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Gene silencing of *CCD7* and *CCD8* in *Phelipanche aegyptiaca* by tobacco rattle virus system retarded the parasite development on the host

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Strigolactones are phytohormones that stimulate seed germination of parasitic plants including *Phelipanche aegyptiaca*. Strigolactones are derived from carotenoids via a pathway involving the *carotenoid cleavage dioxygenases CCD7* and *CCD8*. We report here identification of *PaCCD7* and *PaCCD8* orthologous genes from *P. aegyptiaca*. Expression analysis of *PaCCD7* and *PaCCD8* genes showed significant variation in their transcript levels in seeds and tubercles of *P. aegyptiaca* at different developmental stages. These two parasitic *PaCCD7* and *PaCCD8* genes were silenced in *P. aegyptiaca* using a *trans*-silencing approach in *Nicotiana benthamiana*. The transient knock-down of *PaCCD7* and *PaCCD8* inhibited tubercle development and the infestation process in host plants. Our results suggest an important role of the strigolactone associated genes (*PaCCD7* and *PaCCD8*) in the parasite life cycle.

Phelipanche spp. and *Orobanche* spp. (Orobanchaceae) are economically destructive parasitic weeds, causing large crop losses in sunflower, tomato, faba bean, tobacco and many other field crops in the world.[1] *Phelipanche* plant development is divided into pre-parasitic and parasitic stages. The pre-parasitic stage starts with seed pre-conditioning followed by germination, which is induced by molecules secreted in the rhizosphere by the roots of host plants. Most of the known germination stimulants are strigolactones. Germination leads to the emergence of a radicle which attaches to the host root surface.[2-4] The parasitic phase starts with the penetration of the parasite into the host root through a differentiating haustorium which connects to the host vascular tissues. The haustorium serves as both an attaching organ and a bridge for water and nutrient transfer from the host. The attached parasite first acts as a root, then diverts hormones and photoassimilates from the host plant. The parasite first develops a tubercle carrying numerous adventitious roots; the apical bud of this tubercle give rise to a subterranean shoot (spider) and then to a branched flowering spike after emergence from the soil (**Fig. 1**).[2-4] The flower produces thousands of extremely small (0.15–0.5 mm long) tan-to-brown colored seeds, which blacken with age and can survive more than 15 years in a crop field until the favorable condition obtained.[2-4]

Strigolactones are plant hormones that were initially identified as signaling molecules in the rhizosphere. They are mainly produced in the roots and have been detected in the root exudates of a wide range of monocot and dicot plant species, supporting the ancient origin and importance in nature.[5] In the rhizosphere, strigolactones act as host detection cues for symbiotic arbuscular mycorrhizal fungi and stimulate seed germination of parasitic plants.[6] They are derived from carotenoids through sequential oxidative cleavage by carotenoid cleavage dioxygenases *CCD7* and *CCD8*,[7,8] thus belonging to the apocarotenoid class.[7]

Strigolactones occur in all green lineages of the plant kingdom.[9-11] Although putative *CCD7* was reported to exist in *Phelipanche ramosa*,[12] endogenous strigolactone biosynthetic genes have not been reported in the parasitic plant *Phelipanche aegyptiaca*. Dependence of parasitic plant seed germination on exogenous strigolactones suggests the absence of endogenous strigolactones or a malfunctioning of strigolactone-related signaling in their seeds. Recently, Liu et al.[13] provided the first evidence for strigolactones and strigolactone perception genes of the MAX2-type in the parasite *Striga hermonthica*. These authors also mentioned EST sequences of endogenous *ShCCD7* and *ShCCD8* genes but no further sequence information or accession numbers were published.

