zondend. kast 3 = ex
P13

LABORATORIUM VOOR
BLOEMBOLLENONDERZOEK
BULB RESEARCH CENTRE

Transformation and parasexual recombination in *Fusarium oxysporrum* f.sp. *gladioli*
Intern LBO-Rapport nr: 043
juni 1995

J.R. Bao

1732227

[zwart.pc.wp.doc.intern]voorblad.wp

Transformation and parasexual recombination in *Fusarium oxysporrum* f.sp. *gladioli*
Intern LBO-Rapport nr: 043
juni 1995

J.R. Bao

© **1995 Laboratorium voor Bloembollenonderzoek, Lisse**
Niets uit dit intern rapport mag worden verveelvoudigd en/of openbaar gemaakt door middel van druk, fotokopie, microfilm of op welke wijze ook zonder voorafgaande toestemming van de samensteller.

Het Ministerie van Landbouw, Natuurbeheer en Visserij stelt zich niet aansprakelijk voor eventuele schadelijke gevolgen die kunnen ontstaan bij het gebruik van de gegevens die in dit intern rapport zijn gepubliceerd.

WORK SUMMARY

BAO, Jian-rong, from Zhejiang Agricultural University, Hangzhou of China, worked in Departemnt of Plant Protection, Bulb Research Center(LBO) at Lisse in The Netherlands from July of 1994 to June of 1995 on a project of molecular genetics in fungal plant pathogen *Fusarium oxysporum* f. sp. *gladioli*. The research work was focused on the following subjects:

1. Parasexual recombination of the pathogen using fusion approaches, and the recombinant determinations.

2. Genetic transformation of the fungus using protoplast and electroporate transformation methods, especially with the vector pNE 24 carrying *nia D* gene.

Summaries on these parts will be presented separately. Comments are welcome.

My stay was supported partially by International Agricultural Center at Wageningen in The Netherlands, and partially by LBO. These two agencies should be acknowledged.

I thank Dr. J. van Aartrijk and Drs. Eugene J.A. Roebroeck for their efforts in making the arrangement for my research work at LBO. I also should mention, A. Doornik, Peter Vink, Ted and other members of LBO for their helpfulness and kindness, during my stay in the Netherlands.

BAO, Jian Rong

TRANSFORMATION IN FUSARIUM OXYSPORUM F. SP. GLADIOLI

INTRODUCTION

Fusarium oxysporum f. sp. *gladioli*(Mass.) Snyder & Hansen, the pathogen causing Fusarium yellows and corm rot of gladiolus, was subdivided into two races: race 1, which can infect both "large- and small-flowered" gladiolus cultivars "Peter pears" and "Nymph". Race 2 only infects the "small-flowered" gladiolus cultivar "Nymph"(Roebroeck and Mes. 1992), but within the same vegetative compatibility group: VCG 0340(Mes et al., 1994). The study of such kind of host specialization, at the molecular level requires the development of a convenient and efficient transformation method for the isolation and characterization of the genes involved in the pathogenesis.

Many transformation procedures have been developed, for different kinds of organisms. Protoplast methods was commenly used in fungal transformation(Akins and Lanbowits 1985, Ballance and Turner 1985, Langin et tal.1990, Kistler 1986, van Hartinoveldt et al. 1987, Campbell et al. 1989, Whitehead et al. 1990). Other transformation methods were also developed for filementous fungi(Lorito et al. 1993, Chakraborty et al. 1991, 1990, Becker and Guarente 1990). In the attempts to manipulate the genes involved in the gladiolis hosts, the tries to develop a stable and efficient homologous transformation system for the pathogenic fungus were tested using nitrate reductase gene(nia D), which were cloned and sequenced from a nitrate reductase-deficient strain of Fusarium oxysporum(Diolez et al. 1993).

MATERIALS AND METHODS

FUNGAL STRAINS, GROWTH CONDITIONS AND PLASMID

A nitrate reductase deficient strain G2nit1, a mutant from G2, a race 1 isolate of *Fusarium oxysporum* f. sp. *gladioli*(Roebroeck 1992,n Mes. 1994), was employed in all the tests. Another strain, G6nit1, a mutant from G6, a race 2 isolate, was also involved in some of experiments. These two mutants, the nitrate nonutilizing(nit) mutants, were induced from G2 and G6 using the methods described by Cove(1974) and Puhalla(1985). The stranis were cultured on PDA(Oxoide) medium at 27^{0}C for 7 days.

The plasmid pNA 24, carrying the nia D gene cloned from a Fusarium oxysporum strain, is a 7.2kb EcoR I fragment subcloned into pUC19(Diolez et al. 1993). The extraction of plasmid DNA was conducted using Qiagen kit, or using the standard protocol.

TRANSFORMATION

PROTOPLAST METHOD: Transformation was performed using a PEG/Ca^+ mediation method of protoplasts, described basically by Punt et al. 1992.

1. Generation of protoplast: The spores of strains tested, were harvested from 7-day old cultures on PDA(Oxoid) at 27^{0}C, and incubated in PDB liquid medium(about 1 x 10^7spores per 50ml PDB) at 27°C on a rotary shaker at 140rpm, for 16-18 hr. Mycelium was harvested by centrifuging the culture in a 50ml Falcon tube, at 4500rpm(~3500g) for 10 min, and then washed with MSM(1M $MgSO_4$ in 50mM Maleate, pH 5.8) twice (mix the mycelium well with MSM before centrifugation). 500mg of fresh--weight mycelium was resuspended in 10ml MSM containing novozym 234(5mg/ml), or glucanex(25mg/ml). The mixture was vortexed, and then incubated at 30°C and placed on a rotary shaker at 60rpm, for 1.5 to 2 hr to release the protoplasts. Protoplasts were harvested, by filtering the suspension through sterilized filter paper(Ederol no 261), and then centrifuging supernatant at 1500g for 15 min, and then pippetting the pro-

toplasts floating on the liquid surface, into a clean tube. Protoplasts were washed with SNT(1M sorbitol, 100mM NaCl, 10mM Tris/HCl pH 7.4), and then resuspended in SNT to a concentration of 1 x 10^7 protoplasts/ml.

2. Transformation procedure: The final protoplasts were washed twice with STC1700(1.2M Sorbitol, 10 mM Tris/HCl pH 7.5, 50mM $CaCl_2.2H_2O$, 35mM NaCl), and resuspended in STC1700 to a concentration of 1 x 10^7 protoplasts/ml. 100ul of protoplast suspension was mixed with 5-10ug vector DNA, this was incubated at room temperature for 30 min. The protoplast suspension was mixed gently with 250, 250 and 850ul in three individual steps, with 60% PEG buffer(60% polyethylene glycol 4000, 10mM Tris/HCl pH 7.5, 50mM $CaCl_2.2H_2O$), and incubated on ice for 20 minutes. The suspension was diluted by adding 8ml STC1700.

From here, two procedures were tested for the transformation efficiency: One, called Plating With PEG, was to pippet 100ul of the suspension directly, onto osmotically stablized selection medium(OS-CDA: CDA in 1M sorbitol, MOS-CDA: CDA in 0.5M $MgSO_4.7H_2O$ with Tris/HCl pH 7.4, SAC-CDA: CDA in 20% sucrose), or into 10ml of a melted top agar medium at 45°C(CD in 4% agar). Gently mix the top agar with the suspension, and then pour it onto an osmotically stabilized selective medium.

The other procedure, called Plating Without PEG, was to centrifuge the suspension at 1500g for 10 minutes. The pellet was suspended in STC1700, and the suspension was plated onto osmotically stabilized selective agar medium, or into 10ml of a melted top agar medium at 45°C. The top agar was poured onto a plate with osmotilized selective medium.

These plates were incubated at 27°C. Protoplasts treated with TE buffer, served as controls for spontaneous mutation. The colonies emerged on selective medium with wild-type growth, were regarded as transformants, and some were transferred to fresh CDA medium to test their growth. Some transformants were subjected to monospore culturing on PDA, and the monospore

colonies were tested on CDA medium for the growth appearance.

ELECTROPLATTION PROCEDURE: High voltage electric pulses were delievered by a Gene Pulser apparatus(Bio-Rad model 1652077) equipped with a pulse controller(Bio-Rad model 1652098), capable of generating pulses up to 2500v from 0.25 to 25.0uF capacitors. Cuvettes used were 0.2mm in distance between electrodes with 400ul capacity.

