A peroxidase gene expressed during early developmental stages of the parasitic plant *Orobanche ramosa*

Clara Isabel González-Verdejo1,*, Xabier Barandiaran2, María Teresa Moreno3, José Ignacio Cubero1 and Antonio Di Pietro1

1 Departamento de Genética, Universidad de Córdoba, Campus de Rabanales C5, E-14071 Córdoba, Spain
2 Dominion Biotecnologia, C/ Josefa Valcárcel 3-5, E-28027 Madrid, Spain
3 CIFA, Alameda del Obispo, IFAPA, Area de Mejora y Biotecnología, Apdo. 3092, E-14080 Córdoba, Spain

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Abstract

Broomrapes (*Orobanche* spp.) are holoparasitic weeds that cause devastating losses in many economically important crops. The molecular mechanisms that control the early stages of host infection in *Orobanche* are poorly understood. In the present study, the role of peroxidase has been examined during pre-infection growth and development of *O. ramosa*, using an *in vitro* model system. Peroxidase activity was histochemically localized at the tips of actively growing radicles and nascent attachment organs. Addition of exogenous catalase resulted in a significant reduction in the apical growth rate of the radicle. The *prx1* gene encoding a putative class III peroxidase was cloned from a cDNA library of *O. ramosa* and was found to be expressed specifically during the early stages of the parasitic life cycle. The exogenous addition of sucrose resulted in significantly reduced *prx1* transcript levels and in a dramatic change in radicle development from polarized apical growth to isotropic growth and the formation of tubercle-like structures. The results indicate an important role of peroxidases during the early parasitic stages of *Orobanche*.

Key words: Attachment organ, catalase, cDNA library, germination, *in vitro* culture, polarized growth, sucrose.

Introduction

The ability of plants to parasitize other plants has originated independently at least ten times during the evolution of angiosperms (Wrobel and Yoder, 2001), and approximately 1% of all flowering plants are currently recognized as parasitic (Keyes *et al.*, 2001). The genus *Orobanche* (broomrapes) comprises a number of holoparasitic weeds that cause devastating losses in many economically important crops (Parker and Riches, 1993). The species *O. ramosa* attacks a wide range of host plants, including tobacco, tomato, potato (Kreutz, 1995), as well as the model plant *Arabidopsis thaliana* (Goldwasser *et al.*, 2000).

The infection process of many soil-borne parasitic plants entails a defined sequence of tightly regulated developmental events, including host-induced germination of seeds, directed growth of the radicle towards the host root, and development of an attachment organ that eventually differentiates into an infection organ called the haustorium (Kuijt, 1969; Joel *et al.*, 1995). These pre-infection processes are controlled by endogenous signals as well as by signalling compounds originating from the host plant, so-called xenognosins (Chang and Lynn, 1986; Estabrook and Yoder, 1998; Albrecht *et al.*, 1999; Yoder, 1999; Bouwmeester *et al.*, 2003). During the early stages of infection, the generation of reactive oxygen species (ROS), such as hydrogen peroxide (*H₂O₂*) by peroxidases, is thought to play an important role in mediating the parasite responses to both the endogenous and the xenognosin signals (Kim *et al.*, 1998; Keyes *et al.*, 2001).

Plant peroxidases of the haem type (class III) are bifunctional enzymes that can act in two different catalytic modes (Passardi *et al.*, 2004). In the presence of *H₂O₂* and phenolic substrates, they operate in the peroxidase cycle and are engaged in the synthesis of lignin and other phenolic polymers. However, if the phenolic substrates are replaced by NADPH or related reduced compounds,
a chain reaction starts that provides the basis for the H$_2$O$_2$-producing NADH-oxidase activity of peroxidases (Chen and Schopfer, 1999). Class III peroxidases are apoplastic enzymes that are involved in lignification and cell elongation (Passardi et al., 2004). In the parasitic plant Striga asiatica, two apoplastic class III oxidases (PoxA and PoxB) have been identified and the encoding genes have been cloned (Kim et al., 1998). Both enzymes were capable of oxidizing phenols into benzoquinones, a class of compounds that is known to induce parasitic development in Striga. In Orobanche, the role of class III peroxidases is currently unknown, although their activity has been detected previously in O. cumana and associated with avirulence on resistant sunflower cultivars (Antonova and ter Borg, 1996).

