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Triparental species hybrids from fused zoospores of Phytophthora

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Genetic exchange among three heterothallic *Phytophthora* spp., *P. nicotianae, P. capsici* and *P. citrophthora* each representing mating type A2, was induced via zoospore fusion. Viable offspring colonies that developed following fusion expressed differential drug resistance of each parental mutant. Detection of DNA with species specific sequences and by means of the polymerase chain reaction confirmed somatic hybrid formation in one of three isolates. By overcoming sexual incompatibility of zoosporic fungi, somatic fusion now improves access to direct study of molecular aspects of population variability.

Key words: Genetic markers, PCR, *Phytophthora*, somatic fusion, zoospores.

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Genetická výměna mezi třemi heterothalickými druhy *Phytophthora: P. nicotinae, P. caspici* a *P. citrophthora*, z nichž každý reprezentuje párovací typ (mating-typ) A2, byla realizována pomocí fúze zoospor. Vitální kolonie potomstva, které se vyvíjely po fúzi, vykazovaly expresi diferencující rezistence k antibiotikům u každého rodičovského mutantu. Detekce DNA pomocí specifických druhových sekvencí a pomocí polymerázové řetězové reakce potvrdila tvorbu somatických hybridů u jednoho ze tří izolátů. Díky překonání sexuální inkompability zoosporických hub, poskytuje nyní somatická fúze možnost přesného studia molekulárních aspektů populační variability.

INTRODUCTION

Of great interest is the possibility that related species of pathogenic fungi exchange genetic material when they infect a common host. Genetic exchange among *Phytophthora* species via sexual processes has been suggested or described by several authors (Boccas 1981; Brasier 1992; Goodwin and Fry 1994; Sansome et al. 1991). It also has been suggested that somatic hybridization may occur in nature, and that the process may be a means of bypassing the need for sexual reproduction in species that are heterothallic and lack compatible mating types (Brasier 1992; Érsek et al. 1993). Although somatic hybridization might be an important source of variation in some populations of *Phytophthora* species, such hybrids have not been proven to exist in nature; neither have they been created by conventional methods such as hyphal anastomosis or protoplast fusion (Layton and Kuhn 1988).

In a recent study we described a protocol for creating species hybrids between non-compatible mating types of *Phytophthora capsici* Leonian and *P. nicotianae* Breda de Haan (syn. *P. parasitica*) (Érsek et al. 1993, 1995). The approach used was based on the induced fusion of zoospores. The same technique was applied in the present study to create triparental hybrids of *P. capsici*, *P. nicotianae*, and *P. citrophthora* (Sm. et Sm.) Leonian. These are all pathogenic fungal species that have overlapping host ranges.

MATERIALS AND METHODS

Fungal isolates and culture. Isolates W1, 15399, and P1323 of *P. nicotianae*, *P. capsici* and *P. citrophthora*, respectively, were obtained from J. M. Duniway (University of California, Davis). A unique drug resistant mutant of each isolate was derived by chemical mutagenesis and subsequent screening for drug sensitivity, based on modified methods of Joseph and Coffey (1984). These modifications, as well as methods of maintaining and incubating cultures, and inducing zoospore release, were described previously (Érsek et al. 1994a). Each mutant isolate used in these studies expressed a unique and stable drug resistance phenotype. Mutant isolates, *P. nicotianae* Fpa^r10, *P. capsici* Mex^r5 and *P. citrophthora* Gen^r10 were resistant to p-fluorophenylalanine (Fpa), metalaxyl (Mex) and geneticin (Gen), respectively.

Fusion and regeneration of zoospores. Zoospores were fused using a protocol described previously (Érsek et al. 1995). Equal aliquots of zoospore suspensions (10^6 spores/ml) of each mutant isolate were combined in a fusion solution containing 30% polyethylene glycol (PEG 3350) and 50 mM LiCl. To induce encystment, aggregated and fused zoospores were transferred to encystment solution that consisted of 5 mM $CaCl_2$ and 500 mM KCl in 100 mM sorbitol. Spores in encystment solution were dispersed in molten pea-extract agar without drug amendments and incubated at 25 °C.

Selection of hybrids. After 24 h of incubation, the nonamended pea-extract medium containing zoospores was overlaid with the same medium amended with all three drugs of parental resistance at concentrations of 100, 25 and 15 mgL^{-1} of Fpa, Mex and Gen, respectively. Two to three days later, these plates were overlaid with a final layer of the medium supplemented with 200, 50, and 30 mgL^{-1} of Fpa, Mex and Gen, respectively. These drug concentrations were fully inhibitory to each parental mutant isolate. After 8 to 10 days of incubation on this medium, the fastest growing colonies were transferred to V-8 juice agar that contained the three drugs at the highest concentrations. Colonies that showed abnormal growth, sectoring or other indicators of instability, were discarded. The remaining putative somatic hybrids were stable and expressed the triple drug resistance for over one year in the absence of selection pressure. The hybrids were evaluated for

sporulation and pathogenicity on hosts for parental isolates as described previously (Érsek et al. 1994a, 1995).

