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FUNGAL DISEASES

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# Gene *mpl1*, activated during mating in *Phytophthora infestans*: similar to genes encoding pectate lyases

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**Abstract** Clones of genes activated in mating cultures of A1 and A2 mating-type strains of *Phytophthora infestans* were isolated using the cDNA-representational difference analysis subtraction method. Clone cET58 was selected based on its accumulation in mating cultures and then was used as a probe to isolate cDNA clone cET58L2 from a cDNA library that was constructed from mycelia grown under mating conditions. Sequence analysis revealed that cET58L2 was 1043 bp long and contained a complete open reading frame of 789 bp. The amino acid sequence of the putative protein was similar to a pectate lyase, PLD, of *Fusarium solani* f. sp. *pisi*. The central region of the predicted protein was highly similar to the sequence of other pectate lyases. The gene from which the cDNA clones were derived was designated *mpl1*. A probe corresponding to the protein-coding region of *mpl1* was prepared (probe p58L) for Northern and Southern analyses. The maximum rate of oogonia increase and *mpl1* transcript accumulation reached a maximum after 5 days in mating culture. More than 13 genes with sequences similar to that of *mpl1* were found in the genome, revealing *mpl1* to be a multicopy gene. The *mpl1* may be a pectate lyase gene that is activated in *P. infestans* during mating.

**Key words** *Phytophthora infestans* · Mating conditions · Pectate lyase

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## Introduction

*Phytophthora infestans* (Mont.) de Bary is a well-known plant pathogenic oomycete fungus that causes late blight on potato and tomato. This heterothallic fungus has two mating types, A1 and A2 (Brasier 1992), that form oospores through sexual reproduction. The oospores are important for dormancy and for genetic crossing. Since the discovery of the A2 mating type in Mexico (Gallegly and Galindo 1958), both mating types have been identified around the world (Deahl et al. 1991; El-Korany et al. 1990; Goodwin et al. 1994; Mosa et al. 1989; Tantius et al. 1986). The recent discovery that oospores form on potato leaves in the field has led to the suggestion that oospores have an important ecological role (Drenth et al. 1994, 1995; Kato et al. 1993).

Previous experiments have demonstrated the existence of oosporogenesis-inducible substances in opposite mating types (Ko 1985; Zaki 1983), and there have been several studies of the chemical and physical stimulation of oosporogenesis (Ann and Ko 1989; Brasier 1971; Ho and Zentmeyer 1977; Reeves and Jackson 1974; Zaki 1983). The biochemical events that drive oosporogenesis have not been defined, although stage-specific gene expression patterns during sexual development have been reported (Akino and Ogoshi 1998; Fabritius et al. 2002). Oosporogenesis of *P. infestans* can be artificially controlled, as oospores are formed only when isolates of both mating types are present. The aim of this study was to identify gene expression differences between asexual and sexual reproduction of the A1 and A2 strains using representational difference analysis (RDA) (Niwa et al. 1997). Identifying genes expressed during mating is one way to understand the specific reproductive events of this fungus. We designed experiments to detect gene transcripts expressed in A1 and A2 specifically during mating (Akino and Ogoshi 1998). Here, we describe the isolation of cDNA clones derived from a gene that we designated *mpl1*. Transcriptional regulation of this gene and the predicted amino acid sequence of its product are also presented.

## Materials and methods

### *Phytophthora* strains and culture conditions

Japanese *P. infestans* strains E009 (A1 mating type) and TB201 (A2 mating type) were used for RNA isolation for cDNA-RDA, dot blot analyses, and construction of the mating-specific cDNA library. Strains IB008s (A1) and TB201 were used for RNA isolation for Northern analysis. Strains 98A8 (A1) and TB201 were used for DNA isolation for Southern analysis. All strains were isolated from late blight-diseased leaves of potatoes grown in Japan. The strains were grown in the dark on rye agar medium supplemented with 2% (w/v) sucrose (RS medium) (Caten and Jinks 1968). Agar disks containing E009 and TB201 [cultured on V-8 (Miller 1955) agar medium] were inoculated, placed in contact in the center of a petri dish containing V-8 liquid medium, and incubated for 1 week at 20°C. Oogonia that developed were counted microscopically every day for 12 days. Experiments were repeated six times.