Throughout the early infection stages, Orobanche undergoes a series of striking morphological changes, ranging from highly polarized apical growth toward the host root to attachment organ initiation and, finally, proliferation into round structures denominated haustoria (Kuijt, 1969; Joel et al., 1995). The fact that the latter developmental response occurs after the contact with the root has been established, suggests a possible role of host compounds, including oxidizing phenols into benzoquinones, a class of compounds that is known to induce parasitic development in Striga. In Orobanche, the role of class III peroxidases is currently unknown, although their activity has been detected previously in O. cumana and associated with avirulence on resistant sunflower cultivars (Antonova and ter Borg, 1996).

The aim of the present study was to examine the role of ROS and class III peroxidase during the pre-infection growth and development of O. ramosa. Initially, a histochemical approach was followed to determine the presence of peroxidase activity at the sites of polarized radicle growth. Moreover, the prxl gene, encoding a putative class III peroxidase orthologous to the PoxA and PoxB genes of Striga, was cloned from a cDNA library constructed from O. ramosa. Expression of prxl during different stages of the parasitic life cycle was determined by northern analysis. Finally, the effect of sucrose, a readily assimilated carbon source, on prxl expression and on polarized radicle growth was studied.

**Materials and methods**

**Plant material**

To obtain seedlings for RNA extraction and peroxidase assays, O. ramosa seeds were collected from plants parasitizing tobacco in fields located in Granada (Spain). Seeds were surface-sterilized by treating them for 2 h with a solution of 0.5% formaldehyde and 0.1% Tween 20, followed by a 20 min incubation at 50 °C. Subsequently, seeds were rinsed three times with sterile distilled water (González-Verdejo et al., 2005). Approximately 4000 O. ramosa surface-sterilized seeds were sown in 9 cm Petri dishes containing an autoclaved, moist, glass-fibre, filter paper (Whatman) and maintained at 24 °C in the dark for an 8 d conditioning period, before adding 0.5 ml of a solution of 0.034 mM GR24 (van Hezewijk et al., 1993). GR24 was routinely purchased from the University of Nijmegen, The Netherlands. Germinated seeds (approximately 70%) were used both for in vivo detection of peroxidase activity and for RNA extraction experiments.

To obtain tubercle-like organs in vitro, a stronger seed disinfection protocol was used. Seeds were treated for 10 min with 5% sodium hypochlorite containing 0.1% Tween 20 and rinsed three times with sterile distilled water (González-Verdejo et al., 2005). For sucrose assays, disinfected Orobanche seeds were transferred onto 6 cm Petri plates with solid MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose. Seeds were maintained in the dark in controlled growth chambers at 24 °C and, after 10 d of conditioning, they were treated for 1 d with GR24 (Zhou et al., 2004). Germination was examined after 20 d using a Leica DMR microscope and photographs were recorded with a Leica DC 300F digital camera. All experiments were performed at least three times with similar results. The continuous presence of seeds on sucrose-containing MS medium led to the formation of tubercle-like structures that were used for RNA isolation.

O. ramosa flowers were obtained from mature plants parasitizing Nicotiana tabacum plants. N. tabacum seeds were surface-sterilized by immersing them first in 70% ethanol for 2 min, then in 3.5% sodium hypochlorite containing 0.1% Tween 20 for 10 min, and finally rinsing three times with sterile distilled water. O. ramosa seeds were disinfected with a formaldehyde solution as described above. N. tabacum and O. ramosa were maintained in pots under growth chamber conditions (24 °C, 16 h light).

