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A *Phytophthora sojae* G-Protein α Subunit Is Involved in Chemotaxis to Soybean Isoflavones▽†

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For the soybean pathogen *Phytophthora sojae*, chemotaxis of zoospores to isoflavones is believed to be critical for recognition of the host and for initiating infection. However, the molecular mechanisms underlying this chemotaxis are largely unknown. To investigate the role of G-protein and calcium signaling in chemotaxis, we analyzed the expression of several genes known to be involved in these pathways and selected one that was specifically expressed in sporangia and zoospores but not in mycelium. This gene, named *PsGPA1*, is a single-copy gene in *P. sojae* and encodes a G-protein α subunit that shares 96% identity in amino acid sequence with that of *Phytophthora infestans*. To elucidate the function, expression of *PsGPA1* was silenced by introducing antisense constructs into *P. sojae*. *PsGPA1* silencing did not disturb hyphal growth or sporulation but severely affected zoospore behavior, including chemotaxis to the soybean isoflavone daidzein. Zoospore encystment and cyst germination were also altered, resulting in the inability of the *PsGPA1*-silenced mutants to infect soybean. In addition, the expressions of a calmodulin gene, *PsCAM1*, and two calcium- and calmodulin-dependent protein kinase genes, *PsCMK3* and *PsCMK4*, were increased in the mutant zoospores, suggesting that *PsGPA1* negatively regulates the calcium signaling pathways that are likely involved in zoospore chemotaxis.

The ability to recognize host signals may be critical for the behavior of plant pathogens or symbionts. Chemotaxis to a host-specific signal has been described for several plant-microbe associations (11, 43, 44). In plant-pathogenic *Agrobacterium* species, for example, chemotaxis to plant exudates appears to enhance virulence on soil-grown plants (24). In nitrogen-fixing bacteria, such as *Rhizobium* and *Bradyrhizobium* species, expression of nodulation genes is induced by flavones and isoflavones (19), which are secondary metabolites present in seeds of leguminous plants and are exuded by the roots of those plants. Chemotaxis of zoospores of the oomycete pathogen *Phytophthora sojae* to isoflavones is believed to be an important step in the disease cycle, particularly in recognition of the host and in initiating infection (42, 43). *P. sojae* is a devastating pathogen on soybean that causes "damping off" of seedlings and root rot on older plants. The annual loss worldwide is estimated to be $1 billion to $2 billion (49).

P. sojae zoospores are specifically attracted to the isoflavones daidzein and genistein at concentrations of as low as 10 nM (43). Since zoospores of other *Phytophthora* spp. are not attracted to these compounds, chemotaxis may be part of the mechanism that determines host range (43). Besides motile zoospores, hyphal germ tubes of *P. sojae* also respond chemotropically to soybean isoflavones (42, 51). Although there are indications that calcium influx plays a role in chemotaxis (12), the signal transduction pathways governing the response of *P. sojae* to isoflavones are largely unknown. The first step in unraveling these pathways is identifying genes that control signal transduction. For this study we selected a set of candidate genes with putative roles in chemotaxis and calcium signaling and analyzed their expression in mycelium and zoospores. The candidates included genes encoding proteins with homology to calmodulin, calmodulin- and calcium-dependent protein kinases, ATPase, phospholipases, transcription factors, and the α and β subunits of the heterotrimeric G protein.

Signal transduction pathways involving heterotrimeric G proteins are probably the most ubiquitous and best studied among eukaryotes. The G-protein complex, which is composed of α, β, and γ subunits, is activated by a membrane-bound receptor that senses extracellular ligands. Upon activation, the complex dissociates into the α subunit and the βγ dimer, which can independently modulate downstream targets such as ion channels, adenylyl cyclase, phospholipases, and mitogen-activated protein kinases. G-protein activation eventually leads to changes in gene expression, which allows the cells to adequately respond to extracellular signals (37, 39). In the slime mold *Dictyostelium discoideum*, heterotrimeric G-protein signaling appears to play a critical role in chemotaxis (55). Knockout mutants lacking either an α subunit (*gpaB*) or a β subunit (*gpbA*) lost the ability to aggregate and respond to chemoattractants. Also, the first reports on G-protein signaling in *Phytophthora* pointed to a role in chemotaxis. Phenotypic analysis of *Phytophthora infestans* mutants obtained by silencing the G-protein α (Gα) subunit gene *Pigpa1* demonstrated that zoospores of PiGPA1-deficient mutants had lost the ability to autoaggregate and were no longer attracted by the amino acid glutamic acid (33).

Also, G-protein-independent responses contribute to chemotaxis or zoospore behavior. In *D. discoideum*, for example,

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pharmacological intervention using the competitive calmodulin inhibitors and antagonists trifluoperazine and calmidazolium (R24571) demonstrated that calmodulin is required for both cyclic AMP and folic acid chemotaxis (21). In *P. sojae*, soybean isoflavones can trigger a calcium influx, indicating that calcium-mediated signal transduction is involved in zoospore chemotaxis (12). In *P. infestans*, the calcium channel blocker verapamil and trifluoperazine inhibited zoosporogenesis and encystment, whereas the protein kinase inhibitors K-252a and KN-93 inhibited zoospore release, encystment, and cyst germination. K-252a also reduced zoospore viability (27). Moreover, calcium- and calmodulin-regulated protein kinases were found to be induced during zoosporogenesis in *P. infestans* (27), and a bZIP transcription factor interacting with calcium- and calmodulin-regulated protein kinases was proven to be required for zoospore motility and plant infection (7). Phosphatidic acid, a second messenger that is produced upon hydrolysis of structural phospholipids by phospholipase D (PLD), induces zoospore encystment, and this suggests involvement of PLD in this process (34).