### cDNA-RDA and selection of positive clones

Mycelia of E009 and TB201 (incubated on V-8 liquid medium at 20°C for 1 week) were homogenized (Ace Homogenizer AM-5; Nihonseiki Kaisha, Tokyo, Japan) at 10000 rpm for 1 min under sterile conditions. The resulting suspensions were used to inoculate individual cultures of single strains on V-8 liquid medium. Mating cultures were generated by mixing equal quantities of two cell suspensions in the same petri dish. These cultures were incubated for 6 days at 20°C. Mycelia from the mating culture (the A1 and A2 mating types in the same culture, 2 g) were collected on a Buchner funnel, frozen, and ground into powder in liquid nitrogen using a mortar and pestle. Mycelia from single cultures (pure cultures of individual isolates, 1 g of each) were collected individually by the same procedure and were ground together in a mortar. RNA was isolated using the AGPC method (Chomczynski and Sacchi 1987). Poly(A)$^+$ RNA was purified from the single and mating cultures (mRNA Purification Kit; Pharmacia, Uppsala, Sweden), and double-stranded cDNA was synthesized from each sample (cDNA Synthesis System Plus; Amersham, Buckinghamshire, UK).

cDNA-RDA was performed according to the method described by Niwa et al. (1997), using the cDNA from the mating culture as the tester and cDNA from single cultures as the driver. A subtractive cDNA library was created by transforming (Kobori and Nojima 1993) the cDNAs into *Escherichia coli* XL1-Blue MRF′ (Stratagene, La Jolla, CA, USA). A random selection of 128 colonies from this library was used for further experiments. The DNA inserts of these clones were sequenced (Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza dGTP, Amersham; ALFexpress DNA Sequencer, Pharmacia). Clones with the same inserts were excluded. Sequence data were compared with the DDBJ/GenBank database (Pearson and Lipman 1988) to identify genes with similar sequences.

For RNA dot blot and Northern analyses, total RNA was isolated from mycelia of single and mating cultures of strains IB008s and TB201 by the AGPC method. RNA from A1, A2, and A1/A2 mating cultures was spotted on filters for dot blots or transferred to filters for Northern analysis (Boehringer Mannheim GmbH Biochemica 1993). Probes were isolated from vectors containing selected insert DNAs (Qiagen Plasmid Mini Kit; Qiagen GmbH, Hilden, Germany) and labeled by digoxigenin-UTP (PCR DIG Probe Synthesis Kit; Boehringer Mannheim GmbH Biochemica, Mannheim, Germany). Clones that revealed high transcription levels in mating cultures by dot blot analysis were selected for Northern analysis (Boehringer Mannheim GmbH Biochemica 1993). Three repetitions of RNA isolation and Northern blot analysis were performed.

### Isolation and analyses of cDNA clones

A cDNA library of mating culture cells was constructed according to the manufacturers' instructions (cDNA Synthesis System Plus, Amersham; Uni-Zap XR Cloning Kit, GigaPack III Packaging Extract, Stratagene) from RNA isolated from mycelia of mating cultures as described. cDNA clones were selected using the cET58 probe, the levels of which were shown by Northern analysis to increase under mating conditions. Inserts of isolated clones were subcloned according to the manufacturer's instructions (Uni-Zap XR Cloning Kit, Stratagene). Fragments of the cloned cDNAs were sequenced as described. For Northern and Southern analyses, a digoxigenin-labeled p58L probe was synthesized as described, using PCR primers 58L1 (5′-GCCAAGCTCAAGAACGTC-3′) and 58L2 (5′-TCGTG GACGATGTGGTCT-3′), which amplify a cET58L2 product from nucleotide 358 to 802.

Mycelia of strain IB008s and TB201 were cultured on V-8 medium, and mating cultures between the two strains were prepared. The oogonia that formed were counted microscopically, and total RNA was isolated every day from each mycelium for 10 days, as described. RNA samples were used for Northern blot analysis with a p58L probe (Boehringer Mannheim GmbH Biochemica 1993). Genomic DNA of strains 98A8 and TB201 was isolated as described by Goodwin et al. (1992). Southern blot analysis was performed using *Pst*I, *Sal*I, and *Eco*RI (Takara Biochemicals, Tokyo, Japan) and a p58L probe (Shaw 1987).

