This is a post-print version of:

FERNANDEZ-APARICIO M., RISPAIL N., PRATS E., MORANDI D., GARCIA-GARRIDO J.M., DUMAS-GAUDOT E., DUC G., RUBIALES D., 2010

Parasitic plant infection is partially controlled through symbiotic pathways

Weed Research 50: 76-82.

DOI 10.1111/j.1365-3180.2009.00749.x

The printed version can be visited at:

<http://onlinelibrary.wiley.com/doi/10.1111/j.1365-3180.2009.00749.x/full>

Plant partially controls parasitic plant infection through the symbiotic pathways

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Short title: The symbiotic pathways controls parasitic plant

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Total number of Words: 3603 Number of Figures: 3 Number of tables: 1

Summary

Legumes are the unique genera interacting with *Rhizobium*, arbuscular mycorrhizal (AM) fungi, and parasitic plants. To dissect common parts of these three plant-organism interactions, infection by *Orobancha crenata* was studied in mutants with altered symbiotic phenotypes of *Medicago truncatula* and *Pisum sativum*. *O. crenata* inoculation of mutant lines carrying defective mutation in the genes *dmi2/sym19* and *dmi3* resulted in an increase in *O. crenata* establishment. Similarly, inoculation of mutants carrying mutation in the gene *sun1/sym29* that controls the autoregulation mechanism of the symbiosis, also lead to a significant increase in haustoria formation. Altogether, our results suggest that parasitic plants infection is partly controlled by both the conserved symbiotic pathway that mediates symbiont recognition and establishment and the autoregulation mechanism that regulates the extent of colonisation by rhizobium and AM fungi.

Keywords: *Medicago truncatula*; *Pisum sativum*; *Orobancha crenata*; *Rhizobium*; Mycorrhiza; Symbiosis; Parasitism; *dmi2*; *dmi3*; *sun1*; *sym19*; *sym29*;

1 **Introduction**

2 Plants are constantly interacting with organisms present within their rhizosphere. The
3 arbuscular mycorrhizal (AM) fungi of the genus *Glomeromycota* establish symbiotic
4 relationships with most plant species from mosses to dicots concomitantly with the land
5 colonisation by terrestrial plants (Kistner & Parniske, 2002). More recently during
6 evolution, bacteria from the genus *Rhizobium* evolved the ability to induce the
7 formation of a specific root organ, the nodules, in a more restricted number of plants,
8 the Fabaceae. The nodules fix atmospheric nitrogen in exchange of plant carbohydrates
9 (Kistner & Parniske, 2002). Host mutants impaired in both, nodulation and
10 mycorrhization, indicated that the signalling pathways controlling these symbiotic
11 processes are largely overlapping (Duc et al., 1989; Sagan et al., 1995, Kistner &
12 Parniske, 2002).

13 Apart for these beneficial interactions, plants are also constantly challenged by
14 pathogenic organisms including the parasitic plants of the *Striga*, *Orobanche* and
15 *Phelipanche* genera (Rubiales, 2003; Khan et al., 2008). These parasites have lost their
16 autotrophic way of life during evolution and have evolved to parasitize most mono- and
17 dicotyledon plants. As with symbiotic interactions, a complex dialogue has to occur for
18 efficient parasitisation of the host (Rispaill et al., 2007; Fernández-Aparicio et al.,
19 2008a). This includes 1) the recognition of the host by the symbiont/parasite leading to
20 profound metabolic and morphological changes that orient the organism toward host, 2)
21 attachment of the symbiont/parasite to the host root and simultaneous perception of a
22 putative symbiotic/parasitic associated signal by the host root, 3) penetration and
23 establishment within the host root. In case of parasitic plants, perception by the parasite
24 of exuded host root stimulants triggers seed germination. The germination gives rise to
25 the radicle that elongates toward the host root scouting concentration gradient of the

1 stimulant and adheres to the host root through the formation of an appressorium. Then,
2 *Orobanche* penetrates through the cortex due to mechanical pressure and enzyme
3 activity, grows to the vascular cylinder and develops the haustorium that acts as the
4 endophytic bridge through which all bidirectional transfer between host and parasite is
5 achieved. The outer part of the seedling develops into a nodule that serves as nutrient
6 storage organ and gives rise to a flowering spike that emerges from the soil (reviewed in
7 Pérez-de-Luque et al., 2008).