Here we present evidence of endogenous strigolactone biosynthetic genes *CCD7* and *CCD8* and their induced expression during development stages of *Phelipanche* tubercles. In our study, expression of dsRNA of *Phelipanche CCD7* and *CCD8*

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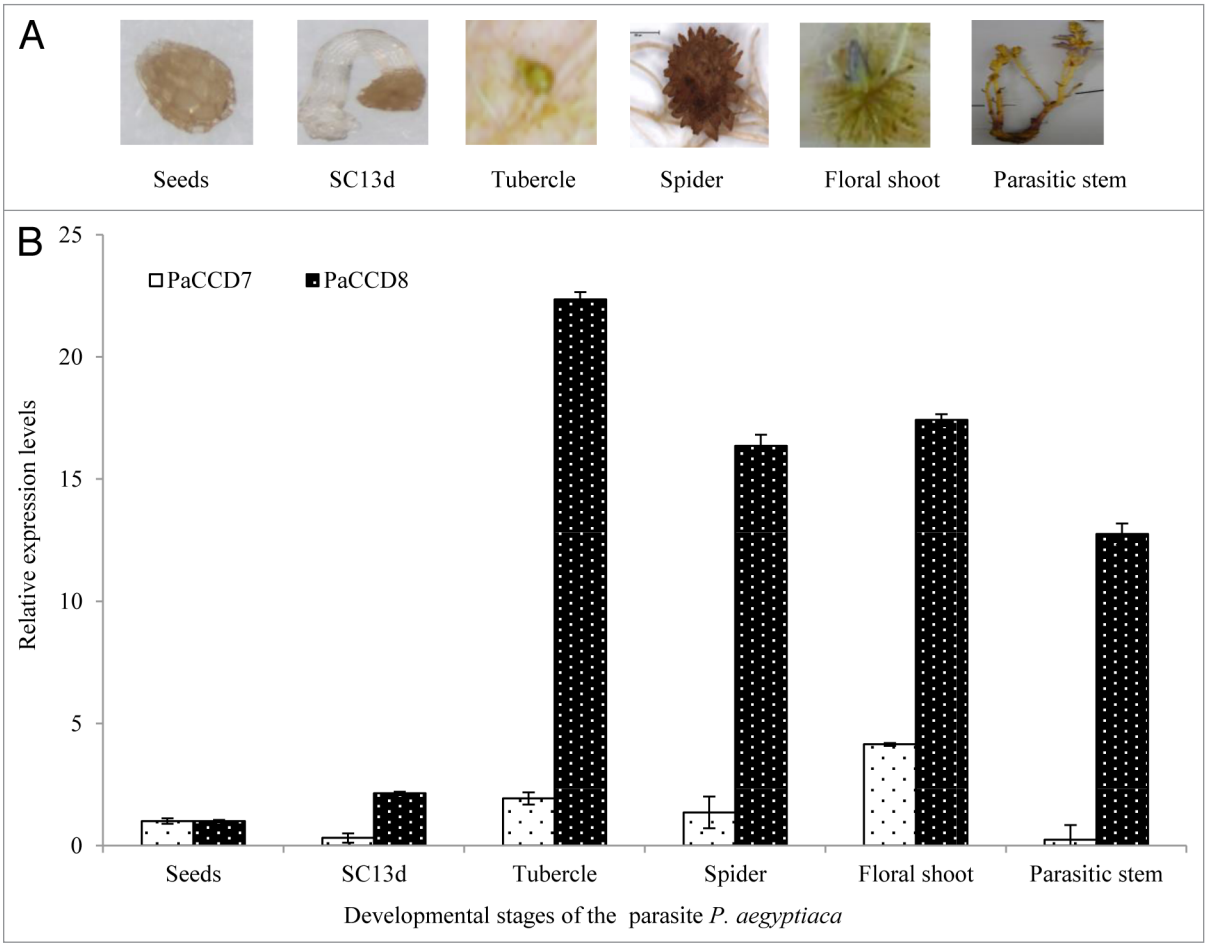