## Results

### Oogonia formation

With the mating of E009 and TB201, oogonia were first identified on day 3 in mating cultures. The maximum number of oogonia was found on day 8 (1059 ± 270 oogonia/cm$^2$ mycelia, mean ± standard error; $n = 6$), and the number of

oogonia formed per day was highest on day 6 (426 ± 180 oogonia/cm$^2$ mycelia). Maturation of the oospore and inner wall formation were observed after the eighth day. Mating cultures formed many oogonia when the A1 strain used was fresh from the original stock culture. The number of oogonia that form may decrease sharply within 1–2 years of repeated generation of certain A1 strains from working cultures. Oogonia formation was examined again in mating cultures of strains IB008s and TB201 for time-course Northern analysis. Hyphal swelling, which was not seen in individual cultures, was observed on the fourth day, and oogonia were observed after day 5. The number of oogonia increased 30% per day through day 7. The maximum rate of oogonia increase occurred on the fifth day (506 ± 201/cm$^2$ mycelia/day).

### Selection of positive clones

A random selection of 128 colonies (clones) from the subtracted cDNA library was used for sequencing and expression analysis. Of the 128 clones sequenced, 74 were identical. RNA dot blot analyses showed that 12 clones gave the strongest hybridization signals during sexual reproduction. Northern analyses confirmed that clone cET58 was derived from a gene that was strongly activated during sexual reproduction. No signal corresponding to cET58 hybridization was found in RNA from single cultures of IB008s (A1), and only a weak signal was detected in RNA from single cultures of TB201 (A2). The strongest hybridization signal was observed in mating cultures (Fig. 1). The size of the corresponding band that hybridized with the probe was approximately 1200 nucleotides. Identical results were seen in three repetitions.

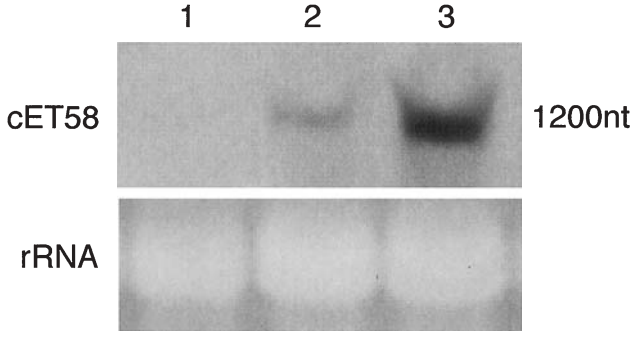


**Fig. 1.** Northern hybridization analysis of cET58 transcript accumulation in *Phytophthora infestans* mycelium grown in single and mating cultures. Total RNA (15 μg/lane) isolated from single cultures of the A1 mating-type strain IB008s (lane *1*), A2 mating-type strain TB201 (lane *2*), and mating cultures of both strains (lane *3*) were blotted onto a nylon membrane and hybridized with the digoxigenin-UTP-labeled cET58 probe. Transcript lengths in nucleotides (*nt*) are indicated on the right. Ribosomal RNA stained with ethidium bromide is also shown (*bottom*)

### Analyses of cDNA clone cET58L2

Two cDNA clones corresponding to the cET58 probe were isolated from the cDNA library of the mating culture. Sequencing revealed that clone cET58L2 contained a continuous 789-bp open reading frame (Fig. 2). The coding sequence begins with an ATG start codon 94 bp downstream from the 5′ end and encodes a putative protein of 262 amino acids. cET58L1 is a partial cDNA clone lacking the N-terminus of the putative protein. The gene from which the cDNA clones were derived was designated *mpl1*. The calculated molecular mass of the protein encoded by *mpl1* (MPL1) is 28 520 Da, and its pI is 8.3.

Comparison of the MPL1 amino acid sequence to known sequences in the database revealed significant similarity with several proteins. The most similar proteins included the following (protein name, homology, and e-value are listed): *Fusarium solani* f. sp. *pisi* PLD (Guo et al. 1996), 43.6%, $5 \times 10^{-33}$; *F. solani* f. sp. *pisi* PLC (Guo et al. 1995a), 44.0%, $3 \times 10^{-30}$; and *F. oxysporum* f. sp. *lycopersici* pectate lyase protein (Huertas-Gonzalez et al. 1999), 40.2%, $6 \times 10^{-29}$. All of the other significantly similar proteins were pectate lyases. The MPL1 amino acid sequence was compared with sequences of pectate lyases from *F. solani* f. sp. *pisi* PLA (Crawford and Kolattukudy 1987), PLB (Guo et al. 1995b), PLC, and PLD (Fig. 3). Residues 51–180 of MPL1 were similar to the *Fusarium* pectate lyases. The rest of the protein did not share significant similarity with the lyases or with any other protein.