**Extraction of total RNA from O. ramosa tissues**

Seedlings from the different treatments (10 plates or approximately 100 mg per treatment), as well as tubercles obtained in vitro and flowers of O. ramosa were used for RNA extraction using Trizol® (Invitrogen) as a reagent according the manufacturer’s protocol. Seeds were harvested by filtration through a cheesecloth (100 μm pore diameter).

**Histochemical detection of peroxidase activity**

Two methods were used for the histochemical staining of peroxidase activity. In the first protocol, O. ramosa seedlings were carefully removed from the germination media and transferred to a solution containing 10 mM sodium phosphate, 0.03% H$_2$O$_2$, and 0.2% pyrogallol (Aldrich). After 15 min incubation at room temperature, oxidation of pyrogallol was detected by the reddish coloration of the tissue (Kim et al., 1998). The stained seedlings were observed using a Leica DMR microscope and immediately photographed with a Leica DC 300F digital camera.

In addition, the method of De Pinto and Ros-Barceló (1997) was used. Seedlings were transferred to a solution containing 0.1 M TRIS-acetate (pH 5), 0.1 mM 4-chloronaphthol (Sigma), and 0.9 mM H$_2$O$_2$. After 15 min incubation at room temperature, peroxidase activity was visible as a dark brown staining.

For catalase experiments, bovine liver catalase (Sigma C-9322) was dissolved in water at different concentrations (0.1, 0.5, 1, 2, and 3 mg ml$^{-1}$) and 500 μl of each solution was added to the conditioned Orobanche seeds simultaneously with the germination stimulant GR24.

**Construction of a cDNA library of O. ramosa**

Total RNA from O. ramosa seedlings, tubercles obtained in vitro, and flowers was isolated using the Trizol protocol described above. mRNA was isolated from total RNA using the Poly(A) Quik mRNA Isolation kit (Stratagene) according to the manufacturer’s instructions. Starting from 1.5 mg of total RNA, 5.5 μg of mRNA was obtained. Library construction was performed using the cDNA

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**Note:** The text continues with additional details not fully transcribed here.
Applied Biosystems PCR System 9700. Degenerate primers of Torres (1993) were used: 40 cycles with denaturation at 94 °C for 35 s, annealing at 64 °C for 35 s, and extension at 72 °C for 1 min. An initial denaturation step of 5 min at 94 °C and a final elongation step at 72 °C for 7 min were performed. The amplified 600 bp DNA fragment was cloned into pGEM-T (Promega), sequenced, and the sequence obtained was used to design the gene-specific primers oliprx-3 (5'-TTGACTGTGAGGCTCGGGAG-3') and oliprx-4 (5'-AC-CATGCTGTTTCTAAGTGCC-3'). The amplified 140 bp fragment was cloned into pGEM-T and used as a probe to screen the λ-ZAP cDNA library of O. ramosa. Library screening, excision of the vector, and other routine procedures were performed as described in the protocols supplied with the kit.

Sequencing of both DNA strands of the complete cDNA clone was performed at the Servicio Centralizado de Apoyo a la Investigación, University of Córdoba, using the DyeDeoxy terminator cycle sequencing kit (PE Biosystems) on an ABI Prism 377 genetic analyser apparatus (Applied Biosystems). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (Bethesda, MD, USA).

Northern analyses

Total RNA was extracted as described above. Northern analysis and probe labelling were carried out as described by Di Pietro and Roncero (1998) using the non-isotopic digoxigenin (DIG) labelling kit (Roche Diagnostics).