Conidia from seven-day old culture on PDA, were harvested, and filtered through 2-layer paper filter(Ederol 261). The spores were spinned down, and suspended in steril water to a concentration of 6 x 10^7 spores/ml. 0.5-1.0ml of the suspension was added into 10ml of PDB liquid medium in 50ml Felcon tube, and then incubated onto a rotary shaker at 140rpm, 27^{0}C for the time rquired. The culture was added with enzyme, and incubated for another 2 hours under the same conditions. The germinated spores were spinned down at 1600g(3500rpm on Henze centrifuger) for 5 minutes, and washed twice with cold HEPES buffer(1mM HEPES, pH 7.5, 50mM mannitol). The pellet of the spores was resuspended in the same buffer to a concentration of about 6-7 x 10^7 spores/ml. 200ul of such suspension was loaded into cuvette, and then plasmid DNA was mixed with the suspension. After kept on ice for 30 minutes, the cuvette was subjected to electroperation, and kept at room temperature for another 30 minutes. The electroperated spore suspension was diluted in sterilized water, and plated onto CDA medium. Controls were set to detect their back mutations. Cell viability was monitored by plating the spores on PDA medium.

RESULTS

1. PROTOPLAST METHOD.

Table 1. Influence of DNA concentration on the transformation efficiency in protoplast method on OS-CDA.

Plasmid DNA (ug)	Regeneration Rate(%)		Total Tr.*		Frequency(Tr./ug DNA)	
	G2nit1	G6nit1	G2nit1	G6nit1	G2nit1	G6nit1
4.2	14.2	4.4	60	55	14	13
2.7	24.5	9.1	55	50	20	19
1.8	13.5	12.5	50	35	28	20
0.9	52.0	16.3	40	45	44	50
0.0	41.6	11.6	0	0	0	0

* Total transformants was based on the volume of original 100ul protoplast suspension.

Table 2. Influence of plating methods on the transformation frequency of the strain G2nit1.

	Plating With PEG						Plating Without PEG					
	OS-CDA		MOS-CDA		SAC-CDA		OS-CDA		MOS-CDA		SAC-CDA	
Item*	-	+Top	-	+Top	-	+Top	-	+Top	-	+Top	-	+Top
Total Tr.	100	1200	25	1000	0	1000	50	50	100	100	0	50
Freq.	7	86	1.5	72	0	72	3.6	3.6	7.2	7.2	0	3.6
Reg-R(%)	8		44		68		8		48		40	

* Tr.: Transformant, Freq.: Frequency(%), Reg-R(%): Regeneration Rate(%).

2. ELECTROPERATION METHOD

Table 3 Spore survival and efficiency of transformation using electroperation method.

Capacitance (uF)	Field Strength (kV/cm)	Time constant (ms)	Incubation (hr.) PDB	+Enzyme	Viability(%)*	Efficiency (Tr./ug DNA)
1	10	0.2	0	0	100(1)	-
			0	2	100(1)	-
			2	0	100(1)	-
			4	0	100(1)	-
			2	2	25(1)	-
	12.5	0.3	0	0	100(2)	0
			0	2	83(1)	0
			2	0	100(1)	-
			4	0	76(1)	0
			2	2	40(1)	0
			4	2	80(1)	-
			6	0	100(1)	25
			4	2-L	100(1)	0
3	10	0.6	0	0	90(3)	0
			0	2	68(1)	-
			2	0	25(1)	-
			4	0	50(1)	-
			2	2	36(1)	-
			4	2	47(3)	800
			6	0	87(2)	30
			4	2-L	55(2)	200
	12.5	0.6	0	0	60(3)	0
			0	2	30(1)	0
			2	0	13(1)	-
			4	0	25(1)	0
			2	2	30(1)	0
			4	2	30(3)	1000
			6	0	43(2)	20
			4	2-L	64(2)	100

25	3	4.0	4	2	49(1)	-
	5	3.9	0	0	55(2)	0
			4	2	55(7)	1200
			6	0	68(2)	100
			4	2-L	60(2)	300
	8	4.3	0	0	15(1)	-
			0	2	7(1)	-
			2	0	3(1)	-
			4	0	20(1)	-
			2	2	20(1)	-
			4	2	37(1)	-
	10	4.3	0	0	2(2)	-
			0	2	6(1)	-
			2	0	1(1)	-
			4	0	12(1)	-
			2	2	4(1)	-
			4	2	5(1)	-
			6	0	2(1)	-
			4	2-L	12(1)	-
	12.5	4.4	0	0	0(1)	-
			0	2	3(1)	0
			2	0	1(1)	-
			4	0	8(1)	0
			2	2	10(1)	0
			4	2	4(1)	-

* The number in parathese is the time tested. -: Not tested. 2-L: Add 1mg/ml of lysis enzyme.
All other were added with 5mg/ml of glucanex enzyme.

Table 4. Survival of protoplasts from G2 after treated with electroporation.

Capacitance (uF)	Field Strength (kV/cm)	Time Constant (ms)	Survival (%)	Transformation Efficiency(Tr./ug DNA)
0.25	1.0	0.1	30	0
	1.6	0.1	37	0
	2.0	0.1	7	9
1.0	0.5	0.3	12	0
	1.0	0.2	15	0
	1.6	0.2	12	17
3.0	0.5	0.6	25	0
	1.0	0.6	10	0

Note: Each cuvette was loaded with 150ul of protoplast suspension at the concentration of 1×10^8 protoplasts/ml, plused with 4.5ul of plasmid DNA at 2.0ug/ul. The protoplasts were suspended in HS buffer(5mM HEPES pH 6.5, 20% Sucrose).

PARASEXUAL RECOMBINATION BETWEEN STRAINS OF RACE 1 AND RACE 2 IN FUSARIUM OXYSPORUM F. SP. GLADIOLI

INTRODUCTION

Fusarium oxysporum f. sp. *gladioli*(Mass.) Snyder & Hansen, the pathogen causing Fusarium yellows and corm rot of gladiolus, was subdivided into two races: race 1, which can infect both "large- and small-flowered" gladiolus cultivars "Peter pears" and "Nymph". Race 2 only infects the "small-flowered" gladiolus cultivar "Nymph"(Roebroeck and Mes. 1992), but within the same vegetative compatibility group: VCG 0340(Mes et al., 1994). Understanding the molecular mechanisms of pathogenicity of the fungus, can be significant not only in understanding the interactions between the pathogen and its hosts. But also in practical work such as detection, and more efficient resistance breeding.

No sexual stage of the pathogenic fungus is known, though intra- and interstrain recombinants could be formed parasexually(Molnar et al.1990). There are several different methods available to produce parasexual recombinants, to obtain the information on genetics of pathogenicity of these races. In the experiments described here, several fusion systems using different combinations of selection markers were examined to get genetic recombinants.

MATERIALS AND METHODS

FUNGAL STRAINS

Two wild-type strains of *Fusarium oxysporum* f. sp. *gladioli* were employed in all tests: G2, a race 1 isolate, and G6, a race 2 isolate(Roebroeck 1992,n Mes. 1994). These two strains were subjected to treatments, generating progenies with selec-

tion markers.

SELECTION MARKERS: All the markered progenies, originated from strains G2 and G6. Several selection markers employed, were integrated into the two parent strains G2 and G6, so that the genetic recombinants could be identified(Table 1).

Auxotrophic mutants were induced by UV irradiation, and selected by replicate plating on CDA(minimal medium) and complete medium(CM: CDA amended with yeast extracts, peptone and cascin hycholysate). Colonies growing on CM, but not on CDA were selected and screened for nutritional requirements.

Nitrate nonutilizing(*nit*) mutants were recovered and identified according to Cove(1976) and Puhalla(1985). All the *nit* mutants used in the experiments, are nitrate reductase-deficit mutants(*nit 1* in phenotype).

Benomyl resistance(BenR) strains were obtained by treating spores with UV irradiation, and then screening them on PDA medium amended with 3ug/ml and 30ug/ml benomyl, for low-tolerant and high-tolerant mutants, respectively. The low-tolerance is determined by a single gene, while high-tolerance is controlled by two genes(Molnar, A. et al. 1985).

Hygromycin B(HygrR) and Phleomycin resistance(PhleR) strains were obtained by transformation with the vectors pAN 7-1 and pAN 8-1, respectively(Punt et al. 1987, Mattern et al. 1988). Vector pAN 7-1 is the carrier of the HygrR gene, while pAN 8-1 is the carrier of the PhleR gene, which were transformed into the parential fungal strains. The transformants were designated with a suffix of "h" for HygrR transformants, or "p" for PhleR transformants, followed by a specific serial number.

Some RAPD markers developed previously(Mes et al. 1994) were also employed to identify recombinants.

TRANSFORMATION

Transformation was performed using a PEG/Ca^{+} mediation method of protoplasts, described basically by Punt et al. 1992.

1. Generation of protoplast: The spores of strains tested, were harvested from 7-day old cultures on PDA(Oxoid) at 27°C, and incubated in PDB liquid medium(about 1 x 10^7spores per 50ml PDB) at 27°C on a rotary shaker at 140rpm, for 16-18 hr. Mycelium was harvested by centrifuging the culture in a 50ml Falcon tube, at 4500rpm(~3500g) for 10 min, and then washed with MSM(1M $MgSO_4$ in 50mM Maleate, pH 5.8) twice (mix the mycelium well with MSM before centrifugation). 500mg of fresh--weight mycelium was resuspended in 10ml MSM containing novozym 234(5mg/ml), or glucanex(25mg/ml). The mixture was vortexed, and then incubated at 30°C and placed on a rotary shaker at 60rpm, for 1.5 to 2 hr to release the protoplasts. Protoplasts were harvested, by filtering the suspension through sterilized filter paper(Ederol no 261), and then centrifuging supernatant at 1500g for 15 min, and then pippetting the protoplasts floating on the liquid surface, into a clean tube. Protoplasts were washed with SNT(1M sorbitol, 100mM NaCl, 10mM Tris/HCl pH 7.4), and then resuspended in SNT to a concentration of 1 x 10^7 protoplasts/ml.