Of the *P. sojae* candidate genes that we tested, only the one encoding the α subunit of the heterotrimeric G protein showed a strong upregulation in zoospores compared to mycelium. Hence, we selected this gene for further analysis. The full-length copy was cloned, and transgenic gene silencing in *P. sojae* was exploited to obtain Gα subunit-deficient mutants. Subsequently, the role of the Gα subunit in chemotaxis to isoflavone daidzein and in virulence on soybean was evaluated, and the expression of putative downstream target genes was analyzed.

MATERIALS AND METHODS

***P. sojae* culture conditions.** *P. sojae* strain P6497, provided by Brett Tyler (Virginia Bioinformatics Institute, Blacksburg, VA), was maintained on V8 juice agar at 25°C in the dark. To obtain axenically prepared mycelium, hyphal tip plugs of P6497 were used to inoculate 30 ml of sterile clarified 10% V8 broth in 90-mm petri dishes. Stationary mycelial cultures were incubated at 25°C in the dark for 3 days. Sporulating hyphae was prepared by repeatedly washing 3-day-old hyphae incubated in 10% V8 broth with sterile distilled water (SDW) and incubating the washed hyphae in the dark at 25°C for 4 to 8 h until sporangia developed on most of the hyphae. Zoospores were filtered with Miracloth (Calbiochem) and collected by centrifugation at 2,000 × *g* for 2 min. Cysts were obtained by vortexing a zoospore suspension for 30 s and then centrifuging at 2,000 × *g*. Cysts were germinated in clarified 5% V8 broth (18) for 2 h. Leaf inoculation was performed as described by Chen et al. (10). Mycelium cultured for 3 days in 10% V8 juice, in Plich medium, or in 4 mM H_2O_2 for 4 h after being cultured for 3 days in 10% V8 juice was vacuum decanted onto filter paper and collected. All of the collected samples were immediately frozen in liquid N_2, lyophilized, and stored at −80°C until being used for RNA isolation.

DNA and RNA extractions. Genomic DNA of *P. sojae* was isolated from mycelium grown in 10% V8 liquid medium by a yeast DNA extraction protocol (3). Total RNA was isolated using the NucleoSpin RNA II RNA extraction kit (Macherey-Nagel) following the procedures described by the manufacturer.

RT-PCR and gene cloning. To remove contaminating genomic DNA from RNA preparations, 10 μg of total RNA was treated with 4 units of RNase-free DNase I (Takara) at 37°C for 1 h. The removal of DNA was verified in a PCR under the same conditions as those used for the reverse transcription-PCR (RT-PCR), except that the 30-min cDNA synthesis step at 37°C was omitted. First-strand cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase (RNase H Minus) and oligo(dT)15 primer (Promega). PCRs were performed with the following programs: for actin A (*ActA*) and *PsCAM1*, 94°C for 1 min followed by 24 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 10 min; for all other genes, 94°C for 1 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 10 min. The primers used in the reactions are listed in Table S2 in the supplemental material. All RT-PCRs were performed at least three times.

Genomic DNA of mycelia and cDNA of zoospores from the *P. sojae* strain P6497 were used as templates in PCRs. The primer sequences of the Gα subunit gene were 5′-ATGGGACTCTGTGCGTCCC-3′ and 5′-CTACATGAAGCCG GAGCCC-3′. The PCR was performed with 30 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C. The PCR products were cloned in pMD18-T vectors and sequenced. Sequence analysis and alignments were performed using the software Bioedit 7.0.

Transformation of *P. sojae*. Antisense *PsGPA1* was amplified with PrimeStar polymerase (Takara) and ligated into vector pHam34 digested with SmaI. This resulted in plasmid pGA1. Stable transformation was performed by the method of McLeod et al. (41). For cotransformation, 25 μg pGA1 DNA and 5 to 10 μg pTH209 DNA were mixed with *P. sojae* protoplasts in 1 ml. For the control transformations, 10 μg pTH209 DNA was mixed with 2×10^6 *P. sojae* protoplasts in 1 ml. The protoplasts with the DNA mixture were kept on ice for 5 min, after which 1.74 ml of 40% polyethylene glycol 4000 in 20 mM $CaCl_2$ and 10 mM Tris-HCl (pH 7.5) was added very slowly. The suspension was gently mixed and placed on ice. Twenty minutes later, 10 ml of pea broth liquid medium containing 0.8 M mannitol was added, and then this mixture was poured into a petri dish which contained 10 ml pea broth liquid medium with 0.8 M mannitol and 50 μg/ml ampicillin. After incubation for 20 to 24 h at 22°C, the mixture, now containing regenerated protoplasts, was gently centrifuged. The supernatant was discarded, and the regenerated protoplast pellets were mixed with 10 ml pea broth agar containing 0.8 M mannitol and 30 μg/ml Geneticin. Transformants appeared within 6 to 12 days at 25°C in the dark and were propagated in pea broth medium containing 30 μg/ml Geneticin. Genomic DNA PCR screening of all of the putative transformants was performed with primers P1(5′-TTCTCCT TTTCACTCTCACG-3′), from the promoter region of the *HAM34* gene, and P2 (5′-ATGGGACTCTGTGCGTCCC-3′), from the *PsGPA1* gene. Zoospores of the genomic DNA PCR-positive transformants were screened for *PsGPA1* silencing by RT-PCR.

Analysis of zoospore behavior. To analyze zoospore release, hyphae of *P. sojae* cultured on V8 juice agar at 25°C for 7 days were soaked with SDW overnight. The following morning, the hyphae were rinsed twice with an equal volume of SDW every 30 min for 4 h. The plates were then transferred to 16°C for 4 h to allow zoospores to release. Subsequently, the number of zoospores of 50 μl SDW was counted under the microscope.