Results

Histochemical detection of peroxidase activity in germinated O. ramosa seeds

To study the presence of peroxidases in O. ramosa, a recently developed in vitro model was used that allows monitoring of the pre-infection stages without the presence of the host plant (Gonzalez-Verdejo et al., 2005). Peroxidase activity was examined histochemically during different stages of germination, radicle growth, and early attachment organ development by staining either with pyrogallol or 4-chloronaphthol (4-CN). A distinct reddish coloration resulting from pyrogallol, which is indicative of peroxidase activity, was visible at the apices of actively growing radicles (Fig. 1A–C) and became somewhat less pronounced during the initiation of attachment organ formation (Fig. 1D, E). Similarly, staining with 4-CN revealed dark staining regions at the tips of radicles and nascent attachment organs (Fig. 1G–L). Removal of the seed coat revealed no activity staining in the inner part of the embryo suggesting the absence of peroxidase activity in this tissue (Fig. 1A). These results indicated that the major peroxidase activity in germinating O. ramosa seeds is restricted to the apical regions of actively growing radicles.

Role of ROS in radicle growth and attachment organ induction

A distinguishing feature of class III peroxidases is their dependence on H2O2 as a co-substrate (Chen and Schopfer, 1999; Passardi et al., 2004). To study further the role of class III peroxidases in germination and apical growth of O. ramosa, the effect of breaking down H2O2 through the exogenous application of catalase was tested. Addition of bovine liver catalase (EC 1.11.1.6) had no effect on the rate of O. ramosa seed germination (results not shown), but resulted in a significant decrease of the apical growth rate of the radicle (Fig. 2). The reduced length of the radicle in the presence of catalase frequently led to the initiation of attachment organ formation in close proximity to the seed (see Fig. 2E, F).

Construction of an O. ramosa cDNA library and cloning of prx1 encoding a putative class III peroxidase

A cDNA library of O. ramosa was constructed with the aim of isolating and characterizing putative class III peroxidase genes. To ensure a broad representation of expressed genes within the library, mRNA was extracted from different developmental stages, including germinated seeds, tubercles obtained in vitro, and flowers. Starting from 1.5 mg of total RNA, 5.5 µg of mRNA was purified and used for cDNA synthesis. The cDNA was packaged into λ-ZAP, a phage that allows excision of the plasmid pBluescript containing the cDNA insert. The final O. ramosa cDNA library contained 2×10^10 plaque-forming units ml^{-1}, with average insert sizes ranging between 0.5 to 4 kb.

For the isolation of the putative O. ramosa peroxidase genes, two degenerate primers (oliprx-1 and oliprx-2) were designed from conserved regions of the S. asiatica peroxidases PoxA and PoxB (Kim et al., 1998). The PCR reaction amplified a 600 bp DNA fragment from O. ramosa genomic DNA that was cloned into the pGEM-T vector (see the Materials and methods for experimental details). After confirming its identity by sequencing, two specific primers, oliprx-3 and oliprx-4 were used to amplify a 140 bp fragment that was used as a probe to screen the O. ramosa cDNA library, leading to the identification of a hybridizing cDNA clone. Sequencing of the insert revealed the presence of an open reading frame (ORF) of 975 nucleotides, which encoded a predicted protein of 325 amino acids with homology to class III plant peroxidases, which was named prx1. The sequence data have been submitted to the Genbank database under accession number DQ073917. The prx1 cDNA clone contained 63 bp of 5' and 168 bp of 3' untranslated sequence. The latter had a possible polyadenylation signal (AATAAA) 111 nucleotides upstream of the poly (A) stretch.
Fig. 1. Histochemical detection, by pyrogallol (A–F) or 4-chloronaphthol (4-CN) (G–L) staining, of peroxidase activity during in vitro seed germination (A–C, G–I) and attachment organ differentiation (D–F, J–L) of Orobanche ramosa. Nascent attachment organs are visible as radicle tip enlargements of variable size. Regions with peroxidase activity stained in a reddish-brown colour are indicated by arrows. (A) An embryo in which the seed testa has been removed before staining (lower part). (E) A seedling with no activity staining. The scale bar represents 100 μm.

