Bacteriophytochromes control conjugation in *Agrobacterium fabrum*

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Key words: type 4 secretion system, DNA transfer, oriT, TraA, codistribution

Abstract

Bacterial conjugation, the transfer of single stranded plasmid DNA from donor to recipient cell, is mediated through the type IV secretion system. We performed conjugation assays using a transmissible artificial plasmid as reporter. With this assay, conjugation in *Agrobacterium fabrum* was modulated by the phytochromes Agp1 and Agp2, photoreceptors that are most sensitive in the red region of visible light. In conjugation studies with wild-type donor cells carrying a pBIN-GUSINT plasmid as reporter that lacked the Ti (tumor inducing) plasmid, no conjugation was observed. When either *agp1*$^{-}$ or *agp2*$^{-}$ knockout donor strains were used, plasmid DNA was delivered to the recipient, indicating that both phytochromes suppress conjugation in the wild type donor. In the recipient strains, the loss of Agp1or Agp2 led to diminished conjugation. When wild type cells with Ti plasmid and pBIN-GUS reporter plasmid were used as donor, a high rate of conjugation was observed. The DNA transfer was down regulated by red or far-red light by a factor of 3.5. With *agp1*$^{-}$ or *agp2*$^{-}$ knockout donor cells, conjugation in the dark was about 10 times lower than with the wild type donor, and with the double knockout donor no conjugation was observed. These results imply that the phytochrome system has evolved to inhibit conjugation in the light. The decrease of conjugation under different temperature correlated with the decrease of phytochrome autophosphorylation.

Keywords

DNA delivery, light effect, Ti plasmid, single stranded DNA, red, far-red

Introduction

In eukaryotic species, gene exchange between individuals takes place during sexual reproduction. Often, biological events around sexual reproduction are modulated by light. In many plants, flower induction is controlled by the length of the day, mediated by phytochrome and cryptochrome photoreceptors [1]. In fungi, the transition of sexual to vegetative reproduction is induced by light, mediated by phytochrome and blue light photoreceptors [2]. Gene delivery in prokaryotes, which are lacking sexual reproduction, takes place during plasmid transfer termed conjugation. Although mechanisms of conjugation have been studied down to the molecular level in several model bacteria [3,4], there is as yet no example for a light effect on conjugation.

We report here about the role of phytochrome photoreceptors on the conjugation of *Agrobacterium fabrum* C58 [5], former *Agrobacterium tumefaciens* C58. This soil bacterium has evolved a mechanism to deliver genes into cells of wounded plant tissue. This infection results in tumor growth, followed by release of amino acid derivatives, termed opines, into the soil. The discovery of plant transformation [6] has made *A. fabrum* to a well-studied model organism [7] and to a widely used vector for plant transformation [8]. Conjugation is also well characterized in *A. fabrum* [9]. Conjugation has many features in common with plant transformation. In both cases, single stranded DNA is delivered to the recipient organism, and the transport occurs through the Type IV secretion system (T4SS). There are three different T4SS machineries in *A. fabrum*, one for plant infection, one for Ti plasmid conjugation and a third one for conjugation of the At plasmid [10]. Expression of these systems is controlled by different environmental stimuli. Phenolic compounds released from a wounded plant, recognized by VirA, result in an induction of the vir system, the T4SS for plant transformation and of other components important for plant transformation [10,11], whereas conjugation can be induced by opine compounds or quorum sensing [10]. Whereas only a segment of the tumor inducing Ti-plasmid, the T-DNA, is transferred during plant transformation, the entire plasmid is transferred during conjugation [9]. The Ti-plasmid of *A. fabrum* harbors genes that are important for plant transformation and genes that are relevant for conjugation, like traA-traD and traF-traH or trbI-J and trbL [12]. Homologous genes exist also on the circular or linear chromosomes or on the At plasmid of *Agrobacterium* [12]. Conjugation is therefore also possible in the absence of the Ti plasmid. An early step in plant transformation is the insertion of nicks at the "right border" and "left border" of the T-DNA by the VirD2 protein followed by cutting out of single stranded T-DNA between both edges [5,13]. Conjugation is initiated by nicking at oriT catalyzed by a relaxase, probably TraA [14]; the nick region has either the same or a very similar sequence as the right border of the Ti plasmid [15,16].

Phytochromes are photoreceptors with a characteristic photoreversible property: two spectrally different forms, termed Pr and Pfr, can be converted into each other by red and far-red light [17]. Phytochromes exist in plants, diverse algae, fungi and bacteria [18,19], where they control a broad array of developmental responses. Although the overall domain organization of bacterial and eukaryotic phytochromes varies, basic phytochrome properties such as spectral characteristics or structures of the N-terminal chromophore binding modules

are similar [20]. Bacterial and fungal phytochromes function as light regulated histidine kinases that transphosphorylate cognate response regulators. *Agrobacterium fabrum* harbors two phytochromes termed Agp1 and Agp2 [21,22], both of which have been studied as model phytochromes for chromophore binding, light induced protein conformational changes, protein structure or kinase activity [23]. Both incorporate biliverdin as natural chromophore which becomes covalently bound to a conserved Cys next to the N-terminus during a rapid assembly reaction [24]. Agp1 and Agp2 have a Pr and Pfr ground state, respectively, due to an antagonistic dark behavior. Agp1 converts slowly from Pfr to Pr in darkness, a feature found for many other typical phytochromes, whereas Agp2 converts from Pr to Pfr in darkness [21,22]. Pr to Pfr dark conversion is an exceptional feature which has been found for few bacterial phytochromes [25–27] termed "bathy phytochromes", to emphasize the long wavelength absorption maximum of their Pfr dark state. Most bathy phytochromes, including Agp2, have a His kinase which belongs to the HWE type and carry a C-terminal response regulator as part of the same protein [27]. The response regulator extension is also found in fungal phytochromes [28] but is absent from typical bacterial phytochromes.

