Curr Genet (1988) 14:567–572

Current Genetics

Diploid construction by protoplast fusion in *Fulvia fulva* (syn. *Cladosporium fulvum*): genetic analysis of an imperfect fungal plant pathogen

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Summary. Auxotrophic and drug resistant mutants have been isolated in five of the seven races of *Fulvia fulva* (syn. *Cladosporium fulvum*) following UV mutagenesis. Drug resistant mutants have also been isolated by genetic transformation using vectors conferring resistance to hygromycin B and phleomycin. Protoplasts of complementing auxotrophs or mutants with different drug resistances have been fused to form diploids as demonstrated by growth characteristics and microfluorimetry. Some of the diploid fusion products remained stable but most went on to haploidise spontaneously. Using this technique it is now possible to perform both complementation and linkage analysis in an imperfect fungal plant pathogen.

Key words: *Fulvia fulva* – Tomato leaf mould – Protoplast fusion – Complementation

Introduction

Fulvia fulva (Cooke) Cif. (syn. *Cladosporium fulvum* Cooke) is a pathogen of tomato (*Lycopersicon esculentum*) causing leaf mould disease. It has a well-characterised race structure (Higgins and de Wit 1985) and the physiology of infection has been studied extensively by de Wit et al. (1985, 1986). *F. fulva* is normally haploid, reproducing by the asexual production of conidia. It appears to lack both sexual and parasexual cycles (Fincham et al. 1979). Since there is no natural way of carrying out conventional genetic mapping, the development of an artificial parasexual cycle might provide an alternative approach. The use of a naturally occurring parasexual cycle in genetic mapping was first developed for *Aspergillus nidulans* by Pontecorvo and Käfer (1958).

The first step in establishing an artificial parasexual cycle is the production of mutants. Mutants blocked in the nitrate assimilatory pathway, conferring chlorate resistance, are particularly easy to obtain in filamentous fungi (Cove 1976). DNA-mediated transformation of *F. fulva* (Oliver et al. 1987) can be used to obtain drug resistant mutants. Such drug resistant transformants carry one or more copies of the transforming vector in their genome.

The next step is to make protoplasts from two complementary auxotrophic or drug resistant strains, fuse them and select for the resulting regenerated heterozygous diploid, or heterokaryotic lines. Protoplast fusion has been achieved in many filamentous fungi (Anné and Peberdy 1976; Peberdy 1987) but to our knowledge in all cases studied, the fungi had sexual or parasexual cycles and protoplast fusion was being used to compare artificial with natural fusion systems.

Since no natural fusion or mating systems are known in *F. fulva* it is not possible to characterise any fusion products by conventional genetic methods. They could be heterokaryons, heterozygous diploids or polyploids. We have recently developed a microfluorimetric method of distinguishing between these (Talbot et al. 1988), based on measuring the fluorescence of single nuclei in mycelial samples stained with 4,6 Diamidino-2-phenylindole (DAPI). Furthermore any stable fusion product should contain the genomes of both parents. If drug resistant transformants are used as parents, then both original transforming plasmids, should be detectable in the genomic DNA of the fusion products by Southern hybridisation (Southern 1975). In fusions using auxotrophic parental strains of different races it might be

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possible to use RFLP markers to demonstrate the presence of parental genomic DNA.

The ability to generate stable heterokaryons or heterozygous diploids or polyploids between races of *F. fulva* would also make it possible to study the dominance relationships of the avirulence/virulence (*dcfR*/*dcfS*) genes which are carried in combinations by the different races of the fungus (Higgins and de Wit 1985). In this paper we report the development of a protoplast fusion system and its use as an artificial parasexual cycle for genetic mapping and complementation analysis.

Materials and methods

Strains

Fulvia fulva races 0, 2, 4, 2.4, 5, 2.4.5 and 2.4.5.9 were obtained from Dr. M. Gerlagh, Institut voor Plantenziektaenkundig Onderzoek (IPO), Wageningen, The Netherlands. Conidia were stored at −80 °C in 10% glycerol (Harling et al. 1988).

Nomenclature

Races of the fungus and mutants were described according to conventions proposed by Yoder et al. (1986). Since each race is distinguished by its ability to cause disease on a line of tomato plants isogenic with respect to the *Cf* locus for resistance to *F. fulva* it was decided to name each race in an unambiguous way to take account of this. Thus for example race 2 is named *dcf*2S because it has the ability to cause disease on tomato plants carrying just the *Cf*2 gene. The designation *dcf*S refers to presumed virulence alleles while *dcf*R refers to avirulence alleles. This nomenclature has been devised for ease of explanation of fusion experiments and may be superceded when (a) virulence gene products are known. Gene designations of mutants in the nitrate assimilatory pathway follow the conventions used for *Aspergillus nidulans* (Cove 1979).