To analyze zoospore encystment, equal volumes (50 μl) of zoospore suspension were pipetted onto a glass plate and incubated at 25°C with 80% humidity. After 2 h, the number of encysted zoospores was counted.

Chemotaxis assay. Chemotaxis assays were performed in an assay chamber that was created by supporting a coverslip with two glass pieces on a glass slide. The weight of the coverslip was sufficient to hold a 250-μl drop of zoospores (10^4/ml) in place, and 0.5 μl agarose containing 30 μM of the isoflavone daidzein or 25 μM glutamic acid was put into the zoospore suspension. After 5 min and 15 min at 25°C, photographs were taken to visualize zoospores and germinating cysts surrounding the agarose. Each strain was tested with at least two different preparations of zoospores. This assay was repeated at least four times.

Analysis of cyst germination. Tubes containing 200 μl zoospore suspension were vortexed to induce encystment and then incubated in 5% V8 liquid medium for 90 min at 25°C. Germination was assessed by vigorously shaking the tubes and transferring drops of the cyst suspension to glass slides for microscopy. At least 100 cysts were examined for each treatment, and all treatments were replicated in three tubes. Cysts were scored as having germinated if the germ tube length equaled or exceeded the cyst diameter (10 μm).

Virulence assays. Detached soybean leaves of HeFeng35, a cultivar that is compatible with *P. sojae* strain P6497, were placed in petri dishes. Each leaflet was inoculated on the abaxial side with a 10-μl droplet of a zoospore suspension containing 100 zoospores or with a 5-mm hyphal plug. The leaves were incubated in a climate room at 25°C under 80% humidity with 16 h of light per 24 h. Pictures of the lesions were taken at 3 and 7 days postinoculation (dpi).

Leaves inoculated for 2, 4, or 12 h were soaked in 0.5% Coomassie brilliant blue for 2 min, destained with alcohol, and washed with SDW three to five times. The infected leaves were examined under the microscope.

Soybean sprouts were immersed in zoospore suspension for 30 min, the epidermis of the sprouts was cut off, and encysted zoospores were observed under the microscope. Each strain was tested with at least two different preparations of zoospores. This assay was repeated at least four times.

Nucleotide sequence accession numbers. The DNA sequence of *PsGPA1* has been submitted to NCBI under accession numbers EU652939 and EU652940.

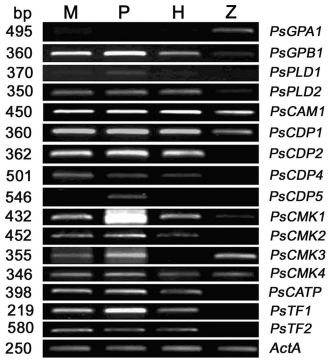

FIG. 1. Expression profiling of the candidate genes. Sixteen genes from zoospores (Z) or mycelia cultured in 10% V8 liquid medium (M), Plich medium (P), and H_2O_2 (H) were analyzed by semiquantitative RT-PCR. RT-PCR products were visualized on ethidium bromide-stained gels, and their sizes are indicated on the left. *PsGPA1*, *P. sojae* Gα subunit 1; *PsGPB1*, *P. sojae* Gβ subunit 1; *PsPLD1*, *P. sojae* PLD1; *PsPLD2*, *P. sojae* PLD2; *PsCAM1*, *P. sojae* calmodulin 1; *PsCDP1*, *P. sojae* Ca^{2+}-dependent protein 1; *PsCDP2*, *P. sojae* Ca^{2+}-dependent protein 2; *PsCDP4*, *P. sojae* Ca^{2+}-dependent protein 4; *PsCDP5*, *P. sojae* Ca^{2+}-dependent protein 5; *PsCMK1*, *P. sojae* Ca^{2+}/calmodulin-dependent protein kinase 1; *PsCMK2*, *P. sojae* Ca^{2+}/calmodulin-dependent protein kinase 2; *PsCMK3*, *P. sojae* Ca^{2+}/calmodulin-dependent protein kinase 3; *PsCMK4*, *P. sojae* Ca^{2+}/calmodulin-dependent protein kinase 4; *PsCATP*, *P. sojae* Ca^{2+} ATPase; *PsTF1*, *P. sojae* transcription factor 1; *PsTF2*, *P. sojae* transcription factor 2; *ActA*, actin A.

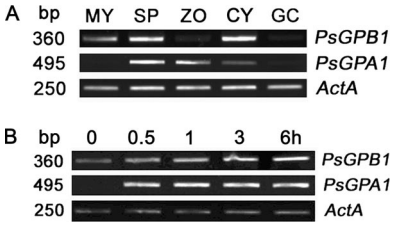

FIG. 2. Expression analyses of *PsGPA1* and *PsGPB1*. (A) Expression during asexual development in vegetative hyphae (MY), sporulating hyphae (SP), zoospores (ZO), cysts (CY), and germinating cysts (GC). (B) Expression during infection of soybean leaves. RT-PCR products were visualized on ethidium bromide-stained gels, and their sizes are indicated on the left.