Figure 1. Expression patterns of *P. aegyptiaca* strigolactone biosynthetic genes during seeds and tubercles development. **(A)** Photography of different developmental stages of *P. aegyptiaca* including: dry seeds, seeds conditioned at 30°C for 13 days (SC13d), tubercle, spider, floral shoot and parasitic stem. **(B)** Quantification of *PaCCD7* and *PaCCD8* transcript levels by real-time RT-PCR analysis normalized to equal levels of actin transcripts, in all different developmental stages of *P. aegyptiaca*. All analyses were performed using three biological replicates.

target genes in the host plants *N. benthamiana* by virus induced gene silencing (VIGS) strategy[14] suppressed the candidate *Phelipanche* genes. VIGS is RNA silencing based technique used for the targeted downregulation of a host gene, allowing the analysis of the gene functions.[15] The dsRNA replication intermediate would be processed so that the siRNA in the infected cell would correspond to parts of the viral vector genome, including any nonviral insert. Thus, if the insert is from a host gene, the siRNAs would target the RNase complex to the corresponding host mRNA and the symptoms in the infected plant would reflect the loss of the function in the encoded protein.[15] VIGS has been used to silence a wide variety of genes in plants.[16] The *trans*-specific RNAi silencing is a genetic interference phenomenon in which double-stranded RNA (dsRNA) induces individual sequence-specific posttranscriptional gene silencing.[17] Trans-specific RNAi silencing was visualized first by transforming roots of the hemiparasite *Triphysaria versicolor* with the β-glucuronidase (GUS) reporter gene and then allowing the transgenic *Triphysaria* spp. to parasitize host roots expressing an RNAi hairpin construction of GUS (hpGUS).[18] VIGS derived dsRNA can apparently transferred from host plant to either herbivores[19] or parasitic plants and suppress the expression of target genes (Dubey et al., 2014, unpublished data). In the current study, silencing of *Phelipanche CCD7* and *CCD8* genes by TRV-VIGS system could lead to retardation of the parasite development.

Potential genes involved in the strigolactone biosynthesis (CCD7, CCD8) in *Phelipanche* were examined in the parasitic plant genome database (http://ppgp.huck.psu.edu/). Data mining of the parasitic plant genome resulted in identification of sequences *PaCCD7* (NCBI Genbank accession number JN412814) and *PaCCD8*, displaying high sequence similarity to *CCD7* and *CCD8* from other species. *PaCCD7* protein displays high sequence similarity to *CCD7* (69% identity) from *Nicotiana tabacum* and *Petunia* x *hybrida*,[20] and to *CCD7* from *Solanum lycopersicum* (65% identity).[21] *PaCCD7* is also similar to *CCD7* (59% identity) from *Arabidopsis thaliana* (**Fig. S1**). The *PaCCD8* protein showed high amino acid similarity to Dad1/*CCD8* (85% identity) from *Petunia* x *hybrida*,[22] to *CCD8* (84% identity) from *Solanum lycopersicum*,[23] to CCD8 (84% identity) from *Nicotiana tabacum*, and also to CCD8 (66% identity) from *Arabidopsis thaliana* (**Fig. S1**).