### Time course of Northern analysis

Northern analysis showed that *mpl1* transcript levels peaked on days 5 and 6 and decreased gradually thereafter. The major *mpl1* hybridizing band was about 1200 nucleotides in size (Fig. 4), the same size as the band that hybridized to the cET58 probe. In addition to this signal, a minor signal of about 900 nucleotides was observed after day 6.

### Southern blot analysis

More than 13 bands hybridized with the probe on DNA digested with *Pst*I, *Sal*I, and *Eco*RI (Fig. 5). Several weak bands appeared on blots of DNA from both strains.

## Discussion

In this study, it was necessary to change the A1 mating-type testing strain from E009 to IB008s and 98A8 because sexual reproduction (oospore formation) decreased over the duration of the study. The decrease may be due to some trait of the A1 mating type strain. Strain 98A8 has not yet exhibited such a decrease. Strains E009 and IB008s were classified into the US-1 group by fingerprint analysis with the RG57 probe (Goodwin et al. 1992) (data not shown). US-1 strains tend to change after only short periods in culture. The

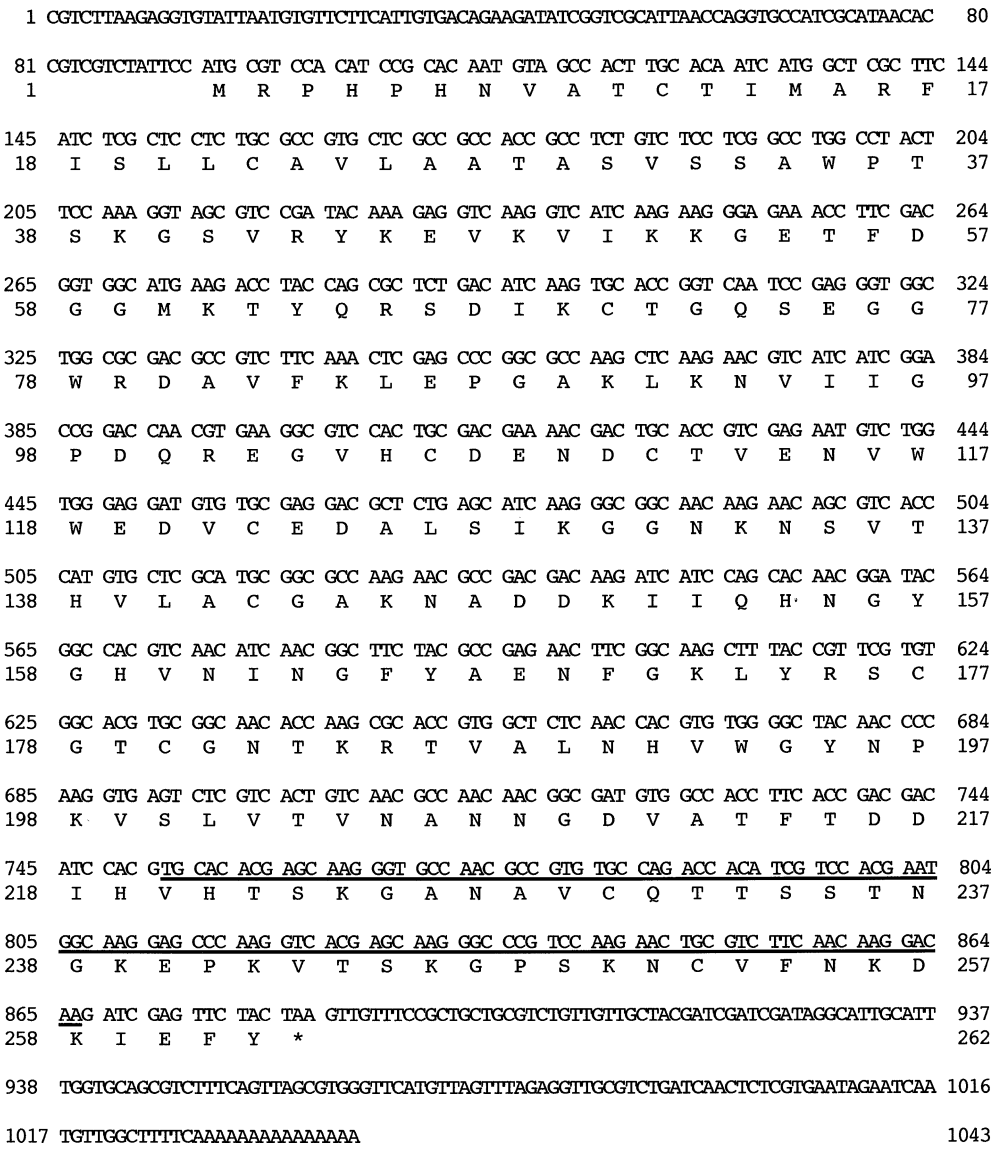


**Fig. 2.** Nucleotide sequence of the *mpl1* cDNA clone cET58L2 from *Phytophthora infestans* mating cultures and the deduced amino acid sequence of MPL1. *Numbers* on the two sides indicate either nucleotide length or amino acid length. The sequence of the RDA clone cET58 is *underlined*

fingerprint patterns of strain 98A8 differ from the patterns of US-1 strains (data not shown), and 98A8 has not yet been classified. Because 98A8 was relatively more stable in culture than were US-1 strains and its sexual reproduction did not differ, 98A8 was used in this study.

Southern blot analysis was performed on the RDA products to confirm that the clones isolated were specific for mating. Weak signals were derived from the second and third enriched product using the driver amplicon (Niwa et al. 1997) as a probe. In contrast, a clear signal was observed when a mixture of the third enriched product was used as a probe (data not shown). This result showed that mating-specific mRNAs were enriched successfully, although there were few specific