Molecular evaluation of hybrids. The hybrid nature of selected isolates was confirmed by detection of parental, species-specific DNA sequences. DNAs were digested with appropriate restriction enzymes, electrophoresed in agarose gel, transferred to nitrocellulose membrane, and hybridized with ^{32}P-labelled probes as described by Sambrook et al. (1989). Plasmids pPP33A and pCIT15A (Érsek et al. 1994b), and pCAP12 (Érsek et al. 1995) were used as species-specific probes for *P. nicotianae, P. citrophthora* and *P. capsici*, respectively. Plasmids pPP33A and pCIT15A were derived from pUC18 into which had been subcloned a 1300-bp or 800-bp sequence specific to repetitive chromosomal DNA from *P. nicotianae* or *P. citrophthora*, respectively. Plasmid pCAP12 was derived as pUC18 containing a 2000-bp insert of repetitive DNA specific to *P. capsici*. DNA sequences of *P. nicotianae* and *P. citrophthora* were amplified by PCR using 24-bp primer-pairs derived from the species specific sequences under conditions reported elsewhere (Érsek et al. 1994b, 1995).

Additionally, 10-base oligonucleotides for RAPD-PCR were selected arbitrarily and used for RAPD-PCR (Williams et al. 1990). Primers were designated as follows: OPG-01, OPG-05 and OPG-10 and OPK-03, OPK-04 and OPK-13 (Operon Technologies, Inc.). In a search for polymorphic DNA sequences representing each parental mutant in the hybrid, reactions were cycled with an automated thermal cycler (Hybaid, model HB TR1). The primers (20 pmoles) were mixed with the reaction buffer, $MgCl_2$ (2 mM), dNTPs (200 mM each), Taq DNA polymerase (2.5 units), fungal DNA (100 ng) and sterile, glass distilled water in a total volume of 50 ml. The thermal cycler was programmed for 44 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 ° min, preceded by one cycle with an extended, 5 min denaturation at 94 ° amplification products were resolved by electrophoresis in 1.2% agarose gels and stained with ethidium bromide.

Results

Phenotypic characterization of putative hybrids. Triple-drug resistant colonies were recovered from amended pea-extract agar at a frequency of $5–8 \times 10^{-6}$. No triple-drug resistant colonies were recovered on plates that had been inoculated with spores that had not been treated with the fusion solution.

Three representative isolates, obtained from two fusion experiments and designated H8, H14 and H20, were retained for further analyses. Morphological traits of each isolate were most similar to those of the *P. nicotianae* Fpa^{r}10 (Fig. 1). On drug-free medium, colony growth rates of hybrids varied from 40 to 50% of those of parental species. On medium amended with the three drugs at highest

concentrations, hybrids grew approximately half as fast as did their respective parental species on medium with appropriate selective drug (Table 1).

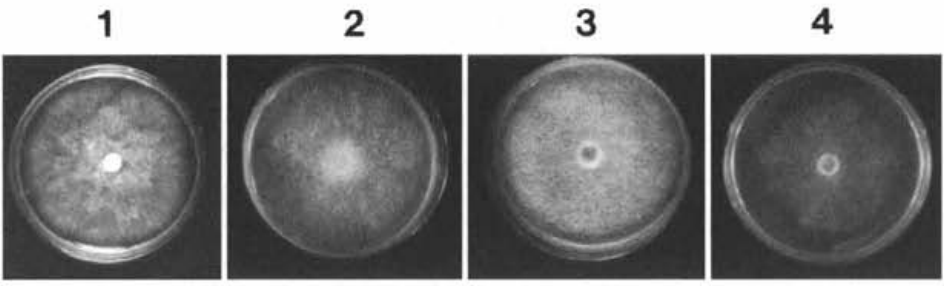

Fig. 1. Colony morphology of parental and hybrid *Phytophthora* isolates. In lanes 1, *P. capsici* Mex^{r}5; 2, *P. nicotianae* Fpa^{r}10; 3, *P. citrophthora* Gen^{r}10 parental mutants and 4, triparental hybrid, H20, after 8 days of growth at 25 °C on V8 medium supplemented with 50 mg l^{-1} of metalaxyl (Mex), 200 mg l^{-1} of fluorophenylalanine (Fpa), 30 mg l^{-1} of geneticin (Gen) and with the three drugs together at the indicated concentrations, respectively.

Table 1. Comparative growth of drug resistant mutants of *Phytophthora* spp. and their triparental hybrids under drug pressure.