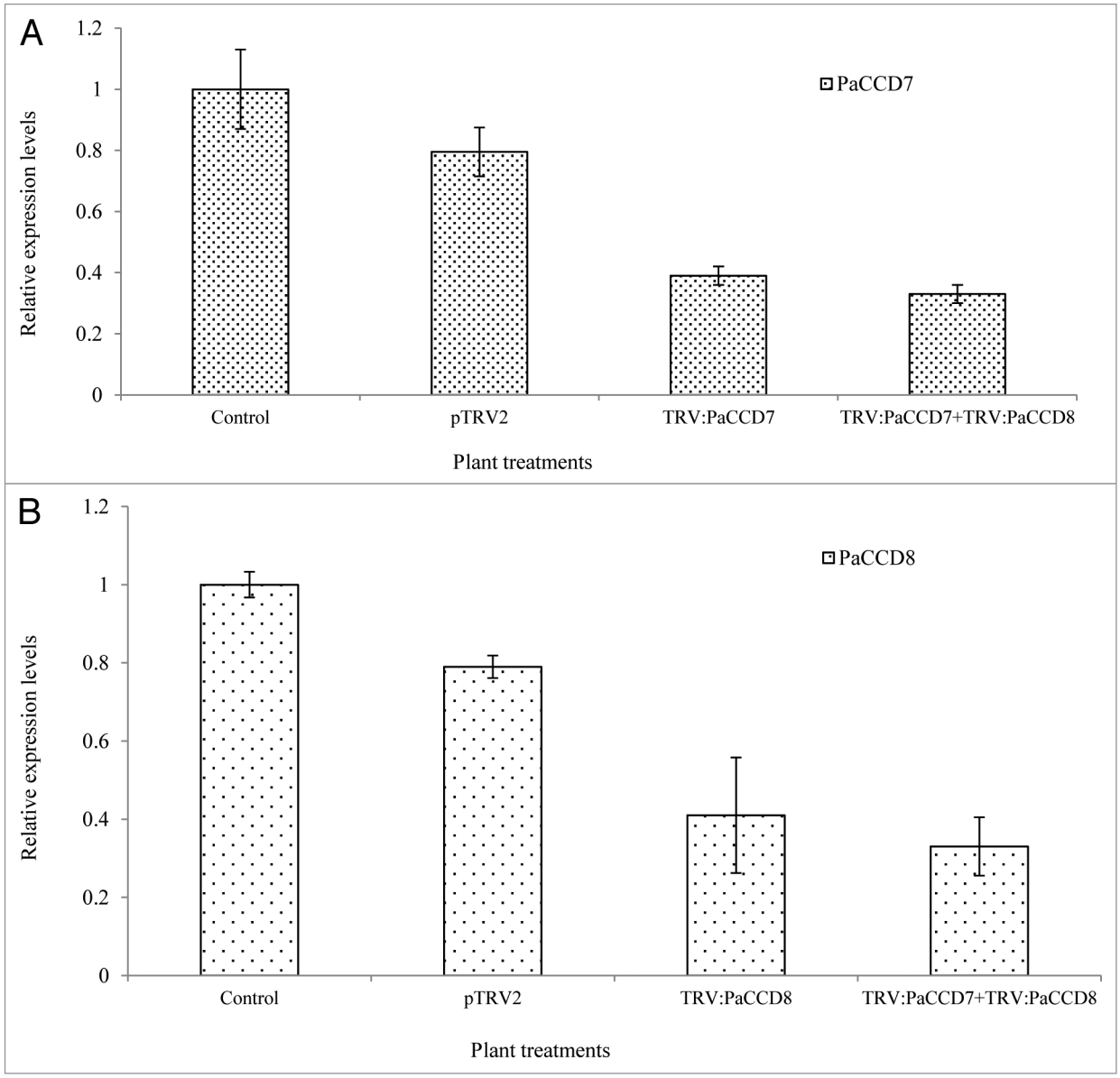

Figure 2. Real-time RT-PCR analysis of *PaCCD7* and *PaCCD8* genes in *P. aegyptiaca* tubercles attached to control and VIGS-treated *N. benthamiana* plants. **(A)** Quantification of *PaCCD7* transcript levels by qRT-PCR analysis normalized to equal levels of actin transcripts in the underground tubercles of *P. aegyptiaca* controls and VIGS-treated *N. benthamiana* plants. **(B)** Quantification of *PaCCD8* transcript levels by qRT-PCR analysis normalized to equal levels of actin transcripts in the underground tubercles of *P. aegyptiaca* controls and VIGS-treated *N. benthamiana* plants. All analyses were performed using three biological replicates.