There are few reports for phytochrome responses in photosynthetic bacteria dealing with the regulation of photosynthesis compounds [26,29], and there is one report about the involvement of phytochrome in biofilm formation of *Pseudomonas aeruginosa* [30]. In view of the broad diversity of bacteria and the broad distribution of phytochromes among bacteria [31] these few reports provide only a minor insight into phytochrome function in the bacterial domain of life. The biological functions of phytochromes in *A. fabrum* remained obscure. Here we show how phytochromes are involved in the conjugation of *A. fabrum*.

Materials and methods

Codistribution analysis

Homologs of all *A. fabrum* proteins were identified by BLAST [32] within a set of 43 selected Rhizobiales proteomes (*Agrobacterium fabrum/tumefaciens str.* C58, *Agrobacterium radiobacter* K84, *Agrobacterium vitis* S4, *Azorhizobium caulinodans* ORS 571, *Bartonella bacilliformis* KC583, *Bartonella henselae str. Houston-1, Bartonella quintana str. Toulouse, Bartonella tribocorum* CIP 105476, *Beijerinckia indica* subsp. indica ATCC 9039, *Bradyrhizobium japonicum* USDA 110, *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium* sp. ORS278, *Brucella abortus bv. 1* str. 9-941, *Brucella abortus S19, Brucella canis ATCC 23365, Brucella melitensis 16M, Brucella melitensis biovar Abortus 2308, Brucella ovis ATCC 25840, Brucella suis 1330, Brucella suis ATCC 23445, Candidatus Pelagibacter ubique HTCC1002, Mesorhizobium loti MAFF303099, Mesorhizobium sp. BNC1, Methylobacterium extorquens PA1, Methylobacterium populi BJ001, Methylobacterium radiotolerans JCM 2831, Methylobacterium sp. 4-46, Nitrobacterhamburgensis X14, Nitrobacterwinogradskyi Nb-255, Ochrobactrumanthropi ATCC 49188, Parvibaculumlavamentivorans DS-1, Rhizobium etli CFN 42, Rhizobium etli CIAT 652, Rhizobium leguminosarum bv. viciae 3841, Rhodopseudomonaspalustris BisA53, Rhodopseudomonaspalustris BisB18, Rhodopseudomonaspalustris BisB5, Rhodopseudomonaspalustris CGA009, Rhodopseudomonaspalustris HaA2, Rhodopseudomonaspalustris TIE-1, Sinorhizobiummedicae WSM419, Sinorhizobiummeliloti 1021, Xanthobacterautotrophicus Py2*) and the homolog distribution for each protein compared with that of Agp1 or Agp2. A codistribution value "P" for was calculated according

to ref. [33]. For Agp1 and Agp2, 202 and 248 groups with different P values were distinguished.

Strains, plasmids and bacterial medium

Agrobacterium fabrum strains were cultivated in LB (20 g L^{-1}, Roth) [34] or AB minimal medium (3 g K_2HPO_4, 1 g NaH_2PO_4, 1 g NH_4Cl, 0. 3 g $MgSO_4$, 0. 15 g KCl, 0. 01 g $CaCl_2$, 2. 5 mg $FeSO_4$, 0. 5% glucose per L) [35]. *Escherichia coli* DH5α were grown at 37 ℃, the growth temperature for propagation *A. fabrum* C58 was usually 26℃. The plasmids pBIN-GUS and pBIN-GUSINT are described in ref. [36]. Both binary vectors are derived from the Ti plasmid with which they share the same left and right border sequences. The pJQ200KS vector is described in ref. [37], the pGEM-T Easy vector is from Promega and pBBR122 from Mobitec.

Mutant construction

The *A. fabrum* strains used here are shown in Table 1. Phytochrome knockout mutants *agp1*$^-$ and *agp2*$^-$were constructed through interrupting the agp1 gene (atu1990, nomenclature according to ref. [38]) and agp2 gene (atu2165) with a kanamycin resistance cassette, respectively [38]. In the *agp1/agp2*$^-$ double knockout mutant, the atu1990 sequence is interrupted by a spectinomycin and the atu2165 by a gentamicin resistance cassette [38]. An ampicillin resistance cassette was introduced in recipient strains as a selection marker between the loci atu2165, the agp2 gene, and atu2166, a sequence with unknown function. Both atu2165 and atu2166 were amplified from genomic DNA by PCR using the primer pairs Bai11 (ACGATACGGTATTTGCCGAATGGGC) / Bai12 (TAAGATCTGGGATGAGGGGAGGCTA) and Bai15 (TGGGTACCTGAAACCGCGCTTCGTT) / Bai16 (CGTCTAGAAACAGCGATCTTCAGGC), respectively. The ampicillin cassette was amplified from the plasmid pGEM-T Easy using the primer pair Bai13 (CGAGATCTGGTTAAAAAATGAGCTG) and Bai14 (TGGGTACCCAGTTACCAATGCTTAA). The PCR parameters were: 30 cycles (95 ℃, 30 s; 50 ℃, 30 s; 68 ℃, 120 s) in all cases. All three fragments were independently cloned into pGEM-T Easy. Following double digestion of the new vectors with KpnI / BglII, NotI / BglII and KpnI / XbaI for the Amp restriction cassette, atu2165 and atu2166, respectively, the three fragments were integrated into the suicide vector pJQ200KS [37] via NotI and XbaI sites. The correct pJQ200KS based plasmid was propagated in *E. coli*DH5α and transformed into *A. fabrum* wild type and mutant cells by electroporation (GenePulser, Biorad, 1 mm cuvette, 2400V, 25 μF, 200 Ω). Ampicillin (100 μg / ml) was used for positive selection, thereafter cells were grown on sucrose to select for double crossover.

