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Specifically isolated cDNA using cDNA-RDA from A2 mating type isolates of *Phytophthora infestans*

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Introduction

The oomycete *Phytophthora infestans* (Mont.) de Bary causes the late blight diseases of potato, tomato, and other Solanaceous plants. The heterothallic species, *P. infestans* produces oospores when the A1 and A2 mating types (sexual compatibility types) are cultured together¹⁾. The compatibility type is determined by hormonal regulation¹¹⁾. The A1 and A2 isolates are bisexual and capable of producing both antheridia and oogonia^{5,10)}. Judelson¹⁰⁾ demonstrated that the sexual preference (sexual compatibility) of most isolates depends on the relative preference of their mating partners. The mating system of heterothallic species of *Phytophthora* is distinguished from those of many other Oomycetes, such as *Achlya bisexualis*¹⁹⁾ and *Pythium sylvaticum*^{16,18)}.

To understand their ecology, it is important to investigate the mating mechanism in *P. infestans*. The objective of this study was to analyze clones of genes that were specifically detected in A2 mating isolates. The clones were by-products from screening clones that were activated in the mating condition. The clones were isolated from a *P. infestans* cDNA library using representational difference analysis (RDA)¹⁴⁾.

Materials and Methods

Isolates and culture conditions

The isolates of *P. infestans* used for cDNA-RDA were E009 (A1) and TB201 (A2). RNA and DNA samples for Northern and Southern analysis were isolated from A1 (E009, IB008s, DN122s and HK2) and A2 (TB201, OB996, F956 and T605) mating isolates from Japan. The A1 mating isolates, CHA-2 (China), TW15-2 (Taiwan) and IND4-1 (India) and A2 mating isolates, CHK-1 (China), K4 (Korea) and 550 (Mexico) were also used in the experiments. All the isolates were isolated from symptomatic host tomato or potato plants, and were maintained on rye-A agar media²⁾ at 20°C.

Screening clones activated in mating condition

An agar plug of each isolate was inoculated on Rye A-agar media (9 cm Petri dish) and incubated at 20°C for 7 days. The agar plugs of A1 and A2 isolates used for the mating culture were inoculated in V-8 broth until they came into contact with each other. Single cultures were established by inoculating V-8 broth with an agar plug of E009 or TB201 separately. The mating and single cultures were incubated at 20°C for 10 days.

Mycelia from a mating culture (2g) of E009 and TB201 were collected using a Buchner funnel, and mycelia from the single cultures (1g each) of E009 and TB201 were collected separately, frozen, and ground together in a mortar. Total RNA samples were isolated using the Acid Guanidinium-Phenol-Chloroform (AGPC) method⁹⁾, and double-stranded cDNA was synthesized with an Amersham cDNA synthesis module (Amersham Life Science, Buckinghamshire, U.K.).

cDNA-RDA was carried out using the mating culture cDNA as the tester and the single culture cDNA as the driver¹⁴⁾. *Sau3AI* digestion of cDNA, adapter ligation, PCR amplification, and *Sau3AI* digestion of the PCR product were used to prepare the tester and driver amplicons. Subtraction was performed by adapter ligation, hybridization of the tester and driver amplicons, PCR amplification, mung bean nuclease treatment, and *Sau3AI* digestion. After three rounds of subtraction, the tester and driver amplicons, DP1 (difference product 1: results of the first subtraction), DP2, and DP3 were checked by electrophoresis and Southern blot analysis, using DP3 as a probe to estimate the specificity of DP3. DP3 probes were prepared using a PCR DIG probe Synthesis Kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) according to the manufacturer's instructions.

Subtraction cDNA library

DP3 was ligated with pBluescript II SK-(Stratagene, La Jolla, USA) and transformed into *Escherichia coli* XL1-Blue MRF' (Stratagene, La Jolla, USA) using electroporation. Three hundred colonies from the library were picked up and maintained on LB agar. The cDNA inserts of 128 clones were sequenced using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Life Science, Buckinghamshire, U.K.) and an ALFexpress DNA sequencer (Pharmacia Biotech, Piscataway, USA). The sequence data were compared with data available in the GenBank DNA database using FASTA¹⁷⁾.

Northern blot analysis

Japanese and foreign isolates were incubated in V-8 broth at 20°C for 7-10 days. Total RNA was isolated from the mycelia of each isolate using TRIzol reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Northern blot analyses were carried out as described by Kohri *et al.*¹²⁾.