Fig. 2. Orobanche ramosa seeds germinated in vitro in the absence (A–C) or presence of 2 mg ml⁻¹ bovine liver catalase (D–F). Photographs were taken at 3 (A, D), 5 (B, E), and 7 d (C, F) after stimulation with GR24.
Figure 3 shows an alignment of the deduced *O. ramosa* Prx1 protein with class III plant peroxidases. The highest overall identity was detected with PoxB (76.5%) and PoxA (74.6%) from *S. asiatica*. The conserved subdomains denominated BoxA, BoxB, and BoxC (Kim et al., 1998) were present in Prx1, as well as the eight cysteine and two histidine residues conserved in class III plant peroxidases (Tyson, 1992; Kim et al., 1998). His72 is located in the Box A subdomain and was suggested to be involved in acid/base catalysis, whereas His199 in the BoxC subdomain is predicted to serve as the fifth ligand for the haem iron (Welinder, 1992). Located between these two subdomains there is a conserved region of seven amino acids, VSCADIV, designated BoxB (Kim et al., 1998).

Expression of the prx1 gene during different developmental stages of *O. ramosa*

Expression of *prx1* during different stages of the *O. ramosa* life cycle was determined by northern hybridization analysis, using total RNA extracted from germinated seeds, early haustorial structures, tubercles grown *in vitro*, and flowers. High levels of *prx1* transcript were detected in germinating seeds, particularly at the onset of attachment organ differentiation (Fig. 4A). By contrast, no *prx1* transcript was detected during the later stages of the *O. ramosa* life cycle, such as *in vitro*-grown tubercles and flowers.

The absence of *prx1* transcript in tubercles which had been grown *in vitro* on sucrose-containing media, prompted an examination of the physiological effects of sucrose on *O. ramosa* development and on *prx1* expression. It was found that seeds germinated in the presence or absence of 3% sucrose exhibited striking morphological differences. Seeds germinated in the absence of sucrose underwent normal radicle growth and attachment organ initiation, followed by an arrest in growth and development, possibly due to the absence of an external carbon source (Fig. 5A–C). In stark contrast, seeds germinated in the presence of sucrose exhibited an isotropic rather than a polarized growth pattern, resulting in the formation of dense, round structures (Fig. 5D–F). These structures continued to grow throughout

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**Fig. 3.** Amino acid sequence alignment of the predicted Orobanche ramosa prx1 gene product (Genbank accession number DQ073917) with class III peroxidases from Striga asiatica (PoxA, AF043234 and PoxB, AF043235), Nicotiana tabacum (AB027752), and Oryza sativa (AF014468). Conserved amino acids are highlighted on a shaded background. Dashes indicate gaps in the alignments. The position of the degenerate primers oliprx-1/prx-2 is indicated (see text for further details). The histidine residues conserved in class III plant peroxidases are marked by arrowheads and conserved cysteine residues are marked by asterisks. The core region of BoxB is indicated.

**Fig. 4.** Northern analysis for *prx1* transcript accumulation in *Orobanche ramosa*. (A) Expression of *prx1* at the indicated developmental stages of the parasitic life cycle. (B) Expression of *prx1* in radicles and nascent attachment organs at the indicated time points after stimulation with GR24, either in the presence or in the absence of 2 mg ml\(^{-1}\) bovine liver catalase or 3% (w/v) sucrose. Upper panels: the filter was hybridized to the *prx1* probe. Lower panels: total RNA was stained directly on the filter with methylene blue.
several weeks and even months, leading to the formation of globular organs somewhat reminiscent of the tubercles produced in the soil during parasitic infection (results not shown). Interestingly, prx1 expression was significantly reduced in the presence of sucrose, coincident with the absence of polarized radicle growth (Fig. 4B). By contrast, the addition of exogenous catalase had no detectable effect on prx1 transcript levels (Fig. 4B).