RESULTS

Gene expression in zoospores and mycelium. The expression of 16 genes (see Table S1 in the supplemental material) potentially involved in G-protein- and calcium-mediated signaling was analyzed in *P. sojae* zoospores and mycelium cultured in different media (10% V8 or Plich medium) and in the presence of H_2O_2 (4 mM in water) (Fig. 1). The treatment with H_2O_2 was meant to mimic the oxidative stress that *P. sojae* may encounter when infecting its host (56). The candidate genes encode Gα and Gβ subunits (*PsGPA1* and *PsGPB1*, respectively), two PLD-like proteins (*PsPLD*), calmodulin (*PsCAM1*), four calcium-dependent proteins (*PsCDP*), four calcium- or calmodulin-dependent protein kinases (*PsCMK*), a Ca^{2+} ATPase (*PsCATP*), and two zinc finger proteins that may function as transcription factors (*PsTF*). *PsTF1* and *PsTF2* are homologs of transcription factor CRZ1, which is the target of the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin in *Saccharomyces cerevisiae* (40). Other CRZ1 homologs, such as *Botrytis cinerea* CRZ1 and *Aspergillus fumigatus* CrzA, have been shown to be involved in fungal pathogenesis (13, 45). As shown in Fig. 1, semiquantitative RT-PCR revealed that with the exception of *PsGPA1*, all genes were expressed in mycelium. Expression of *PsPLD1* and *PsCDP5* was undetectable in mycelium cultured in 10% V8 medium, but both genes were expressed in mycelium cultured in Plich medium. Expression of *PsTF1*, *PsCMK1*, *PsCMK3*, *PsPLD1*, *PsGPB1*, and *PsCDP5* was also higher in Plich medium than in V8. Oxidative stress did not seem to affect the 15 tested genes, except *PsCMK3*, whose expression was completely repressed by H_2O_2. In zoospores, expression of only half of the candidate genes, including *PsGPA1*, *PsGPB1*, *PsCAM1*, *PsCDP1*, *PsCMK1*, *PsCMK3*, and *PsCMK4*, was detectable, and the ones that were expressed often had lower expression levels in zoospores than in mycelium. Interestingly, *PsGPA1* was the only candidate gene that was expressed in zoospores but not in mycelium.

***PsGPA1* is a single-copy gene and is highly conserved.** In the *P. sojae* genome sequence (50), there is one copy of a gene that is homologous to Gα subunit genes in other organisms. Consistent with the JGI *P. sojae* database (http://genome.jgi-psf.org/Physo1_1/Physo1_1.home.html; protein ID 108814), the gene was named *PsGPA1*, and full-length cDNA was obtained by RT-PCR amplification (see Table S1 in the supplemental material). Genomic Southern blot hybridization confirmed that *PsGPA1* is a single-copy gene in *P. sojae* (data not shown); however, Gα subunit genes are multicopy in the fungi, such as *Ustilago maydis* and *Aspergillus nidulans* (see Fig. S2 in the supplemental material). *PsGPA1* and *Pigpa1*, the Gα subunit gene in *P. infestans* (35), share 90% identity at the DNA level, and the encoded proteins are 96% identical (see Fig. S1 in the supplemental material). Comparison of cDNA and genome sequences revealed that *PsGPA1* contains an intron between positions 172 and 253. The intron is somewhat shorter than that in *Pigpa1*. The position of the intron in *PsGPA1* and *Pigpa1* is conserved but differs from the intron position in Gα subunit genes in other organisms, such as *Arabidopsis thaliana* (38). The first six and the last three nucleotides of the intron are similar to the consensus sequences for 5′ and 3′ intron splice sites of oomycete genes (GTRNGT and YAG, respectively) (see Fig. S3 in the supplemental material).

***PsGPA1* is differentially expressed during the *P. sojae* life cycle.** To determine the expression pattern of *PsGPA1* in distinct developmental stages and during the infection stage, mRNA accumulation was analyzed by RT-PCR and compared with that of the Gβ subunit gene *PsGPB1*. The highest levels of expression were found in sporulating hyphae and zoospores. *P. sojae* cysts also contained *PsGPA1* mRNA, but, as described above, no *PsGPA1* mRNA could be detected in mycelium. *PsGPB1* mRNA was found in all developmental stages, but in zoospores and germinating cysts the levels were very low (Fig. 2A). In infected soybean leaves, mRNAs of both *PsGPA1* and *PsGPB1* were detectable in different stages and up to 6 h postinoculation, with no changes found in levels of expression (Fig. 2B). These results show that *PsGPA1* and *PsGPB1* have different expression patterns in mycelia and zoospores (Fig. 2A), though both are expressed in sporangia, cysts, and during infection (Fig. 2A and B).

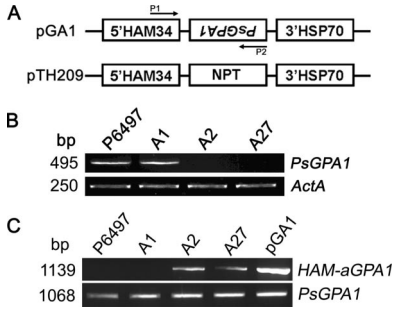

FIG. 3. Transgene silencing of *PsGPA1* in *P. sojae.* (A) Plasmids used for transformation of *P. sojae.* The maps are presented in linearized form, and the pBluescript backbone is not shown. The plasmid DNA that was used for transformation was circular. Plasmid pGA1 contains the coding region of *PsGPA1* in antisense orientation and fused to the constitutive *HAM34* promoter and the *HSP70* terminator. Plasmid pTH209 contains the neomycin phosphotransferase gene (NPT) for selecting transformants. P1 and P2 indicate the primers from the *HAM34* promoter region and *PsGPA1*, respectively. (B) Expression of *PsGPA1* in zoospores of the wild-type strain P6497, the control transformant A1, and the *PsGPA1*-silenced mutants A2 and A27. RT-PCR products were visualized on ethidium bromide stained gels, and their sizes are indicated on the left. (C) Genomic PCR analysis of plasmid integrations in *PsGPA1*-silenced mutants. Primers P1 and P2 were used to specifically amplify the plasmid sequences integrated in the genomes of strains P6497, A1, A2, and A27. Plasmid pGA1 was used as a positive control. The PCR products from *HamaGPA1* were 1,139 bp with the full-length gpa1 primer (see Table S2 in the supplemental material) and were also used to confirm the quality of genomic DNA.