8 Flavonoids and strigolactones exuded by the host are known to mediate the early
9 recognition step of nitrogen fixing and mycorrhiza symbiosis, respectively (Bécard et
10 al., 1992, Chabot et al., 1992, Akiyama et al., 2005, Cooper, 2007). During coevolution
11 of host and symbiont, strigolactones were selected as a host location signal which
12 promotes branching of germinative hyphae of AM. Strigolactones are also a
13 germination stimulant of the *Orobanche* seeds suggesting that this more recent evolved
14 parasitic plant have hijacked this established communication for its own purpose
15 (Akiyama & Hayashi, 2008, Akiyama et al., 2005). Since AM fungi and *Rhizobium*
16 share common genetic pathways, it is tempting to speculate that these genetic pathways
17 at least partially overlap with that driving plant parasitism.

18 Legumes uniquely interact with *Rhizobium*, AM fungi and parasitic plants. The
19 model legume *Medicago truncatula* (Barker et al., 1990) is natural host of the
20 *Rhizobium*, *Sinorhizobium meliloti*, as well as various AM fungal species including
21 *Glomus mosseae* and *G. intraradices*, and it can be infected by the parasitic plants *O.*
22 *crenata*, *O. foetida*, *P. aegyptiaca* (syn. *O. aegyptiaca*) and *P. ramosa* (syn. *O. ramosa*)
23 (Fernández-Aparicio et al., 2008b). *dmi1*, *dmi2* and *dmi3* are *M. truncatula* genes
24 required both for nodulation and mycorrhization (Catoira et al., 2000). *M. truncatula*
25 gene *dmi2* and its orthologues in *L. japonicus* and pea, SYMRK and *sym19*

1 respectively, encodes a leucine-rich repeat-like receptor kinase acting near a point of
2 molecular convergence of AM and legume-rhizobium signalling (Endre et al., 2002,
3 Stracke et al., 2002). This leucine-rich repeat-like receptor kinase is also required for the
4 actinorhizal symbiosis involving *Frankia* bacteria (Markmann et al., 2008) and
5 facilitates root colonisation of the root-knot nematode *Meloidogyne incognita*
6 (Weerasinghe et al., 2005). *dmi2* is located at the head of a convergent pathway
7 integrating all symbiotic signals that is activated once the specific symbiotic signal is
8 perceived by its specific receptor. Upon activation, *dmi2* induces a still poorly
9 understood transduction pathway that recruits the putative nuclear potassium channels
10 encoded by *dmi1* (Ané et al., 2004) and its *L. japonicus* and pea (*Pisum sativum*)
11 orthologues, CASTOR/POLLUX (Imaizumi-Anraku et al., 2005) and *sym8* respectively
12 (Imaizumi-Anraku et al., 2005, Edwards et al., 2007) which ultimately induces calcium
13 spiking in the cell nucleus. This spiking is sensed by the *M. truncatula* gene *dmi3* also
14 known as CCaMK that encodes a calcium/calmodulin dependent kinase and induces
15 symbiotic gene expression that finally leads to symbiont establishment within the host
16 root (Lévy et al., 2004). On the other hand, the *M. truncatula sunn* gene and its pea
17 orthologue *sym29* (Levy et al., 2004) encode another leucine-rich repeat-receptor like
18 kinase that is required for the symbiotic autoregulation system, which regulates the
19 extent of host colonisation by the symbiont (Schnabel et al., 2005). In association with a
20 supernodulation phenotype, Morandi et al. (2000) observed more colonisation by the
21 mycorrhizal fungi *Glomus mosseae* of a *sym29* mutant of pea and the majority of *sunn*
22 mutants of *Medicago truncatula*, than their corresponding wild types suggesting
23 common factors of regulation for both symbioses. The objective of the present study
24 was to dissect common parts of these three plant-organism interactions. To this aim we

monitored the susceptibility to the plant parasite *O. crenata* of several *M. truncatula* and pea mutants deficient for *dmi2/sym19*, *dmi3* and *sun1/sym29* genes.