**Discussion**

In the present study, the role of peroxidase during the early stages of development of the parasitic plant O. ramosa was examined. To this end, an in vitro system was used in which surface-sterilized and conditioned Orobanche seeds on moist filter paper were stimulated with the synthetic strigol analogue GR24, resulting in germination rates of around 70% and the initiation of attachment organ development in approximately half of the germinated seeds (Gonzalez-Verdejo et al., 2005). The system allows seed germination, radicle elongation, and attachment organ initiation to be monitored in the absence of the host plant. Peroxidase activity, as detected by histochemical staining with two different substrates, specifically localized to the tips of the growing radicle and of nascent attachment organs. Localization of peroxidase at the tips of actively growing roots has been previously reported in seedlings of different dicots and monocots and has been associated with root elongation (Cordoba-Pedregosa et al., 2003) and lignification (de Obeso et al., 2003). In the parasitic plant S. asiatica a model was proposed for attachment organ differentiation, whereby peroxidases would use H2O2 as a co-substrate for the oxidative release of benzoquinone xenognosins from host roots, which, in turn, should trigger attachment organ formation (Kim et al., 1998). In Orobanche such a mechanism appears unlikely, because (i) the presence of the host is not required for attachment organ initiation (Gonzalez-Verdejo et al., 2005), and (ii) removal of H2O2 by exogenous catalase had no effect on the rate of attachment organ initiation (this study). However, treatment with catalase resulted in a significant reduction in radicle elongation, which led to the initiation of attachment organ formation in close proximity to the seed. Together with the histochemical data, these results provide evidence for a role of peroxidase activity and the generation of H2O2 in the apical growth of the radicle in Orobanche. While the exact mechanism whereby H2O2 promotes radicle tip growth remains to be elucidated, this hypothesis is supported by the recent finding that ROS regulate polarized cell growth in A. thaliana through the activation of Ca2+ channels (Foreman et al., 2003; Mori and Schroeder, 2004).

To study further the role of peroxidase during differentiation and infection of O. ramosa, the prx1 gene encoding a putative class III peroxidase of O. ramosa was cloned. Prx1 was isolated from a cDNA library constructed in λ-ZAP, representing different developmental stages of O. ramosa including germinated seeds, tubercles obtained in vitro, and flowers. Besides the prx1 gene, the cDNA library has been used for isolating full-length clones of a number of additional O. ramosa genes (CI Gonzalez-Verdejo et al., unpublished results), suggesting that it represents a useful tool for the future molecular analysis of this species. Significant quantities of prx1 transcript were detected in germinated seeds and during attachment organ initiation, but not in tubercles and shoots. This expression pattern suggests that prx1 may have a specific role during the early pre-infection stages of Orobanche. Interestingly, the poxA and poxB genes of S. asiatica were also found to
be specifically expressed during seed germination, but not in the aerial parts of the plant (Kim et al., 1998).

The absence of prxl transcript in tubercles, which had been obtained in vitro on sucrose-containing media, prompted the testing of the effect of sucrose on O. ramosa development and on prxl expression. Interestingly, exogenous addition of sucrose to germinating O. ramosa seedlings resulted in dramatic changes in growth pattern, from highly polarized apical growth, attachment organ initiation and growth arrest, to indefinite isotropic growth into round tubercle-like structures. This differential developmental response suggests a possible role of sucrose in Orobanche seedling development. Soluble sugars such as glucose and sucrose are known to act as regulators of tuberization (Gibson, 2004). In the case of Orobanche, the exogenous supply of sucrose might mimick the later stages of infection when the parasite is connected to the host vascular system and obtains its carbohydrate nutrients mostly in the form of sucrose (Aber, 1984). The fact that prxl transcript levels were significantly reduced in the presence of sucrose suggests that expression of prxl may be specifically associated with polarized but not isotropic radicle growth, although the existence of a causal relationship remains to be determined.

Taken together, the results from the present study suggest that class III peroxidases could have an important function during early developmental stages of Orobanche. Further studies will be required to elucidate the exact role of peroxidases throughout the infection of the host plant.

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