synthesis, this is the first report showing the role of bacteriophytochrome in A. brasilense in tolerating the red light mediated photodynamic stress, without inducing carotenoids.

### Methods

**Bacterial strains, plasmids, chemicals and growth conditions.** Bacterial strains and plasmids used in this study are listed in Supplemental Table S1. Plasmid pT7-HO1-1, expressing heme oxygenase gene hox1, was maintained in E. coli strains grown in Luria Bertani (LB) medium with kanamycin (40 μg/ml). Plasmid pET15b expressing AbBphP1 was maintained in E. coli DH5α and E. coli BL21, and grown in LB medium having ampicillin (100 μg/ml). A. brasilense Sp7 was grown in minimal maltate (MM) medium or LB broth, and maintained on MM Agar plates. E. coli and A. brasilense Sp7 cultures were grown at 37 °C and 30 °C, respectively.

Bioinformatic tools/softwares are described in the Supplemental Material.

### RT-PCR analysis of co-transcription of AbBphP1 gene with downstream genes.

RNA was isolated from the cultures of A. brasilense Sp7 as described earlier. The cDNA was prepared using RNaseH minus MMLV reverse transcriptase and random hexamers primer. PCR amplifications were performed using primers from the BphP intragenic region and 3' end RR gene (RT1-RT2) in one set, and from BphP intragenic region and sequences at the 5' end of the gene encoding multisensor histidine kinase (RT1-RT3) in the other set. PCR reactions were carried out as recommended by manufacturer (NEB). Primers used in this study are listed in Supplemental Table S2.

### Cloning, expression and purification of AbBphP1.

The coding region for AbBphP1 was PCR-amplified from A. brasilense strain Sp7 by using primers designed to introduce NdeI and BamHI sites before the start- and stop codons of AbBphP1, respectively. The Ndel-BamHI digested PCR product was cloned into similarly digested pET15b (Novagen), resulting in the addition of a 6×His-tag at the N-terminus. E. coli DH5α was used as host for cloning whereas E. coli BL21 (DE3) pLYS was used for protein expression. The pET15b clone having a gene encoding AbBphP1 was transformed into E. coli BL21 (DE3) harboursing the plasmid pT7-ho1-1, which expresses the heme oxygenase gene hox1 of Synechocystis sp. It was expected that the

![Figure 7](https://www.nature.com/scientificreports/)

**Figure 7** Sensitivity of A. brasilense Sp7, ΔAbBphP1 and ΔΔAbBphP1 (pSNk5) to 5 mM toluidine blue plus light shown by zone of inhibition diameter on 1.5 % LB agar. Each bar represents the mean diameter of the zone of inhibition recorded in three independent assays performed in triplicate. Error bars indicate SD.

---

**Table 1** Identification of differentially expressed proteins in response to red light in A. brasilense and ΔAbBphP1 mutant by MALDI-TOF/TOF

| Spot No | Protein | Matched Accession # | Function | Fold change | PI | M.W. | Mascot Score |
|---------|---------|---------------------|----------|-------------|----|------|--------------|
| S1      | DnaK    | gi|302381499 | chaperon DnaK/Hsp70 | 3.0 | 4.77 | 67718 | 67 |
| S2      | RpsA    | gi|288957134 | small subunit ribosomal protein S1 | 2.0 | 5.13 | 62367 | 293 |
| S3      | GroEL   | gi|288957187 | chaperon GroEL /Hsp60 | 8.14 | 4.96 | 57702 | 212 |
| S4      | AcoB    | gi|209963467 | pyruvate dehydrogenase subunit beta | 2.2 | 4.84 | 49523 | 166 |
| S5      | AtpD    | gi|288959333 | F-type H+ transporting ATPase beta chain | 2.0 | 4.93 | 50036 | 527 |
| S6      | OmaA    | gi|10798859 | major outer membrane protein OmaA | 2.5 | 4.78 | 40901 | 93 |
| S7      | TufB    | gi|288957406 | elongation factor EF-Tu | 1.5 | 5.36 | 43169 | 139 |
| S8      | RfaG    | gi|46241709 | glycosyl transferase-like protein | 3.0 | 6.11 | 153539 | 57 |
| S9      | LivK    | gi|288962866 | Branched chain amino acid transport system substrate-binding protein | 1.75 | 6.47 | 40510 | 202 |