Materials and Methods

Plants and inoculation

The *M. truncatula* genotypes used were: the wild type line J5, its non-nodulating and non-mycorrhizal mutants TR89 (defective for *dmi2* gene) (Sagan et al., 1995) and TRV25-25 (defective for *dmi3* gene) and its supernodulated mutant TRV17 (defective for *sun1* gene). These mutants were selected after gamma ray irradiation and described in Morandi et al. (2000, 2005) and Schnabel et al. (2005). The pea genotypes used were: the wild type Frisson, its non-nodulating and non-mycorrhizal mutant P4 (defective for *dmi2* orthologue), and its supernodulated mutants P116 and P87 (defective for *sun1* orthologue; Table 1). These mutants were selected after EMS mutagenesis and described in Duc & Messenger (1989) and Krussel et al. (2002).

Experiments were performed in mini-rhizotrons (Fernández-Aparicio et al., 2008) in the absence of Rhizobia and Mycorrhiza. Seeds of *M. truncatula* and pea were induced to germinate by means of scarification and cold stratification for 48 h in Petri dish. One 4 days old seedling was transplanted per square Petri dish filled with sterile perlite and covered in the upper surface, by a sheet of glass-fiber filter paper (GFFP; Whatman International Ltd, England) (12x12 cm). Petri dishes were previously punctured to allow development of host seedling stems. The dishes were sealed with parafilm and wrapped in aluminium foil and vertically stored during 15 days in a growth chamber (20± 2°C, 12/12h day/night rhythm, 200 µmol m⁻² s⁻¹).

Orobancha crenata seeds were surface-sterilized by immersion in 0.5% formalin with 1% Tween® for 2 h and 20 min at 50°C, followed by three rinses in distilled water. Seeds were air-dried before use. Fifty seeds per square centimetre were sown on sheets of GFFP wetted with sterile distilled water. Each sheet was placed in one square Petri dish (12x12 cm). The Petri dishes were sealed with parafilm and incubated in the dark during 11 days at 20± 2°C to promote necessary warm stratification of seeds to germinate.

Then, the *Orobancha* seeds were evenly distributed at a density of 50 seeds cm⁻² on the GFFP on which the plants were developing its roots. *M. truncatula* accessions are known to induce very low percentages of germination of *O. crenata* seeds (Fernández-Aparicio et al., 2008b). Thus, the synthetic germination stimulant GR24 (provided by Dr Zwanenburg, University of Nijmegen, The Netherlands) was added simultaneously to *Orobancha* seed distribution at concentration of 10 ppm. to increase the number of germinated parasites with the chance of start the parasitic process. The experiment consisted on six replications per accession. Plants were irrigated using a Hoagland's nutrient solution (Hoagland & Arnon, 1950) twice per week.

Parasitic plant development monitoring

After thirty five days of plant growth in Petri dish, germinated *Orobancha* seeds that were close (< 3 mm) to the host roots were observed under a stereoscopic microscope at ×30 magnification to establish percentage of radicle contact as the number of *Orobancha* germlings that established contact with host root surface. The percentage of established haustorium and total number of *Orobancha* nodules per host plant were then recorded five and ten days later respectively.

Statistical analysis

Assays were developed using a completely randomised design. Data recorded as percentages were transformed to arcsine square roots (transformed value = $180/\pi \times \arcsin [\sqrt{(\%/100)}]$) to normalize data and stabilize variances throughout the data range, and subjected to analysis of variance using GenStat 7.0, after which residual plots were inspected to confirm data conformed to normality. For comparison between a specific mutant and the wild type, a contrast analysis (Scheffe's F) for each parameter was performed.

Results and Discussions

1. The DMI pathway controls *O. crenata* establishment

None of the *dmi2* and *dmi3* deficient mutants of *M. truncatula* tested had a different percentage of germinated *O. crenata* seeds that contacted with the host roots compared to the wild type (Fig. 1A). This suggests that the mutations in *M. truncatula* do not modify its capacity to attract the germinated parasitic seed radicles. Surprisingly, mutations in the pea *dmi2* orthologues, *sym19*, increased the number of germinated *O. crenata* seeds contacting the host roots (Fig. 2A). Pea is highly susceptible to *O. crenata* while *M. truncatula* is an inappropriate host that can only slightly be infected (Fernández-Aparicio et al., 2008b, Rubiales et al., 2009). Induction of germination of *O. crenata* by *M. truncatula* accessions in absence of GR24 is low (below 15%) (Fernández-Aparicio et al., 2008b), what contrast with the high induction by pea accessions (above 43%) (Pérez-de-Luque et al., 2005). Thus, the parasite germination inducer and chemo attractant produced by these legumes are likely to be different,

1 which may explain the difference in their capacity to attract *O. crenata* seeds and the
2 effect of the mutation.