clones in the subtractive library. The library contained some clones of genes transcribed specifically in the A2 strain used for the RDA (Fujii et al. 1999). Enrichment efficiency in this experiment was not high. The time and temperature of hybridization of the tester and driver amplicons should be coordinated. Our hybridizations were performed at 67°C for 12 h. However, it may be effective to hybridize under more stringent conditions. One report on stage-specific gene expression during sexual development (Fabritius et al. 2002) identified genes whose products affect RNA stability or resemble elicitor proteins. Thus, genes other than the ones we identified are probably expressed during the sexual stage of this fungus.

We hypothesized that the protein product of *mpl1* (MPL1) might participate in sexual reproduction. The highest level of transcript accumulation of *mpl1* was observed on days 5 and 6 in culture, in good agreement with the timing of oogonia formation in mating cultures. Additionally, a smaller transcript (about 900 nucleotides) was identified by Northern analysis after day 6 in mating cultures. Thus, another gene homologous to *mpl1* appears to have been induced in mating cultures after *mpl1*. The timing of the appearance of this small mRNA suggests a relation between these two transcripts.

**Fig. 3.** Multiple sequence alignment of the deduced amino acid sequences of the *Phytophthora infestans* MPL1 peptide and *Fusarium solani* f. sp. *pisi* PLA (Crawford and Kolattukudy 1987), PLB (Guo et al. 1995b), PLC (Guo et al. 1995a), and PLD (Guo et al. 1996). *Asterisks* represent amino acids that are identical in all five peptides. *Black boxes* represent amino acids that are identical in MPL1

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PLA     1:M-KF----TAAFVAA-LVGTSSAAV--T----KTLPKSAGATSFPTAVPV-KG-SYDGGM  46
PLB     1:M-K-----ASALIIA-AVTGASAAV--T----TVLPASAGVQSEPTAIPVRKGDKYNGGM  47
PLC     1:----------------------MACLGYT----GGVPKPTDHISNSKVIEVKAGQVYDGKW  35
PLD     1:M-HAP---SIVTVLA-ALPAAMACLGYT----GGVPKATGSKSLSAPKTLKKGEVFDAGW  51
MPL1    1:MRPHPHNVATCTIMARFISLLCAVLAATASVSSAWPTSKGSVRYKEVKVIKKGETFDGGM  60
                                  *    *    *         *