Total RNA (20 μg) from each isolate was separated by electrophoresis on formaldehyde gels and hybridized to probes. The probes of cET clones (from mating condition of E009 and TB201) were labeled with a PCR DIG probe Synthesis Kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) according to the manufacturer's instructions.

Southern blot analysis

Japanese and foreign isolates were incubated as described in the method for Northern blot analysis. A genomic DNA sample of each isolate was isolated from mycelia as described by Husted⁶. Southern blot analyses were carried out as described by Onoue *et al.*¹⁵. Genomic DNA (10 μg) was digested with *Hind*III, resolved by electrophoresis in agarose gels and hybridized to probes. The probes of cET clones were labeled as described above.

Results

Northern blot analysis of cET clones

One hundred and twenty-eight clones were sequenced and 74 of these overlapped various clones. The overlapping clones were excluded and digoxigenin-labeled probes were synthesized from 54 cET clones. In the Japanese isolates, Northern blot analysis using these probes showed that cET59 and cET110 were A2 mating type-specific transcriptions. When the cET59 clone was used as a probe, approximately 1.1 kb transcripts were specifically detected in A2 isolates (TB201, OB996 and F956) (Fig. 1A). When the cET110 clone was used as a probe, it detected approximately 0.9 kb transcripts, while no transcripts were detected in any of the A1 isolates (E009, IB008s, and DN122s) (Fig. 1B). In the foreign isolates, 1.1 kb transcripts were detected in A2 isolates (CHK-1, K4, and 550) and an A1 isolate (CHA-2) using cET59 probe, although the signal was weak in CHA-2 and 550 (Fig. 2A). The cET110 probe detected approximately 0.9 kb transcripts in A2 isolates (CHK-1 and K4) and an A1 isolate (CHA-2), although the signal was weak in CHA-2 and CHK-1 (Fig. 2B). In the A2 isolate T605 (control), the cET59 and 110 probes detected transcripts of the same size as seen in various Japanese A2 isolates (Fig. 2A, 2B).

Southern blot analysis of cET clones

In the Japanese isolates, the same pattern of multiple bands was obtained with *Hind*III-digested genomic DNA of each isolate using cET59 or 110 probes (Fig. 3A, B). Of note, a band larger than 23 kb was observed in all the A1 mating type isolates and 19 kb bands were observed in all the A2 isolates using the cET59 probe. Moreover, 20.2 kb bands were detected in all the A2 isolates using the cET110 probe. In the foreign isolates, the same pattern of multiple bands was obtained with *Hind*III-digested genomic DNA from each isolate using either cET59 or cET110 as the probe (Fig. 4A, B). A band larger than 23 kb was