3 On the other hand, infection of *M. truncatula* TR89 (defective for *dmi2* gene) and
4 TRV25-25 (for *dmi3* gene) mutants led to a highly significant increase in the parasitic
5 nodule establishment compared to wild type infection both in terms of haustoria
6 formation, estimated as the number of established parasites from 100 parasitic
7 individuals which were in contact with host root ($p < 0.05$ and $p < 0.01$, respectively; Fig.
8 1B), and total number of *O. crenata* nodules per plant ($p < 0.05$ and $p < 0.001$,
9 respectively; Fig. 1C). Similarly, pea P4 (defective for *sym19*) showed a significant
10 increase in the level of parasitic nodule establishment in terms of haustoria formation
11 and nodule number per plants ($p < 0.05$ and $p < 0.001$; Figs. 2B and 2C). These data
12 suggest that mutants in these two genes are more susceptible to the parasite infection
13 than the wild type and that these two genes may play an opposite role in response to
14 symbionts or to parasitic plants. As far as we know, this is the first time that the DMI
15 pathway is described as negative regulator of plant infection in legume. Indeed, this
16 pathways so far have been shown to be required for the establishment of several
17 beneficial symbionts within the host roots including the nitrogen-fixing bacteria
18 *Rhizobium*, the cyanobacteria *Frankia* and the AM fungi (Endre et al., 2002, Stracke et
19 al., 2002, Markmann et al., 2008). In addition, the *L. japonicus* SYMRK have also been
20 shown to facilitate root colonisation of the root-knot nematode *Meloidogyne incognita*
21 in a Nod factor signalling-like manner (Weerasinghe et al., 2005). The *dmi2/symRK*
22 mutants have been shown to have a higher sensitivity to touch (Esseling et al., 2004)
23 and an aberrant defence reaction during symbiont establishment (Limpens et al., 2005).
24 Thus this receptor in legumes has been recently described as a “guard” that converts
25 basal defence reaction to benefit microbial accommodation (Holster, 2008). The

negative role of *dmi2* and *dmi3* on *O. crenata* infection detected here and the high conservation of *dmi2* in plant found even in species that do not form symbiotic interaction led us to speculate a much more general function of the DMI pathways and in particular of DMI2/SYMRK during plant root-organism interaction. Indeed, it may be possible that DMI2 and its subsequent signalling pathways is the integrator of the secondary signals generated by the recognition of interacting organisms allowing switching basal defence according to the nature of the interacting organism. The extreme conservation of DMI2/SYMRK in all plant species including species that do not form symbiosis such as *Arabidopsis* (Markmann et al., 2008), which do not form any symbiotic interaction, is in favour of this more general function of this receptor as basal defence switch

2. The autoregulation mechanism control the extent of *O. crenata* infection

The extent of host root colonisation by its symbiont is permanently and strictly controlled by the host to balance the resulting carbohydrate cost with the beneficial effect of the symbiont (Fig. 3). This regulatory mechanism of symbiotic invasion, the autoregulatory mechanism, is controlled by a still unknown shoot-derived signal and is mediated by a leucine-rich repeat-receptor like kinase encoded by the orthologue of the *Arabidopsis thaliana clv1* gene, called *sun* in *M. truncatula* and *har1* in *L. japonicus*. *sun* and *har1* deficient mutants show a hypernodulant and hypermycorrhization phenotype (Table 1) (Morandi et al., 2005, Nishimura et al., 2002, Schnabel et al., 2005). The TRV17 mutant harbouring the *sun4* mutation that leads to a dead allele of the SUNN protein was used to determine whether this symbiotic autoregulation system also regulates the response to parasitic plants (Morandi et al., 2000). This mutant

