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RESEARCH ARTICLE

microRNA-encoded peptides inhibit seed germination of the root parasitic plant *Orobanche cumana*

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Societal Impact Statement

The root parasitic plant *Orobanche cumana* (sunflower broomrape) is one of the major pests of sunflower crops. Despite intense efforts to develop effective agricultural practices and breeding programs, selective control of broomrapes is still rare and ineffective in terms of sustainability. It is thus essential to develop new specific control methods against those pests. miRNA-encoded peptides (miPEPs) are a new class of peptides regulating the expression of miRNAs and their corresponding target genes. This study demonstrates that certain miPEPs strongly inhibit the germination of broomrape seeds by regulating their miR gene, making them good candidates for use as biocontrol agents against this pathogen.

Summary

- Root parasitic plants of the Orobanchaceae family are a constant and growing threat to agriculture worldwide. Among them, the parasitic weed *Orobanche cumana*, the sunflower broomrape, causes significant losses to sunflower production in European-Asian and North African countries. Despite the use of several conventional control methods against this pathogen, none has proved effective or durable, underlining the need to develop innovative strategies. miRNA-encoded peptides (miPEPs) are regulatory peptides stimulating the expression of their own primary transcript of miRNA, and plant watering with those molecules leads to down-regulating specifically miRNA target genes and altering plant physiology.
- Through seed germination assays and qRT-PCR analysis, we investigated the impact of exogenous treatments of synthetic miPEPs on broomrape seed germination.
- First, we report that the conserved miRNA repertoire of *O. cumana* consists of 39 members. Thirty-nine miPEPs were designed, synthetized, and assayed, 11 of which strongly inhibited *O. cumana* seed germination. Interestingly, miPEP319a showed the strongest inhibiting effect while miPEP319b did not. Three out of the four corresponding miR319 target genes showed upregulation after treatment

with a germination stimulant, which was impaired by treatment with miPEP319a. This downregulation of expression is associated with an increase in the expression of the corresponding pri-miR319a.

- We reveal thus that the use of miPEPs can increase our knowledge of key molecular mechanisms underlying a complex parasite interaction and should provide a new phytosanitary method to control broomrape parasitism with highly specific and biodegradable natural substances.

KEYWORDS

broomrapes, germination, germination stimulants, miPEP, miRNA, *Orobanche cumana*, parasitic plant, sunflower

1 | INTRODUCTION

The sunflower broomrape, *Orobanche cumana*, is a root parasitic plant from the Orobanchaceae family that entirely depends on its unique host, the sunflower, for water, mineral, and carbohydrate supplies (Delavault, 2015). This pest is the most serious biotic constraint to sunflower in countries where it is cultivated (Euro-Asia and North Africa) with the exception of North and South America (Amri et al., 2012; Eizenberg et al., 2004; Molinero-Ruiz et al., 2015; Shi et al., 2015). Yield reductions due to the infection by *O. cumana* frequently reach 100% in heavily infested fields (Domínguez, 1996; Shi & Zhao, 2020). Several control strategies have been employed against this parasitic plant, but none of them have led to unequivocal success (Fernández-Aparicio et al., 2020; Pérez-De-Luque et al., 2009). Currently, the only efficient way of preventing its expansion is the selection for crop resistance. However, to avoid the emergence of new pathogenic races overcoming the prevailing resistance genes, the production of new resistant lines cumulating several resistance traits associated with an integrative pest management approach is strongly recommended (Velasco et al., 2016).

Among the sophisticated mechanisms that allow the obligate parasite to detect the presence of a suitable host plant and to coordinate its lifecycle with it, its particular germination process is probably the most important (Brun et al., 2021). Indeed, this early stage of development is critical to its survival because a germinated seedling that fails to connect to a host will quickly exhaust its energy reserves and die. Broomrapes compensate for this by having strict requirements for germination, involving the presence of specific biochemical molecules exuded by the roots of the host, the germination stimulants (Bouwmeester et al., 2020; Nelson, 2021). Thus, *O. cumana* seeds, whose sole host is sunflower, germinate in response to the sunflower specific-strigolactone, heliolactone (Ueno et al., 2014), but also other compounds known as sesquiterpene lactones such as dehydrocostus lactone (DCL) and costunolide (Joel et al., 2011; Raupp & Spring, 2013). Irrespective of the type of germination stimulant, broomrape seed response involves an ABA catabolism via a rapid and strong up-regulation of the Abscisic acid (ABA) 8′-hydroxylase-encoding gene *CYP707A* (Brun et al., 2019; Lechat et al., 2012). This decrease in the amount of ABA thus leads to a release of dormancy and is therefore at the origin of the triggering of the germination itself, beginning with a metabolic recovery (i.e., resumption of cellular respiration, mitochondrial biogenesis, and mitochondrial enzymatic activities) followed by the protrusion of the radicle (Pouvreau et al., 2013). This key stage of development occurs before irreparable damage has occurred and is therefore a good target for control methods.

Behind the linear and binary scheme of genes encoding protein sequences via mRNA translation lie multiple, finely-tuned molecular processes. Among the regulatory molecules involved in these processes, the microRNAs (miRNAs) play a critical role. They are 20–22 nucleotides regulatory RNA molecules participating in the regulation of numerous biological processes in plants and animals. In plants, miRNAs are generally organized in multigenic families that regulate key target genes, such as transcription factors. The transcription of miRNA genes first leads to the production of a "long" primary transcript of miRNAs (pri-miRNAs) further processed into an active mature miRNA. Once loaded in a protein complex containing Argonaute, generally Argonaute RISC Component 1 (AGO1), the miRNAs negatively regulate the expression of specific target genes based on the sequence complementarity between the miRNA and the mRNA target. An original mechanism controlling miRNA expression was discovered a few years ago (Lauressergues et al., 2015). Briefly, this mechanism involves a new class of small peptides called miRNA-encoded peptides (miPEPs) encoded by pri-miRNAs. The first open reading frame (miORF) on the pri-miRNA is frequently translated and produces a miPEP (Lauressergues et al., 2022) that activates the transcription of its encoding pri-miRNA. As a result, it indirectly increases the level of its mature miRNA and induces a stronger downregulation of the expression of the corresponding target genes. The presence of miORFs encoding miPEPs in pri-miRNAs is a common feature in plants. Whereas miRNAs are well conserved in the green lineage, miPEP sequences are extremely polymorphic. This means that orthologous miRNAs in different plant species are regulated by species-specific miPEPs. Then, specific physiological or developmental responses can be triggered following specific miRNA activation. Thereby, treatments with well-chosen miPEPs were shown to improve plant development (Chen et al., 2020; Ormancey et al., 2021), regulate metabolite biosynthesis (Sharma et al., 2020; Vale et al., 2021), regulate response to stress conditions (Kumar et al.,

2023), and stimulate symbiotic interactions (Couzigou et al., 2016; Couzigou et al., 2017).