Culture conditions and media

F. fulva was grown on V8 juice agar at 23 °C and conidia harvested after 10 days. Mycelium for protoplast production was produced by inoculation of potato dextrose broth (Difco) and incubation at 23 °C in the dark for 48 h. *F. fulva* was also grown on the following media. MM; Czapek-Dox medium (Oxoid). CM; 3.3% Czapek-Dox medium, 0.1% yeast extract (Oxoid), 1.2% potato dextrose broth (Difco), 0.1% mycological peptone (Oxoid), 0.1% acid casein hydrolysate. These were supplemented for mutant isolation as described later. All media were adjusted to pH 6.0 with HCl or NaOH unless stated. Solid media were supplemented with 2% agar. Protoplast regeneration media were supplemented with 0.8 M sucrose as osmotic stabiliser.

Mutagenesis

Suspensions of conidia at a concentration of 10^7 ml^{-1} in distilled water containing 0.1% (w/v) Tween 80 were irradiated with UV light to give a 90–99% kill. Conidia were continuously agitated with a magnetic stirrer to ensure uniform irradiation.

Table 1. Table showing minimum inhibitory concentrations and mutant resistant levels of anti-fungal agents used in *F. fulva* mutagenesis experiments

Anti-fungal agent	Min inhibitory concentrations	Mutant resistance limit
Benomyl	1 μg/ml	10 μg/ml
Hygromycin	50 μg/ml	1,000 μg/ml
Phleomycin	20 μg/ml	500 μg/ml
Cadmium	5 mM	50 mM
Lead	100 mM	150 mM
Cycloheximide	1 μg/ml	10 μg/ml
Dichlofluanid	1 μg/ml	10 μg/ml

Isolation of mutants

Auxotrophic mutants. Irradiated conidia were plated onto MM to give a density of approximately 50 colonies per plate. Plates were incubated in the dark for 5 days and then overlaid with CM and incubated for a further 5 days. New colonies arising after the overlay were subcultured and their auxotrophic requirements determined by the method of Holliday (1956).

Nitrate assimilatory pathway mutants. Irradiated conidia were plated onto MM with arginine as sole nitrogen source (24 mM w.r.t N) supplemented with 500 mM $KC10_3$ and plates were incubated for 10 days in the dark in an adaptation of the method of Cove (1976). Mutants were phenotypically analysed by growth tests in liquid culture using MM supplemented with the following nitrogen sources; ammonia (as ammonium tartrate), sodium nitrite (at pH 7.0), hypoxanthine and adenine (all at 24 mM w.r.t N) as described by Coddington (1976).

Drug and heavy metal resistant mutants. Mutants were isolated on MM supplemented with metal salts and anti-fungal agents at between five and ten times their minimum inhibitory concentrations. For details of these see Table 1.

Pathogenicity testing

Mutants were screened for pathogenicity on near isogenic lines of tomato seedlings differing only at the *Cf* locus for hypersensitive resistance to *F. fulva* grown in axenic culture using modified Hoaglands nutrient solution (Mitchell 1976) supplemented with 1% agar. A solution of 10^5 ml^{-1} conidia was used to inoculate the underside of the cotyledons on 14 day old seedlings and symptoms were apparent after another 14 days.

Protoplast production and fusion

Protoplasts were produced as described by Harling et al. (1988) and fusion was promoted by an adaptation of the method of Anne and Peberdy (1975). Protoplasts were incubated in 30% polyethylene glycol 4000 (PEG) supplemented with 0.01 M $CaCl_2$ in 0.01 M Tris-HCl buffer (pH 7.5) for 15 min at 30 °C. In the fusions involving drug resistant mutants, fused protoplasts were plated onto non-selective regeneration media and a selective overlay was applied after 18 h. In fusions involving auxotrophic mutants, protoplasts were applied directly to the selective minimal media. Control regeneration plates of selective

Table 2. Mutants produced in *F. fulva* by UV mutagenesis and transformation. Mutants were tested for pathogenicity and conidia from them stored in 10% glycerol at −80 °C.