In order to obtain further information about the presence of *PaCCD7* and *PaCCD8* in parasitic plant development, the expression pattern of *P. aegyptiaca PaCCD7* and *PaCCD8* genes was examined during different developmental stages of the parasite [dry seeds, seeds conditioned designated SC13d, and tubercle development] by quantitative RT-PCR (**Fig. 1**). *PaCCD7* and *PaCCD8* transcripts were detected in dry seeds, conditioned seeds, and in all *P. aegyptiaca* tubercle developmental stages. The abundance of *PaCCD7* transcripts increased with the growth of parasite tubercle, reaching maximum levels at floral shoots followed by decreased levels in the parasite stems (**Fig. 1B**). Surprisingly, expression of *PaCCD8* transcripts remained continuously high up to the last monitored stage, the fully grown parasite stems (**Fig. 1B**). To ensure that *PaCCD7* and *PaCCD8* primers are specific only for the parasite and do not amplify *CCD7* and *CCD8* from the *Nicotiana benthamiana* host plants, RT-PCR was performed on total RNA from the host roots. No amplification of these genes was detected when primers designed for the *P. aegyptiaca* genes were used. However, total RNA from VIGS-treated host plants revealed a strong amplification signal by the same primers (**Fig. S2**). Additionally, to ensure that the host tobacco plant *CCDs* (*CCD7*, *CCD8*) were not also suppressed by the VIGS gene silencing, qRT-PCR was performed on total root RNA from all