The objectives of this study were to evaluate for the first time whether miPEPs are able to control interaction between a host plant and its pathogen, in this case, a parasitic plant, by focusing on their germination process. For these peptides to act in broomrape, functional processing of sRNAs is required. A first answer to this question was provided by a study performed in *O. cumana* which revealed that most components of the machinery for post-transcriptional gene silencing are present in the transcriptome (e.g., DCLs and AGOs) (Jiang et al., 2020). Thus, we determined the repertoire of the conserved miRNAs of *O. cumana* and, by monitoring the expression of the miRNA target genes following treatment with a germination stimulant, we identified miRNAs potentially involved in the chemically-activated germination of *O. cumana* seeds. A full set of miPEPs corresponding to this miRNA repertoire was chemically synthetized and assessed for their activity on seed germination. Our results demonstrate that miPEP319a strongly inhibits the germination of seeds through the regulation of miR319 expression.

As farmers and sunflower seed companies have high expectations concerning this pest, whose expansion is threatening sunflower production, this study is timely, as these miPEPs are good candidates to be used as biocontrol agents against this pathogen. Potential applications of this new technology in sunflower fields are discussed.

2 | METHODS

2.1 | Plant material, seed sterilization, and root exudate production

O. cumana seeds (seed lot Oc49) were collected in 2017 in a sunflower field (*Helianthus annuus*) at Longeville-sur-Mer (France). Seed lot OcIN23, collected in 2010 in Guadalquivir Valley (Spain), was provided by Laboratoire des Interactions Plantes-Microbes-Environnement (LIPME) from Toulouse (France). Sunflower seeds (accession 2603 susceptible to *O. cumana*) were provided by LIPME.

Broomrape and sunflower seeds were surface-sterilized according to Lechat et al. (2012) using 2.4% (v/v) sodium hypochlorite for 7 min. Seeds were successively rinsed three times for 30 s and three times for 5 min with sterile distilled water.

For the production of root exudates, seedlings of sunflower were disposed into a drained plastic pot (700 mL, 9 × 11 × 12 cm [W × L × H], Nicoplast) nested in an undrained pot and filled with glass beads (Ø 3 mm) covered by a 1 cm layer of vermiculite. The pot systems were deposited in a growth chamber at 21°C with a 16 h light/8 h dark photoperiod. Plantlets were first watered with 200 mL of half-strength TT medium (Tadano & Tanaka, 1980) and the volume was renewed every 2 days. Exudates were collected after 6 weeks, then concentrated on Strata-X 33u Polymeric Reversed Phase (Phenomenex) and eluted with 6 mL of 100% acetonitrile. Eluate was evaporated and the remaining liquid was diluted with 1 volume of 50% acetonitrile.

2.2 | Peptide assays

Peptides were synthesized by sb-PEPTIDE (www.sb-peptide.com) and dissolved at 10 mM in water or in 50% acetonitrile (stock solution), aliquoted and conserved at −80°C. Seeds were treated with a 1–100 µM final concentration of miPEP diluted in water before use.

2.3 | Seed germination assays and miPEP treatments

Surface-sterilized parasitic seeds were incubated in the incubation solution (1 mM HEPES buffer; pH 7.5 and 0.1% p/v of Plant Preservative Mixture) in 50 mL tubes for 7 days in the dark at 25°C. This period is called the conditioning phase required for the seeds to be able to perceive the germination stimulants. Once conditioned, 50 µL of seed (about 150 seeds) were distributed in 96-well plates. Ten microliters of germination stimulants ((±)-GR24, a synthetic strigolactone, DCL, a sequiterpene lactone or exudates) and 10 µL of miPEP solution were added, and the final volume was adjusted to 100 µL with sterile water. The incubation time was 54 h. Seed germination starts with an early metabolic resumption and continues with the protrusion of the radicle. Because some molecules inhibit radicle protrusion without inhibiting metabolic resumption, both parameters were systematically assessed to characterize broomrape seed germination. Metabolic resumption and radicle protrusion of seeds were observed 18 h after Methylthiazolyldiphenyl-tetrazolium bromide (MTT) staining that reveals mitochondrial dehydrogenase activities (Pouvreau et al., 2013). When the aim of the assays was to extract RNA in sufficient quantity for gene expression analysis, upscaling was carried out by performing germination stimulation and miPEP treatments in 24-well plates with 7 mg of seeds in a final volume of 500 µL per well.

2.4 | RNA extraction and qRT-PCR

Total RNA was extracted from 14 mg of seeds (two wells of 24 well-plates each containing 7 mg of seeds were pooled) using the NucleoSpin RNA Plant kit (Macherey-Nagel) with small modifications. The steps corresponding to DNAse treatment and subsequent washing were omitted. RNA was eluted with 50 µL of RNase-free water and a DNase I digestion was then performed (Qiagen). RNA was finally purified using NucleoSpin RNA Clean-up XS (Macherey-Nagel) and collected in 30 µL of RNase-free water. cDNAs were synthesized from 400 ng of total RNA using qScript cDNA supermix (Quantabio) for miRNA target genes expression analysis and from 1 µg using qScript cDNA Flex synthesis (Quantabio) for precursor miRNA expression analysis. qPCR was performed with the PerfeCTa SYBR Green SuperMix ROX (Quantabio). The polyubiquitin gene *UBI10* was used as the reference gene and primer sequences for qPCR are listed in Table S1.

2.5 | Identification and analysis of *O. cumana* miRNAs

Conserved sunflower broomrape miRNAs were identified in the *O. cumana* genome (www.heliagene.org/OcIN23-20190821/) by searching by blast for sequences highly homologous to *Arabidopsis thaliana* miRNAs. Blast was performed using *Arabidopsis* mature miRNAs, with default parameters, and accepting max 4 mismatches. A blast of the sequence against *Arabidopsis* proteins allowed to remove target genes. For positive hits, partially complementary miRNA (miR*) was next searched around (100 bases) the miRNA sequence, according to the 5′-3′ scheme of the family. Finally, the 3D RNA structure was analyzed using the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

2.6 | Statistical analyses

Statistical analyses were done with R Software (www.rproject.org) and were performed using analysis of variance (ANOVA) and multiple confidence interval testing.