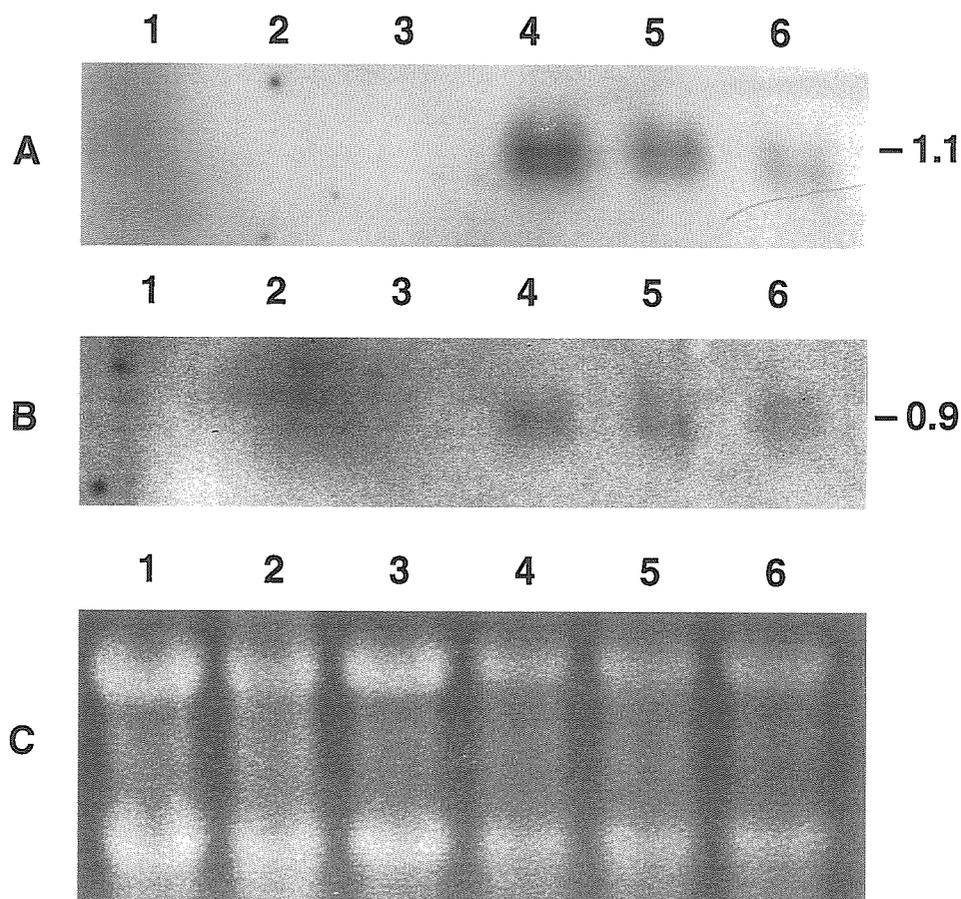


Fig. 1. RNA blotting analysis of Japanese isolates. Total RNA (20 μ g) isolated from *Phytophthora infestans* was separated by electrophoresis in a formaldehyde gel and hybridized with the digoxigenin-labeled probe. The probes used in the hybridization: A: cET59; B: cET110. The sizes in kilobases are shown to the right of each panel. The amount of RNA in each lane was further assessed by examining the amount of rRNA present (C). Lane 1, E009; 2, IB008s; 3, DN122s; 4, TB201; 5, OB996; 6, F956 (lanes 1-3, A1 mating type; lanes 4-6, A2 mating type).

observed in one isolate (TW15-2) and 15.3 kb bands were observed in two isolates (CHA-2 and K4) using the cET59 probe. In addition, 13.8 kb bands were observed in two isolates (CHA-2 and K4) and 16.6 kb bands were observed in two isolates (IND4-1 and TW15-2) using cET110.

Database searching

The sequences of the A2 isolate-specific clones were compared with the data available in GenBank. The results are shown in Table 1. The sequence of