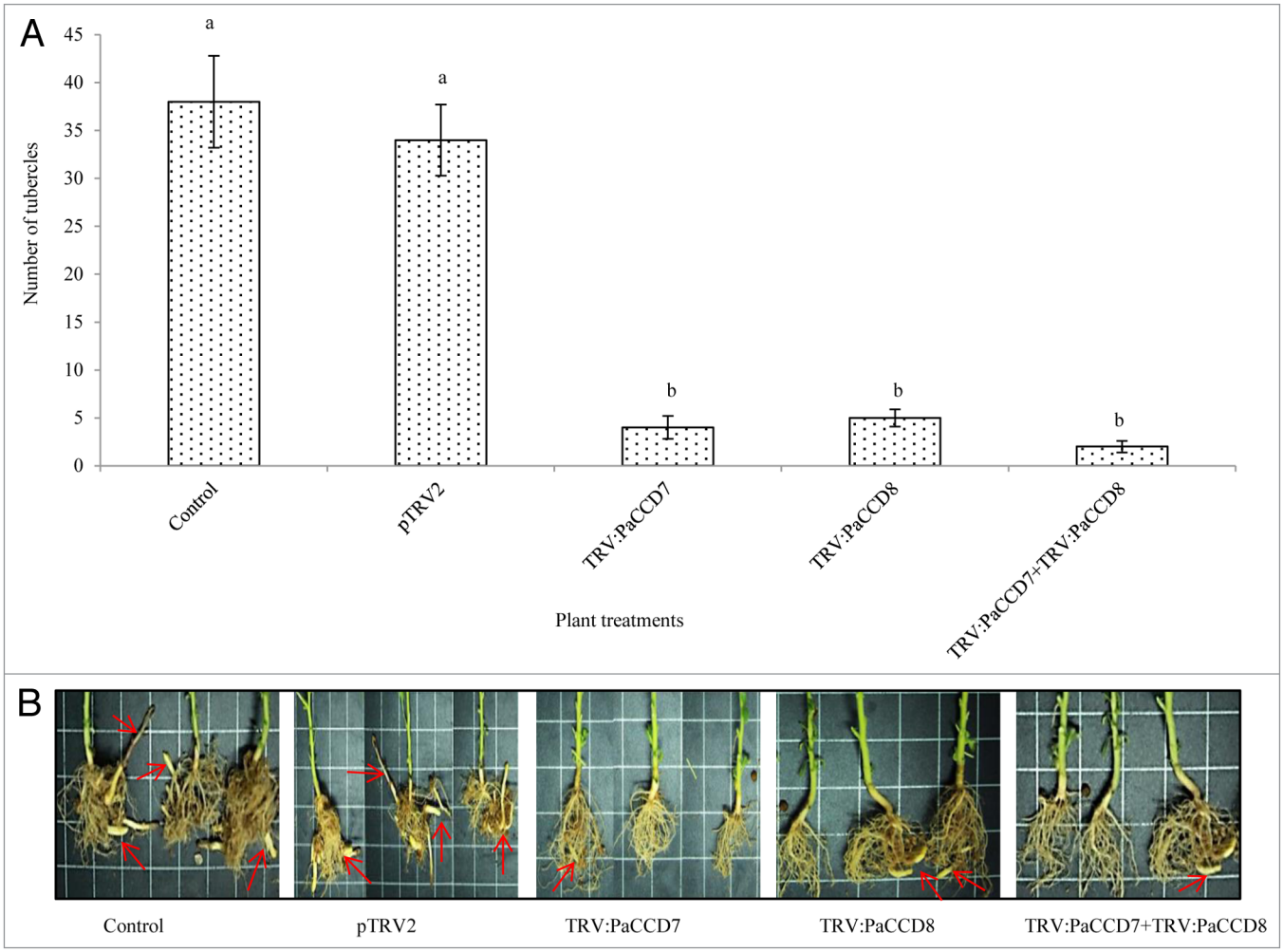

Figure 3. Effect of *P. aegyptiaca* target genes (*PaCCD7* and *PaCCD8)* silencing on development of the parasite in *N. benthamiana* host plants. **(A)** Effect of *trans*-specific gene silencing of the *PaCCD7* and *PaCCD8* on the tubercle numbers attached to *N. benthamiana* plants. **(B)** *P. aegyptiaca* tubercles and shoots attached to the controls and VIGS treated *N. benthamiana* plants indicated by red arrows. Mean ± SE were obtained from 20 independent plants. Bars labeled with different letters indicate the significant differences as determined by JMP statistic software ($P \leq 0.05$).

N. benthamiana plant treatments. Transcripts of *NbCCD7* and *NbCCD8* in all four measurements, showing that the two investigated genes were expressed in all root of *N. benthamiana* plant treatments (control wild type plants, and plants infiltrated with TRV:*PaCCD7*, TRV:*PaCCD8*, or a mixture of TRV:*PaCCD7* and TRV*:PaCCD8*) (**Fig. S3**). In general, no significant difference in the *NbCCD7* and *NbCCD8* transcripts expression levels were observed in all plant treatments (**Fig. S3**).

In conclusion, these data suggest presence of endogenous strigolactone biosynthesis genes and these genes were expressed in *P. aegyptiaca* seeds and tubercles. Those, suggest that might be parasitic plant produces its own strigolactones and those they are important for the development of the parasite.

In order to determine whether *PaCCD7* and *PaCCD8* are functional in the parasite and whether they are indeed involved in the parasite strigolactone biosynthesis, the two genes were transiently knocked-down by the VIGS-mediated strategy.[15] Systemic expression of VIGS vector in the host roots followed by Agro-infiltration was confirmed by RT-PCR (**Fig. S4**). These experiments demonstrated that endogenous *PaCCD7* and *PaCCD8* transcripts from *P. aegyptiaca* tubercles grown on *N. benthamiana* plants infiltrated with TRV:*PaCCD7,* TRV:*PaCCD8* or a mixture of TRV:*PaCCD7* and TRV*:PaCCD8* were significantly reduced as compared to control plants (wild type plants or plants transformed with empty pTRV plasmids) (**Fig. 2A and B**).

Additionally, the number of parasite tubercles attached to the roots of host plants treated with TRV:*PaCCD7*, TRV:*PaCCD8*, or a mixture of TRV:*PaCCD7* and TRV*:PaCCD8* was significantly reduced (by 95%) as compared to control plants (**Fig. 3A and B**).