Data corresponding to the inhibition rate of germination were subjected to pairwise comparison analysis (Tukey-HSD) with confidence intervals of 99.9%, 99%, and 95%. The mean values were ±SEM with $n = 3$ independent bioassays. All raw data relating to germination assays are provided in Dataset S1.

Target gene expression data from seeds treated or not with (±)-GR24 were subjected to pairwise comparisons (Tukey-HSD) between treatment and negative control (solvent treatment with 0.1% (v/v) of acetonitrile) and with a confidence interval of 99.9% and 99%. Means are values ± SD with $n = 3$ independent bioassays, each corresponding to three technical replicates.

Gene expression data from seeds treated with (±)-GR24 and miPEP were subjected to ANOVA with a 95% confidence interval. Means are values ± SD with $n = 3$, each corresponding to three technical replicates.

3 | RESULTS

3.1 | Identification of miRNA, their targets, and miPEPs in *Orobanche cumana*

We focused our analysis on miRNA families conserved in most green plants, that is, from miR156 to miR399. We were able to identify 39 miRNAs, corresponding to miR156 to miR396 (accession numbers OP961563 to OP961601). Interestingly, we were not able to identify miRNAs from the families 157, 158, 161, 162, 163, 391, 397, 398, and 399.

Due to the lack of available data concerning the expression of the miRNA gene, we identified 5′ genomic sequences close to pre-miRNAs and searched for ORFs corresponding to miPEPs (Lauressergues et al., 2015, 2022). Previous analyses in *A. thaliana* revealed a leader sequence in the 5′ arm of the pri-miR with a median length of 363 bases. We searched miPEP ORF located close to the pre-miRNA within this window. Thus, 39 miPEPs were identified and synthetized (Table S2).

The target genes of miRNA conserved in *A. thaliana* (Sarkar Das et al., 2018) were searched in the genome of *O. cumana* using blast modules. The miRNA binding sites were searched in the transcripts of the target genes by sequence alignment. Only target genes whose binding site had a maximum of five mismatches and no more than two in a row were retained. Additional verification was performed on *O. cumana* target gene sequences using psRNATarget software (Dai et al., 2018) by blasting *A. thaliana* miRNAs. By applying this strategy, 37 mRNA targets for 13 of the 16 identified miRNA families were identified (Table S1).

3.2 | Expression of miRNA target transcripts during strigolactone-induced seed germination in *O. cumana*

Analysis of the transcriptome changes during strigolactone-dependent germination of broomrape seeds has been the subject of several studies (Bao et al., 2017; Lechat et al., 2012; Okazawa et al., 2020). Gene expression regulated by miRNAs during plant seed germination has also been widely studied (Das et al., 2015; Tognacca & Botto, 2021). We therefore investigated this miRNA-associated expression pattern in the particular case of a chemically induced seed germination. For this, the relative expression of the target genes of the 13 miRNA families was determined 48 h after treatment of conditioned *O. cumana* seeds with 0.1 µM (±)-GR24 (Figure 1). Among the 37 target genes studied, 23 were significantly up-regulated, 3 were down-regulated, and 11 did not show differential expression. Four genes are overexpressed more than 20 times compared with the control: *OcSPL10* target of miR156, *OcARF10* target of miR160, *OcCUC1/2b* target of miR164, and *OcAP2b* target of miR172 exhibiting a 368.7 ± 0.21-fold upregulation.

3.3 | Some miPEPs affect the chemically-induced seed germination of *O. cumana*

Thus, most of the miRNA target genes are up-regulated during chemically-induced germination of *O. cumana* seeds. Therefore, it can be expected that the corresponding miRNAs are down-regulated during this biological process. If miPEPs stimulate the expression of their respective miRNAs and thus inhibit those of their target genes, an exogenous addition may then impact germination and probably inhibit it. To test this hypothesis, the 39 miPEPs generated from the *O. cumana* genome analysis were assayed on germination. As a preamble, the first screening consisted of testing the 39 miPEPs on conditioned but unstimulated (±)-GR24 seeds to assess their ability to mimic the effect of germination stimulants on seeds. None of them induced seed germination of this root parasitic plant (data not shown).

