Plasmodiophora brassicae, the Causal Agent of Clubroot Disease, May Penetrate Plant Cell Walls via Cellulase

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Abstract

Plasmodiophora brassicae causes the clubroot disease of *Brassicaceae* by its obligate biotrophic lifestyle within host roots. The pathogen enters the root cortex, but is soon found near the vascular tissue. There are basically two possibilities for the distribution of *Plasmodiophora* within the root tissue: 1. distribution by simultaneous division with the host cell or 2. by active migration from cell to cell. It has been shown that plasmodia of the pathogen are indeed able to penetrate plant cell walls. We have therefore begun to isolate a cellulase gene from the pathogen by using degenerated primers to different fungal cellulases. It was possible to amplify specifically a fragment from infected roots 14 days after inoculation which was not present in healthy roots and later time points of infection. The fragment was sequenced and showed high homology to various fungal cellulases. Cloning of the complete cDNA and expression analysis of the putative *Plasmodiophora* cellulase is in progress.

Keywords: *Brassica rapa* ssp. *pekinensis*; cellulase; host cell wall lysis; *Plasmodiophora brassicae*

INTRODUCTION

The clubroot disease of the *Brassicaceae*, caused by the obligate pathogen *Plasmodiophora brassicae*, is world-wide one of the most damaging diseases within this plant family. About 10% of *B. oleracea* field plots in Northwestern Europe, Japan, North America and Australia are infested with clubroot and the disease is difficult to control by either chemical or cultural means (for a recent review see LUDWIG-MÜLLER 1999). The growth of clubroot-infected plants is stunted compared to healthy plants and when such plants are pulled out of the soil, the root system shows typical gall formation. At maturity, the galls turn brown and soft and the resting spores are liberated from the plant tissue. The life cycle of the pathogen is divided into the primary phase, which is restricted to root hairs of the infected plant, and the secondary phase which occurs in the cortex and stele of hypocotyl and roots (INGRAM & TOMMERUP 1972). Once the pathogen enters the root cortex, there is little information on how the plasmodia are distributed within the root tissue. The plasmodia may be either passively distributed by the synchronized division of host cells (BUCZACKI 1983), or they could be actively migrating by destroying the host cell walls (IKEGAMI *et al.* 1978; MITHEN & MAGRATH 1992; KOBELT 2000). The authors speculated that *P. brassicae* may produce enzymes which are able to digest cell wall components. We have therefore used degenerated PCR to amplify putative cellulase cDNA fragments from *P. brassicae*.

MATERIALS AND METHODS

Brassica rapa ssp. *pekinensis* seeds were sown onto compost and grown under controlled conditions at 23°C with additional lighting for 16 h per day. The seeds were directly inoculated with a resting spore suspension of 10^8 spores per ml of a field isolate of *Plasmodiophora brassicae* as described by LUDWIG-MÜLLER *et al.* (1999). The roots of control and infected plants were harvested 14, 21, and 28 days

after inoculation (dai), washed in deionized water and frozen in liquid nitrogen. Histological examinations were performed according to KOBELT (2000). Isolation of total RNA followed the protocol of LOGEMAN *et al.* (1987). First strand cDNA was prepared from total RNA using AMV reverse transcriptase. For the isolation of the putative cellulase fragment described here, the following primer pair was used: Cel 2 forward 5'-AGATGGAYATCTGGGAGGCCAAC-3'; Cel 4 reverse 5'-CTGGAYGTAGARRCGSTKGATCT-3'; (where K=G/T, R=A/G, S=C/G, Y=C/T). PCR was performed according to standard procedures, using the following program: initial denaturation at 96°C for 5 min, followed by 30 cycles of 96°C/45s – 55°C/45s – 72°C/45s in a Stratagene Robocycler. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide and purified for direct sequencing using the Stratagene gel slice kit (Stratagene Europe, Amsterdam, The Netherlands). Direct sequencing in both directions was performed by SEQLAB (Göttingen, Germany). Sequence homologies were searched using BLAST (ALTSCHUL *et al.* 1997).

RESULTS AND DISCUSSION

The infection cycle of the obligate pathogen *Plasmodiophora brassicae* can be divided into two phases: the primary or root hair phase and the secondary phase which occurs in the root cortex and results in the abnormous swellings of the roots. After penetrating the root cortex (stage I), the pathogen develops into plasmodia which are then distributed within the root tissue. Two ways are possible as shown in Figure 1A: 1) active movement through cells (stage II) or 2) passive distribution by simultaneous cell division with the host (stage III). Previous work by IKEGAMI *et al.* (1978) and MITHEN and MAGRATH (1992) have given indications that plasmodia of *P. brassicae* are able to migrate through host cell walls using light and electron microscopy. Employing the histochemical techniques developed by KOBELT (2000), visualizing the cytoskeleton of the pathogen with anti-actin antibodies/FITC-coupled secondary antibodies, these movements through host cell walls were confirmed (Figure 1B). Since one of the major constituents of the plant cell wall is cellulose, it was hypothesized that the pathogen must contain cellulase to degrade these cell walls. Cellulase genes can be found in a variety of organisms including bacteria, fungi and plants. Although the systematics of *P. brassicae* is not yet clear, it is becoming more obvious that according to morphological criteria and sequence analysis (BRASELTON 1995), *P. brassicae* should be placed in the kingdom of Protoctista (MARGULIS *et al.* 1989). However, since we were recently able to amplify a trehalose phosphate synthase (TPS) fragment from *P. brassicae* (BRODMANN *et al.* 2002) using sequence homologies to fungal TPS genes, we have used the same approach to attempt the isolation of cellulase