2. Transformation procedure: The final protoplasts were washed twice with STC1700(1.2M Sorbitol, 10 mM Tris/HCl pH 7.5, 50mM $CaCl_2.2H_2O$, 35mM NaCl), and resuspended in STC1700 to a concentration of 1 x 10^7 protoplasts/ml. 100ul of protoplast suspension was mixed with 5-10ug vector DNA, this was incubated at room temperature for 30 min. The protoplast suspension was mixed gently with 250, 250 and 850ul in three individual steps, with 60% PEG buffer(60% polyethylene glycol 4000, 10mM Tris/HCl pH 7.5, 50mM $CaCl_2.2H_2O$), and incubated on ice for 20 minutes. The suspension was diluted by adding 8ml STC1700, and centrifuged at 1500g for 10min. The pellet was suspended in STC1700 for plating on osmotically stabilized selective agar medium(PDA in 1M sorbitol with antibiotics: hygromycin B at

100ug/ml, or phleomycin at 50ug/ml), these plates were incubated at 27°C. Protoplasts treated with TE buffer, served as controls for spontaneous mutation. The fast-growing colonies which emerged on the plates with PEG-treated protoplasts, were transferred to fresh selective medium, and a block of medium from the rim of the colony, was placed onto the same kind medium for re-selection. Stable fast-growing colonies were monocultured, and the subsequent monospore colonies were tested for growth rate on selective medium, and stored on both PDA and sand tubes at 4°C.

FUSION SYSTEM

Different pairing systems were conducted between G2 and G6 progeny strains, carrying on them different selection markers to produce presumed genetic recombinants(Table 5).

1. GENERATION OF PROTOPLAST: The protoplast generation procedure, was the same as that in transformation described above.

2. FUSION OF PROTOPLASTS: Polyethylene glycol(PEG, mol.wt 4000. 30% PEG, 10mM $CaCl_2$, 10mMTris/HCl pH 7.5) was used as the fusiogenic agent to treat the mixtures of parental protoplasts. The protoplast mixture pellet after centrifugation, was resuspended in ice-cold PEG buffer and kept on ice for 30 minutes. The protoplasts were spinned down at 1500g for 5 minutes, and resuspended in SNT for the dilution series, and then plated directly on selective medium. Plates were incubated at 27°C. In all experiments, the combinations of parental protoplasts treated with SNT, and parental protoplasts alone treated with either SNT or PEG were set for the controls, to check for spontaneous mutations, and for the comparisons with PEG treatments. Fast-growing colonies developed from PEG treated protoplasts on selective medium, these were considered as fusion products, while those colonies showing restricted

growth on selective medium, were regarded as heterokaryotic colonies. All the tested dishes were incubated at 27^{0}C for at least 4 weeks.

3. HYPHAL FUSION: Hyphal fusion tests were only carried out for the auxotrophic strains. Spores were collected from 7-day old culture on PDA to a concentration of 4 x 10^6 spores/ml. Spores from different parents were mixed in 2% PDA solution, and the mixture was transferred to reserviors(60mm in diameter) in CDA plates with non-mixed parent spores alone as controls. The plates were incubated at 27 °C. Fast-growing sectors from the sides of the reserviors, were cultured on fresh selective medium for further selection and monospore manipulations. The codes for hyphal fusion products were started with a letter "h".

SELECTION PROCEDURE FOR RECOMBINANTS

1. SELECTED BY SELECTIVE MEDIA: In fusion experiments, fast-growing colonies developed from the mixed PEG-treated protoplasts on selective media, were regarded as fusion products, and transferred to fresh selective medium(Some of them were selected twice on fresh selective medium)(Table 2). From the rim of stable vigorously growing colony on fresh selective medium, a block of the colony was cut off for monospore isolation, and placed onto non-selective medium(PDA). Several monospore colonies from the same fusion products were transferred to both selective and non-selective media(10ml per plate of 9cm in diameter), to measure and compare their growth rates at 27^{0}C. Growth rates were also determined for their parental strains, on both selective and non-selective media for comparison. Those showing both parent markers, were selected as fusion products, and stored on PDA and sand tubes.

In hyphal fusion tests, a block of the fast-growing sector was cultured on a selective medium to examine its growth. Wild-type and stable colonies on selective medium were subjected to

monospore culturing. The monospore colonies were re-selected on the same selective medium, to distinguish stable recombinants from heterokaryons.

2. SELECTED BY RAPD: Several 10-mer primers, which generated random amplified polymorphic DNAs(RAPDs) for the parental strains G2 and G6, were used as genomic DNA markers to analyse fusion products(Table 3).

A. Genomic DNA Extraction: The basic procedure was described by Tennis et al.(1990). About 50mg of fresh weight mycelium was collected from 3-4-day old PDB culture, from Falcon tube at 27°C, these were digested using lysis buffer(50mM Tris/HCl pH 7.2, 50mM EDTA, 3% SDS, 2% DTT) at 65°C for 60min. DNA from the digested suspension, was extracted using an equal volume of chloroformal/phenol/isoamylalcohol (PCI, 25:24:1) by centrifugation, at 12,000g for 5min. The DNA was precipitated with 0.1 volume 3M sodium acetate and 0.6 volume isopropanol, from the upper aqueous phase of PCI extracted suspension, and pelleted at 12,000g for 5min, and washed once with cold 70% ethanol, and resuspended in 50 ul TE buffer, and stored at -20°C.

B. PCR Reaction and Electrophoresis: The basic performance of PCR reaction for the fusion product DNA, was described by Mes et al. (1992). 50ul of PCR reaction mixture contained 20 pmol primer, 10mM Tris/HCl pH 9.0, 1.5mM $MgCl_2$, 50mM KCl, 0.01% gelatine, 0.1% Triton X-100, 40uM each of dATP, dCTP,dGTP and dTTP(Perkin Elmer Cetus), and including 5ul extracted fungal DNA. These were amplyfied in thermocycler(Omnigene HBTR3CM, Hybaid, UK) for 35 cycles, after an initial denaturation for 4 minutes at 93.4°C. Each cycle consisted of 93.4°C for 30 seconds, 35.2°C for 40 seconds, 71°C for 2 minutes and the last cycle has a final extension at 71°C for 10 minutes. The holding temperature was 25°C. The amplified products were run on 1.5% agarose gel, and the bands were stained with ethidium bromide. DNA from the parent strains, were also amplyfied and run on the gels.

RESULTS

1. PROTOPLAST FUSION: Different fusion systems tested, had different results for the generation of fusion products(Table 5).

In the system, pairings with strains carrying BenR marker did not generate fusion products. Even though the heterokaryon colonies could be formed abundantly on selective medium. No fast-growing colonies were observed from these heterokaryon colonies during the whole culturing period(at least 7 weeks). The heterokaryon colonies were developed within a week, but hardly any growth was seen afterwards. The size of these colonies were also smaller(about 2mm in diameter), compared with those developed in other pairing systems, which can be expanded to 5mm or more in diameter. On any of the control plates, no heterokaryotic colonies were observed in these experiments,

In the system of HygrR-nit x Wild type, fusion products were obtained from pairing of 6h9nit1 x 2W, but not from the pairings of 2hnit x 6W.

The results from the auxotroph pairings, were almost the same as the results from those in hyphal fusion tests: The pairing of 2aux2 x 6aux2 produced fusion products, easier than pairings such as 2aux1, with other G6 auxotrophes. In the later pairings, some wild-type colonies appeared on original selective medium, but the single spore progenies from most of these colonies, grew into an auxotrophic state on fresh selective medium. Meanwhile sometimes, some back ground growth problems existed in the auxotrophes G6aux4 and G6aux6, during their culturing period on selective medium , but not in the auxotroph G6aux8. Even though, the background growth colonies on selective medium from these two parents, were still distinguishable from the presumed fusion colonies. The background growth colonies, were wild growth types with more dense aerial mycelium, than the fusion product colonies. The presumed fusion product colonies, grew more or less abnormal in appea-

rance. Their aerial mycelium were not so dense, and usually, the mycelium expanding on selective medium, became more or less robust. Meanwhile, the expanding of background colonies were shown to be restricted on the selective medium.