**Proteins upregulated by red light in A. brasilense Sp7**

**Proteins upregulated by red light in ΔAbBphP1**
heme oxygenase synthesized by pT7-hot-1 would convert intracellular heme into biliverdin, which will covalently bind to the apoprotein to produce the AbBphP1 holoprotein. The transformants were selected on LB-agar plates containing appropriate antibiotics (50 μg/mL kanamycin or 100 μg/mL ampicillin).

A primary culture was inoculated in LB medium supplemented with appropriate antibiotics (50 μg/mL kanamycin or 100 μg/mL ampicillin). The secondary culture was prepared by inoculating an overnight grown primary culture into the terrific antibiotics (50 μg/mL ampicillin). Growth curves of the plates with aluminum foil. Growth curves of the plates with aluminum foil. Growth curves of the plates with aluminum foil. Growth curves of the plates with aluminum foil.

plates were incubated for 48 h in the dark or under the red light. The size of the chemotactic ring in the swarm plates was recorded by a digital camera.

**Plate assay for sensitivity to reactive oxygen species.** Precultures of A. brasilense, ΔAbBphP1 mutant and ΔAbBphP1(pSnK5) were grown up to the linear exponential phase in LB medium with respective antibiotics. 100 μl pre-culture of each strain was mixed with 5 ml of LB agar, having only 0.5 % agar, poured on top of the LB agar plates (0.2 %) plates, and allowed to solidify. After 15 min, a 10 μl drop of 5 mM toluidine blue (TB), 2 mM H2O2 or 1 mM paraquat was placed in the centre of each agar plate, incubated for 3 days in white light at 30 °C, and the zone of inhibition by each oxidative stress agent was measured.

**Proteome analysis by 2D-gel electrophoresis.** A. brasilense Sp7 and its ΔAbBphP1 mutant were grown in 50 ml M9MM medium in a 250 ml flask up to mid log phase (O.D600nm 1.5). These cells were harvested by centrifugation, and the pellet was used to prepare the protein sample. The detailed description of the procedure for proteome analysis is given in the Supplemental Material.

