Quinone oxidoreductase 2 is involved in haustorium development of the parasitic plant *Phtheirospermum japonicum*

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**ABSTRACT**

The family Orobanchaceae includes many parasitic plant species. Parasitic plants invade host vascular tissues and form organs called haustoria, which are used to obtain water and nutrients. Haustorium formation is initiated by host-derived chemicals including quinones and flavonoids. Two types of quinone oxidoreductase (QR) are involved in signal transduction leading to haustorium formation; QR1 mediates single-electron transfers and QR2 mediates 2-electron transfers. In the facultative parasite *Triphysaria versicolor*, QR1 is involved in haustorium induction signaling, while this role is played by QR2 in the model plant *Phtheirospermum japonicum*. Our results suggest that there is functional diversification in haustorium signaling molecules among different species of the Orobanchaceae.

Parasitic plants in the Orobanchaceae include harmful pests targeting a large range of crops. The parasites interact with their hosts via haustorium, a unique organ connecting own vasculature to the host conducting system to obtain water, nutrients, and small substances. To develop strategies for controlling plant parasites, it is important to understand the intricate molecular mechanisms that control the interactions between host and parasite. Parasitic plants initiate haustorium development upon perception of host-derived haustorium-inducing factors (HIFs). HIFs include quinones and flavonoids, which are broadly distributed in nature. The quinone 2,6-dimethoxy1,4-benzoquinone (DMBQ), which was originally identified in sorghum root extracts, is an active HIF for many Orobanchaceae species. Recognition by the parasites is associated with the redox potential of DMBQ. The quinone oxidoreductase enzymes (EC 1.6.5) catalyze quinone redox changes via the transfer of one or 2 electrons. The NADPH-dependent quinone oxidoreductase, QR1, catalyzes the transfer of single electrons from quinones to generate the highly reactive free radicals semiquinones, leading to the formation of reactive oxygen species. Another type of quinone oxidoreductase (QR2) catalyzes the divalent reduction of quinones to hydroquinones, possibly working as a scavenger of reactive quinone molecules. In the facultative parasite *Triphysaria versicolor*, both QR1 and QR2 transcripts are upregulated during haustorium induction triggered by DMBQ. However, only *Tv-QR1* is differentially regulated in response to host contact. The knockdown of *QR1* but not *QR2* expression in *T. versicolor* roots significantly reduced the frequency of haustorium formation. These observations suggest that *QR1* is involved in haustorium initiation signaling in *T. versicolor*.

The facultative parasite *Phtheirospermum japonicum* also belongs to the Orobanchaceae and has become a model for studies of parasitic plants. *P. japonicum* responds to HIFs in a similar way to other parasitic Orobanchaceae and forms lateral haustoria (i.e., haustoria formed on the lateral side of parasitic roots). To expand our understanding of the molecular events associated with haustorium development, we sequenced the transcriptomes of *P. japonicum* roots and haustorial tissues and generating a list of genes that are actively expressed during infection. Gene expression patterns were investigated after exposure of parasite roots to DMBQ over a period ranging from 30 minutes to 48 hours. Although most genes in the haustorium transcriptome are similar among parasitic plant species in the Orobanchaceae, there are some differences. For example, our analyses revealed that in *P. japonicum* the expression patterns of *QR1* and *QR2* differ from those in *T. versicolor*. In *P. japonicum*, *Pj-QR1* expression is not altered by contact with host root exudates or DMBQ treatments. In contrast, *Pj-QR2* is highly upregulated in response to both treatments. To investigate the expression patterns of *QR1* and *QR2* in other parasitic species, we analyzed the *QR1* and *QR2* homologs in *S. hermonthica*. The homologs *Sh-QR1*, *Sh-QR2*, *Pj-QR1*, and *Pj-QR2* were amplified from a cDNA library using primers shown in Table 1, and the full length sequence was confirmed by a combination of RACE® and Sanger-based strategies as described previously. A phylogenetic analysis of the full length putative QR proteins from *T. versicolor*, *P. japonicum*, and *S. hermonthica* clearly assigned the *QR1* and *QR2* homologs from all 3 species into distinct nodes with high support values (Fig. 1). Transcription profiles for the *Sh-QR* genes were obtained by mapping the RNA-Seq reads from different
developmental stages onto the Sh-QR1 and Sh-QR2 sequences (Fig. 2). As a control we included the housekeeping gene β-tubulin 1 (TUB1, Unigene accession StHe1GB1_52449). Both Sh-QR1 and Sh-QR2 showed basal transcriptional levels at the seedling stage. Similar to Pj-QR2, the transcriptional level of Sh-QR2 was increased during the haustorial development stages, while the Sh-QR1 expression was stably maintained in both parasitic and non-parasitic vegetative tissues. Both genes showed increased expression with the development of reproductive structures. Our data indicate that although each group of QR homologs in T. versicolor, P. japonicum, and S. hermonthica shares the same origin, their expression patterns differ during haustorial development.