In the system PhleR x HygrR, not all pairings generated fusion products. The generation of fusion products relied on the strains involved in the pairings. The pairing of 6h1 x 2p22 for example, produced many fusion colonies on selective medium, while other pairings produced only a few, or even no fusion products. This was evidenced that the strains 6h1 and 2p22, produce many fusion products when paired together, but don't easly generate fusion colonies when paired with other strains on their own(Table 5). The formation and number of heterokaryon colonies on selective medium, didn't mean the formation and correspondent number of fusion colonies developed. Though they were associated with in the pairings, from which fusion colonies were formed.

Usually, two types of fusion product colonies were observed on selective medium in the pairing system of Hygr-R x Phle-R: typical wild-type colonies with dense aerial mycelium, and colonies with very sparse aerial mycelium. The morphological type of fusion product colonies, usually was dependent on the parental strains involved in the pairings. Most of pairings, able to generate fusion products, produced the first type of colonies, such as 6h1 x 2p22, and some pairings, like 2h1 x 6p2, inclined to produce the later type of colonies.

The protoplast regenerations varied from experiment to experiment, but no evidences were found, that the regeneration rates did not correlate with the frequency of heterokaryon's formation. The regeneration rates were also influenced by the stabilizer used in the medium. The use of $MgSO_4.7H_2O$, for example, led to protoplast regeneration rates several times higher than that when sorbitol was used. However, this stabilizer influenced on the effectiveness of both antibiotics: phleomycin and hygromycin B(Table 6), which made it difficult to pick up the fusion colonies from heterokaryon colonies on original

selective medium.

In most of the control plates, protoplast mixtures treated with SNT, had no heterokaryon-like colonies developed on the selective media. However, a few heterokaryon-like colonies could be developed from some mixture plates, but the growth of these kind colonies were inhibited, while they were cultured on fresh selective media. Some small colonies were also observed on some control plates with parent protoplasts alone, treated with either SNT or PEG, especially from the strains with HygrR markers, but none of them tested, grew as well as the fusion colonies did, when subjected to fresh selective media. The colonies, develoed from parental strain protoplasts on selective medium, usually were emerged 2 or 4 days later than the heterokaryon colonies, developed from mixture protoplasts treated with PEG. However, it was hard to tell the differece of the colonies on control plates from those on treated plates in morphological appearance.

2. HYPHAEL FUSION

For the fusion via hyphal anamotose, it was showed that different pairings of auxotrophic mutatants had different abilities to form fusion products. This was the same as the performance in protoplast fusion tests. In most cases, recombinant-like sectors were developed in about 2-week. The outcomes, however, were quite different when the sectors from different pairings were transferred to fresh selective medium. Fast-growing sectors from the pairing of 2aux1 x 6aux8, for example, were produced on selective medium, but most of them shew parental-type growth after transferred to fresh selective medium or after monoculturing.

Hyphal fusion pairing between BenR and hygrR strains produced fusion products within 2-3 weeks on selective medium, though no fusion colonies were generated in several protoplast fusion tests with the same pairing system.

4. ANALYSIS OF FUSION PRODUCTS

A. By selective Medium

In the selective procedures, fast-growing colonies from original selective plates were transferred to fresh selective media. In the fusion system Hygr-R x Phle-R, over 90% such kind of colonies could be analysed as recombinants after screened through several steps. In this system, the well-growing colonies from original plates on fresh selective medium were monocultured on PDA, and then one or more single spore colony was further tested at the same time on CDA, CDA amended with hygromycin B and CDA with phleomycin. The growth rates were compared with those of parent strains on the same media at the same conditions(Table. Growth). However, fast-growing colonies were also not so easy to be picked up when PDA was used as the basic forum for selective medium, because heterokaryon colonies could grew biger with culturing. In those early experiments, no accurate calculation of recombination progenies is possible.

In the hygrRnit1 x Wild-type system, it became difficult to get rid of the hygrRnit parent from the fusion colonies, because the parent hygrRnit1 could also accompany the fusion colony growth at the same rate on the selective medium-CDA amended with hygromycin B(CDA +h). The separation of fusion colonies from hygrRnit parent could be achieved by monosporulating the selected colony on PDA after the presumed fusion product shew wild-type growth on fresh selective medium, so that both the progenies could grow on PDA, the nonselective medium. Fusion product colonies were collected by re-screening them on CDA+h, on which colony from recombinant grew in wild-type, while colony from the parent grew in mutant type.

In the auxotroph pairing system, the selection procedure was principally the same as the system HygrR x PhleR, except the selection medium, which CDA was used only. Meanwhile, many colonies, which were originally regarded as presumed fusion

products on original selective medium, were found not to be recombinants after subcultured to CDA medium, or afetr mono-cultured on PDA and futher cultured on CDA. The recombination frequency for these pairing became somewhat unfeasible.

For the hyphal fusion products, the same basic procedures were also implemented to select the fusion products. There were the same problems as those in selecting protoplast fusion products of the same fusion system, that is: many original fast-growing sectors were not recombinants in the futher subculturings.

B. BY RAPD MARKERS

The primers were selected for the RAPD-PCR to perform the re-combinant markers specifing both parent's genomic DNA. Among the primers tested, G2 markers were more easy to be performed and detected using any fusion products from any fusion system. The markers specifing G6 parent became much difficult for some fusion products. At beginning, two well-established primers: B14 and E20, were selected for PCR performance. E20 primer had a 1100bp band for G6 and its derivative strains, but the band was usually invisible in the fusion product gels. In the other primers screened, H09 primer could be a reliable marker for G6 for its 1250bp band(Table. Marker).

C. PIGMENT MARKER

The existance of difinit pigment in G2, greyish white, and G6, purple-redish in stromata on PDA gave the pigmental marker du-ring culturing. Some fusion products followed one of their parent pigment, and others produce some different pigmentation which differed from their parents.

The pigmentation of fusion products from HygrRnit1 x Wild-type fusion system usually followed that of G6 parent. However, the G6 markers from E20 and B14 primers were usually absent.

DISCUSION

Different pairings using different combinations of genetic markers had different outcomes of fusion generation. Several systems in this experiment failed to generate fusion products. The protoplast fusion system using benomyl resistance marker seemed to be not desirable to produce fusion products. However, even within the same system, the generation of fusion products could be quite different. It could be showed from the results of pairings of 2aux1 with 6aux4 or 6aux8, which, unlike the pairing 2aux2 with 6aux2, only generated very few fusion products. Another example was the results from PhleR x HygrR pairings, in which the mixtures from different strains could get totally different fusion production. In this system, mixture of 6h1 x 2p22 was more effective in produceing heterokaryons, and in about 5-day or more , some of them became fast-growing colonies, while the mixtures 6h5 x 2p17 and others hardly produced any fusion product.

In the protoplast fusion system Hygr-R x Phle-R, at beginning, PDA was used as the basic component for selective medium, which Sorbitol stabilized PDA was amended with Hygromycin B and Phleomycin. With this selective medium, the growth of presumed heterokaryotic and parental colonies was not so restrictively inhibited, so the real fusion product colonies were hard to be identified on the plates. Later, CDA was used as the basic forum in selective medium, and the heterokaryon colony growth were restricted more effectively.

The G2 RAPD markers, were shown to be quite stable, and easy to be detected on electrophoresis gels, in the detection pro-

cedure, while G6 RAPD markers sometimes became difficult to detect. The well-established G6 marker band from the primer E20, was easy to be detected for G6 and its derivative strains on the electropholysis gels. But, the bands were usually absent in the fusion product gels, even though the fusion products had other prominant G6 markers, like pigmentation on PDA. In the primers tested for G6 markers, primers H09, B14 and G15, were recommended as G6 markers in the fusion product analysis by RAPD approaches.

REFERENCE

Buxton, E.W.1956, Heterokaryosis and parasexual recombination in pathogenic strains of Fusarium oxysporum. J. Gen. Microbiol. 15:133-139

Mattern, E., P.J.Punt and C.A.M.J.J.van den Hondel. Fungal gen. Newsletter. 35:25, 1988.

Mes, J.J., J.van Doorn, E.J.A.Roebroeck, E.van Egmond, J.van Aartrijk and P.M. Boonekamp. 1994. Restriction fragment length polymorphisms, races and vegetative compatibility groups within a worldwide collection of Fusarium oxysporum f.sp.gladioli. Plant Pathology43:362-370

Mes, J.J., J.van Doorn, E.J.A.Roebroeck and P.M. Boonekamp. 1994. Detection and Identification of Fusarium oxysporum f. sp. gladioli by RFLP and RAPD analysis. In"Modern Assays for plant pathogenic fungi: Identification, detection and quantification." Edited by A.Schots, F.M.Dewey and R.Oliver. CAB International, Oxford.

Molnar,A, M.Pesti and L.Hornok.1985. Isolation, Regeneration, and fusion of Fusarium oxysporum protoplasts. Acta Phytopathologica Academiae Scientiarum Hungaricae.20(1-2):175-182.

Molnar, A. Hornok, L., and Pesti, M. 1985. The high level of benomyl tolerance in Fusarium oxysporum is determined by the synergistic interaction of two genes. Experimental Mycology 9:326-333.