Name	Mutagen	Pathogenicity	Phenotype	No. Isolated
*dcf*4S *ben*R	UV	+	Benomyl resistant	1
*dcf*4S *hyg*R	pAN7-1	+	Hygromycin res.	2
*dcf*4S *hyg*R *red*	UV	+	Red hygromycin res.	1
*dcf*4S *cyh*R	UV	+	Cycloheximide res.	1
*dcf*4S *dco*R	UV	+	Dichlofluanid res.	1
*dcf*4S *cyh*R *cad*R	UV	+	Cycloheximide/cadmium resistant	1
*dcf*4S *pbm*R	UV	+	Lead resistant	1
*dcf*4S *ben*R *bio*	UV	+	Benomyl resistant/biotin auxotroph	1
*dcf*5S *met*2	UV	+	Methionine auxotroph	1
*dcf*5S *bio*	UV	+	Biotin auxotroph	1
*dcf*5S *nia*	UV	+	Chlorate resistant	2
*dcf*5S *nir* (or *nii*)	UV	+	Chlorate resistant	17
*dcf*5S *cnx*	UV	+	Chlorate resistant	3
*dcf*4S *nia*	UV	+	Chlorate resistant	1
*dcf*4S *nir* (or *nii*)	UV	+	Chlorate resistant	4
*dcf*4S *cnx*	UV	+	Chlorate resistant	3
*dcf*2S *dcf*4S *nia*	UV	+	Chlorate resistant	4
*dcf*2S *dcf*4S *nir* (or *nii*)	UV	+	Chlorate resistant	4
*dcf*2S *dcf*4S *cnx*	UV	+	Chlorate resistant	3
*dcf*4S *hyg*R *nia*	UV	+	Hygromycin/chlorate resistant	7
*dcf*4S *hyg*R *nir* (or *nii*)	UV	+	Hygromycin/chlorate resistant	4
*dcf*4S *hyg*R *cnx*	UV	+	Hygromycin/chlorate resistant	6
*dcf*2S *cad*R	UV	−	Cadmium resistant	2
*dcf*2S *met*	UV	+	Methionine auxotroph	1
*dcf*2S *ura*	UV	+	Uracil auxotroph	1
*dcf*2S *dcf*4S *dcf*5S *dcf*9S *phl*R	pAN8-1	+	Phleomycin resistant	1

and non-selective media were inoculated with fused and unfused protoplasts for each fusion performed, in order to compare regeneration. As a control, hyphal anastamosis was attempted using the method of Hastie (1973).

Transformation

Transformation of protoplasts with plasmids pAN7-1 and pAN8-1 (Mattern et al. 1987) which carry the genes for hygromycin and phleomycin resistance respectively, and Southern blot analysis were performed as described by Oliver et al. (1987).

Ploidy determination

Ploidy determination was performed by measuring nuclei-associated fluorescence of DAPI-stained mycelia, using a computer-controlled imaging system as described by Talbot et al. (1988). Mycelial samples for ploidy determination were prepared by inoculating 10 ml of potato dextrose broth with 10 µl of a conidial suspension at a concentration of 10^7 ml^{-1} and incubating overnight at 23 °C in the dark on an orbital shaker. An 8 µl aliquot of the culture was stained by adding DAPI solution (5 µg/ml in 0.01 M Tris-HCl pH 7.0) to give a final concentration of 1 µg/ml^{-1}. 4 µl of "Citifluor" antifade glycerol mountant (City University, London) was added and the slide covered. The mycelium was viewed with a high sensitivity video camera attached to a Zeiss universal microscope and interfaced to a framestore (GEMS Mk III; GEMS of Cambridge Ltd., Carlyle Road, Cambridge, UK) and a VAX 11/750 mini-computer. Images of the mycelium were displayed on a colour monitor and optical densities of individual nuclei integrated under computer control and values written to a data file for processing using the program MINITAB (MINITAB Inc., State College, Pa) to produce histograms of fluorescence yield of populations of nuclei. Variation in staining between slides was overcome by normalising the fluorescence yield to an internal standard of *Chlorella* cells which were added at a concentration of 10^4 ml^{-1} to each slide before staining.

Results and discussion

Mutants

A full list of the mutants is given in Table 2. Auxotrophs were isolated with requirements for uracil, biotin (2) and methionine (2). One of the methionine mutants could grow on MM supplemented with homocysteine a biosynthetic precursor of methionine but not when supplemented with homoserine indicating that the mutant was analogous to the MET2 mutants of *S. cerevisiae* (Langin et al. 1986). All strains of *F. fulva* have a partial requirement for thiamine showing enhanced sporulation in its presence. The ability of *F. fulva* to

Table 3. A summary of the results obtained in protoplast fusion experiments. The sample size for ploidy determination was 50 nuclei in all cases