The roles of CCD7 and CCD8 enzymes in the biosynthesis of strigolactones have been reported for several plant species.[13,21,23] Recently, it has been shown that endogenous strigolactone biosynthesis and signaling are also present in the parasitic plant *Striga hermonthica* during their development.[13] Specifically, partial sequences of two related *CCD* genes were identified in *Striga* EST database and designated as *ShCCD7*

and *ShCCD8*.[13] Similarly, a full length parasite sequence for *ShMAX2* was also reported, which is closely related to host plant *MAX2*-type genes.[13] In this paper we describe two sequences of parasite strigolactone biosynthesis genes, *PaCCD7* and *PaCCD8* (**Fig. S1**) which display high sequence similarity to other plant *CCD7* and *CCD8*.[20,21,22,23]

The *PaCCD7* and *PaCCD8* genes are expressed in *P. aegyptiaca* seeds and different development stages of *P. aegyptiaca* tubercles (**Fig. 1**), but they differ from the expression profiles of *ShCCD7* and *ShCCD8*.[13] Both *ShCCD7* and *ShCCD8* are expressed specifically in the pre-conditioned seeds of in *Striga hermonthica*.[13] By contrast, in our study, expression of *PaCCD8* was relatively high in developed tubercles, but low levels for *PaCCD7* in parasitic stem stage of *P. aegyptiaca* (**Fig. 1B**). Our results showed that the transcript levels of *PaCCD8* were more abundant than those of *PaCCD7* in all examined tissues (**Fig. 1B**). Similar results were also reported by Liu et al.[13]

Vogel et al.[21] showed that tomato *SlCCD7* antisense lines display up to 90% reduction in strigolactone content. In this case the host *SlCCD7* gene is expressed in all tissues, including roots, stems and fruits. Also, generating *SlCCD8* knock-down tomato lines led to the reduction of strigolactone level.[23] Based on the results of these antisense and knock-down analyses, we postulate that expression of *CCD7* and *CCD8* in the parasite is amenable to suppression approaches via VIGS-mediated and transgenic approaches. Interestingly, in our experiments using *trans*-silencing approaches by expressing parasite gene suppression in *N. benthamiana,* the infection by *P. aegyptiaca* was reduced by up to 95% in the VIGS-treated plants (**Fig. 2**). Reduction in the parasite seed germination and infection could be suggested due to the lack of the endogenous strigolactone of the parasite, which was inhibited following suppression of *P. aegyptiaca* endogenous strigolactone biosynthesis genes (*PaCCD7* and *PaCCD8*). Liu et al.[13] also suggested that *Striga* endogenous strigolactones possibly play a role in seed germination.

In conclusion, the *trans*-silencing of the parasitic *P. aegyptiaca PaCCD7* and *PaCCD8* genes significantly reduced the number of parasite tubercles attached to the host roots of *N. benthamiana*. To the best of our knowledge, this is the first report using the TRV-VIGS system for gene silencing of strigolactone biosynthetic genes from the parasitic weed *P. aegyptiaca.* The efficacy of the *trans*-silencing of *PaCCD7* and *PaCCD8* as a control strategy against parasitic plant management still needs to be determined in the field by using stable transgenic lines.