Molnar, A, L.Sulyok and L.Hornok. 1990, Parasexual recombination between vegetatively incompatible strains in Fusarium oxysporium. Mycol. Res. 94(3):393-398

Punt, P.J.,R.P.Oliver, M.A.Dingemanse, P.H.Pouwels and C. A. M. J. J. van den Hondel. Gen56: 117(1987)

Punt,P.J. and Cees A.M.J.J.van den Hondel. 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. In "Methods in Enzymology" 216:447-457.

Roebroeck, E.J.A. and J.J.Mes. 1992. Physiological races and vegetative compatibility groups within Fusarium oxysporum f.sp.gladioli. Neth. J.Pl.Path. 98:57-64.

Steven B. Lee and J.W.Taylor. 1990. Isolation of DNA from Fungal Mycelia and single spores. In "PCR Protocols" edited by M.A. Innis, D.H. Gelfaud, J.J.Sninsky, T.J.White. pp:282-287. Academic Press.

Table 1. Parents and their derivatives involved in fusion tests.

Designation	Parent	Identity	Reference
G2		Wild-type for Race 1	Roebroeck.1992
2ben6	G2	Benomyl resistance	Induced by UV.
2ben12	"	" "	"
2aux1	"	Adenine requirement	"
2aux2	"	Leucine requirement	"
2ben12nit1b	"	Ben-R with nit mutation	nit mutant from 2ben12
2h1	"	Hygromycin B resistance	Transformant
2h2	"	" "	"
2h10	"	" "	"
2h11	"	" "	"
2h12	"	" "	"
2h11nit1	"	Hygr-R with nit mutation	nit mutant from 2h11
2h12nit1	"	" "	"
2p15	"	Phle-R	Transformant
2p18	"	"	"
2p21	"	"	"
2p22	"	"	"
G6	G6	Wild-type for Race 2	Roebroeck.1992
6ben13	"	Ben-R	Induced by UV
6aux2	"	Adenine requirement	"
6aux4	"	?	"
6aux6	"	Proline requirement	"
6aux8	"	Lysine requirement	"
6h1	"	Hygr-R	Transformant
6h2	"	"	"
6h3	"	"	"
6h5	"	"	"
6h9	"	"	"
6h10	"	"	"
6h9nit1	"	Hygr-R with nit mutation	nit mutant from 6h9
6p2	"	Phle-R	Transformant
6p5	"	"	"
6p6	"	"	"
6p7	"	"	"
6p8	"	"	"

Table 2. Fusion systems and their selective media

Fusion system	Selective Medium*		Non-selective medium*	
	Fusion test	Selection test	Fusion test	General Culture
Phle-R x Hygr-R	OS-PDA p+h	PDA p+h	OS-PDA	PDA
	OS-CDA p+h	CDA p+h	OS-CDA	
Hygr-Rnit1 x Wild	OS-CDA	CDA	OS-PDA	PDA
Ben-R x Phle-R	OS-PDA b+p	PDA b+p	OS-PDA	PDA
Ben-R x Hygr-R	OS-PDA b+h	PDA b+h	OS-PDA	PDA
Aux x Aux**	OS-CDA	CDA	OS-PDA	PDA
Ben-R x Wild	OS-PDA +b	PDA +b	OS-PDA	PDA

*. OS: 1M Sorbitol as stabilizer, and the suffix b, h, p, represent benomyl(3ug/ml), hygromycin B(100ug/ml) and phleomycin(50ug/ml).

**. Aux for auxotrophes, and only CDA was used in hyphal fusion generation.

Table 3. Primers employed in the RAPD detection

Primer	Sequence	Marker band(bp)	
		G2	G6
A18	5'-AGGTGACCGT	1000	-
B14	5'-TCCGCTCTGG	2000	1600
E20	5'-AACGGTGACC	1020,1010	1100,700
F02	5'-GAGGATCCCT	1700,1100	3000
F13	5'-GGCTGCAGAA	1100, 2000,700	-
F14	5'-GGCTGCAGAA	2000,930,700	-
G09		1600	1200
G12	5'-CAGCTCACGA	1100,550	-
G15	5'-ACTGGGACTC	1200	550
H01	5'-GGTCGGAGAA	-	1700
H09	5'-TGTAGCTGGG	1100	1250

Table 4. Inflence of magnetium sulphate on the expression of antibiotic genes --Hygromycin and Phleomycin

Code	Growth rate in diameter(mm/4-day)									
	M-PDA					PDA				
	-	+H	+P	P+H	IR(%)	-	+H	+P	P+H	IR(%)
2h-11	33	31	17	18	45.2	45	48	20	20	58.3
2p-21	36	22	21	21	-4.5	44	0	39	0	100.0
6h-9	40	39	28	13	28.2	47	49	20	23	59.2
6p-6	38	14	34	14	58.8	43	0	37	0	100.0
2h-11(Pe)	28	16	15	13	6.3	38	0	19	0	100.0
2p-21(Pe)	32	17	29	14	41.4	39	0	35	0	100.0
6h-09(Pe)	26	27	15	14	44.4	38	38	22	22	42.1
6p-06(Pe)	35	16	31	13	48.4	40	5	28	5	82.1
2h-11(S)	27	25	19	22	24.0	42	0	21	6	100.0
2p-21(S)	33	32	30	27	6.3	39	0	35	0	100.0
6h-09(S)	28	31	19	19	38.7	40	41	22	22	46.3
6p-06(S)	32	20	30	17	33.3	40	6	29	6	79.3

M-PDA: PDA plus 0.5M Magnetium Sulphate in 10mM Tris,pH 7.4.
H: Hygromycin 100ppm, P: Phleomycin 50ppm.
Pe: Colonies tested are recovered from protoplasts treated with PEG, and (S): with SNT.
IR: Inhibited Ratio: IR = (D1 - D2) x 100 / D1, where D1 and D2 are colony diameters on media with Hygromycin B or Phleomycin.

Table 5. Protoplast regeneration, formation of heterokaryon and fusion colonies in fusion tests.

Fusion System	Fusion Pair	Number of test	Protoplast regeneration(%)	Ratio of H/P(%)*	Ratio of F/H(%)**
BenRnit x Wild-type	2ben12nit1b x 6w	4	8.5	0	0
	6ben6nit1b x 2w	4	2.8	0	0
BenR x HygrR	2ben12 x 6h3	2	16	0.6	0
	2ben6 x 6h9	1	26	0.02	0
	6ben12 x 2h3	2	38-50	0.013	0
	6ben13 x 2h11	2	10-15	0.01	0
HygrRnitxWild-type	6h9nit1 x 2w	1	8	0.3	2
	2h11nit1 x 6w	1	12	nc	0
	2h12nit1 x 6w	1	5	nc	0
Aux x Aux	2aux2 x 6aux2	1	3	20	1.6
	2aux1 x 6aux4	2	0.5-8	6	0.12
	2aux1 x 6aux6	1	10	2	nc
	2aux1 x 6aux8	1	10	2	nc
HygrR x PhleR	6h3 x 2p21	1	7	2.5	0
	2h11 x 6p3	1	34	0.4	0
	2h11 x 6p6	2	11-15	0.071	nc
	6h9 x 2p21	3	1.2-27	0.5	nc
	2h12 x 6p6	1	3.5	0.8	nc
	2h10 x 6p2	1	6	1	0.1
	" x 6p5	1	7	0.8	0.15
	" x 6p8	1	6	1	0.2
	2p17 x 6h1	1	9	0.01	0
	" x 6h5	1	8	0.003	0
	" x 6h10	1	10	0.0025	0
	6h1 x 2p15	1	10	0.04	0
	" x 2p18	1	11	0.05	0.5
	" x 2p22	3	5-15	0.75-1	1.6
	6p7 x 2h1	2	2.5-8	0.03-0.04	0
	" x 2h2	1	6	0.08	0
	" y 2h10	1	8	0.6	0.5
	6h10 x 2p22	1	11.0	1.2	0
	2h1 x 6p2	1	10.0	0.04	1

*. Fusion Frequency(H/P): Heterokaryon colonies/protoplasts regenerated, and nc: not counted.
**. Recombination Frequency(F/H): Fusion colonies/Heterokaryon colonies.

Growth------1

TRANSFORMANTS

Code	Growth rate in diameter(mm)							
	PDA		PDA+Hygro		PDA+Phle		PDA+Ben	
	42h	24h	42h	24h	42h	24h	42h	24h
2h-1		13.0		10.8		4.2		0.0
2h-2		11.3		10.0		4.8		0.0
2h-10		/Dry		11.0		4.0		0.0
2h-11		13.2		13.8		4.4		0.0
2h-12		14.5		13.8		4.4		0.0
2p-9		13.5		0.0		8.0		0.0
2p-12		13.2		0.0		7.5		0.0
2p-15		13.5		0.0		8.2		0.0
2p-16		13.0		0.0		7.8		0.0
2p-17		13.0		0.0		12.2		0.0
2p-18		13.2		0.0		8.0		0.0
2p-20		11.8		0.0		8.5		0.0
2p-21		12.8		0.0		10.3		0.0
2p-22		14.5		0.0		10.5		0.0
2p-23		9.8		0.0		7.5		0.0

* Hygromycin: 100ppm, Phleomycin: 100ppm, Benomyl:3ppm. The growth rates were measured after 42-hour initial growth on the media.