pBBR122 driven expression

For complementation and overexpression, the pBBR122-*agp1* and pBBR122-*agp2* plasmids were created. The *agp1* and *agp2* promoter sequences were determined by a Promotor Prediction server (http://www. fruitfly. org/seq_tools/promoter. html), the lengths were 865 and 750 base pairs, respectively. The promotor-agp1 and promotor-agp2 sequences were amplified by using the primers gr_agp1_prom-> (GCGGCCGCGTATGCAGGCCGTAAAGATG), gr_agp1_prom<- (CTCGAGCAGAATGGGTCTGAGTTCAG) and gr_agp2_prom-> (AGTACTCAGAACCGAATGTTCAGCAG), gr_agp2_prom<- (GCGGCCGCCTGTTACAAGCACAGGGATG). The PCR products were cloned into

pGEM-T Easy to obtain pGEMagp1 and pGEMagp2. pGEMagp1 and pBBR122 were digested with NotI and ligated to obtain the Agp1 expression vectorpBBR122-agp1. pGEMagp2 and pBBR122 were double digested with NotI and ScaI and ligated to obtain the Agp2 expression vector pBBR122-agp2. Cloning of the Agp2 expression vector results in a loss of the Cm cassette. The expression vectors were transformed into DH5α and selected by kanamycin. Plasmids were then transferred into *A. fabrum* donor cells. Modified versions of expression vectors were constructed for recipient strains because the kanamycin resistance of pBBR122 is not compatible with the conjugation protocol. (The kanamycin resistance of pBBR122-agp1 or -agp2 in the recipient cell will not allow selecting for pBIN-GUS which carries also a kanamycin resistance cassette.) Therefore we interrupted the kanamycin cassette of pBBR122-agp1and pBBR122-agp2 by insertion of a chloramphenicol cassette to produce the pBBR122-agp1 (Cm) and pBBR122-agp2 (Cm). The primers bai17 (TCCCGGGATAAATACCTGTGACGG) and bai18 (TACGCTCGAGCACCAATAACTGCC) were used to amplify the chloramphenicol resistance cassette sequence from pBBR122. The PCR parameters were: 95 ℃, 30 s; 62 ℃, 30 s; 68 ℃, 1 min for 30 cycles. The fragment was cloned into pGEM-T. This plasmid, pGEM-*Cm*, was double digested with SmaI and XhoI and ligated with pBBR122-*agp1* (Km) or pBBR122-*agp2* (Km) that were cut by the same restriction enzymes. The positively selected pBBR122-*agp1* (Cm) and pBBR122-*agp2* (Cm) were transformed into *A. fabrum* recipient cells.

Bacteria without Ti plasmid

Propagation of the Ti plasmid is slowed down at elevated temperature. Bacteria were cultivated on LB plates at 37 °C and subcultivated every 2nd day. After 10 days the presence or absence of the Ti plasmid was tested in several colonies by PCR using primers Bai01 (TCACGGTACAGATTTTCC) and Bai02 (TTAGCCCTGAGCCTGATT). Colonies in which the Ti plasmid was missing were used for subsequent experiments.

Conjugation assay

For donor strains, wild type and mutants either without or with Ti plasmid were used. Cells without Ti plasmid were transformed with the pBIN-GUSINT plasmid while cells with Ti plasmid were transformed with the pBIN-GUS plasmid [36]. Both plasmids are only distinguished by the presence of a plant intron in pBIN-GUSINT which interrupts the GUS coding region [36]. The plasmids carry a kanamycin cassette and the oriT origin of transfer which is identical with the right border of the T-DNA. The temperature during cultivation was usually kept at 26 °C. Donor and recipient strains were grown in LB medium [34] with half concentrations of appropriate antibiotics to an $OD_{600\ nm} = 1$. A defined starting volume of cells were washed separately three times in AB medium [35] and finally suspended in the same volume AB medium. For conjugation, 100 µl of donor and 100 µL of recipient cells were mixed and another 600 µL AB medium were added. The solution was inoculated for 24 h at 26 °C with or without shaking at 100 rpm (as indicated) under different light conditions (darkness, red light and far-red light). After a series of dilution, spots of 100 µL were spread on LB agar plates with kanamycin and ampicillin or chloramphenicol. After another 3 days at 26 °C and different light conditions, the conjugation efficiency was determined by the number of colonies. For each treatment three independent experiments were made. Significance of differences between all conditions of a set of experiments was tested by the T-test. In most cases the differences were significant with $p<0.01$. For temperature variations, the cocultivation in liquid medium occurred at various temperature as given in the results section. Subsequent selection on agar medium occurred at 26 °C.

Light treatments

For red light, an 80 × 80 cm array of 655 nm light emitting diodes (LEDs) was used. The light intensity was adjusted to 50 $\mu M \cdot m^{-2} \cdot s^{-1}$. For far-red light, a 25 cm x 21 cm array of 735 nm LEDs was used, the light intensity was also adjusted to 50 $\mu M \cdot m^{-2} \cdot s^{-1}$. During the co-cultivation in liquid medium, the samples were continuously illuminated. Selection by double resistance on agar plates occurred under the same light.

In vivo difference spectra

Phytochrome measurements *in vivo* were performed as described in [27]. The cells were initially cultivated in 10 ml LB with appropriate antibiotics at 26 °C for 16 h. This pre-culture was added to 1 L fresh LB medium and inoculated until the $OD_{600\ nm}$ reached 0.6. After centrifugation at 5000 g for 10 min at 4 °C, the pellet was washed three times with water followed by centrifugation at 10000 g, 5 min, at 4 °C. The cell pellets were stored in -80 °C. Measurement were performed using a Jasco-Photometer V550 with a scan speed of 200 nm/min. Photoconversion was performed by LEDs of 655 nm, 140 $\mu M \cdot cm^{-2} \cdot s^{-1}$ for 4 min or 780 nm, 1000 $\mu M \cdot cm^{-2} \cdot s^{-1}$ for 1 min. Red and far-red irradiations and subsequent spectral scans after each irradiation were repeated three times. Difference spectra were calculated by subtracting spectra after far-red illumination from those after red illumination. The final difference spectrum was always the average from three difference spectra.