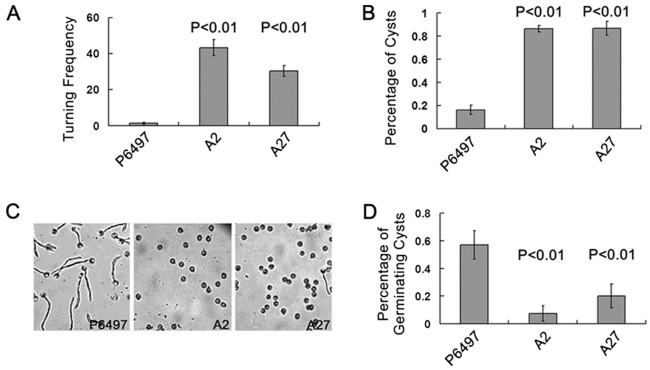

FIG. 4. Zoospore behavior and cyst germination of wild-type *P. sojae* (P6497) and Gα-silenced mutants (A2 and A27). (A) Turning frequencies of zoospores. Individual zoospores were monitored for 1 min and the number of turns counted. The means and standard deviations for multiple zoospores from four different zoospore isolations are presented. (B) Zoospore encystment rates. Swimming zoospores were incubated on a glass plate at 25°C for 2 h. Numbers of swimming zoospores and encysted zoospores were countered under the microscope, and the ratio of the number of encysted zoospores to the total number of zoospores (swimming and encysted) was calculated. (C) Zoospores were encysted by vortexing for 30 s, and cysts were incubated in 5% V8 liquid medium for 2 h at 25°C. Pictures were taken under the microscope. (D) Cyst germination rates. Zoospores were encysted by vortexing for 30 s, and cysts were incubated in 5% V8 liquid medium for 2 h at 25°C. Numbers of germinated cysts and ungerminated cysts were countered under the microscope, and the ratio of the number of germinated cysts to the total number of cysts (germinated and ungerminated) was calculated.

Silencing of the *PsGPA1* gene. To obtain mutants that lack the Gα subunit, we used a gene silencing approach. *P. sojae* strain P6497 was transformed with pGA1, a construct containing the antisense *PsGPA1* coding region, under the control of the *HAM34* promoter and terminator (Fig. 3A). pTH209, a plasmid carrying the Geneticin resistance gene *NPT*, was used as a selection marker (28). To select *P. sojae* transformants in which expression of the *PsGPA1* gene is silenced, we first screened for pGA1-integrated transformants from 82 putative clones which were Geneticin resistant by genomic PCR with primers P1 and P2. Initially, 62 pGA1-integrated transformants were obtained (data not shown), and these were then used to evaluate the levels of *PsGPA1* mRNA accumulation by RT-PCR. Only two transformants (which we named A2 and A27) failed to give amplicons when the normal number of PCR cycles was applied with the zoospore RNA as initial template (Fig. 3B). Genomic PCR analysis also showed that pGA1 was introduced in A2 and A27 (Fig. 3C); however, A1, a control transformant with plasmid pTH209 but not pGA1, had accumulated the normal levels of *PsGPA1* mRNA (Fig. 3B) and the integrated pGA1 was absent (Fig. 3C). These results show that transformants A2 and A27 lack *PsGPA1* mRNA, most likely due to silencing of the *PsGPA1* gene.

***PsGPA1*-silenced transformants show aberrant zoospore behavior.** Overall, the colony morphology of the *PsGPA1*-silenced mutants A2 and A27 was similar to that of the wild-type recipient strain P6497 and the control transformant A1. The hyphae seemed to be a bit more compact, but this was hardly significant (data not shown). Since sporulation was not impaired and the sporangia had a normal morphology, it seemed that vegetative development can proceed in the absence of the Gα subunit. Moreover, oospores were formed normally, and this implied that sexual development was not disturbed in the *PsGPA1*-silenced mutants. However, when examining the behavior of the zoospores, we observed aberrant phenotypes. In suspension, zoospores from A2 and A27 turned much more frequently than zoospores from the wild-type strain P6497. The latter changed direction only when they encountered obstacles and thus had low turning frequencies. In contrast, A2 and A27 zoospores changed their swimming direction at least 30 times per minute (Fig. 4A). Another difference was the time span between zoospore release and encystment. Wild-type zoospores continued swimming for at least a few hours, but the majority of the zoospores from the two *PsGPA1*-silenced mutants was encysted within 1 hour after release from the sporangia (Fig. 4B).

Once zoospores are encysted and if conditions are optimal, they start to germinate. However, the germination rate of the *PsGPA1*-silenced mutants was drastically reduced. Zoospore suspensions of the two *PsGPA1*-silenced mutants and the wild-type strain P6497 were vortexed to stimulate rapid encystment. The cysts were then incubated at 25°C. After 2 h, more than 50% of the cysts obtained from the wild-type strain had germinated, but only 10% of the cysts of mutant strains A2 and A27 had germ tubes (Fig. 4C and D).

***PsGPA1* silencing changes zoospore chemotaxis.** Prior to infection, zoospores of *P. sojae* swim chemotactically toward soybean roots, and upon touching the root surface they encyst. The zoospores are attracted by isoflavones that are released by soybean roots. We tested chemotaxis to the isoflavone daidzein

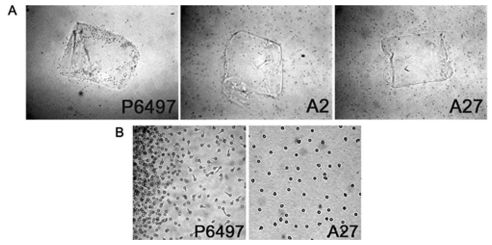

FIG. 5. Zoospore chemotaxis of wild-type *P. sojae* (P6497) and the *PsGPA1*-silenced mutants (A2 and A27) to the isoflavone daidzein (30 μM). (A) Wild-type zoospores swim toward the agarose containing daidzein and encyst in 5 min; in contrast, zoospores of *PsGPA1*-silenced mutants show reduced chemotaxis to daidzein. The agarose containing daidzein is in the center of each panel. (B) In the vicinity of the agarose containing daidzein, cysts of the wild-type strain germinate within 15 min, but cysts of the *PsGPA1*-silenced mutants do not germinate.