Growth-------2

TRANSFORMANTS

Code	Growth rate in diameter(mm/24h)			
	PDA	PDA+Hygro	PDA+Phle	PDA+Ben
6h-1	13.8	6.8	5.0	0.0
6h-2	13.3	10.5	5.8	0.0
6h-3	12.5	9.5	5.0	0.0
6h-4	12.5	10.8	5.8	0.0
6h-5	12.5	8.3	6.0	0.0
6h-8	11.5	6.5	6.3	0.0
6h-9	14.3	14.3	5.8	0.0
6h-10	11.0	9.5	6.0	0.0
6h-11	13.3	6.5	6.0	0.0
6h-13	10.8	7.5	3.8	0.0
6h-14	13.3	6.0	5.5	0.0
6h-15	13.0	5.8	5.3	0.0
6p-1	13.0	0.0	11.0	0.0
6p-2	14.8	0.0	11.0	0.0
6p-3	12.5	0.0	12.3	0.0
6p-4	14.2	0.0	8.2	0.0
6p-5	/dry	0.0	8.2	0.0
6p-6	13.8	0.0	10.0	0.0
6p-7	13.5	0.0	9.3	0.0
6p-8	13.3	0.0	10.0	0.0
6p-9	7.2	0.0	5.3	0.0
6p-10	13.3	0.0	7.5	0.0

*Hygromycin and Phleomycin: 100ppm, Benomyl:3ppm. With 42-hour initial growth.

Growth----3

FUSION PRODUCTS---Hygro x Phle
Growth on selective media

Code	Parents	Monoculture	Morphology	Growth in Diameter (mm/24-hr.)		
				CDA	CDA+h	CDA+p
	6h1	11-04-95	6	14	14	TG
	2p22	"	2	14	0	14
	6p7	"	6	14	0	14
	2h1	"	2	14	14	TG
	2h12	"	2	14	14	TG
	6p6	"	6	14	0	14
	2p21	"	2	14	0	14
	6h9	"	6	14	14	TG
f1409-1	6h1 x 2p22	"	2	14	13	14
f1409-2	"	"	2	14	12	14
f1409-3	"	"	2	13	13	13
f1409-4	"	"	2	14	11	14
f1409-5	"	"	2	15	14	14
f1409-6	"	"	2	15	14	14
7	"	18-04-95	2	15	14	14
8	"	"	2	15	12	15
9	"	"	2	15	11	12
10	"	"	6	15	15	14
11	"	"	2	15	13	14
12	"	"	2	15	13	14
13	"	"	2	15	14	14
14	"	"	6	15	14	14
15	"	"	6	15	12	15
16	"	21-04-95	6	14	14	14
17	"	"	2	15	15	15

2: G2 type, 6: G6 type. TG: Tyne Growth.

Growth-----4

FUSION PRODUCTS----Hygro x Phle

Growth on Selective Media

Code	Parents	Monoculture	Morphology	Diameter (mm/24hr.)		
				CDA	CDA+h	CDA+p
f1401-1	2h10 x 6p2	21-04-95	2	13	13	13
3	"	"	6	13	13	13
4	"	"	2	15	15	15
5	"	"	6	14	13	14
6	"	"	2	14	13	13
f1402-1	2h10 x 6p5	18-04-95	2	14	13	10
2	"	"	2	13	13	11
3	"	"	2	14	14	13
f1403-2	2h10 x 6p8	"	2	9	10	9
3	"	"	6	12	12	12
4	"	"	6	13	13	13
7	"	21-04-95	2	13	13	13
f1408-1	6h1 x 2p18	18-04-95	2	14	14	13
3	"	"	2	14	14	14
4	"	21-04-95	2	14	14	14
5	"	"	2	14	14	14
f1412-1	6p7 x 2h10	18-04-95	2	12	13	12
2	"	"	2	13	13	13
5	"	"	2	12	12	12
6	"	"	2	13	13	13
7	"	21-04-95	2	13	13	13

FUSION PRODUCTS---Hygr-R x Phle-R(Fusion-15)
10-20, 05-1995

Code	Parent	Morphology	Growth rate(mm/24hr. in diameter)		
			CDA	CDA +h	CDA +p
f151-1	2h1 x 6p2	2	14	13	14
2	"	6	13.5	13.5	13.5
3	"	6	14	14	14
4	"	6	14	14	13
5	"	6	14	14	14
6	"	2	14	14	14
7	"	2	14	14	14
8	"	2	14	14	14
9	"	2	14	14	14
10	"	6	14	14	14
f152-1	2h1 x 6p7	2	14	13	14
3	"	2	13.5	13.5	13
4	"	2	14	14	14
5	"	2	14	14	14
6	"	2	14	14	14
7	"	2	14	14	14
8	"	6	14	14	14

Growth---6 Fusion Products Phle-R x Hygr-R

Code	Parent	Morpgology	Growth rate(mm in diameter/24hr)		
			CDA	CDA+h	CDA+p
f154-1	6h1 x 2p22	6	14	14	14
2	"	2	14	14	13
3	"	2	14	14	14
4	"	6	14	14	14
5	"	2	14	14	14
6	"	2	14	14	14
7	"	6	14	14	14
8	"	2	14	14	14
9	"	2	14	14	14
10	"	2	14	14	14
11	"	6	14	14	14
12	"	2	14	14	14
13	"	2	14	13	14
14	"	2	14	14	14
15	"	2	14	14	14
16	"	2	14	14	14
17	"	2	14	14	14
18	"	2	14	14	14
19	"	2	14	14	14
20	"	2	14	14	14
21	"	2	14	14	14
22	"	2	14	14	14
23	"	2	14	14	14

Growth -----7 Fusion Products Phle-R x Hygr-R

Code	Parent	Monospore	Morphology	Growth rate on CDA(mmin diameter/24hr)			
				-	+h	+p	p+h
f121-01	2h12 x 6p6	18/04-95	2	12	12	10	11
02	"	"	2	12	12	11	10.5
f121-09	2h12 x 6p6	18-04-95	2	10	11	10	
f122-01	2p21 x 6h9	"	6	16	16	16	16
02	"	"	6	16	16	16	16
03	"	"	6	16	16	16	16
04	"	"	6	16	16	16	16
f122-21	2p21 x 6h9	"	6	14	14	14	
f122-23	"	"	6	14	14	14	
f122-26	"	"	6	14	14	14	
f122-31	"	"	6	14	14	14	
	2h12			15	15	0	tg
	2p21			16	0	18	0
	6h9			16	16	0	tg
	6p6			16	0	16	0

tg: Tine Growth.

Growth-------8 FUSION PRODUCTS----6h9nit1 x 2w

Growth on selective media

Code	Monoculture	Morphology	Growth Diameter(mm/24-hr.)	
			CDA	CDA+h
6h9nit1		6	15	15(on PDA)
2w		2	15	0
6hn2w-1	05-04-95	6	15	15
2	"	6	15	15
3	"	6	15	15
4	"	6	15	15
5	11-04-95	6	15	15
6	"	6	15	14
7	"	6	15	15
8	"	6	15	15
9	"	6	15	15
10	"	6	15	15
11	"	6	15	15
12	"	6	15	15
13	"	6	15	15
14	"	6	15	14
16	"	6	15	15
17	"	6	14	13
18	"	6	14	14
19	"	6	15	15
20	"	6	15	15
34	"	6	15	15
35	"	6	14	14

Marker---1 FUSION PRODUCTS----AUXTROPH MUTANTS---21:2aux1(ade-), 22:2aux2(leu-), 62:6aux2(ade-), 64:6aux4(?).

Code	M	G2 Marker								G6 Marker				
		CDA	B14 2000	E20 1010	E20 1020	G12 550	G12 1100	F13 1100	F13 2000	CDA	B14 1600	E20 700	E20 1100	F13 700
21		-	+	+	+	+	+	+	+		-	-	-	-
64			-	-	-	-	-	-	-	-	+	+	+	+
p2164-2	6	+	+	+	+	n	n	n	n	+	+	-	-	n
" 3	6	+	+	+	+	n	n	n	n	+	+	-	-	n
" 11	6	+	+	+	+	n	n	n	n	+	+/-	+	+	n
" 101	2	+	+	+	+	+	+	+	+	+	?	-	-	-
" 102	2	+	+	+	+	+	+	+	+	+	+	-	-	+
22		-	+	+	+	+	+	+	+		-	-	-	-
62			-	-	-	-	-	-	-	-	+	+	+	+
p2262-103	2-6	+	n	n	n	n	n	n	n	+	n	n	n	n
105	6	+	n	n	n	n	n	n	n	+	n	n	n	n
109	6	+	+	+	+	n	n	n	n	+	+	-	-	n
110	2	+	n	n	n	n	n	n	n	+	n	n	n	n
201	2	+	+	+	+	n	n	n	n	+	-	-	-	n
203	2	+	n	n	n	n	n	n	n	+	+	-	-	n
205	6	+	n	n	n	n	n	n	n	+	n	n	n	n
207	2	+	+	+	+	n	n	n	n	+	+	+	-	n

*2:G2, 6:G6, M: Morphology of Colony, CDA: CDA medium, +: Positive, -: Negative. +/- : Fade band. n: not tested.