Estimation of Pr/Pfr levels

Purified Agp1 and Agp2 holo-protein [39] were incubated overnight in darkness at 26 °C and absorbance spectra were recorded using a Uvikon 931 photometer with a scan speed, 500 nm/min. Subsequently, the samples were incubated under the red or far-red LED arrays for 2 hours. The Agp1 or Agp2 containing cuvettes were immediately placed in the photometer; the time for dark conversion was < 20 sec. After measurements of the absorption spectra, the Pr or Pfr states were calculated under the assumption that in the measurement after darkness, the 100% of Agp1 is in the Pr form and 100% Agp2 is in the Pfr form using the formula $A=A_0 \times f + A_1 \times (1-f)$ (A_0 and A_1 stand for the spectra of Pr or Pfr and A for the measured spectrum, f is the fraction of Pr). For Agp1, A and A_0 are known, for Agp2, A and A_1 are known. To obtain f, the unknown spectra A_1 or A_0 were calculated for different f until the shapes of the spectra were comparable with those of known Pr or Pfr spectra.

Results

Codistribution

Our motivation to investigate phytochrome effects on conjugation was driven by a codistribution analysis. Such a study has been performed earlier on the basis of 138 bacterial species belonging to diverse phylogenetic groups. It has been found that bacterial phytochromes and glutamate synthase or methionine synthase are present in a similar subset of species, i. e. closely codistributed [33]. The reliability of such a study is however lower if the proteins under investigation acquired different functions during evolution or if sequence changes of functionally related partners proceed at different speed. Thus, the predictive power

is expected to be higher if closer related species are chosen for a codistribution analysis. We therefore performed another study that was restricted to 43 rhizobial species. All *A. fabrum* protein sequences were screened for BLAST homologs in a local database generated with these 43 proteomes. Then, the codistributions of each protein with Agp1 and with Agp2 were determined. The best codistribution with Agp2 was found for the conjugal transfer protein TraA (Fig. 1), cellulose synthase, a transcriptional regulator and a chromate transporter protein. The codistribution results for Agp1 were slightly different with TraA and cellulose synthase in the group of proteins with the 3rd best match (out of 202 groups). The close codistribution of Agp1 or Agp2 with TraA might point to functions of the phytochromes in the conjugation process. TraA proteins are large multidomain proteins of about 1000 to 1500 amino acids with helices and relaxase domains [14]. TraA is most likely the relaxase that cleaves the plasmid at the origin of transfer (oriT) site to prepare for conjugal transfer of single stranded DNA. The genome of *A. fabrum* contains three *traA* genes, one located on the linear chromosome, a second one located on the At plasmid and a third one on the Ti plasmid [12,40]. The TraA identified in the present study, Atu5111, is encoded by the At plasmid and has a length of 1542 amino acids. The other two TraA proteins have homologs in more species and appear thus further down in the codistribution table.

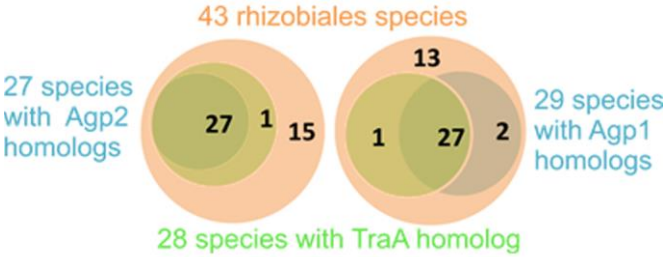


Figure 1. Codistribution of TraA and Agp1 / Agp2 in 43 Rhizobiales, Venn diagram.

Conjugation

The conjugation assays were performed with donor cells without and with Ti-plasmid. This comparison shows in which way the conjugation is dependent on the Ti-plasmid-encoded Tra proteins. Because in our computer study we found a possible link between phytochromes and the At-encoded TraA, we expected that phytochrome effects are easier to be recognized in the absence of the Ti-encoded Tra proteins. In order to generate donor cells, wild type *agp1*$^{-}$, *agp2*$^{-}$ knockout and *agp1/2*$^{-}$double knockout mutants of *A. fabrum* were transformed with the pBIN-GUS or pBIN-GUSINT plasmid, which serve as reporter plasmids [36]. These binary vectors are constructed for plant transformation. They contain the left and right border sequences of the C58 Ti-plasmid but lack the other genes [41]. The right border sequences are identical with the oriT of conjugation. It is therefore expected that both plasmids are mobile in a similar way as the Ti-plasmid. Recipient strains were produced by cloning an Amp resistance cassette into the circular chromosome of the same strains by homologous recombination. The Ti-plasmid was removed from all recipient strains and from those donor strains that carry the pBIN-GUSINT plasmid.