and observed that zoospores of strain P6497 were attracted to agarose plugs containing daidzein at concentrations of as low as 100 nM. At concentrations of 15 to 30 μM, the zoospores swam rapidly toward the attractant and encysted within 5 min. Germ tubes emerged from the cysts within 10 min (see Video S1 in the supplemental material). In contrast, zoospores of the *PsGPA1*-silenced mutants failed to respond to the presence of low concentrations of daidzein, and their chemotactic response to 30 μM daidzein was clearly less than that of the wild-type zoospores (Fig. 5A). Cysts of strain P6497 that were near 30 μM daidzein germinated very fast, but the *PsGPA1*-silenced mutants failed to germinate (Fig. 5B). In the vicinity of the agarose, though, they began to swim faster and their turning frequency was reduced, but they did not encyst. Only after 30 min was some encystment observed. The majority of the zoospores continued swimming for up to 6 h or longer. In the presence of daidzein, the turning frequency and life span of mutant zoospores resembled those of wild-type zoospores in the absence of a chemotactic compound (see Video S2 in the supplemental material). We also observed a clear difference in the zoospore response to glutamic acid between the wild-type and mutant strains. The wild-type zoospores swam rapidly toward 25 μM glutamic acid, and the response was similar to that to 30 μM daidzein; however, the attractant had no effect on the zoospores of the silenced mutants (data not show).

Zoospores of *PsGPA1*-silenced mutants have lost the potential to infect soybean. Hefeng35 is a cultivar of soybean (*Glycine max*) that is susceptible to *P. sojae* strain P6497. At 3 dpi with zoospores of P6497, the leaves showed the typical disease symptoms, and at 7 dpi the water-soaked lesions had spread all over the leaf (Fig. 6A). In contrast, when zoospores of the *PsGPA1*-silenced mutants were inoculated on Hefeng35 leaves, no disease symptoms were observed at 3 dpi, and at 7 dpi there was a very small lesion at the site of inoculation (Fig. 6A). Figure 6A shows the results of virulence assays with wild-type strain P6497 and mutant strains A27 and A2. To investigate whether the aberrant swimming behavior of the zoospores of *PsGPA1*-silenced mutants was the cause of the change in virulence, leaves were inoculated with mycelium plugs instead of zoospores. In this assay there was no clear difference in the spread of disease symptoms; the *PsGPA1*-silenced strains were as virulent as the wild type (Fig. 6B). This implied that virulence itself is not impaired

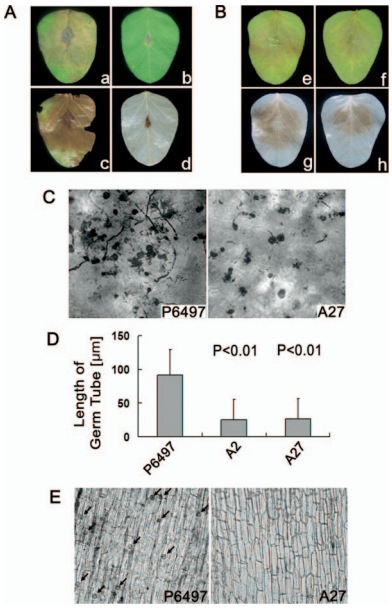

FIG. 6. Virulence of wild-type *P. sojae* (P6497) and *PsGPA1*-silenced mutants (A2 and A27). (A) Leaves of 10-day-old soybean plants (cultivar HeFeng35) were spot inoculated with equal numbers of zoospores from P6497 (a) and A27 (b) and incubated for 7 days. Panels c and d show the same leaves as in panels a and b but after destaining in alcohol. (B) Leaves of 10-day-old soybean plants (cultivar HeFeng35) were inoculated with hyphal tip plugs from P6497 (e) and A27 (f) and incubated for 48 h. Panels g and h show the same leaves as in panels e and f but after destaining in alcohol. (C) Detailed pictures of germinated cysts on soybean leaves that were inoculated with zoospores and incubated at 25°C for 4 h. The leaves were then put into ethanol to destain the chlorophyll and subsequently into 0.5% Coomassie brilliant blue for 2 min. After the leaves were washed in water for 10 min, the pictures were taken. (D) Average lengths of germ tubes from encysted zoospores on soybean leaves. Error bars indicate standard deviations. (E) Infection ability of zoospores from P6497 and A27. Germinated soybean seeds were immersed in zoospore suspensions of P6497 or A27 for 30 min. The epidermis of soybean sprouts was stripped, and zoospore encystment was observed under a microscope.

but, more likely, that preinfection events were disturbed. To test whether the mutant zoospores were simply not able to encyst or to germinate, we analyzed the efficiency of germination after inoculation on soybean leaves. Similarly to those illustrated in Fig. 5C, many of the encysted zoospores of wild-type strain P6497 had germinated at 4 h after inoculation, but zoospores of the mutants either were not encysted or had not germinated (Fig. 6C). Only a few germ tubes were observed, and overall they were much shorter (Fig. 6D).

To further analyze the infection ability of zoospores, germinated soybean seeds were immersed in zoospore suspensions of wild-type *P. sojae* and the Gα mutants for 30 min. The epidermis of soybean sprouts was stripped, and zoospore encystment was observed under a microscope. As shown in Fig. 6E, many zoospores of the wild-type strain could encyst on the epidermis, whereas no zoospore encystment was observed for the *PsGPA1* mutants.