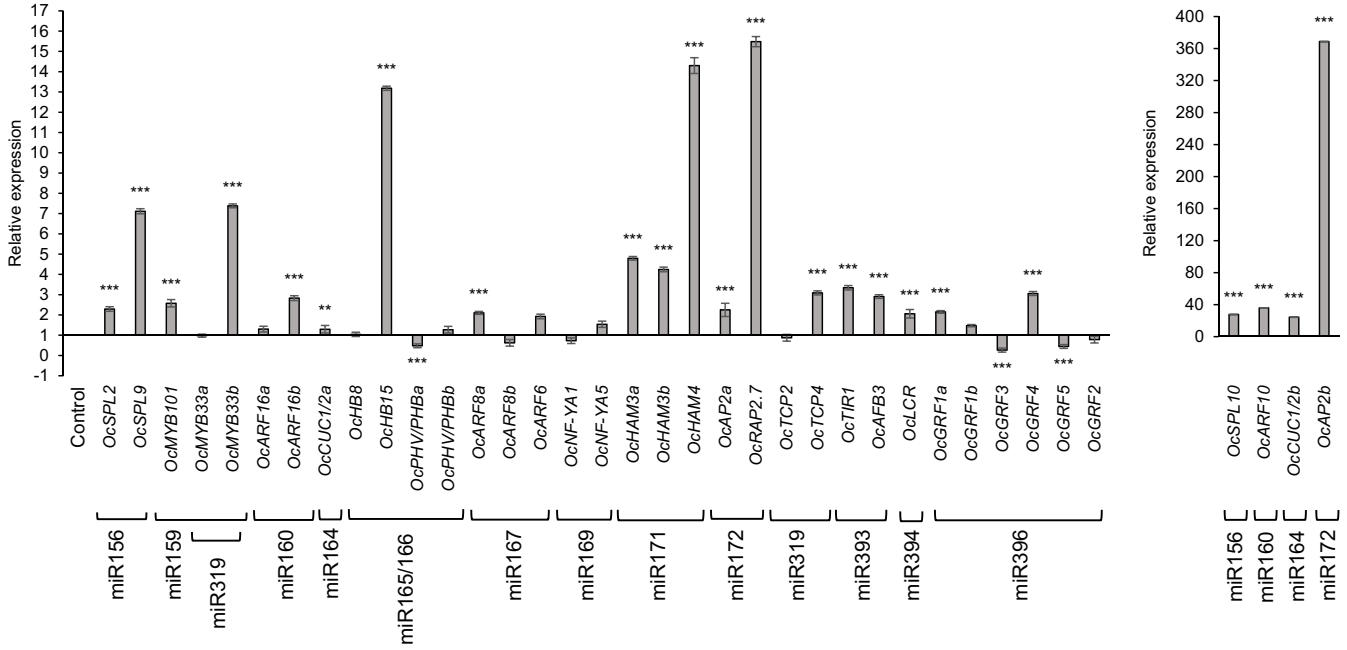

FIGURE 1 Stimulant-induced seed germination of *O. cumana* involves regulation of microRNA (miRNA) target gene expression. Relative expressions were measured by quantitative PCR using RNA isolated from *O. cumana* seeds treated with mock (control) or 0.1 μM (±)-GR24 during 48 h. *UBI10* was used as the reference gene and expression in GR24-treated seeds was normalized to gene expression in mock-treated seeds (ΔΔCt). Data are means ± standard deviation ($n = 3$). Asterisks indicate statistical differences compared with mock-treated seeds (Tukey honestly significant difference; $^{**}p < .01$ and $^{***}p < .001$).

Then, seeds were treated concomitantly with (±)-GR24 and each miPEP at 100 μM. The concentration of 1 μM in (±)-GR24 usually used for germination assays (Brun et al., 2019) leads to a saturation of the germination process and does not allow us to visualize the potential inhibitory effect of a miPEP. In order to overcome this saturation effect, the germination assay was performed at lower concentrations of germination stimulant. The half maximal effective concentration (EC50) of GR24 is equal to 4 nM as previously determined (Pouvreau et al., 2013, 2021). Thus, three concentrations of (±)-GR24 (0.1, 1, and 10 nM), around this EC50, were used to screen the effects of miPEPs on seed germination. Two parameters of seed germination were evaluated 54 h after co-treatment, the metabolic activity recovery and the radicle protrusion by measuring their inhibition by a miPEP treatment. The 0.1 nM (±)-GR24 concentration is the one allowing the highest inhibitory activity of the tested miPEPs (Figure 2). However, a compilation of the data obtained for the three concentrations (Figures 2 and S1) reveals that a core set of 11 miPEPs (miPEP159, miPEP160a,b,c,d, miPEP164, miPEP169b, miPEP172a,b, miPEP319a, and miPEP393b) related to 7 miRNA family (miR159, miR160, miR164, miR169, miR172, miR319, and miR393) exhibits inhibitory activity only on radicle protrusion while only miPEP319a and miPEP393b inhibit both metabolic activity recovery and radicle protrusion, whatever the (±)-GR24 concentration. Overall, these results indicate that miPEP319a has the strongest inhibitory activity on the germination of *O. cumana* seeds. miR319a and miR319b are two different pri-miRNAs producing the same mature miRNA (Figure S2). It is interesting to note that miPEP319b has no effect on this process demonstrating the miRNA-specific nature of the mode of action of miPEPs. The impact of a miPEP on the expression of its miRNA may result in the inhibition of the genes targeted by said miRNA, however, does not predict its inhibitory effect on the phenotype.

It is interesting to note the miRNA-specific nature of the action of miPEPs because of the two members of the miPEP319 family, one is strongly inhibitory while the second is almost inactive. This was confirmed by performing germination assays with varying concentrations of (±)-GR24 and miPEP (Figure 3). At a concentration of 100 μM, miPEP319a induced a total inhibition of radicle protrusion regardless of (±)-GR24 concentration, accompanied by a partial or total inhibition of metabolic recovery. In contrast, miPEP319b had only a weak effect, and only at low (±)-GR24 concentration and high peptide concentration. In order to verify that the effects induced by these miPEPs during the germination of *O. cumana* seeds are not specific to the seed stimulation by a strigolactone, the same experiment was performed with a natural germination stimulant present in sunflower exudates, DCL, a guaianolide sesquiterpene lactone (Joel et al., 2011) (Figure S3). The range of DCL concentrations is the same as the one used in (±)-GR24 for the previous tests. The application of DCL results in the same inhibition profiles by miPEP319a as those obtained with strigolactones, indicating that probably the same metabolic pathway was targeted by the peptide. Root exudates from the sunflower accession 2603, known to be susceptible to broomrape (Labrousse et al., 2001), were also used in order to simulate as much as possible the natural conditions of germination induction of *O. cumana* seeds by the host plant. Indeed, these root exudates are