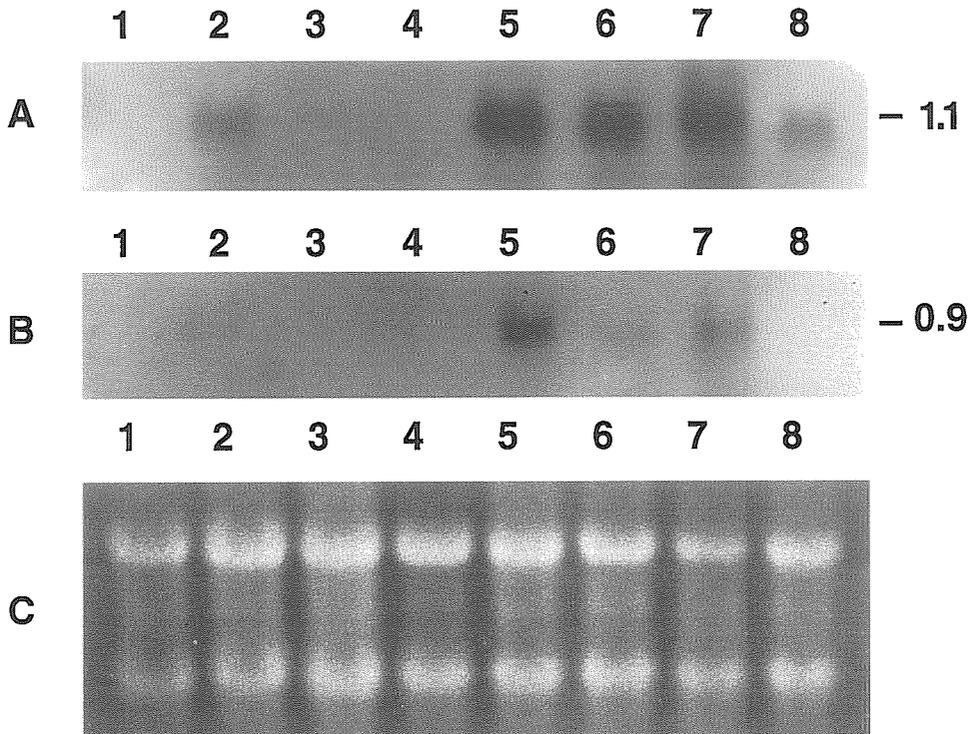


Fig. 2. RNA blotting analysis of foreign isolates. Total RNA (20 μ g) isolated from *Phytophthora infestans* was separated by electrophoresis in a formaldehyde gel and hybridized with the digoxigenin-labeled probe. The probes used in the hybridization: A: cET59; B: cET110. The sizes in kilobases are shown to the right of each panel. The amount of RNA in each lane was further assessed by examining the amount of rRNA present (C). Lane 1, HK2; 2, CHA-2; 3, IND4-1; 4, TW15-2; 5, T605; 6, CHK-1; 7, K4; 8, 550 (lanes 1-4, A1 mating type; lanes 5-8, A2 mating type).

cET59 was similar to 94 bp of *Lytechinus variegatus* beta-catenin mRNA (E value=0.087) and the sequence of cET110 was similar to 140 bp of *Arabidopsis thaliana* DNA chromosome 4, BAC clone T12H17 (E value=0.046).

Table 1. Characterization of the cDNA inserts of cET clones

cET clone	be	High homology genes ^{a)}	E value ^{b)}
59	136	<i>Lytechinus variegatus</i> beta-catenin mRNA	0.087
110	140	<i>Arabidopsis thaliana</i> DAD chromosome 4, BAC clone T12H17	0.046

a) The sequences in the GenBank data bank were searched for homology.

b) This value indicates the significance of the results of the homology search.
(<0.001 significant; 0.1-0.001 subtle; >0.1 not significant).

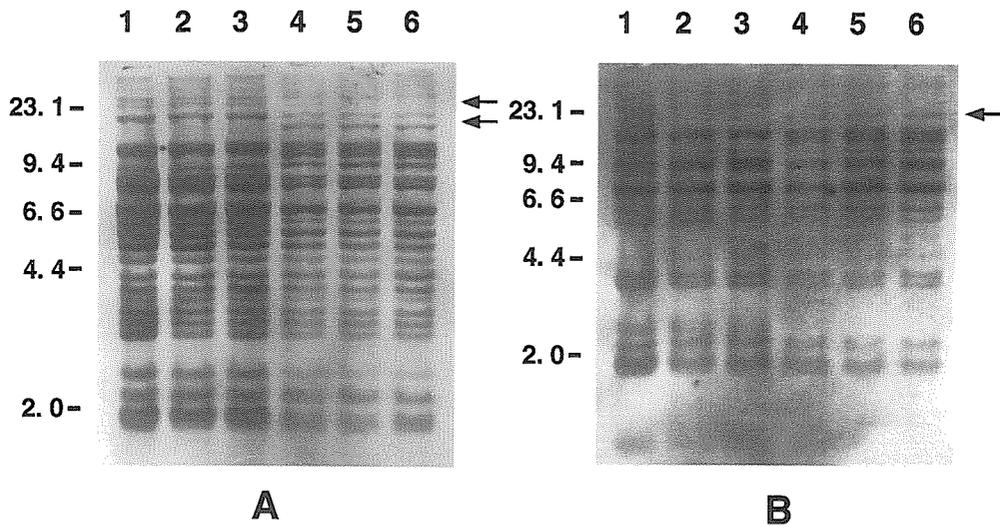


Fig. 3. DNA hybridization analysis of Japanese isolates. DNA (10 μ g) isolated from *Phytophthora infestans* was fractionated by electrophoresis in a 0.8% agarose gel and hybridized with the digoxigenin-labeled probe. DNA digested with *Hind*III and hybridized with cET59 (A) or cET110 (B) probes. Lane 1, E009; 2, IB008s; 3, DN122s; 4, TB201; 5, OB996; 6, F956 (lanes 1-3, A1 mating type; lanes 4-6, A2 mating type). Arrows in the right margins of A and B denote bands that produce a different pattern. Size standards (in kb) are indicated in the left margin.