Marker----2 FUSION PRODUCTS-----Phle x Hygro

Code	Parents	M	H	P	G2 marker					G6				
					E20 1010	1020	B14 2000	G15 1200	H9 1100	B14 1600	E20 1100	G15 550	H9 1250	H01 1700
	2p22	2	-	+	+	+	+	+	+	-	-	-	-	-
	6h1	6	+	-	-	-	-	-	-	+	+	+	+	+
f1409-1	6h1 x 2p22	2	+	+	+	+	+	+	+	+	-	-	+	-
2	"	2	+	+	+	+	+	+	+	+	-	+	+	+
3	"	2	+	+	+	+	+	+	+	-	-	+	+	+
4	"	2	+	+	+	+	+	+	+	-	-	+	+	+
5	"	2	+	+	+	+	+	+	+	-	-	-	+	-
6	"	2	+	+	+	+	+	+	+	-	-	-	+	-
	2h12	2	+	-	+	+	+	+	+	-	-	-	-	-
	2p21	2	-	+	+	+	+	+	+	-	-	-	-	-
	6h9	6	+	-	-	-	-	-	-	+	+	+	+	+
	6p6	6	-	+	-	-	-	-	-	+	+	+	+	+
f121-9	2h12 x 6p6	2	+	+	+	+	+	n	n	-	-	n	n	n
f122-21	2p21 x 6h9	6	+	+	+	+	+	n	n	+	-	n	n	n
23	"	6	+	+	+	+	+	n	n	+	-	n	n	n
26	"	6	+	+	+	+	+	n	n	+	-	n	n	n
31	"	6	+	+	+	+	+	n	n	?	-	n	n	n

-: negative; +: positive; n: not tested.

Marker----- 3

FUSION PRDUCTS------Hygro-R X Phle-R

Code	Parents	M	G2 Markers H	P	B14 2000	E20 1010	E20 1020	G12 550	G12 1100	F13 1100	F13 2000	G6 Marker H	P	B14 1600	E20 700	E20 1100
	2p21		-	+	+	+	+	+	+	+	+	-	+	-	-	-
	6h9		+	-	-	-	-	-	-	-	-	+	-	+	+	+
fph-601	2p21x6h9	2	+	+	+	+	+	n	n	n	n	+	+	-	-	-
602	" "	2	+	+	+	+	+	n	n	n	n	+	+	-	-	-
603	" "	2	+	+	+	+	+	n	n	n	n	+	+	-	-	-
604	" "	2	+	+	+	+	+	n	n	n	n	+	+	-	-	-
617	" "	6	+	+	+	+	+	n	n	n	n	+	+	+/-	+	+
f122-01	" "	6	+	+	+	+	+	n	n	n	n	+	+	+	-	-
02	" "	6	+	+	+	+	+	n	n	n	n	+	+	+	+	+
03	" "	6	+	+	+	+	+	n	n	n	n	+	+	+	-	-
04	" "	6	+	+	+	+	+	n	n	n	n	+	+	+	-	-
	2h11	2	+	-	+	+	+	n	n	n	n	+	-	-	-	-
	2h12	2	+	-	+	+	+	+	+	+	+	+	-	-	-	-
	6p6	6	-	+	-	-	-	-	-	-	-	-	+	+	+	+
fph-605	2h11x6p6		+	+	+	+	+	n	n	n	n	+	+	-	-	-
630	" "	2-6	+	+	+	+	+	n	n	n	n	+	+	+	-	-
f121-01	2h12x6p6	2	+	+	+	+	+	+	+	+	+	+	+	-	-	-
02	" "	2	+	+	+	+	+	+	+	+	+	+	+	-	-	-

2: G2, M: Colony Morphology, H: Hygro-R +: Positive
6: G6 N: Not tested, P: Phle-R -: Negative
Primer bands: B14-2000, E20-1010 E20-1020
B14-1600 E20-700 E20-1100
G12-550 G12-1100 F13-1100

Marker----4 FUSION PRODUCTS-----G6h9nit 1 x G2w

Code	M				G2 Marker								G6 Marker						
		H	CDA	B14 2000	E20 1010	E20 1020	G12 550	G12 1100	F13 1100	F13 2000	G09 1600	G15 1200	H	CDA	B14 1600	E20 700	E20 1100	F13 700	G9&G15 12&5.5
G2		-	+	+	+	+	+	+	+	+	+	+			-	-	-	-	--
G6h9nit1				-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	++
pf6hn2w-1	6	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	--
" 2	6	+	+	+	+	+	+	+	+	+	+	+	+	+	+/-	-	-	-	--
" 3	6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	--
" 4	6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	--
5	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
6	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
7	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
8	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
9	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
10	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
11	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
" 12	6	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	--
13	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
16	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
17	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
19	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
20	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
34	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn
35	6	+	+	+	+	+	n	n	n	n	n	n	+	+	-	-	-	n	nn

* : 2:G2, 6:G6, M: Morphology of Colony, H:Hygro-R, CDA:CDA medium, +: Positive, -: Negative, n: not tseted.

Marker---5 Hyphael Fusion Products----2ben x 6h

Code	Parent	M	B	H	G2 marker B14 2000	G2 marker E20 1020	G2 marker E20 1010	G2 marker A18 1000	G6 marker B14 1600	G6 marker E20 1100	G6 marker H01 1700	G6 marker H01 ?
hf101	2b12 x 6h1		+	+	+	+	+	+	+?	-	+	-
104	"		+	+	+	+	+	+	-	-	-	-
105	2b12 x 6h2		+	+	+	+	+	+	+?	-	-	+
107	"		+	+	+	+	+	+	-	-	-	+/-
108	"		+	+	+	+	+	?	-	-	-	+
109	"		+	+	?	+	+	?	-	-	+	-
110	6b12 x 6h3		+	+	+	+	+	+	+?	+?	-	-
111	"		+	+	+	+	+	?	-	-	-	-
	2b12		+	-	+	+	+	+	-	-	-	-
	6h1		-	+	-	-	-	-	+	+	+	+
	6h2		-	+	-	-	-	-	+	+	+	+
	6h3		-	+	-	-	-	-	+	+	+	+

B: Benomyl resistance. H: Hygromycin B resistance. M: Morphology on PDA medium. +: Positive. -: Negative. +? & ?: Fading bands.

Strain List-----1 TRANSFORMANTS

Parent strain: G2 Vector: pAN 8, Phle-R gene. Experiment: T-01, 09-1994

Transformant	Pre-test on PDA +Phle*	Monospore / Date	Stored
2TP-1	+ +	no	14-09-94
-2	+ +	no	"
-3	+ +	"	"
-4	+ +	"	"
-5	+ +	"	"
-6	+ +	"	"
-7	+ +	"	"
-8	+ +	"	"
-9	+ +	13-09	06-10-94
-10	+ +	no	14-09-94
-11	+ +	no	"
-12	+ +	13-09	06-10-94
-13	+ +	no	14-09
-14	+	no	"
-15	+ +	13-09-94	06-10-94
-16	+ +	13-09	06-10-94
-17	+ +	13-09	06-10-94
-18	+ +	13-09	06-10
-19	+ +	no	14-09
-20	+ +	13-09	06-10
-21	+ +	13-09	"
-22	+ +	13-09	"
-23	+ +	13-09	"
-24	+	no	14-09

*: The growth rate on selective medium were presented on a separate table. +: Positive growth; no: Not done.

Strain List------2. TRANSFORMANTS

Parent Strain : G2
Vector: pAN 7, Hygromycin - R gene
Experiment: 01, 09-09-1994

Transformant and Sandy Tube	Pre-test on	Monospore / Date	Stored on PDA	PDA +h*
2Th-01	+ +	19-09-94	21-09-94	
-02	+ +	19-09	"	
-03	+	no	14-09	
-04	+	no	"	
-05	+ +	no	"	
-06	+ +	no	"	
-07	+	no	"	
-08	+ +	no	"	
-09	+ +	no	"	
-10	+ +	19-09	21-09	
-11	+ +	19-09	21-09	
-12	+ +	19-09	21-09	
-13	+/+ +	no	14-09	
-14	+/+ +	no	14-09	

* Growth rate is presented on a separate table. no: No monospore culturing done.