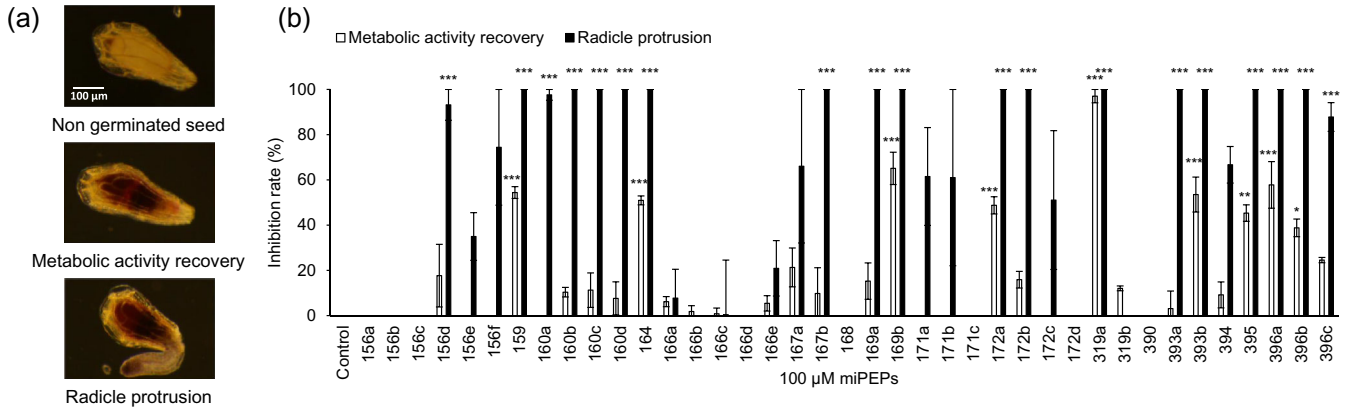

FIGURE 2 Several miRNA-encoded peptides (miPEPs) affect the germination of *O. cumana* seeds. (a) Visualization through a stereo microscope of metabolic activity recovery and radicle protrusion after MTT staining of seeds. (b) Percentage inhibition of metabolic activity recovery (white bars) and radicle protrusion (black bars) of seeds 54 h after a co-treatment with 0.1 nM (±)-GR24 and 100 µM miPEPs. The control corresponds to GR24 treatment without miPEP. Error bars represent SEMs; asterisk indicate a significant difference between test conditions and controls according to Tukey-HSD test ($n = 3$; $^*p < .05$, $^{**}p < .01$, and $^{***}p < .001$).

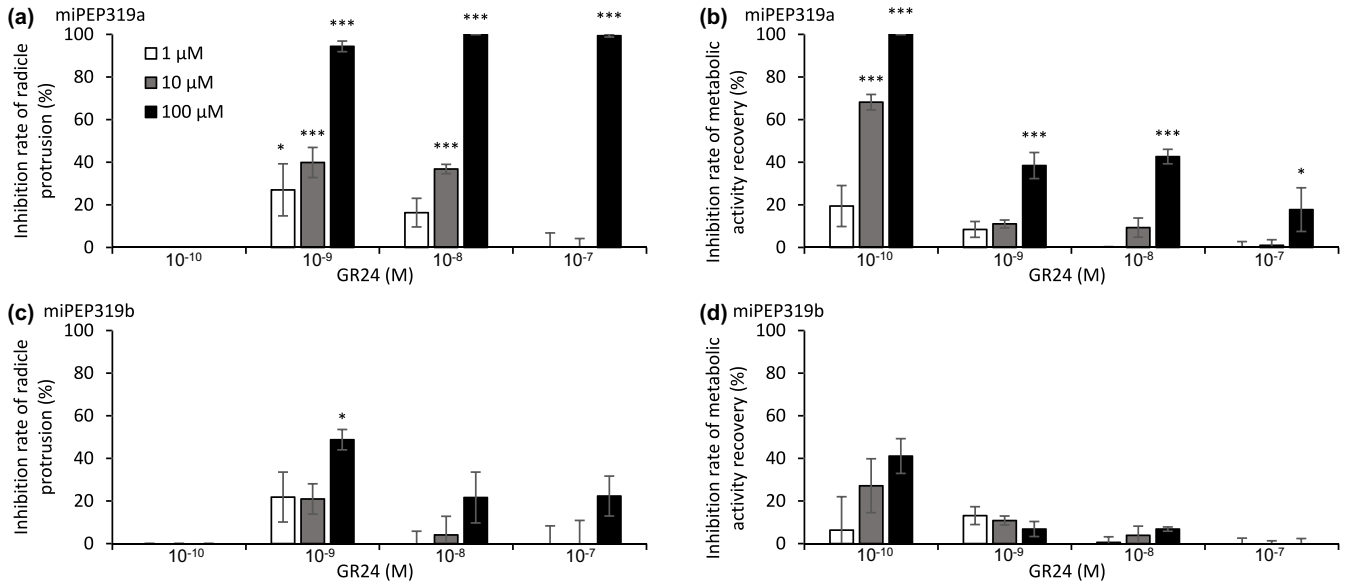

FIGURE 3 miPEP319a affects broomrape seed germination. The percentages of radicle protrusion (a and c) and of metabolic activity recovery (b and d) of *O. cumana* seeds were determined after treatment with the relevant miPEP319a (a and b) or miPEP319b (c and d). Means and standard errors were determined from three biological replicates. Asterisk indicate a significant difference between test conditions and controls according to Tukey-HSD test ($n = 3$; $^*p < .05$ and $^{***}p < .001$); error bars represent SEMs. GR24, a synthetic analogue of strigolactones.

likely to contain the two types of germination stimulants, strigolactones, and sesquiterpene lactones (Raupp & Spring, 2013; Ueno et al., 2014). Three successive dilutions to the tenth in root exudate were used. Exudates induce a response similar to that of (±)-GR24 or DCL following treatment with miPEP319a (Figure S3). The inhibitory effect on radicle protrusion and metabolic recovery is strong at the highest miPEP concentration.

The effect of miPEP319 was also characterized on another race of *O. cumana* to confirm that the germination inhibitory effects observed for miPEP319a are not race-specific. The OcIN23 population was collected in Spain in the Guadalquivir Valley and corresponds to the race F, while the French Oc49 population is a race E. (±)-GR24 was not used on this seed lot because they germinate poorly with this synthetic stimulant, making visualization of a potential inhibitory effect of miPEP very difficult. Inhibition of radicle protrusion is observed for all concentrations of miPEP319a and DCL used (Figure S3). MiPEP319a at the highest concentration also induces inhibition of radicle protrusion when root exudates from susceptible sunflowers are used instead of DCL. Inhibition of metabolic recovery is observed for all concentrations of DCL, or root exudates used with the highest peptide concentration. miPEP319b has almost no effect on germination of the seeds of this lot, used in co-treatment whether with DCL or root exudates. Only a slight inhibitory effect can be observed, but only at the highest concentration of miPEP319b.