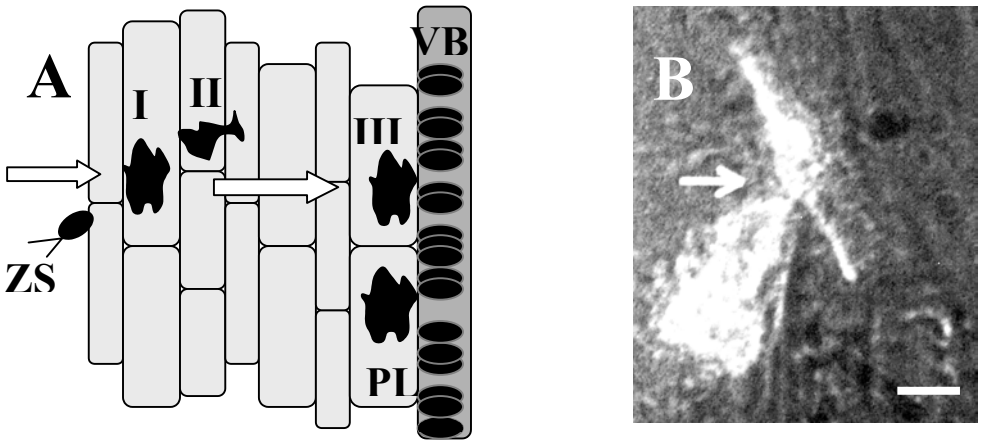

Figure 1. A. Hypothetical pathways for the distribution of *Plasmodiophora brassicae* in host root tissue. Zoospores (ZS) enter the root cortex (I), plasmodia (PL) migrate possibly by host cell lysis (II) or by simultaneous division with host cells (III). Many plasmodia are found in the vicinity of the vascular bundle (VB). B. Plasmodium migrating through host cell wall (indicated by an arrow). *P. brassicae* was visualized with an antibody against actin and immuno uorescence using FITC coupled secondary antibodies was carried out according to KOBELT (2000). The bar represents 2 μm

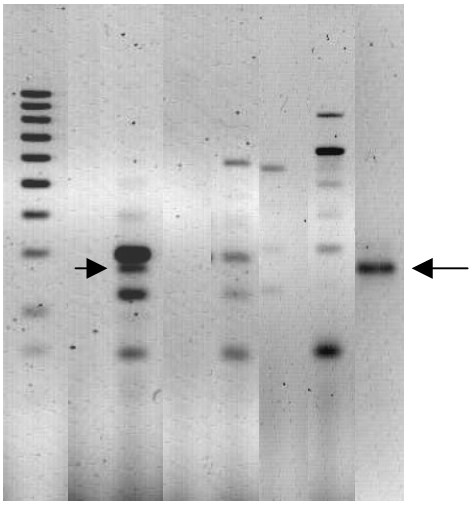

Figure 2. Amplification of a putative cDNA cellulase fragment (arrow) from infected Chinese cabbage roots 14 days after inoculation (dai) using degenerated primers Cel 2 and Cel 4. Lane 1: 100 bp marker; lanes 2 and 3: control and infected roots 14 dai; lanes 4 and 5: control and infected roots 21 dai; lanes 6 and 7: control and infected roots 28 dai; lane 8: re-amplification of putative cellulase cDNA fragment

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Pb      CTGTAXGGP- -TNRYGSICD PDGXDFXSYR QGNKTYYGPG
Hg      CEGDSCGGTY SNERYAGVCD PDGCDFNSYR QGNKTFYGKG
Fo      CTGDSCGGTY SSDRYGGTCD ADGCDFNAYR QGNKTFYGPG

Pb      -FSGVNTNQK FSVVTQFI-- -GSPVTEINR XYIQ
Hg      -MT-VDTTKK ITVVTQFLKD ANGDLGEIKR FYVQ
Fo      SNFNIDTTKK MTVVTQFHKG SNGRLSEITR LYVQ
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Figure 3. Amino acid sequence comparison of the putative cellulase cDNA fragment from *Plasmodiophora brassicae* (Pb) with cellulase (β-1,4-cellobiosidase, EC 3.2.1.91) from *Fusarium oxysporum* (Fo) (accession no. L29379) and *Humicola grisea* (Hg) (accession no. X17258) showing the highest homology (BLAST E values 1e-13 and 9e-13, respectively). Gray marker: homology between all three sequences, black marker: homology between two of the sequences

genes from this pathogen. Twelve different fungal cellulase (β-1,4-cellobiosidase, EC 3.2.1.91) sequences were aligned and used for the design of three forward and three reverse degenerated primers homologous to fungal but not to plant cellulase sequences, which were used in all possible combinations. A 260 basepair size fragment was specifically amplified from cDNA of infected roots 14 dai using the primer combination Cel 2 and Cel 4 (Figure 2). This early stage of infection may be the phase where the major part of migration by the pathogen occurs (KOBELT 2000). The cDNA fragment was re-amplified and subjected to direct sequencing. It showed high homology to different fungal cellulase genes, including *Fusarium oxysporum*, *Humicola grisea* (Figure 3), *Trichoderma harzianum* and *Aspergillus niger*. The amino acid sequence homology of *P. brassicae* cellulase with both *F. oxypsorum* and *H. grisea* was 50%. Future research will concentrate on cloning the gene from *P. brassicae* and to show whether it is functional as cellulase.

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