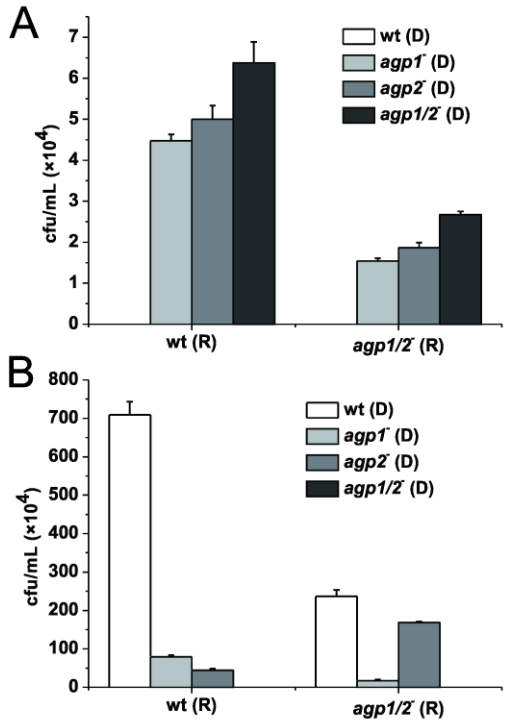


Figure 2. Conjugation in darkness at 26 °C. A) Assay with wild type and phytochrome knockout mutant donor strains without Ti plasmid, cocultivation under shaking. Wild type (left group) or phytochrome double knockout (right group). Note that the values for wild type donor cells were always zero. B) Assay with wild type and phytochrome knockout mutant donor strains containing the Ti plasmid, cocultivation without shaking. Wild type (left group) or phytochrome double knockout (right group). Mean values ± SE of 3 experiments.

Despite our expectations, we observed no conjugation when Ti-plasmid-free wild-type donor cells were used (Fig. 2A). High conjugation rates were however obtained when wild-type donor cells with Ti-plasmid were used (Fig. 2 B). The negative result without Ti-plasmid is thus not due to inappropriate medium or growth conditions. Surprisingly, conjugal transfer occurred in experiments with Ti-plasmid-free donor strains when Agp1 or Agp2 or both phytochromes were knocked out, irrespective of whether wild type or a mutant was used as recipient (Fig. 2A). These results show that in Ti-plasmid-free wild-type donor cells, Agp1 and Agp2 inhibit conjugation and that both proteins are required for this inhibition. With wild-type recipient strains, the number of colonies that grew after cocultivation was higher than with phytochrome-knockout recipient strains (Fig. 2 A). Therefore, Agp1 and Agp2 have a positive effect on conjugation in the recipient cell.

In donor cells with Ti-plasmid, phytochrome knockout resulted in a reduction of conjugation. The high conjugation rates of the wild type donor were reduced to below one tenth in the *agp1*$^-$ or *agp2*$^-$ mutants, and there was no conjugation in the double knockout mutant (Fig. 2 B). The loss of both phytochromes in the recipient strain resulted again in lower conjugation rates when wild-type or *agp1*$^-$ donor was used (Fig. 2 B). The differences between with and without Ti-plasmid (Fig. 2 A and B) must result from the set of Ti-plasmid-encoded Tra proteins. PCR based assays showed that the Ti plasmid migrated always together with the

pBIN-GUS vector because in all randomly selected colonies, always both sequences were detected (Fig. 3).

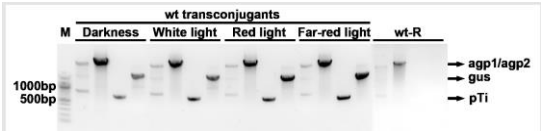


Figure 3. Polymerase chain reaction with primers for *agp1*, *agp2*, Ti plasmid and pBIN-GUS (1st, 2nd, 3rd and 4th lane in each group, respectively), after conjugation under the indicated light conditions and double selection. The wild type without plasmid (wt-R) was used as negative control. These experiments were performed with three replicas for each condition with the same outcome.

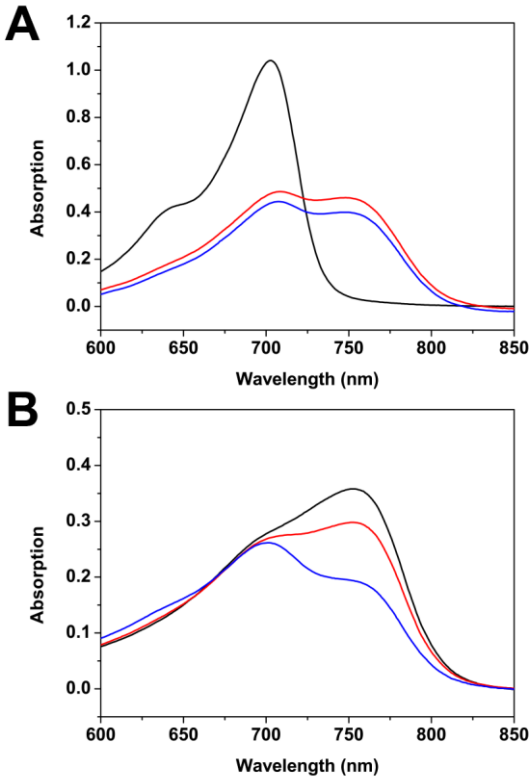


Figure 4. UV/vis absorbance spectra of purified Agp1 (A) and Agp2 (B) in the dark state (black line) after continuous red (red line) or far-red light (blue line). The estimated Pfr contents of Agp1 after red and far-red were 60% and 55%, respectively. For Agp2, the Pfr levels under red and far-red were 80% and 45%, respectively.

Light effect

Phytochromes are most sensitive in the red and far-red region of visible light. In many phytochrome effects of plants, red and far-red act antagonistically. We used red and far-red light from LED sources for irradiation experiments. With recombinant phytochromes placed

under these light sources, the relative Pfr contents were estimated. Under continuous red and far-red irradiation, the relative Pfr contents of Agp1 were 60% and 55%, respectively. This difference is only small, due to the inefficient Pfr to Pr photoconversion of Agp1 [21]. For Agp2 this difference was larger; these values were 80% and 45%, respectively (Fig. 4). In long term irradiation experiments, the magnitudes of phytochrome effects do often not match with the Pr/Pfr ratio because cycling and adaptation or other long term effects can be relevant.