3.4 | Impact of miPEP on gene expression

To know whether the inhibition of *O. cumana* seed germination by some miPEPs was mediated through a miRNA process, we first analyzed the expression of their corresponding target genes following a peptide treatment. Thus, a treatment of the miPEP159, miPEP164, miPEP172a,b, and miPEP319a, which strongly inhibited seed germination of *O. cumana*, induced concomitantly a strong downregulation of at least one of their associated target genes (Figure S4), suggesting a post-transcriptional regulation by miRNAs. However, in the absence of data on the expression of mature miRNAs and/or pri-miRNAs, transcriptional regulation of these targets without the intervention of miRNA cannot be excluded as has been demonstrated in *A. thaliana* (Das et al., 2015; Sarkar Das et al., 2018). To address this, we focused our study on miPEP319a, the most effective peptide, by analyzing the expression of the pri-miR319a and its corresponding target genes *OcTCP2*, *OcTCP4*, *OcMYB33a*, and *OcMYB33b* (Figure 4). Quantification of expression was done for up to 48 h after treatment with 0.1 nM (±)-GR24 concomitantly or not with 100 µM miPEP319a or miPEP166c as negative control. First of all, it should be noted that (±)-GR24 leads to an accumulation of the target gene mRNAs as early as 6 h after treatment which is maintained or increases up to 48 h with the exception of *OcTCP2* which shows a transient upregulation. In each case, the application of the active synthetic miPEP319a induces a significative decrease of the corresponding mRNA, which is not the case with the inactive miPEP166c. Regarding the pri-miR319a, a decrease in accumulation is observed 6 h after (±)-GR24 addition, which coincides interestingly with the upregulation of all four target genes. Again, the addition of miPEP319a (but not of miPEP166c) impacts the expression profile because it clearly limits the downregulation of its pri-miRNA. Altogether, these results suggest that the chemically induced seed germination of *O. cumana* involved a regulation of the expression of some transcription factors mediated by their miRNA gene. Indeed, throughout the first hours of the chemically induced seed germination of *O. cumana*, the accumulation of pri-miR319a is synchronized and inversely proportional to that of its target genes. Moreover, the effects of a miPEP319a treatment on the accumulation of pri-miR319a, downregulation of its target genes, and seed germination rate further support this hypothesis.

It is known that seed dormancy release and thus germination in plants rely mainly on ABA catabolism by specific ABA 8′-hydroxylases encoded by the cytochrome *CYP707A* gene family (Okamoto et al., 2006). In root parasitic plants, it has been shown that the activation of the expression of these genes is dependent on germination stimulants (Brun et al., 2019). To investigate whether this molecular process leading to seed germination is under the control of miR319 and its associated target genes in *O. cumana*, the expression of *CYP707A1* was studied after treatment with miPEP319a (Figure 5). First, as expected, 0.1 µM (±)-GR24 induces a strong and rapid accumulation of *CYP707A1* mRNA (Figure 5a). To observe a potential effect of miPEP319a, seed germination was induced with a lower (±)-GR24 concentration of 0.1 nM explaining a global lower *CYP707A1* expression level (Figure 5b). Treatment of *O. cumana* seeds with synthetic miPEP319a decreases the accumulation of *CYP707A1* 3 h after application suggesting that expression of this gene is directly or indirectly under the control of transcription factors, themselves under the control of miR319.

4 | DISCUSSION

O. cumana, the sunflower broomrape, is the most serious biotic constraint to sunflower in all countries where it is cultivated (Euro-Asia and North Africa) with the exception of North and South America (Kaya, 2014; Molinero-Ruiz et al., 2014). Yield reductions due to the infection by *O. cumana* depend on several factors (Molinero-Ruiz et al., 2015). As an average, sunflower seed losses caused by broomrape can be quantified above 50% when susceptible hybrids are grown, and they frequently reach 100% in heavily infested fields (Domínguez, 1996; Shi & Zhao, 2020). Although this has been a problem for many years, there is a real concern that it will increase during the next years.

Thus, understanding the biology of the interaction between the parasite and its host is a necessary step toward the development of selective and socially acceptable control methods. In this sense, obtaining the complete genome sequence of *O. cumana* (Gouzy et al., 2017) associated with the discovery of miPEPs and their physiological effects (Lauressergues et al., 2015) were major advances to contribute to this goal. Analysis of the *O. cumana* genome revealed that most miRNA families conserved in most plant species are present in the *O. cumana* genome (39 miRNAs grouped in 16 families). An analysis of the miRNA repertoire in *Phelipanche aegyptiaca*, another broomrape species, was also performed and revealed divergences with *O. cumana* (Zangishei et al., 2022). Thus, while our analysis of the *O. cumana* genome revealed the absence of the miR genes from the families 157, 162, 398, and 399, the corresponding miRNA was identified in *P. aegyptiaca*. Conversely, no 390, 393, and 394 miRNAs were identified in *P. aegyptiaca* while the miR genes are present in the *O. cumana* genome. However, in the absence of a genome sequence in *P. aegyptiaca*, it is difficult to make definitive conclusions.

In our study, we identified 37 mRNA targets for 13 of 16 identified miRNA families. Indeed, it was not possible to find potential target sequences for miR168, miR390, and miR395. At first, the relative expression of the 37 target genes was determined 48 h after treatment of conditioned *O. cumana* seeds with 0.1 µM (±)-GR24 (Figure 1). Among the target genes studied, 23 were significantly upregulated, 3 were down-regulated, and 11 did not show differential expression. The fact that the expression of the three genes *OcGRF3*, *OcGRF5*, and *OcPHV/PHBa* is down-regulated may suggest that the corresponding miRNAs, miR396 and miR165/166, may naturally be involved in the chemically-dependent process of seed germination in *O. cumana*. Because these miRNAs are not known to be involved in seed germination in autotrophic plants such as *A. thaliana* (Das et al., 2015), these results require further study.