1. Elias-Arnanz, M., Padmanabhan, S. & Murilo, F. J. Light-dependent gene regulation in nonphototrophic bacteria. *Curr. Opin. Microbiol.* 14, 128–135 (2011).
2. Ziegelhofer, E. C. & Donohue, T. J. Bacterial responses to photo-oxidative stress. *Appl. Environ. Microbiol.* 7, 856–860 (2009).
3. Sayed, Z., Harris, F. & Phoenix, D. A. A study on the bacterial photo-toxicity of phenothiazinium based photosensitizers. *FEMS Immunol. Med. Microbiol.* 43, 367–372 (2005).
4. Hellingwerf, K. J. The molecular basis of sensing and responding to light in microorganisms. *Antonie Van Leeuwenhoek* 81, 51–59 (2002).
5. Glaser, J. & Klug, G. Photo-oxidative stress in Rhodobacter sphaeroides: protective role of carotenoids and expression of selected genes. *Microbiol.* 151, 1927–1938 (2005).
6. Rockwell, C. N., Su, Y. S. & Lagarias, J. C. Phytochrome structure and signaling mechanisms. *Annu. Rev. Plant. Biol.* 57, 837–858 (2006).
7. Rockwell, N. C. & Lagarias, J. C. A brief history of phytochromes. *Chemphyschem.* 11, 1172–1180 (2010).
8. Fankhauser, C. The phytochromes, a family of red/far-red absorbing photoreceptors. *J. Biol. Chem.* 276, 11453–11456 (2001).
9. Bellini, D. & Papiz, M. Z. Structure of a bacteriophytochrome and light–stimulated proton pumping with a common respiratory E1 center. *Structure* 20, 1436–1446 (2012).
10. Vierstra, R. D. & Karniol, B. Phytochromes in microorganisms. In: Handbook of photosensory receptors. (W.R. Briggs and J. L. Spudich, eds). Wiley-VCH Press, Heidelberg, Germany 197–195 (2010).
11. Yang X, Stojkovic E. A, Kuk J. & Moffat K. Crystal structure of the chromophore binding domain of an unusual bacteriophytochrome. *RbBphP3*, reveals residues that modulate photoconversion. *Proc. Natl Acad. Sci. USA* 104, 12571–12576 (2007).
12. Yang, X., Kuk, J. & Moffat, K. Crystal structure of *Pseudomonas aeruginosa* bacteriophytochrome: photoconversion and signal transduction. *Proc. Natl Acad. Sci. USA* 105, 14719–14724 (2008).
13. Cho, M. H., Yoo, Y., Blaho, S. H. & Lee, S. W. Purification and characterization of a recombinant bacteriophytochrome of *Xanthomonas oryzae* pathovar oryzae. *Protein J.* 30, 120–131 (2011).
14. Barkovits, K., Schubert, B., Heine, S., Scheer, M. & Frankenberg-Dinkel, N. Function of the bacteriophytochrome BphP in the RpoS/Las quorum-sensing network of *Pseudomonas aeruginosa*. *Microbiol.* 157, 1651–1664 (2011).
15. Essen, L. O., Mailllet, J. & Hughes, J. The structure of a complete phytochrome sensory module in the Prn ground state. *Proc. Natl Acad. Sci. USA* 105, 14709–14714 (2008).
16. Ulijasz, A. T. et al. Cyanochromes are blue/green light photoreversible photoreceptors defined by a stable double cysteine linkage to a phycocyanobilin-type chromophore. *J. Biol. Chem.* 284, 29757–29772 (2009).
17. van der Horst, M. A., Key, J. & Hellingwerf, K. J. Photosensing in chemotrophic, non-phototrophic bacteria: let there be light sensing too. *Trends Microbiol.* 15, 57 (2007).
18. Giraud, E. et al. A new type of bacteriophytochrome acts in tandem with a classical bacteriophytochrome to control the antenna synthesis in *Rhodopseudomonas palustris*. *J. Biol. Chem.* 280, 32389–32397 (2005).
19. Wilde, A., Fiedler, B. & Borner, T. The cyanobacterial phytochrome Cph2 inhibits phototaxis towards blue light. *Mol. Microbiol.* 44, 981–988 (2002).
20. Rottwinkel, G., Oberpichler, I. & Lamparter, T. *Bathy* phytochromes in rhizobial soil bacteria. *J. Bacteriol.* 191, 5114–5133 (2009).
21. Oberpichler, I., Rosen, R., Rasouly, A., Vugman, M., Ron, E. Z. & Lamparter, T. Light affects motility and infectivity of Agrobacterium tumefaciens. *Env. Microbiol.* 10, 2020–2029 (2008).
22. Davis, S. J., Vener, A. V. & Vierstra, R. D. Bacteriophytochromes: photoreceptive-like photoreceptors from non photosynthetic eubacteria. *Science* 286, 2517–2520 (1999).
23. Karniol, B. & Vierstra, R. D. The pair of bacteriophytochromes from *Agrobacterium tumefaciens* is histidine kinases with opposing photobiological properties. *Proc. Natl Acad. Sci. USA* 100, 2807–2812 (2003).