When Ti-plasmid-free wild-type donor cells were used, there was also no conjugation under red or far-red light conditions (Fig. 5 A). With *agp1*$^{-}$ donor cells, red and far-red resulted in a decrease of conjugation. With the *agp2*$^{-}$ donor cells, red resulted in an increase and far-red in a decrease of conjugation (Fig. 5 A). With the double knockout recipient (Fig. 5 B) and the *agp1*$^{-}$ donor there was an inhibition of conjugation by red light but far-red induced no effect. Conjugation of the *agp2*$^{-}$ donor was inhibited by red and by far-red. These data show that red and far-red light have an impact on conjugation but there is no clear correlation with the steady state Pfr levels or Pr/Pfr cycling rates. The comparison of wild type and mutants shows that phytochromes affect conjugation in the donor and recipient in an antagonistic way. Light hits donor and recipient at the same time and the outcome can thus be complex.

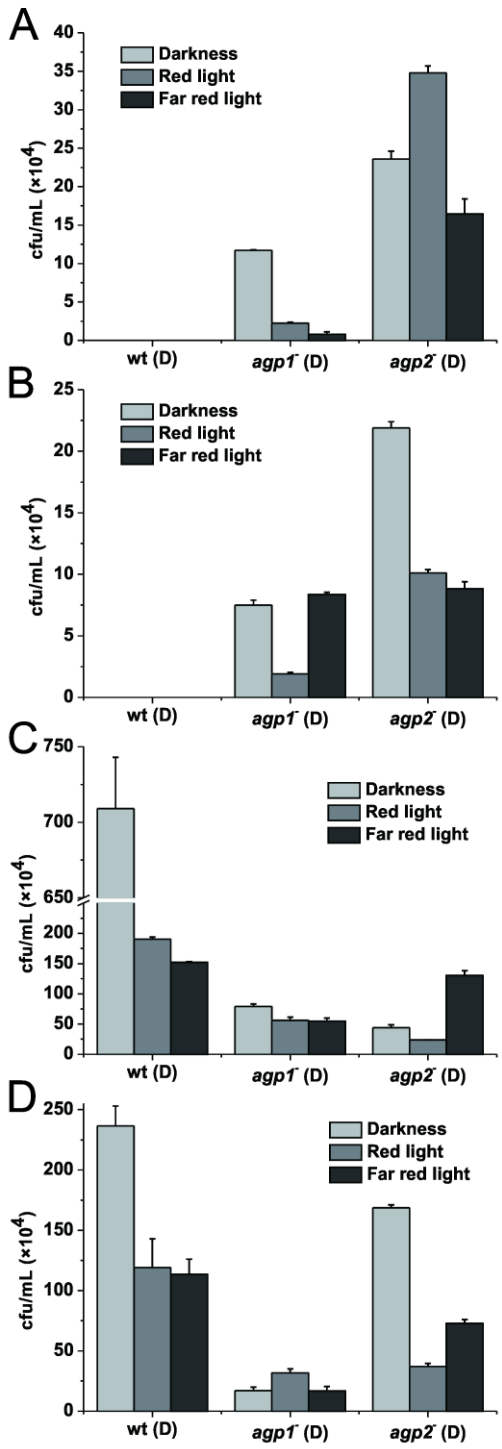


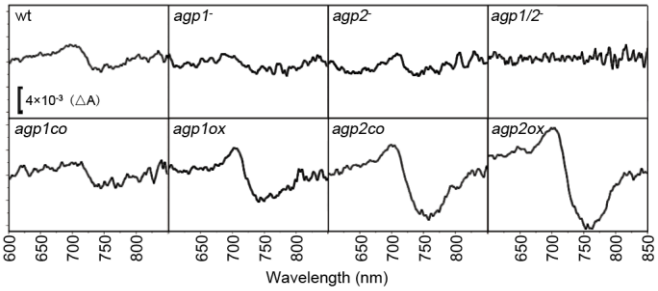
Figure 5. Effects of red and far-red light on conjugation of *A. fabrum*. (A) and (B), donor cells without Ti-plasmid, (C) and (D), donor cells with Ti-plasmid. (A) and (C), wild type recipient; (B) and (D), *agp1*$^{-}$/ *agp2*$^{-}$ recipient.

Clearer light responses were observed when donor cells with Ti-plasmid were used (Fig. 5 C and D). The high level of conjugation with wild-type donor cells (combined with wild type recipient) was reduced by red or far-red to less than a third. There was no such light regulation with the *agp1*$^{-}$ donor and with the double knockout donor, which does not induce conjugation (see above) under all light conditions. When the double knockout recipient was

used, a down regulation of conjugation by light was observed with wild-type donor but not with the *agp1*$^{-}$ knockout donor. The finding of a down regulation by light when donor cells with Ti-plasmid were used is consistent with the effect of a phytochrome knockout in the donor. Histidine kinase activity of Agp1 or Agp2 is down regulated by light *in vitro* [21,22].

Complementation and overexpression

In order to check for possible second site mutation effects, we introduced Agp1 or Agp2 genes under the control of their natural promotors into donor or recipient cells. The wild type in which Agp1 was expressed is termed *agp1ox* (overexpression), and the *agp1*$^{-}$ strain in which Agp1 is expressed is termed *agp1co* (complementation); the Agp2 overexpressors are termed accordingly. Spectroscopic analyses of phytochrome photoreversibility *in vivo* [27] indicated that all strains carrying an expression plasmid had higher mean ΔΔA values than the wild type, due to the presence of several copies of the expression plasmid (Fig. 6).



Strain	wt	*agp1*$^{-}$	*agp2*$^{-}$	*agp1/2*$^{-}$	*agp1co*	*agp1ox*	*agp2co*	*agp2ox*
△△A (10^{-3})	3±1	3±1	3±1	<0.5	5±1	8±3	10±5	15±3

Figure 6. ***In vivo*** **phytochrome difference spectra** (examples) and mean ΔΔA value (difference between ΔA at 700 nm and at 750 nm) of 3 independent measurements ± SE.