Drug, mg l^{-1}	Radial growth (mm) of isolate[a]					
	PpFpa^{r}10	PcMex^{r}5	PciGen^{r}10	H8	H12	H20
None	19[b]	22	18	7	10	10
Fpa, 200	15	0	0	7	8	8
Mex, 50	0	22	0	7	9	9
Gen, 30	0	0	16	7	8	8
Fpa/Mex/Gen[c]	0	0	0	6	8	8

[a]Abbreviations: Pp, Pc and Pci denote *P. nicotianae, P. capsici* and *P. citrophthora*, respectively; H8, H12, H20 are hybrids; Fpa, Mex and Gen denote fluorophenylalanine, metalaxyl and geneticin, respectively.

[b]Growth measurements were made after 4 days of growth at 25 °C on V-8 medium.

[c]Concentration of each drug is the same as that of individual drugs.

None of the hybrids could be induced to produce sporangia, and thus, zoospore progeny could not be evaluated for similarities to parent isolates. The pathogenicity phenotypes of parental mutants were not retained in hybrids. Neither tomato (the common host of all parental microorganisms), or lemon fruit (common host of *P. citrophthora* and *P. nicotianae*), nor the storage taproot of radish (host of

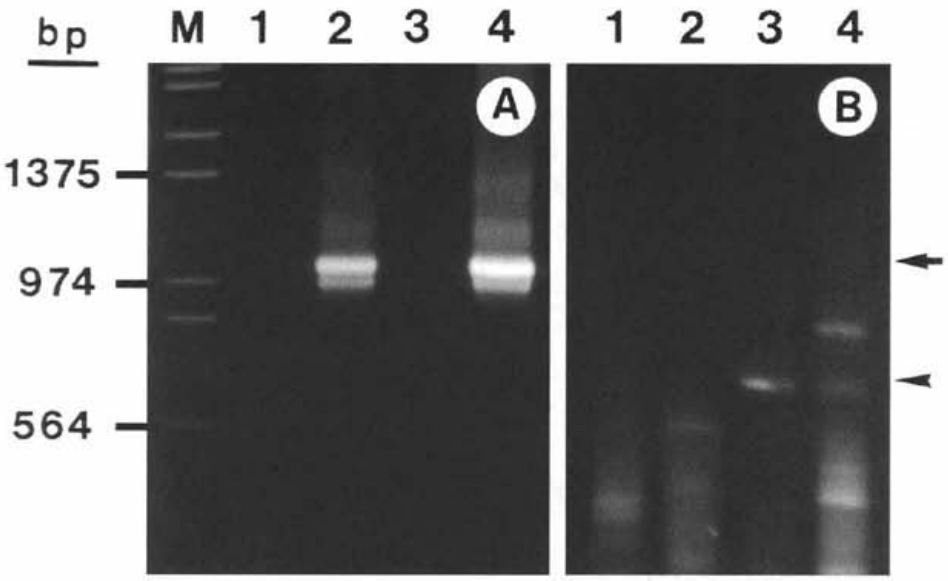

Fig. 2. Parental DNA sequences in triparental hybrid following PCR amplification of 1 μg template (total genomic) DNA with the respective species specific primer pairs. (A) Occurrence of the *P. nicotianae*-specific, 1000-bp DNA sequence (arrow). (B) Occurrence of the *P. citrophthora*-specific, 650-bp sequence (arrowhead). Sources of DNA in lanes 1, *P. capsici* Mex^{r}5; 2, *P. nicotianae* Fpa^{r}10; 3, *P. citrophthora* Gen^{r}10 and 4, hybrid H20.

P. capsici) exhibited any disease symptoms or a hypersensitive resistance response following inoculation with the hybrids.

Genotypic characterization of hybrids. The formation of hybrids was confirmed by detection of parental DNA sequences. When total genomic DNAs from hybrids and parent organisms were digested with EcoRI/XhoI and probed with pPP33A containing the *P. nicotianae*-specific, repetitive DNA sequence, multiple bands were visualized in *P. nicotianae* and, at low intensities, in the hybrid isolates. In contrast, when DNA was digested with PstI and probed with pCIT15A containing the *P. citrophthora*-specific repetitive sequence, hybridization was detected in P. citrophthora Gen^{r}10 only. Similarly, pCAP12 containing the *P. capsici*-specific repetitive sequence hybridized only with total DNA from the *P. capsici* Mex^{r}5 after digestion with HaeIII; hybridization with putative hybrids was not observed (data not shown).