For the experiment setup, *N. benthamiana* plants were grown in polyethylene bags as described by Aly et al.[24] Sterilized and conditioned seeds of *P. aegyptiaca* were spread into the properly established root. Tubercles of different stages (tubercle, spider, floral shoot, and parasitic stem) (**Fig. 1**) were isolated from the *N. benthamiana* roots. RNA from dry seeds, seeds conditioned at 30°C for 13 days (SC13d), and tubercles of different stages was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). Five hundred ng of total RNA was reversed transcribed to cDNA by using reverse transcriptase (Verso cDNA kit, AB-1453/A, Thermo), followed by 5 fold dilution with molecular grade water. Quantitative RT-PCR (qRT-PCR) was performed by using the sequences of forward 5'-AAGACACCAC CACCATCAAT GC-3' and reverse primer 5'-GGGACGGGTC TGTTGGTTTC-3' in case of *PaCCD7* and forward 5'-CACCCGATCG TCACGGATA-3' and reverse primer 5'-CATCACCTTC CTCTCGTTCG TT-3' in case of *PaCCD8*. Thermo-SYBR Green Master Mix (AB4162) was used in qRT-PCR according to manufacturer protocol, on the platform of ABI-Prism 7000 Real-Time PCR Detection System (Applied Biosystems). The expressions of target candidate gene were normalized with Actin gene of following forward and reverse primer sequences Actin-F: 5'-AATGATCGGA ATGGAAGCTG-3', and Actin-R: 5'-TCCACTGAAG GACGATGTTT C-3'. The relative gene expression was calculated by using the $2^{-\Delta\Delta}$Ct method.[25] The qRT-PCR experiment was performed in three biological replicates.

All sequences in the parasitic plant genome database (http://ppgp.huck.psu.edu/) that were similar to *Arabidopsis thaliana* CCD7 (accession number NM_130064) and CCD8 (accession number NM_119434) were translated using the bioinformatics resource "ExPASy" server (http://www.expasy.org) with several carotenoid cleavage dioxygenase 7 and 8 sequences being reassembled using the CAP3 sequence assembly program.

In this study, two *carotenoid cleavage dioxygenases* (*CCD*) genes were tentatively identified as *P. aegyptiaca PaCCD7* and *PaCCD8*. For VIGS experiment, unique N-terminal regions about 300 bp of *PaCCD7* and *PaCCD8* (**Fig. S5**) were selected and amplified from the cDNA prepared from tubercles followed by cloning into pJET1.2 vector (Thermo Scientific). *PaCCD7* was amplified by using the forward primer of 5'-GAGCTCATGC AGTCTGCCAC AGCTTGC-3' sequence overhang with restriction site of Sac I and reverse primer of 5'-CTCGAGGGGA TGGACGGTAG ACCCG-3' with the overhang of Xho I, respectively. Similarly, *PaCCD*8 was amplified by using the forward primer of sequence 5'-GAATTCTACA TTCAGCCCAC CCGATC-3' with overhang of EcoR I and reverse primer with sequence of 5'-GGATCCCATG CATGTAACCT TTAGAGTTAG-3' with BamH I restriction site. Both *PaCCD*7 and *PaCCD*8 genes were cloned separately into pTRV2 (Tobacco Rattle Virus vector 2) at the respective site and designated as TRV:*PaCCD7* and TRV:*PaCCD8,* respectively. The VIGS assay was performed as described by the Bachan et al.[26] The experiments were performed in *N. benthamiana* plants grown in the "Newe Yaar" Research Center in northern Israel, under standard field irrigation and fertilization conditions.[4] *Agrobacterium* (strain EHA105) containing pTRV1 with TRV:*PaCCD7* and/or TRV:*PaCCD8* were mixed and infiltrated into the leaves of *N. benthamiana*. Non-infiltrated *N. benthamiana* plants, and plants infiltrated with mixtures of *Agrobacterium* transformed with empty plasmid of pTRV1 and pTRV2 were used as controls.

N. benthamiana plants were pre-challenged with parasitic seed about ten days before Agro-infiltration. After ten days of co-cultivation, RNA was isolated from leaves and roots of tobacco plants and systemic expression of TRV virus was analyzed. About 25 days after Agro-infiltration, tubercles attached

to the VIGS-treated plants were isolated and the total number of tubercles attached to the VIGS-treated and control plants were counted.[24] Additionally, the level of expression of the *PaCCD7* and *PaCCD8* genes was examined by qRT-PCR. All analyses were performed using three biological replicates.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/psb/article/29376/

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