Protoplast fusion pair	Regeneration frequency of selective phenotype	Maintenance of selective phenotype through sub-culture	Ploidy of fusion fusion products retaining selective phenotype
*dcf*5S *nir*-73 x *dcf*5S *cnx*-69	0.0024%	53.3%	n
*dcf*5S *cnx*-57 x *dcf*5S *cnx*-66	0.016%	100%	n
*dcf*5S *cnx*-57 x *dcf*5S *cnx*-69	0.069%	33%	2n
*dcf*5S *cnx*-66 x *dcf*5S *cnx*-69	0.0%	–	–
*dcf*4S *ben*R-06 x *dcf*4S *hyg*R-07	0.0048%	0.04%	n
*dcf*4S *cyh*R-87 x *dcf*4S *hyg*R-07	0.0016%	20%	n
*dcf*2S *dcf*4S *dcf*5S *dcf*9S *phl*R-01 x *dcf*4S *hyg*R-07	0.045%	42%	2n

scavenge trace substances in the media meant that many putative auxotrophs had to be abandoned due to their leakiness. This could be reduced by using Noble agar (Difco) in solid media and in the case of biotin auxotrophs by adding 0.1 mg/ml avidin to MM.

In general mutants in the nitrate assimilatory pathway have been the most easy auxotrophs to isolate (Table 2). Phenotypes consistent with mutations in the structural gene for nitrate reductase (*nia*), the regulatory protein for the nitrate assimilation pathway (*nir*), the structural gene for nitrite reductase (*nii*) and in the genes for the molybdenum containing co-factor for nitrate reductase (*cnx*) have been isolated. Phenotypes were determined by growth tests and mutants were isolated in three race backgrounds. Loss of hygromycin resistance sometimes followed mutagenesis of transformants to chlorate resistance.

Mutants resistant to a range of anti-fungal agents and heavy metals have been isolated. With the exception of cadmium resistant mutants which exhibited a very unusual morphology, all retained full pathogenicity and race specificity. This concurs with the findings of Miao and Higgins (1986) who isolated mutants resistant to benomyl and cycloheximide in *F. fulva*.

The isolation of hygromycin and phleomycin resistant mutants by transformation is now routine (Oliver et al. 1987) and has proved the most reliable method of producing lines with positively selectable markers.

A number of colour mutants were isolated but most were unstable and reverted to wild type colouration on sub-culture. One stable red mutant was isolated following mutagenesis of a hygromycin resistant transformant. It has red conidia compared to the wild type dark green colouration and is similar to mutants described by Day (1957).

Pathogenicity

All mutants isolated (except cadmium resistant ones) retained full pathogenicity and their original race specificity. This is uncommon in plant pathogenic fungi particularly with auxotrophic mutations (Garber et al. 1983).

Hyphal anastamosis

Attempts to cross pairs of mutants by hyphal anastamosis (Hastie 1973) were unsuccessful in all cases. This confirms the findings discussed by Fincham et al. (1979). One report of hyphal anastamosis in *F. fulva* exists (Barr and Tomes 1961) but we have been unable to reproduce their results.

Protoplast fusions

A summary of the fusions achieved is given in Table 3. Protoplast regeneration frequencies varied between 40 and 50% before PEG treatment (Harling et al. 1988). This figure fell to 1% regeneration of non-selected phenotypes following PEG fusion of protoplasts. PEG treatment of protoplasts caused clumping and fusion to occur as witnessed by light microscopy. Ploidy determination of protoplasts proved difficult as the fluorescence filled the whole of the cells, thus although histograms of integrated optical densities of fused protoplasts show haploid, diploid and polyploid amounts of DNA it is impossible to say whether karyogamy directly follows plasmogamy in fused protoplasts. For this reason ploidy determination was performed using mycelial samples of fusion products where individual nuclei were easily seen and ploidy differences could be clearly shown.

Protoplast fusions between nitrate assimilatory pathway mutants proved to be the most successful