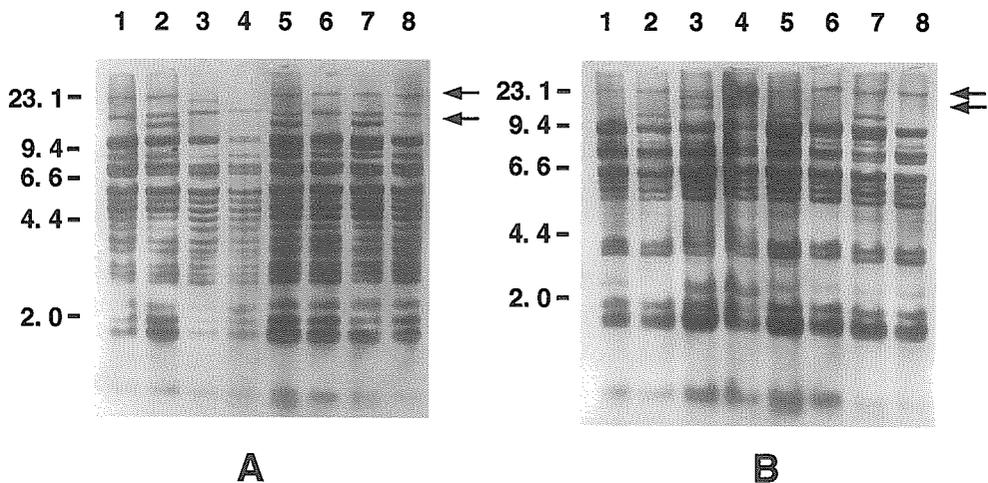


Fig. 4. DNA hybridization analysis of the foreign isolates. DNA (10 μ g) isolated from *Phytophthora infestans* was fractionated by electrophoresis in a 0.8% agarose gel and hybridized with the digoxigenin-labeled probe. DNA digested with *Hind*III and hybridized with cET59 (A) or cET110 (B) probes. Lane 1, HK2; 2, CHA-2; 3, TW15-2; 4, IND4-1; 5, T605; 6, CHK-1; 7, K4; 8, 550 (lanes 1-4, A1 mating type; lanes 5-8, A2 mating type). Arrows in the right margins of A and B denote bands that produce a different pattern. Size standards (in kb) are indicated in the left margin.

Discussion

In the Northern blot analyses, the probes cET59 and 110 detected many transcripts in the Japanese A2 mating type isolates isolated from a cDNA library using RDA. It is thought that these clones were residual cDNA resulting from incomplete subtraction. Although the subtraction experiment produced these clones as background, they had a very interesting property. In the foreign isolates, the cET59 and 110 probes detected transcripts in most of the A2 isolates (respectively, CHK-1, K4, and 550, and CHK-1 and K4). It is possible that these cloned genes are transcribed dominantly in A2 mating type isolates. In the Southern analysis, with the exception of a few differences, the same pattern of bands was obtained for each mating type with these probes. Presumably, these genes were regulated in transcription. Since the same pattern of multiple bands was detected in all the isolates using cET59 or cET110 in the Southern blot analysis, it is possible that these genes are members of a multigene family like the hsp70 gene family²⁰.

Although we cannot definitely explain why these genes were transcribed in the A2 mating type specifically, Northern blot analysis indicated the possible contribution of these genes to the mating type characteristics. Since mating type loci in *P. infestans* have been described in previous studies^{4,7-9}, we must examine the relationship between this loci and the A2-specific transcripts obtained in this study.

In the database search, cET59 was similar to *Lytechinus variegatus* beta-catenin mRNA¹³, and cET110 was similar to the *Arabidopsis thaliana* DNA chromosome 4, BAC clone T12H17 (Bevan *et al.*, unpublished data). It is not clear why this gene is specifically transcribed in A2 isolates. The results are preliminary data using short cET59 and 110 clones, which were completely digested by *Sau3A1*. The full-size cDNA clones of cET59 and 110 should be isolated from the cDNA library to investigate the structure and function of these genes. It will be very interesting to determine whether these genes are related to the differences in mating type. Further investigations to characterize the mating type in *P. infestans* are in progress.

Summary

In the oomycete fungus *Phytophthora infestans*, two A2 mating-specific cDNA clones were isolated from a cDNA library using cDNA-representational difference analysis (cDNA-RDA). These clones (cET59 and cET110) were used as probes for Northern and Southern blots. In Northern blots, 1.1 kb transcripts were only detected in Japanese and foreign A2 mating type isolates using cET59 probe. Furthermore, 0.9 kb transcripts were only detected in Japanese and foreign A2 mating type isolates using cET110 probe. In Southern blots, the same pattern of multiple bands was obtained with *HindIII*-digested genomic DNA for

both Japanese and foreign isolates using cET59 probe. In addition, the same pattern of multiple bands was obtained with cET110 probe. The sequence of cET59 was similar to the mRNA of *Lytechinus variegatus* beta-catenin, and that of cET110 was similar to *Arabidopsis thaliana* DNA chromosome 4, BAC clone T12H17.

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