Strain List-------3 TRANSFORMANTS

G6, 1994

Transformant	Pre-test on PDA +p*	Monospore/Date	Store
G6p-1	++	13-09	06-10
2	++	"	"
3	++	"	"
4	++	"	"
5	++	"	"
6	++	"	"
7	++	"	"
8	++	"	"
9	+	"	"
10	++	"	"
G6h-1	++	"	"
2	++	"	"
3	++	"	"
4	++	"	"
5	++	"	"
8	++	"	"
9	++	"	"
10	++	"	"
11	++	"	"
12	++	"	"
13	++	"	"
14	++	"	"
15	++	"	"

* Growth rates were repersented on a separate table.

Strain List-----4

Nit mutants and auxotrophes

Code	Parent	Character	Monospore	Store
6h9nit1	6h9	Hygr-R/Chlorate-R	12-1994	12-1994
2h11nit1	2h11	"	01-1995	01-1995
2h12nit1	2h12	"	"	"
6nh	G6nit1	"	01-1995	03-1995
2nh	G2nit1	"	01-1995	03-1995
6aux6	G6	Pro -	03-1995	03-1995
6aux8	G6	Lys-	03-1995	03-1995

Strain List----5-1 FUSION PRODUCTS---Hygro x Phle

Code	Parents	Monoculture	Morphology	Growth in Diameter(mm/24-hr.)		
				CDA	CDA+h	CDA+p
f1409-1	6h1 x 2p22	"	2	14	13	14
f1409-2	"	"	2	14	12	14
f1409-3	"	"	2	13	13	13
f1409-4	"	"	2	14	11	14
f1409-5	"	"	2	15	14	14
f1409-6	"	"	2	15	14	14
7	"	18-04-95	2	15	14	14
8	"	"	2	15	12	15
9	"	"	2	15	11	12
10	"	"	6	15	15	14
11	"	"	2	15	13	14
12	"	"	2	15	13	14
13	"	"	2	15	14	14
14	"	"	6	15	14	14
15	"	"	6	15	12	15
16	"	21-04-95	6	14	14	14
17	"	"	2	15	15	15

Strain List-----5-2 Fusion products Phle-R x Hygr-R

Code	Parent	Monospore	Morphology	Growth Rate on CDA (mm/24hr at 27^{0}C)		
				-	+h	+p
f154- 1	6h1 x 2p22	10-05-95	6	14	14	14
2	"	"	2	14	14	14
3	"	"	2	14	14	13
4	"	"	6	14	14	14
5	"	"	2	14	14	14
6	"	"	2	14	14	14
7	"	"	6	14	14	14
8	"	"	2	14	14	14
9	"	"	2	14	14	14
10	"	"	2	14	14	14
11	"	"	6	14	14	14
12	"	12-05-1995	2	14	14	14
13	"	"	2	14	13	14
14	"	"	2	14	14	14
15	"	"	2	14	14	14
16	"	"	2	14	14	14
17	"	"	2	14	14	14
18	"	"	2	14	14	14
19	"	"	2	14	14	14
20	"	"	2	14	14	14
21	"	"	2	14	14	14
22	"	"	2	14	14	14
23	"	"	2	14	14	14

Strain List-----5-3

FUSION PRODUCTS----Hygro x Phle

Code	Parents	Monoculture	Morphology	Diameter (mm/24hr.)		
				CDA	CDA+h	CDA+p
f1401-1	2h10 x 6p2	21-04-95	2	13	13	13
3	"	"	6	13	13	13
4	"	"	2	15	15	15
5	"	"	6	14	13	14
6	"	"	2	14	13	13
f1402-1	2h10 x 6p5	18-04-95	2	14	13	10
2	"	"	2	13	13	11
3	"	"	2	14	14	13
f1403-2	2h10 x 6p8	"	2	9	10	9
3	"	"	6	12	12	12
4	"	"	6	13	13	13
7	"	21-04-95	2	13	13	13
f1408-1	6h1 x 2p18	18-04-95	2	14	14	13
2	"	"	2	14	13	13
3	"	"	2	14	14	14
4	"	21-04-95	2	14	14	14
5	"	"	2	14	14	14
f1412-1	6p7 x 2h10	18-04-95	2	12	13	12
2	"	"	2	13	13	13
5	"	"	2	12	12	12
6	"	"	2	13	13	13
7	"	21-04-95	2	13	13	13

Strain List-----5-4

FUSION PRODUCTS---Hygr-R x Phle-R(Fusion-15)
10-20, 05-1995

Code	Parent	Monospore	Morphology	Growth rate(mm/24hr. in diameter)		
				CDA	CDA +h	CDA +p
f151-1	2h1 x 6p2	12/05-95	2	14	13	14
2	"	"	6	13.5	13.5	13.5
3	"	"	6	14	14	14
4	"	"	6	14	14	13
5	"	"	6	14	14	14
6	"	"	2	14	14	14
7	"	"	2	14	14	14
8	"	"	2	14	14	14
9	"	"	2	14	14	14
10	"	"	6	14	14	14
f152-1	2h1 x 6p7	"	2	14	13	14
3	"	"	2	13.5	13.5	13
4	"	"	2	14	14	14
5	"	"	2	14	14	14
6	"	"	2	14	14	14
7	"	"	2	14	14	14
8	"	"	6	14	14	14

Strain List-----5-5 FUSION PRODUCTS Hygr-R x Phle-R

Code	Parent	Monoculture	Morphology	CDA (Diameter mm/24hr at 27C)			
				CDA	+h	+p	p+h
f121-01	2h12 x 6p6	18/04-95	2	12	12	10	11
02	"	"	2	12	12	11	11
f121-09	2h12 x 6p6	18-04-95	2	10	11	10	
10	"	"	2	10	10	10	
f122-01	2p21 x 6h9	"	6	16	16	16	15
02	"	"	6	16	16	16	16
03	"	"	6	16	16	16	16
04	"	"	6	16	16	16	16
f122-21	2p21 x 6h9	"	6	14	14	14	
f122-23	"	"	6	14	14	14	
f122-26	"	"	6	14	14	14	
f122-31	"	"	6	14	14	14	
f601	"	11/94		13	13	10	
602	"	"		16	16	13	
603	"	"		15	16	14	
604	"	"		15	14	14	
630	"	"		13	12	11	
617	"	"		14	15	13.5	
605	2h11 x 6p6	"		15	15	13	
	2h11			14	13	7 (PDA)	
	2p21			11	0	10 (PDA)	
	6h9			11	11	6.5 (PDA)	
	6p6			12	0	11 (PDA)	

2: G2 type, 6: G6 type.

Strain List------5-6 FUSION PRODUCTS----6h9nit1 x 2w

Code	Monoculture	Morphology	Growth Diameter(mm/24-hr.)	
			CDA	CDA+h
6h9nit1		6	15	15(on PDA)
2w		2	15	0
6hn2w-1	26/1-95	6	15	15
2	"	6	15	15
3	"	6	15	15
4	"	6	15	15
5	11-04-95	6	15	15
6	"	6	15	14
7	"	6	15	15
8	"	6	15	15
9	"	6	15	15
10	"	6	15	15
11	"	6	15	15
12	26/1-95	6	15	15
13	11/04-95	6	15	15
14	"	6	15	14
16	"	6	15	15
17	"	6	14	13
18	"	6	14	14
19	"	6	15	15
20	"	6	15	15
32	"	6	14	14
34	"	6	15	15
35	"	6	14	14

Strain List----6 Protoplast Fusion Product------Auxotrophes

Code	Growth on CDA	Monospore	Store
f2262-103	+	03-95	04-95
105	+	"	"
109	+	"	"
110	+	"	"
201	+	"	"
203	+	"	"
205	+	"	"
207	+	"	"
fp101(2164)	+	01-95	01-95
102 "	+	"	"

Strain List----7

HYPHAL FUSION PRODUCTS

Code	Parents	Growth on			Monospore	Store
		CDA	B+H	PDA		
hf-101	2b12 x 6h1		+++	+++	26-09-94	12-10-94
104	" "		"	"	"	"
105	2b12 x 6h2		"	++	"	"
107	" "		++	+++	"	"
108	" "		++	+++	"	"
109	" "		++	++	"	"
110	2b12 x 6h3		+++	+++	"	"
111	" "		+++	+++	"	"
h2164-2		+			02-95	03-95
3		+			"	"
8		+			"	"
11		+			"	"
15		+			"	"
h2166-2		+			04-95	05-95
3		+			"	"
6		+			"	"
7		+			"	"
20		+			"	"
21		+			"	"
22		+			"	"
23		+			"	"
24		+			24/4-95	30/4-95
25		+			"	"
26		+			"	"
29		+			"	"
h2168- 2		+			04-95	05-95
3		+			"	"
4		+			"	"
22		+			05-95	05-95

LABORATORIUM VOOR
BLOEMBOLLENONDERZOEK
BULB RESEARCH CENTRE

VENNESTRAAT 22, POSTBUS 85
2160 AB LISSE, THE NETHERLANDS
TEL. (+31) 2521-62121, FAX (+31) 2521-17762