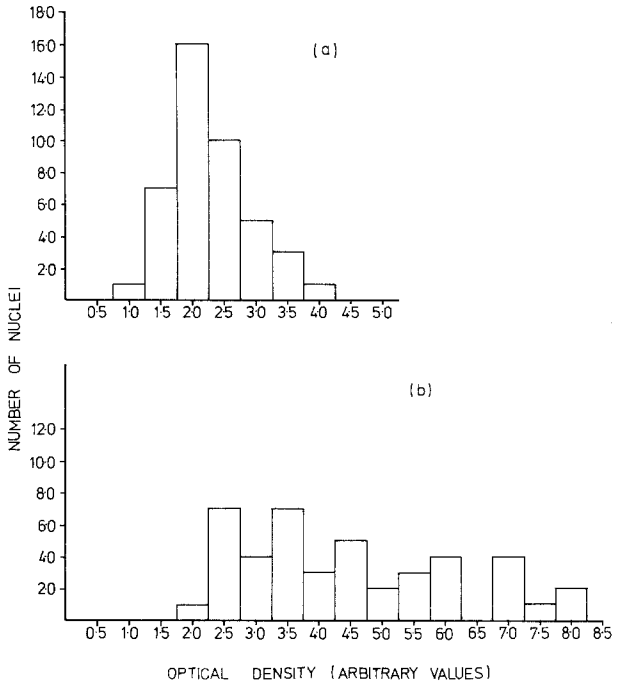

Fig. 1. Histograms showing normalised optical densities of DAPI stained nuclei in *F. fulva* mycelium. **a** *dcf*5S *cnx*-57 parent strain, **b** fusion product [*dcf*5S *cnx*-57/*dcf*5S *cnx*-69]-2. Ploidy differences may be clearly seen. A Mann-Whitney two sample test showed a highly significant difference between the parental and fusion product distributions

and complementation was achieved between *dcf5S nir*-73 and *dcf5S cnx*-69, *dcf5S cnx*-57 and *dcf5S cnx*-66 and between *dcf5S cnx*-57 and *dcf5S cnx*-69 but was not possible between *dcf5S cnx*-66 and *dcf5S cnx*-69. Products were shown to be diploid in the fusion between *dcf5S cnx*-57 and *dcf5S cnx*-69 (see Fig. 1) and remained stably diploid throughout three sub-cultures. In the other fusions where complementation was achieved fusion products were shown to be haploid. These may be heterokaryons or haploid recombinants since they maintain the selective phenotype throughout successive sub-cultures. There is some preliminary evidence for them being haploid recombinants as nuclei appear to be evenly distributed throughout the mycelium in *F. fulva* and this distribution is identical in parent strains and fusion products. Where complementation was not achieved between *dcf5S cnx*-66 and *dcf5S cnx*-69 a light background growth occurred on nitrate media which on sub-culture retained chlorate resistance. On the basis of the fusions performed between *cnx* mutants, they may be allocated to two complementation groups *cnx1* (*dcf5S cnx1*-57) and *cnx2* (*dcf5S cnx2*-66 and *dcf5S cnx2*-69).

The use of chlorate resistance as a double check on fusion products selected for their ability to grow on nitrate made the screening of putative fusion products far more accurate since *F. fulva* appears to be a very effective scavenger of trace nitrogen.

Protoplast fusions between drug resistant mutants often resulted in the loss of hygromycin resistance during the initial post fusion sub-cultures. In the fusion between *dcf4S benR*-06 and *dcf4S hygR*-067 and between *dcf4S cyhR*-87 and *dcf4S hygR*-07 loss of the doubly resistant phenotype on sub-culture always resulted from a loss of the hygromycin resistance marker. Loss of insert plasmid DNA was confirmed by Southern blotting of genomic DNA preparations of fusion products. The instability of transforming DNA in fusion products may be due to a similar mechanism to that causing instability in sexual crosses involving fungal transformants as discussed by Fincham (1988). The loss of foreign genes may however be a consequence of spontaneous haploidization since both fusions resulted in haploid fusion products and in the fusion between *dcf2S dcf4S dcf5S dcf9S phlR*-01 and *dcf4S hygR*-07 stable resistance to both antibiotics is seen. The numbers of stable diploids resulting from this fusion fell from 42% of all fusion products as shown in Table 3 to 24% and then 12.5% on successive sub-cultures. Other fusion products lost one or other of the resistance markers in roughly equal proportions and were haploid. Protoplast fusion seems to result in stable diploids or in haploid recombinants. The diploid state appears to be transitory with spontaneous haploidisation occurring between the initial post-fusion sub-cultures giving rise to one or other of the parental phenotypes or retention of the recombinant phenotype.

The ability to construct stable diploids is the first step in producing a genetic map in this imperfect fungus. Parasexual analysis should allow us to gain insight into the genetic determinants of pathogenicity and in particular permit the study of the hitherto unknown dominance relationships of the virulence (*dcfS*) and avirulence (*dcfR*) alleles.

Acknowledgements. We are grateful to Robert Harling and Lawrence Kenyon who supplied the cycloheximide and benomyl resistant mutants and to the AFRC and the Gatsby Charitable Foundation for financial support. The method for pathogenicity testing was developed by John Turner, Robert Harling and Lawrence Kenyon of this department.

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Communicated by K. Esser

Received August 29, 1988 / Revised October 31, 1988