PLA    47:KRFEREPKVCKGQDETGEKDAMFILENGATLSNVIIGASQAEGVHC-KGTCTLNNVWWAD  105
PLB    48:KRFVRNPTTCKDQYETGEKDASFILEDGATLSNVIIDRSSGEGVHC-KGTCTLNNVWWAD  106
PLC    36:AKYDRGSGACKGQNEGGDKDAVFLLHEGATLKNVIIGKDQSEGVHC-KGHCTLEFVWFED  94
PLD    52:VRYDRG-VKCSGQAEGGSKDAVFILEEGATLRNVIIGANQREGIHC-KGSCNIEFAWFED  109
MPL1   61:KTYQRSDIKCTGQSEGGWRDAVFKLEPGAKLKNVIIGPDQREGVHCDENDCTVENVWWED  120
             *    *  * * *  ** * *  ** * ****     ** **    *     * *

PLA   106:VCEDAVTLK--QTSGTSYINGGGAFHASDKIIQFNGRGIVHVKDFYAEDYGKLSRSCGNC  163
PLB   107:VCEDAATFK--QKSGTSTINGGGAFSAQDKVLQFNGRGTLNVNDFYVQDYGKLVRNCGNC  164
PLC    95:VCEDAISI---AGK-ESWIIGGGAYHASDKVVQHNGCGTVNIINFYVEDYGKLYRSCGNC  150
PLD   110:VCEDAISI---LGSGTANIIGGGAYHASDKVIQHNGCGHVNIVNFYANDYGKVYRSCGNC  166
MPL1  121:VCEDALSIKGGGNKNSVTHVLACGAKNADDKIIQHNGYGHVNINGFYAENFGKLYRSCGTC  180
          *****                ** * ** * ** *      **    **  * ** *

PLA   164:KDNGG-PRNVIVENSVAVDG-GVLCGINTNYGDTCKVINSCQDKGKYCDRYEGNSSGKEP  221
PLB   165:EGNGG-PRNINIKGVVAKNG-GELCGVNHNYGDVCTITDSCQNKGKSCQAYIGNDQKKEP  222
PLC   151:-SK-QCKRNVYIEGVTAKNG-GELAGINANYGDTATLKNVCADAKQKCTMYNG--C--A-  202
PLD   167:KGNTNCKRSVHMEGTTAVKG-GELIGINTNYGDKATYSNNCY-PKTQCQGYKG--CDKSK  222
MPL1  181:GNTKRTVALNHVWGYNPKVSLVTVNANNGDVATFTDDIHVHTSKGANAVCQITSSTNGKE  240
                                        *

PLA   222:TKIG-SGPDGKYCTVTGSTTSC  242
PLB   223:PKFGPAGDNGKSCLVKSLRTNC  244
PLC   203:GGCEP--KKIGACPA-------  215
PLD   223:GECEP--SKAAKC---------  233
MPL1  241:PKVTSKGPSKNCVFNKDKIEFY  262
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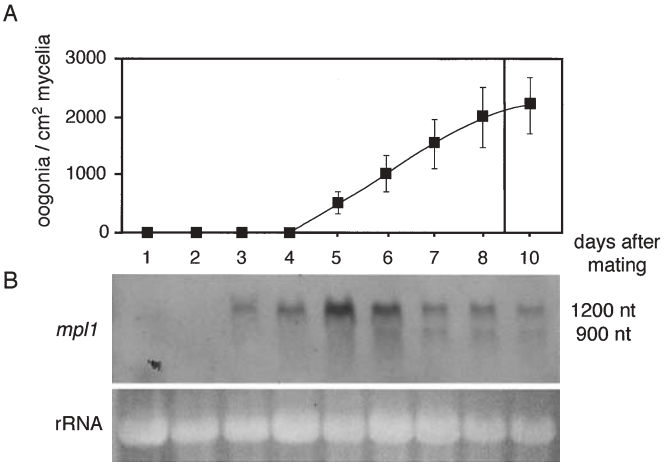


**Fig. 4.** Oogonia formation and simultaneous expression of gene *mpl1* in mating cultures of strains IB008s (A1 mating-type) and TB201 (A2 mating-type). **A** Oogonia formation in liquid mating cultures of strains IB008s and TB201. Mating cultures were grown in V-8 media at 20°C for 10 days. Error bars represent the standard error. **B** Expression of *mpl1* during oogonia formation in mating cultures of strains IB008s and TB201. Chemiluminescence was used to detect hybridization of a digoxigenin-labeled *mpl1* probe (p58L) on a Northern Blot of total RNA (15 µg) from mating cultures of *Phytophthora infestans* strains IB008s and TB201. Transcript lengths in nucleotides (*nt*) are indicated on the right. Ribosomal RNA stained with ethidium bromide is also shown (*bottom*)