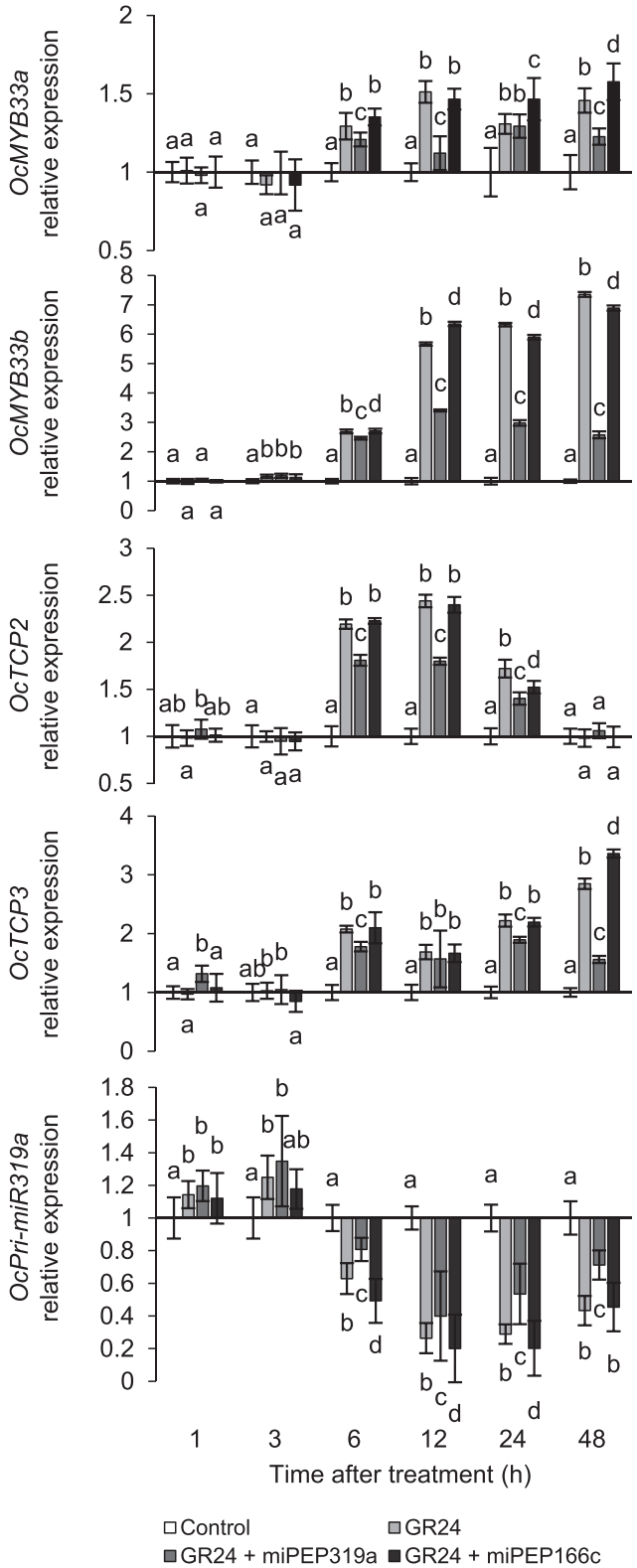

FIGURE 4 miPEP319a regulates expression of its associated microRNA (miRNA) target genes and primary transcript of miRNA (pri-miRNA) during seed germination of *O. cumana*. Relative expressions were measured by quantitative PCR using RNA isolated from *O. cumana* seeds treated with mock (control) or 0.1 nM (±)-GR24 with or without 100 µM miRNA-encoded peptide (miPEP) during 48 h. *UBI10* was used as the reference gene and expression in GR24-treated seeds was normalized to gene expression in mock-treated seeds (ΔΔCt). Data are means ± standard deviation ($n = 3$). Different letters indicate significantly differences with negative control according to analysis of variance (ANOVA) test ($p < .05$). GR24, a synthetic analogue of strigolactones.

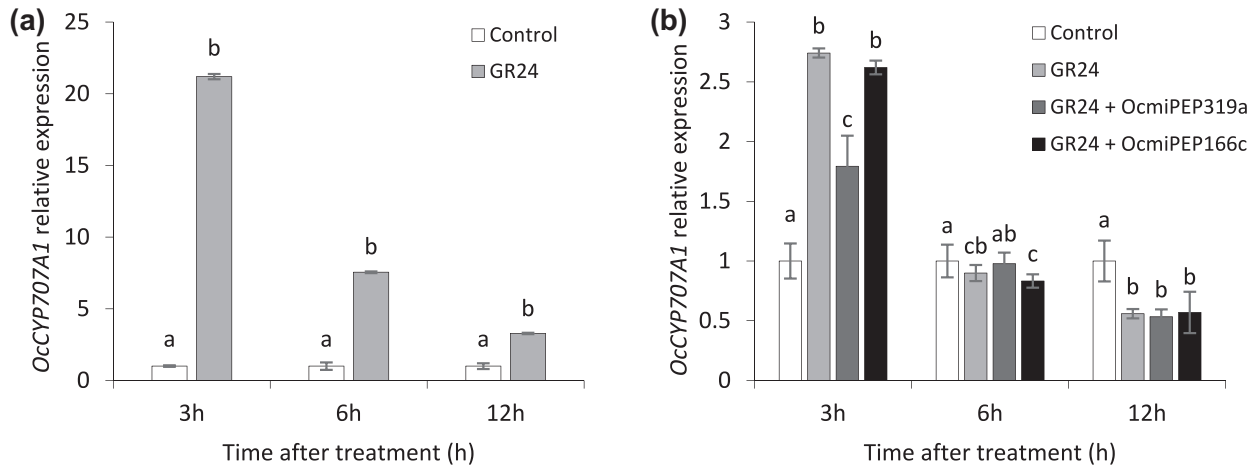

FIGURE 5 miPEP319a down-regulates the *CYP707A1* expression induced by a (±)-GR24 treatment during seed germination of *O. cumana*. Relative expressions of *CYP707A1* were measured by quantitative PCR using RNA isolated from *O. cumana* seeds treated (a) with mock (control) or 0.1 µM (±)-GR24 and (b) with mock (control) or 0.1 nM (±)-GR24 with or without a 100 µM miRNA-encoded peptide (miPEP) treatment during 12 h. *UBI10* was used as the reference gene and expression in GR24-treated seeds was normalized to gene expression in mock-treated seeds (ΔΔCt). Data are means ± standard deviation ($n = 3$). Different letters indicate significantly differences with negative control according to analysis of variance (ANOVA) test ($p < .05$). GR24, a synthetic analogue of strigolactones.

Thirty-nine miPEPs were generated from the *O. cumana* genome analysis and assayed on germination. None of them were able to induce alone the seed germination while a core set of 11 miPEPs belonging to the 7 miRNA family (mir159, mir160, mir164, mir169, mir172, mir319, and mir393) exhibits a strong inhibitory activity on the seed germination induced by (±)-GR24. This suggests that these miRNAs may be involved in the seed germination process of *O. cumana*, as previously demonstrated in non-parasitic plants (Curaba et al., 2014; Das et al., 2015; Nonogaki, 2010).