The species-specific primer-pairs derived from pPP33A amplified the 1000-bp, P. nicotianae-specific sequence in all the hybrids, but the primer-pairs from pCIT15A amplified the 650-bp, *P. citrophthora*-specific sequence in the parental isolate and only hybrid isolate H20 (Fig. 2). With two exceptions, all of the

tested 10-base primers that produced various levels of polymorphisms of randomly amplifying DNA sequences of parental isolates, amplified only *P. nicotianae*-characteristic sequences in the hybrids. However, primer OPK-04 amplified DNA sequences characteristic of both *P. nicotianae* and *P. citrophthora* in the hybrids (data not shown). Additionally, amplification of DNA from one of the hybrids, H20, with primer OPG-05 resulted in detectable sequences of *P. nicotianae* and *P. capsici* (Fig. 3).

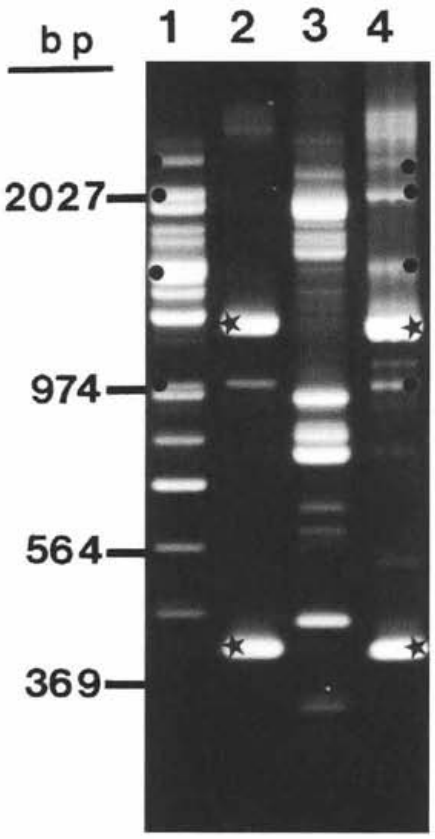

Fig. 3. RAPD patterns of parental and hybrid isolates following PCR with arbitrary 10-base primer OPG-05. Sources of DNA in lanes 1, *P. capsici* Mex^{r}5; 2, *P. nicotianae* Fpa^{r}10; 3, *P. citrophthora* Gen^{r}10 and 4, triparental hybrid H20. DNA bands in the hybrid that correspond to those of *P. nicotianae* and *P. capsici* are differentially marked.

Discussion

Phytophthora species vary in their abilities to reproduce sexually and generate genetic variability. Some species, such as *P. cactorum* or *P. sojae*, are homothallic organisms that reproduce by selfing. Other species, including *P. nicotianae, P. capsici* and *P. citrophthora* are heterothallic and outcross if compatible mating types come into contact with each other. Often in nature, only one mating type of a species will occur at a specific geographic location. Under these circumstances, interspecific somatic hybridization has been suggested as a mechanism of importance in generating genetic variability within a single mating type of a species (Brasier 1992). Evidence for this phenomenon has been provided only recently (Goodwin and Fry 1994; Sansome et al. 1991).

It is not known to what extent somatic hybridization occurs among the species in this study, but we have been able to examine the consequences of the event by creating interspecific hybrids *in vitro*. We reported the first proof that somatic hybrids of this sort between *P. capsici* and *P. nicotianae* can be created via induced fusion of zoospores (Érsek et al. 1993; Érsek et al. 1995). Zoospore fusion was achieved by a novel technique utilizing Li^+ as a key component in the procedure (Érsek et al. 1991). The present study extends the utility of zoospore fusion methods to create hybrids from three parents, *P. nicotianae, P. capsici* and *P. citrophthora*.

On the basis of morphological traits and drug resistance patterns, several putative triparental hybrids were created. Goodwin and Fry (1994) stressed the importance of molecular evidence to confirm the hybrid nature of such organisms. This proved to be an important step in our study, in that only one of three putative hybrids, based on morphology and drug-resistance, contained detectable sequences of all three parental organisms. Specifically, molecular analyses revealed that only DNA sequences specific to *P. nicotianae* and *P. citrophthora* could be detected in the restriction patterns of all hybrids using radiolabelled, species-specific probes, or PCR. This suggested that species-specific DNA sequences of *P. capsici* represented by pCAP12 were lost during hybrid formation. However, in one hybrid organism, other sequences of *P. capsici* were detectable using 10-base random primers in RAPD-PCR. It is apparent that further analyses are required to determine the manner in which genetic material of parent organisms are combined and phenotypic traits are expressed in hybrids.

At present, the reasons for loss of sporulation and virulence in the triparental hybrids are unknown. It is likely that triparental hybrids derived from zoospore fusion represent an array of new genetic combinations. Many of these combinations would be deleterious to the fitness of hybrid individuals and to their abilities to compete with other members of the fungal population. The zoospore fusion technique used in this study may open up new avenues for examination of the

processes that are involved in the success or extinction of widely variable new genotypes in nature.

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