showed more haustoria ($p < 0.001$) and nodules ($p < 0.001$) formation, with around four fold more healthy *O. crenata* nodules observed on the mutant roots, indicating that this mutant has also a hyperparasitisation phenotype (Figs. 1B and 1C, Table 1). Interestingly, a separate study of the TRV17-*O. foetida* interaction also showed a higher level of haustoria and nodule formation (data not shown). Additionally, the analysis of two *sym29* pea mutant lines, P119 and P87, carrying a mutation in the *sun* orthologue had significantly more haustoria formation ($p < 0.01$ and $p < 0.001$, respectively) although this did not lead to a higher number of mature nodules probably due to early abortion of the infection events (Fig. 2B and C). Similarly, *har1* deficient mutant of *L. japonicus* was also hyperinfected by the root-knot nematode *Meloidogyne incognita* (Lohar & Bird, 2003). Thus the autoregulation mechanism seems to be a general pathway to control the extent of “parasitisation” performed by the plant symbionts or real parasites. In this more general context, this autoregulation mechanism, would be a second level of defence acting once the basal defence mechanism have been overwhelmed or specifically switched off (Fig. 3).

Conclusion

In the present study, we show for the first time that two different pathways initially described for the symbiotic interaction and the closely related root-knot nematode interaction are also involved in response to parasitic plant infection. On one hand, we determined that the DMI pathways and in particular DMI2 play a negative function during *O. crenata* infection in the legume species *M. truncatula* and pea by limiting the number of infection events. This fact together with the extreme conservation and structural complexity of the DMI2 protein in plant species favour a more complex role of this pathway as a general switch of basal defence than initially thought. In this sense,

our finding open new perspective to the highly complex function of this pathway in plant and bring preliminary evidence explaining the diversity of the DMI2 protein. On the other hand, we also found that the autoregulation mechanism limiting host root colonisation by symbiont below detrimental level is also part of the defence mechanism against *O. crenata*. This findings indicate that this autoregulatory mechanism would be a second general defence mechanism acting after the basal defence mechanism.

Acknowledgement

This research was supported by projects AGL2008-01239/AGR and P07-AGR-02883.

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Tables

Table 1. Phenotypes and mutations of the different *M. truncatula* and pea genotypes used in this study. +: normal phenotype, -: completely resistant phenotype, -/+: leaky resistant phenotype with rare infection events, +++: hypersusceptible phenotype

Species	Genotype	Mutated gene	Gene Function	Phenotype		
				<i>Nod</i>	<i>Myc</i>	<i>Orobanche</i>
<i>M. truncatula</i>	J5 (wild type)	-	-	+	+	+
<i>M. truncatula</i>	TR89	<i>Mtsym2 (dmi2)</i>	LRR receptor kinase	-/+	-/+	++
<i>M. truncatula</i>	TRV25-25	<i>Mtsym13 (dmi3)</i>	CcaM kinase	-	-	++
<i>M. truncatula</i>	TRV17	<i>Mtsym12 (sunn4)</i>	CLV1-like kinase	+++	++	+++
<i>P. sativum</i>	Frisson (wild type)	-	-	+	+	+
<i>P. sativum</i>	P4	<i>sym19</i> (orthologue for <i>dmi2</i>)	LRR receptor kinase	-/+	-/+	++
<i>P. sativum</i>	P87	<i>sym29</i> (orthologue for <i>sunn</i>)	CLV1-like kinase	+++	+++	+++
<i>P. sativum</i>	P116	<i>sym29</i> (orthologue for <i>sunn</i>)	CLV1-like kinase	+++	+++	+++

Figure legend

Figure 1. Assessment of susceptibility of *M. truncatula* genotypes to *Orobanch*
crenata. (A) Percentage of germinated *O. crenata* seeds that contacted host root, (B)
Percentage of contacting *O. crenata* that form an efficient haustorium and (C) Total
number of mature parasite nodule. Vertical bars represent standard error for n=6. Stars
indicate statistically significant difference between the wild type and the mutant at the
level of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ as determined by contrast analysis.

Figure 2. Assessment of susceptibility of *P. sativum* genotypes to *Orobanch* *crenata*.
(A) Percentage of germinated *O. crenata* seeds that contacted host root, (B) Percentage
of contacting *O. crenata* that form an efficient haustorium and (C) Total number of
mature parasite nodule. Vertical bars represent standard error for n=6. Stars indicate
statistically significant difference between the wild type and the mutant at the level of
 $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ as determined by contrast analysis

Figure 3. Schematic representation integrating the genetic control of symbiotic and
parasitic interaction.

Figures

Figure 1.

Figure 2.

Figure 3.