An ABA treatment of *Arabidopsis* or broomrape seeds inhibits their germination (Pouvreau et al., 2013; Reyes & Chua, 2007). In *Arabidopsis*, it has been shown that this treatment induced a miR159 up-regulation associated with *MYB33* and *MYB101* mRNA cleavage (Reyes & Chua, 2007). In *O. cumana*, miPEP159 induced an inhibition of seed germination through a total inhibition of radicle protrusion (Figure 2), concomitantly to a down-regulation of the expression of the three miR159 target genes, *OcMYB101*, *OcMYB33a*, and *OcMYB33b* (Figure S4). Similarly, miPEP319a induces inhibition of seed germination, marked by activation of pri-miR319a expression and repression of expression of both *OcMYB33a* and *OcMYB33b* genes (Figure 4). Both miPEP treatments of *O. cumana* seeds would therefore act by mimicking the effects of an ABA treatment. Germination assays with a range of *O. cumana* miPEP319 concentrations, coupled with a range of concentrations of (±)-GR24, DCL or root exudates, confirmed the inhibitory effect of miPEP319a on radicle protrusion regardless of the germination stimulant and the lot of *O. cumana* seeds used (Figure S3). Thus, there would be a common response pathway to the different germination stimulants, involving miR319. The miPEP319b of *O. cumana* shows only a very weak inhibitory effect on radicle protrusion at the lowest concentration of (±)-GR24 or DCL coupled with the highest concentration of peptide. In addition, this miPEP has no effect on the metabolic recovery of seeds reflecting a lesser effect compared with miPEP319a.

miPEP319a can then disrupt the germination process of broomrapes by specifically reducing the expression of its associated miRNA target genes. Molecular events governing the chemically-induced seed germination of root parasitic plants are poorly understood but are being characterized. Thus, *P. aegyptiaca* and *Striga hermonthica* perceive strigolactones via several α/β hydrolases of the HTL/KAI2 family (HYPOSENSITIVE TO LIGHT/KARRIKIN INSENSITIVE2) (Conn et al., 2015; Toh et al., 2015). More recently, in *Phelipanche ramosa*, it was shown that at least one of the five members of these KAI2 receptors, PrKAI2d3, was able to accept in addition to strigolactones another class of germination stimulant, the isothiocyanates (de Saint Germain et al., 2021). Downstream of these receptors, the induced signaling pathway has been extensively studied in non-parasitic plants. It involves the F-box protein MORE AXILLARY GROWTH2 (MAX2) that subsequently ubiquitinates transcriptional regulators such as SUPPRESSOR OF MAX2-1 (SMAX1) (Nelson, 2021). It is likely that this pathway is responsible for the induced germination of parasites, although this remains to be demonstrated experimentally. In all cases, this leads in parasitic seeds to the activation of ABA catabolism via a rapid and strong up-regulation of the ABA 8′-hydroxylase-encoding gene *CYP707A1* (Brun et al., 2019; Lechat et al., 2012). In *Arabidopsis*, activation of the CYP707A genes by chemical signals, such as GR24 and KAR1, is also partially dependent on the canonical karrikin signaling pathway KAI2/MAX2/SMAX1 (Brun et al., 2019). Interestingly, in this study, we demonstrated that the GR24-dependent *CYP707A1* expression can be downregulated by a treatment with miPEP319a, suggesting that the corresponding miR319 may interact with this signaling pathway in *O. cumana*. Analysis with the psRNAtarget server (Dai et al., 2018) revealed that *CYP707A1* is not a primary target of miR319a. This implies that

miR319a could induce the mRNA degradation of at least one of the transcription factors Teosinte branched1/Cincinnata/Proliferating (TCP) or Myeloblastosis (MYB), leading to a modulation of the expression level of its target genes, and then finally to the downregulation of *CYP707A1*. In the absence of effective functional genomics tools for root parasitic plants, the demonstration of this hypothesis remains unlikely. However, it has been shown that transcription factors of the TCP family are components of strigolactones signaling pathway in pea (*PsBRC1*), bread wheat (*TaTB1)*, and *Arabidopsis* (*AtBRC1* and *AtTCP1*) for other physiological processes than germination (Braun et al., 2012; Liu et al., 2017; Wang et al., 2020). It is therefore tempting to propose that a TCP transcription factor targeted by miR319a is a positive actor in the germination signaling pathway of *O. cumana*, which could explain the inhibitory effect of miPEP319a on this process.

The control of plant pathogens is an ongoing effort that requires constant investment by scientists, especially as the effective tools available are increasingly limited because of growing governmental and societal expectations to reduce the use of chemical pesticides and fertilizers. This is especially true for parasitic weeds, against which chemical treatments are in addition mostly ineffective due to the lack of selectivity and the difficulty of employing a strict protocol of application that needs to consider many kinetic factors, such as seed germination, root infection, and post-attachment development. Despite intense efforts to develop effective agricultural practices, selective control of various parasitic plants is still scarce and ineffective in terms of sustainability. The discovery of the action of miPEPs and the decrypting of the genome of the root parasitic plant *O. cumana* have been two major and concomitant events that have contributed to initiate the development of a completely new and cutting-edge technology: the use of peptides (miPEPs) as natural molecules to modulate specific gene expression (without genetic transformation or mutation) in the parasite for its biocontrol. Moreover, in order to commercialize such molecules to protect sunflowers against broomrape, several studies will be conducted on their dosage optimization, ecotoxicity, formulation, and production, as well as economic feasibility and field validation studies. Regarding the miPEP formulation study, product development strategies could include sunflower-coated seeds with miPEPs increasing sunflower's resistance against broomrape or freeze-dried miPEPs inhibiting broomrape growth that could be diluted and directly sprayed in fields. Seed coating is an approach that has already been used to protect sunflowers from the damaging effects of *O. cumana*. For example, seed coating with herbicides or salicylic acid has been shown to be effective in reducing *O. cumana* pressure on sunflowers (Jorge et al., 2003; Yang et al., 2016).

Thus, this study lays the first foundations of a new phytosanitary method to control broomrape parasitism with highly specific and biodegradable natural substances, the miPEPs (Farrell et al., 2011). It is expected that the increasing availability of genomic data will allow the extension of this control method to other damaging parasitic pest species from the *Striga* and *Phelipanche* genera.

AUTHOR CONTRIBUTIONS

Sabine Tourneur and Philippe Delavault conceived and designed the research. Sabine Tourneur performed most of the experiments. Jean-Philippe Combier and Serge Plaza identified the sequence of miRNAs and miPEPs in *O. cumana*. Jean-Philippe Combier, Serge Plaza, and Stéphane Muños provided helpful discussions. Philippe Delavault wrote the manuscript with input from all authors.

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CONFLICT OF INTEREST STATEMENT

Authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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