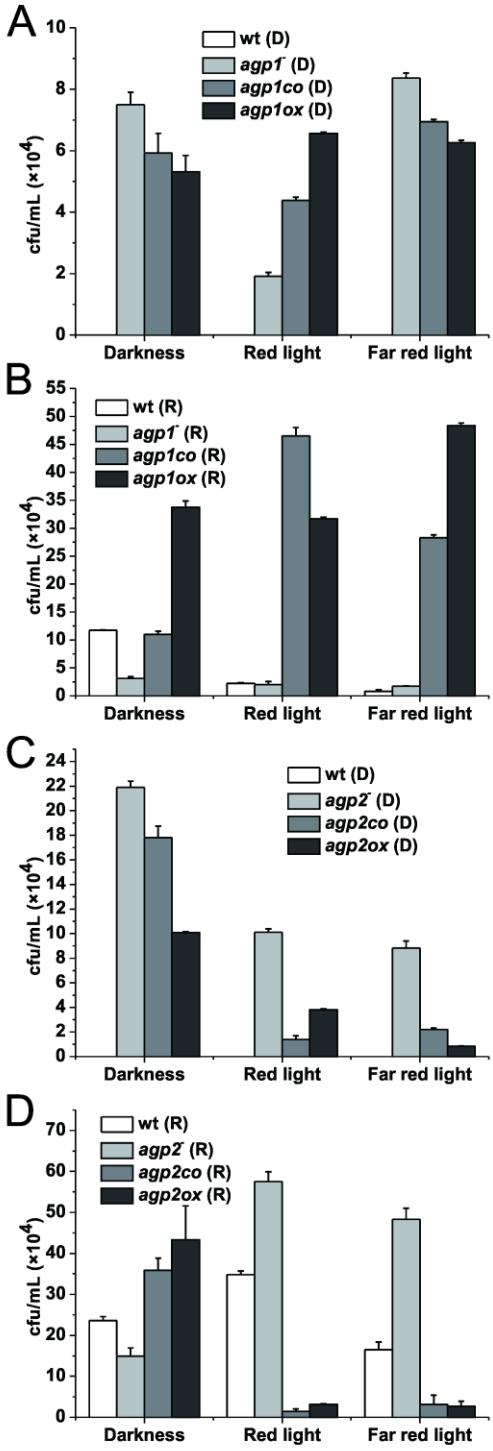


Figure 7. Conjugation of wild type and mutant strains and of the same strains with overexpressed phytochrome. In all cases, donor and recipient cells without Ti plasmid were used. In the legends, "co" and "ox" stand for complementation (mutant with pBBR122-agp1 or -agp2) and overexpression (wild type with pBBR122-agp1 or -agp2), respectively. (A) Variation of Agp1 in donor strain, *agp1/2*$^-$ double knockout recipient. (B) *agp1*$^-$ knockout donor and variation of Agp1 in recipient strains (C) variation of Agp2 in donor strain, *agp1/2*$^-$ double knockout recipient (D) *agp2*$^-$ knockout donor and variation of Agp2 in recipient

strains. Note that in (A) and (B) the values for wild type donor were always zero. Mean values ± SE of 3 experiments.

The conjugation rate of *agp1co* donor was just slightly lower than that of the *agp1*$^-$ donor. With the *agp1ox* donor, conjugation was similar to that of the *agp1co* strain (Fig. 7A). Although the expected result - no conjugation with the *agp1co* - was not obtained, the similar level of both overexpressor strains suggests that the effect is linked to phytochrome. We assume that the pBBR122 based expression of Agp1 leads to derepression of conjugation that overlays the complementation effect. A similar pattern was obtained in a series with *agp2*$^-$ *agp2co* and *agp2ox* donor strains (Fig. 7C).

When overexpression plasmid was introduced into the recipient strain, a typical pattern of complementation was obtained for the series *agp1-agp1co* and *agp1ox* (*agp1*$^-$ donor). The *agp1co* strain yielded the same conjugation rate as the wild type; the overexpressor had higher conjugation rates (Fig. 7B). The pattern with the *agp2-agp2co* and *agp2ox* recipients (*agp2*$^-$ donor) was similar (Fig. 7D).

In experiments with overexpressor strains, light effects were in most cases stronger than in the other experiments. The *agp1* expression plasmid in the recipient cells resulted in a strong induction under far-red light (Fig. 7B). A strong repression under red or far-red was obtained with *agp2* overexpression in donor (Fig. 7C) or recipient (Fig. 7D). In other cases (Fig. 7A), light effects were less pronounced.

Temperature effects

Different histidine kinases including Agp1 have a reverse temperature behavior *in vitro*. In the case of Agp1, the autophosphorylation maximum is at 25 °C, whereas at 37 °C phosphorylation is almost undetectable [42,43]. In order to see whether there could be a link between this effect and conjugation, we varied the temperature during conjugation between 20 °C and 37 °C. These experiments were performed in darkness with donor cells containing a Ti plasmid. The overall conjugation rates had a maximum at 25 °C, these values decreased continuously with increasing temperature. At 37 °C only very low conjugation rates were obtained. This pattern follows the phosphorylation pattern of Agp1 and is therefore consistent with the role of Agp1 as temperature sensitive regulator of conjugation. With *agp1*$^-$ or *agp2*$^-$ donor cells, conjugation rates at 20 °C, 25 °C and 32 °C were reduced as compared to the wild type donor. With the double knockout donor, no conjugation was obtained at any temperature.