28. Noack, S., Michael, N., Rosen, R. & Lamparter, T. Protein conformational changes
38. Guisbert, E., Herman, C., Lu, C. Z. & Gross, C. A. A chaperone network controls
36. Mohammadi, M., Burbank, L. & Roper, M. C. Biological role of pigment
35. Maisch, T., Baier, J., Franz, B., Maier, M., Landthaler, M., Szeimies, R. M. &
34. Wang, X., Wang, H. & Huang, Z. Photosensitization induced by exposure to
33. Wilson, M. Lethal photosensitisation of oral bacteria and its potential application
31. Lysenko, V. S. SCIENTIFIC
32. Briviba, K., Klotz, L. O. & Sies, H. Toxic and signaling effects of photochemically
29. König, K., Teschke, M., Sigusch, B., Glockmann, E., Eick, S. & Pfister, W. Red light
28. Ko¨nig, K., Teschke, M., Sigusch, B., Glockmann, E., Eick, S. & Pfister, W. Red light
27. Wagner, J. R., Zhang, J., Brunzelle, S. J., Vierstra, R. D. & Forest, K. T. High
26. Li, H., Zhang, J., Vierstra, R. D. & Li, H. Quaternary organization of a
25. Tien, T. M., Gaskins, M. H. & Hubbell, D. H. Plant growth substances produced by
24. Scheerer, P. et al. Light induced conformational changes of the chromophore and
23. Chen, E. C., Broadfoot, B. P. & Dyer, A. J. Dimer TOF-MS analysis of
22. Cresci, M., Chambers, C. T., Kallioniemi, O., Dyrker, D. & Eaves, C. P. Malignant
21. Burcharth, L. & Roper, M. C. RpoHII activates oxidative stress defense
20. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
19. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
18. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
17. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
16. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
15. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
14. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
13. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
12. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
11. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
10. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
9. Nuss, A. M., Glaeser, J. & Klug, G. RpoHII activates oxidative stress defense
8. Gamer, J., Multhaup, G., Tomoyasu, T., McCarty, J. S., Ru¨diger, S., Scho¨nfeld,
7. Mary, I., Tu, C. J., Grossman, A. & Vaulot, D. Effects of high light on transcripts of
6. Mary, I., Tu, C. J., Grossman, A. & Vaulot, D. Effects of high light on transcripts of
5. Mary, I., Tu, C. J., Grossman, A. & Vaulot, D. Effects of high light on transcripts of
4. Nuss, A. M., Glaeser, J., Berghoff, B. A. & Klug, G. Overlapping alternative sigma
3. Nuss, A. M., Glaeser, J., Berghoff, B. A. & Klug, G. Overlapping alternative sigma
2. Nuss, A. M., Glaeser, J., Berghoff, B. A. & Klug, G. Overlapping alternative sigma
1. Nuss, A. M., Glaeser, J., Berghoff, B. A. & Klug, G. Overlapping alternative sigma
Acknowledgements
This work was supported by a grant from the Department of Biotechnology, New Delhi to AKT. SK was supported by a fellowship from Indian Council of Medical Research, New Delhi. We thank Mark Gomelsky for providing the plasmid pT7-ho1-1. This paper is dedicated to the memory of Pt. Madan Mohan Malaviya, the founder of Banaras Hindu University, on his 150th birth anniversary.

Author contribution
1'SK, VSS, MT, SA and HS performed the experiments. IPK and DVA provided some of the experimental facilities and inputs to the manuscript. 2SK organized spectral characterization work and contributed in manuscript writing. AKT conceived the idea, coordinated the whole project and wrote the manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports
Competing financial interests: The authors declare no competing financial interest.
License: This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/
How to cite this article: Kumar, S. et al. Bacteriophytochrome controls carotenoid-independent response to photodynamic stress in a non-photosynthetic rhizobacterium, Azospirillum brasilense Sp7. Sci. Rep. 2, 872; DOI:10.1038/srep00872 (2012).