Putative downstream targets of the Gα subunit. Previous studies with other organisms have reported that silencing of a Gα subunit gene could influence the activity of the Gβ subunit and other downstream targets (16). Based on the phenotypes

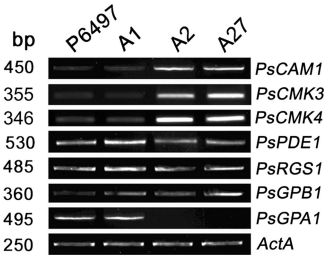

FIG. 7. Putative downstream targets of the Gα subunit. Expression of *PsGPA1* and genes encoding putative downstream targets of the Gα subunit in zoospores of the wild-type strain P6497, the control transformant A1, and the *PsGPA1*-silenced mutants A2 and A27 is shown. RT-PCR products were visualized on ethidium bromide-stained gels, and their sizes are indicated on the left.

that were observed in the two *PsGPA1*-silenced mutants in this study, we hypothesized that intermediates in calcium signaling pathways are potential downstream targets of the Gα subunit in *P. sojae*. To test this hypothesis, we analyzed the expression of genes that may have a function in G-protein- and calcium-mediated signaling. These included the Gβ subunit gene *PsGPB1*, the calmodulin gene *PsCAM1*, the *PsRGS1* gene encoding a regulator of G protein signaling, two *PsCMK* genes encoding calmodulin-dependent protein kinases, and the phosphodiesterase gene *PsPDE1.* When comparing the expression in zoospores of the wild-type strain P6497, the control transformant A1, and the mutants A2 and A27, we found that *PsCAM1*, *PsCMK3*, and *PsCMK4* were clearly upregulated in the two *PsGPA1*-silenced mutants (Fig. 7), pointing to a potential negative regulator activity of Gα. Expression of *PsGPB1*, *PsRGS1*, and *PsPDE1* was the same in all strains, and hence their regulation by the Gα subunit at the transcription level seems unlikely.

DISCUSSION

Many pathogens and parasites possess ingenious mechanisms to successfully locate their hosts. When zoospores of *P. sojae* sense isoflavones secreted by soybean roots, they swim toward the attractant and use the attractant as a guide to find the roots (33, 42, 43, 49). They then attach to the roots, encyst, and start the infection cycle. This study provides insight into mechanisms governing chemotaxis in *P. sojae.* Expression analysis of genes with putative roles in chemotaxis showed that the Gα subunit gene *PsGPA1* was one whose expression pattern pointed toward a function in zoospores. We showed that *P. sojae* exploits heterotrimeric G-protein signaling pathways to sense isoflavones and to control zoospore behavior. Silencing the *PsGPA1* gene severely affected zoospore motility and abolished chemotaxis to isoflavones.

G-protein signaling in *Phytophthora*. G-protein signaling is the most evolutionarily conserved signaling pathway in eukaryotes. *Phytophthora* has genes encoding basic components of the G-protein signaling pathway, including G-protein-coupled receptors (GPCRs), a Gα subunit, and a Gβ subunit (50). However, apart from a few studies describing the effects of activators and inhibitors of G-protein signaling (1, 34), there is hardly any knowledge about the mechanisms underlying the G-protein signaling events in *Phytophthora.* Unlike most eukaryotes, *Phytophthora* species possess only one Gα subunit gene and one Gβ subunit gene (35, 50). As was observed in our study and in a study by Laxalt et al. (35) with *P. infestans*, both the Gα and the Gβ genes are differentially expressed during development. *PsGPA1* and *Pigpa1* are expressed in zoospores but not in mycelium, whereas *PsGPB1* and *Pigpb1* show the opposite pattern, being expressed in mycelium but not in zoospores. Only in sporangia were mRNAs of both Gα and Gβ detectable, suggesting that this is the only developmental stage where the two subunits are produced simultaneously and could participate in forming the heterotrimeric G protein. As yet, the nature, or even the occurrence, of a heterotrimeric G protein in *Phytophthora* is unclear, since no conserved Gγ gene was found in any of the sequenced oomycete genomes. There are indications that in yeast (*S. cerevisiae*) certain G-protein subunits participate in signaling pathways independent from other subunits (29, 36, 47). This may also occur in *Phytophthora* and may explain why the expression patterns of the two single-copy genes are different. It may also explain why the phenotypes of *Pigpa1*- and *Pigpb1*-silenced mutants of *P. infestans* are different. Transformants lacking the Gα subunit had a normal colony morphology and produced sporangiophores and sporangia (33), whereas those lacking Gβ failed to sporulate (32, 33).

GPCRs are transmembrane receptors that transmit extracellular signals over the plasma membrane to the interior of the cell, thus allowing an organism to respond adequately and in a timely manner to changes in its environment. Genome mining revealed that *P. sojae* has 24 GPCRs, 12 of which have a unique architecture. The latter have a seven-transmembrane domain fused to a phosphaditylinositol phosphate kinase (PIPK) domain and are thus named GPCR-PIPKs (5, 50). Among all sequenced eukaryotes only one other GPCR-PIPK has been found, i.e., RpkA in *Dictyostelium.* Interestingly, RpkA plays a crucial role in cell density sensing (4, 5), and this is likely a process that involves chemotaxis. As yet there are no published data revealing a role for any of the *Phytophthora* GPCRs, including the GPCR-PIPKs.