We performed an RNA interference (RNAi) experiment to investigate the relevance of Pj-QR2 in P. japonicum haustorial development. Fragments of Pj-QR2 were amplified with the RNAi primers shown in Table 1 and inserted into the silencing vector pHG8-YFP. The transcript levels of Pj-QR2 were quantified by real-time qPCR in the roots of plants transformed with the silencing construct (pHG8-QR2), and roots harbouring the empty vector (pHG8-YFP) were used as negative controls. In the pHG8-QR2 plants the transcript levels of Pj-QR2 were reduced to about one tenth of the levels in the control plants (Fig. 3A). The total numbers of roots, numbers of lateral roots, and root lengths were similar between the pHG8-QR2 and pHG8-YFP plants (Table 2). However, the percentages of haustoria formed after to exposure to host root exudates were significantly lower in the pHG8-QR2 roots than in the pHG8-YFP plants.

Table 1. Primers sequences.

| Primer Name | sequence | Primer size | Amplicon size | Application |
|-------------|----------|-------------|---------------|-------------|
| ShQR1-F     | CCCAATTGCCAACTTTATTCA | 21 | 1375 bp | Full length |
| ShQR1-R     | AGTAGAAGCTCTGAGGGCCTGCG | 20 | Full length |
| ShQR2-F     | CCACCTTCACACACCAACAC | 22 | 702 bp | Full length |
| ShQR2-R     | TTCCGCCATTGCATCAAAATAAA | 20 | Full length |
| PjQR1-F     | CAAGCTCTCTGATAAACACACAAAG | 26 | 1204 bp | Full length |
| PjQR1-R     | TTATGCTGATTITATTATCTGCGATCA | 30 | Full length |
| PjQR2-F     | CCAACCAATCTACTAACCAC | 24 | 856 bp | Full length |
| PjQR2-R     | GATGACCAATGATTCTTGCG | 19 | Full length |
| PjQR2-RNAi-F | GGCAGGTCAGAAACACTCTG | 21 | 448 bp | RNAi target |
| PjQR2-RNAi-R | AAACCTCTGATGAGGATG | 20 | RNAi target |
| PjQR2-qPCR-F | ATGTACATCGCAGGATCGAC | 20 | 73 bp | qRT-PCR |
| PjQR2-qPCR-R | GATGACCAATGATTCTTGCG | 20 | Full length |

Figure 1. Phylogenetic analysis of the QR homologs of T. versicolor, P. japonicum, and S. hermonthica. The tree was generated with the MEGA7 software using the maximum likelihood statistical method. The putative QR proteins were aligned using the CLUSTALW algorithm with default settings. The bootstrap percentages of 10000 replicates are shown on the internal nodes. The topology of the tree was also confirmed by the UPGMA and neighbor-joining methods.

Figure 2. Expression profiles of Sh-QR1 (orange) and Sh-QR2 (gray) at different developmental stages in the S. hermonthica life cycle. Transcript levels were determined as log2 (RPKM +1) values from an RNA-Seq analysis. For reference, we also included the expression profile of the S. hermonthica TUB1 housekeeping gene (blue).
roots (Fig. 3B). This result indicated that *Pj-QR2* is involved in haustorium formation in *P. japonicum*.

In summary, our results suggest that the expression patterns and functions of *QR1* and *QR2* homologs during haustorium formation are diversified among parasitic plant species. In *T. versicolor*, *Tv-QR1* has important roles in haustorium induction after exposure to HIFs, while in *P. japonicum*, haustorium formation is mediated by *Pj-QR2*. The high expression levels of the *S. hermonthica* homolog *Sh-QR2* during the parasitic stages suggest that, like *Pj-QR2*, *Sh-QR2* may also be involved in haustorium development. The biologic function of *Tv-QR1* may not be specific for haustorium induction since this gene is recruited for floral development and is responsive to non-HIF quinones.20 These observations suggest that *Tv-QR1* may play a role in oxidative stress signaling. Genetics studies of natural populations of *T. versicolor* from Northern California indicated that the *Tv-QR1* alleles retain a high level of molecular diversity at the nucleotide and amino acid levels, with the highest non-synonymous substitution rates in the catalytic domain.22 Such sequence diversity was not found in *Tv-Pirin*, another gene that is involved in *T. versicolor* parasitism.22 Such plasticity in *QR1* and *QR2* may confer evolutionary advantage for parasitic plants that recognize different factors from a wide range of hosts. Thus, the functions of *QR1* and *QR2* may have been diversified during evolution, depending on the species. Future studies will help to elucidate the diverse roles of these quinone oxidoreductases in plant parasitism.

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No potential conflicts of interest were disclosed.

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Table 2. Root morphology in RNAi (pHG8-QR2) and control (pHG8-YFP) *P. japonicum* lines.

|                          | pHG8-YFP | pHG8-QR2 |
|--------------------------|----------|----------|
| Number of lateral roots  | 1.16 (± 0.5) | 1.0 (± 0.2) |
| Root length (mm)         | 10.26 (± 1.7) | 7.6 (± 5.1) |
| Total number of transgenic roots (N) | N = 90 | N = 113 |

Data are means and standard error (±) of 3 to 5 biologic replicates with 5–15 independent transgenic roots per experiment.
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