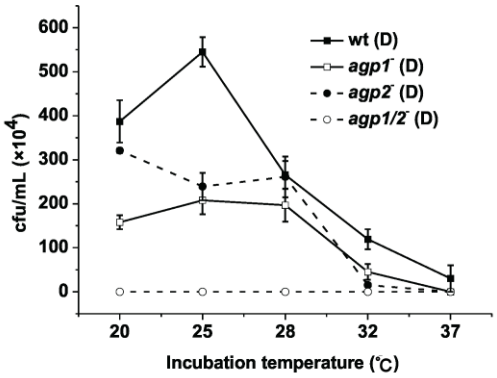


Figure 8 Conjugation at different temperature

Discussion

The present experiments show that phytochromes and red / far-red light play an important role in the conjugation of *Agrobacterium fabrum*. Although complementation of knockout mutants by phytochrome bearing pBBR122 plasmids did not always lead to the expected wild type like phenotypes, we propose that the differences between mutants and wild type are always based on the differences in phytochrome levels and not on second site effects: (i) transformation of mutant and wild type with phytochrome gene bearing pBBR122 plasmids resulted in similar phenotypes. In both cases the phytochrome levels were above wild type. We assume that the inhibition of conjugation in wild type donor cells lacking Ti plasmids is based on balanced Agp1 and Agp2 levels. Misbalanced levels, i. e. too little or too much of either phytochrome, results in derepression of conjugation in Ti-plasmid free donor cells. (ii) Red or far-red light had a different impact on wild type, knockout mutants and overexpression strains, suggesting that phytochromes are the photoreceptors of these light effects.

Three outcomes provide insight into molecular action of phytochromes in the conjugation process: (i) There is a major difference of the light and mutant effects on conjugation between donor cells with and donor cells without Ti-plasmid. These differences are most likely due to the Ti-plasmid encoded Tra proteins. (ii) Both phytochromes are involved in the regulation of conjugation, the phenotypes of *agp1*$^{-}$ and *agp2*$^{-}$ knockouts are similar, and their roles are not redundant. There are many examples for coaction of phytochromes in plants [44] and different types of plant phytochromes can form heterodimers [45]. However, phytochrome coaction is so far unknown for bacteria. Although the evolutionary distance between Agp1 and Agp2 is much larger than e.g. between different types of plant phytochromes [31], we propose that the coaction of Agp1 and Agp2 has evolved specifically in *A. fabrum*. (iii) Conjugation is regulated in the donor as well as in the recipient cells by light and phytochromes.

The protein identified by the codistribution assay, TraA, is encoded by one gene on the chromosome, another gene on the At plasmid and a third gene on the Ti plasmid. According to domain homology, TraA could function as a relaxase and perform the first step during conjugation [14]. A possible scenario for the phytochrome effects could be that the Ti-encoded TraA induces conjugation of pBIN vectors in donor cells more efficiently than both other TraA proteins and that the three TraA proteins are differently regulated through light

and phytochromes. The two TraA proteins encoded by the chromosome and At plasmid could be inhibited by Agp1 and Agp2, whereas the Ti-plasmid encoded TraA could be stimulated by both phytochromes. It is possible that these regulations occur by direct interaction. An interaction of Agp2 with yet unknown components in the cell extract has been monitored by spectral modifications [38,46]. Another option, the transcriptional regulation of conjugation factors via light and phytochromes is less likely. Microarray studies performed with wild type and double knockout in the light and in darkness [47] gave no evidence for such an effect. Light and phytochrome effects on conjugation in the recipient could be related to the docking success or to the proper procession or integration of the delivered DNA. We would like to emphasize that with wild type donor cells without Ti plasmid or with double knockout donor cells with Ti plasmid conjugation was completely lost, whereas modifications of the recipient cells never led to a complete loss of conjugation.

The temperature effect on conjugation is consistent with a role of Agp1 as temperature sensor: the temperature pattern of Agp1 autophosphorylation *in vitro* [42,43] is comparable with that of the conjugation. In the *agp1*$^{-}$ donor, Agp2 could be responsible for temperature dependence or this effect is regulated in the recipient. However, many other factors that are involved in conjugation could as well be the cause for such a temperature behavior. The present experiments must be continued in order to clarify the role of phytochromes as temperature sensors.

What could be the evolutionary advantage of light control of conjugation? As a soil bacterium, *A. fabrum* is not necessarily exposed to the sunlight, but these bacteria can live on top of the soil or on the surface of plant stems or leaves [48]. *A. fabrum* has two photolyases [49,50] and enzymes for nucleotide excision repair (UvrABC) [51]. The presence of these proteins that repair UV damaged DNA indicates that *A. fabrum* has evolved to survive in the light. The photolyase PhrB of *A. fabrum* repairs (6-4) TT lesions more efficiently in double stranded than in single stranded DNA [49]. Nucleotide excision repair is not possible in single stranded DNA because an undamaged opposite strand is required for the insertion of correct nucleotides. We therefore propose that the single stranded DNA that is transferred to the recipient strain during conjugation is more sensitive against UV-light as compared to double stranded DNA. The down regulation of conjugation by light (e. g. in donor cells with Ti plasmid) could thus be a protective mechanism against UV induced mutagenesis.

The distribution of TraA proteins and of phytochromes in Rhizobiales suggests that in other species of this group conjugation is also modulated by phytochrome. Because of the high UV doses in the pre-oxygen era it seems plausible that such a light regulation has evolved before the evolution of oxygenic photosynthesis, in line with the proposed early evolution of phytochromes [31].

Because mechanisms of conjugation are well understood at the molecular level, it seems to be possible to find phytochrome interaction partners and investigate agrobacterial phytochrome signal transduction at the molecular level in the near future. It should be noted that cognate response regulators that are transphosphorylated by phytochrome histidine kinases do not contain a DNA binding motif [52], a property that distinguishes them e. g. from the VirG response regulator, a transcription factor for virulence genes in *A. fabrum*. This points to a mode of signal transduction that is not based on transcriptional regulation. Our results also show that the codistribution assay can help finding functions of proteins that have otherwise not been suggested.

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