Gene silencing as a tool for functional analysis. For determining the role of *PsGPA1* in chemotaxis, we generated mutants that lack the Gα subunit. Since the diploid nature of oomycetes hampers directed mutagenesis by gene disruption, we chose gene silencing as a method to obtain mutants. Gene silencing has been demonstrated in *P. infestans* and *Phytophthora parasitica* and was used for functional analysis of a few genes (7, 20, 22, 52). For *P. sojae*, however, this is the first report describing successful silencing of a target gene. An alternative method for directed mutagenesis in *P. sojae* is TILLING (*t*argeting *i*nduced *l*ocal *l*esions *in g*enomes), but this requires investments in library construction and maintenance, and the procedure to select the mutants is quite laborious and costly (31). By using an improved DNA transformation procedure, we were able to generate sufficient transformants to select a number of *PsGPA1*-silenced mutants, and this might serve as a robust technique for functional analysis of other interesting genes in *P. sojae*.

***PsGPA1* is important for zoospore encystment and cyst germination.** Silencing of the Gα subunit gene in *P. infestans* severely affected zoospore mobility and virulence and resulted in reductions in zoospore release and appressorium formation (33). Some of the phenotypes of the *PsGPA1*-silenced mutants

are similar to those of the *Pigpa1*-silenced mutants, indicating that Gα subunit functions are conserved in *Phytophthora.* Gα silencing did not have an obvious effect on mycelium growth in either of the two species, and this is in line with the observation that the genes are not expressed in mycelium. Oospore formation also was not disturbed. Despite the fact that *PsGPA1* is highly expressed in sporangia, sporangium formation in the *P. sojae* mutants was normal, as was the morphology of the sporangia. In *P. infestans* the absence of PiGPA1 caused aberrant cytoplasmic cleavage in sporangia and a higher proportion of "large" aberrant zoospores (33). We have not observed these defects in the *P. sojae* mutants, but nevertheless it seems that, overall, Gα plays a more prominent role in cleavage and zoospore release than sporangium formation per se. As observed in *P. infestans* (32), we expect that Gβ is more important for sporangium formation than Gα.

As in *P. infestans* Gα mutants, the most prominent defects in the *P. sojae* mutants were observed in zoospores. For example, they encysted very quickly, a phenomenon that could be due to inappropriate activation of calcium signaling (14, 26, 54). Zoospores can be induced to encyst by a variety of external stimuli, including mechanical shock, pectin, calcium, and isoflavones (17, 25, 43). Calmodulin is a typical calcium sensor, which binds calcium and activates downstream kinases and phosphatases. Upregulation of the expression of a calmodulin gene in zoospores of *PsGPA1*-silenced mutants suggested that the Gα subunit may function as a negative regulator in calcium signaling.

In contrast to the rapid encystment, the germination of the cysts was extremely slow or even aborted, which we envision as a main reason for the reduced pathogenicity of the *P. sojae* Gα mutants. The inhibition of cyst germination indicates that signals which induce *P. sojae* cysts to germinate are transmitted by Gα. For fungi, similar findings were reported. In *Aspergillus nidulans*, for example, the Gα subunit GanB controls a rapid transient cyclic AMP increase in response to glucose during early germination (30), and in *Botrytis cinerea*, induction of conidial germination by a carbon source requires the Gα protein BGC3 and an adenylyl cyclase (15). This suggests that Gα-dependent signaling is important to transmit signals for cyst germination and, indirectly, to promote infection.

Zoospore motility and chemotaxis are dependent on G-protein signaling. The influence of physical and chemical stimuli on the swimming behavior of zoospores has been studied intensively (2, 53). Already in 1981 it was reported that zoospores swim more smoothly in the presence of an attractant and turn more frequently in the presence of a repellent (9). The importance of external calcium for the swimming behavior of zoospores also is well known (9, 17, 23), but how the external stimuli are perceived and transmitted into the zoospores is far less understood. This study on *P. sojae* and that of Latijnhouwers et al. (33) on *P. infestans* clearly demonstrate that zoospore motility is controlled by Gα-mediated signaling. In comparison with wild-type zoospores, mutant zoospores swam slower, encysted faster, and changed their swimming direction more frequently. The novel finding in this study, and of particular importance for understanding the pathogenicity of *P. sojae*, was the chemotaxis to isoflavones. In contrast to wild-type zoospores, mutant zoospores were not attracted by low concentrations of daidzein, and at higher concentrations, they swam faster without encystment and seldom changed the swimming direction.

It is not likely that soybean roots produce isoflavones with the aim to attract the propagules of a pathogen. Soybean probably produces isoflavones for other purposes, and *P. sojae* simply exploits this for its own well being. One beneficial effect of isoflavones is their role as inducers of nodulation (*nod*) genes in rhizobia, the nitrogen-fixing bacteria that live in symbiosis with leguminous plants. *nod* gene expression in rhizobia results in the production of Nod factors, which in turn induce the development of root nodules, the structures that encapsidate the rhizobia and form the niche where the atmospheric N_2 is fixed (8, 46). Since prokaryotes lack G proteins, the mechanisms by which rhizobia perceive isoflavones must be different. It is possible that isoflavones attract other beneficial eukaryotic microorganisms, but to our knowledge this has not been reported. What has been reported is an adverse effect of isoflavones, namely, inhibition of spore germination and hyphal growth of some mycorrhizal fungi that colonize legumes (6, 48). The mechanism underlying this inhibition is unknown, but it would be interesting to investigate whether this inhibition is governed by G-protein signaling in the mycorrhiza and thus has similarity with the chemotactic responses in *Phytophthora*.

In conclusion, this study offers novel insights into chemotaxis in an important group of plant pathogens and shows that elimination of a ubiquitous signaling component can severely disturb pathogenicity. The mutants that we have generated can now be exploited to find downstream effectors and upstream receptors of the Gα subunit. The first attempts revealed a calmodulin gene as a potential target, hence pointing to calcium or calcium signaling. Elucidating additional targets under the control of Gα will help in unraveling the signaling networks that underlie the chemotaxis and pathogenicity of *Phytophthora* pathogens.

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