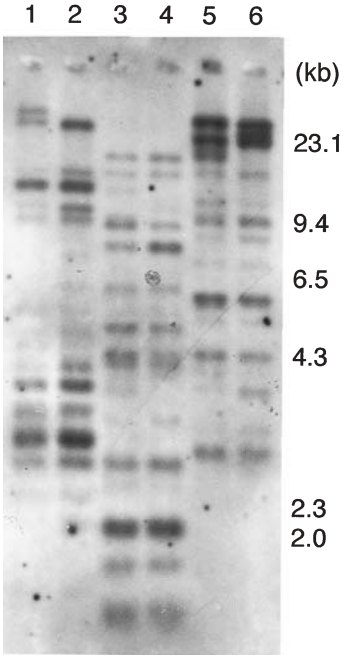


**Fig. 5.** Southern hybridization of genomic DNA of *Phytophthora infestans* with the *mpl1* cDNA probe p58L. A Southern blot containing genomic *P. infestans* DNA of strain 98A8 (4 µg/lane) digested with *Pst*I (lane *1*), *Sal*I (lane *3*), and *Eco*RI (lane *5*) and strain TB201 (4 µg/lane) digested with *Pst*I (lane *2*), *Sal*I (lane *4*), and *Eco*RI (lane *6*). Molecular marker sizes are indicated in kilobases

The amino acid sequence of the MPL1 putative peptide was similar to pectate lyases, particularly those of *Fusarium* species. Pectate lyases degrade pectin, a complex heteropolysaccharide found in higher plants (McNeil et al. 1984) and produced by many plant pathogens. The involvement of pectinolytic enzymes in plant pathogenesis has been investigated (Barras et al. 1994; Collmer and Keen 1986; Cooper 1984). Pectate lyases of *Fusarium* have been hypothesized to be involved in plant infection. MPL1 may be a pectate lyase by its homology. However, it is not clear why the gene of such an enzyme was activated in mating cultures in the absence of a host plant. The role of MPL1 is unidentified because there was no information on the role of pectate lyase in the process of mating or in the cell wall components of *Phytophthora* species. It may be involved in an unknown event during the process of mating and oosporogenesis. The significance of the stage-specific expression of *mpl1* should be investigated further.

The portion of the MPL1 putative amino acid sequence that is similar to PLA, PLB, PLC, and PLD lies near the center of the protein (residues 51–180). This portion is similar in four *Fusarium* pectate lyases and is a conserved region of pectate lyase proteins (Huertas-Gonzalez et al. 1999). Amino acids 1–50 and 181–262 of MPL1 were not similar to pectate lyases and appear to be MPL1-specific regions. No other conclusions can be drawn because these two regions were not similar to any known proteins.

Southern blot analysis using a p58L probe was performed to confirm that *mpl1* is found in the genome. Many hybridizing bands were found in both mating-type strains, suggesting that more than one sequence similar to p58L was present. The presence of weak hybridization signals on Southern blots indicated that there may be related sequences with low homology to p58L. The *mpl1* is a multicopy gene; we identified more than 13 bands with similarity to *mpl1* on the Southern blots. This contrasts with *Fusarium pelC* (Guo et al. 1995a), which is a single-copy gene. Additionally, each strain produced different hybridization patterns. We interpret this to indicate differences in their genomic structures. Of course, additional strains must be examined to investigate this point. The information available for *mpl1* is not yet sufficient to discuss the relation of this gene to the mating-type loci in *P. infestans* (Fabritius and Judelson 1997; Judelson et al. 1995; Judelson 1996a, 1996b).

The cDNA library from which clone cET58L2 was derived was constructed from poly(A)$^+$ RNA of mycelia from mating cultures. Thus, which mating-type strain activated *mpl1* is unclear. Southern blot analysis showed that the genomes of both strains include *mpl1*. Northern blot analysis revealed that *mpl1* transcripts were found slightly in single cultures of the A2 strains but were not found in A1. Therefore, we do not know which strain caused the increased transcript accumulation in the mating cultures. To characterize *mpl1* fully, it is important to determine which mating-type strain increases *mpl1* transcript accumulation. Additionally, the ecological and pathological significance of MPL1 (e.g., expression in planta) should be investigated in the future.

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